PH responsive polymers for controlled release of liposomal contents

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Doctor of Philosophy

Aston University

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Summary

A growing interest has been seen in mechanisms to control the delivery and release of many active agents, such that by manipulating the site and timing of their action it optimises their activity. Hydrophobically modified polyamides and amine modified glycidyl methacrylate pre-polymer were synthesised to confer pH-responsive conformational changes and investigated for controlled release/delivery applications. Applications of interest included the use of these polymers as endosomolytic agents and use to cause pH-responsive liposomal content release. To assess these areas of interest the release of haemoglobin from pH adjusted erythrocytes that were pre-incubated in the presence and absence of polymer were compared. Poly(lysine butyl malonamide), poly(lysine diethyl malonamide), poly(lysine *iso*-phthalamide), poly(lysine dodecandiamide), poly(ornithine dodecandiamide) and poly(ornithine sebacamide) were shown to exhibit pH-responsive erythrolysis. The pH-responsive membrane disruption properties were assessed further by measuring calcein released from pH adjusted liposomes with encapsulated calcein that were pre-incubated in the presence or absence of polymer. Using this methodology polymers poly(lysine butyl malonamide), poly(lysine dodecandiamide) and poly(ornithine sebacamide) were shown to have pH- responsive disruption properties, while, polymers poly(lysine diethyl malonamide), poly(ornithine dodecandiamide) and poly(lysine *iso*-phthalamide) did not. As both hydrophobically modified polyamides and amine modified glycidyl methacrylate pre-polymer exhibited membrane disruption properties they were also assessed for use as anti-microbial agents. However, on the range of micro-organisms tested, no anti-microbial properties were observed. In addition, both polymer groups were investigated for use as solid particle coatings. The pH-responsive uncoating of polymer coated solid particles was then quantified using dye release and conductivity measurement systems. Both systems showed that a coating of *N,N*-diethylamine modified glycidyl methacrylate polymer controlled the release of solid particles from within the polymer coating as a function of pH. However, the results suggested there were imperfections in the coating as release was not completely prevented at any pH value.

Key words: **polymers, liposomes, responsive-release, pH, solid-coating.**

Dedication

To my grandmother Tydfil Williams and the memory of my grandparents Robert Williams and Rhys and Nancy Morcom.

To my mum and dad, Eirlys and John Morcom respectively, for the sacrifices they have made which have allowed me to come this far this soon.

To my sister, Amanda Morcom, for her encouragement and understanding.

Finally, to my partner, Vaughan Shilton, for all his emotional support and belief in my abilities.

Research communication and publications

Morcom, A. (2002) PH-responsive release of cell contents using polyamide and polyacrylic acid polymers. Poster presentation, International conference on polymer synthesis (2002), University of Warwick.

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Chapter 1

Introduction and literature review

1.0: Introduction.

This thesis describes research conducted at Aston University, involving two groups of polymers that show pH-responsive conformational changes and therefore, have potential controlled release or controlled delivery applications. The two groups investigated were hydrophobically modified polyamides and a glycidyl methacrylate pre-polymer modified with different amine groups.

Polymers which show large property changes in response to small physical or chemical stimuli such as temperature, pH, ions, electric fields, solvents, reactants, light, stress or magnetic fields (Zareie *et al.*, 2000, Chen *et al.*, 1995; Chen *et al.*, 1993; Feil *et al.*, 1992) have been defined as "intelligent", "smart", "stimuli-responsive" and "environmentally sensitive" polymers (Hoffman, 1995).

The application of such polymers has recently been of increasing interest in biomedical and biotechnology fields. For example, uses include drug delivery systems (Duncan, 2003; Dong *et al.*, 1991) biosensors (Sershen *et al.*, 2002; Aoki *et al.*, 1995), control of protein ligand recognition (Stayton *et al.*, 1995), catheters, wound dressings and separation membranes (Peppas, 1981; Bell *et al.*, 1996; Shin *et al.*, 1997).

Controlled delivery and or release in general refer to optimising an agents activity by manipulating the site and timing of action of the agent. The motivation behind this thesis has been to improve understanding of the properties of these two groups of polymers in the hope that their properties will prove advantageous for controlled delivery or release applications.

Three main polymer properties are the focus of this thesis. The first property is pH-responsive disruption of cell membranes, such that these polymers can be used as endosomolytic agents. The second property is when used in conjunction with liposomes they cause pH-responsive disruption of liposomal membranes and

therefore, content release. Such properties could aid the delivery of agents encapsulated within liposomes. The final property is when used as solid coatings they show pH-responsive uncoating of the polymer coated agent, such that they can be used to release solid agents in response to pH change.

In this chapter the main controlled release industries will firstly be described. The technologies that have been developed and currently exist will then be discussed. The review will then focus upon three main sections, which include polymers that disrupt membranes in relation to use as endosomolytic agents, disruption of liposomes to cause liposomal content release and finally pH-responsive solid polymer coatings will be discussed. As the use of liposomes forms a large part of this thesis their classification, preparation and their applications will also be discussed.

1.1: Controlled release industries.

The controlled release technologies currently available, which are described in section 1.2 have an application to a wide range of industries including those intended for human, veterinary and agricultural use.

1.1.1: Products intended for human use.

Products intended for human use must undergo rigorous testing before use. However, a large potential market for high tariff products exists, making research in to such products economically feasible. For example, large amounts of research have focused on systems that allow prolonged and improved control of drug administration. In traditional drug delivery systems, the drug is taken and the drug concentration in the body rises, peaks and then declines. Commercial devices, currently available are able to maintain drug concentration in the desired therapeutic range with just a single dose, localise delivery to a particular body compartment reducing the systemic drug level and reducing the need for follow up care, preserve medications that are rapidly destroyed by the body and increases patient comfort and thus compliance (Kost *et al.*, 2001). Although great advances have been made with such constant state drug delivery systems there are a number of clinical situations where this is insufficient.

That is, conditions which require pulsed or self-regulated delivery. Examples include, delivery of insulin for patients with diabetes mellitus, antiarrhythmics for patients with heart rhythm disorders, gastric acid inhibitors for patients with ulcers, nitrates for patients with angina pectoris, selective β-blockade, birth control, general hormone replacement, immunization and cancer chemotherapy (Kost *et al.*, 2001). Consequently, many research groups have been developing responsive systems that can respond to normal physiological processes, such that drug release is affected according to the physiological needs (Simo *et al.*, 2003). It is thought evaluation of new responsive polymers will help to advance this field.

1.1.2: Veterinary.

Economic considerations play a larger influence on research and development in veterinary medicine compared to that in human medicine. The treatment of disease in commercial animals is controlled almost entirely by financial constraints. Therefore, products used to combat disease must be cost effective so that animal health is improved, while financial return is at least equalled. The ethical considerations that apply to products intended for human use are not as strict when tested on animals. This means there is greater flexibility to test products used against life-threatening disease, such that many are now in routine use.

In veterinary medicine, the main reasons for developing a drug in the form of a controlled and/or prolonged drug release system are to reduce animal stress from restraint, handling and dosing and to reduce cost in terms of both money and time (Magruder, 1999). Several markets exist for the application of controlled release technologies in the veterinary field. These include fertility control, growth promotion and oestrus synchronisation by delivery of hormones, supplementation of nutrients by delivery of trace nutrients and also the delivery of anti-microbial and anti-parasitic agents (Rathbone *et al.*, 1999; Duncan *et al.*, 1989). Other minor markets also exist, such as wildlife management. For example, by using controlled delivery technologies attempts have been made to disrupt the reproduction of pests, which cause problems to natural fauna and flora (Hurley *et al.*, 2002; Mcleod *et al.*, 1998).

A wide variety of methods are already available for controlled delivery to animals. These include oral formulations, such as ruminal and pulsatile boluses, expanding devices and high density devices; injectable and implantable devices such as intra-vaginal drug delivery systems; topical dosage forms, such as oil based systems; collars and ear-tags and also ophthalmic devices (Rothen-Weinhold *et al.*, 2000).

While many advances have already been made with the polymeric systems (Matschke *et al.*, 2002) for controlled release in animals, it is thought improvements can still be made by evaluation of new and further development of the currently available polymers.

1.1.3: Agricultural.

Factors governing pharmaceutical and veterinary research vary considerably from those concerned with agriculture. Preparations need to be cheap so that large-scale use is possible. Additional factors, such as reluctance to use new preparations and the potential of total crop failure, hold back research. However, controlled release still has various applications in agriculture including seed coatings, along with herbicide, pesticide and fertiliser release. Seed coatings can be utilised to incorporate a variety of active agents that encourage germination and growth, thus increasing potential productivity. For example, Extender ® (Grow Tech Inc. Nisku, Alta) is a coating for canola seed used to increase germination rates (Zaychuk *et al.*, 2000). Partial or uneven coatings are easily lost before sowing, making systems for coating seeds evenly of great interest.

The main problems concerned with herbicide use is the fact that many are unstable in free form and they move away from the required site, thus making their use futile and potentially harmful to the surrounding environment. A number of systems have been investigated to date to combat such problems, including encapsulation in pellets formed from various polymers. A recent study involving co-precipitation of the herbicide diuron and the polymer poly(lactic acid), using a supercritical antisolvent technique has been described to achieve controlled delivery (Taki *et al.*, 2001). Various sustained release pesticides are currently available (Chandra *et al.*, 1998).

Examples include polymeric microcapsules that contain pesticides. When added to water, the active agent is slowly liberated over a period of weeks. Thus, timing of application does not need to be as accurate since the pesticide remains active for a prolonged period of time.

Similar to pesticides, a sustained delivery of fertilisers is important, so that plants receive a constant supply. For example, Duvdevani *et al.*, (1996) describe a polymer coated vegetation enhancement composite that releases fertiliser slowly, enhancing plant growth.

1.2: Controlled release technologies and devices.

As explained, controlled delivery systems are required for many applications. A number of technologies and devices have been investigated to achieve controlled delivery, each having their own advantages and disadvantages for particular applications. The procedures that have been investigated and discussed below can be subdivided in to polymer and non-polymeric systems and are described under the appropriate subheadings. Those outlined will be discussed mainly in relation to drug delivery applications.

1.2.1: Non polymer systems.

1.2.1.1: Oil droplet formulations.

Bioactive agents are dispersed with oil droplets to form an emulsion. When the dispersion is placed in an aqueous environment the bioactive agent is slowly released as it moves towards equilibrium with the aqueous phase. The release of particulate agents from an oil suspension is dependent upon the particulate solubility and the oil and water partition coefficient. Emulsions are extensively used, especially as topical treatments. In addition, they are widely utilised to deliver drugs orally, parenterally and as aerosols for inhalation. More recently they have been evaluated for gene delivery (Hara *et al.*, 1997; Liu *et al.*, 1996) and also have been of interest to the food industry for controlled aroma and flavour release (Malone *et al.*, 2003).

1.2.1.2: Iontophoretic systems.

Iontophoresis is a system for administering charged drugs using an electric current usually at an order of magnitude of less than 0.5 mA/cm^2 (Nair *et al.*, 2003). Drugs are delivered via an electrode that has the same charge as the drug to an electrode having an opposite charge, which is placed at a neutral site in the body (Figure 1.1).

Figure 1.1: Diagrammatic representation of a simple iontophoretic system (Adapted from Duncan *et al.*, 1989).

Most developments have focused on the use of aqueous solutions to deliver drugs using iontophoresis (Tomohira *et al.*, 1997). However, over latter years gels have been becoming an attractive alternative since they are easily fabricated into the iontophoresis device, they are flexible so follow the skin contours, they have better occlusion, and better stability. Also, the high proportions of water employed in gel formulations have been shown to be an advantageous electro-conductive base for clinical use (Fang *et al.*, 1999).

1.2.1.3: Infusion pumps.

Infusion pumps can be driven mechanically, by battery, by peristalsis or using chemical based technologies. They are used for a number of clinical applications and exist as three principal types, including external non-portable devices, external portable devices and implanted pumps (Mikkelsen-Lynch *et al.*, 2000). They are often used to deliver medications when the oral route is ineffective or impractical. For example, for patients who require very high doses or who have difficulties with swallowing, nausea, vomiting, or bowel obstruction (Moulin *et al.*, 1991; Lang *et al.*,

1991). They can also be used to administer medications in an outpatient setting, eliminating the need for inpatient visits and intravenous access (Moulin *et al.*, 1992).

1.2.1.4: Prodrugs.

The term 'prodrug' was originally used to describe low molecular weight derivatives of a parent drug, such that only the parent drug is active at the desired site of action. However, the term 'prodrug' now incorporates macromolecular drug-carriers that deliver drugs following enzymatic cleavage at specific target sites. Prodrugs typically do not show activity; therefore it is essential that conversion back to the active form occurs efficiently at the desired site (Wermuth *et al.*, 1996). Such systems have been evaluated to improve the solubility and thus absorption of medications. For example, Matsumoto *et al.* (2001) designed and synthesised a series of highly water-soluble prodrugs of an HIV protease inhibitor. These prodrugs showed significantly increased water-solubility compared to that of the parent drugs.

1.2.1.5: Liposomes.

Liposomes are vesicle structures consisting of hydrated lipid bilayers. They have been extensively investigated as carrier vesicles owing to their ability to entrap a wide range of compounds, which can be released in response to a number of environmental conditions (Lian *et al.* 2001). The properties of liposomes and their application in conjunction with polymers for controlled release, is discussed in detail in Section 1.4.

1.2.2: Polymer systems.

The main polymer based systems that exist in relation to drug delivery include stimuli-responsive, soluble macromolecules, membrane based systems and matrix based systems, each of which will be described in turn.

1.2.2.1: Stimuli-responsive systems.

Also true of non-polymeric systems, polymeric systems used for responsive delivery of pharmaceuticals can be classified in to open loop systems, which are externally regulated and closed loop systems, which are self regulated (Rennard, 2002). The externally regulated systems can be triggered by magnetic, ultrasonic, thermal, electrical and light stimuli. Whereas, self regulated systems are stimulated by feedback information, without external intervention. For example, devices designed to deliver insulin to patients with diabetes mellitus are designed to respond to changes in glucose concentration (Zhang *et al.*, 2002).

An extensively investigated polymer is poly(*N*-isopropylacrylamide) which presents a low critical solution temperature and thus shows reversible transition close to physiological temperature (Kikuchi *et al.*, 1998). As already mentioned systems whereby polymers are sensitive to glucose are being researched to produce a self regulated insulin delivery systems for patients with diabetes (Zhang *et al.*, 2002; Kim *et al.*, 2001; Shiino *et al.*, 1994).

Polymers that are responsive to pH are also of great interest and the focus of this thesis. This interest stems from a variation in pH that is known to occur at several body sites, including the gastrointestinal tract (Kobayashi *et al.*, 2001) vagina (Lourens *et al.*, 2002) and blood vessels (Alverez-Lorenzo *et al.*, 2002) and can provide a suitable base for pH-responsive drug release. Also, local pH changes in response to specific substrates and pH variation in cellular compartments, which will be discussed later, can be used to stimulate drug release.

Hydrogel polymers have been synthesised and extensively investigated to exploit these differences in pH. That is, the charge density of these polymers, which contain weakly acidic or basic groups in their polymer backbone is dependent upon the pH or ionic composition of their surrounding environment. A number of researchers have seen the potential of such a property for pH-responsive drug release. For example, polyacidic polymers will be unswollen at a low pH, since their acidic groups are protonated and thus unionised. However, as pH increases the polymer swells. The reverse is seen with polybasic polymers as the ionization of basic groups increases

with decreasing pH. Therefore, drug release from reservoir or matrix device made from these polymers will display release rates that are pH-dependent (Gupta *et al.*, 2002). The most commonly studied polymers which fall in to this category include poly(acrylamide) (PAAm), poly(acrylic acid) (PAA), poly(methacrylic acid) (PMAA), poly(diethylaminoethyl methacrylate) (PDEAEMA) and poly(dimethylaminoethyl methacrylate) (PDMAEMA) (Lowman *et al.*, 1999). A number of advances have been made with these polymers. To illustrate the advances that have been made some of the more recent ones are outlined below.

For example, Kim *et al.* (2003) describe two novel methacrylic acid (MAA) based copolymers with pendant glucose. The first is a copolymer of MAA and 2-methacryloxyethyl glucoside (MEG), henceforth designated as P(MAA-co-MEG) and the second is a copolymer of MAA and poly(ethylene glycol) monomethyl ether monomethacrylate (PEGMA), henceforth designated as P(MAA-g-EG). The feasibility of these hydrogels as oral protein delivery carriers was evaluated. The pH-responsive release of insulin was analysed from both P(MAA-co-MEG) and P(MAA-g-EG) hydrogels. In acidic media (pH 2.2), insulin release from the hydrogels was very slow. However, as the pH of the medium was changed to 6.5, a rapid release of insulin occurred. In both cases, the biological activity of insulin was retained. For P(MAA-co-MEG) hydrogels, the biological activity of insulin decreased when the pendent glucose content increased. In P(MAA-g-EG) hydrogels, when the grafted PEG molecular weight increased, the insulin biological activity decreased. Finally, hydrogels of P(MAA-co-MEG) prepared with an initial ratio of 1:4 MEG:MAA and P(MAA-g-EG) hydrogels containing PEG chains of molecular weights of 200 showed the greatest change in insulin release rate from acidic to basic pH solutions and the greatest protective effect for insulin in simulated gastrointestinal tract conditions.

Soppimath *et al.* (2001) have developed chemically modified polyacrylamide-g-guar-gum based anionic spherical hydrogels of micron size. This polymer has been tried as a pH and ionic sensitive drug delivery system for the delivery of diltiazem hydrochloride and Nifedipine, drugs that characteristically exhibit a short elimination half-life (Sood *et al.*, 2003). The system was found to respond rapidly to a changing environment.

A number of researchers have attempted to devise pH-responsive polymeric networks for insulin delivery. For example Klumb *et al.* (1992) immobilised the enzymes glucose oxidase and catalase in a pH-responsive hydrogel of hydroxyethyl methacrylate based copolymer enclosing a saturated solution of insulin. The pH-responsive swelling of the hydrogel is triggered by the diffusion of glucose in to the hydrogel, which results in the enzyme catalysed conversion of glucose to gluconic acid, thereby lowering the pH of the microenvironment of the hydrogel and causing swelling. A decrease in glucose concentration caused the hydrogel to contract and thus decrease the rate of insulin delivery.

Although research in to these pH-responsive hydrogel based drug delivery systems has been widespread and still continues few have achieved commercial success. Nevertheless, their success in other biomedical applications such as usage in surgical implants, catheters and wound dressings (Hoffman, 2002; Stoy, 1999) and exploitation of rapid responsiveness still make these polymers a promising area of research. PH-responsive polymers as enteric coatings have also been widely investigated (Harianawala *et al.*, 2002; Eriksson *et al.*, 2003; Gonzalez *et al.*, 2003) and polymers that respond to differences in pH are currently of interest as endosomolytic agents (Murthy *et al.*, 2003; Christie *et al.*, 2003), both of which will be discussed in more detail later.

1.2.2.2: Soluble macromolecules.

This system uses macromolecular conjugates for site-specific delivery of drugs. There are two main types of macromolecular drug-conjugate systems, classified in to those that are linked by biodegradable or non-biodegradable spacers. The drug-conjugates having non-biodegradable spacers can only exert their activity if they come in to direct contact with their target receptor. Owing to the larger combined size of drug and conjugate, both types prevent random access of drug to the cell interior. Many conjugated systems have been designed and evaluated (Ichikawa *et al.*, 2002; Gallardo *et al.*, 2001; Steyger *et al.*, 1996; Duncan, 1992). Most are designed based on two conjugates based on the work by Duncan *et al.* (1984) and Duncan *et al.* (1992). These two systems are based on hydroxypropyl methacrylamide (HMPA) and styrene-co-maleic anhydride (SMA) copolymer, respectively. They have been

designed so that they can attach to target cells and become internalised by pinocytosis. Following internalisation, the drug is released within the lysosomal compartment, passes across the lysosomal membrane and finally interacts with its intracellular receptors to exert its pharmacological activity. For example, copolymers of HMPA, containing doxorubicin bound via a lysosomally degradable spacer Gly-Phe-Leu-Gly were shown to be more cytotoxic to mouse melanoma cells than doxorubicin bound via a non-degradable spacer (O'Hare *et al.*, 1993).

Maeda (2001) evaluated aqueous SMA copolymers formulations for potential treatment of patients with solid tumours of the ovary, oesophagus, lung, stomach, adrenal gland and the brain. Formulations based on SMA copolymers were shown to be effective for therapeutic use in solid tumours where the formulations are given arterially via a catheter. That is, in a pilot study a reduction in size of solid tumours of liver was observed for about 90% of cases when an adequate amount of the macromolecular drug was administered.

1.2.2.3: Membrane based systems.

For drug delivery there are two main membrane based systems, which include diffusion controlled devices and osmotic systems.

Diffusion controlled devices - These are devices where release of drug is achieved by diffusion of drug across a polymeric membrane. These membranes can be porous or non-porous and biodegradable or non-biodegradable. Many commercial products are based on biodegradable systems. For example, Ocusert to deliver pilocarpine in ocular therapies and Progestasert used to deliver contraceptive progesterone are based on biodegradable ethylene vinyl acetate copolymers (Kakish *et al.*, 2002).

Osmotic systems - The most representative of the osmotic systems is an osmotic pump known as Oros (Dash *et al.*, 1998). This system was first described by Theeuwes *et al.* (1976) and then released for use by Alza Corporation (Martin *et al.*, 1993). This pump consists of a drug reservoir surrounded by a semi-permeable membrane. The surrounding membrane allows a steady influx of water and biological fluid into the reservoir through the process of osmosis. The hydrostatic pressure build-

up from this influx causes a steady release of the drug from an opening in the membrane called the drug portal. The rate of drug release is constant or zero-order until the drug within the reservoir is completely depleted.

1.2.2.4: Matrix based systems.

Matrices formed by direct compression offer advantages such as, controlled release of low molecular weight drugs and application with unstable substances, such as peptides since no heating (Chandy *et al.*, 1992) or contact with organic solvents (Sansdrap *et al.*, 1993) is required. Also, the release rate can be controlled easily by effective selection of polymer type and formulation. The different matrix systems that exist are outlined below.

1.2.2.4.1: Drug diffusion from non-degradable polymeric matrix.

This approach enables release-control by diffusion of an active agent out of a matrix containing it. Such devices have been widely used to achieve sustained-release of drugs administered orally (Sanchez-Lafuente *et al.*, 2001). Thus, giving therapeutically effective concentrations of drug over an extended period of time.

1.2.2.4.2: Drug diffusion from degradable polymeric matrix and erosion of polymeric matrix.

The release of drug in this system is controlled by both diffusion of the drug from the polymer matrix and the degradation of the matrix. The most studied systems from this group are those based on poly(lactic-co-glycolic acid) with commercial products already available for delivery of hormones (Heller, 1993). Zhu *et al.* (2002) recently investigated microparticles made from blends of poly(lactic-co-glycolic acid) and poly(ethylene glycol) as controlled delivery devices. Nuclear factor-kappa B (NF_KB), which regulates cell proliferation in tumour cells was incorporated in to the polymer matrix. The release profiles of NF_KB when dispersed in buffer showed an initial burst for 2 days, a linear release for a further 16, with 85% of the total NF_KB released after 28 days. Similarly, sustained release of fentanyl, an anaesthetic drug, from poly(L-

lactide-co-glycolide) compressed wafers could be precisely controlled by modifying factors in the preparation conditions (Seo *et al.*, 2002).

1.2.2.4.3: Erosion of degradable polymeric matrix.

Biodegradable polymers such as poly(anhydrides) and poly(orthoesters) are highly hydrophobic and therefore, undergo surface erosion when placed in to aqueous solutions. The release of drugs dispersed in matrices of such polymers will therefore be controlled by rate of polymer erosion (Heller, 1996). Gliadel is an example of a commercially available device that consists of poly(anhydride) discs and is used to treat tumours in the brain (Wang *et al.*, 2002).

1.2.2.4.4: Hydrophilic matrices that swell.

Hydrophilic matrices that swell consist of polymers that undergo a glass/rubber transition to form hygrogel like materials on contact with aqueous solutions. Release of drugs that are incorporated in such systems are controlled by the degree of swelling and extent of uncoated areas (Conte *et al.*, 1992). Geomatrix is a commercially available device based on this system and used for oral delivery of drugs (Prisant *et al.*, 2003; Colombo, 1993).

1.2.2.4.5: Hydrophilic matrices that degrade.

Poly(vinyl pyrrolidone-co-hydroxyethyl methacrylate) is a well known biocompatible hydrogel with broad applications in the biomedical area (Akashi *et al.*, 1990; Bell *et al.*, 1995; Laporte *et al.*, 1997). Their biocompatibility and hydrophilic nature has been proposed as an interesting carrier for drug delivery (Blanco *et al.*, 1997). Drugs with poor water solubility can be released in a controlled way by the unfolding and disentanglement of polymer chains of such polymers (Narasimhan *et al.*, 1997).

1.3: PH-responsive disruption of cell membranes in relation to use as endosomolytic agents.

The advent of sophisticated molecular biology techniques has allowed advances in gene identification and cloning, permitting a clearer appreciation of the mechanisms of diseases, such as cancer (Lebediva *et al.*, 2003; Jensen *et al.*, 2003; Murthy *et al.*, 2003). Consequently, several gene therapy approaches have now been realised, including genetic correction therapy, suicide gene therapy, antiangiogenic therapy and gene based immunotherapy. A few have even reached the stage of clinical trials, most of them phase I while some antisense strategies have advanced to phase II and III studies (NIH OBA Gene transfer clinical trials database, 2002). These therapies act at specific sites within cells. Such therapeutics initially are taken up by cells through the process of fluid-phase or receptor mediated endocytosis (Murthy *et al.*, 2003). A large fraction of endocytosed therapeutics are subsequently trafficked to the lysosomal compartment and degraded. Here, the therapeutic must be released from this compartment in to the cytosol before excessive degradation or cell re-expulsion (exocytosis) occurs (Christie *et al.*, 2003). Thus, escape from this biological barrier remains a significant limitation for intracellular delivery of DNA, RNA, and proteins (Berg *et al.*, 1999; Bijsterboch *et al.*, 1997). Endosomes and lysosomes exist at acidic pH values between 5.0 and 6.4, in contrast to the cytoplasm, which is at pH 7.4 (Mellman, 1996).

Organisms such as viruses and pathogenic bacteria have evolved pH-responsive surface proteins that exploit the pH gradient between the endosome and the cytoplasm. These surface proteins change their conformation and physical properties at the low pH of the endosome and become membrane destabilising. This enhances transport of active proteins or DNA from the endosome to the cytoplasm (Subbarao *et al.*, 1987).

Fusogenic peptides structurally derived from viruses have been designed in order to specifically disrupt intracellular endosomal or liposomal compartments (Plank *et al.*, 1998). The most commonly used gene delivery system for both viral (Galanis *et al.*, 2001) and non-viral (Schatzlein, 2001) systems are adenoviruses. However, the

toxicity and immunogenicity of such viral systems limits their effectiveness and presents a major barrier in exploiting the full potential of gene therapy. Consequently, non-viral strategies are becoming an attractive alternative to provide a safe and efficient delivery system for nucleic acids. Although non-viral strategies demonstrate adequate safety profiles, their low transfection efficiency, which can be attributed to specific systemic and subcellular obstacles, remains a major barrier (Merden *et al.*, 2002). Therefore, the aim of a number of researchers is to create systems that act as targeted synthetic viruses displaying high specificity, such as for cancer tissue, while showing high transfection efficiencies and controllable safety risks (Zuber *et al.*, 2001; Kamiya *et al.*, 2001). Since non-viral gene therapy systems are currently not optimal, it is thought by studying their biological and physiochemical properties this will provide valuable knowledge for the future design of more sophisticated systems. To date, alternatives to viral systems that have been investigated for the delivery of nucleic acid include cationic lipids (Pedrosa de Lima, 2001), such as polycation liposomes (Oku *et al.*, 2001), naked DNA (Gharwan *et al.*, 2003; Abo-Auda *et al.*, 2003), particle bombardment and ultrasound transfection (Newman *et al.*, 2001) and synthetic and natural polymers. Presently, cationic polymers and cationic lipids are by far the most widely used non-viral systems (Matsuura *et al.*, 2003). This can largely be explained by the spontaneous formation of complexes that can form between these positively charged agents and the negative charge on biological membranes.

1.3.1: Polymers as endosomolytic agents.

Drug delivery research is currently evaluating the potentials and benefits of synthetic gene carriers. Polymeric drug carriers were originally proposed by Ringsdorf (1975). They exhibit distinct advantages as vectors for delivery of therapeutics. For example, modifications to the polymer, such as molecular weight changes, architectural changes or linking with cell/tissue targeting moieties can confer upon them specific physiological or physiochemical properties. Also important properties such as their relatively easy large-scale production make them of great interest.

Two main classes of polymers have been studied, which include cationic and anionic polymers. Cationic polymers, which are described in more detail below, generally

have a high density of protonable amino nitrogen atoms, which makes them an effective 'proton sponge' causing lysosomal swelling and rupture, and provides subsequent escape for molecules entrapped in lysosomes or endosomes (Boussif *et al.*, 1995; Haensler *et al.*, 1993; Richardson *et al.*, 1999; Pack *et al.*, 2000; Midoux *et al.*, 1999). Cationic polymers, while being able to mask negative charges on DNA have also been able to condense large genes in to smaller structures (Smedt *et al.*, 2000). However, these cationic polymers have also been associated with major cytotoxicity, especially when high intracellular accumulation occurs, which is typical of phagocytic cells (Takagi *et al.*, 2000; Filion *et al.*, 1997; Farhood *et al.*, 1992; Walter *et al.*, 2002; Erni *et al.*, 2002; Steinman *et al.*, 1983; Wetering *et al.*, 1998).

A newer class of polymers that have also been investigated and are also discussed in more detail below are anionic pH-sensitive polymers. These have a potentially lower cytotoxicity and are based on α-alkyl acrylic acids (Kusonwiriya *et al.*, 2003).

1.3.1.1: Cationic polymers.

The main polymers from the cationic group of polymers which have been investigated to date include poly(ethylenimine) (branched and linear), poly(L-lysine), dendrimers, imidazole modified poly(L-lysine) and chitosan, which are outlined below:

1.3.1.1.1: Poly(ethylenimine) (PEI).

PEI with M_w 25-KDa (Aldrich) and 800-KDa (Fluka) is a commonly used polycation and enters the nucleus whether accompanied by DNA or not (Matsuura *et al.*, 2003; Goodbey *et al.*, 1999). Low molecular weight PEI is highly efficient for gene therapy, although less efficient than high M_w PEI (Lee *et al.*, 2003). However, low M_w PEI has a comparably lower cytotoxicity at a broad range of concentrations (Fischer, 1999). Earlier studies have shown PEI polymers effectively complex even large DNA molecules (Marschall *et al.*, 1999; Campeau *et al.*, 1999), producing condensed particles with a size of around 100 nm or less that are capable of transfecting cells efficiently *in vitro* as well as *in vivo*. PEI is a better condensing reagent than polycations such as poly(L-lysine) and consequently protects DNA from shearing and endosomal degradation (Marschall *et al.*, 1999). The high positive charge exhibited by

PEI is also thought to afford protection against nuclease degradation (Merdan *et al.*, 2002). However, it results in a rather high toxicity, which is one of the major limiting factors especially for *in vivo* use. Every third atom on PEI is an amino nitrogen, which can be protonated at weakly acidic pH, therefore, this polymer can act as an efficient 'proton sponge' at endosomal pH, which can explain the high transfection efficiency of PEI (Gosselin *et al.*, 2001; Boussif *et al.*, 1995).

High and low M_w PEI exhibit high transfection efficiency and low cytotoxicity, respectively. Highly improved transfection with only a moderate increase in toxicity has been achieved by attempting to combine these properties using small PEIs crosslinked via biodegradable disulphide bonds (Gosselin *et al.*, 2001).

As well as size, structural properties of the polymer are important when considering their biological properties. For example, ExGen^{TM} 500 (Euromedex, France), a linear PEI derivative is able to transfer reporter genes more efficiently than cationic lipids (Ferrari *et al.*, 1997). However, in a recent study conducted by Smith *et al.* (2003) it was demonstrated ExGen^{TM} showed evidence of mutagenic effects in transfection studies, indicating additional testing is required to assess this risk. Liposomes modified with cetylated PEI have shown promising results since they have been shown to be superior to both PEI and cetyl PEI for gene delivery (Matsuura *et al.*, 2003).

1.3.1.1.2: Poly(L-lysine).

Poly(L-lysine), being one of the first has now been investigated for non-viral gene delivery applications for over a decade (Zauner *et al.*, 1998; Wu *et al.*, 1987). Properties including its peptide structure and biodegradability have made it desirable for *in vivo* use. However, use has been limited since it has modest to high toxicity and is poorly endosomolytic, such that it requires another endosomolytic agent or needs to be chemically modified to increase its lytic activity (Zenke *et al.*, 1990; Erbacher *et al.*, 1996). It has also been the most widely investigated for attaching ligands (Smedt *et al.*, 2000). For example, the efficiency of lactosylated poly(L-lysine) as an agent for gene transfer in to epithelial cells was shown to be equivalent to that of viral vectors

when agents that enhance endosomal escape such as chloroquine were present (Kollen, 1999; Klink, 2003).

1.3.1.1.3: Dendrimers.

Dendrimers are synthetic macromolecules with a well-defined globular structure (Esfand *et al.*, 2001). They can be synthesised using either a divergent approach developed by Tomalia *et al.* (1985) and Newkome *et al.* (1985) or a convergent approach developed by Hawker *et al.* (1990). The level of branching is expressed as the generation number of the dendrimer such that, with increasing generation number the size and molecular weight increase (Kolhe *et al.*, 2003). Dendrimers are able to encapsulate molecules within their core and have the ability to form complexes or conjugates with molecules due to free functional groups on their surface. Such properties make them an attractive approach for delivery of molecules such as drugs and nucleic acids. Sixth generation Starburst™ polyamidoamine (PAMAM) dendrimers have attracted most interest (Merdan *et al.*, 2002) due to their stability, controllable methods of synthesis and wide availability (Kolhe *et al.*, 2003). There are doubts for the clinical application of amine terminated PAMAMs (Malik *et al.*, 2000), since at physiological pH the terminal amines are protonated. Consequently, they attract and bind negatively charged cells resulting in their haemolysis. However, Kojima *et al.* (2000) found that it is possible to synthesise PAMAM dendrimers with a hollow core for encapsulation of drugs with a biocompatible surface comprised of poly(ethylene) grafts. Also, more recently, Beezer *et al.* (2003) describe the synthesis of neutral water-soluble PAMAM derivatives for drug delivery and demonstrated the dendrimers were able to release hydrophobic molecules when in contact with a biological cell.

1.3.1.1.4: Imidazole containing polymers.

Polymers have been developed that incorporate basic imidazole groups in to polymer vehicles, which act as a 'proton sponge' to mediate endosomal escape without the cytotoxic effects of poly(L-lysine) and PEI (Pichon *et al.*, 2001). Polymers with an imidazole content of 86.5% allowed for gene expression comparable to PEI but with little cytotoxicity (Putnam *et al.*, 2001). Similarly, a number of studies have shown

modification of ε-amino groups of poly(L-lysine) using histidine or other imidazole containing structures significantly enhance reporter gene expression compared to poly(L-lysine) (Fajac *et al.*, 2000; Benns, 2000; Midoux, 1999). Therefore, imidazole-containing polymers provide a potential option to improve gene delivery without high cytotoxicity.

1.3.1.1.5: Chitosans.

Chitosan is the deacetylated form of chitin, a natural polymer purified from the exoskeletons of crustaceans (Chew *et al.*, 2003). It has shown promising results as a gene carrier (Borchard, 2001; Singla *et al.*, 2001; van der Lubben, 2001). It has advantageous properties including being non-toxic, non-immunogenic, biodegradable, and the ability to form complexes with DNA easily (Illum, 1998; Singla *et al.*, 2001; Borchard *et al.*, 2001). Studies have shown that small (1.2 kDa) and very large chitosan polymers lead to low levels of reporter gene expression (Sato *et al.*, 2001), whereas, chitosan polymers with a M_w range between 30 and 170 kDa provide levels of gene expression comparable to PEI (Koping-Hoggard *et al.*, 2001). It has also been demonstrated incorporation of a pH-sensitive endosomolytic peptide into chitosan/plasmid complexes, significantly increases reporter gene expression (MacLaughlin *et al.*, 1998), suggesting endolysosomal escape is a limiting factor in gene delivery with chitosans. Chitosan has been assessed in recent studies for novel applications such as oral delivery of genes and peptides to overcome the need for invasive strategies such as intramuscular injections (Chew *et al.*, 2003; Secil *et al.*, 2002) and evade obstacles such as the acidic pH of the stomach.

1.3.1.2: Anionic polymers.

The second category of synthetic polymers are anionic polymers, which are based on α-alkyl acrylic acids. These polymers bear pendent carboxylic acid groups and destabilise membrane bilayers by pH triggered conformational change. They collapse from an expanded hydrophilic coil at physiological pH to a hydrophobic globule in an acidic environment (Seki *et al.*, 1984; Borden *et al.*, 1987; De Oliveira *et al.*, 1996; Olea *et al.*, 1999). Such membrane lytic properties have made them of interest for the

liposome delivery applications (Christie *et al.*, 2003; Thomas *et al.*, 2000; Chen *et al.*, 1999; Mills *et al.*, 1999), which are discussed in detail later. Also, due to the difference in pH between the endosome and cytosol, pH-responsive anionic polymers have been designed and evaluated as endosomolytic agents to enhance non-viral transfection and cytoplasmic delivery of proteins (Thomas *et al.*, 1992; Murthy *et al.*, 1999; Mourad *et al.*, 2001).

Anionic polymers, poly(ethyl acrylic acid) and poly(propyl acrylic acid) have been demonstrated to display pH-responsive membrane disruptive properties in liposomes and red blood cells dependent upon their molecular weight (Murthy *et al.*, 1999; Lackey *et al.*, 2001; Thomas *et al.*, 1994). Also, a copolymer composed of ethyl acrylate and acrylic acid has been suggested to display a significant pH-responsive membrane disruptive effect on red blood cells (Murthy *et al.*, 1999; Lackey *et al.*, 2001; Thomas *et al.*, 1994). Recently, Kusonwiriya *et al.* (2003) have investigated a large variety of anionic copolymers and analogues of poly(acrylic acid) and compared them to a cationic poly(acrylic acid) copolymer. The pH-responsive membrane disruptive properties were characterised by utilising three different *in vitro* models. These included measuring liposome leakage, lysis of red blood cells and also the pH-dependent shift of pyrene fluorescence, a commonly used fluorescent probe for measuring environmental polarity, which is used because its emission intensity is dependent upon solvation. The pH-dependent increase in polarity and membrane disruption in the different models was in good agreement for all tested poly(acrylic acid) polymers. Among the various anionic poly(acrylic acid) polymers, only medium and low molecular weight poly(ethyl acrylic acid) and poly(propyl acrylic acid) were identified to display significant pH-responsive disruptive activity, which were placed in relation to the cationic poly(butyl methacrylate-co-[2-dimethyl aminoethyl]methacrylate-co-methyl methacrylate) in the order of poly(ethyl acrylic acid) < poly(propyl acrylic acid) < poly(butyl methacrylate-co-[2-dimethyl aminoethyl]methacrylate-co-methyl methacrylate). The efficacy of polymer induced membrane disruption was also shown to be concentration dependent and significantly affected by the composition of the membrane.

As already mentioned poly(ethyl acrylic acid) (PEAAc) has membrane destabilising properties. This was first demonstrated by Tirrell and coworkers (1985) in elegant

studies that detailed the pH-responsive mechanism, of lipid vesicle disruption (Thomas *et al.*, 1992; Ferrito *et al.*, 1992; Chung *et al.*, 1996). PEAAc was subsequently investigated as a component to enhance transfection efficiencies. Proteins and peptides that disrupt endosomal membranes have shown moderate success (Brown *et al.*, 2001). Work carried out by Plank *et al.* (1994) showed a correlation between the haemolytic activity of endosomal disruptive peptides and transfection efficiency *in vitro*.

Since the composition of these proteins and peptides share similarities with PEAAc pendant carboxylate and ethyl groups, Tirrell and co-workers began to explore whether polymers containing other related hydrophobic moieties inspired by the amino acid side-chains such as leucine, isoleucine could provide advantageous properties.

In a RBC haemolysis assay PEAAc was compared to mellitin a membrane-disruptive peptide that has been shown to be extremely efficient at haemolysis. Approximately $5x10^6$ molecules of PEAAc vs. $11x10^6$ molecules of mellitin were required to disrupt a single red blood cell (Tosteson *et al.*, 1985). It was also shown poly(propyl acrylic acid) PPAAc, displays a remarkable increase in activity with the addition of a single methylene unit and disrupts red blood cells 15 times more efficiently than PEAAc. PPAAc and PEAAc are not haemolytic at pH 7.4 and the two polymers display distinct pH dependencies (Stayton *et al.*, 2000). That is, PEAAc reaches maximum haemolysis at approximately pH 6 and below, whereas the maximum for PEAAc is reached at pH 5.0 and below. This group has therefore suggested that these properties can be used to enhance membrane transport at different endosome development points since the endosomal pH drops gradually over time as the early endosome develops in to the late endosome. In addition, gene transfection studies have been performed by this group to investigate whether the addition of PPAAc enhances the delivery of genes in non-viral delivery systems. The initial *in vitro* studies tested whether the pinocytosis of poly(lysine)/PPAAc nanoparticles could increase the transfection efficiency of poly(lysine)/plasmid nanoparticles when both are concurrently taken up in to NIH3T3 fibroblast cells. The transfection efficiencies of this non-targeted system are extremely low as a consequence of inefficient pinocytic particle uptake, which provides a good test for whether PPAAc can enhance transfection. There was no

observable toxicity associated with control exposures of PPAAc to the cells, although some toxicity was observed with the control poly(lysine)/plasmid particles alone. Low numbers of cells were transfected by the pinocytosis mechanism controls containing no PPAAc or with controls where PPAAc was added without prior particle formation with poly(lysine). When particles of PPAAc and poly(lysine) were added concurrently with poly(lysine):plasmid particles, a 20-fold increase in transfected cells was observed at the optimal 1:1 poly(lysine):PPAAc particles. The free PPAAc did not enhance transfection efficiency, while PPAAc in particle form did.

Work performed with PPAAc has also led to the development of a class of versatile pH-responsive, endosomal disruptive polymeric carriers for biomolecules called 'encrypted polymers' (Boletta *et al.*, 1997; Fischer *et al.*, 1999). These polymeric drug carriers combine a cell targeting, pH-responsive membrane-disruptive component, and serum-stabilising poly(ethylene glycol) (PEG) grafts. They have been shown to direct the uptake and endosomal release of oligonucleotides in a primary hepatocyte cell line. The ability of the encrypted polymers to deliver rhodamine-labelled oligonucletides or PEG-FITC (a model macromolecular drug) in to the cytoplasm of hepatocytes was investigated by fluorescence microscopy. Kunath *et al.* (2003) have also recently synthesised two new encrypted polymer derivatives that contain lactose to target hepatocytes. The results of the fluorescence microscopy experiments show that these encrypted polymers direct vesicular escape and efficiently deliver oligonucleotides and macromolecules in to the cytoplasm of hepatocytes.

1.4: Disruption of liposomes to cause liposomal content release.

A system that triggers the release of liposomal contents has enormous potential in food, pharmaceutical and cleaning product industries. For example, in the food industry, routes to achieve triggered release of agents such as flavours and vitamins are sought (Annesini *et al.*, 2000). The natural pH variation along the digestive system could be exploited to trigger release of liposomal contents that are ingested with foods.

Similarly, pH-responsive delivery of tumour chemotherapy might be accomplished by exploiting pH differences in the microenvironment of tumour and normal cells. That is, the microenvironment of tumour cells is comparatively more acidic (ca.6.5) due to larger amounts of neuramic acid on the cell surface and also their high metabolic rate vigorously produces acidic compounds (Kitano *et al.*, 1991). Alternatively, endosomes characteristically have a pH value one to two units lower than physiological pH (Murthy *et al.*, 1999). Therefore, pH-sensitive liposomes internalised by cells via the endocytic pathway, offer an attractive approach to facilitate delivery of membrane impermeable drugs in to the cytoplasm before enzymatic degradation in lysosomes (Meyer *et al.*, 1998) (Figure 1.2).

Finally, the fact that washing powders take on an alkaline pH when dissolved could be utilised to control the release of conditioning agents and active ingredients that have been encapsulated within liposomes and are required at particular stages of the cleaning process.

Figure 1.2: Diagrammatic representation of the endocytic pathway, showing the main stages involved in achieving digestion of liposomes within the cell (Adapted from Meyer *et al.*, 1998).

1.4.1: PH-responsive disruption of liposomes.

Various external stimuli have been investigated for their ability to trigger release of liposome contents, including temperature (Hayashi *et al.*, 1996; Chanderoy *et al.*, 2001; Gaber *et al.*, 1996), light (Thompson *et al.*, 1996) and pH (Kitano *et al.*, 1991; Kono *et al.*, 1997; Osanai *et al.*, 2000). To date, four main approaches have been utilised to give pH-triggered release from liposomes including polymorphic lipids, polymorphic lipid derivatives, pH-sensitive peptides or proteins and pH-titratable polymers.

1.4.1.1: Polymorphic lipids.

The first approach involves liposomes that are composed of polymorphic lipids such as phosphatidylcholine (PC) with an acid sensitive constituent such as *N*-palmitoyl-L-homocysteine (PHC). PHC is in a charged form at neutral pH and mixes with other bilayer components. Whereas, at an acidic pH, PHC has a neutral thiolactone structure causing phase separation and destabilisation of the bilayer structure (Crommelin *et al.*, 1994; Yatvin *et al.*, 1980) (Figure 1.3). Such liposomes have been successfully used for *in vitro* cytoplasmic delivery of protein toxins (Collins *et al.*, 1987; Chu *et al.*, 1990), antitumour drugs (Connor *et al.*, 1986[1]), antigens (Reddy *et al.* 1991; Lee *et al.*, 1996), anti-sense oligonucleotides (Couvreur *et al.*, 1997) and plasmid DNA (Wang *et al.*, 1989; Budker *et al.*, 1996). A small amount of success has also been achieved *in vivo* with pH-sensitive immunoliposomes (antibody conjugated liposomes) administered to mice via the intra peritoneal route (Wang *et al.*, 1987). That is, through the thin membrane that lines the walls of the abdominal cavity and encloses the abdominal organs. The low plasma stability of immunoliposomes, especially large unilamellar vesicles (LUVs) (Liu *et al.*, 1989; Connor *et al.*, 1986[2]), has hindered their clinical use. Although progress has been made using small unilamellar vesicles (SUVs), which are more stable in plasma (Liu *et al.*, 1989[2]: Liu *et al.*, 1990), the extraction of a single-chain acidic amphiphile by plasma albumin results in an abrupt loss of pH-sensitivity. The rate pH-sensitive moieties are lost can be reduced by using double chain amphiphiles such as 1-2-di-palmitoyl-sn-3-succinylglycerol (DPSG) (Leventis *et al.*, 1987; Collins *et al.*, 1990). However, it has

been demonstrated that although small liposomes formulated from dioleoylphosphatidylethanolamine (DOPE) and containing DPSG maintain their pH-sensitivity following incubation in plasma, the pH at which destabilisation occurs shifts from 5.3 to 4.2 (Collins *et al.*, 1990).

Figure 1.3: Equilibrium between the uncharged thiolactone and charged open form of *N*-palmitoyl-L-homocysteine (Adapted from Yatvin *et al.*, 1980).

1.4.1.2: Polymorphic lipid derivatives.

A second approach involves incorporation of polymorphic lipid derivatives in to the lipid bilayer that undergo pH-responsive conformational changes, again causing destabilisation of the bilayer (Drummond *et al.*, 2000; Drummond *et al.*, 1995; Gerasimov *et al.*, 1997). For example, Kirpotin *et al.*, (1996) synthesised liposomes with DOPE and a disulphide linked PEG phospholipid conjugate. Cleavage of the grafted PEG chains facilitated complete release of liposomal contents in response to low pH, while at neutral pH liposomes remained stable.

Although pH-responsive release of liposomal contents can be achieved using the latter approaches, it may prove technically difficult to synthesise liposomes that are disrupted within narrow pH ranges. For example, those exhibited between the extracellular fluid of tumour and normal cells. The factors that cause this variation are not fully explained as yet, but some possible reasons are illustrated in figure 1.4. Also, from a delivery viewpoint, a drawback of certain liposome formulations such as those formulated from mildly acidic amphiphiles, such as oleic acid and unsaturated phosphatidylethanolamines, such as dioleoylphosphatidylethanolamine, is that they are destabilised in plasma or serum (Allen *et al.*, 1980: Connor *et al.*, 1986[2]; Liu *et al.*, 1989[2]; Zignani *et al.*, 2000). Therefore, small molecules such as fluorophores and antisense oligonucleotides encapsulated within liposomes are effectively delivered to

the cytosol of cells in culture, but when used *in vivo*, the presence of plasma or serum, causes liposomes to become leaky and release their contents, therefore, severely hampering their use.

1.4.1.3: pH-sensitive peptides or proteins.

Liposome destabilisation can be performed via pH-sensitive peptides or modified proteins (Turk *et al.*, 2002; Gautman *et al.*, 1996; Alverez *et al.*, 2001). These proteins and peptides are designed to mimic pH-sensitive viral fusion peptides (Jiricek *et al.*, 1997). The route a virus takes to gain entry in to their hosts cell is virus-specific. However, one of the main routes, and one that will be discussed, is via pH-dependent endocytosis. That is, the virus first binds to cell surface receptors, then is endocytosed through clathrin-coated pits and vesicles. The virus is then delivered to endosomes and fuses with the endosomal membrane. This fusion is induced by the low pH of the endosome compartment and is mediated by pH-sensitive viral spike or fusion proteins (Hernandez *et al.*, 1996; Kielian *et al.*, 1990; Martin *et al.*, 1999; Plank *et al.*, 1994). Such a pH-sensitive trigger for intracellular delivery of viral components can be applied in designing liposomal or lipid-based delivery systems using either full proteins or peptides to induce membrane destabilisation. In addition, certain toxin components are also very efficient at pH-dependent endosomal escape and are presently being used in conjunction with lipid-based delivery systems (Lee *et al.*, 1996). Examples of pH-dependent viral fusion peptides and toxins are displayed in Table 1.1.

Figure 1.4: Factors affecting intracellular (pH_i) and extracellular (pH_e) (Taken from Stubbs *et al.*, (2000)).

H^+ produced by metabolism is pumped from the intracellular compartment and subsequently flows into the blood. The concentration of H^+ in the interstitial compartment is relatively high in tumours (low pH_e) compared with normal tissue. At steady state, the flow (f) of H^+ between the compartments is equal ($f_1=f_2$). The increased interstitial acidity of tumour cells could be caused by (a) an increase in H^+ pumping by, for example, expression in the plasma membrane of vacuolar-type H^+ pumps or (b) an increase in resistance induced either by altered gene expression, or by action of cytokines on cells at the interstitial/vascular interface.

Table 1.1: pH-dependent viral fusion peptides and toxins.

Virus or toxin	**Family**	**Fusion peptide**
Influenza virus	Orthomyxovirus	HA2
Semliki forest virus	Togavirus	E1
Uukuniemi virus	Bunyavirus	G1/G2
Lymphocytic chorio-meningitis virus	Arenavirus	G1/G2
Vesicular stomatitis virus	Rhabdovirus	G
Tick-borne encephalitis virus	Flavivirus	E
Diptheria toxin B fragment	*Corynebactium diptheriae*	α helices TH8 and TH9
Listeriolysin O toxin	*Listeria monocytogenes*	Not applicable
Tetanus toxin	*Clostridium tetanii*	Not applicable
Colicin A toxin	*Escherichia coli*	Not applicable
Exotoxin A	*Pseudomonas aeruginosa*	Not applicable

1.4.1.4: pH-titratable polymers.

The fourth method currently receiving considerable interest uses pH-titratable polymers that undergo conformational changes in response to pH, causing liposome membranes to destabilise. In the past, polymers have been used to improve particular properties of conventional liposomes, which make them unsuitable for *in vivo* use, such as poor *in vivo* stability, due to destabilisation in the presence of serum.

Synthetic polymers offer a number of favourable characteristics, which include simple large-scale preparation, structure versatility, low toxicity, easy association with the liposome surface and also utilisation with various liposome formulations.

Similar to polymer based endosomolytic agents, there are two main categories of polymer that have been investigated for their ability to give pH-responsive membrane destabilisation. These include cationic and anionic polymers (Drummond *et al.*, 2000).

1.4.1.4.1: Cationic polymers.

There is a large overlap of work with the cationic polymers used as endosomolytic agents and cationic polymers used to disrupt liposome membranes, since in effect they both are used to disrupt negatively charged lipid membranes. That is, the cationic polymers used to disrupt endosomal membranes have also been investigated for disruption of liposome membranes. In addition, a large amount of work has been performed for cationic polymers that are used in conjunction with liposomes for gene delivery applications. These cationic polymers exhibit neutral charges at high pH but acquire a positive charge as the pH decreases. In solution they are ionised, which allows them to interact with negatively charged membranes and cause a disturbance in the lipid packing, promoting aggregation and fusion of liposomes.

Synthetic polypeptides such as poly(L-lysine) and poly(L-histidine) were the first polymers identified to exhibit such pH-responsive destabilisation properties (Hong *et al.*, 1983; Walter *et al.*, 1986; Gad *et al.*, 1982). It has been demonstrated polymers with a higher pK_a destabilise liposomes at higher pH values. For example, a pH value

of 5.8 was required to destabilise liposomal suspensions with poly(L-histidine) added (Uster *et al.*, 1985). Whereas, poly(L-lysine), which is a stronger base, resulted in destabilisation at a neutral pH (Hong *et al.*, 1983; Walter *et al.*, 1986; Gad *et al.*, 1982). It was explained earlier that along with cationic polymers, cationic liposomes are the most widely used non-viral gene delivery systems. These cationic liposomal gene delivery systems have previously shown low transfection efficiency. Whereas, when used in conjunction with polycationic polymers such as poly(L-lysine) their transfection efficiency can be increased by 2–28 fold in a number of cell lines both *in vitro* and *in vivo* (Lee *et al.*, 2003). Recently, Koslova *et al.* (2001) examined the interaction between cationic polymer, poly(*N*-ethyl-4-vinylpyridinium bromide) (PEVP) and proteoliposomes, prepared from neutral egg yolk lecithin and negatively charged chymotrypsin. This interaction was compared with egg lecithin vesicles containing an anionic phospholipid, cardiolipin. Binding of PEVP to both types of vesicles was electrostatic in nature with the polymer manifesting a higher affinity to the cardiolipin relative to the enzyme. PEVP had no effect on the permeability of the bilayer membranes to sodium chloride. On the other hand, PEVP increased the transmembrane permeability of the non-ionic anti-tumour drug, doxorubicin. The more negatively charged component in the membrane the greater the PEVP effect. Binding of cationic polymer to the vesicles was accompanied by clustering of the chymotrypsin molecules within the membrane. It was hypothesised that the protein clustering was responsible for the increase in the doxorubicin permeation. Enzymatic activity of the membrane-associated chymotrypsin remained unchanged upon PEVP binding. The findings for chymotrypsin showed similar effects as what were exhibited for cellular membranes in the presence of cationic polymers. In addition, cationic polymers, such as synthetic amphiphilic derivatives of poly(vinyl pyrrolidone) have been shown to give good steric protection to liposomes *in vivo* (Torchilin *et al.*, 2001).

Gene delivery, using both liposomal and cationic polymer based systems has been shown to be effective *in vitro* and *in vivo* (Kabanov, 1999). Again the success of using cationic polymer based systems is a result of the charge interactions between negatively charged cellular membranes and positively charged cationic polymers (Drummond *et al.*, 2000). However, the use of cationic polymers to produce pH-sensitive liposomes is in its early stages. It has recently been demonstrated poly(L-

lysine) groups linked by copolymerisation with hydrophobic dicarboxylic moieties have pH-responsive cell lysis properties (Eccleston *et al.*, 2000). Such polymers have a potential use as systems that trigger pH-responsive liposome lysis.

1.4.1.4.2: Anionic polymers.

Like cationic polymers, there is a large overlap in the anionic polymers studied for the application as endosomolytic agents and for disruption of liposomal membranes. Anionic polymers are weakly acidic polyelectrolytes, and unlike cationic polymers can cause destabilisation of both neutral and charged liposomes. In addition, during the destabilisation process fusion is not always involved. The mechanism of destabilisation is largely dependent upon polymer structure. A common characteristic of the pH-responsive anionic polymers, which have been investigated so far, is that they convey carboxylic acid groups. The polymers ability to destabilise or interact with lipid bilayers is dependent upon the charge on these carboxylic acid groups. To date, three main anionic polymer systems have been investigated, which include poly(acrylic) derivatives, succinylated poly(glycidol)s of and copolymers of *N*-isopropylacrylamide (NIPAM) (Figure 1.5), each of which is described in turn.

Poly(acrylic acid) derivatives.

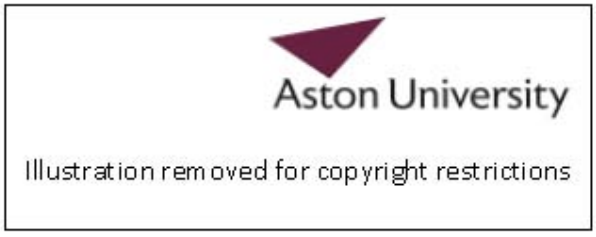
Derivatives of poly(acrylic acid) undergo pH-responsive conformational changes. For example, PEAAc in aqueous solutions exhibits an expanded hydrophilic coil structure at high pH but on acidification exhibits a compact globular structure (Maeda *et al.*, 1988). Seki *et al.* (1984), investigated carboxylic acids, poly(acrylic acid), poly(methacrylic acid) and poly(α-ethylacrylic acid) for their ability to modify phospholipid vesicles. They showed structural modification by complexation with these polymers is dependent on the pH of the surrounding medium and can be controlled by variation of polymer structure and tacticity. At high pH, complexation with MLVs (multilamellar vesicles) was absent. However, decreasing pH in the presence of PEAAc resulted in the disappearance of vesicles. Later studies showed that PEAAc at high concentrations (0.1% w/v) resulted in micellisation of liposomes above their phase transition temperature (Tirrell *et al.*, 1985), whereas PEAAc at low

concentrations, caused pore formation in the lipid bilayer (Chung *et al.*, 1996; Thomas *et al.*, 2000). The pH at which disruption of lipid bilayers occurs can be altered by using polymers of different molecular weights, such that, those having a higher molecular weight cause disruption at higher pH values (Schroeder *et al.*, 1989).

Alternatively, the pH at which disruption of liposomes occurs, can be altered by substituting PEAAc moieties with more hydrophilic or hydrophobic acrylic acid derivatives (Needham *et al.*, 1998; Thomas *et al.*, 1994). For example, substituting hydrophilic methacrylic acid in to the polymer structure reduces the pH at which destabilisation occurs. As already mentioned, with reducing pH, PEAAc undergoes a coil to globule transition. When PEAAc is in aqueous solutions this transition occurs at pH 6.2 (Borden *et al.*, 1987). However, this transition occurs at pH 6.5 when PEAAc is in the presence of 1,2-palmitoyl-sn-3-phosphatidylcholine (DPPC) vesicles (Borden *et al.*, 1987; Thomas *et al.*, 1992). The difference in pH value at which PEAAc undergoes a transitional change is explained by the presence of hydrogen bonding between the polymer and liposome surface (Tirrell *et al.*, 1985).

Figure 1.5: Chemical structures of (A) poly(2-ethylacrylic acid), (B) hydrophobically modified succinylated poly(glycidol), and (C) poly(NIPAM-co-methacrylic acid-co-octadecyl acrylate) (Adapted from Drummond *et al.*, 2000).

A.



B.

C.

It has also been shown that disruption of liposome membranes occurs at the same pH at which PEAAc undergoes a transitional change (Tirrell *et al.*, 1985). Therefore, it would be thought that the phase transition is the causative event leading to membrane disruption since the globular polymer provides a hydrophobic site for solubilisation. However, an alternative hypothesis was suggested by Thomas *et al.* (1992), when they showed destabilisation at higher pH values can be achieved by simply increasing the polymer concentration and need not be achieved by adjusting pH. Thus, polymer becomes increasingly hydrophobic as the carboxylic acid groups are protonated, such that adsorption of polymer to liposome surface increases. Therefore, possibly leading to an increased lateral compression of the lipid bilayer, resulting in its disruption.

Later studies have indicated the liposome micellisation rate may be related to the polymer adsorption rate, which is maximal near bilayer phase transition temperatures (Thomas *et al.*, 1996). However, when polymer is covalently attached to the vesicle surface or is at low concentrations, the latter theory may not hold-true. For example, under the latter conditions permeabilisation occurs without micelle formation (Maeda *et al.*, 1988; Chung *et al.*, 1996; Kitano *et al.*, 1994). It has been shown that PEAAc coupled to egg PC vesicles via thiol functions exhibit rapid calcein release at pH 6.5 (Maeda *et al.*, 1988). Further studies have shown a pH of 5 destabilises and promotes micellisation of surface linked PEAAc egg PC-cholesterol vesicles (Chen *et al.*, 1999). This effect was explained by the insertion of hydrophobic segments of PEAAc in to adjacent vesicles leading to close vesicle-vesicle contact, which facilitates local dehydration at contact site causing disturbance of the lipid membrane and eventually fusion. The release of liposomal contents utilising PEAAc is largely dependent upon the liposome composition. For example, the addition of cholesterol increases vesicle stability and tends to decrease the pH at which vesicle contents are released (Mills *et al.*, 1999).

Although studies involving poly(acrylic acid) derivatives have largely been to demonstrate their interaction with lipid bilayers their use to aid cellular uptake is of interest. For example, Fujiwara *et al.* (1996) showed conjugating poly(acrylic acid) to egg PC liposomes, increased their uptake by macrophages 5-fold. Also recently, the inclusion of poly(acrylic acid) with DNA/polycation and DNA/cationic lipid complexes prevented the serum inhibition of the transfection complexes in cultured cells, which was observed with DNA/polycation and DNA/cationic lipid complexes (Trubetskoy *et al.* 2003).

Succinylated poly(glycidol)s.

Succinylated poly(glycidol)s are derivatives of poly(glycidol), prepared by reaction of poly(glycidol) with succinic anhydride to give a structure bearing hydroxyl and carboxylic acid groups (succinic acid) on the side chain. The fusion ability of egg PC liposomes bearing succinylated poly(glycidol)s increases under weakly acidic and acidic conditions, since ionised carboxylate groups on the polymer strongly interact with the liposome membrane (Kono *et al.*, 1994). Later studies have shown that

succinylated poly(glycidol) modified liposomes enhance cytoplasmic delivery of their contents following internalisation by African green monkey cells, compared to non-modified liposomes (Kono *et al.*, 1997). Subsequently, tumour specific immunoliposomes have been prepared by coupling anti-BCG monoclonal antibodies to liposomes modified with succinylated poly(glycidol). These liposomes show efficient binding and endocytotic internalisation in to tumour cells and also exhibit fusion abilities under acidic conditions (Mizoue *et al.*, 2002).

Copolymers of N-isopropylacrylamide (NIPAM).

Liposomes incorporating NIPAM have been shown to have pH-sensitivity. NIPAM is physically characterised by its Lower Critical Solution Temperature (LCST), which is approximately 32° C in aqueous solutions (Heskins *et al.*, 1968). Thus, the polymer is soluble below its LCST and undergoes phase transition when heated above it. When liposomes are coated with co-polymers of NIPAM bearing long alkyl chains they can exhibit temperature sensitive properties (Kono *et al.*, 1994; Kim *et al.*, 1997; Hayashi *et al.*, 1996). The alkyl chain acts as an anchor to allow the polymer to interact with the liposome membrane (Ringsdorf *et al.*, 1993). It has been suggested that temperatures above the LCST cause poly(NIPAM) bearing C18 alkyl chains to become increasingly hydrophobic and therefore, contract. Consequently, spaces are produced in the exoliposomal leaflet, which stimulates lipid flip-flop (Bhattacharya *et al.*, 1993) or transverse diffusion, which describes the transfer of a lipid molecule across a bilayer. Nevertheless, during collapse some polymer remains anchored to the liposomes, therefore, they are not destroyed (Ringsdorf *et al.*, 1993; Franzin *et al.*, 1998; Winnik *et al.*, 1995). It has been demonstrated that fusion of DPPC SUVs (Hayashi *et al.*, 1998) and budding of giant unilamellar 1,2-myristoyl-sn-3-phosphatidylcholine (DMPC) liposomes occurs when polymer changes from a coil to globular structure at the LSCT (Simon *et al.*, 1995). Such temperature sensitivity has been utilised for a number of studies, which destabilise liposomes and consequently cause them to release their contents (Kono *et al.*, 1994; Kim *et al.*, 1997; Hayashi *et al.*, 1996). It is possible to increase the LCST of poly(NIPAM) and also render them pH-sensitive by introducing small amounts of a titratable comonomer in to their structure (Chen *et al.*, 1995; Hirotsu *et al.*, 1987). For example, copolymers of

NIPAM containing the pH-sensitive moiety methacrylic acid and a hydrophobic anchor octadecylacrylate, trigger content release from egg PC and egg PC/PEG-distearoylphosphatidylethanolamine (DSPPE) LUVs in response to acidic conditions (ca. 4.9-5.5) (Meyer *et al.*, 1998). This was achieved by primarily incubating the polymer (molecular weight ≈ 9000) with liposomes overnight to allow the polymer to be incorporated in to the liposomal bilayer. It has also been shown that even sterically stabilised liposomes, that is liposomes with 6 mol% PEG-DSPE, are destabilised when acidified, so long as the molecular weight of polymer does not exceed that which allows diffusion through the steric barrier (Zignani *et al.*, 2000). It has been hypothesised that the pH at which polymer collapses is related to that which causes release of liposome contents (Figure 1.6). In this study the copolymer phase transition was measured as an increase in light scattering at 480 nm. HPTS release was measured as an increase in HPTS fluorescence following release from liposomes (λex = 413 nm, λem = 512 nm) and expressed as a fraction of the fluorescence upon liposome solubilisation with Triton X100.

Recently, it was demonstrated that liposomes having a low copolymer-lipid ratio efficiently released the cytotoxic anticancer drug doxorubicin under acidic conditions (Leroux *et al.*, 2001). NIPA copolymer bearing glycine as a pH-sensitive moiety have also been shown to interact strongly with both positive and negatively charged non-phospholipid n-octadecyldiethylene oxide vesicles principally via hydrogen bonding (Polozova *et al.*, 1999). Further studies have shown the interaction of positively charged POPC LUVs was not as strong and predominantly an electrostatic interaction. In addition, as the polymer undergoes phase transition the interaction with POPC is weaker than that with non-phospholipid vesicles. The use of NIPAM bearing copolymers for intracellular delivery is at an early stage (Drummond *et al.*, 2000). However, they seem to be well tolerated both *in vitro* (Taillefer *et al.*, 2000) and *in vivo* (Molinaro *et al.*, 1999).

Figure 1.6: Comparison of the rate of HPTS release from liposomes (egg PC) modified with NIPAM-containing copolymers and the phase transition of the copolymer (NIPAM/MAA/ODA 93:5:2 mol%). (Adapted from Zignani *et al.*, 2000).

1.5: pH-responsive release of solids from polymer coatings.

Film coating to the surface of pharmaceutical solid-dosage forms, particularly tablets, has been practiced for more than 100 years (Guo *et al.*, 1998). Film coating has been used for a variety of reasons including use to mask unpleasant taste and odours, improve product stability and to facilitate handling and modify drug release characteristics (Gennaro, 1995). A number of factors influence the release of a film coated active agent, including, transport of solvent from the bulk vessel to the active agent core, dissolution of the active agent, mass transfer of the dissolved active agent in the core, swelling of polymer film and thickness of polymer film. Numerical models have been designed to determine release profiles using experimental data acquired for some, but not all of these parameters. For example, Borgquist *et al.*, (2002) recently designed a model to fit the film thickness and the drug loading to the experimental release data. Such models can be used to optimise conditions for controlled release of active agents from polymer film coatings (Narasimhan *et al.*, 2001).

Polymers that form solid coatings and show pH-dependent dissolution have successfully been used to provide protection required to achieve delivery of orally

administered therapeutics. However, it is important that the search and investigation of new polymers that exhibit such properties continues to improve site-specific delivery of therapeutics. Most research to date has been performed with enteric coatings. Enteric coatings are polymer coatings that can be used to protect solid oral dosage forms for specific drug delivery to the colon. Polymer coated drugs delivered to the colon have been of value for the topical treatment of diseases of the colon such as Crohn's disease, ulcerative colitis and colorectal cancer (Rodriguez *et al.*, 2001). These polymer coatings have pH-dependent solubility. Therefore, they are used to protect the drug in the acidic environment of the stomach. They then rely on the difference in pH of the proximal and distal colon to cause pH-dependent dissolution and drug release. That is, they exploit the difference in pH of the proximal colon, which is slightly acidic (pH 6.0 to 6.4) and the distal colon, which is slightly alkaline (pH 7.0 to 7.4). Drug release from many of these enteric coatings will also depend on the transit time through the small intestine, such that time controlled release can be achieved by selecting an appropriate coating level (Habib *et al.*, 1998). It has been suggested sustained colonic release of drugs may also be useful in the treatment of other conditions such as diabetes, nocturnal asthma, angina and arthritis (Reddy *et al.*, 1999). That is, orally administered peptides, proteins, oligonucleotides and vaccines used to treat these conditions are potential candidates of interest for colon specific drug delivery if they could be provided with protection from gastric acid and digestive enzymes in the upper parts of the alimentary tract (Wilding *et al.*, 1994; Rubenstein, 1995). For example, early studies to by Bronsted *et al.* (1991) to achieve this resulted in the development of hydrogel capsules based on acrylic acid. That is, *N*,*N*-dimethylacrylamide and *N*-tert-butylacrylamide were crosslinked with 4,4'-di(methacryloylamino)azobenzene to produce hydrogels that do not swell in the stomach. However, with increasing pH through the small intestine the polymer swells. In the colon the degree of swelling exposes cross-links to bacterial azoreductases resulting in breakdown of the hydrogel and release of the drug. Work following on from this work has been in developing genetically engineered protein polymers. For example Capello *et al.* (1990) designed a silk-elastinlike class of genetically engineered protein polymers (Cappello *et al.*, 1990; Capello *et al.*, 1997). These polymers are composed of tandemly repeated silk-like and elastin-like amino acid blocks (Megeed *et al.*, 2002). Strategic placement of charged amino acid residues in genetically engineered silk-elastinlike protein block copolymers has permitted more

precise control of pH and temperature-sensitivity (Nagarseker *et al.*, 2002; Nagarseker *et al.*, 2003). The swelling properties (Dinerman *et al.*, 2002) and transport properties (Dinerman *et al.*, 2002) of hydrogels formed by association of silk-elastinlike polymers is being evaluated in relation to controlled drug and gene delivery. However, these early results indicate that they may be more promising for *in situ* gel forming implants for localised drug delivery where stimuli-sensitivity is not required.

Early work by Touitou and co-workers (1986) showed insulin coated with Eudragit S when administered to rats produced a hypoglycaemic response. The Eudragit S polymer is insoluble at acidic pH values, therefore protects the insulin as it passes through stomach. At neutral/alkaline conditions the polymer is soluble, therefore the insulin is released in to the colon and absorbed to produce a response. More recently, Colo *et al.* (2002) have investigated the release pattern of the antiglycaemic agent metaformin from compressed matrix tablets of pH-sensitive poly(ethylene oxide) with Eudragit L100, when placed in simulated gastric fluid. Oral absorption of metaformin is usually confined to the upper part of the intestine. This matrix has shown promise for the gradual and complete release of metaformin from stomach to jejunum.

Eiamtrakarn *et al.* (2002) have also recently designed and tested a gastrointestinal mucoadhesive patch system (GI-MAPS) for the oral delivery of the protein, granulocyte colon stimulating factor (G-CSF). The system consisted of a patch preparation containing an enteric capsule. The patches were composed of four layers: (1) a water-insoluble backing layer which prevents attack from intestinal luminal proteolytic enzymes, (2) a middle layer in which G-CSF, an organic acid and a surfactant were formulated, (3) a pH-sensitive surface layer attached to the middle layer with (4) an adhesive layer made of carboxyvinyl polymer. Three different enteric polymers were used as a surface layer. These were HP-55, Eudragit L100 and S100. The same three kinds of GI-MAPSs containing 125 μg of recombinant human G-CSF were prepared and orally administered to dogs and the increase in total white blood cell counts were measured as the pharmacological index for G-CSF. Eudragit L100 showed the highest pharmacological index for G-CSF and showed promising results for the development of an oral peptide/protein delivery system.

To date pH-sensitive enteric polymer coatings which have been most intensively investigated are Eudragit® polymers (copolymers of methacrylic acid and either methylmethacrylate or ethyl acrylate) (Cole *et al.*, 2002). These polymers are and have been used to coat many commercial drug formulations. For example, Claversal®, Asacolitin®, Salofalk® and Budenofalk®, which are used for the oral treatment of inflammatory bowel disease (Leopold, 1999). Eudragit® polymers have a dissolution pH of between pH 6 and pH 7 and are intended to release drug as soon as intestinal pH exceeds pH 6 or pH 7, respectively.

PH-sensitive enteric coatings and site-specific release of coated drugs is reliant on the surrounding pH being at a specific value, therefore, any deviation from these pH values will affect drug release patterns. For example, an elevation in gastric pH has been observed in elderly patients, in individuals who have taken antacids and those who have been administered H_2 receptor antagonists (Kao *et al.*, 1997). In addition, patients with severe inflammatory bowel disease show a dramatic drop in colonic pH (Leopold *et al.*, 1998). Thus, the traditional formulations do not provide adequate drug release. Leopold *et al.* (1998) developed a system to overcome the latter, whereby the drug core was coated by an acid-soluble basic polymer layer, such as Eudragit® E, which was then coated in a gastroresistant enteric coating.

Recent studies in this field have looked at improving the residency time of drugs at the delivery site. For example, Sangalli *et al.* (2001) have evaluated an oral system, namely, Chronotopic™, which is designed to achieve time and/or site-specific release. The system consisted of a drug-containing core, coated by a hydrophilic swellable polymer that was responsible for a lag phase in the onset of release and an outer gastroresistant film. Yamada *et al.* (2001) have also evaluated and shown Eudragit® microparticles coated with ethylcellulose and carboxymethylethylcellulose can be used for sustained release of ketoprofen, which is used to treat rheumatism. Other studies are evaluating methods to optimise the enteric coat formulation (Crotts *et al.*, 2001).

A search for new polymers with similar or enhanced properties is still required, since it has been shown that suitable combinations of more types of polymer enables

appropriate modifications of the release characteristics of active agents (Rodriguez *et al.*, 1993; Rey *et al.*, 2001).

1.6: Liposomes.

Since the use of liposomes is a large part of this thesis, their classification and preparation, their applications, the methods used to separate non-encapsulated material from liposomes, the advantages of liposome encapsulation and finally sizing of liposomes are described.

1.6.1: Classification and preparation.

Liposomes, first characterised by Bangham (1965), were developed as models for biological membranes. The classification of liposomes is either based upon the method by which they were prepared or on their structural properties (Table 1.1) (Figure 1.7). The original, most commonly used method and one that will largely be used in this study to prepare liposomes, is via thin film hydration (Rongen *et al.*, 1997; Szoka *et al.*, 1980). Lipid is dried under vacuum as a thin film on the walls of a rotary evaporator flask. This is followed by the addition of an aqueous suspension with gentle shaking causing the lipid to swell spontaneously and fall in to the aqueous suspension as multilamellar vesicles (MLVs).

Figure 1.7: Structure of different liposomes (Adapted from Rongen *et al.*, 1997).

Table 1.2: Liposome classification (Adapted from Crommelin *et al.*, 1994).

1.6.2: Applications of liposomes.

Until 1971 liposome research focused upon their application as models for biological membranes (Tirrell *et al.*, 1976). Subsequently, their applications have broadened, being of interest to many disciplines including medicine, immunology, diagnostics, cosmetics, ecology, cleansing and the food industry. The most investigated practical application is as carrier vesicles in drug delivery systems (Lasic, 1998). The two main properties that make them of interest for this application are their similarity to biomembranes (Gier *et al.*, 1978) and their ability to entrap a wide range of compounds. Similar to biomembranes, liposomes are biodegradable, thus do not accumulate in the body. The degradation of liposomes to substances that can be released from body is rapidly achieved via the reticulo-endothelial system, mainly in

the liver and spleen (Liu *et al.*, 1993; Kono *et al.*, 1997; Horwitz *et al.*, 1997). In addition, their natural lipid composition, gives them low toxicity and low antigenicity when used *in vivo* (Chonn *et al.*, 1995; Rahman *et al.*, 1993).

As mentioned, various compounds can be entrapped within liposomes. For example, water-soluble compounds are entrapped within liposomes by spontaneous formation of lipid bilayers on hydration of anhydrous phospholipids. This vesicle formation is due to the hydrophilic interaction of lipid head groups of double chained lipids, such as phosphatidylcholine have with the water (Vemuri *et al.*, 1995; Crommelin *et al.*, 1994). Lipid soluble compounds such as lipophilic drugs (Gulati *et al.*, 1998) can also be incorporated in to liposomes. However, these are incorporated within the lipid bilayer, as oppose to the aqueous space (Figure 1.8).

Figure 1.8: Illustration of a liposome (Adapted from Gulati *et al.*, 1998).

1.6.3: Methods used to separate non-encapsulated material from liposomes.

The encapsulation efficiency by liposomes varies depending on the compound being encapsulated. For example, lipophilic compounds generally have a high affinity for the bilayer, so are totally liposome associated, whereas, encapsulation of other compounds is not one hundred per cent (Crommelin *et al.*, 1994). A range of

techniques are can be used to separate non-encapsulated material from liposomes, some of which are outlined.

1.6.3.1: Dialysis and ultra filtration.

Conventional dialysis membranes with specific molecular 'cut-offs' can be used to remove 'free' compounds having a molecular weight between this range. Also, by selecting particular experimental conditions, separation is fast and concentration of the liposome dispersion can be achieved (Crommelin *et al.*, 1994). However, using dialysis membranes to remove free compounds from a heterogeneous sized sample of liposomes has been shown to increase the percentage of small liposomes and reduce the percentage of large liposomes. In addition, as a consequence of water uptake, the diameter of the remaining large vesicles is increased (Dams *et al.*, 1999).

1.6.3.2: Ultra centrifugation.

High-speed centrifugation can be used to completely separate liposomes from non-encapsulated material (Purohit *et al.*, 2001; Kim *et al.*, 1996; Dabrowska *et al.*, 1998), with the advantage of not diluting the liposome preparation.

1.6.3.3: Ion exchange reactions.

The potential of ion exchange resins to separate liposomes from non-encapsulated material has been investigated. For example, Dowex 50W-X4, was tested with a range of adsorbents and shown to give successful separation without dilution of the liposome preparation (Storm *et al.*, 1985). The use of ion exchange resins to separate liposomes from non-encapsulated material is now an established method (Harashima *et al.*, 1999; Chung *et al.*, 2002).

1.6.3.4: Gel permeation chromatography.

Gel permeation chromatography, also known as gel filtration, has been used to purify sensitive bio-molecules for nearly forty years. Its widespread use can be attributed to it being a reliable simple method. In order to separate non-encapsulated material from

liposomes by gel filtration, columns are primarily packed with swollen Sephadex (Grabielle-Madelmont *et al.*, 2003), a bead-formed gel prepared by cross-linking dextran with epichlorohydrin (Phamacia Biotech). It is supplied in dry form and swells in aqueous solutions. The degree of cross-linking determines the extent to which macromolecules can permeate the beads. Thus, large molecules such as liposomes are excluded first, while smaller molecules, such as dyes, enter the beads and are eluted from the column at a slower rate.

1.6.4: Advantages of liposome encapsulation.

Using liposomes as carrier vehicles of active agents has been shown to give a number of benefits. One advantage is the reduction of toxicity of potent compounds for site-specific delivery by incorporation within liposomes. For example, the use of doxorubicin, an anthracycline antibiotic that shows anti-tumour properties, has been limited since it can cause cardiac muscle damage, venous sclerosis, alopecia, myelosuppression, gastrointestinal disturbances and tissue burns after extravasation. Phase II trials with doxorubicin have shown many of these toxic effects are reduced by encapsulation within liposomes, without the loss of anti-tumour activity (Rahman *et al.*, 1993; Judson *et al.*, 2001). Similarly, administration of certain antibiotics is limited by dose-dependent nephrotoxicity. The cytotoxic effects are markedly reduced by encapsulation of the antibiotics within liposomes (Zager, 2000).

As well as protecting the individual receiving treatment from toxic effects, liposomes can also protect the encapsulated agent from the natural defence mechanisms of the body. Where they would ordinarily be metabolised when taken in to the body, they are provided with protection and have improved efficiency since they are not detected. For example, many free drug eye drops for ophthalmic therapy are found to have poor penetration in to ocular tissue (Monem *et al.*, 2000). However, it has been demonstrated that when the drugs are administered entrapped within liposomes their bioavailability is improved (Ding, 1998; Lasic, 1998).

Liposomes can also beneficially alter the biodistribution of compounds administered *in vivo*. For example, as explained doxorubicin, displays potent anti-neoplastic

activity against a range of tumours and leukaemia (Carter, 1975; Young *et al.*, 1981), but in its free form displays numerous acute toxicities, including cardio toxicity. Numerous studies showing a reduction in toxicity by encapsulation within liposomes (Mehta, 1996; Boman *et al.*, 1995) can in part be explained by the biodistribution of liposomes that are largely taken up in to the liver via the reticulo-endothelial system. Although liposomes have been seen to accumulate in the liver, further trials have shown the toxicity of encapsulated drugs is reduced, while their therapeutic index is increased (Mahew *et al.*, 1987).

1.6.5: Sizing of liposomes.

It is accepted that the size of liposomes is an important factor in their biodistribution (Kostarelos, 2003). Adjustments to the size of liposomes can give particular advantages. For example, large liposomes have a short circulation half-life as they are eliminated very rapidly from circulation by a complement-mediated phenomenon (Bradley *et al.*, 1998; Szebeni, 1998; Szebeni *et al.* 1999) and accumulate in the organs of reticuloendothelial system (RES). Whereas, small liposomes (<200 nm) circumvent RES uptake due to their reduced recognition by circulating opsonins (Heldt *et al.*, 2001). It has been shown poly(ethylene glycol) coated liposomes further inhibit liposome-induced complement activation (Ahl *et al.*, 1997; Bradley *et al.*, 1998). Nevertheless, it has been observed that large liposomes have better encapsulation efficiency because as the size of the liposomes increases, the entrapped volume increases for constant lamellarity (Perkins *et al.*, 1993; Berger *et al.*, 2001). Therefore, it is important to be able to control the size of prepared liposomes in order to impart particular properties for different applications.

Several methods are available to determine the size of liposomes. The most appropriate method is dependent on the sample characteristics. For example, the methods that are most appropriate to size small vesicles include nuclear magnetic resonance spectroscopy (NMR), analytical centrifugation and light scattering techniques.

1.6.5.1: Nuclear magnetic resonance spectroscopy.

NMR spectroscopy relies upon the use of paramagnetic ions to shift or broaden the NMR signal from the phosphate moiety of the phospholipid (Szoka *et al.*, 1980; Peleg-Shulman *et al.*, 2001; Heldt, 2001; Touitou, 2000).

1.6.5.2: Analytical centrifugation.

Centrifugation can be used to assess vesicle size by determination of the sedimentation rate of the vesicles (Mason *et al.*, 1978). Using this principle, vesicles of differing size can be separated from each other (Fry *et al.*, 1978).

1.6.5.3: Light scattering techniques.

Light scattering techniques are widely used to determine particle size (Chanderoy *et al.*, 2002; Matsuzaki *et al.*, 2000; Meiden *et al.*, 2001; Imura *et al.*, 2001). Mastersizer and Zetasizer equipment utilise the passage of light through the liposome sample to determine the size of particles, the principles of which are detailed in Chapter 4.

Conversely, light microscopy, negative stain electron microscopy and freeze-etch and freeze-fracture electron microscopic techniques can be useful to determine the size of larger liposomes.

1.6.5.4: Light microscopy.

Light microscopy is useful for determining the gross size distribution of a sample of liposomes (Szoka *et al.*, 1980). However, the resolution of light microscopy limits this technique from determining a full size distribution. Nevertheless, it is often used in parallel with other methods to give a clearer idea of sample characteristics (Dorovska-Taran *et al.*, 1996; Rades *et al.*, 1997; Shangguan *et al.*, 2000).

1.6.5.5: Negative stain electron microscopy.

Negative staining transmission electron microscopy is useful to determine the size of liposomes since it has an image resolution 200 times better than a standard light microscope (Jones *et al.*, 1994). Liposomes are stained with compounds such as uranyl acetate (Schneider *et al.*, 1995), which have a high atomic number and scatter electrons strongly. Visualisation of TEM images show as dark and light areas where the stain has and has not attached, respectively.

1.6.5.6: Freeze-etch and freeze-fracture electron microscopic techniques.

Freeze-fracture and freeze-etch is extensively used to study vesicle size and structure (Sternberg *et al.*, 1998; Clary *et al.*, 1999; Jaaskelainen *et al.*, 1998). The liposomes are rapidly frozen and cleaved along their fraction plane (the point between the lipid bilayer). The fractured sections are then coated with a heavy metal (etched). A replica is made of this surface, which is viewed by electron microscope.

1.7: Aims of research project.

Polymers that have alternating charged and hydrophobic pendant groups can show conformational changes with loss of charge from their charged pendant groups. That is, when the pendant groups are charged they show an extended linear conformation as a result of charge repulsion. Neutralisation of the charged groups causes hydrophobic association such that the polymer is hydrophobically asymmetric and agglomerated.

There are two groups of polymers used in thesis, which have been synthesised based on the latter structure. The first group which are synthesised to have an anionic charge on their pendant charged groups include poly(lysine *iso*-phthalamide), poly(lysine dodecandiamide), poly(ornithine sebacamide), poly(lysine butyl malonamide) and poly(lysine diethyl malonamide). The second group which are synthesised to have a cationic charge on their pendant charged groups include *N,N*-diethylamine, *N,N*-

diethyl ethylene diamine and 3-(diethylamino)propylamine modified glycidyl methacrylate polymers.

Polymers that have this structure have potential controlled release applications. For example, it is anticipated such polymers could be used to rupture liposomal membranes, subsequently releasing their contents in to the surrounding environment. It is thought this could be achieved by embedding or attaching polymer to the liposome membrane. That is, as polymer changes conformation the lipid packing of the liposome membrane is disturbed and consequently disrupted.

Alternatively, endocytosed drug/gene conjugates of these polymers could potentially be used to improve the efficiency of drug/gene delivery. It is thought that the low pH of the endosome could be exploited to produce a conformational change in the polymer. Thus, polymer in contact with the endosomal membrane would cause disruption of the membrane and deliver the conjugated drugs/genes in to the cytoplasm.

To assess the potential of these two groups of polymers for such applications, a series of aims were set out which included:

- Undertaking potentiometric titrations for the described polymers to determine their apparent pK_a values with degree of ionisation and establish any conformational changes they exhibit.

- Measuring release of haemoglobin from erythrocytes to determine whether erythrocyte membranes are disrupted as a function of pH when pre-incubated in the presence or absence of the first group of polymers, which have negatively charged pendant groups.

- Assessing the relationship between apparent pK_a values of the latter polymers and pH values at which membrane disruption of erythrocytes occurs.

- Determining the visual changes of liposomes at a range of pH values when pre-incubated in the presence and absence of both groups of polymers, since visual changes of liposome suspensions can be used as an indicator of liposome disruption.

- By measuring the release of an encapsulated marker from liposomes, quantify and determine whether liposome disruption varies as a function of pH by pre-incubating in the presence or absence of both groups of polymers.

Further aims:

- Agents that are able to disrupt lipid membranes could have anti-microbial properties by causing disruption of the prokaryotic cell membrane. Therefore, it was aimed to use MIC experiments to investigate the anti-microbial properties of both groups of polymers.

Polymers that show pH-responsive conformational changes could also be used as solid coatings to encapsulate and subsequently release active agents in response to changes in pH. Two main aims were made to assess the polymers used in this thesis for such an application. These included:

- Determining the rate of dissolution of polymer films in different pH solutions.

- Determine rate of release of polymer coated markers in different pH solutions.

Chapter 2

Synthesis and determination of the conformational changes of polyamides and amine modified glycidyl methacrylate polymers

2.0: Aims and Overview.

Aim: Production of polyamides that have alternating charged (e.g. carboxyl) and hydrophobic functional groups, which can be used to modulate 'switch' hydrophobic association by varying the extent of ionisation of the charged group.

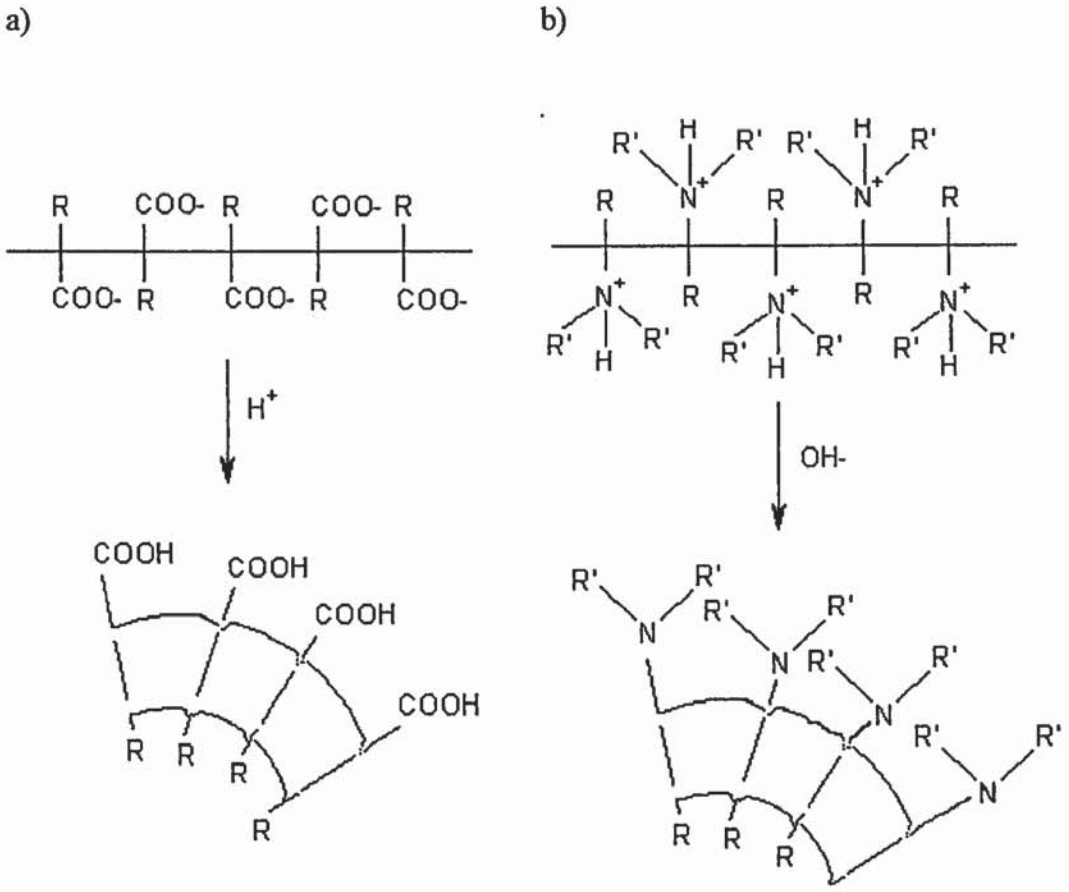
The described principle can be illustrated by Figure 2.1(a). This illustration with hydrophobic functional groups omitted for clarity shows the polymer to be in a linear conformation as a result of anionic charge repulsion of pendant carboxyl groups. Neutralisation of the charged carboxyl groups causes hydrophobic association, such that the polymer is hydrophobically asymmetric and agglomerated or 'coiled'. This principle can also be applied when the functional groups are cationic as illustrated in Figure 2.1(b). That is, the anionic groups are replaced with cationic groups, such that the extended polymer conformation is a result of cationic as opposed to anionic charge repulsion. As with anionic groups, neutralisation of cationic groups causes agglomeration of the polymer.

Polymers that exhibit hydrophobic association can be utilised to interact and disrupt lipid membranes as discussed in Chapter 1. Earlier studies conducted at Aston University showed that polyamides formed by copolymerisation of amine and hydrophobic dicarboxylic acid moieties exhibited cell lysis properties at reduced pH values (Eccleston *et al.*, 2000).

To achieve the aim of this chapter it was subdivided in to sections. Firstly, the structures that enable the hydrophobic association of polymers with pH change are discussed. Then, the methods used, based on methods used by Eccleston (1995) to synthesise polyamides and amine modified glycidyl methacrylate polymers, that have such structures are described. The molecular weight of these polymers was determined. In addition, potentiometric titrations were performed for these polymers

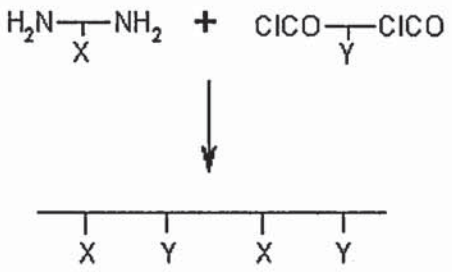
to determine their pK_a values and study their conformational changes. The pK_a values are also used in later chapters to determine if there is any correlation between membrane disruption and the pK_a values of polymer.

Figure 2.1: Illustration of 'switch' hydrophobic association of polymers caused by neutralisation of anionic (a) / cationic (b) functional groups.



Polyamides with alternating pendant anionic and hydrophobic groups were synthesised by interfacial polycondensation as illustrated in the Figure 2.2.

Figure 2.2: Interfacial polycondensation reaction used to synthesise polyamides with alternating pendant anionic and hydrophobic groups.



The amino acids, lysine and ornithine, were used to replace pendant carboxyl groups (Figure 2.2) at X positions. The introduction of pendant hydrophobic groups at Y positions was achieved in three ways:

1) By making an alkyl group substitution; as was for poly(lysine butyl malonamide) (Figure 2.3) and poly(lysine diethyl malonamide) (Figure 2.4).
2) Enchainment of an aromatic group; as was for poly(lysine *iso*-phthalamide) (Figure 2.5).
3) By incorporation or enchainment of a long alkyl group; as was for poly(lysine dodecandiamide) (Figure 2.6), poly(ornithine dodecandiamide) (Figure 2.7) and poly(ornithine sebacamide) (Figure 2.8).

Figure 2.3: Poly(lysine butyl malonamide).

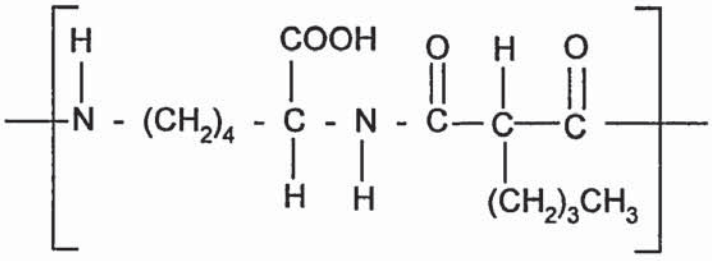


Figure 2.4: Poly(lysine diethyl malonamide).

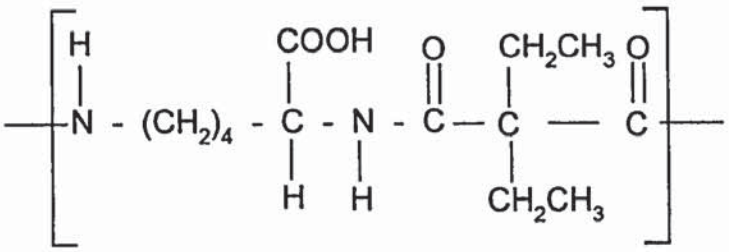


Figure 2.5: Poly(lysine *iso*-phthalamide).

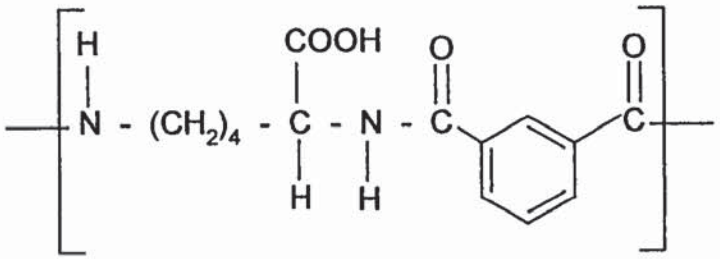


Figure 2.6: Poly(lysine dodecandiamide).

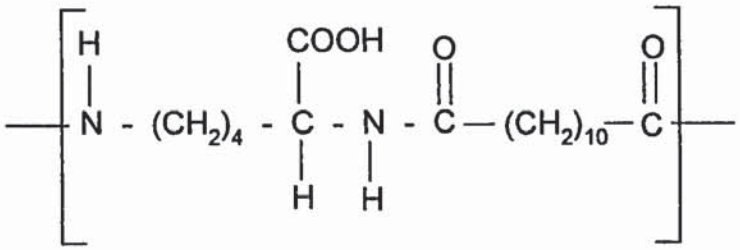


Figure 2.7: Poly(ornithine dodecandiamide).

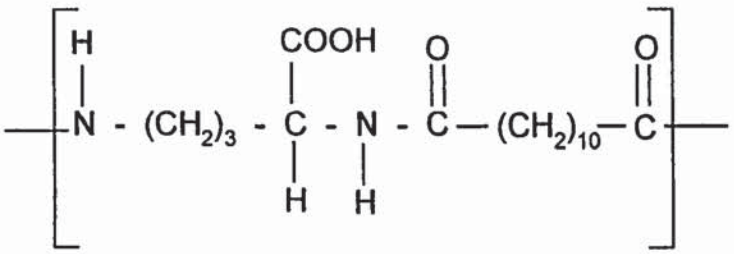
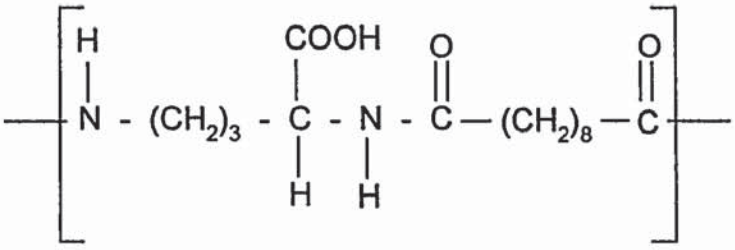


Figure 2.8: Poly(ornithine sebacamide).



Cationic polymers were synthesised by amination of a glycidyl methacrylate pre-polymer to give an aminated glycidyl methacrylate polymer (GMP) as illustrated in Figure 2.9 using the diamines *N,N*-diethylamine (Figure 2.10), *N,N*-diethyl ethylene diamine (Figure 2.11) and 3-(diethylamino)propylamine (Figure 2.12) to give *N,N*-diethylamine (Figure 2.13), *N,N*-diethyl ethylene diamine (Figure 2.14) and 3-(diethylamino)propylamine (Figure 2.15) modified glycidyl methacrylate polymers, respectively.

Figure 2.9: Amination of glycidyl methacrylate pre-polymer.

Glycidyl methacrylate pre-polymer Aminated GMP

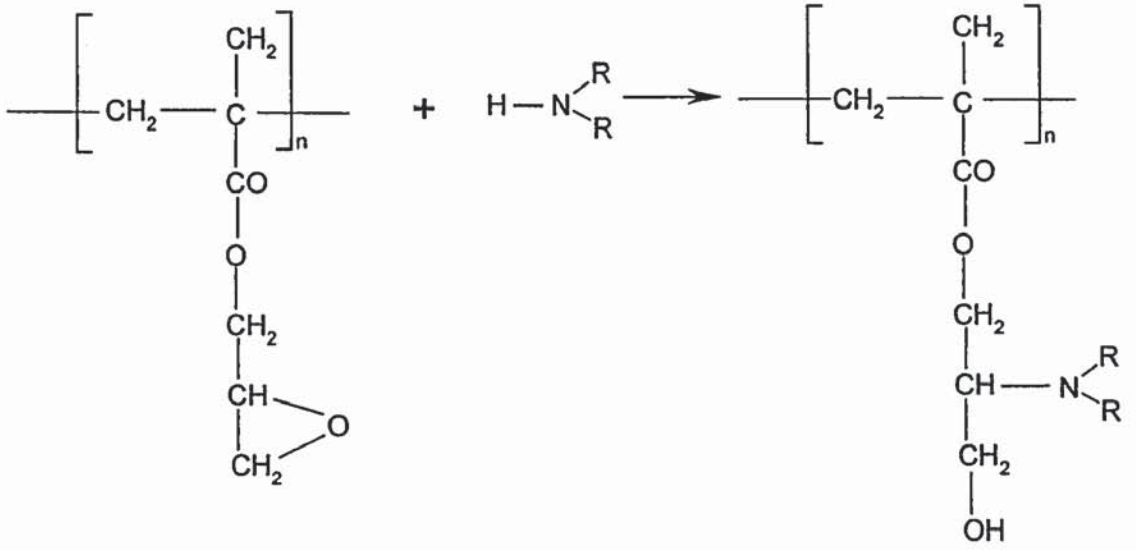


Figure 2.10: *N,N*-diethylamine.

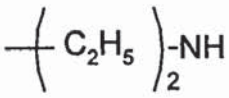


Figure 2.11: *N,N*-diethyl ethylene diamine

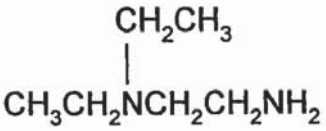


Figure 2.12: 3-(diethylamino)propylamine.

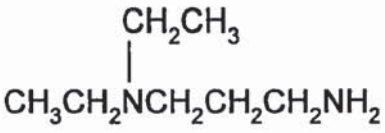


Figure 2.13: *N,N*-diethylamine modified GMP.

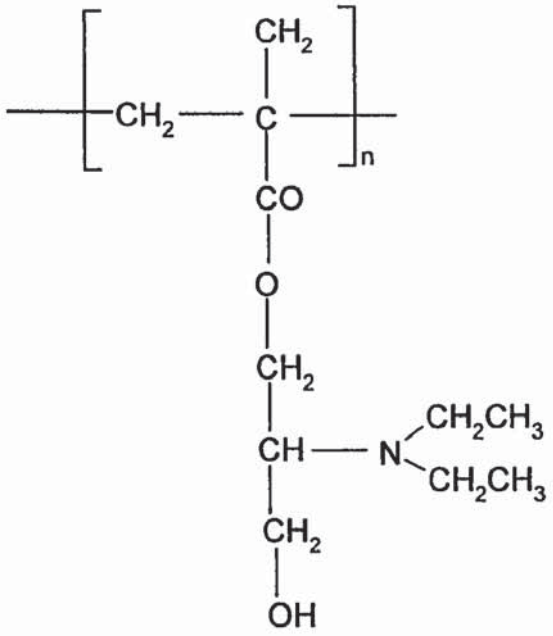


Figure 2.14: *N,N*-diethyl ethylene diamine GMP.

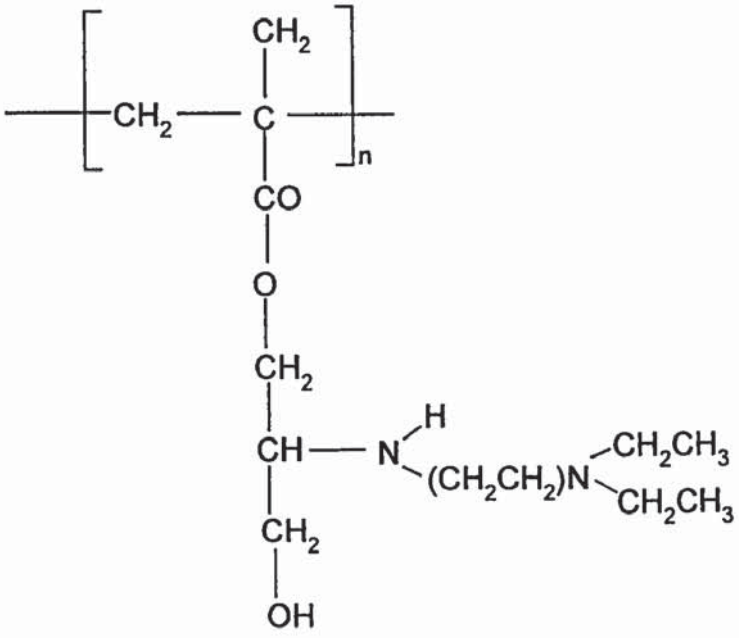
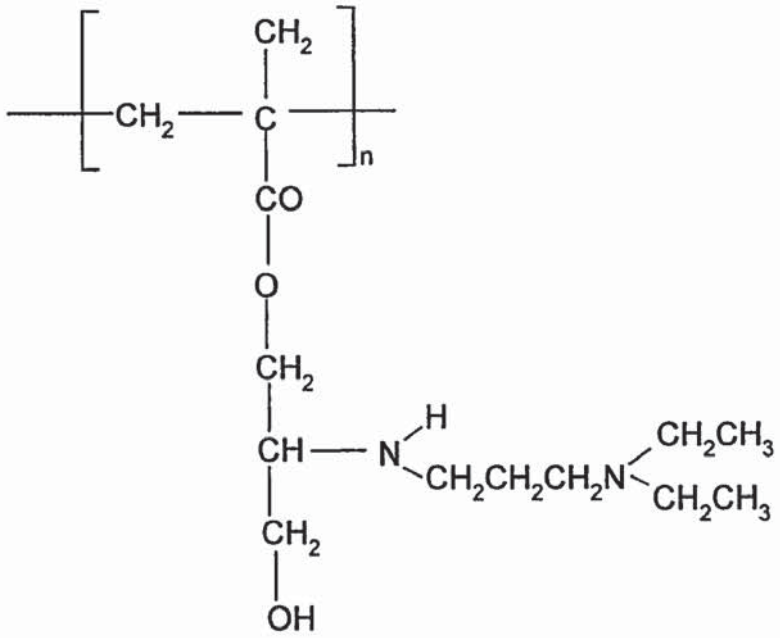


Figure 2.15: 3-(diethylamino)propylamine GMP.



2.1: Methods.

2.1.1: Synthesis of polyamides.

The methods used to synthesise polyamides including poly(lysine *iso*-phthalamide), poly(lysine dodecandiamide), poly(ornithine dodecandiamide), poly(ornithine sebacamide), poly(lysine butyl malonamide) and poly(lysine diethyl malonamide) which are illustrated in Figures 2.3 to 2.8 are described below.

2.1.1.1: Poly(lysine *iso*-phthalamide).

To synthesise poly(lysine *iso*-phthalamide), firstly a 200 ml volume of de-ionised water was placed in to a 250 ml conical flask. This flask was placed in a freezer and the contents periodically checked for the formation of ice crystals. When ice crystals started to form the flask was removed from the freezer and the correct quantities of L-lysine monohydrate (Figure 2.17) and potassium carbonate, that is 6.5648 g and 16.582 g, respectively (Calculation 2.1), were transferred to the flask and allowed to dissolve. The solution was then placed back in to the freezer to cool and then

transferred to a Waring blender containing a thin layer of ice at the bottom. The blender was set at low speed. To a 250 ml conical flask, 8.081 g of *iso*-phthaloyl chloride (Figure 2.16) (Calculation 2.1) was transferred and melted using a hot air blower. Once melted 150 ml of acetone removed from a 200 ml volume was transferred to the conical flask. The *iso*-phthaloyl chloride in acetone was then transferred to the blender. The remaining 50 ml of acetone was used to remove any residual *iso*-phthaloyl chloride from the conical flask to the blender. Finally the total contents were blended at high speed for approximately 30 minutes until a colour change from white through yellow and back to white was observed. The contents of the blender were then transferred to a beaker and left overnight to allow evaporation of acetone. To separate high and low molecular weight polymer, the crude polymer solution was concentrated to a volume of 75 ml and dialysed with 500 ml of water using an ultra filtration machine set at master flex speed 4 and a pressure of 20 psi. Low and high molecular weight polymer were separated via a Millipore Miniplate™ containing a cellulose diafiltration membrane with a molecular 'cut off' of 3000 Da. The solution containing high molecular weight polymer was placed in freezer and allowed to freeze then lyophilised to form solid poly(lysine *iso*-phthalamide).

Calculation 2.1: Poly(lysine iso-phthalamide).

A 0.2 M solution of poly(lysine *iso*-phthalamide) was required. For this experiment, 200 ml of water was used to dissolve the amine and acid acceptor and 200 ml of acetone was used to dissolve the acid chloride. As 200 ml volumes of water and acid chloride were used 0.04 moles of both amine and acid chloride were required. That is, 200 ml/1000 ml x 0.2 M = 0.04 moles. Therefore, to a 200 ml volume of water, total lysine in the form of L-lysine monohydrate added was 0.04 moles x 164.12 (formula weight) = 6.5648 g. In addition, the acid acceptor potassium carbonate (K_2CO_3) was added to the aqueous phase to allow subsequent polymerisation reactions to occur. The amount required is based on the structure of the amines. That is, the structure of L-lysine monohydrate allows hydrochloric acid (HCl) formation due to loss of hydrogen from both amino ($-NH_2$) groups and the carboxylic acid group (-COOH). Therefore, for lysine 0.04 moles x 3 x 138.21 (FW of K_2CO_3) = 16.5852 g of acid acceptor is required.

To 200 ml of acetone, 0.04 moles of *iso*-phthaloyl chloride (Figure 2.16) is required. Therefore, the mass added is 0.04 moles x 202.2 (FW of *iso*-phthaloyl chloride) = 8.0812 g.

Figure 2.16: Structure of *iso*-phthaloyl chloride.

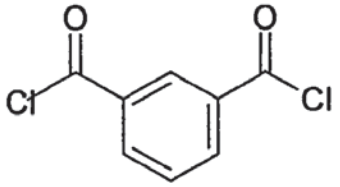


Figure 2.17: Structure of L-lysine monohydrate ($C_6H_{14}N_2O_2.H_2O$).

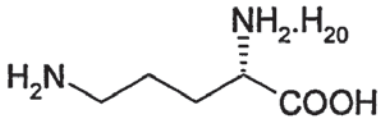


Figure 2.18: Structure of dodecanedioyl dichloride ($C_{12}H_{20}Cl_2O_2$).

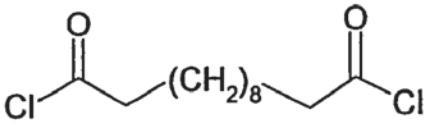


Figure 2.19: Structure of ornithine hydrochloride ($C_5H_{12}N_2O_2.HCl$).

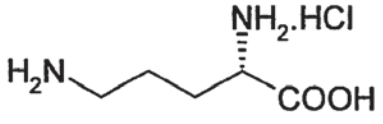
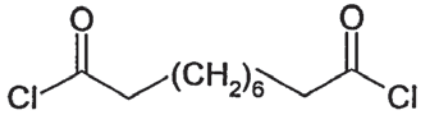


Figure 2.20: Structure of sebacoyl chloride $C_{10}H_{16}Cl_2O_2$.



The procedures, which are described to synthesise poly(lysine *iso*-phthalamide) were repeated to synthesise poly(lysine dodecandiamide), except different reactants were used as described in Section 2.1.1.2. Polyamides poly(ornithine dodecandiamide), poly(ornithine sebacamide), poly(lysine butyl malonamide) and poly(lysine diethyl malonamide) used in this study were synthesised by Dr Mark Eccleston (Aston University, Birmingham). The reactants and quantity used are described in Sections 2.1.1.3 to 2.1.1.6.

2.1.1.2: Poly(lysine dodecandiamide).

A 0.2 M solution of poly(lysine dodecandiamide) was required. This was achieved by transferring 0.04 moles (0.2 M x 200 ml/1000 ml) of lysine to 200 ml of water. That is, using solid L-lysine monohydrate (Figure 2.17), 0.04 x 164.12 (FW of L-lysine monohydrate) = 6.5648 g was required. Formation of HCl can occur via loss of hydrogen from both -NH_2 groups and also from the -COOH group. Therefore, 0.04 moles x 3 of acid acceptor K_2CO_3 was required. That is, 0.12 moles x 138.21 (FW of K_2CO_3) =16.5852g.

Unlike *iso*-phthaloyl chloride, which is an aromatic group, dodecanedioyl dichloride (Figure 2.18) is an aliphatic group and therefore, dissolved in chloroform as oppose to acetone. Thus, to 200 ml of chloroform 0.04 moles x 218.77 (FW of dodecanedioyl dichloride) = 8.7508 g was required.

2.1.1.3: Poly(ornithine dodecandiamide).

A 0.2 M solution of poly(ornithine dodecandiamide) was required. This was achieved by transferring 0.04 moles (0.2 M x 200 ml/1000 ml) of ornithine to 200 ml of water. Using the solid ornithine hydrochloride (Figure 2.19) 0.04 x 168.62 (FW of ornithine hydrochloride) = 6.744 g was required. Formation of HCl can occur via loss of hydrogen from both -NH_2 groups, from the -COOH group and also loss of the attached HCl group from the amine. Therefore, 0.04 moles x 4 of acid acceptor, K_2CO_3 was required. That is, 0.16 moles x 138.21 (FW of K_2CO_3) = 22.1136 g.

As dodecanedioyl dichloride is an aliphatic group it needs to be dissolved in chloroform. Therefore, to 200 ml of chloroform 0.04 moles x 218.77 (FW of dodecanedioyl dichloride) = 8.7508 g was required.

2.1.1.4: Poly(ornithine sebacamide).

A 0.2 M solution of poly(ornithine sebacamide) was required. This was achieved by transferring 0.04 moles (0.2 M x 200 ml/1000 ml) of ornithine to 200 ml of water. That is, using solid ornithine hydrochloride 0.04 x 168.62 (FW of ornithine hydrochloride) = 6.744 g was required. Formation of HCl can occur via loss of hydrogen from both -NH2 groups and also from the -COOH group and also loss of the attached HCl group from the amine. Therefore, 0.04 moles x 4 of acid acceptor K_2CO_3 was required. That is, 0.16 moles x 138.21 (FW of K_2CO_3) = 22.1136 g.

As sebacoyl chloride (Figure 2.20) is an aliphatic group it was dissolved in chloroform as oppose to acetone. Therefore, to 200 ml of chloroform 0.04 moles x 239 (FW of sebacoyl chloride) = 9.56 g was required.

2.1.1.5: Poly(lysine butyl malonamide).

A 0.2 M solution of poly(lysine butyl malonamide) was required. This was achieved by transferring 0.04 moles (0.2 M x 200 ml/1000 ml) of lysine to 200 ml of water. That is, using the solid L-lysine monohydrate, 0.04 x 164.12 (FW of L-lysine monohydrate) = 6.5648 g was required. Formation of HCl can occur via loss of hydrogen from both -NH_2 groups and also from the -COOH group. Therefore, 0.04 moles x 3 of acid acceptor, K_2CO_3 is required. That is, 0.12 moles x 138.21 (FW of K_2CO_3) =16.5852 g.

Butyl malonyl chloride ($C_7H_6ClO_3$), an aliphatic group and pre-formed from butyl malonic acid was dissolved by chloroform. Thus, to 200 ml of chloroform, 0.04 moles x 173 (FW of butyl malonyl chloride) = 6.92 g was required.

2.1.1.6: Poly(lysine diethyl malonamide).

A 0.2 M solution of poly(lysine diethyl malonamide) was required. This was achieved by transferring 0.04 moles (0.2 M x 200 ml/1000 ml) of lysine to 200 ml of water. Thus, using solid L-lysine monohydrate 0.04 x 164.12 (FW of L-lysine monohydrate) = 6.5648 g was required. Formation of HCl can occur via loss of hydrogen from both $-NH_2$ groups and also from the -COOH group. Therefore, 0.04 moles x 3 of acid acceptor K_2CO_3 was required. That is, 0.12 moles x 138.21 (FW of K_2CO_3) = 16.5852 g.

Diethyl malonyl chloride ($C_7H_{10}Cl_2O_2$) is an aliphatic group and therefore was dissolved in chloroform. Thus, to 200 ml of chloroform 0.04 moles x 196 (FW of Diethyl malonyl chloride) = 7.84 g was required.

2.1.2: Synthesis of amine modified glycidyl methacrylate polymers.

The synthesis of amine modified glycidyl methacrylate polymers which include *N,N*-diethylamine GMP (Figure 2.13), *N,N*-diethyl ethylene diamine GMP (Figure 2.14) and 3-(diethylamino)propylamine GMP (Figure 2.15) is a two-step process. The first step involves the preparation of glycidyl methacrylate polymer and the second involves attachment of the amine, each of which will be described in succession.

2.1.2.1: Polymerisation of glycidyl methacrylate.

Under nitrogen 600 ml of chloroform and 300 ml of a 1 M glycidyl methacrylate (2,3 epoxy propyl methacrylate) solution (FW142.16) (Aldrich, UK) was stirred for 60 minutes. To initiate the polymerisation 0.246 g of azo-*iso*-butyronitrile (FW164) (Fluka, UK) was added, which is 0.5% of the total molar amount of glycidyl methacrylate. The solution was then heated to 60° C and the level of nitrogen reduced to a level that allowed refluxing of the solution. Stirring was continued for 2 hours at this temperature until the solution became viscous, indicating polymer formation. The nitrogen flow was then increased to allow removal of the majority of chloroform. The glycidyl methacrylate polymer was then recovered by precipitation in to an excess of

diethyl ether. This white precipitate was re-dissolved with chloroform and recovered again in diethyl ether three times to remove residual monomer. Residual solvent was allowed to evaporate at room temperature for a period of 72 hours.

2.1.2.2: Amine modification.

A gram of glycidyl methacrylate polymer prepared in Section 2.1.2.1 was transferred to a glass beaker with approximately 200 ml of acetone (Sigma) and dissolved by stirring overnight. This gives a relatively dilute solution, which avoids cross-linking reactions. With stirring, distilled water was slowly added to the polymer solution until an opaque colour change remained. Acetone was then slowly added until the opaqueness cleared. At this stage, 1.03 g of *N,N*-diethylamine (FW 731.14) (Lancaster) was transferred to polymer solution, giving a 2:1 molar ratio of *N,N*-diethylamine: glycidyl methacrylate polymer. This solution was stirred and heated at 45° C ± 2° C to speed up the amine modification reaction but avoid evaporation of *N,N*-diethylamine (boiling point 55° C). The volume of the solution was kept constant by addition of acetone. At two hour intervals a 1 ml volume was removed from the bulk solution and transferred to 9 ml of 0.1 M sulphuric acid (Fischer chemicals, UK) to check for precipitate formation. No precipitate formed after 4 hours, indicating the amine modification stage was complete. The solvent was then removed under vacuum, using rotary evaporation. To the remaining solution, with stirring, a 0.2 ml volume of 1.8 M sulphuric acid (Fischer chemicals, UK) was added to acidify and allow hydrolysis of any residual epoxide groups. Stirring was continued for a period of 1-hour. The pH of the solution was then increased by addition of 1 M sodium hydroxide until the polymer precipitated from solution. The precipitate was collected using a Whatman filter paper number 1 and vacuum pump. The collected precipitate was rinsed using distilled water to remove residual sodium hydroxide, then dried at room temperature in a sealed container with silica gel.

N,N-diethyl ethylene diamine and 3-(diethylamino)propylamine modified glycidyl methacrylate polymers were synthesised by Dr Mark Eccleston (Aston University, Birmingham) using the procedures described in Section 2.1.2.1 and 2.1.2.2 but using

N,N-diethyl ethylene diamine and 3-(diethylamino)propylamine respectively, as opposed to *N,N*-diethylamine for the amine modification.

2.1.3: Determination of the molecular weight of polyamides and amine modified glycidyl methacrylate polymers.

The molecular weight of poly(lysine *iso*-phthalamide) was determined to represent the polyamide group of polymers. Lyophilised poly(lysine *iso*-phthalamide) was insoluble in Dimethylformamide (DMF), which is commonly used for analysis of polar samples. Therefore, aqueous gel permeation chromatograms were obtained using a Viskotek GPC system (Viscotek Europe, Hampshire, UK), consisting of a VE 7510 degasser, a VE 1122 pump, a VE 3580 refractive index detector, a DM 400 data manager and 2 x 30 cm Viscotek GPC (Gel permeation chromatography) columns with 0.1 M sodium nitrate/15% methanol as eluent at 30° C. The molecular weight was determined relative to poly(ethylene glycol) standards. Under these conditions relatively low equivalent molecular weights were obtained which was thought to be due to peak broadening caused by retention of material on the columns and structural differences between the analysed samples and the calibrants. Poly(lysine *iso*-phthalamide) sample was determined to have a Mn of 8990 Da and a M_w of 18610 Da giving a polydispersity of 2.07. The apparent molecular weight was extremely sensitive to sample preparation and the concentration of added salts. This was even more apparent in the case of amine modified glycidyl methacrylate polymers. The polymers were all prepared from a stock supply of a glycidyl methacrylate pre-polymer. The molecular weight of the glycidyl methacrylate pre-polymer was determined using 2 x 30 cm styrene divinyl benzene columns with Tetrahydrofuran (THF) as the eluent at 30° C giving a Mn of 16027 Da and a M_w of 35411 Da with a corresponding polydispersity of 2.2. Attempts to analyse the lyophilised amine modified glycidyl methacrylate polymers by aqueous GPC suggested there was a strong interaction between the polymer and the column material with the sample eluting as a shoulder on the solvent/salt peak at the end of the chromatogram.

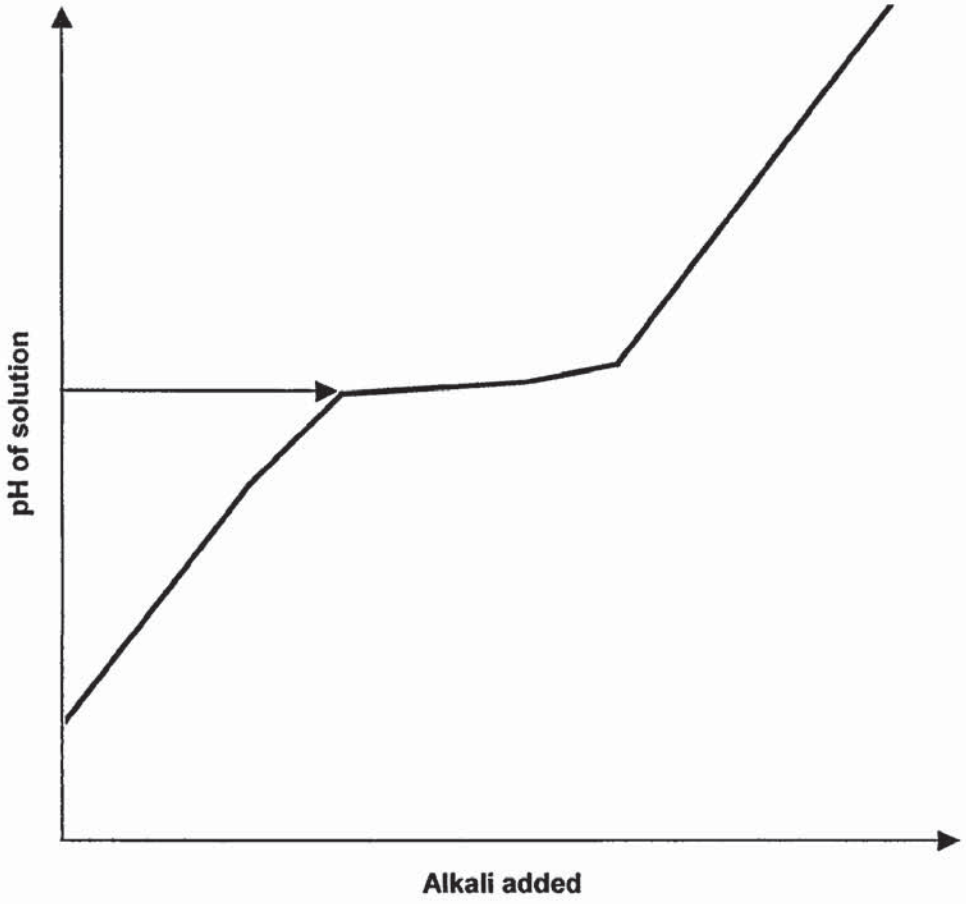
2.1.4: Potentiometric titrations of polyamides.

pK_a is the pH at which there is an equivalent distribution of acid and conjugate base or base and conjugate acid in solution. Unlike mono basic acids that have a single pK_a value (Figure 2.21), polyacids have a range of pK_a values, termed apparent pK_a values, which increase with increasing degree of polymer ionisation. The increase in pK_a value is a result of electrostatic repulsion experienced by the carboxylate ions along the polymer backbone as successive carboxylic acid groups are ionised. Potentiometric titrations can be used to determine the apparent pK_a values of polyamides as a function of degree of ionisation (α). They were performed by first preparing a 0.1012 M NaOH solution by dissolving 4.073 g of NaOH (FW 40) in 1 L of de-ionised water. 50 mg of the polyamides, poly(lysine *iso*-phthalamide), poly(lysine dodecandiamide), poly(lysine diethyl malonamide), poly(lysine butyl malonamide), poly(ornithine dodecandiamide) and poly(ornithine sebacamide) were dissolved in 5 ml volumes of the prepared NaOH solution. Once dissolved and thus dissociated the polyamide solutions were made up to 20 ml using deionised water, then transferred to a glass beaker. With continual stirring 0.05 ml volumes of a 0.102 M HCl solution was titrated in to each polyamide solution past precipitation with pH readings taken after each addition. The described procedure was performed in triplicate at a temperature of 25 ± 1° C

2.1.5: Potentiometric titrations of amine modified glycidyl methacrylate polymers.

Conversely, glycidyl methacrylate pre-polymer modified with *N,N*-diethylamine, *N,N*-diethyl ethylene diamine and 3-(diethylamino)propylamine precipitate from solution at alkaline pH values. Therefore, each was made up to 20 ml with distilled water to give a concentration of 2.5 mg ml^{-1}. Then with continual stirring 0.05 ml volumes of 0.101 M NaOH was titrated in to the polymer solution past precipitation with pH readings taken after each addition. The described procedure again was performed in triplicate at a temperature of 25 ± 1°C.

Figure 2.21: Theoretical titration curve of a monobasic acid, with pK_a value indicated by arrow.



2.1.6: Deriving apparent dissociation (pK') and degree of dissociation (α) of polymers.

Bronsted (1923) was the first to show the advantage of expressing the ionisation of both acids and bases on the same scale. A distinction was made between strong and weak acids and bases. Such that, strong acids and bases are defined as completely ionised in pH range 0-14. Whereas, weak acids and bases are defined as incompletely ionised in the pH range 0-14. The pK_a is defined as the negative logarithm of the equilibrium coefficient of the neutral and charged forms of the compound. This allows the proportion of neutral and charged species at any pH to be calculated, as well as the basic or acidic properties to be defined. If the polyamides (Table 2.1) are in their associated form and are thought of as acid/base conjugates denoted H/Polymer, then apparent pK' and degree of dissociation (α) of each polymer can be

calculated using principles described by Bronsted (Lehinger, 1975). The tendency of an acid to dissociate is given by its thermodynamic dissociation constant K at a specific temperature where:

$K = [H^+][Polymer^-] / [HPolymer]$, where brackets indicate moles L^{-1} **Eqn. [2.1]**

When K is determined using analytical measurements such as concentration of reactants under a given set of experimental conditions it is denoted apparent dissociation constant, K′, in order to distinguish it from its thermodynamic dissociation constant, K. The logarithmic transformation of K′ is p*K*′ and given by:

$pK' = -\log K'$ **Eqn. [2.2]**

The Hendersson-Hasselbach equation also states:

$pK' = pH + \log ([HA]/[A^-])$ **Eqn. [2.3]**

Substituting HPolymer and $Polymer^-$ for HA and A^- gives,

$pK' = pH + \log ([HPolymer]/[Polymer^-])$ **Eqn. [2.4]**

The pH values of dissolved polymers with increasing addition of HCl were determined experimentally using a pH meter. If the concentration of HPolymer is equal to 1-α, then degree of polymer dissociation is α. That is,

$HPolymer \leftrightharpoons H^+ + Polymer^-$ **Eqn. [2.5]**

is equal to,

$1-\alpha \leftrightharpoons \alpha + \alpha$ **Eqn. [2.6]**

From equation 2.6,

$$[\text{HPolymer}] = (1-\alpha)\,[\text{Polymer}]_o \qquad \textbf{Eqn. [2.7]}$$

where $[\text{Polymer}]_o$ indicates starting concentration of polymer.

$$[\text{Polymer}^-] = \alpha\,[\text{Polymer}]_o \qquad \textbf{Eqn. [2.8]}$$

In these experiments a known weight of polymer was dissolved in a known volume of a NaOH solution and subsequently made up to a known and increased volume using deionised water. Sequential volumes of HCl were added to this solution and the pH taken after each addition. Thus, the concentration of polymer, Na^+, Cl^-, OH^- and H^+ after each addition of HCl could be calculated.

By electronegativity,

$$[Na^+] + [H^+] = [Cl^-] + [OH^-] + [\text{Polymer}^-] \qquad \textbf{Eqn. [2.9]}$$

From equation 2.8 and 2.9,

$$([Na^+] + [H^+] - [Cl^-] - [OH^-]) = \alpha([\text{Polymer}]_o \times (V_o / V_{total})) \qquad \textbf{Eqn. [2.10]}$$

where V_o is the starting volume of polymer solution and V_{total} is the volume of polymer solution after each sequential addition of HCl solution. Therefore,

$$\alpha = ([Na^+] + [H^+] - [Cl^-] - [OH^-]) / [\text{Polymer}] \qquad \textbf{Eqn. [2.11]}$$

Thus, using the potentiometric data and molecular formulae (Table 2.1), α and apparent pK_a values could be calculated.

Table 2.1: Molecular formula and formula weight of polymers.

Polymer	Molecular formula	Formula weight of monomer
Poly(lysine *iso*-phthalamide)	$C_{14}H_{16}N_2O_4$	276
Poly(lysine dodecandiamide)	$C_{18}H_3N_2O_4$	340
Poly(lysine diethyl malonamide)	$C_{13}H_{22}N_2O_4$	270
Poly(lysine butyl malonamide)	$C_{13}H_{22}N_2O_4$	270
Poly(ornithine dodecandiamide)	$C_{17}H_{30}N_2O_4$	326
Poly(ornithine sebacamide)	$C_{15}H_{26}N_2O_4$	298
N,N-Diethylamine modified GMP	$C_{18}H_{31}NO_6$	357
N,N-Diethyl ethylene diamine modified GMP	$C_{20}H_{35}N_2O_6$	399
3-(Diethylamino)propylamine modified GMP	$C_{21}H_{38}N_2O_6$	414

GMP – Glycidyl methacrylate polymer

2.2: Results.

The pK_a values were plotted against both pH and α (Figures 2.22 to 2.39.). The data points for apparent pK_a against degree of dissociation for polyamides and amine modified glycidyl methacrylate polymers were plotted on Figure 2.40 and Figure 2.41, respectively. This was performed to determine if there was any relationship between structural properties of polymer and trend in their plots.

Figure 2.22: Variation of apparent pK_a of poly(lysine *iso*-phthalamide) with degree of dissociation.

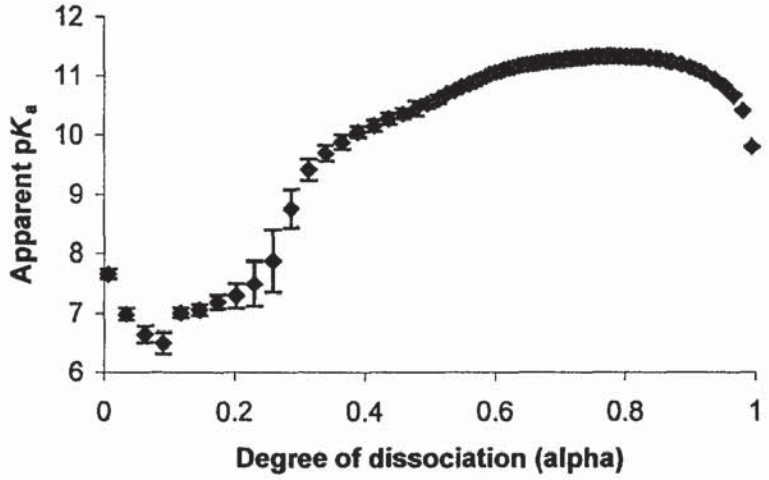


Figure 2.23: Variation of apparent pK_a of poly(lysine *iso*-phthalamide) with pH of polymer solution.

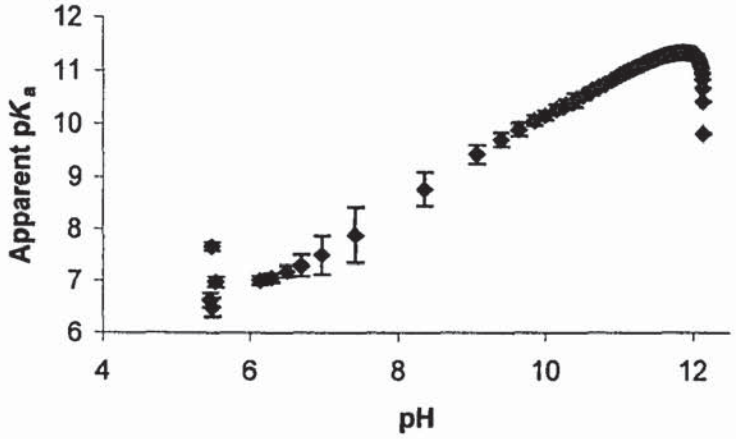


Figure 2.24: Variation of apparent pK_a of poly(lysine dodecandiamide) with degree of dissociation.

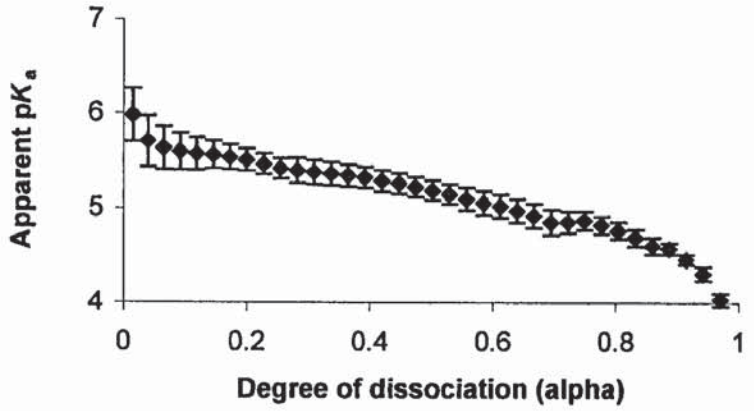


Figure 2.25: Variation of apparent pK_a of poly(lysine dodecandiamide) with pH of polymer solution.

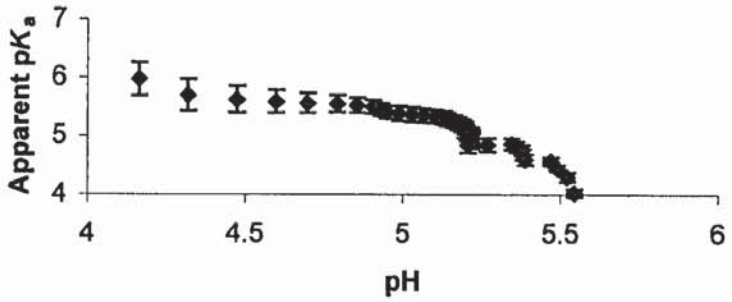


Figure 2.26: Variation of apparent pK_a of poly(lysine diethyl malonamide) with degree of dissociation.

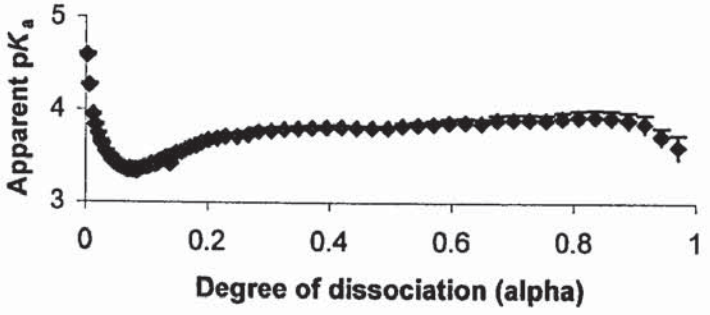


Figure 2.27: Variation of apparent pK_a of poly(lysine diethyl malonamide) with pH of polymer solution.

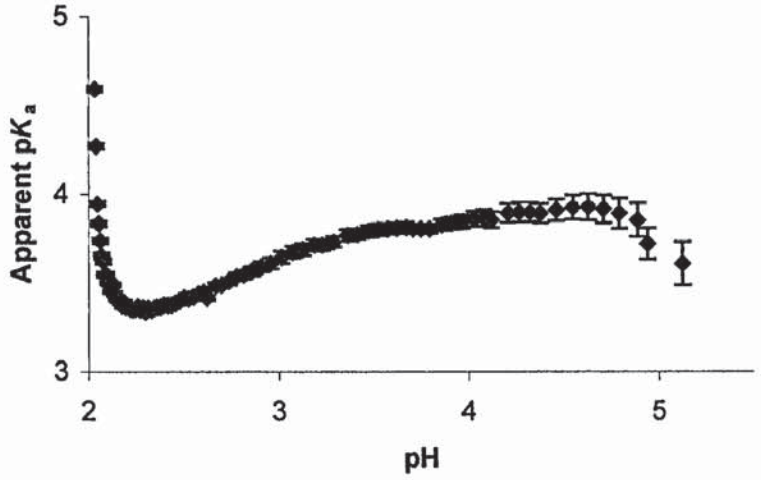


Figure 2.28: Variation of apparent pK_a of poly(lysine butyl malonamide) with degree of dissociation.

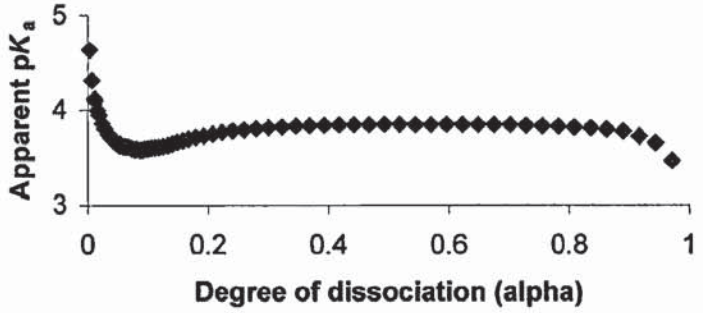


Figure 2.29: Variation of apparent pK_a of poly(lysine butyl malonamide) with pH of polymer solution.

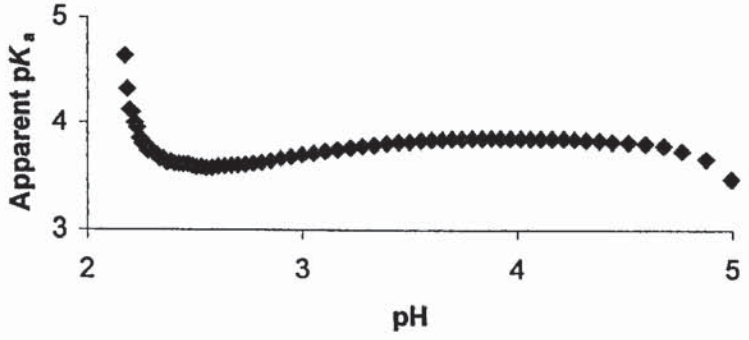


Figure 2.30: Variation of apparent pK_a of poly(ornithine dodecandiamide) with degree of dissociation.

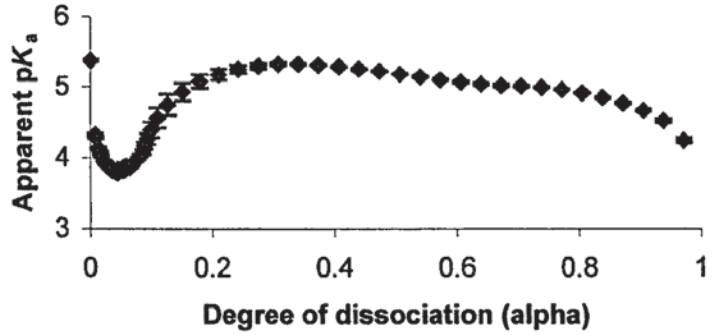


Figure 2.31: Variation of apparent pK_a of poly(ornithine dodecandiamide) with pH of polymer solution.

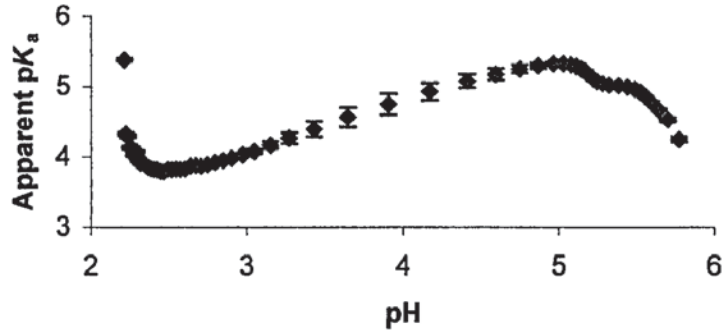


Figure 2.32: Variation of apparent pK_a of poly(ornithine sebacamide) with degree of dissociation.

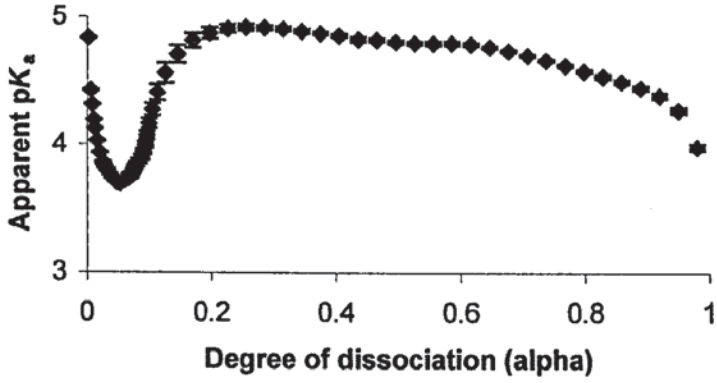


Figure 2.33: Variation of apparent pK_a of poly(ornithine sebacamide) with pH of polymer solution.

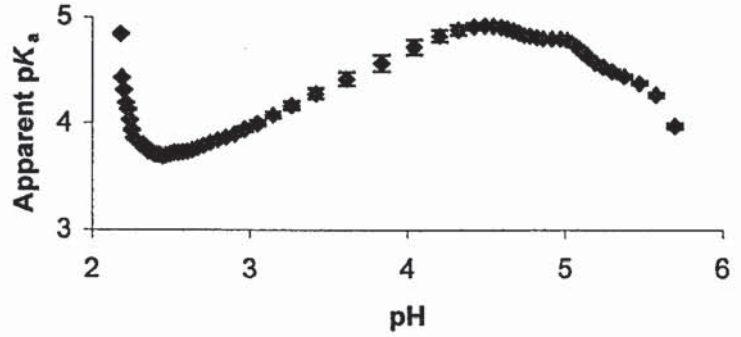


Figure 2.34: Variation of apparent pK_a of *N,N*-diethylamine modified glycidyl methacrylate polymer, with degree of dissociation.

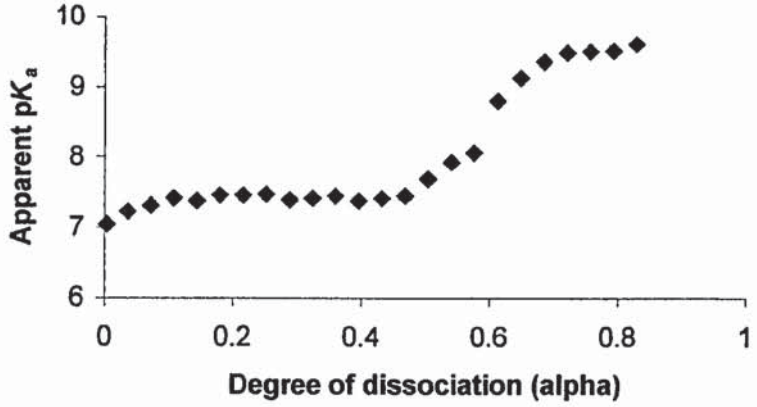


Figure 2.35: Variation of apparent pK_a of *N,N*-diethylamine modified glycidyl methacrylate polymer, with pH of polymer solution.

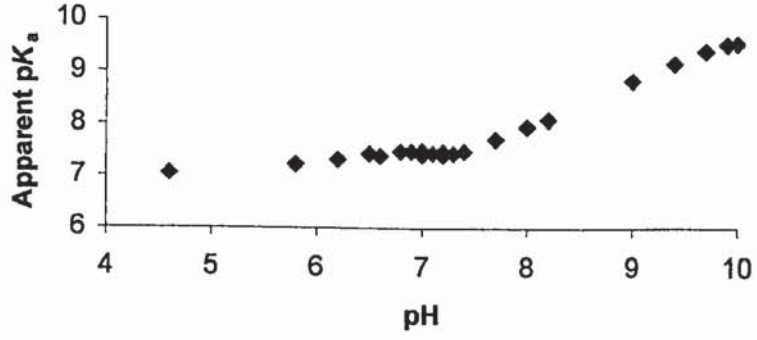


Figure 2.36: Variation of apparent pK_a of 3-(diethylamino)propylamine modified glycidyl methacrylate polymer, with degree of dissociation.

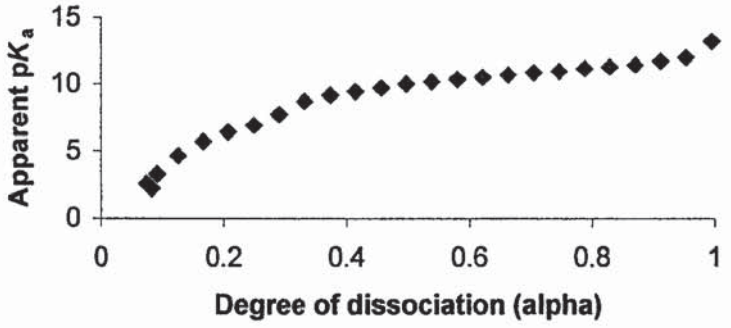


Figure 2.37: Variation of apparent pK_a of 3-(diethylamino)propylamine modified glycidyl methacrylate polymer, with pH of polymer solution.

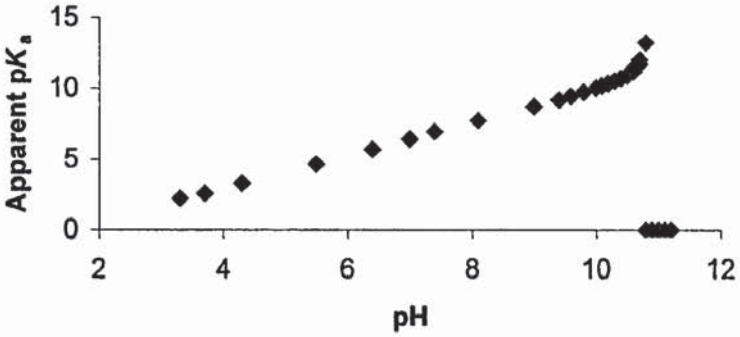


Figure 2.38: Variation of apparent pK_a of *N,N*-diethyl ethylene diamine modified glycidyl methacrylate polymer, with degree of dissociation.

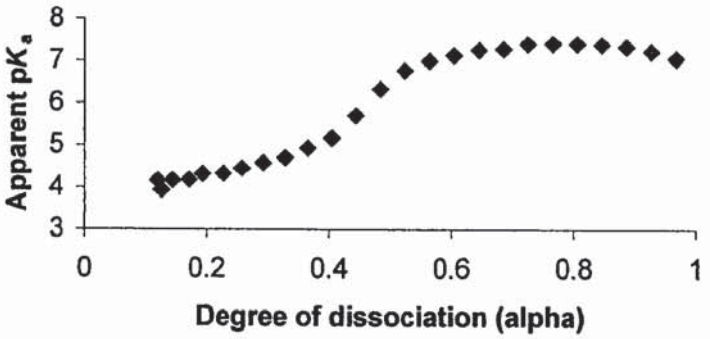


Figure 2.39: Variation of apparent pK_a of *N,N*-diethyl ethylene diamine modified glycidyl methacrylate polymer, with pH of polymer solution.

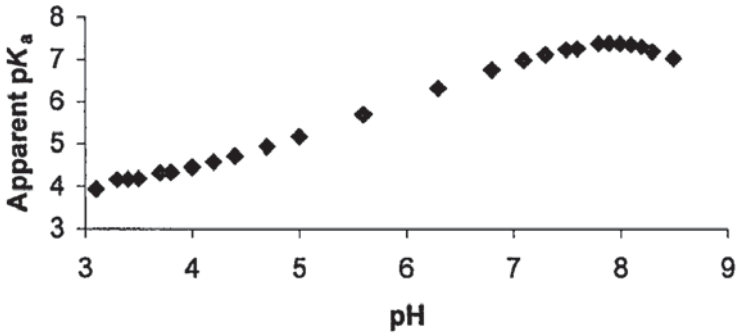


Figure 2.40:Variation of apparent pK_a of poly(lysine *iso*-phthalamide) ♦, poly(lysine diethyl malonamide) Δ, poly(lysine butyl malonamide) •, poly(ornithine sebacamide) □, poly(ornithine dodecandiamide) ■ and poly(lysine dodecandiamide) ○ with degree of dissociation.

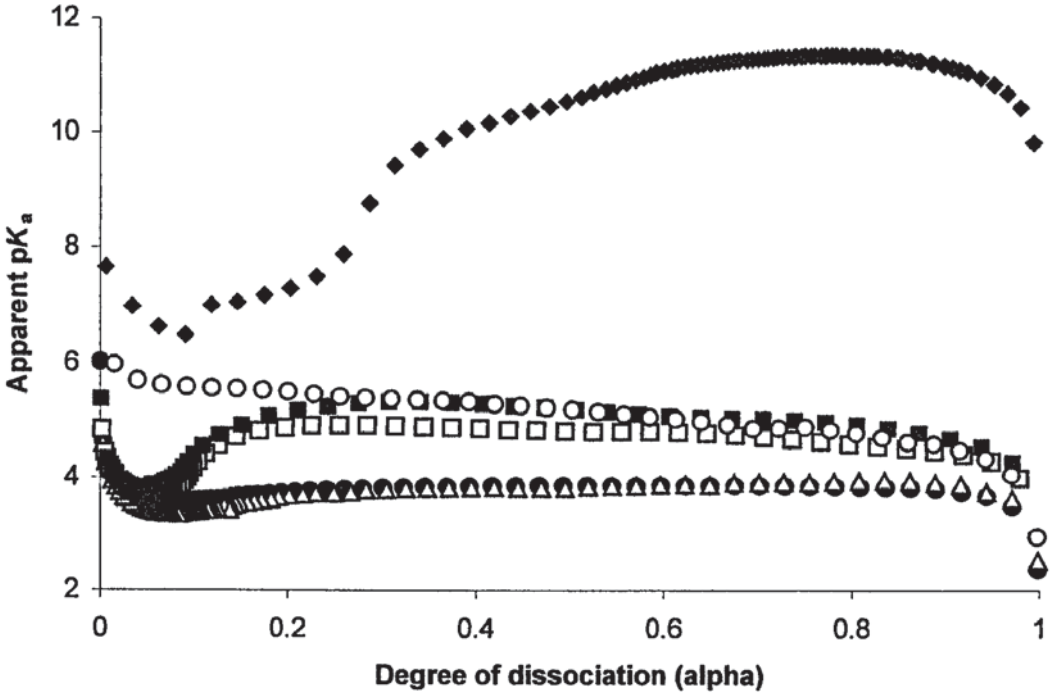
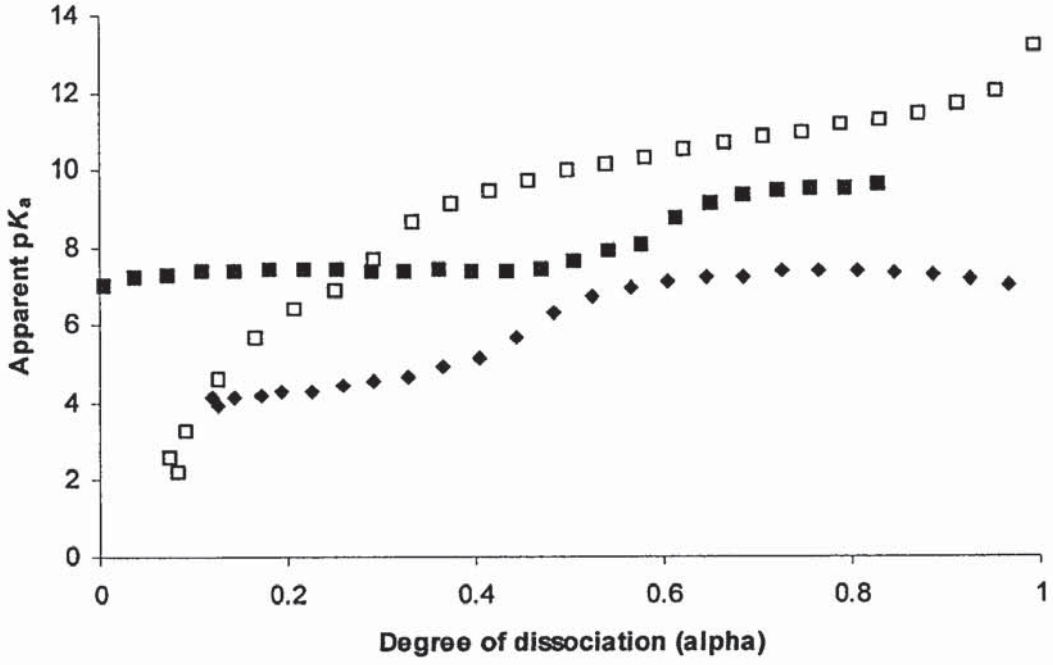


Figure 2.41: Variation of apparent pK_a of *N,N*-diethylamine ■, 3-(diethylamino)propylamine □ and *N,N*-diethyl ethylene diamine ♦ modified glycidyl methacrylate polymers with degree of dissociation.



2.3: Discussion.

2.3.1: Molecular weight of polyamides and amine modified glycidyl methacrylate polymers.

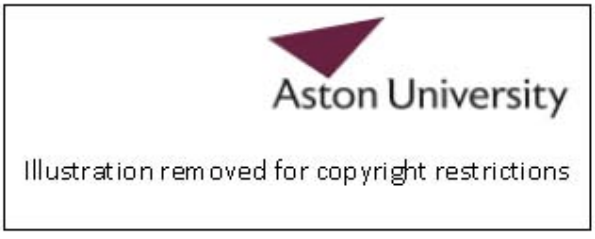
Relative to poly(ethylene glycol standards), the molecular weight of poly(lysine *iso*-phthalamide) and poly(glycidyl methacrylate) pre-polymer were found be 18160 Da and 35411 Da. It is thought the actual molecular weights of these polymers are likely to be higher than those analysed. That is, the low readings are thought to be a consequence of peak broadening due to retention of polymer on column material used to analyse polymer molecular weight. However, further analysis would be required to confirm this. Nevertheless, the analysed molecular weights of these polymers are comparable to molecular weights of polymers used for gene delivery, which are discussed in detail in Chapter 1. For example, low molecular weight poly(ethylenimine) has a molecular weight of approximately 25K Da. Low molecular weight PEI is highly efficient for gene therapy, although less efficient than high M_w

PEI. It is thought modifications to the molecular weight of polymers synthesised in this thesis could alter the properties that they exhibit, for example the conformational changes that they exhibit. Thus, adjustments in their molecular weight could be made to impart particular advantageous properties.

2.3.2: Potentiometric titrations.

As already discussed, potentiometric titrations can be used to determine pK_a and α. When plotted these values can be used to study the conformational changes of macromolecules (Tonge *et al.*, 2001; Nishio, 1998). Such that, deviations from the titration curve represent particular conformational changes in the macromolecule. For example, a typical potentiometric curve of an ionic polypeptide, such as poly(L-glutamic acid), was demonstrated by Nagasawa *et al.*, (1964) (Figure 2.42). They equated the deviations from a curve to particular conformational changes of the polypeptide. That is, coil formation, through a transitional stage, helix formation and finally aggregation.

Figure 2.42: Potentiometric titration curve of poly(L-glutamic acid) with conformational changes indicated (adapted from Nagasawa *et al.*, 1964).



The first group of polymers studied are classified as polyamides and are synthesised via an interfacial polycondensation reaction using amine and acid chloride groups. However, there are key differences in their structure. These differences include

variation in chain length of the aliphatic acid chloride groups, the use of an aromatic as opposed to aliphatic groups. Finally, variation in amine groups used. Since their structures are not vastly different it would be expected that the trends visualised on their titration curves would be very similar. It would be thought that any variations could be explained by the key differences in their structure. The second group of polymers are synthesised by amination of a glycidyl methacrylate polymer and thus only vary by the amine group used.

2.3.2.1: Polyamides.

The deviations from a curve to represent conformational changes shown in Figure 2.42 will be primarily used to describe the titration curves obtained for each polyamide and amine modified GMPs in turn. The similarities and differences in the plots for structurally similar polymers will then be discussed.

2.3.2.1.1: Poly(lysine iso-phthalamide).

It can be observed that the titration curve for poly(lysine *iso*-phthalamide) (Figure 2.22) takes the same general trend as poly(L-glutamic acid) (Figure 2.42). That is, pK_a values plotted at alpha values less than 0.1 show trends of aggregation or precipitation of polymer. The trends typical of helix formation can be observed at alpha values between 0.1 and 0.4. A possible transition stage is observed between alpha values of 0.4 and 0.8, although this is not as clear as what can be observed in the curve of poly(L-glutamic acid). Also, points that can be equated to coil formation are not observed.

2.3.2.1.2: Poly(lysine dodecandiamide).

If the titration curve for poly(lysine dodecandiamide) (Figure 2.24) is observed it can be seen that the trends that are apparent in Figure 2.42 are not obvious. However, an aggregation stage is possible at alpha values less than 0.2. The helix stage is not clear, however, it is thought a transitional stage occurs between alpha values 0.2 and 0.7. The blip observed at alpha values between 0.7 and 0.77 may represent coil formation.

2.3.2.1.3: Poly(lysine diethyl malonamide).

The trends seen on the titration curve plotted for poly(lysine diethyl malonamide) are more obvious (Figure 2.26). That is, aggregation occurs at alpha values less than 0.07. A helix stage occurs at alpha values between 0.07 and 0.23. A transitional stage occurs at alpha values between 0.23 and 0.67. It is thought a possible coil stage occurs at alpha values greater than 0.67.

2.3.2.1.4: Poly(lysine butyl malonamide).

The titration curve for poly(lysine butyl malonamide) (Figure 2.28) shows an aggregation stage at alpha values less than 0.08. A helix stage occurs at values between 0.2 and 0.8, however, a coil stage is not apparent.

2.3.2.1.5: Poly(ornithine dodecandiamide).

If the titration curve of poly(ornithine dodecandiamide) (Figure 2.30) is observed the conformational changes can be made out easily. That is, alpha values of less than 0.05 are typical of aggregation. A helix stage can be made out at alpha values between 0.05 and 0.24. A transitional stage is seen at alpha values between 0.22 and 0.77. Again a coil stage is not observed.

2.3.2.1.6: Poly(ornithine sebacamide).

The same general trends can be observed on the titration curve plotted for poly(ornithine sebacamide) (Figure 2.32) as were seen on the titration curve plotted poly(ornithine dodecandiamide). Thus, an aggregation stage is seen at alpha values less than 0.49. A helix stage is seen at alpha values between 0.049 and 1.96. A transitional stage is seen at alpha values between 0.196 and 0.64, while a coil stage is again not observed.

2.3.2.2: Amine modified glycidyl methacrylate polymers.

The trends observed on the titration plots of the polyamides are not as obvious for amine modified GMPs. However, if the titration curve of *N,N*-diethylamine modified GMP (Figure 2.34) is observed a transitional stage can be observed between alpha values of 0.2 and 0.8. Similarly, for alpha values greater than 0.4 for 3-(diethylamino)propylamine modified GMP (Figure 2.36). If the titration curve of *N,N*-diethyl ethylene diamine modified GMP (Figure 2.38) is observed a helix stage between 0.4 and 0.6 is apparent, with a transitional stage occurring at alpha values between 0.6 and 0.9.

2.3.3: Similarities and differences observed for polymers.

2.3.3.1: Polyamides.

Plots of degree of dissociation vs. apparent pK_a for poly(lysine diethyl malonamide) and poly(lysine butyl malonamide) are shown in figure 2.26 and 2.28, respectively. The polymers are very similar in structure since they both have the amine group, lysine. Also, their alkyl groups have the same number of carbon and hydrogen atoms. If the plots for these polymers are compared it can be seen they follow almost exactly the same trends, indicating replacement of butyl malonyl chloride with diethyl malonyl chloride has little effect.

Plots of degree of dissociation vs. apparent pK_a for poly(ornithine dodecandiamide) and poly(ornithine sebacamide) are shown in Figure 2.30 and 2.32, respectively. These two polymers are very similar in structure to each other since they both have ornithine as an amine group and have long alkyl groups, which only vary by two CH_2 groups. These structural similarities again explain why their plots follow almost exactly the same trends.

Poly(lysine diethyl malonamide) and poly(lysine butyl malonamide) vary structurally to poly(ornithine dodecandiamide) and poly(ornithine sebacamide) since they have different amine groups and also the length of the alkyl groups of the latter two

polymers is longer than the first two. If the plots of these sets of polymers are compared, that is Figures 2.26 and 2.28 compared with Figures 2.30 and 2.32 it can be seen there are differences in their plots. If the plots are compared to Figure 2.42 it can be seen the position on Figure 2.42 that is labelled as the helix position is steeper on Figures 2.30 and 2.32 compared to Figures 2.26 and 2.28. This potentially indicates that more profound structural changes may be occurring with poly(ornithine dodecandiamide) and poly(ornithine sebacamide) compared to poly(lysine diethyl malonamide) and poly(lysine butyl malonamide) with small changes in pH. Also, it can be observed that the position labelled as the transition stage on Figure 2.42 has a slight incline on Figures 2.26 and 2.28, whereas a slight decline is seen on Figures 2.30 and 2.32. Since the two sets of polymers vary by both their amine group and the length of their alkyl groups, it is not clear which is the causative factor in the variation of the plots.

A plot of degree of dissociation vs. apparent pK_a for poly(ornithine dodecandiamide) and poly(lysine dodecandiamide) is shown in Figures 2.30 and Figure 2.24, respectively. These plots can be used to show the differences caused by using a different amine group. That is, if the plot for poly(ornithine dodecandiamide) and poly(lysine dodecandiamide) are compared to that shown for poly(glutamic acid) (Figure 2.42), apart from the stage representing coil formation, each conformational stage can clearly be distinguished for both. However, the shift between stages is not as obvious for poly(lysine dodecandiamide) as discussed earlier. Figure 2.22 shows a plot of degree of dissociation vs. apparent pK_a for poly(lysine *iso*-phthalamide). This polymer is different to poly(lysine dodecandiamide) in that an aromatic acid chloride group *iso*-phthaloyl chloride is used in its synthesis as oppose to an aliphatic group, namely dodecanedioyl dichloride. If the plot for poly(lysine *iso*-phthalamide) is compared to that shown for poly(glutamic acid), unlike poly(lysine dodecandiamide) the trends indicative of conformational changes can be clearly distinguished. Thus the trends observed for poly(lysine *iso*-phthalamide) are closer to those observed for poly(ornithine dodecandiamide) and poly(ornithine sebacamide) rather than those observed for poly(lysine dodecandiamide). However, the plot for poly(lysine *iso*-phthalamide) does have differences to poly(ornithine dodecandiamide) and poly(ornithine sebacamide) in that the point indicative of a transitional stage starts at a higher alpha value. The aggregation stage for poly(lysine *iso*-phthalamide) is also not

so steep and occurs over a wider range of alpha values compared to those of poly(ornithine dodecandiamide) and poly(ornithine sebacamide).

2.3.3.2: Amine modified GMPs.

Degree of dissociation vs. apparent pK_a for *N,N*-diethylamine, 3-(diethylamino)propylamine and *N,N*-diethyl ethylene diamine modified GMPs is shown in Figures 2.34, 2.36 and 2.38, respectively. It was expected that since they only vary by the attached amine group they would have shown very similar trends. However, if the plots are compared it can be seen they vary considerably. While a transition stage can be made out for all three polymers, the degree of dissociation over which this occurs is different. Also, only *N,N*-diethyl ethylene diamine modified GMP has a visible helix stage. None of the polymers have visible aggregation or coil formation stages.

2.4: Conclusions.

The aim of this chapter was to produce polymers that showed conformational changes in response to pH. In order to achieve this polyamides and amine modified glycidyl methacrylate polymers were synthesised since the structure of these polymers potentially enables hydrophobic association with pH change. The molecular weight of these polymers was determined. In addition, potentiometric titrations were performed for these polymers to determine their pK_a values and study their conformational changes.

The molecular weight of poly(lysine *iso*-phthalamide) was determined and used to represent the molecular weight of polyamides synthesised in this thesis. The molecular weight was analysed as 18160 Da. The molecular weight of glycidyl methacrylate pre-polymer was determined and found to be 35411 Da. It is thought the actual molecular weight of these polymers is higher than the analysed values. That is, it is thought the methodology used did not give a true indication of the polymers molecular weight. However, further analysis would be required to confirm this. It is important that the molecular weight information is gained since by adjusting the

polymers molecular weight the conformational changes that polymers exhibit can be altered and improved for particular applications.

Potentiometric titrations were used to plot pK_a values against degree of dissociation. These plots were used to study the conformational changes of polymers. A range of polyamides were synthesised using varying amine groups and acid chloride groups, which exhibited a variation in their conformational changes. It was concluded that polyamides that had the same amine groups and also alkyl groups of similar length exhibited the same conformational trends. It was noted trends, which indicated more profound conformational changes were exhibited for polyamides with the amine group ornithine, if a short alkyl group was replaced for a longer alkyl group. However, replacing ornithine with lysine in polyamides with a long alkyl group also caused a dramatic difference in conformational trends. That is, transitions between conformational changes were not as obvious for the polyamide with lysine groups. Finally, replacing an alkyl group for an aromatic group in a polyamide with the amine group, lysine group shows conformational changes similar to polyamides with the amine group ornithine and a long alkyl group. However, the degree of dissociation at which these changes occurred varied.

A second group of polymers, amine modified glycidyl methacrylate polymers, only varied in their attached amine group. Nevertheless, when their apparent pK_a was plotted against degree of dissociation they exhibited quite different trends.

Thus, it was observed for both sets of polymers that small structural alterations caused dramatic changes to the conformational trends the polymers exhibited. It was thought that the possible conformational changes occurring as a function of pH, indicated on the potentiometric titration plots could have pH-responsive membrane disruption applications. Thus, giving motivation to the following two chapters.

Chapter 3

Disruption of erythrocyte and microbial cell membranes using polyamides

3.0: Aims and Overview.

Aim 1: To determine whether polymers, with alternating anionic and hydrophobic functional groups that were studied in Chapter 2 and show pH-responsive conformational changes, can be used to disrupt cell membranes as a function of pH.

In Chapter 2 polymers that have alternating charged and hydrophobic functional groups were synthesised. A range of polymers were synthesised that had small structural variations. These polymers included poly(lysine butyl malonamide), poly(lysine diethyl malonamide), poly(lysine *iso*-phthalamide), poly(lysine dodecandiamide), poly(ornithine dodecandiamide) and poly(ornithine sebacamide).

Potentiometric titrations were used to study the conformational changes of these polymers. It was concluded that the small structural changes had dramatic effects on their pH-responsive conformational changes. Such conformational changes have a potential application to disrupt membranes as already discussed in Chapter 2. It would be expected that structurally similar polymers would show similar erythrocyte disruption properties.

Erythrocytes are relatively cheap and when lysed release their contents, which contain the pigment haemoglobin. Haemoglobin can be measured using a light spectrophotometer when set at certain wavelengths. Therefore, it was thought that using light spectrophotometry to measure haemoglobin release from erythrocytes could be a potentially useful way to quantify the pH-responsive membrane disruption properties of polymers. In summary, this was achieved by pre-incubating each of the polymers used in Chapter 2 with a suspension of erythrocytes. Acid was titrated in to this suspension to reduce the pH value of the suspension. The release of haemoglobin was then measured using light spectrophotometry at each pH value. Maximum release of haemoglobin from erythrocytes at each pH value was determined by exposing an

aliquot of the polymer-erythrocyte solution to either Triton X100 or a freeze-thaw process. As a control, haemoglobin release in the absence of polymer was also determined. That is, erythrocytes were pre-incubated with buffer solution. Acid was again titrated in to the solution. Released haemoglobin was measured at the same pH values as erythrocytes in the presence of polymer. Maximum release of haemoglobin from this suspension of erythrocytes at each pH value was again determined by exposing the buffer-erythrocyte solution to either Triton X100 or a freeze-thaw process. It was expected that if released haemoglobin from an erythrocyte-buffer solution and an erythrocyte-buffer solution exposed to Triton X100 or freeze-thaw were plotted against pH, the trend shown in Figure 3.1 would be observed. It was also expected that a similar trend would be observed for erythrocytes in the presence of polymer and erythrocytes in the presence of polymer exposed to Triton X100 or freeze-thaw. However, in addition it would be expected that at a certain pH value a peak in absorbency would be seen for erythrocytes in the presence of polymer compared to erythrocytes alone. This expected peak is where a conformational change in the polymer takes place leading to the disruption of erythrocyte membranes causing a sudden release of haemoglobin. As can be seen from Figure 3.1 the theoretical absorbencies of erythrocytes and erythrocytes plus polymer, exposed to Triton X100 or freeze-thaw should be at their maximum at the start of the experiment. The addition of HCl to erythrocytes and erythrocyte plus polymer solution to cause a reduction in pH should decrease the absorbency readings as erythrocytes become increasingly diluted. Therefore, it would be expected that prior to lysis of erythrocytes, observed as an increase in absorbency readings, a decrease in absorbency would be observed due to a dilution effect.

If the results shown in Figure 3.1 are used to calculate percentage erythrolysis with reducing pH, where maximum erythrolysis is taken as the absorbency of erythrocytes exposed to Triton X100 or freeze-thaw, the trends observed in Figure 3.2 would be observed.

Although not shown on Figure 3.2 it is expected that a similar trend would be observed with reducing pH for erythrocytes in the presence of polymer. However, as previously explained, it would be expected at a certain pH value a peak in percentage erythrolysis would be seen for erythrocytes in the presence of polymer compared to

erythrocytes alone. The plot of percentage erythrolysis of erythrocytes and erythrocytes in the presence of polymer should show a continual increase with reducing pH until maximum erythrolysis is achieved. The plot should then plateau since when maximum erythrolysis has been achieved, no further erythrolysis can occur.

Figure 3.1: Theoretical release of haemoglobin from erythrocytes ■ and erythrocytes exposed to Triton X100 or a freeze-thaw process □.

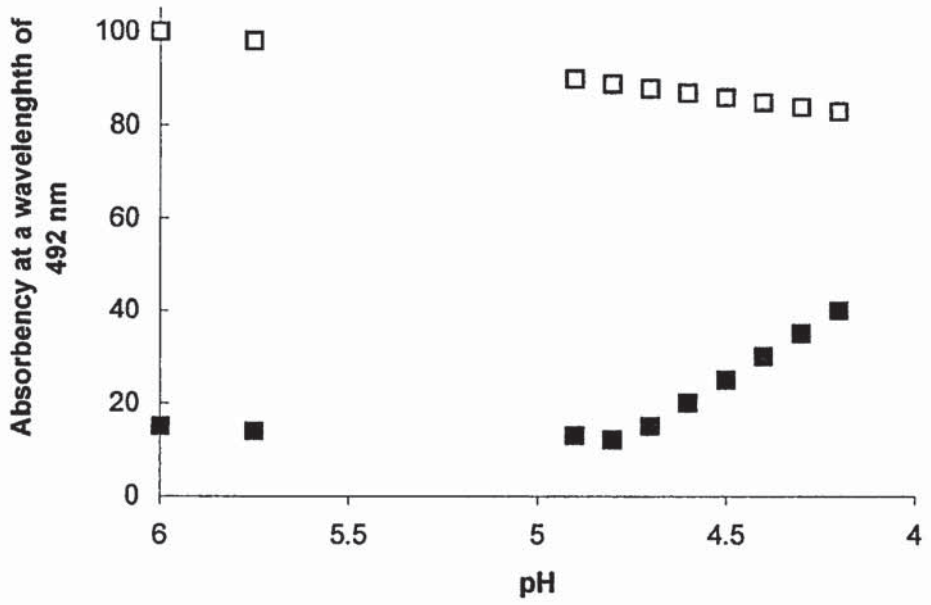
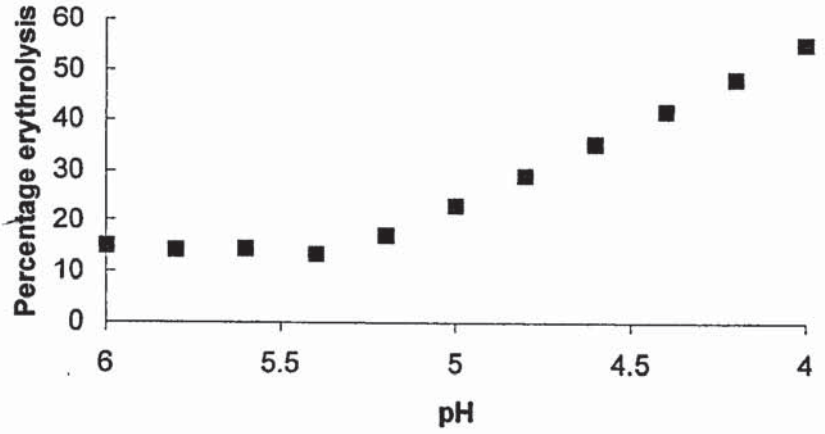


Figure 3.2: Percentage erythrolysis ■ with reducing pH.



The exact methodology used to determine percentage erythrolysis in the presence of each of the polymers will be explained in Section 3.1.1. The results of these experiments showed when percentage erythrolysis in the presence of polymer was calculated and plotted, anomalous values were observed at certain pH values. That is, certain values appeared to be greater than 100%. Since this is not actually possible it was attempted to clarify the reasoning for these anomalous readings. A plot similar to Figure 3.1 was produced and additional experiments were performed to help achieve this.

Firstly, an experiment was used to determine the effect of pH on absorbency readings of each constituent of the experiment. These constituents included polymer dissolved in a buffer solution, haemoglobin, haemoglobin treated with Triton X100, haemoglobin exposed to freeze-thawing, haemoglobin plus polymer dissolved in a buffer solution, haemoglobin plus polymer dissolved in a buffer solution then treated with Triton X100 and finally haemoglobin plus polymer dissolved in a buffer solution exposed to freeze-thaw. This is explained fully in Section 3.1.2. The second experiment assessed whether a wavelength of 492 nm, which was used to measure released haemoglobin was suitable at the full range of pH values used. This is also fully explained in Section 3.1.3. Finally, the effect of Triton X100 concentration on the absorbency of haemoglobin was determined and is discussed in Section 3.1.4.

Aim 2: To determine whether the polymers used in Chapter 2 have anti-microbial properties by disturbing the microbial cell membrane as a function of pH.

To assess the anti-microbial properties of polymers, three main types of microbes were chosen that represented a range of microbes that have similar properties. These microbes included *Candida albicans*, *Escherichia coli* and *Staphylococcus aureus*. *Candida albicans* was chosen to represent the yeasts. Yeasts are surrounded by a cell wall. This cell wall is a rigid but dynamic structure essential for their viability. *E. coli* was chosen to represent gram negative bacteria. *E. coli* are enteric bacteria that are facultatively anaerobic gram negative rods and part of the *Enterobacteriacae* family. *Staph. aureus*, which are gram positive cocci were chosen to represent gram positive bacteria. The cell wall of gram positive and gram negative bacteria vary in their structure, which is more clearly indicated in Figure 3.3. It is thought there might be a

variation in the properties of these microbial groups as a result of a variation between differences in their cell wall structure.

Minimum inhibitory concentration (MIC) experiments were used to determine whether polymers used in Chapter 2 have anti-microbial properties. The methodology of which is described fully in Section 3.1.5. It has been well documented that the polypeptide poly(L-lysine) exhibits anti-microbial properties exerted via pore formation in microbial cell membranes (Pollock *et al.*, 1984; Blondelle *et al.*, 1992; Perez-Paya *et al.*, 1995; Helmerhorst *et al.*, 1997). It is demonstrated in this chapter that possible conformational changes in polymer cause membrane disruption of erythrocytes. Therefore, it was thought they might also have an application as bactericidal or fungicidal agents, by disruption of the prokaryotic cell/plasma membrane (Figure 3.4) in response to pH change. An anti-microbial polymer might provide an alternative to current practices. For example, quaternary ammonium salts of low molecular weight are extensively used as disinfectants or biocidal coatings to prevent growth of micro-organisms on the surface of materials such as antifouling paints. Protection is achieved by leaching of bioactive molecules from the coating. However, this has disadvantages including harmful effects on the environment and short-lived protection due to difficulty in controlling leaching rate. Kenawy *et al.*, (1998) suggested these problems could be overcome by anchoring the toxic compound via a polymer backbone by covalent non-hydrolysable bonds or direct attachment of a polymer possessing anti-microbial activity.

Figure 3.3: Structure of the cell wall of Gram positive and Gram negative bacteria.

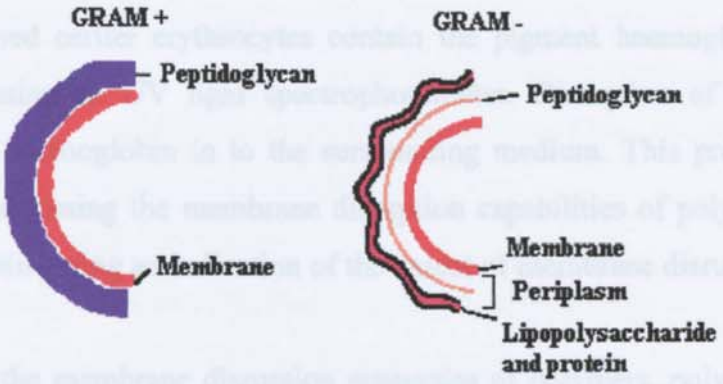
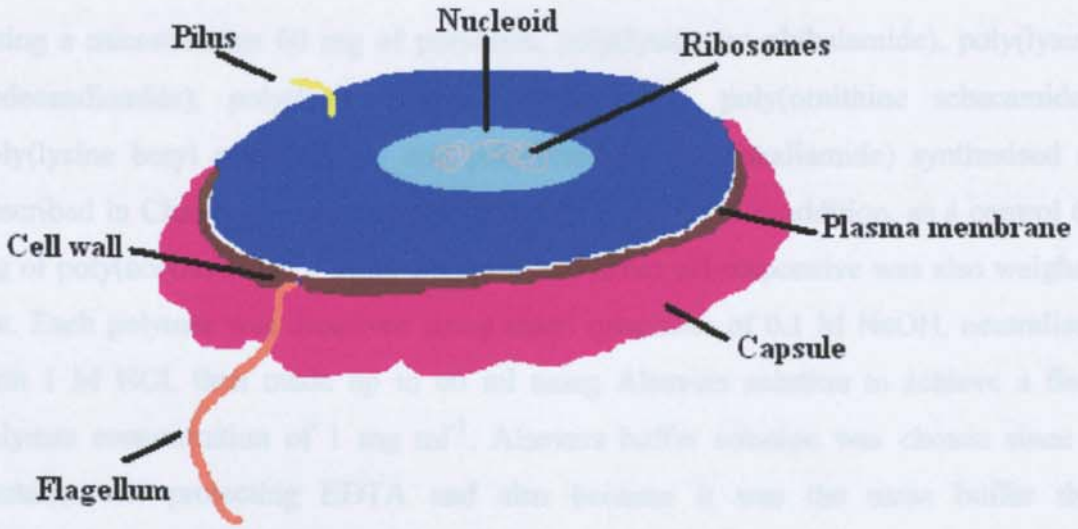


Figure 3.4: Diagrammatic representation of a bacterial cell.



3.1: Methods.

3.1.1: Disruption of erythrocyte membranes at a range of pH values in the presence and absence of polymers.

As explained earlier erythrocytes contain the pigment haemoglobin, which can be detected using an UV light spectrophotometer. Disruption of erythrocytes causes release of haemoglobin in to the surrounding medium. This property is a potential means of assessing the membrane disruption capabilities of polymers, with released haemoglobin giving an indication of the extent of membrane disruption.

To assess the membrane disruption properties of polymers, polymer and erythrocyte solutions were prepared, then pre-incubated together before adjusting their solution pH to a range of values. Haemoglobin release was measured using an UV spectrophotometer to indicate extent of membrane disruption.

3.1.1.1: Preparation of polymer solutions.

Using a microbalance 60 mg of polymers, poly(lysine *iso*-phthalamide), poly(lysine dodecandiamide), poly(lysine diethyl malonamide), poly(ornithine sebacamide), poly(lysine butyl malonamide) and poly(ornithine dodecandiamide) synthesised as described in Chapter 2, were weighed in to glass beakers. In addition, as a control 60 mg of poly(acrylic acid), a linear polymer that is not pH-responsive was also weighed out. Each polymer was dissolved using small quantities of 0.1 M NaOH, neutralised with 1 M HCl, then made up to 60 ml using Alsevers solution to achieve a final polymer concentration of 1 mg ml^{-1}. Alsevers buffer solution was chosen since it contains cell protecting EDTA and also because it was the same buffer that erythrocytes were received in.

3.1.1.2: Erythrocyte preparation.

Defibrinated sterile sheep blood was received in glass bottles suspended in Alsevers solution (TCS, Biosciences, Buckingham). Before use bottles of blood were gently

rolled across a flat surface to obtain a homogenous solution. To remove any remaining serum proteins and platelets and obtain a pure solution of erythrocytes a series of washing steps were applied to the blood solution. The initial washing step was performed by placing 6 ml volumes of blood in to 50 ml centrifuge tubes, centrifuging for ten minutes at 1500 revolutions per minute (rpm), then removing the obtained supernatant. Further washing steps were performed by re-suspending the blood pellet in a 6 ml volume of Alsevers solution, centrifuging for ten minutes at 1500 rpm, then removing the obtained supernatant. This process was repeated three times until a clear supernatant was obtained.

3.1.1.3: pH adjustment of polymer-erythrocyte solutions.

Each polymer solution (60 ml) was transferred to a glass beaker using a pipette. Some of the 60 ml volume of polymer solution was used to re-suspend the washed erythrocyte pellet, which was also transferred to the glass beaker. With constant mixing a titra-lab titration machine was used to transfer a continual flow of 0.1 M HCl to the beaker. When the solution reached pH values 6.0, 5.75, 5.5, 5.0, 4.9, 4.8, 4.7, 4.6, 4.5, 4.4, 4.3, 4.2, eight 200 µl volumes were transferred to one clear round-bottomed micro titre plate and four 200 µl volumes were transferred to a second plate (Figure 3.5).

When the titration process was complete, to each of the bottom 48 wells of plate one (Figure 3.5), 20 µl of a 10% v/v mixture of Triton X100 in Alsevers solution was added. This was performed to completely lyse erythrocytes. So that all wells remained at the same volume 20 µl of Alsevers solution was added to each of the top 48 wells of plate one and each of the top 48 wells of plate two. The second plate was placed in to a minus 80° C freezer. The contents of the wells were allowed to freeze and thaw three times to completely lyse erythrocytes by an alternative method. The freeze-thaw process was used as an alternative to Triton X100 addition since it has been reported that Triton X100 can affect fluorescence readings (Hamity *et al.*, 2000; Ceron *et al.*, 1999). Thus, it was expected any anomalies due to Triton X100 would be made apparent from the results when the freeze-thaw method was used. Plates one and two were then transferred to a bench centrifuge and centrifuged at 4000 rpm for 4 minutes.

This was performed to pellet any solid material and intact erythrocytes at the bottom of the wells. The obtained supernatant in each well containing any released haemoglobin due to erythrolysis was then transferred to flat-bottomed micro titre plates. The absorbency of each well was determined by placing the flat-bottomed micro titre plate in to an UV absorbency plate reader set at 492 nm, giving quadruplicate readings at each pH value.

Figure 3.5: Diagrammatic representation of micro titre plate with pH at which 200 µl volumes transferred indicated.

Plate One

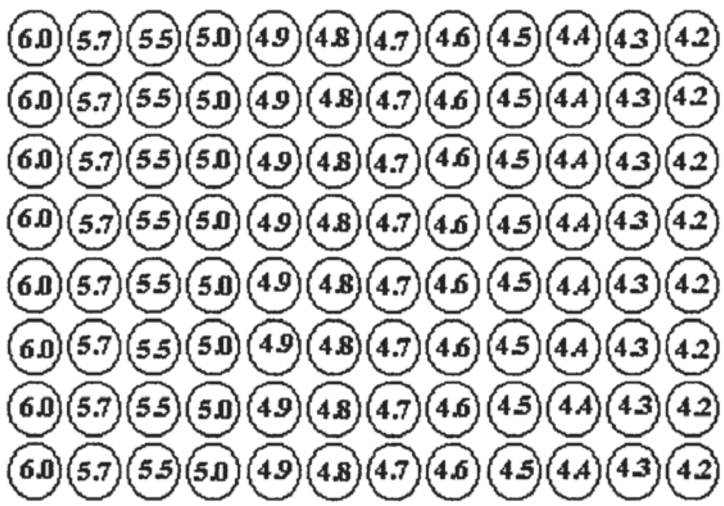
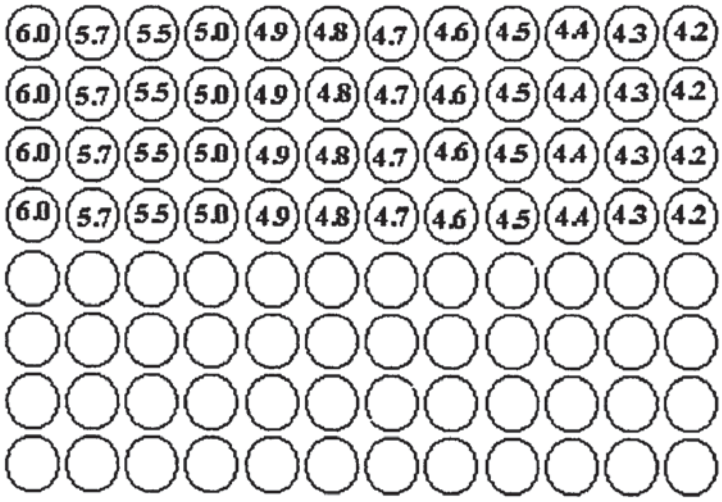


Plate Two



The mean percentage lysis of erythrocytes in the presence of polymer at each pH was determined by using the following equation:

$$[A /(B \text{ or } C)] \times 100,$$

where,

A = Mean absorbency of supernatant that was removed from centrifuged polymer-erythrocyte solution at the appropriate pH.

B = Mean absorbency of supernatant that was removed from centrifuged polymer-erythrocyte solution at the appropriate pH and lysed by Triton X100.

C = Mean absorbency of supernatant that was removed from centrifuged polymer-erythrocyte solution at the appropriate pH and lysed by applying a freeze-thaw process.

The results of these calculations were plotted as relative erythrolysis against pH of solution for each of the polymers (Figure 3.7 to Figure 3.13). The reasoning behind plotting pH of solution against relative erythrolysis as oppose to percentage erythrolysis is explained in section 3.3.1.

The mean percentage erythrolysis of erythrocytes in the presence of buffer was determined using the same procedures already described in this section except, instead of the erythrocyte pellets being initially re-suspended with polymer solution, they were re-suspended with Alsevers solution (60 ml). The absorbency for supernatant removed from erythrocytes, erythrocytes exposed to Triton X100 and erythrocytes exposed to freeze-thaw, at reducing pH values is plotted in Figure 3.6. Using the results of this plot percentage lysis of erythrocytes could be calculated as described in the calculation above and the results are presented in Table 3.1.

3.1.2: Determination of the effect on absorbency readings with reducing pH on each constituent of the erythrolysis experiment.

The results obtained for the effect of reducing pH in the presence of specific polymers on total erythrolysis gave certain anomalies. For example, erythrolysis values were sometimes greater than one hundred percent. As this is not possible, an assessment of the absorbency readings for each interacting constituent of the erythrolysis experiment with reducing pH was performed. The absorbency readings with reducing pH for each of the constituents listed below was determined:

- Poly(lysine dodecandiamide) dissolved in Alsevers solution.
- Haemoglobin.
- Haemoglobin which is then treated with 10% Triton X100.
- Haemoglobin which is then exposed to freeze-thawing.
- Haemoglobin plus poly(lysine dodecandiamide) dissolved in Alsevers solution.
- Haemoglobin plus poly(lysine dodecandiamide) dissolved in Alsevers solution which is then treated with 10% Triton X100.
- Haemoglobin plus poly(lysine dodecandiamide) dissolved in Alsevers solution which is then exposed to freeze-thaw.

3.1.2.1: Absorbency with reducing pH of poly(lysine dodecandiamide), poly(lysine dodecandiamide) treated with Triton X100 and poly(lysine dodecandiamide) exposed to freeze-thaw.

60 ml of a 1 mg ml^{-1} poly(lysine dodecandiamide) in Alsevers solution was prepared and transferred to a glass beaker. With constant mixing a titra-lab titration machine was used to transfer a continual flow of 0.1 M HCl to this beaker. When the solution reached pH values 6.0, 5.75, 5.5, 5.0, 4.9, 4.8, 4.7, 4.6, 4.5, 4.4, 4.3 and 4.2 eight 200 µl volumes were transferred to one round-bottomed micro titre plate and four 200 µl volumes were transferred to another round-bottomed micro titre plate. To each well of the top four horizontal rows of the first micro titre plate, 20 µl of Alsevers solution was transferred. To each of the wells of the bottom four horizontal rows of the first micro titre plate, 20 µl of 10% Triton X100 was transferred.

To each well of the top four horizontal rows of the second plate 20 µl of Alsevers solution was transferred. This plate was then exposed to freeze-thawing a total of three times. Both plates were then centrifuged at 4000 rpm for 4 minutes. From each well 150 µl of the obtained supernatant was transferred to the corresponding well of a flat-bottomed micro titre plate. The absorbency of each well was determined by placing the flat- bottomed micro titre plate in to an UV absorbency plate reader set at 492 nm. The absorbency results are plotted as pH against absorbency (Figure 3.14).

3.1.2.2: Absorbency with reducing pH of haemoglobin, haemoglobin treated with Triton X100 and haemoglobin exposed to freeze-thaw.

Haemoglobin in Alsevers solution was prepared by washing 6 ml of sheep blood in Alsevers solution, as described in section 3.1.1.2. The obtained pellet was re-suspended in 6 ml of Alsevers solution, which was then exposed to freeze-thaw three times. The lysed blood was then transferred to a 50 ml centrifuge tube and centrifuged at 4000 rpm for four minutes. 6.5 ml of the supernatant (haemoglobin in Alsevers solution) was transferred to a glass beaker and made up to 60 ml using fresh Alsevers solution. With constant stirring 0.1 M HCl was transferred to the beaker. When the solution reached pH values 6.0, 5.75, 5.5, 5.0, 4.9, 4.8, 4.7, 4.6, 4.5, 4.4, 4.3, 4.2 eight 200 µl volumes were transferred to one round-bottomed micro titre plate and four 200 µl volumes were transferred to another. Then to each well of the top four horizontal rows, 20 µl of Alsevers solution was transferred. To each well of the bottom four horizontal rows, 20 µl of a 10% v/v Triton X100 in Alsevers solution was transferred. To the second plate, each well of the top four horizontal rows had a 20 µl volume of Alsevers solution transferred. This plate was then exposed to freeze-thawing a total of three times. Both plates were then centrifuged at 4000 rpm for 4 minutes. From each well 150 µl of the obtained supernatant was transferred to the corresponding well of a flat-bottomed micro titre plate and the absorbency of each well was determined and again plotted as pH against absorbency (Figure 3.15).

3.1.2.3: Absorbency with reducing pH of haemoglobin plus poly(lysine dodecandiamide), haemoglobin plus poly(lysine dodecandiamide) treated with 10% Triton X100 and haemoglobin plus poly(lysine dodecandiamide) exposed to freeze-thawing.

Haemoglobin and poly(lysine dodecandiamide) in Alsevers solution was prepared by washing a 6 ml volume of sheep blood in Alsevers solution as described in Section 3.1.1.2. The obtained pellet was re-suspended in 6 ml of Alsevers solution, then exposed to freeze-thawing three times. The lysed blood was then transferred to a 50 ml centrifuge tube and centrifuged at 4000 rpm for four minutes. 6.5 ml of the supernatant was removed and transferred to 53.5 ml of Alsevers solution with 60 mg of dissolved poly(lysine dodecandiamide). The haemoglobin and poly(lysine dodecandiamide) in Alsevers solution was transferred to a glass beaker. Again with constant stirring 0.1 M HCl was transferred to the beaker. When the solution reached pH values 6.0, 5.75, 5.5, 5.0, 4.9, 4.8, 4.7, 4.6, 4.5, 4.4, 4.3, 4.2 eight 200 µl volumes were transferred to one round-bottomed micro titre plate and four 200 µl volumes were transferred to another. Then each well of the top four horizontal rows had 20 µl of Alsevers solution transferred. To each of the wells of bottom four horizontal rows, 20 µl of 10% v/v Triton X100 in Alsevers solution was transferred. To the second plate again to each well of the top four horizontal rows, 20 µl of Alsevers solution was transferred. This plate was then exposed to freeze-thawing a total of three times. Both plates were then centrifuged at 4000 rpm for 4 minutes. From each well 150 µl of the obtained supernatant was transferred to the corresponding well of a flat-bottomed micro titre plate and the absorbency of each well was determined and plotted (Figure 3.16).

3.1.3: Analysis of the ultra violet spectrum of erythrocytes with poly(lysine dodecandiamide) before and after addition of hydrochloric acid.

In the previous investigations it was explained that with decreasing pH, total erythrolysis in the presence of a polymer was greater than total erythrolysis in the absence of that particular polymer (Section 3.1.1). However, at lower pH values, percentage erythrolysis values in the presence of polymers were greater than 100%.

This is not actually possible since further erythrolysis cannot occur once total erythrolysis has been achieved. From the results it was observed that these anomalies were due to a decrease in the denominator value (the value used to represent total erythrolysis), rather than an increase in the numerator (the value used to represent erythrolysis in presence of polymer). It was thought that this could have been because the plate reader being set at a wavelength of 492 nm was inappropriate to read the absorbency both at high and low pH values. For example, a low pH could result in a reduction of haemoglobin absorbency readings. To determine if this was true a scan of the ultra-violet spectrum of erythrocytes in the presence of poly(lysine dodecandiamide) before and following addition of HCl was obtained. This was undertaken by preparing a 60 ml volume of a 1 mg ml^{-1} poly(lysine dodecandiamide) in Alsevers solution then transferring this to a glass beaker. As described in Section 3.1.1.2, 6 ml of sheep blood in Alsevers was washed and the obtained pellet re-suspended using polymer solution. A 750 µl volume of this solution was transferred to three Eppendorfs (2 ml volume). To the first Eppendorf, 75 µl of Alsevers was also transferred and the contents freeze-thawed three times. To the second Eppendorf, 75 µl of a 10% v/v Triton X100 in Alsevers solution was transferred. Finally, the remaining Eppendorf had 75 µl of Alsevers solution transferred. All tubes were then centrifuged at 4000 rpm for 4 minutes. A 500 µl volume of the obtained supernatants were transferred to separate cuvettes and made up to 1 ml using Alsevers solution. After setting the UV spectrophotometer to scan between a wavelength range of 525 nm and 375 nm and using Alsevers solution as a blank the absorbency readings for each cuvette at the wavelengths 405 nm, 431 nm, 450 nm and 492 nm were recorded. Using Alsevers solution as a diluent, the contents of the cuvettes were diluted by a factor of 2 a further four times, taking absorbency readings for each wavelength at each dilution.

Absorbency readings were obtained at a pH of 4.2 by transferring a continual flow of a 0.1 M HCl solution in to the beaker with constant mixing using a titra-lab titration machine. When the mixture reached pH 4.2, three 750 µl volumes were transferred to separate 2 ml Eppendorfs. A 75 µl volume of Alsevers was transferred to the first tube and the contents were freeze-thawed a total of three times. To the second, 10% v/v Triton X100 in Alsevers solution was transferred. Finally, to the remaining Eppendorf tube, 75 µl of Alsevers solution was added. The same procedures were then followed

as described above except Alsevers solution titrated with 0.1 M HCl to reach pH 4.2 was used as a diluent instead of Alsevers solution alone. The absorbency readings for contents of each Eppendorf were plotted against wavelength (Figures 3.17 to 3.22).

3.1.4: Effect of increasing volumes of 10% Triton X100 on absorbency readings of haemoglobin and poly(lysine dodecandiamide) solution with decreasing pH.

To check the effect Triton X100 had on the absorbency readings of haemoglobin the following procedures were performed. First, 6 ml of sheep blood in Alsevers solution was washed as described previously (Section 3.1.1.2). To lyse erythrocytes and release haemoglobin in to solution the obtained pellet was re-suspended in 10 ml of Alsevers solution, then exposed to freeze-thaw three times and centrifuged at 4000 rpm for 4 minutes to pellet any solid material. A 9.5 ml volume of the obtained supernatant was transferred to 51.5 ml of Alsevers solution with 60 mg of dissolved poly(lysine dodecandiamide) to give a 1 mg ml^{-1} polymer solution. From this solution 200 µl volumes were removed and transferred to 12 horizontal wells of one clear flat-bottomed micro titre plate and 4 horizontal wells of a second flat-bottomed micro titre plate. To the remaining polymer-haemoglobin solution, with constant mixing, a titra-lab titration machine was used to transfer 0.1 M HCl in to the solution. When the solution reached pH 5.8, 200 µl volumes were transferred to the next 12 horizontal row of wells of one plate and the next 4 horizontal wells of a second plate. This was repeated when the pH reached 5.6, 5.4, 5.2, 5.0, 4.8 and 4.6. After all pH values had been reached, to each well of the first three vertical columns of the first plate 10 µl of 10% Triton X100 was transferred. Similarly, to the next three columns 25 µl of Triton X100 was transferred. To the next three columns 50 µl of Triton X100 was transferred and to the last three columns 100 µl of Triton X100 was transferred. No Triton X100 was added to the wells of the second plate. Each well of the first plate and second plate was made up to a volume of 300 µl using Alsevers solution. The plates were centrifuged at 4000 rpm for four minutes. A 200 µl volume of supernatant from each well was then transferred to the corresponding well of a new clear flat-bottomed micro titre plate. The absorbency of each of the new plates was read at a wavelength of 492 nm. The mean absorbency readings for polymer-haemoglobin solutions were

plotted against volume of Triton X100 added (Figure 3.23) to determine if there was a variation in absorbency of haemoglobin with reducing pH and increasing amount of Triton X100.

3.1.5: Determination of minimum inhibitory concentration (MIC) of hydrophobically modified polyamide and amine modified GMPs compared to poly(L-lysine).

To determine if polyamide and amine modified GMPs exhibit bactericidal and fungicidal properties, MIC studies were performed for a range of micro-organisms. This was achieved by first preparing a litre of Mueller-Hinton broth (MHB) according to the manufacturer's instructions. A 500 ml volume of the prepared broth was adjusted to pH 7 and pH 5 using 0.1 M NaOH and 0.1 M HCl, respectively. Then, 5 ml volumes of each pH-adjusted broth was dispensed in to 52 test tubes and autoclaved at 121° C for 15 minutes. Simultaneously, a 10 mg ml^{-1} stock solution of poly(lysine dodecandiamide) was prepared by dissolving solid polymer in minimum amount of 0.1 M NaOH, neutralising with 1 M HCl, then making up to a final volume of 10 ml with distilled water. This polymer solution was then filter-sterilised using a 0.2 μm cellulose acetate sterile filter. Aseptically, 1 ml volumes of the sterilised polymer solution were made up to 5 ml using pH 7 and pH 5 MHB to give 2 mg ml^{-1} polymer solutions. These were transferred to a further 5 ml of pH 7 and pH 5 MHB respectively, to give 1 mg ml^{-1} polymer solutions. These solutions were diluted by a factor of two a further ten times using the respective pH adjusted MHB, so that each test tube remained with a 5 ml volume. 5 ml volumes of pH 7 and pH 5 MHB containing no polymer were also set up. This procedure was repeated a further three times to obtain three more sets of test tubes. Overnight cultures of *Staphylococcus aureus* NCTC 6571, *Escherichia coli* DCO and *Candida albicans* were diluted by a factor of 100 using MHB. Then, to each set of prepared test-tubes 100 μl volumes of the diluted culture was transferred. All tubes were incubated at 37° C for 24 hours before visualising for cloudiness, indicative of microbial growth. Tubes that exhibited cloudiness were noted, with the MIC for growth displayed in Table 3.2. The described procedure was then repeated for 3-(diethylamino)propylamine modified GMP and

also poly(L-lysine), a known anti-bacterial and anti-fungal agent, using MHB adjusted to pH values 7 and 9. Again the MIC for microbial growth was noted (Table 3.2).

3.2: Results.

The results presented were obtained in order to assess the properties of a range of polymers, namely poly(ornithine sebacamide), poly(lysine diethyl malonamide), poly(ornithine dodecandiamide), poly(lysine *iso*-phthalamide), poly(lysine dodecandiamide) and poly(lysine butyl malonamide).

Presented in Figure 3.6 are the absorbency readings for supernatant removed from erythrocytes exposed to reducing pH values, therefore indicating haemoglobin released from erythrocytes with addition of acid. Also, presented are the absorbency readings of supernatant removed from erythrocytes exposed to reducing pH values and treated with either Triton X100 or exposure to a freeze-thaw process, therefore indicating haemoglobin released from erythrocytes with addition of acid and exposure to Triton X100 or a freeze-thaw process, respectively. These three sets of absorbency readings are also presented in Table 3.1. From these readings the percentage erythrolysis at each pH value was calculated and the results obtained are also presented in Table 3.1.

Percentage erythrolysis with reducing pH was determined for erythrocytes that were pre-incubated with polymer. The polymers used included poly(ornithine sebacamide), poly(lysine diethyl malonamide), poly(ornithine dodecandiamide), poly(lysine *iso*-phthalamide), poly(lysine dodecandiamide), poly(lysine butyl malonamide) and poly(acrylic acid). The percentage erythrolysis values obtained for these polymers are presented in Figure 3.7 to 3.13. Percentage erythrolysis caused by the latter polymers at certain pH values were unexpected. To determine the reasoning for such unexpected results the absorbency with reducing pH for each constituent of the erythrolysis experiment was determined. These absorbencies are presented in Figures 3.14 to 3.16.

A wavelength of 492 nm was used to measure the absorbency of haemoglobin released from erythrocytes in both the presence and absence of polymers with reducing pH. Therefore, it was thought important to check a wavelength of 492 nm was appropriate for measuring the absorbency of haemoglobin at the range of pH values used and was not the cause of anomalous readings. Therefore, the absorbency readings of each constituent of the erythrolysis experiment taken at 492 nm were compared to absorbency readings obtained when wavelengths of 405 nm, 431 nm and 450nm were used. The absorbencies obtained are presented in Figure 3.17 to 3.22.

The results presented in Figure 3.23 show the effect of Triton X100 concentration on absorbency of haemoglobin.

It was thought the polymers used in this thesis might have anti-microbial properties. To assess these properties MIC values of poly(lysine dodecandiamide) and 3-(diethylamino)propylamine modified glycidyl methacrylate pre-polymer were compared with MIC values of poly(L-lysine) for micro-organisms *E. coli*, *Staph. aureus* and *C. albicans*. These MIC values are presented in Table 3.2.

Figure 3.6: Absorbency of supernatant removed from: erythrocytes at reducing pH values ■; erythrocytes at reducing pH values and exposed to Triton X100 □; erythrocytes at reducing pH values and exposed to a freeze-thaw process •.

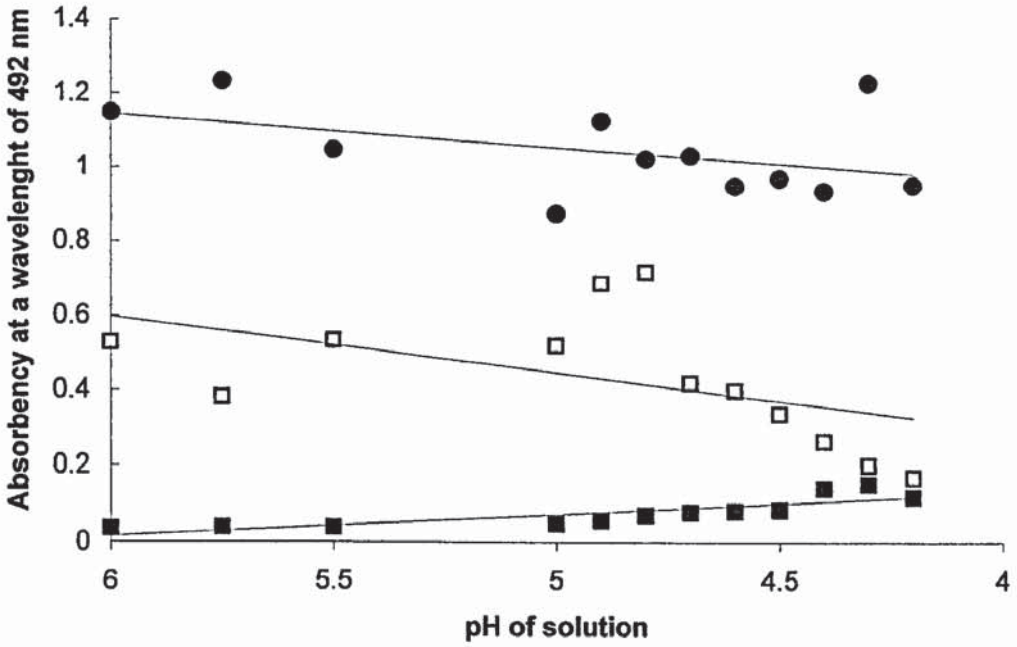


Table 3.1: Mean absorbency readings of supernatant from erythrocytes in the absence of polymer.

Sol^n pH	Mean absorbency at wavelength 492 nm of supernatant from:			Percentage erythrolysis using:	
	RBCs	RBCs exposed to Triton X100	RBCs exposed to freeze-thaw	Triton X100 for 100% lysis	Freeze-thaw for 100% lysis
6.0	0.036	0.527	1.149	6.739	3.091
5.75	0.039	0.382	1.234	10.151	3.141
5.5	0.039	0.533	1.048	7.364	3.746
5.0	0.047	0.519	0.874	9.052	5.379
4.9	0.056	0.686	1.122	8.166	4.992
4.8	0.071	0.715	1.019	9.895	6.941
4.7	0.079	0.418	1.027	18.888	7.696
4.6	0.082	0.398	0.945	20.477	8.622
4.5	0.085	0.335	0.965	25.336	8.787
4.4	0.141	0.263	0.931	53.707	15.176
4.3	0.153	0.201	1.221	75.871	12.490
4.2	0.118	0.169	0.946	69.881	12.447

Figure 3.7: Lysis of red blood cells (RBCs) in the presence of poly(ornithine sebacamide) compared to lysis of RBCs in the presence of buffer using Triton X100 or a freeze-thaw process for complete lysis where, ■ indicates RBCs in the presence of poly(ornithine sebacamide) and Triton X100 is used for complete lysis, □ indicates RBCs in the presence of poly(ornithine sebacamide) and freeze-thaw is used for complete lysis, • indicates RBCs in the presence of buffer and Triton X100 is used for complete lysis, ○ indicates RBCs in the presence of buffer and freeze-thaw is used for complete lysis.

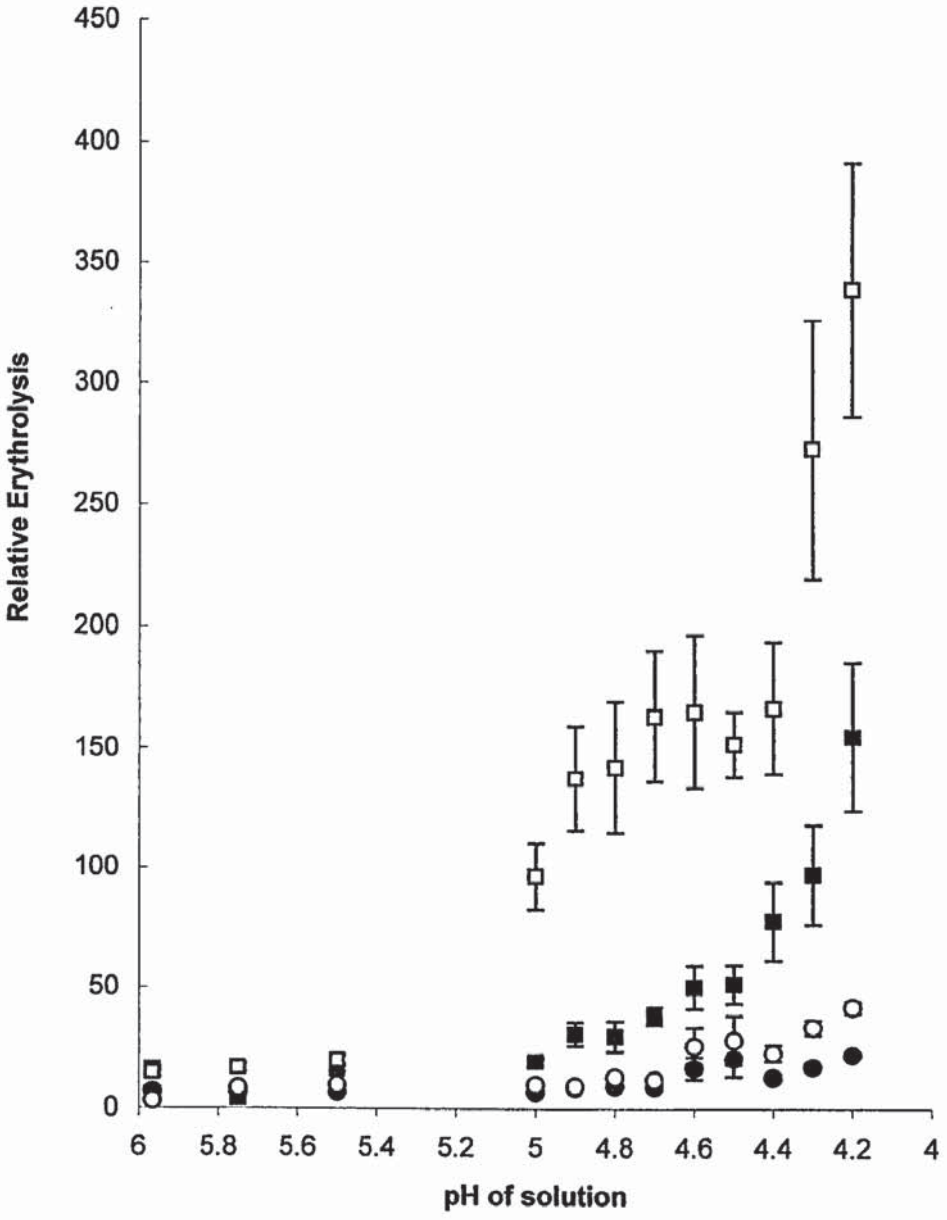


Figure 3.8: Lysis of RBCs in the presence of poly(lysine diethyl malonamide) compared to lysis of RBCs in the presence of buffer using Triton X100 or a freeze-thaw process for complete lysis where, ■ indicates RBCs in the presence of poly(lysine diethyl malonamide) and Triton X100 is used for complete lysis, □ indicates RBCs in the presence of poly(lysine diethyl malonamide) and freeze-thaw is used for complete lysis, • indicates RBCs in the presence of buffer and Triton X100 is used for complete lysis, ○ indicates RBCs in the presence of buffer and freeze-thaw is used for complete lysis.

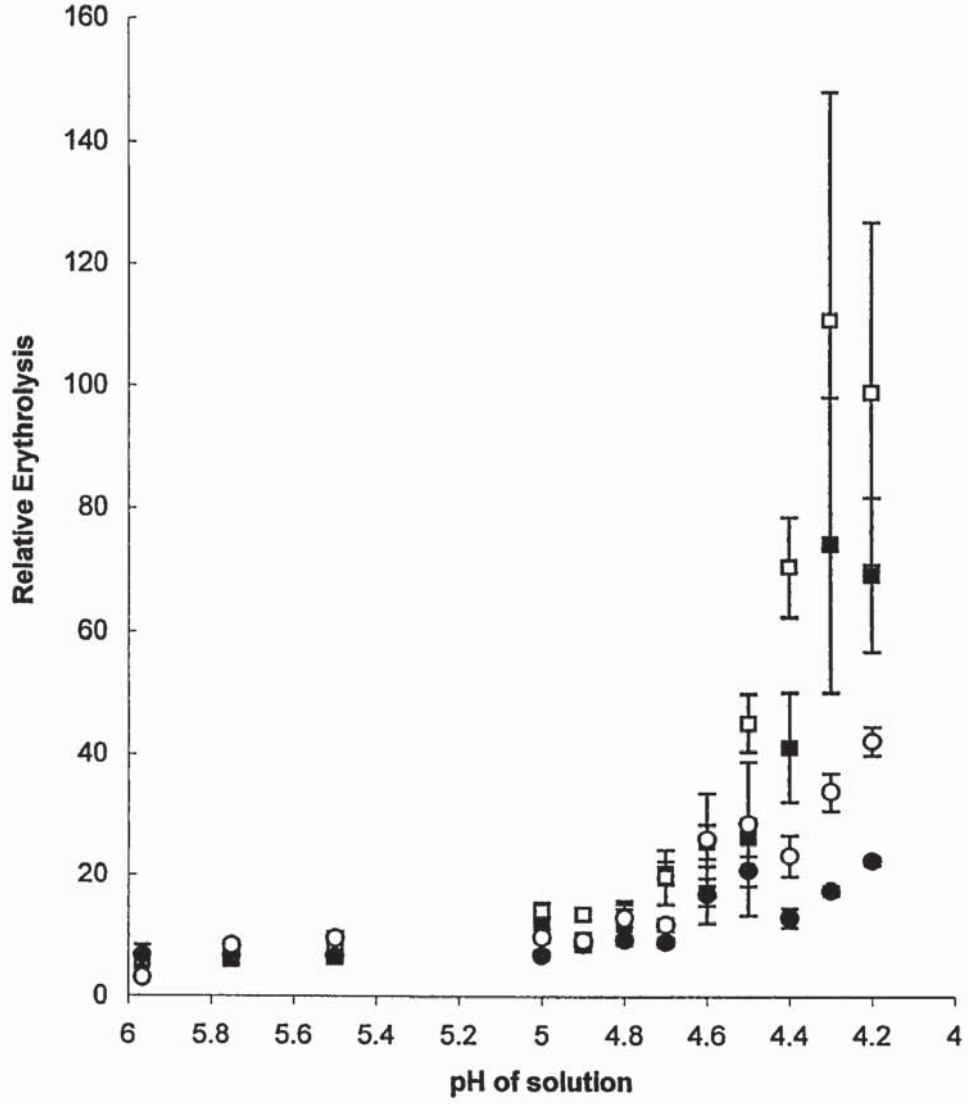


Figure 3.9: Lysis of RBCs in the presence of poly(ornithine dodecandiamide) compared to lysis of RBCs in the presence of buffer using Triton X100 or a freeze-thaw process for complete lysis where, ■ indicates RBCs in the presence of poly(ornithine dodecandiamide) and Triton X100 is used for complete lysis, □ indicates RBCs in the presence of poly(ornithine dodecandiamide) and freeze-thaw is used for complete lysis, ● indicates RBCs in the presence of buffer and Triton X100 is used for complete lysis, ○ indicates RBCs in the presence of buffer and freeze-thaw is used for complete lysis.

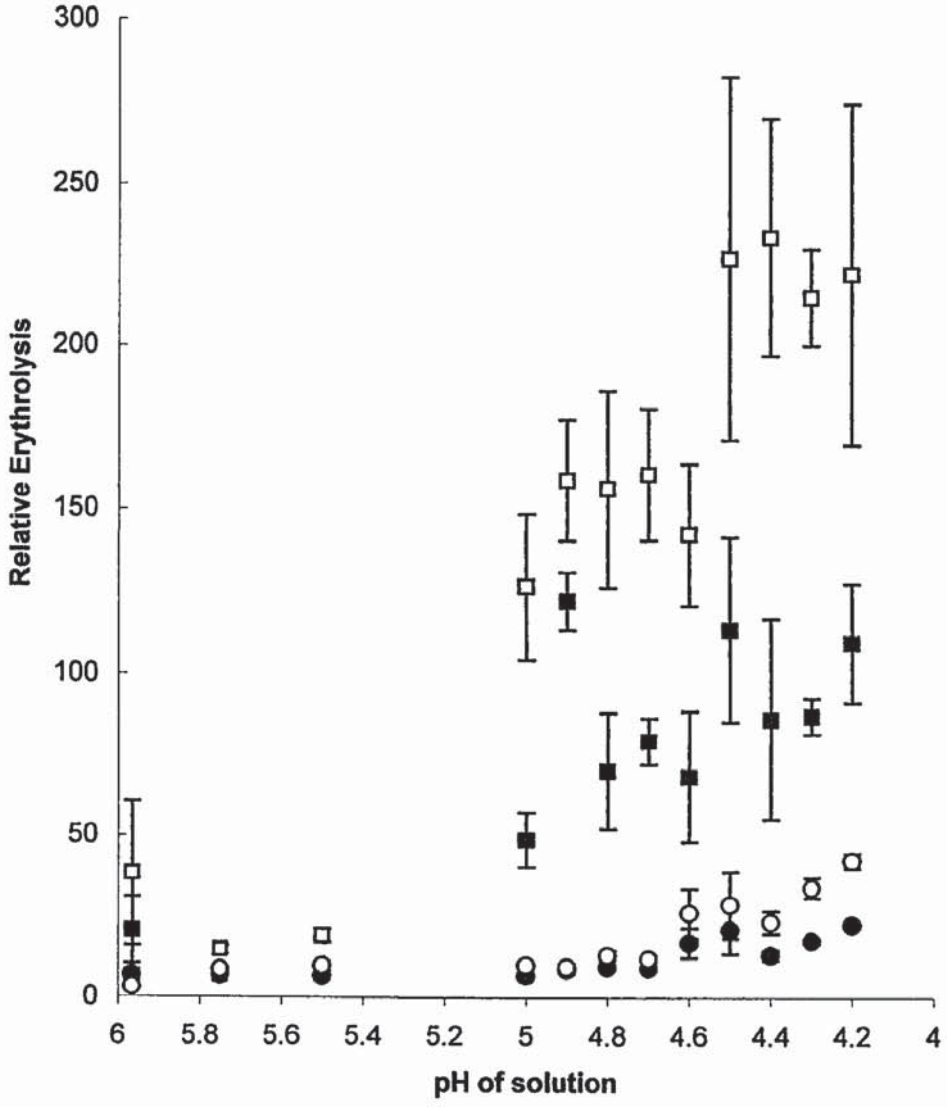


Figure 3.10: Lysis of RBCs in the presence of poly(lysine *iso*-phthalamide) compared to lysis of RBCs in the presence of buffer using Triton X100 or a freeze-thaw process for complete lysis where, ■ indicates RBCs in the presence of poly(lysine *iso*-phthalamide) and Triton X100 is used for complete lysis, □ indicates RBCs in the presence of poly(lysine *iso*-phthalamide) and freeze-thaw is used for complete lysis, • indicates RBCs in the presence of buffer and Triton X100 is used for complete lysis, ○ indicates RBCs in the presence of buffer and freeze-thaw is used for complete lysis.

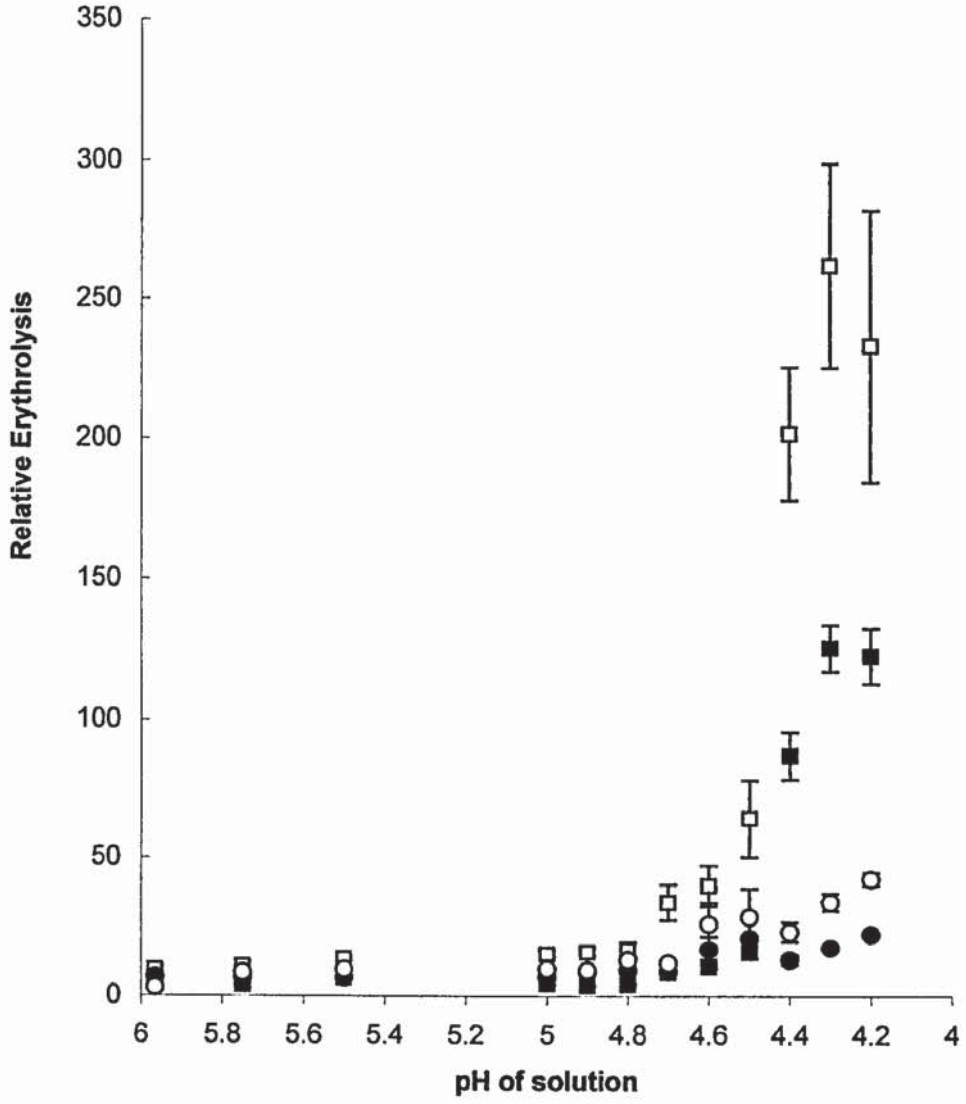


Figure 3.11: Lysis of RBCs in the presence of poly(lysine dodecandiamide) compared to lysis of RBCs in the presence of buffer using Triton X100 or a freeze-thaw process for complete lysis where, ■ indicates RBCs in the presence of poly(lysine dodecandiamide) and Triton X100 is used for complete lysis, □ indicates RBCs in the presence of poly(lysine dodecandiamide) and freeze-thaw is used for complete lysis, ● indicates RBCs in the presence of buffer and Triton X100 is used for complete lysis, ○ indicates RBCs in the presence of buffer and freeze-thaw is used for complete lysis.

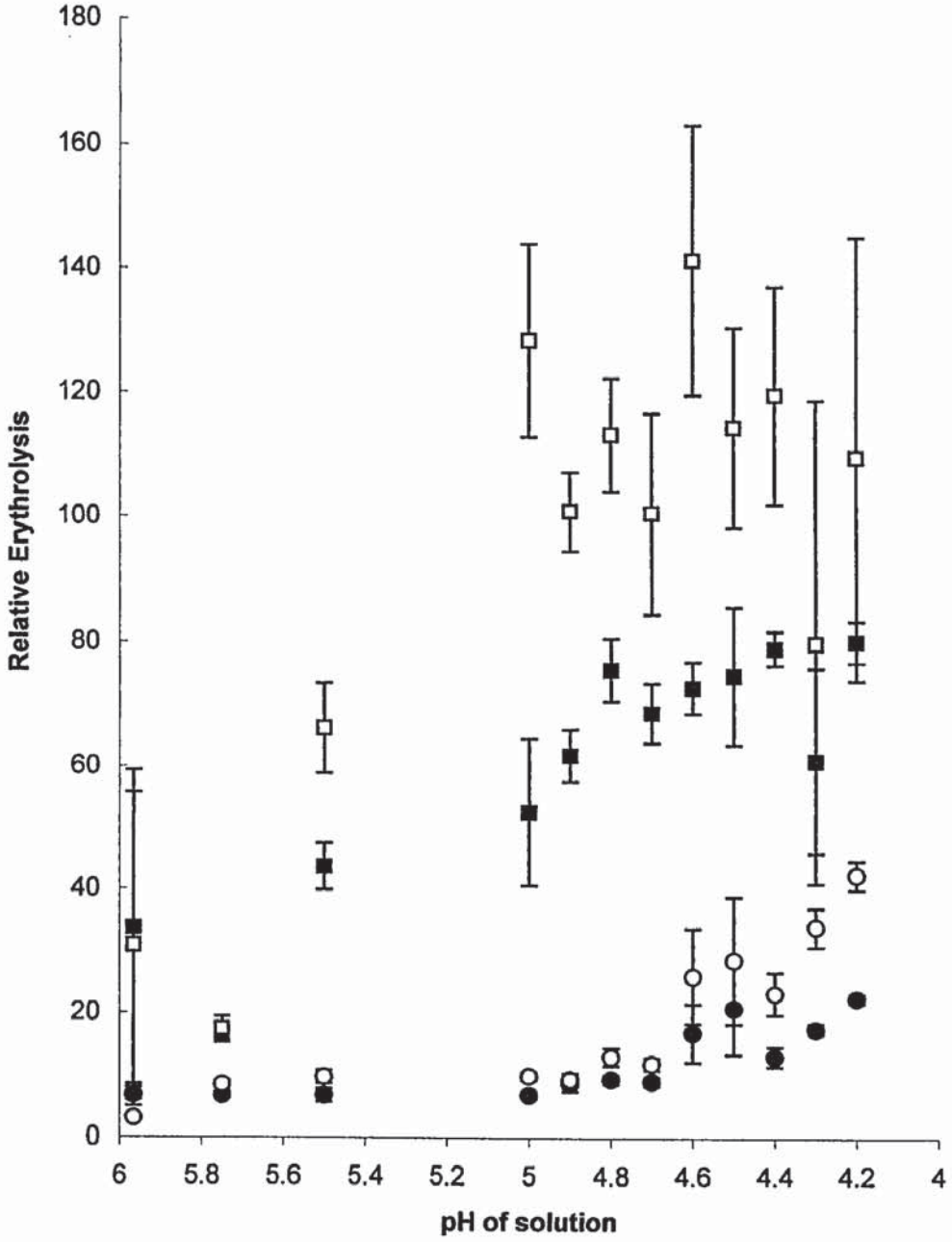


Figure 3.12: Lysis of RBCs in the presence of poly(lysine butyl malonamide) compared to lysis of RBCs in the presence of buffer using Triton X100 or a freeze-thaw process for complete lysis where, ■ indicates RBCs in the presence of poly(lysine butyl malonamide) and Triton X100 is used for complete lysis, □ indicates RBCs in the presence of poly(lysine butyl malonamide) and freeze-thaw is used for complete lysis, • indicates RBCs in the presence of buffer and Triton X100 is used for complete lysis, ○ indicates RBCs in the presence of buffer and freeze-thaw is used for complete lysis.

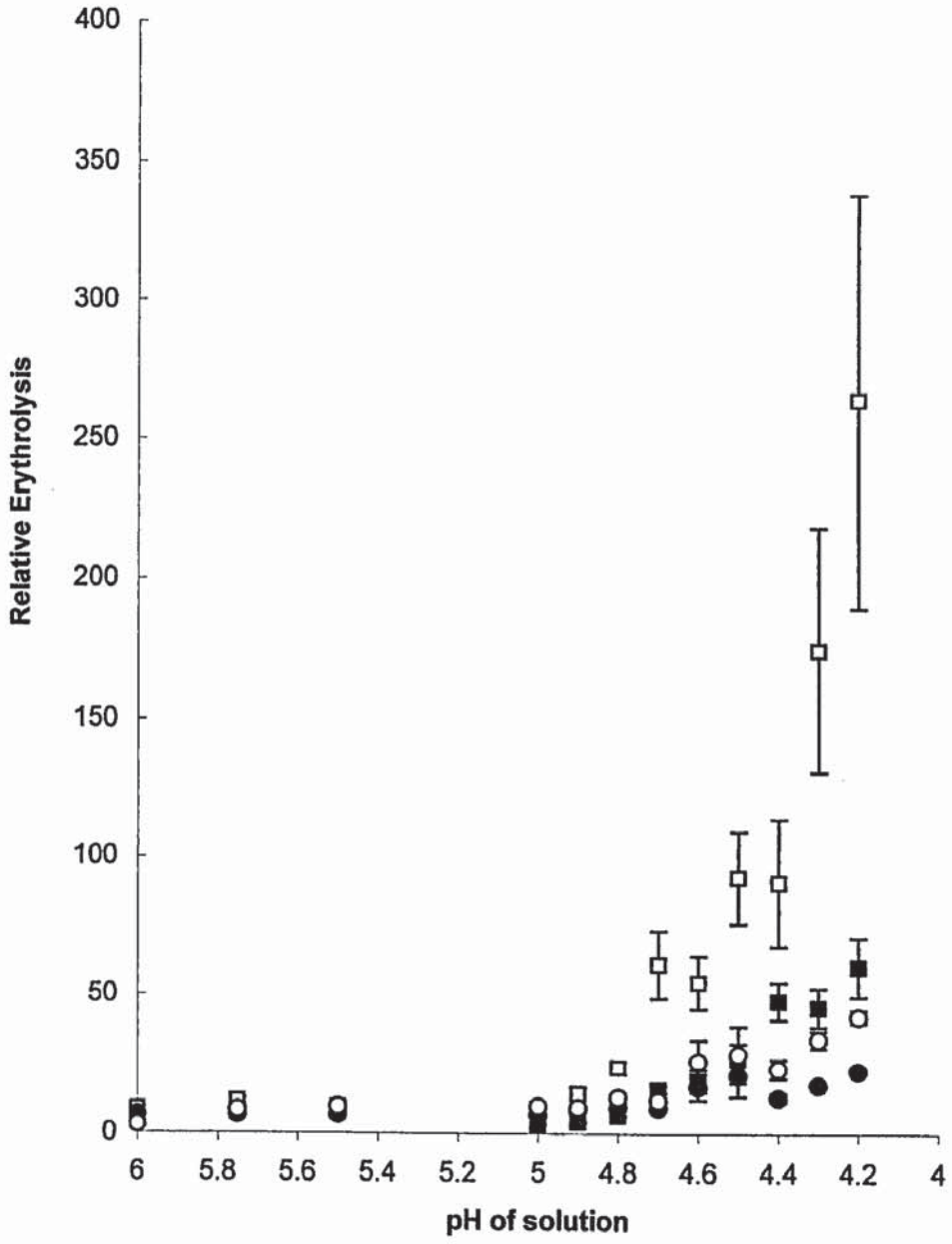


Figure 3.13: Lysis of RBCs in the presence of poly(acrylic acid) compared to lysis of RBCs in the presence of buffer using Triton X100 or a freeze-thaw process for complete lysis where, ■ indicates RBCs in the presence of poly(acrylic acid) and Triton X100 is used for complete lysis, □ indicates RBCs in the presence of poly(acrylic acid) and freeze-thaw is used for complete lysis, • indicates RBCs in the presence of buffer and Triton X100 is used for complete lysis, ○ indicates RBCs in the presence of buffer and freeze-thaw is used for complete lysis.

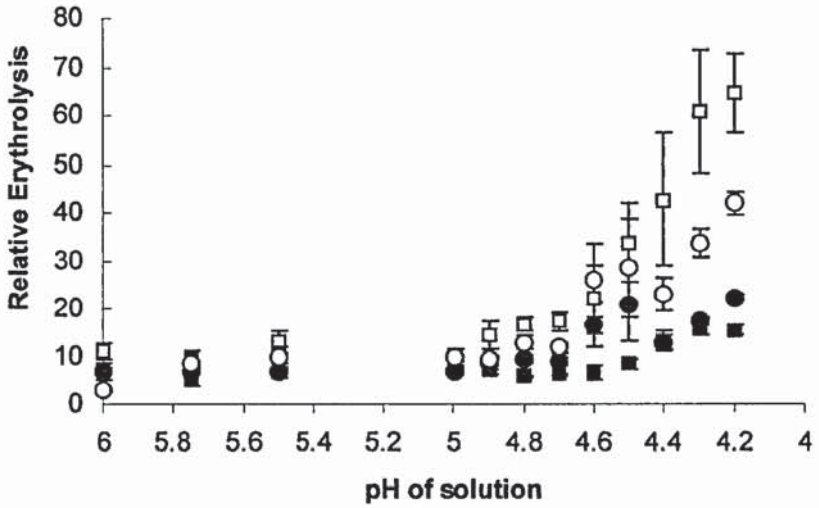


Figure 3.14: Mean absorbency with reducing pH of buffer plus poly(lysine dodecandiamide) ■ ; buffer plus poly(lysine dodecandiamide) and Triton X100 □; buffer plus poly(lysine dodecandiamide) exposed to freeze-thaw •.

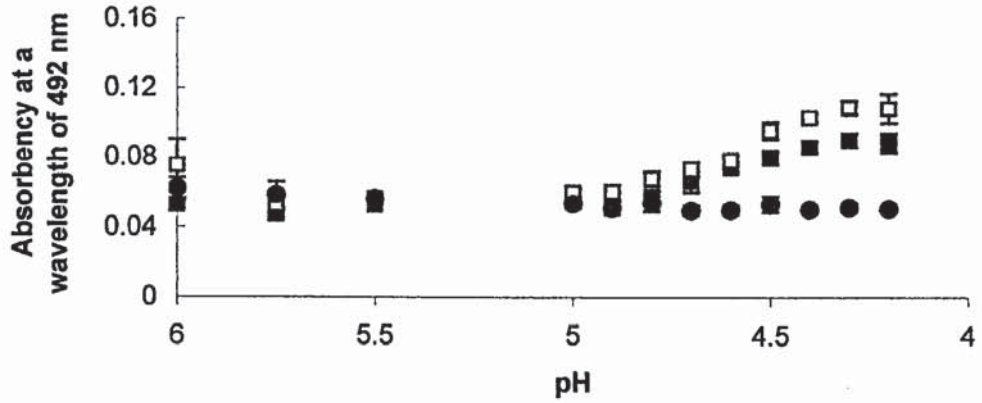


Figure 3.15: Mean absorbency with reducing pH of buffer plus haemoglobin ■; buffer plus haemoglobin and Triton X100 □; buffer plus haemoglobin exposed to freeze-thaw ●.

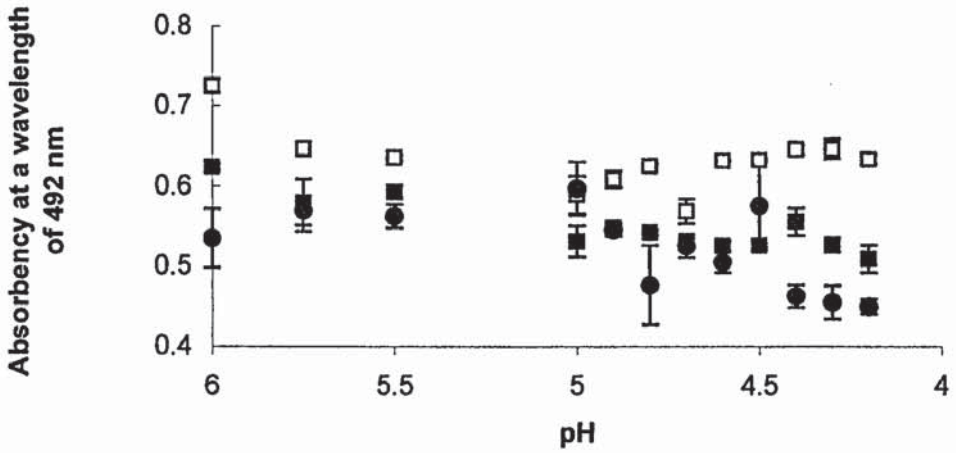


Figure 3.16: Mean absorbency with reducing pH of poly(lysine dodecandiamide) plus haemoglobin ■; poly(lysine dodecandiamide) plus haemoglobin and Triton X100 □; poly(lysine dodecandiamide) plus haemoglobin exposed to freeze-thaw ●.

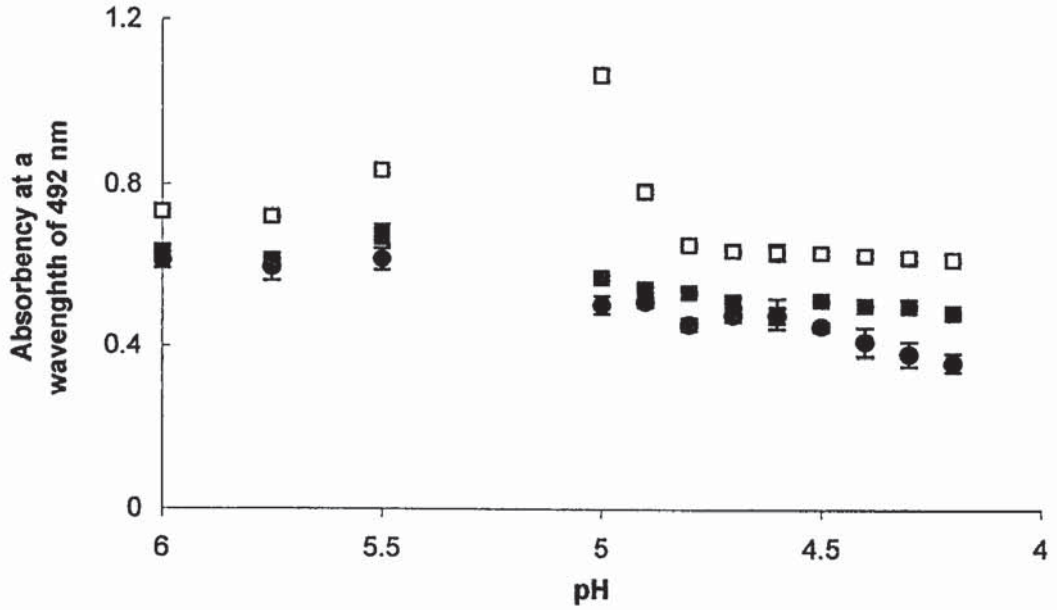


Figure 3.17: Relative absorbency readings at wavelengths 405 nm ■, 431 nm □, 450 nm ● and 492 nm ○ for supernatant obtained from erythrocytes plus poly(lysine dodecandiamide) diluted by Alsevers solution.

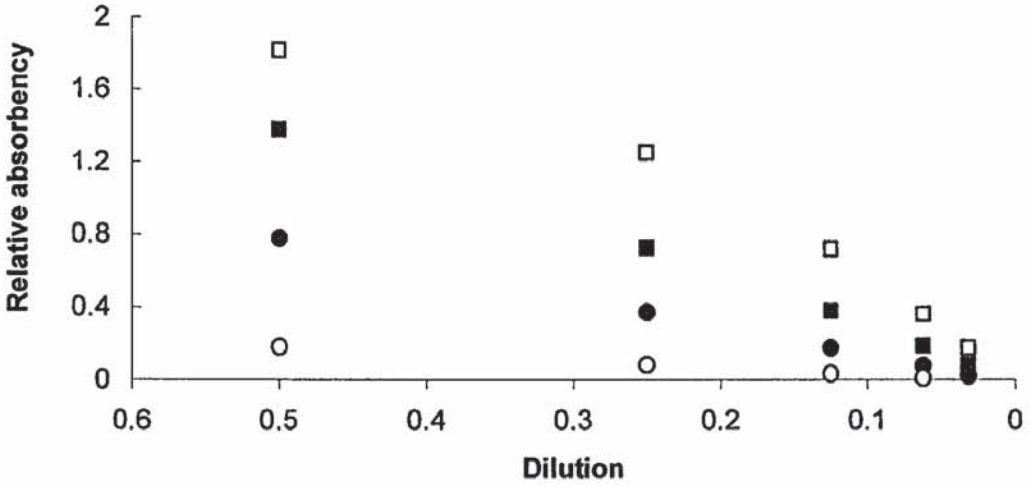


Figure 3.18: Relative absorbency readings at wavelengths 405 nm ■, 431 nm □, 450 nm ● and 492 nm ○, for supernatant obtained from erythrocytes plus poly(lysine dodecandiamide) diluted with Alsevers solution and treated with 10% v/v Triton X100.

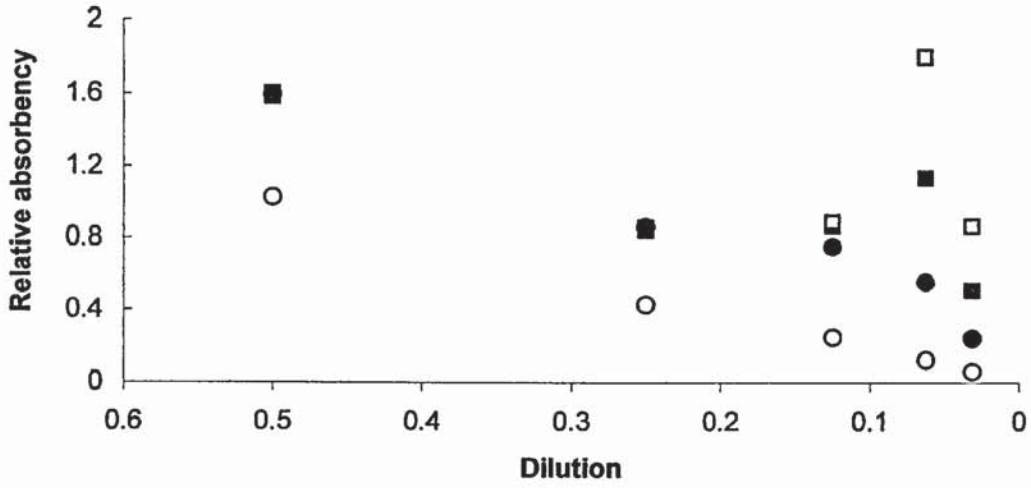


Figure 3.19: Relative absorbency readings at wavelengths 405 nm ■, 431 nm □, 450 nm ● and 492 nm ○ for supernatant obtained from erythrocytes plus poly(lysine dodecandiamide) diluted in Alsevers solution and exposed to freeze thawing.

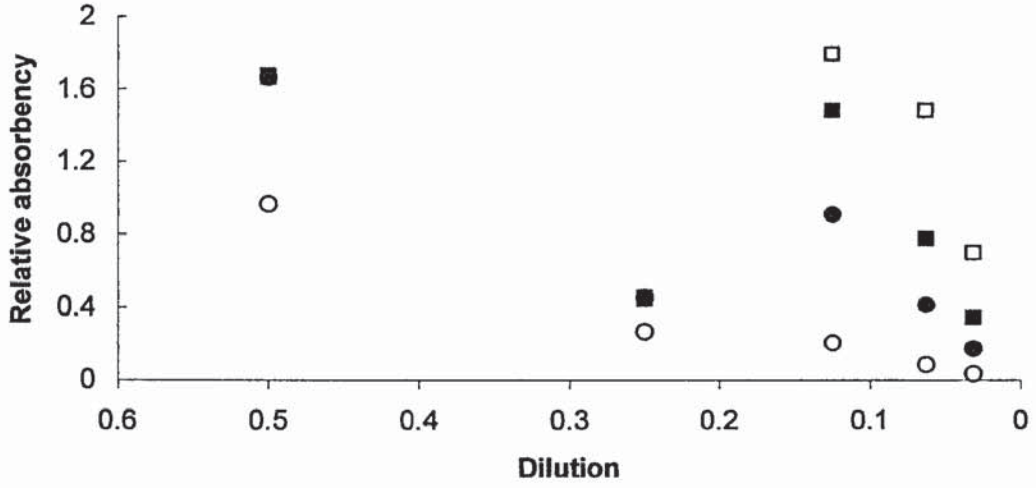


Figure 3.20: Relative absorbency readings at wavelengths 405 nm ■, 431 nm □, 450 nm ● and 492 nm ○, for supernatant obtained from erythrocytes plus poly(lysine dodecandiamide) diluted in Alsevers and titrated down to pH 4.2.

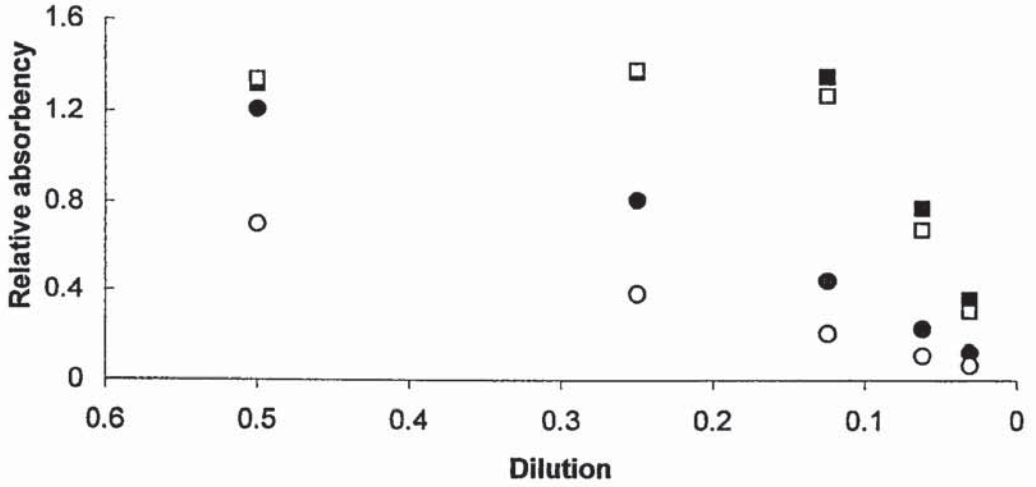


Figure 3.21: Relative absorbency readings at wavelength 405 nm ■, 431 nm □, 450 nm • and 492 nm ○, for supernatant obtained from erythrocytes plus poly(lysine dodecandiamide) dissolved in Alsevers and titrated down to pH 4.2 and treated with 10% v/v Triton X100.

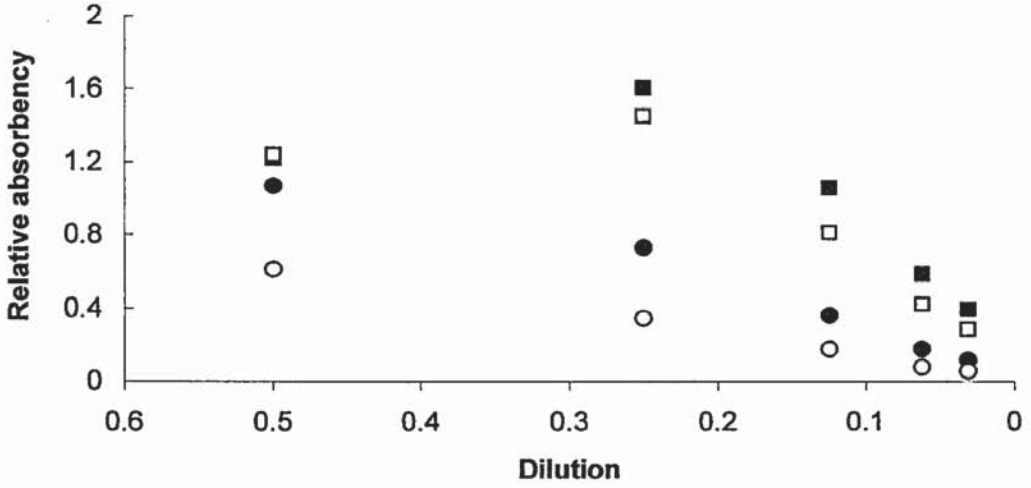


Figure 3.22: Relative absorbency readings at wavelengths 405 nm ■, 431 nm □, 450 nm • and 492 nm ○, for supernatant obtained from erythrocytes plus poly(lysine dodecandiamide) diluted in Alsevers and titrated down to pH 4.2 and exposed to freeze-thaw.

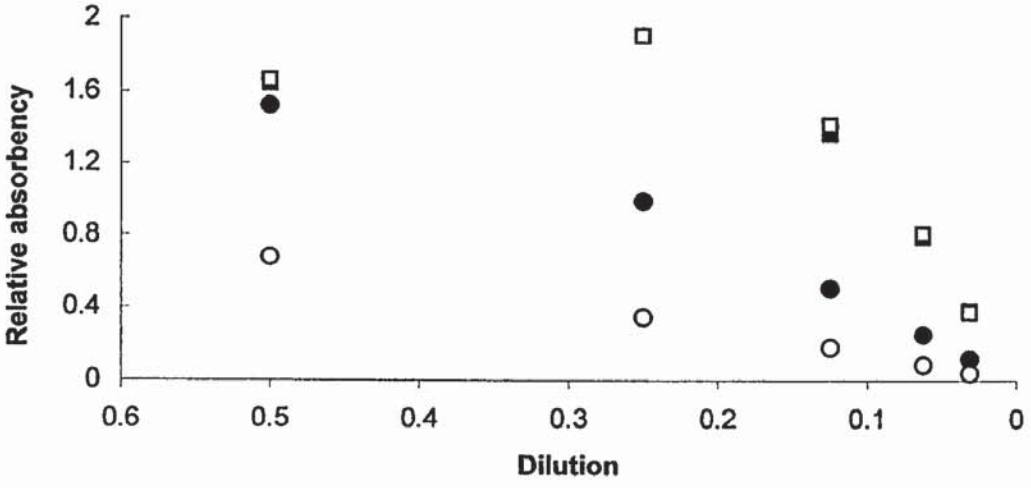


Figure 3.23: Absorbency with reducing pH of a solution of polymer-haemoglobin with 33.3% ■, 16.6% ○, 8.3% ♦, 3.3% □ and none ■, of a 10% v/v Triton X100 solution constituting the total volume.

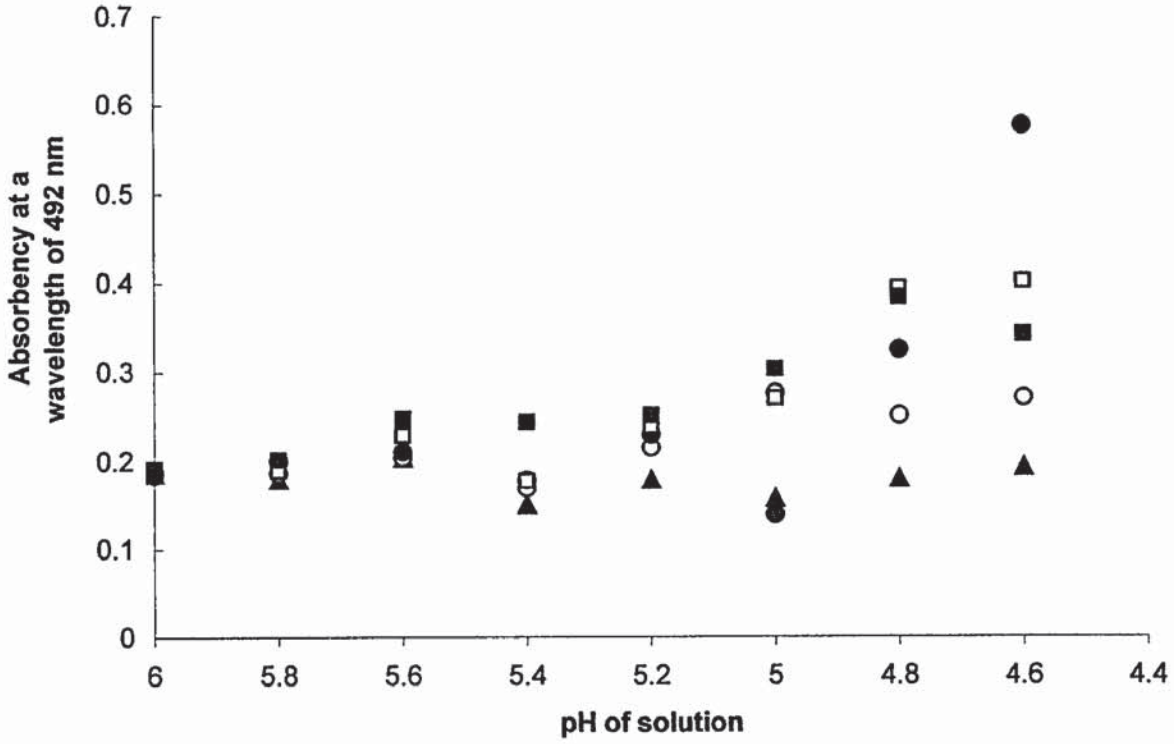


Table 3.2: MIC (mg/100 ml) values for poly(L-lysine), poly(lysine dodecandiamide) and 3-(diethylamino)propylamine modified glycidyl methacrylate pre-polymer at pH values 7 and 5.

Potential anti-microbial constituent	pH of inoculum	MIC with *E. coli*	MIC with *C. albicans*	MIC with *S. aureus*
Poly(L-lysine)	7	12	>50	50
Poly(L-lysine)	5	>50	>50	25
Poly(lysine dodecandiamide)	7	>100	>100	>100
Poly(lysine dodecandiamide)	5	>100	>100	>100
3-(diethylamino)propylamine modified GMP	7	>100	>100	>100
3-(diethylamino)propylamine modified GMP	9	>100*	No growth at pH 9	>100

*Presence of polymer appears to allow growth of this micro-organism as without polymer micro-organism has not grown

3.3: Discussion.

3.3.1: Disruption of erythrocyte membranes at a range of pH values in the presence and absence of polymers.

The mean absorbencies of supernatant from erythrocytes, erythrocytes exposed to Triton X100 and erythrocytes exposed to freeze-thaw with reducing pH are shown in Figure 3.6. The trends observed are not as smooth as those expected (Figure 3.1). However, the general trend shown by the inserted regression-lines are the same. A possible explanation for the points not being as smooth as expected may be due to measuring inaccuracies. For example, exact 200 µl volumes of the erythrocyte solution not being transferred to the micro titre plate when the specific pH values between 6.0 and 4.2 are reached. This would cause a variation in erythrocyte concentration between comparable wells. Consequently, causing a variation in the amount of erythrocytes available to lyse and thus release haemoglobin. Similarly after centrifugation, exact 150 µl volumes of supernatant containing haemoglobin may not be transferred to the flat-bottomed micro titre plate. This would give a false indication of released haemoglobin. Also, any transferral of whole erythrocytes along with the supernatant to flat-bottomed micro titre plate would result in false absorbency readings. The centrifugation process may cause further lysis of erythrocytes not exposed to Triton X100 or freeze-thaw, which does not give a true indication of haemoglobin release as a result of reducing pH, since centrifugation is applied following pH adjustment. If the absorbency readings of erythrocytes exposed to freeze-thaw and erythrocytes exposed to Triton X100 are compared it can be seen that Triton X100 causes a reduction in absorbency readings. This can be explained by the fact that Triton X100 absorbs light in the UV region of the spectrum. In addition, it was noted that Triton X100 can cause the well constituents to bubble, which is likely to affect absorbency readings.

As already explained, with reducing pH a general decline in the absorbency of supernatant removed from erythrocytes exposed to Triton X100 or freeze-thaw is observed (Figure 3.6). However, the individual points do not show a smooth decline. This is also true for the absorbency results obtained for supernatant removed from

erythrocytes in the presence of polymer and exposed to Triton X100 or freeze-thaw. Therefore, when these results are used to calculate percentage erythrolysis a smooth increase with reducing pH is not always observed (Figure 3.7 to Figure 3.13). Possible reasons for these anomalies can be explained using the same reasons as what were described for erythrocytes in the absence of polymer. However, in addition at pH values lower than ≈ pH 5 the polymers start to precipitate from solution. This could also potentially prevent transfer of light through the supernatant and affect absorbency readings. At certain pH values the absorbency readings of supernatant removed from erythrocytes in the presence of polymer exceeds the absorbency reading of supernatant removed from erythrocytes in the presence of polymer that have been exposed to Triton X100 or freeze-thaw to achieve complete erythrolysis. Percentage erythrolysis as a result of polymer interactions is calculated by multiplying by one hundred the value obtained by dividing (A) absorbency of supernatant from erythrocytes in presence of polymer by (B) absorbency of supernatant from erythrocytes in presence of polymer exposed to Triton X100 or freeze-thaw. Therefore, when the absorbency of (A) exceeds absorbency of (B), then percentage erythrolysis will appear to be greater than one hundred percent. Therefore, rather than plotting percentage erythrolysis against pH where maximum erythrolysis is taken as one hundred percent, the results were plotted as relative erythrolysis against pH where maximum erythrolysis is taken as the highest erythrolysis value for that set of experiments. By plotting the results in this format the trends in erythrolysis with pH for each polymer can be observed and compared to erythrolysis in the absence of polymer. However, different polymers cannot be compared to determine the most effective in causing erythrolysis at specific pH values.

The trends for each of the polymers is described in turn and the similarities observed for structurally similar polymers is then discussed:

3.3.1.1: Poly(ornithine sebacamide).

At each pH value, relative erythrolysis obtained when Triton X100 is used to achieve complete erythrolysis is lower than when a freeze-thaw process is applied (Figure 3.7). This trend is also observed for all other polymers analysed (Figures 3.8 to 3.12).

The degree of error when freeze-thaw is used for complete lysis for this polymer and all other polymers in this set of experiments is greater than those on the plotted points when Triton X100 is used for complete lysis. This suggests results when Triton X100 is used for complete lysis may be more reliable. Although the results obtained when using freeze-thaw are higher and possibly less reliable than those for Triton X100 the same general trends are seen for both. That is, erythrolysis in the presence of poly(ornithine sebacamide) is greater than erythrolysis in the absence of this polymer at all pH values. This is true even at the starting pH, indicating poly(ornithine sebacamide) lyses erythrocytes even without any conformational change. Although no peak in erythrolysis is seen at a particular pH, the difference between relative erythrolysis in the presence and absence of polymer is widened at pH values of 5 and below. This can be explained by conformational changes of the polymer at reduced pH values, causing increased lysis of erythrocytes. The suggestion that conformational changes cause increased lysis, is supported by observing the earlier titration curve of this polymer, plotted as pH against pK_a (Figure 2.33). A transitional stage is apparent between pH values 5 and 4.4. Thus, corresponding to the pH values where relative erythrolysis in the presence and absence of polymer is widened at pH values of 5 and below (Figure 3.7).

3.3.1.2: Poly(lysine diethyl malonamide).

It can be seen that relative erythrolysis in the presence and absence of the polymer are similar at the starting pH down to approximately pH 5 (Figure 3.8). At pH values below pH 5, relative erythrolysis becomes increasingly widened in the presence of polymer compared to in its absence. If the titration curve of this polymer (Figure 2.27) is observed, a possible transitional stage begins at pH 5, which again corresponds to pH values where erythrolysis in the presence of polymer is greater than erythrolysis in the absence of polymer. Therefore, indicating erythrolysis is likely to be caused by conformational changes of the polymer. These results also indicate this polymer is more pH specific than poly(ornithine sebacamide), since increased erythrolysis only occurs at lower pH values. Therefore, such a polymer may be more useful for biological applications since it does not appear to have lytic effects at the higher pH values.

3.3.1.3: Poly(ornithine dodecandiamide).

Similar to poly(ornithine sebacamide) (Figure 3.7), relative erythrolysis in the presence of poly(ornithine dodecandiamide) (Figure 3.9) is greater than in its absence at all pH values analysed. Although, no obvious peak in erythrolysis is observed at a particular pH value, again at pH values below 5 the difference between relative erythrolysis in the presence and absence of polymer becomes widened. The results can again be related to a conformational change in polymer, which is seen on the titration curve of this polymer (Figure 2.31) as a transitional stage occurring between pH values 5.5 and 5.0.

3.3.1.4: Poly(lysine *iso*-phthalamide).

When freeze-thaw is used to achieve complete erythrolysis, relative erythrolysis is only marginally higher in the presence of polymer compared to in its absence at pH values down to 4.8 (Figure 3.10). Below pH 4.8 relative erythrolysis becomes increasingly greater in the presence of polymer compared to in its absence. When Triton X100 is used to achieve complete lysis an increase in relative erythrolysis in the presence of polymer compared to in its absence is only seen at pH values less than 4.4. At pH values above this, relative erythrolysis is similar or slightly less than in the absence of polymer. Again these results indicate conformational changes in polymer cause increased erythrolysis. However, the titration curve of poly(lysine *iso*-phthalamide) (Figure 3.10) does not give any further indication towards this explanation since the calculated pK_a values for pH values below 5.5, do not give true results. The variation in results when using Triton X100 and freeze-thaw to achieve complete lysis can be explained by the absorbency effects of Triton X100, which have already been discussed (Section 3.3.1).

3.3.1.5: Poly(lysine dodecandiamide).

At all pH values, relative erythrolysis is higher in the presence of polymer compared to in its absence (Figure 3.11). With reducing pH, relative erythrolysis becomes increasingly greater in the presence of polymer compared to in its absence down to a pH value of approximately 4.5. Below this pH no further increase is seen. As for

poly(lysine *iso*-phthalamide) the titration curve for poly(lysine dodecandiamide) (Figure 2.25) does not give any further insight in to the whether erythrolysis is due to conformational changes of polymer. However, there are deviations from a curve at pH values between 5.3 and 5.0.

3.3.1.6: Poly(lysine butyl malonamide).

Haemoglobin release from erythrocytes is similar when erythrocytes are pre-incubated in the presence of buffer or poly(lysine butyl malonamide) when adjusted to pH values between pH 6 and 4.8 (Figure 3.12). However, haemoglobin release from erythrocytes that are pre-incubated in the presence of poly(lysine butyl malonamide) then adjusted to pH values below 4.8 show a higher haemoglobin release compared to erythrocytes pre-incubated with buffer.

The solution pH of poly(lysine butyl malonamide) was plotted against apparent pK_a (Figure 2.29). The plot shows similar trends to those plotted for poly(lysine diethyl malonamide) (Figure 2.26). An increase in pK_a is observed for pH values between pH 5 and pH 4.5, indicating the polymer becomes increasingly ionised with this reduction in pH and possibly changes conformation. Thus, this possible conformational change corresponds to pH values where presence of polymer increases erythrolysis. The polymer shows a buffering effect or transition stage between pH 4.5 and pH 3, indicating the charge on polymer neutralises the H^+ ions of the added acid. At pH values below pH 3 a polymer aggregation effect is observed with the sharp rise in pK_a likely to be due to excess acid addition.

3.3.1.7: Poly(acrylic acid).

In the presence of poly(acrylic acid) (Figure 3.13) the relative erythrolysis values follow a similar trend to those in the absence of polymer. Thus, indicating erythrolysis is a result of pH effects rather than interaction with this non-responsive polymer.

3.3.2: Similarities and differences in erythrolysis trends observed for polymers with similar structures.

As explained earlier it was expected that all polymers studied in this chapter, which include poly(ornithine sebacamide), poly(lysine diethyl malonamide), poly(ornithine dodecandiamide), poly(lysine *iso*-phthalamide), poly(lysine dodecandiamide) and poly(lysine butyl malonamide) had potential erythrocyte disruption properties. All the polymers studied showed similar trends when adjusted to low pH values. That is, lysis of erythrocytes pre-incubated with each of the polymers is greater than lysis of erythrocytes pre-incubated with buffer and adjusted to the same pH values.

It was also expected that polymers that were structurally similar would show similar membrane disruption properties. That is, the disruption of erythrocytes would occur at similar pH values and with similar effect. As explained in Chapter 2, polyamides were synthesised based on an alternating pendant amino acid (either lysine or ornithine) and hydrophobic group structure. The introduction of pendant hydrophobic groups was achieved in 3 ways: 1) by making an alkyl group substitution; 2) enchainment of an aromatic group; 3) by enchainment of a long alkyl group.

As mentioned above, it is thought the results obtained when Triton X100 was used for complete lysis are more reliable than those obtained when freeze-thaw was used for complete lysis. Therefore, the following summary describing the results for the three main polymer structures are based on the results obtained when Triton X100 was used for complete lysis.

3.3.2.1: Alkyl group substitution.

Poly(lysine butyl malonamide) and poly(lysine diethyl malonamide) were synthesised by making an alkyl group substitution. The results for these two polymers, which are explained above are very similar. That is, erythrolysis is similar when erythrocytes are pre-incubated with buffer or poly(lysine butyl malonamide) and poly(lysine diethyl malonamide), when adjusted to pH values down to 4.8 and 5.0, respectively. Lysis of erythrocytes that are pre-incubated with poly(lysine butyl malonamide) and

poly(lysine diethyl malonamide) then adjusted to pH values below 4.8 and 5.0 respectively, is greater than the lysis of erythrocytes that are pre-incubated with buffer, then adjusted to the same pH values.

The lysis of erythrocytes following pH adjustment can be compared for erythrocytes pre-incubated with buffer and erythrocytes pre-incubated with poly(lysine butyl malonamide) or poly(lysine diethyl malonamide). The maximal difference for poly(lysine butyl malonamide) is seen at pH 4.2 with a difference of approximately 36%, whereas the maximum difference for poly(lysine diethyl malonamide) is seen at pH 4.3 with a difference of approximately 56%. Thus, indicating poly(lysine diethyl malonamide) lyses erythrocytes with greater efficiency than poly(lysine butyl malonamide) at low pH values.

3.3.2.2: Enchainment of an aromatic group.

Poly(lysine *iso*-phthalamide) was the only polymer in this thesis to be synthesised by enchainment of an aromatic group. Nevertheless, compared to the other polyamides synthesised in Chapter 2, poly(lysine *iso*-phthalamide) is structurally most similar to poly(lysine dodecandiamide), since both polymers have the same amine group. However, they do vary in that the hydrophobic groups of poly(lysine dodecandiamide) are long aliphatic groups, whereas, poly(lysine *iso*-phthalamide) has aromatic groups.

If the results of these two polymers are compared differences can be seen. That is, at all pH values poly(lysine dodecandiamide) causes considerable lysis of erythrocytes and this lysis is increased at low pH values. Whereas, at pH values down to 4.8 lysis of erythrocytes is only marginally higher when pre-incubated with poly(lysine *iso*-phthalamide), compared to when pre-incubated with buffer. Like poly(lysine dodecandiamide), the erythrolysis at lower pH values when erythrocytes are pre-incubated with poly(lysine *iso*-phthalamide) is greater than erythrolysis of erythrocytes pre-incubated with buffer.

The difference between lysis of erythrocytes can be compared following pH adjustment of erythrocytes pre-incubated with buffer and erythrocytes pre-incubated

with poly(lysine dodecandiamide) or poly(lysine *iso*-phthalamide). The maximum difference for poly(lysine dodecandiamide) is seen at pH 4.4 with a difference of approximately 66%, whereas the maximum difference for poly(lysine *iso*-phthalamide) is seen at pH 4.3 with complete erythrolysis achieved. Thus, indicating although poly(lysine dodecandiamide) lyses erythrocytes at all pH values, poly(lysine *iso*-phthalamide) is more pH specific and lyses erythrocytes with greater efficiency at low pH values.

3.3.2.3: Enchainment of a long alkyl group.

Poly(lysine dodecandiamide), poly(ornithine dodecandiamide) and poly(ornithine sebacamide) were synthesised by enchainment of a long alkyl group. The results for poly(ornithine dodecandiamide) and poly(ornithine sebacamide) are similar. That is, erythrolysis in the presence of poly(ornithine dodecandiamide) and poly(ornithine sebacamide) is greater than erythrolysis in the absence of this polymer at all pH values and although no peak in erythrolysis is seen at a particular pH, the difference between relative erythrolysis in the presence and absence of polymer is widened at pH values below pH 5. Poly(ornithine dodecandiamide) and poly(ornithine sebacamide) like poly(lysine *iso*-phthalamide) are more pH specific than poly(lysine dodecandiamide) in causing erythrolysis.

Again the difference between lysis of erythrocytes can be compared following pH adjustment of erythrocytes pre-incubated with buffer and erythrocytes pre-incubated with poly(lysine dodecandiamide), poly(ornithine dodecandiamide) or poly(ornithine sebacamide). The maximum difference for poly(lysine dodecandiamide) is seen at pH 4.4 with a difference of approximately 66%, whereas complete erythrolysis is achieved for both poly(ornithine dodecandiamide) and poly(ornithine sebacamide) at pH values 4.9 and 4.2, respectively.

3.3.3: Determination of the effect on absorbency readings with reducing pH on each constituent of the erythrolysis experiment.

A number of factors in the experiment to determine the effect of decreasing pH in the presence and absence of specific polymers on total erythrolysis may have resulted in false absorbency readings. With reducing pH a slight increase in absorbency of poly(lysine dodecandiamide) plus Alsevers is seen (Figure 3.14). This trend was expected, since at low pH polymer precipitates from solution preventing the transmission of UV light. This trend is also seen for poly(lysine dodecandiamide) plus Alsevers solution with added Triton. However, the absorbency values for poly(lysine dodecandiamide) plus Alsevers exposed to freeze-thaw did not follow this trend, instead they remained relatively constant with reducing pH. It was thought that a low pH value might affect absorbency readings of haemoglobin. However, absorbency readings of poly(lysine dodecandiamide) plus haemoglobin gave a general decrease with decreasing pH (Figure 3.16), which can be explained by the dilution effect (Figure 3.1). Similarly, haemoglobin plus Alsevers solution (Figure 3.15), haemoglobin plus Alsevers solution plus Triton X100 (Figure 3.15), poly(lysine dodecandiamide) plus haemoglobin exposed to freeze-thaw (Figure 3.16), and haemoglobin plus Alsevers solution exposed to freeze-thaw (Figure 3.15) all showed a general decrease in absorbency readings with decreasing pH.

Absorbency readings for poly(lysine dodecandiamide) plus haemoglobin plus Triton X100 showed a sharp increase from pH 5.75 down to pH 5.0, a sharp decline from pH 5.0 to 4.8, followed by a general decrease from pH 4.8 down to pH 4.2 (Figure 3.16). The sharp increase in absorbency can be explained by a possible interaction between polymer, haemoglobin and Triton X100 between these pH values. As the pH drops further it is thought this interaction is disrupted as a result of polymer precipitation.

3.3.4: Analysis of the ultra violet spectrum of erythrocytes with poly(lysine dodecandiamide) before and after addition of hydrochloric acid and before and following exposure to freeze-thaw.

It can be observed that serially diluting supernatant obtained from erythrocytes in the presence of polymer gave a proportional decline in relative absorbency at all wavelengths tested, that is 405 nm, 431 nm 450 nm and 492 nm (Figure 3.17). Similarly, serially diluting supernatant obtained from erythrocytes in the presence of polymer and treated with Triton X100 (Figure 3.18) or exposed to freeze-thawing (Figure 3.19) gave a proportional decline in relative absorbency at a wavelength of 492 nm. However, a proportional decline was not seen at wavelengths 405 nm, 431 nm and 450 nm. Since serially diluting supernatant from erythrocytes in the presence of polymer gave a proportional decline in relative absorbency at a wavelength of 492 nm and a steady decline is not always observed when absorbency readings are taken at lower wavelengths of 405 nm, 431 nm and 450 nm, this indicates a wavelength of 492 nm was optimum for experiments conducted earlier. By observing Figure 3.20 it can be seen that the absorbency of supernatant obtained from erythrocytes plus polymer and titrated down to 4.2 gives a proportional decline in absorbency with increasing dilution when using a wavelength of 492 nm. This is also true when using a wavelength of 450 nm. Using lower wavelengths of 405 nm and 431 nm do not give a proportional decline. Similarly, the absorbency of supernatant obtained from erythrocytes plus polymer titrated down to 4.2, then treated with Triton X100, give a proportional decline with increasing dilution when measured at a wavelengths 492 nm and 450 nm (Figure 3.21). Whereas, a proportional decline is not seen for the lower wavelengths analysed. Finally, if Figure 3.22 is observed the same trend is observed for supernatant obtained from erythrocytes plus polymer titrated down to 4.2 and exposed to freeze-thaw. Thus, the latter results indicate a decrease in pH alone does not cause a deviation from a proportional decline in the absorbency of haemoglobin, even when treated with Triton X100 or exposed to freeze-thaw.

3.3.5: Effect of increasing volumes of 10% Triton X100 on absorbency readings of haemoglobin and poly(lysine dodecandiamide) solution with decreasing pH.

It was expected that the addition of increasing volumes of acid to the polymer-haemoglobin solution to reduce the solution pH would cause a decrease in the absorbency of the solution. If Figure 3.23 is observed this trend is true for the polymer-haemoglobin solution with no Triton X100 added, between pH values between 6 and 5, with the absorbency remaining fairly constant for pH values below 5. The addition of Triton X100 to polymer-haemoglobin solution causes an increase in absorbency readings with reducing pH. In general an increasing percentage of Triton X100 added causes an increase in the absorbency of the polymer-haemoglobin solution as the pH of the polymer-haemoglobin is reduced. Thus, giving a possible explanation to why erythrolysis values described in Section 3.3.1 were greater than one hundred percent.

3.3.6: Determination of minimum inhibitory concentration (MIC) of hydrophobically modified polyamide and amine modified GMPs compared to poly(L-lysine).

From Table 3.2, it can be seen that poly(lysine dodecandiamide) and 3-(diethylamino)propylamine modified GMP do not exhibit bactericidal or fungicidal properties, even at high concentrations of 1 mg ml^{-1}. Unlike eukaryotic cells, prokaryotic cells possess a cell wall and capsule. It is thought that these structures protect the cell membrane of the prokaryotes, thus allowing their growth.

Polymers are widely used in conjunction with anti-microbial agents. For example, anti-microbial agents can be incorporated in polymer food packaging such as poly(ethylene) (Appendini *et al.*, 2002) or incorporated in to catheters synthesised from materials such as latex or silicone for medical application (Stickler *et al.*, 2002). However, fewer are used directly to impart anti-microbial activity. Kenawy *et al.*, (1998) have shown linear poly(glycidyl methacrylate) modified with triethyl ammonium, tributyl phosphine and triphenyl phosphine salts show anti-microbial

activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella sp. Salmonella typhae*, *Bacillus subtilis* and *Bacillus cereus*. Also, more recently Tew *et al.*, (2002), describe simple, inexpensive and biomimetic facially amphiphilic acrylamide AB (A and B indicating different aromatic groups within the repeat unit) oligomers and polymers that have a broad antibacterial activity.

Adjustments to the structure of polyamides and amine modified GMPs or a means of transferring polymer through capsule and cell wall may show the polymers examined in this study to exhibit the expected anti-microbial activity. Also, an assessment of a wider range of micro-organisms may show that the polymers have anti-microbial properties.

3.4: Conclusions.

The first aim of this Chapter was to determine whether polymers with alternating anionic and hydrophobic functional groups that were studied in Chapter 2 and showed pH-responsive conformational changes, could be used to disrupt cell membranes as a function of pH.

To achieve this haemoglobin release from erythrocytes was used as an indicator of membrane disruption. The lysis of erythrocytes as a function of pH when pre-incubated in the presence and absence of polymers was determined by measuring the absorbency of haemoglobin released from erythrocytes. Some anomalous absorbency readings were observed for polymers, poly(ornithine sebacamide), poly(lysine diethyl malonamide), poly(ornithine dodecandiamide), poly(lysine *iso*-phthalamide), poly(lysine dodecandiamide) and poly(lysine butyl malonamide) at certain pH values and this is discussed further under the 'anomalous absorbency values' section 3.4.8 below. However, it was concluded that each of the polymers used in this chapter showed pH-responsive membrane disruption properties. The conclusions drawn for each polymer are described in turn:

3.4.1: Poly(ornithine sebacamide).

Poly(ornithine sebacamide) shows pH-responsive membrane disruption properties. Although at all pH values analysed between 6 and 4, more erythrocytes are disrupted when erythrocytes are pre-incubated with poly(ornithine sebacamide) compared to when erythrocytes are pre-incubated with buffer prior to pH adjustment, the difference between lysis of erythrocytes when pre-incubated with poly(ornithine sebacamide) and buffer is widened at pH values 5 and below.

3.4.2: Poly(lysine diethyl malonamide).

This polymer showed pH-responsive membrane disruption properties with more specificity than poly(ornithine sebacamide). That is, lysis of erythrocytes is similar when erythrocyte suspensions pre-incubated with polymer or buffer and adjusted to pH values down to pH 5. Lysis of erythrocytes when the suspension pH is adjusted to pH values below pH 5 is greater when erythrocytes are pre-incubated with polymer compared to when erythrocytes are pre-incubated with buffer.

3.4.3: Poly(ornithine dodecandiamide).

Poly(ornithine dodecandiamide) showed similar pH-responsive membrane disruption properties as poly(ornithine sebacamide). That is, at all pH values analysed between 6 and 4 more erythrocytes are disrupted when erythrocytes are pre-incubated with poly(ornithine dodecandiamide) compared to when erythrocytes are pre-incubated with buffer prior to pH adjustment. However, the difference between lysis of erythrocytes when pre-incubated with poly(ornithine dodecandiamide) and buffer is widened at pH values 5 and below.

3.4.4: Poly(lysine *iso*-phthalamide).

Poly(lysine *iso*-phthalamide) also shows pH-responsive membrane disruption properties. That is, when poly(lysine *iso*-phthalamide) is pre-incubated with erythrocytes and adjusted to pH values down to approximately 4.8 only marginally

more lysis of erythrocytes is observed than for erythrocytes pre-incubated with buffer. When the pH of these suspensions are adjusted below 4.8, erythrocytes pre-incubated with poly(lysine *iso*-phthalamide) show higher lysis of erythrocytes than erythrocytes pre-incubated with buffer.

3.4.5: Poly(lysine dodecandiamide).

Poly(lysine dodecandiamide) also shows pH-responsive membrane disruption properties similar to poly(ornithine sebacamide) and poly(ornithine dodecandiamide), although not as specific. That is, much higher lysis of erythrocytes is seen when erythrocytes are pre-incubated with poly(lysine dodecandiamide) and adjusted to values between 6 and 4.5 when compared to lysis of erythrocytes that are pre-incubated with buffer.

3.4.6: Poly(lysine butyl malonamide).

Poly(lysine butyl malonamide) again shows pH-responsive membrane disruption properties, which are similar to poly(ornithine sebacamide) and poly(ornithine dodecandiamide). That is, erythrolysis is similar when erythrocytes are pre-incubated in the presence of buffer or poly(lysine butyl malonamide) when adjusted to pH values between pH 6 and 4.8. However, lysis of erythrocytes that are pre-incubated in the presence of poly(lysine butyl malonamide) then adjusted to pH values below 4.8 show a higher haemoglobin release compared to erythrocytes pre-incubated with buffer.

3.4.7: Poly(acrylic acid).

Poly(acrylic acid) was used as a control since it is does not show pH-responsive properties. This polymer, as expected and unlike poly(ornithine sebacamide), poly(lysine diethyl malonamide), poly(ornithine dodecandiamide), poly(lysine *iso*-phthalamide), poly(lysine dodecandiamide) and poly(lysine butyl malonamide), did not show pH-responsive disruption properties.

The pH-responsive erythrolysis properties of the polymers discussed above are comparable to those found for other pH-responsive polymers studied by researchers in this field. For example, studies analysing membrane disruptive synthetic polymers, have shown poly(ethyl acrylic acid) (PEAAc) exhibit pH-responsive disruption of RBCs and with greater efficiency than mellitin, a known membrane disruptive peptide (Stayton *et al.*, 2000). That is, 5×10^6 molecules of PEAAc compared to 11×10^6 molecules of mellitin were required to disrupt a single RBC. In addition, studies showed poly(propyl acrylic acid) (PPAAc) synthesised by addition of a single methylene unit to PPAAc to make the pendant alkyl group more hydrophobic lysed RBCs 15 times more efficiently. PPAA was found to completely lyse a 1 ml suspension of approximately 10^8 RBCs at pH 6.1 in 1 hour using PEAAc concentrations as low as 3-5 $\mu g\ ml^{-1}$ (Murthy *et al.*, 1999; Kyriakides *et al.*, 2002). PEAA was not haemolytic at pH 7.4 and displayed a pH-responsive haemolysis that rose sharply as pH dropped below 6.5. It also has been shown PPAA enhanced haemolysis of RBCs at acidic pH when complexed via biotin to the protein strepavidin (Lackey *et al.*, 1999).

3.4.8: Anomalous absorbency values.

The percentage erythrolysis caused by polymers, poly(ornithine sebacamide), poly(lysine diethyl malonamide), poly(ornithine dodecandiamide), poly(lysine *iso*-phthalamide), poly(lysine dodecandiamide) and poly(lysine butyl malonamide), when polymer-erythrocyte suspensions were adjusted to particular pH values were calculated to be over one hundred percent. An experiment was set up to determine the reasoning for this and thus the effect of absorbency readings with reducing pH on each constituent of the erythrolysis experiment. Poly(lysine dodecandiamide) was used as a representative of the other polymers used for erythrolysis studies. It was concluded that anomalous absorbency readings were not caused by pH effects on polymer alone, polymer exposed to Triton X100 or a freeze-thaw process, haemoglobin alone, polymer plus haemoglobin, haemoglobin exposed to Triton X100 or a freeze-thaw process or polymer plus haemoglobin exposed to a freeze-thaw process. However, polymer plus haemoglobin exposed to Triton X100 showed unexpected absorbency readings with reducing pH.

It was concluded that a wavelength of 492 nm used to measure released haemoglobin was optimal compared to other wavelengths tested (405 nm, 431 nm, 450 nm). That is, when a wavelength of 492 nm was used a linear decline in absorbency was observed for diluted samples of haemoglobin in the presence of polymer at neutral and at an acidic pH of 4.2. A linear decline was also observed for diluted samples of haemoglobin in the presence of polymer at neutral and at an acidic pH of 4.2 when treated with Triton X100 or exposed to a freeze-thaw process.

In general an increasing percentage of Triton X100 added causes an increase in the absorbency of the polymer-haemoglobin solution as the pH of the polymer-haemoglobin is reduced. Thus, giving a possible explanation to why erythrolysis values were greater than one hundred percent.

The pH-responsive properties shown for each of these polymers is promising since polymers with such properties have a potential application as endosomolytic agents. The conclusions drawn from this chapter led to the work carried out in Chapter 4, which was undertaken to gain further insight in to the properties of poly(ornithine sebacamide), poly(lysine diethyl malonamide), poly(ornithine dodecandiamide), poly(lysine *iso*-phthalamide), poly(lysine dodecandiamide) and poly(lysine butyl malonamide) and to attempt to gain more reliable results by overcoming the anomalous readings, which have been discussed.

The second aim of this chapter was to determine whether the polymers used in Chapter 2 have anti-microbial properties by disturbing the microbial cell membrane as a function of pH. However, the results from this study showed poly(lysine dodecandiamide), *N,N*-diethylamine and 3-(diethylamino)propylamine glycidyl methacrylate polymers did not exhibit anti-microbial properties. However, it is thought anti-microbial properties may have been observed if the polymer could break through the cell wall and thus allowing access to the plasma membrane.

Chapter 4

Disruption of liposomal membranes using polyamides and amine modified glycidyl methacrylate polymers

4.0: Aims and overview.

The aim of this chapter, similar to Chapter 3 is to determine whether polymers studied in Chapter 2 can be used to disrupt membranes as a function of pH.

Two main types of polymer are investigated, which include polyamides and amine modified glycidyl methacrylate polymers. Dissimilar to Chapter 3, liposome membranes are used to investigate membrane disruption properties of polymers as oppose to the natural lipid membranes of erythrocytes or microbes. To achieve this aim and gain further insight in to these polymers mode of action, each of the following studies were performed and the reasoning described in turn.

1) Determination of liposome solubilisation properties of polymers as a function of pH.

MLVs synthesised by hydration of L-α-dilauroylphosphatidylcholine (DLPC) afford a turbid suspension. Tirrell *et al.*, (1986) has shown such suspensions can be solubilised to clear suspensions in the presence of poly(α-ethylacrylic acid), on production of gluconic acid via an enzymatic degradation of glucose by glucose oxidase. Control experiments were also performed, where glucose oxidase plus glucose, glucose oxidase plus poly(α-ethylacrylic acid) or glucose oxidase was added to the DLPC MLVs. In all the controls they showed solubilisation did not occur. Based on the methods used by Tirrell *et al.*, (1986) it was attempted to determine whether the polymers studied in Chapter 2 disrupt liposomes as a function of pH. To achieve this liposome suspensions were synthesised and pre-incubated in the presence and absence of polymers studied in Chapter 2. That is polyamides including poly(lysine butyl malonamide), poly(lysine diethyl malonamide), poly(lysine *iso*-phthalamide), poly(lysine dodecandiamide), poly(ornithine dodecandiamide) and poly(ornithine sebacamide). Also, amine modified glycidyl methacrylate polymers

including *N,N*-diethylamine, *N,N*-diethyl ethylene diamine and 3-(diethylamino)propylamine modified glycidyl methacrylate polymers. These suspensions were observed for solubilisation before and following pH adjustment. Thus, solubilised liposomes would be indicative of liposome disruption.

2) Preparation of liposomes with encapsulated calcein for the application of quantifying membrane disruption properties of polymers.

It was attempted to quantify the pH-responsive membrane disruption properties of the polymers studied in Chapter 2 using a more sensitive method than erythrolysis. To achieve this liposomes with encapsulated calcein were synthesised. The detection limits of calcein were assessed for the application of membrane disruption studies.

3) Determination of calcein release from liposomes in the presence and absence of polymers as a function of pH.

Liposomes with encapsulated calcein were prepared then pre-incubated in the presence and absence of the polymers studied in Chapter 2. The pH of suspensions of polymer and liposomes was adjusted to a range of values and calcein release measured. The release of calcein was used as a marker indicative of membrane disruption.

4) Ascertain the properties of polymers, which enable the rupturing of lipid membranes.

To achieve this, primarily the charge on liposome membranes as a function of pH was measured. This was performed to determine whether membrane rupture is a result of charge interactions between polymer and liposomes. Secondly, it was attempted to determine whether polymer becomes permanently bonded to liposome membranes after a period of incubation time. This was undertaken to determine whether polymer needs to be in free form or attached to the liposome membrane to cause membrane rupture.

5) Applicability of liposomes for *in vivo* use.

Some preliminary studies were performed to assess the usefulness of liposomes prepared in thesis for *in vivo* applications. Firstly, the size range of the liposomes was assessed. Secondly, it is useful if preparations for *in vivo* use can be stored for an

extended period of time. Therefore, the ability of the liposomes preparations to withstand lyophilisation was assessed.

4.1: Methods.

4.1.1: Determination of liposome solubilisation properties of polymers as a function of pH.

To determine whether liposomes are solubilised as a function of pH by polyamides including poly(lysine dodecandiamide), poly(lysine *iso*-phthalamide), poly(lysine butyl malonamide) and glycidyl methacrylate pre-polymer modified with amines including *N,N*-diethylamine, *N,N*-diethyl ethylene diamine and 3-(diethylamino)propylamine, the following methods were undertaken.

Firstly, a 50 ml volume of each polyamide was prepared at a concentration of 2 mg ml^{-1}. This was undertaken by dissolving solid polymer in a minimal amount of 0.1 M NaOH, adjusting to pH 7 with 0.1 M HCl and making up to its final volume using 10 mM HEPES buffer that was adjusted to pH 7 using 0.1 M NaOH. Simultaneously, 50 ml of MLVs were prepared using a thin film hydration method (Szoka, 1980). That is, 50 mg of egg PC was transferred to a round-bottomed flask and dissolved in an excess of diethyl ether (≈250 ml). Using a rotary evaporator under vacuum, the organic solvent was completely removed leaving a thin lipid film around the walls of the flask. The aqueous phase, that is, a 50 ml volume of pH 7 adjusted HEPES was then transferred to the flask and vortexed for 12 hours. In triplicate, for each polyamide, test tubes were labelled A7, A5, A4, B7, B5, B4, C7, C5 and C4. To all tubes labelled A, 2 ml of MLVs and 2 ml of polymer were transferred. To all tubes labelled B, 2 ml of MLVs and 2 ml of pH 7 HEPES buffer was transferred. Finally, to all tubes labelled C, 2 ml of pH 7 HEPES buffer and 2 ml of polymer was transferred. Then, by addition of 1 M HCl, the pH of each tubes contents were adjusted to either pH 7, 5 or 4 as indicated by the number labelled on the test tube. The tubes were then visualised for solubilisation of MLVs and photographed to show the observed results (Plate 4.1 to 4.3), and the results noted (Table 4.1). The latter experiment was repeated for amine modified glycidyl methacrylate pre-polymers. However, polymer solutions and MLVs were made up using pH 6 adjusted HEPES buffer. Also, test

tubes A, B and C were adjusted using 1 M NaOH to pH 6, 7.5 and 9 as oppose to 7, 5 and 4. Again the tubes were visualised and photographs taken to show the observed results (Plates 4.4 to 4.6).

This whole experiment was then repeated but using positively charged MLVs synthesised from egg PC, stearylamine and cholesterol at molar ratios of 63, 18 and 9 respectively, per ml of pH 6 and pH 7 HEPES buffer.

4.1.2: Preparation of liposomes with encapsulated calcein for the application of quantifying membrane disruption properties of polymers.

Although the results are not included in this thesis liposomes with encapsulated glucose were initially prepared for membrane disruption studies as an alternative to erythrocytes. However, it was found that the quantity of glucose released from completely lysed liposomes was insufficient for detection using a YSI 2300 STAT PLUS machine. Consequently, an alternative was sought and therefore, the aim of the following investigation was to prepare liposomes with encapsulated calcein so that released calcein could be measured and used to quantify the disruption properties of polymer. Calcein is a water-soluble molecule that is weakly fluorescent at high concentrations due to self-quenching. A calcein solution at such high concentrations appears orange in colour. The fluorescence of calcein increases at lower concentrations as quenching is reduced. Therefore, calcein within liposomes is at high concentrations so is weakly fluorescent, but calcein that leaks from the liposomes is highly fluorescent. As a result of these properties, liposomes with encapsulated calcein have been widely used for membrane permeability assays since calcein leakage can be measured *in situ* without any separation of liposomes from the assay medium (Katsu, 1999). Such characteristics make liposomes with encapsulated calcein a potentially useful means of determining the membrane disruption properties of polymers synthesised in Chapter 2.

Before liposomes with encapsulated calcein were prepared for this study some preliminary investigations were performed. The optimal excitation and emission wavelengths of calcein were determined. Then the detection limits of a Spectra Max

Gemini XS fluorescent plate reader for calcein were determined. Finally the effect on calcein fluorescence of pH and Triton X100, which is used to completely lyse liposomes, was determined.

4.1.2.1: Determination of the optimal excitation and emission wavelengths of calcein.

It is widely stated in the literature that the optimal excitation and emission wavelengths for calcein are close to 490 nm and 520 nm, respectively (Kono *et al.*, 1999; Hara *et al.*, 2000). To conclude this a full fluorescence spectra of a 1 μM calcein solution was performed using a Spectra Max Gemini XS fluorescent plate reader (Molecular Devices). That is, relative fluorescence was measured for excitation wavelengths between 350 nm and 500 nm when using an emission wavelength of 520 nm. The results were plotted as excitation wavelength against relative fluorescence (Figure 4.2). Similarly, relative fluorescence was determined for emission wavelengths between 500 nm and 650 nm, when using an excitation wavelength of 490 nm. These results were plotted as emission wavelength against relative fluorescence (Figure 4.3).

When the excitation spectra (Figure 4.2) is observed it can be seen there is a clear peak in relative fluorescence at a wavelength of 490 nm. If the emission spectra (Figure 4.3) is observed a 'shoulder' is seen at 520 nm. A distinctive peak is not seen since the excitation and emission wavelengths are very close. Therefore, fluorescence from the excitation spectra overlaps with fluorescence from the emission spectra. To reduce this overlap a 'cut off ' filter can be introduced which reduces emission fluorescence above a certain wavelength. Different 'cut off' points were introduced between the excitation and emission wavelengths. Excitation wavelength is plotted against relative fluorescence for the optimal 'cut off' wavelength (Figure 4.4), which was found to be 515 nm.

4.1.2.2: Determination of the detection limits of Spectra Max Gemini XS fluorescent plate reader (Molecular Devices).

In order for released calcein from liposomes with encapsulated calcein to be useful as a means of quantifying the extent of liposome disruption it must be detectable by the fluorescent plate reader. Thus, the detection limits of the plate reader for calcein were determined. To achieve this, a 10 ml volume of a 100 mM solution of calcein (FW 662.5) was prepared. This was achieved by pre-dissolving solid calcein in a minimum volume of 1 M NaOH. This solution was neutralised with 1 M HCl before making it up to its final volume with Dulbecco's Phosphate Buffered Saline (DPBS). In quadruplicate, the calcein solution was decimally diluted ten times by transferring 100 µl volumes of the more concentrated dilution to 900 µl volumes of fresh DPBS. A 200 µl volume of each dilution was transferred to a black flat-bottomed 96-well micro titre plate. The fluorescence of each dilution was measured using the fluorescent plate reader set at excitation and emission wavelengths of 490 nm and 520 nm, respectively and with an emission 'cut off' wavelength introduced at 515 nm. Relative fluorescence was plotted against calcein concentration to show the plate readers detection limits (Figure 4.5).

4.1.2.3: Determination of pH effect on calcein fluorescence.

In later experiments, calcein release from liposomes as a function of pH is determined. Therefore, it was important to determine whether fluorescence of calcein was affected by changes in pH. This was achieved by preparing, Hydrion pH-buffers 4, 5, 7, 9, 10 and 12 according to the manufacturer instructions. In quadruplicate, 990 µl of each buffer was transferred to Eppendorf tubes. To each of these Eppendorfs, 10 µl of 1 mM calcein in DPBS was transferred, giving a final calcein concentration of 0.01 mM. A concentration of 0.01 mM was used since earlier investigations indicated this was within the linear detection range of the fluorescence plate reader (Figure 4.5). The contents of each Eppendorf were agitated to obtain a homogenous suspension. Then, a 200 µl volume was transferred from each Eppendorf to a well of a black 96-well micro titre plate. The fluorescence of each well was determined using the fluorescent plate reader set at excitation and emission wavelengths of 490 nm and 520 nm, respectively. An emission 'cut off' wavelength was also introduced at a

wavelength of 515 nm. The mean fluorescence readings were plotted against pH of the 0.01 mM calcein solutions (Figure 4.6).

4.1.2.4: Determination of the effect of Triton X100 concentration on calcein fluorescence.

Triton X100 is used in later experiments to completely lyse liposomes. Therefore, it was important to determine whether Triton X100 affected calcein fluorescence. To determine this primarily a 100 mM calcein in HEPES buffer solution was decimally diluted with fresh HEPES buffer to obtain a 0.1 mM solution, which is within the linear detection range of the fluorescent plate reader. In triplicate, 990 µl, 900 µl, 800 µl and 500 µl of the diluted calcein solution were transferred to test tubes labelled 1 to 4, respectively. Simultaneously, a 10% Triton X100 solution was prepared by dissolving 10 ml of Triton X100 in 90 ml of HEPES buffer. The prepared 10% Triton X100 solution was used to make test tubes labelled 1 to 4 up to 1000 µl. In quadruplicate, a 200 µl volume was transferred from each test tube to a well of a black 96-well plate. The described process was repeated, however, HEPES buffer instead of Triton X100 was used to make up the volume of test tubes 1 to 4 to 1000 µl. The relative fluorescence of each well was determined using excitation and emission wavelengths of 490 nm and 520 nm, respectively with an emission 'cut off' filter introduced at 515 nm. The percentage difference between fluorescence of a calcein solution with Triton X100 added and fluorescence of a calcein solution with buffer added was calculated (Figure 4.7).

4.1.2.5: Preparation of multilamellar vesicles (MLVs) with encapsulated calcein and separation from free calcein using various sizes of gel filtration columns.

4.1.2.5.1: Gel filtration column (10 mm width by 100 mm length).

MLVs with encapsulated calcein were prepared using a thin film hydration technique. That is, a 100 mM calcein solution was prepared as described in Section 4.1.2.2, except the solution was made up to its final volume with buffer solution containing 10 mM HEPES, 150 mM NaCl and 1 mM EDTA (Ruysschaert *et al.*, 1998). Simultaneously, 5 mg of egg PC was transferred to a round-bottomed glass flask and

dissolved using 25 ml of diethyl ether. A thin lipid film was obtained using rotary evaporation under vacuum for 2 hours. When all organic solvent had been removed 5 ml of the calcein solution was transferred to the flask and vortexed overnight to form a mixture of MLVs with encapsulated calcein plus free calcein.

For the MLVs with encapsulated calcein to be useful for assay investigations non-encapsulated calcein must be removed. This can be achieved by passing the solution of MLVs with encapsulated calcein plus free calcein through a gel filtration column, the principles of which are described in Chapter 1. As liposomes are relatively large they are eluted from the column first, while smaller molecules of calcein are eluted more slowly. The gel filtration column was prepared according to the manufacturers instructions by firstly adding a gram of solid Sephadex (Course G-50, Sigma) to 10 ml of HEPES buffer to form a slurry. Then, avoiding the formation of air bubbles, the slurry was carefully poured in to a glass column with a width of 10 mm and length of 100 mm. The Sephadex was then allowed to swell for 48 hours before use. A 0.5 ml sample of the MLVs with encapsulated calcein plus free calcein mixture was loaded on to the column. The tap was opened until the top of the sample was in line with the indent of the column. Fresh buffer was loaded on top, avoiding disruption of the Sephadex bed. As it was not known at which point free MLVs would leave the column, the tap was opened and fifty sequential 0.125 ml volume fractions were collected. Throughout this procedure fresh buffer was added to the column to prevent the top of the Sephadex bed drying out. To determine which fractions contained MLVs, the relative fluorescence was determined for these fractions, and the same fractions diluted by a factor of 2, 4 and 8 before and after addition of 25 µl of 10% Triton X100 (Figure 4.8 and 4.9). A fraction containing the MLVs should show an increase in fluorescence on addition of Triton X100 as MLVs are ruptured and calcein is released in to the surrounding medium.

4.1.2.5.2: Gel filtration column (20 mm width by 260 mm length).

The 10 mm (width) by 100 mm (length) sized gel filtration column used in Section 4.1.2.5.1 was too small to separate free calcein from liposomes with encapsulated calcein, such that a certain volume of liposomes with encapsulated calcein were voided with free calcein. Thus, these voided liposomes were futile for assay

applications. It was thought this problem would be overcome by using a larger sized column, such that all free calcein could be separated from liposomes with encapsulated calcein.

To achieve this, liposomes with encapsulated calcein were prepared using thin film hydration with 1 mg of egg PC per ml of 100 mM calcein. A gel filtration column was set up which had an inner diameter and length of 20 mm and 260 mm, respectively. A 0.5 ml volume of MLVs with encapsulated calcein plus free calcein was loaded on top of the column and allowed to pass through using HEPES buffer as an eluent. All eluate excluded from the column prior to exclusion of free calcein was collected. It is expected that any excluded MLVs would be present in this eluate. To confirm this, size analysis was performed on this sample using a Malvern Mastersizer and HEPES buffer as a blank. To give further confirmation 200 µl of eluate plus 50 µl of buffer and 200 µl of eluate plus 50 µl of 10% Triton X100 were transferred in triplicate to columns A and B of a 96-well black micro titre plate. To check Triton X100 was not affecting fluorescence, 250 µl of buffer and 200 µl of buffer plus 50 µl of 10% Triton X100 were transferred in triplicate to columns C and D. Using excitation and emission wavelengths of 490 nm and 520 nm, respectively and a 'cut off' filter of 515 nm the relative fluorescence of the contents of each well were determined (Figure 4.10). It was expected that wells containing eluate plus Triton X100 would show an increased fluorescence compared to wells containing eluate plus buffer, since encapsulated calcein is released from MLVs in to the surrounding medium on rupturing of MLVs by Triton X100.

In Section 4.1.2.5.1, a column of size 10 mm width by 100 mm length was loaded with liposomes with encapsulated calcein and free calcein. The fluorescence of the fractions excluded from the Sephadex column was determined. These procedures were repeated for a column of size 20 mm width by 260 mm length. That is, a 3 ml volume of MLVs with encapsulated calcein was prepared using thin film hydration using 1 mg egg PC per ml of 100 mM calcein, as described previously. A 0.5 ml volume of the prepared MLVs was loaded on to Sephadex column. Then a 200 ml volume of HEPES buffer was placed carefully on top of the column, preventing disturbance of the Sephadex bed. The column tap was opened to allow exclusion of the sample with sequential 2 ml fractions collected. This was performed until free

calcein began to leave column. A 450 µl volume of each fraction was transferred to test tubes labelled 1 to 3. A 50 µl volume of 10% Triton X100 in HEPES, 20% Triton X100 in HEPES and HEPES buffer with no added Triton X100 were transferred to test tubes labelled 1 to 3, respectively. Therefore, test tubes 1 and 2 had an overall Triton X100 concentration of 1% and 2%, respectively. A 2% concentration was used to confirm whether a concentration of 1% was sufficient to cause complete lysis of MLVs. In triplicate, 150 µl volumes of the untreated fraction were transferred to a 96-well black micro titre plate. Similarly, in triplicate 150 µl volumes of the fractions treated with 1% and 2% Triton X100 were transferred to a black 96-well micro titre plate. It was expected that a fraction containing MLVs would show a higher fluorescence after treatment with Triton X100 compared to an untreated fraction. The described experiment was repeated on the following day using the same sample of MLVs with encapsulated calcein, which were stored at 4° C until use. The obtained results for day 1 were plotted as relative fluorescence of each fraction against fraction number (Figure 4.11). Similarly, the results were plotted in the same way for day 2 (Figure 4.12).

To increase the concentration of MLVs in collected fractions MLVs were prepared with 5 mg as oppose to 1 mg of egg PC per ml of 100 mM calcein solution. Fluorescence analysis of the fractions excluded from the Sephadex column for this preparation were again determined and the results obtained again plotted as relative fluorescence of fractions against fraction number (Figures 4.13 and 4.14).

4.1.2.5.3: MLVs for assay.

It was observed that fractions 12-19 collected from the Sephadex column, which was loaded with MLVs with encapsulated calcein synthesised from 5 mg of egg PC per ml of 100 mM calcein showed an obvious increase in fluorescence after treatment with Triton X100 (Figures 4.13 and 4.14). For these fractions to be useful for biological assays an increase in fluorescence after rupture must also be detectable when the fractions have been diluted further than what will be performed during the assay. To determine this, a 3 ml volume of MLVs with encapsulated calcein was synthesised using 5 mg of egg PC per ml of 100 mM calcein. A 0.5 ml volume was removed and loaded on to the Sephadex column. As before the column tap of the Sephadex column

was opened and twenty-two 2 ml fractions were collected. All orange coloured fractions were placed in to a glass flask and gently mixed to obtain a homogenous suspension. An orange colour indicating the presence of MLVs with encapsulated calcein. This suspension was then decimally diluted six times by transferring 0.5 ml volumes to 4.5 ml of fresh HEPES buffer. In quadruplicate 150 µl volumes of each dilution were transferred to a black 96-well micro titre plate. The suspension of MLVs was also decimally diluted six times by transferring 0.5 ml volumes to 4 ml of fresh HEPES buffer and 0.5 ml of 10% Triton X100 to cause complete liposomal rupture. Again in quadruplicate 150 µl volumes of each dilution were transferred to a black 96-well micro titre plate. The fluorescence of the micro titre plates were determined using excitation and emission wavelengths of 490 nm and 520 nm, respectively. For each decimal dilution the log of relative fluorescence was plotted (Figure 4.15).

4.1.3: Determination of calcein release from liposomes in the presence and absence of polymers as a function of pH.

The synthesis of liposomes with encapsulated calcein for the application of quantifying disruption properties of polymers was described in Section 4.1.2. It was found liposomes synthesised using 5 mg egg PC per ml of 100 mM calcein and collected off a gel filtration column with size 20 mm width by 260 mm length were suitable for measuring membrane disruption properties of polymers and therefore, were used in the following studies.

4.1.3.1: Release of calcein from MLVs with encapsulated calcein in the presence and absence of poly(lysine dodecandiamide) with reducing pH.

MLVs with encapsulated calcein were synthesised with 5 mg of egg PC per ml of a 100 mM calcein solution using thin film hydration as described previously. A 0.5 ml volume of the synthesised MLVs were loaded on to the Sephadex column and fractions 12-19 were collected, each fraction being 2 ml in volume and containing free MLVs with encapsulated calcein. Simultaneously, a 20 ml volume of 2 mg poly(lysine dodecandiamide) per ml of HEPES solution was prepared by pre-dissolving solid polymer with small amounts of 1 M NaOH, neutralising with 1 M

HCl, then making the solution up to its final volume. A 10 ml volume of the prepared polymer solution was then transferred to 10 ml of the collected MLVs and incubated at room temperature for 10 minutes to allow interaction of polymer with MLVs. After incubating, the pH of the polymer and liposome solution was adjusted by transferring 1 ml volumes to test tubes containing 9 ml of pH 7 HEPES buffer and 9 ml volumes of pH 6, 5, and 4 Hydrion buffers. A 900 µl volume of each solution of pH adjusted buffer and MLVs was removed from the test tubes and transferred to both 100 µl of pH 7 HEPES buffer and 100 µl of 10% Triton X100 in HEPES. In triplicate, 150 µl volumes of these solutions were transferred to a 96-well black micro titre plate and their fluorescence determined. The latter procedures were repeated, however, instead of initially transferring 10 ml of MLVs to 10 ml of poly(lysine dodecandiamide) in HEPES, 10 ml of MLVs were transferred to 10 ml of pH 7 HEPES. The obtained fluorescence readings were plotted as relative fluorescence of MLVs with encapsulated calcein in the presence and absence of polymer against pH of the solution (Figure 4.16).

The results in Figure 4.16 showed that liposomes are almost completely lysed when adjusted to pH values 5 and 4. It was thought this might have been due to insufficient liposomes available to lyse. Therefore, the procedures described above were repeated but using fractions 15-17 collected from the Sephadex column instead of fractions 12-19. These fractions were used as visually they were the more concentrated and it was thought this might overcome complete lysis problems. The results were plotted as pH adjustment of liposomal solution against fluorescence (Figure 4.17).

It was found the results shown in Figure 4.17 were the same as those shown in Figure 4.16. Therefore, it was thought that an alternative reason for complete rupture of MLVs at low pH was due to a combination of low pH and use of Hydrion buffers, which do not contain any cell protecting EDTA. Therefore, the experiment was repeated. However, instead of using Hydrion pH buffers to reduce the pH of solutions of polymer plus MLVs with encapsulated calcein, pH adjusted HEPES buffer (10 mM) containing NaCl (150 mM) and EDTA (1 mM) was used. Again, relative fluorescence of MLVs with encapsulated calcein in the presence and absence of polymer was plotted against pH of the solution (Figure 4.18).

The results displayed in Figure 4.18 were used to calculate the percentage of total calcein released in the presence and absence of polymer. These calculations are undertaken by multiplying by one hundred the value obtained when the fluorescence of polymer plus MLVs is divided by fluorescence of polymer plus MLVs with added Triton X100. Similarly, percentage of total calcein released in the absence of polymer at each pH is calculated by multiplying by one hundred the value obtained when the fluorescence of HEPES plus MLVs is divided by fluorescence of HEPES plus MLVs with added Triton X100. These percentage calcein release values in the presence and absence of polymer were plotted against pH of the solution (Figure 4.19).

4.1.3.2: Calcein release from liposomes with encapsulated calcein pre-incubated with poly(lysine diethyl malonamide), poly(ornithine dodecandiamide), poly(lysine *iso*-phthalamide), poly(lysine butyl malonamide) and poly(ornithine sebacamide), following pH change compared to liposomes with encapsulated calcein alone, following the same pH changes.

Using thin film hydration, as described previously MLVs with encapsulated calcein were synthesised with 5 mg of egg PC per ml of a 100 mM calcein solution. A 0.5 ml volume of the synthesised MLVs was loaded on to the Sephadex column and all orange fractions were collected. Simultaneously, a 20 ml volume of 2 mg poly(lysine diethyl malonamide) per ml of HEPES solution was prepared by pre-dissolving solid polymer with small amounts of 1 M NaOH, neutralising with 1 M HCl, then making the solution up to its final volume. A 10 ml volume of the prepared polymer solution was then transferred to 10 ml of the collected MLVs and incubated at room temperature for 10 minutes to allow interaction of polymer with MLVs. After incubating, the pH of the polymer and liposome solution was adjusted by transferring 1 ml volumes to test tubes containing 9 ml of HEPES buffer (10 mM) containing NaCl (150 mM) and EDTA (1 mM) and adjusted to pH values 7, 6, 5 and 4. A 900 µl volume of each solution of pH buffer plus MLVs was removed from the test tubes and transferred to both 100 µl of pH 7 HEPES buffer and 100 µl of 10% Triton X100 in HEPES. In triplicate, 150 µl volumes of these solutions were transferred to a 96-well black micro titre plate and their fluorescence determined. This experiment was then repeated, except instead of initially transferring 10 ml of MLVs to 10 ml of

poly(lysine diethyl malonamide) in HEPES, 10 ml of MLVs were transferred to 10 ml of pH 7 HEPES alone.

All procedures described in this section were repeated for poly(ornithine dodecandiamide), poly(lysine *iso*-phthalamide), poly(lysine butyl malonamide) and poly(ornithine sebacamide). The obtained fluorescence readings for each polymer were plotted as relative fluorescence of MLVs with encapsulated calcein in the presence and absence of polymer against pH of the solution (Figures 4.20 and 4.21)

The results shown on Figures 4.20 and 4.21 could be used to calculate the percentage difference in fluorescence in the presence of polymers compared to in their absence after each pH change. This was achieved for each pH value by subtracting the fluorescence value in the presence of buffer from fluorescence value in the presence of polymer. The results of these calculations were plotted as pH of polymer and liposome suspension against difference in released calcein from MLVs with encapsulated calcein incubated in the presence and absence of polymer (Figures 4.22 and 4.23).

4.1.3.3: Release of calcein from cationic liposomes incubated with poly(lysine dodecandiamide) followed by decreasing pH.

Cationic liposomes with encapsulated calcein were prepared using thin film hydration as described for anionic liposomes in Section 4.1.3. However, 19.2 mg of egg PC, 1.94 mg of stearylamine and 1.36 mg of cholesterol was transferred to the round-bottomed flask with 25 ml of chloroform per ml of liposome suspension to be prepared. A 0.5 ml volume of prepared MLVs was loaded on to the Sephadex column. All orange fractions were collected using pH 6 adjusted HEPES as an eluent. Simultaneously, a 20 ml volume of a 2 mg ml^{-1} poly(lysine dodecandiamide) in HEPES solution was prepared by pre-dissolving solid polymer in small amounts of 1 M NaOH then neutralising with 1 M HCl before making the solution up to its final volume. A 10 ml volume of the prepared polymer solution was transferred to 10 ml of the collected liposomes and incubated at room temperature for 10 minutes to allow interaction of polymer with liposomes. After incubating, 1 ml volumes of the solution of polymer and liposomes were transferred to test tubes containing 9 ml of pH 7, 6, 5

and 4 adjusted HEPES buffer. A 900 µl volume of each solution of pH buffer and MLVs was removed from the test tubes and transferred to both 100 µl of pH 7 HEPES buffer and 100 µl of 10% Triton X100 in HEPES. In triplicate, 150 µl volumes of these solutions were transferred to a 96-well black micro titre plate and their fluorescence determined. The above process was then repeated, However, instead of initially transferring 10 ml of liposomes to 10 ml of poly(lysine dodecandiamide) in HEPES buffer, 10 ml of liposomes were transferred to 10 ml of pH 7 HEPES only. The obtained fluorescence readings were plotted as relative fluorescence of MLVs with encapsulated calcein in the presence and absence of polymer against pH of the solution (Figure 4.24).

4.1.3.4: Release of calcein from anionic liposomes with encapsulated calcein in the presence and absence of amine modified glycidyl methacrylate pre-polymer with increasing pH.

With reducing pH, anionic MLVs with encapsulated calcein that are pre-incubated with polyamides show an increased release of calcein compared to MLVs pre-incubated with buffer alone. It was thought that the rupturing of liposomes was a result of conformational changes of polymer with reducing pH. Conversely, it is thought amine modified glycidyl methacrylate pre-polymers lose their extended conformation with increasing pH. Therefore, it was thought anionic liposomes pre-incubated with amine modified glycidyl methacrylate polymers would show an increased calcein release with increasing pH. To determine if this trend was seen, anionic liposomes with encapsulated calcein were synthesised using thin film hydration and 5 mg of egg PC per ml of 100 mM calcein in pH 6 adjusted HEPES buffer. A 0.5 ml volume of liposomes with encapsulated calcein plus free calcein was loaded on top of the column and allowed to pass through using pH 6 adjusted HEPES buffer as an eluent, with all orange fractions collected. Simultaneously, 20 ml volumes of *N,N*-diethylamine, *N,N*-diethyl ethylene diamine, 3-(diethylamino)propylamine modified GMPs were dissolved in pH 6 adjusted HEPES solution at a concentration of 2 mg ml^{-1}. A 10 ml volume of each polymer solution was transferred to 10 ml of the collected MLVs and incubated at room temperature for 10 minutes to allow the polymer and MLVs to interact. After incubating, 1 ml volumes of each polymer and liposome solution were transferred to 9 ml of pH 6, 7, 8

and 9 HEPES buffer. A volume of 900 µl of each solution of the pH buffer and MLVs was transferred to both 100 µl of pH 6 HEPES buffer and 100 µl of 10% Triton X100 in HEPES. Then, in triplicate 150 µl volumes of these solutions were transferred to a 96-well black micro titre plate and their fluorescence determined. The above process was repeated, however, instead of initially transferring 10 ml of MLVs to 10 ml of a polymer solution, 10 ml of MLVs were transferred to 10 ml of pH 6 HEPES only. Relative fluorescence of MLVs with encapsulated calcein in the presence and absence of polymers was plotted against pH of the solution (Figure 4.25).

4.1.3.5: Release of calcein from cationic liposomes with encapsulated calcein in the presence and absence of amine modified glycidyl methacrylate polymers with increasing pH.

Cationic liposomes with encapsulated calcein were synthesised again using thin film hydration using a total of 22.5 mg of egg PC, stearylamine and cholesterol at molar ratios of 63, 18 and 9, respectively (Recommended by Sigma, U.K) per ml of pH 6 adjusted 100 mM calcein solution. In addition, chloroform was used instead of diethyl ether to dissolve lipids since stearylamine is insoluble in diethyl ether. A 0.5 ml volume of liposomes was loaded on to the Sephadex column and all orange fractions were collected. Simultaneously, 20 ml volumes of 2 mg ml^{-1} *N,N*-diethylamine, *N,N*-diethyl ethylene diamine and 3-(diethylamino)propylamine modified GMPs in pH 6 HEPES solution were prepared. 10 ml volumes of the prepared polymer solutions were transferred to 10 ml of the collected liposomes and incubated at room temperature for 10 minutes to allow interaction of polymer with liposomes. After incubating, 1 ml volumes of the polymer and liposome solution were transferred to test tubes containing 9 ml of pH 6, 7, 8 and 9 HEPES buffer. Then, a 200 µl volume of each solution of pH buffer plus MLVs was transferred in duplicate to wells of a black micro titre plate. 20 µl volumes of pH 7 HEPES buffer and 20 µl volumes of 10% Triton X100 in pH 7 HEPES buffer were transferred to the first and second set of wells, respectively and the fluorescence of each well was determined. The described procedures were performed in triplicate.

The procedures described in this section were repeated except instead of initially transferring 10 ml of liposomes to 10 ml of polymer in HEPES, 10 ml of MLVs were

transferred to 10 ml of pH 6 HEPES. The percentage of total calcein fluorescence due to complete liposomal rupture at each pH value was determined by multiplying by 100 the value obtained when the fluorescence of wells with added buffer are divided by fluorescence of wells with added Triton X100 (Figure 4.26).

4.1.3.6: Release of calcein from neutrally charged MLVs with encapsulated calcein in the presence of anionic and cationic polymers with decreasing and increasing pH, respectively.

Using thin film hydration, as described previously, 10 mg of L-α-phosphatidylcholine-β-oleoyl-γ-palmitoyl (FW 760.1) per ml of 100 mM calcein in pH 7 HEPES was used to synthesise neutrally charged MLVs with encapsulated calcein. This was then repeated using 100 mM calcein in pH 6 HEPES. A 0.5 ml volume of the prepared pH 7 adjusted MLVs were loaded on to a Sephadex column and all orange fractions were collected using pH 7 HEPES buffer as eluate. Simultaneously, a 20 ml volume of a 2 mg ml^{-1} poly(lysine dodecandiamide) in pH 7 HEPES solution was prepared. A 10 ml volume of the prepared polymer solution was transferred to 10 ml of the collected liposomes and incubated at room temperature for 10 minutes. After incubating, 1 ml volumes of the solution of polymer and liposomes were transferred to test tubes containing 9 ml of pH 7, 6, 5 and 4 adjusted HEPES buffer. A 900 µl volume of each solution of pH buffer and MLVs was removed from the test tubes and transferred to both 100 µl of pH 7 HEPES buffer and 100 µl of 10% Triton X100 in HEPES. In triplicate, 150 µl volumes of these solutions were transferred to a 96-well black micro titre plate and their fluorescence determined.

The above process was then repeated, however, instead of initially transferring 10 ml of liposomes to 10 ml of poly(lysine dodecandiamide) in HEPES buffer, 10 ml of liposomes were transferred to 10 ml of pH 7 HEPES alone The obtained fluorescence readings were plotted (Figure 4.27).

Similarly, 0.5 ml of the prepared MLVs with encapsulated calcein in pH 6 adjusted buffer were loaded on to a Sephadex column and all orange fractions were collected using pH 6 HEPES buffer as an eluate. Simultaneously, a 20 ml volume of a 2 mg ml^{-}

[1] *N,N*-diethylamine GMP in pH 6 HEPES solution was prepared. A 10 ml volume of the prepared polymer solution was transferred to 10 ml of the collected liposomes and incubated at room temperature for 10 minutes. After incubating, a 1 ml volume of the solution of polymer and liposomes was transferred to test tubes containing 9 ml of pH 6, 7, 8 and 9 adjusted HEPES buffer. From each solution of pH buffer plus MLVs a 900 µl volume was removed and transferred to both 100 µl of pH 6 HEPES buffer and 100 µl of 10% Triton X100 in HEPES. In triplicate, 150 µl volumes of these solutions were transferred to a 96-well black micro titre plate and their fluorescence determined (Figure 4.28).

The above process was then repeated, however, instead of initially transferring 10 ml of liposomes to 10 ml of *N,N*-diethylamine GMP in HEPES buffer, 10 ml of liposomes were transferred to 10 ml of pH 6 HEPES buffer only (Figure 4.28).

4.1.3.7: Release of calcein from small liposomes with encapsulated calcein in the presence and absence of polymers.

To determine whether similar trends in release of calcein from small liposomes with pH change are observed when compared to release from MLVs, the following study was undertaken. Using thin film hydration negatively charged liposomes with encapsulated calcein were prepared using 5 mg of egg PC per ml of a 100 mM calcein in pH 7 HEPES solution. Negatively charged liposomes with encapsulated calcein were separated from non-encapsulated calcein using gel filtration as described in Section 4.1.2.5.2. Similarly, positively charged liposomes with encapsulated calcein were prepared using 19.2 mg of egg PC, 1.94 mg of stearylamine and 1.3 mg of cholesterol per ml of 100 mM calcein in pH 6 adjusted HEPES solution and separated from non-encapsulated calcein using gel filtration. To obtain suspensions of small liposomes with encapsulated calcein, the prepared liposome suspensions were then passed through 0.2 µm cellulose acetate filters three times. The mean vesicle diameter for each filtered liposome suspension was determined by transferring a 3 ml volume to clear cuvettes that had been rinsed with distilled water and dried previously. These cuvettes were then inserted in to a Zetasizer (Brookhaven Instruments) and the mean vesicle diameter of the suspensions determined. This was undertaken in triplicate for both positively and negatively charged liposomal suspensions. The mean diameter of

negative and positively charged liposomes was found to be 153.76 nm (standard error = 1.24) and 160.56 nm (standard error = 1.68), respectively. The release of calcein from small negatively charged liposomes with encapsulated calcein was determined in the presence and absence of poly(lysine dodecandiamide) with reducing pH by using the methodology described for MLVs in Section 4.1.3.1. These results are presented as mean percentage change in calcein fluorescence of small liposomes with encapsulated calcein in the presence and absence of poly(lysine dodecandiamide) followed by a change in pH (Figure 4.29). The release of calcein from small positively charged liposomes with encapsulated calcein was determined in the presence and absence of *N,N*-diethylamine modified GMP with increasing pH using the methodology described for cationic liposomes in Section 4.1.3.5. These results are presented as mean percentage change in calcein fluorescence of small liposomes with encapsulated calcein in the presence and absence of *N,N*-diethylamine modified GMP followed by a change in pH (Figure 4.30).

4.1.3.8: Determination of calcein release from MLVs with encapsulated calcein incubated in the presence of varying concentrations of poly(lysine dodecandiamide) followed by a change in pH.

All previous studies using poly(lysine dodecandiamide) have been performed at a concentration of 1 mg ml^{-1} of MLVs. It is not known whether this concentration is optimal for calcein release from MLVs. It would be beneficial to use the lowest possible concentration of polymer for maximum calcein release. That is, from a financial viewpoint, lowering polymer usage reduces production costs. Also, from a medical viewpoint, minimising the polymer concentration to cause membrane rupture internally would minimise toxicity to the human body. Therefore, an experiment was set up to determine calcein release from MLVs with encapsulated calcein in the presence of poly(lysine dodecandiamide) at concentrations of 2 mg, 1 mg and 0.5 mg per ml of MLVs. This was undertaken using the procedures described in Section 4.1.3.1, where pH adjusted HEPES buffer was used to reduce the pH of suspensions of polymer and MLVs. From the obtained results the mean percentage of total calcein released from MLVs that were pre-incubated with varying concentrations of poly(lysine dodecandiamide), followed by a pH change was calculated. However, in addition all fluorescence results obtained for samples in the presence of Triton X100

were increased by 9.27% since earlier experiments showed fluorescence of calcein was reduced by this value in the presence of 1% Triton X100 (Figure 4.31).

The release of calcein from anionic MLVs pre-incubated with a wider range of polymer concentrations was also determined. This was performed by firstly preparing a 10 ml volume of 4 mg ml^{-1} poly(lysine dodecandiamide) in pH 7 adjusted HEPES buffer. In triplicate, eight doubling dilutions of this solution were undertaken, by transferring 0.5 ml of the more concentrated solution to a test-tube containing 0.5 ml of fresh pH 7 HEPES buffer. A 0.5 ml volume was discarded from the eighth tube so that all tubes remained with a total volume of 0.5 ml. In addition, 0.5 ml of pH 7 HEPES buffer was transferred to test tubes in triplicate. A 0.5 ml volume of MLVs with encapsulated calcein was transferred to each test-tube. After 10 minutes, the volume of each test tube was made up to 5.5 ml using pH 4.0 adjusted HEPES buffer. This was carried out to reduce the pH of the solution of polymer and MLVs. In quadruplicate, 200 µl volumes were transferred from each test-tube to wells of a black micro titre plate. The fluorescence of each well was determined and the results obtained were plotted as fluorescence of calcein released from MLVs at pH 4 against polymer concentration (Figure 4.32).

4.1.3.9: Determination of calcein release from MLVs with encapsulated calcein incubated in the presence of varying concentrations of *N,N*-diethylamine modified glycidyl methacrylate polymer followed by a change in pH.

To determine the amount of calcein released from cationic MLVs when pre-incubated with increasing concentrations of *N,N*-diethylamine modified GMP the following study was performed. That is, cationic MLVs were firstly prepared by transferring 19.2 mg of egg PC, 1.94 mg of stearylamine and 1.36 mg of cholesterol to a round-bottomed flask with 25 ml of chloroform. The organic solvent was removed by rotary evaporation to obtain a thin lipid film and 1 ml volumes of 100 mM calcein solution were transferred to the flask and vortexed overnight. Free calcein was removed by gel filtration using pH 6 HEPES as eluent. Simultaneously, a 10 ml volume of a 4 mg ml^{-1} of *N,N*-diethylamine GMP in pH 6 adjusted HEPES buffer was prepared. In triplicate, eight doubling dilutions of this solution were then prepared, by transferring 0.5 ml of the more concentrated solution to a test-tube containing 0.5 ml of fresh pH 6 HEPES

buffer. A 0.5 ml volume was discarded from the eighth tube so that all tubes remained with a total volume of 0.5 ml. In addition, 0.5 ml of pH 6 HEPES buffer was transferred to test tubes in triplicate. A 0.5 ml volume of MLVs with encapsulated calcein obtained as described above was transferred to each test-tube. After 10 minutes, the volume of each test tube was made up to 10 ml using pH 9.0 adjusted HEPES buffer. This was undertaken to increase the pH of the polymer and liposome solution. In quadruplicate, 200 µl volumes were transferred from each test-tube to wells of a black micro titre plate. The fluorescence of each well was determined and the results obtained were plotted as fluorescence of calcein released from MLVs at pH 9 against polymer concentration (Figure 4.33)

4.1.3.10: Determination of calcein release from MLVs with encapsulated calcein incubated in the presence of poly(lysine dodecandiamide) for increasing incubation times prior to pH reduction.

It was thought more calcein would be released from MLVs following pH reduction if the time MLVs were incubated with polymer prior to pH reduction was increased. That is, it would be expected that an increased incubation time would allow more polymer to interact with the membrane of MLVs, consequently causing more to be ruptured when polymer changes confirmation at low pH. To determine this, MLVs were synthesised from 5 mg of egg PC per ml of 100 mM calcein. These MLVs were then incubated with poly(lysine dodecandiamide) for 10, 30 and 60 minutes before the pH of the suspension was reduced using HEPES buffer adjusted to pH values 7, 6, 5 and 4 as described in Section 4.1.3.1. The mean percentage change in calcein fluorescence of MLVs following pH change when pre-incubated with poly(lysine dodecandiamide) was determined (Figure 4.34).

4.1.4: Polymer properties which enable the rupturing of lipid membranes.

4.1.4.1: Charge Interactions.

It was shown that calcein release from anionic (Figure 4.19) and neutral liposomes (Figure 4.27) when pre-incubated with poly(lysine dodecandiamide), which has a

negative charge at neutrality, increases with reducing pH. Whereas, when anionic or neutral liposomes are incubated with amine modified GMPs, which have a positive charge at neutrality, a high calcein release is observed at pH 6 with no further increase observed with increasing pH (Figure 4.25). When cationic liposomes are incubated with amine modified GMPs, calcein release increases with increasing pH (Figure 4.26). These results are indicative of charge interactions between polymer and liposomes. Positive and negatively charged polymers lose charge with increasing and decreasing pH, respectively. However, the surface charge on liposomes is also dependent upon pH. That is, liposomes synthesised from egg PC have a strong negative charge at pH values of pH 7 and below. With reducing pH, the negative charge on the liposome surface becomes weaker and eventually becomes positive (Figure 4.1a). Liposomes synthesised from egg PC and stearylamine have a positive surface charge at pH values less than pH 7.5. However, with increasing pH the positive charge becomes weaker and eventually becomes negative (Figure 4.1b). It is thought that liposomal rupture will occur when the charge of polymer and surface charge on liposomes is opposite. To confirm liposomes used in this study followed the same trends in zeta potential with pH as described by Washington (2001) the following procedures were undertaken.

A pH 7.0 adjusted 10 mM HEPES buffer solution with 1 mM EDTA was prepared. No NaCl was added as this interferes with charge measurements. Positive MLVs were prepared by transferring 96 mg of egg PC, 9.7 mg of stearylamine and 6.8 mg of cholesterol to a round-bottomed flask and dissolving with 20 ml of chloroform. Chloroform was removed under vacuum by rotary evaporation for 2 hours at 25° C. To the dry lipid film 5 ml of the prepared HEPES buffer was added and agitated overnight. Volumes of 0.5 ml of the prepared MLVs were adjusted to pH values 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 using 0.1 M NaOH, with their final volume made up to 1.5 ml using HEPES buffer. Each pH adjusted suspension of MLVs was transferred to a 1 cm by 3 cm cuvette. Zeta potential measurements of each suspension were determined at 25° C by inserting the zeta potential probe in to the cuvettes. The obtained zeta potential readings were plotted against pH of liposome suspension (Figure 4.35). The above procedures were repeated for neutral liposomes prepared

using 10 mg of L-α-phosphatidylcholine-β-oleoyl-γ-palmitoyl (FW 760.1) per ml of pH 7 HEPES.

Figure 4.1a: Surface charge on egg PC liposomes (—) as a function of pH (adapted from Washington, 2001).

Figure 4.1b: Surface charge on egg PC/Stearylamine liposomes (—) as a function of pH (adapted from Washington, 2001).

Negative MLVs were synthesised using the same methodology as for positive MLVs but using 20 mg of egg PC per ml of pH 7.0 adjusted HEPES. 0.5 ml volumes of the prepared MLVs were adjusted to pH values 7.0, 6.5, 6.0, 5.5, 5.0, 4.5 and 4.0 using 0.1 M HCl with their final volume made up to 1.5 ml using HEPES buffer. Each suspension of pH adjusted MLVs was transferred to a 1 cm by 3 cm cuvette and the zeta potential of each suspension determined at 25° C by inserting the zeta potential

probe in to the cuvettes. The zeta potential readings were plotted against pH of the liposomal suspension (Figure 4.36). The above procedures were repeated for neutral liposomes prepared using 10 mg of L-α-phosphatidylcholine-β-oleoyl-γ-palmitoyl (FW 760.1) per ml of pH 7 HEPES.

4.1.4.2: Determination of whether polymer attaches to surface of MLVs.

Anionic MLVs with encapsulated calcein synthesised using 5 mg of egg PC per ml of 100 mM calcein in pH 7 adjusted HEPES were separated from free calcein using gel filtration and a column with an inner diameter and length of 20 mm and 260 mm, respectively as described in Section 4.1.2.5.2. A 5 ml volume of collected MLVs were transferred to 5 ml of pH 7 adjusted HEPES buffer and to a 5 ml volume of a 2 mg ml^{-1} poly(lysine dodecandiamide) in pH 7 HEPES solution. These solutions were incubated at 20° C for 10 minutes. Both solutions were then centrifuged at 5000 rpm for 10 minutes using a Beckman J2-MC centrifuge. The supernatant for each solution was removed and the liposomal pellets re-suspended in fresh pH 7 adjusted HEPES buffer. The centrifugation process was repeated a further two times. The pellets were finally re-suspended in 5 ml of HEPES buffer. 1 ml volumes of the re-suspended solutions were transferred to 9 ml of pH 7, pH 6, pH 5 and pH 4 buffers. Volumes of 900 µl were removed from each solution of pH adjusted buffer plus MLVs and transferred to both 100 µl of pH 7 HEPES buffer and 100 µl of 10% Triton X100 in HEPES. In triplicate, 150 µl volumes of these solutions were transferred to a 96-well black micro titre plate and their fluorescence determined. The obtained fluorescence values were used to calculate the mean percentage of total calcein released from MLVs, which have or have not been pre-incubated with polymer. These calculations were undertaken by multiplying by one hundred the value obtained when the fluorescence of MLVs exposed to polymer is divided by fluorescence of MLVs exposed to polymer with Triton X100 added. Similarly, percentage of total calcein released from MLVs not pre-incubated with polymer was calculated by multiplying by one hundred the value obtained for fluorescence of MLVs that have not been pre-incubated with polymer divided by fluorescence of MLVs that have not been pre-incubated with polymer with Triton X100 added. These percentage calcein release

values for MLVs exposed and not exposed to polymer were then plotted against pH of the solution (Figure 4.37)

4.1.5: Applicability of liposomes for *in vivo* use.

4.1.5.1: Liposome size studies.

As explained in Chapter 1, the size of liposomes largely governs their biodistribution. Therefore, the following experiments were undertaken to determine the effects of sonication and extrusion methods on the size of liposome preparations and determine whether homogenous suspensions could be obtained. The variation of liposome sizes was determined using Mastersizer and Zetasizer 1000 instruments (Malvern Instruments, UK), the principles of which are described in Appendices 1 and 2, respectively.

4.1.5.1.1: Sonication and extrusion studies.

As explained in Section 4.1.2 liposomes with encapsulated glucose were not suitable for membrane disruption studies. However, they were used in liposome size studies. Primarily, liposomes with encapsulated 2 M glucose in DPBS were prepared using a beaker method. That is, for each 1 ml of liposome suspension prepared, 33 μmol of egg PC dissolved in chloroform and 33 μmol of cholesterol dissolved in 5 ml of diethyl ether were transferred to a 25 ml glass beaker. For this investigation 3 ml of liposomes were required, therefore, the quantities stated above were increased threefold. A 3 ml volume of a 2 M glucose in DPBS solution (aqueous phase) was transferred to the dissolved lipids. Using a Stuart scientific magnetic stirrer and hotplate the mixture was vigorously stirred and heated (20-25°C) to remove the organic solvent and allow liposome encapsulation of aqueous phase. When all the organic solvent had been removed free glucose was removed from the liposome preparation by repeating the process of centrifugation at a speed of 11,000 rpm and re-suspension in fresh DPBS. 1 ml volumes of the prepared liposome suspensions were transferred to three separate test tubes labelled 1, 2 and 3. No further procedures were performed on the contents of test tube 1. Test tube 2 was exposed to sonication

for a period of 30 minutes as described, using a temperature of 0-5° C, which is above the transition temperature (Tc) of the lipids and using a bath type sonicator. It has been suggested sonication at a temperature below the Tc produces defects in the lipid bilayer of liposomes (Szoka *et al.*, 1980). The contents of test tube 3 were diluted 1 in 6 using DPBS, since the original suspension would be too viscous to pass through a pressure extruder. The diluted liposome suspensions were passed through an extruder three times using a cellulose filter with a pore size of 0.8 μm. To prevent filter blockage a pressure of 40-50 PSI and a temperature of 50° C was applied to the liposome suspension during extrusion. This process was then repeated using 0.4 μm and 0.2 μm pore size filters as described by Cortesi *et al.*, (1999). Particle size analysis was undertaken on the contents of test tube 1, using a Mastersizer (Malvern Instruments, Malvern UK) (Table 4.2 and Figure 4.42). Particle size analysis was undertaken on the contents of test tubes 2 (Table 4.2) and 3 (Table 4.2 and Figure 4.42) using a Zetasizer 1000 (Malvern Instruments, Malvern UK), which detects sub-micron sized particles. Figure 4.38 shows a typical Mastersizer volume histogram obtained in determining size distribution of a preparation of liposomes with encapsulated glucose synthesised using the beaker method. Figure 4.40 shows the typical Zetasizer 1000 intensity and volume histograms obtained in determining size distribution of a preparation of liposomes with encapsulated glucose synthesised using a beaker method and exposed to extrusion.

The latter experiment was repeated for liposomes with encapsulated glucose synthesised using reverse phase evaporation, adapted from the method described by Szoka *et al.*, (1978). That is, for each 1 ml of liposome suspension prepared, 33 μmol of egg PC dissolved in 5 ml of chloroform and 33 μmol of cholesterol dissolved in 5 ml of diethyl ether was transferred to a 50 ml round-bottomed glass flask with long extension neck. For this investigation a 3 ml volume of liposomes was required, therefore the quantities stated above were increased threefold. Using a Buchi rotary evaporator under reduced pressure, set at speed 9 and a temperature of 20-25° C a thin lipid film around the flask was achieved. The lipid film was re-dissolved using 5 ml of diethyl ether. Then 3 ml of the aqueous phase (2 M glucose in DPBS) was added. The solution was placed in a sonication-bath for 5 minutes and placed back on to the Buchi rotary evaporator in order to remove all the organic solvent. When all the

organic solvent had been removed free glucose was removed from the liposome preparation by repeat centrifugation at a speed of 11,000 rpm and re-suspension in fresh DPBS until all free glucose was removed, determined by taking readings of the supernatant using a YSI 2300 STAT PLUS machine. Similar to the beaker method, the liposome preparation was divided equally between three separate glass vials and labelled 1, 2 and 3. No further treatment was applied to the contents of test tube 1. The contents of test tube 2 were exposed to sonication for a period of 30 minutes at a temperature of 0-5° C. Finally, using a pressure of 40-50 PSI and a temperature of 50° C the contents of test tube 3 were passed through an extruder three times using a 0.8 μm pore size filter. Then three times through 0.4 μm and 0.2 μm pore size filters. Particle size analysis was again performed on the contents of test tube 1 (Table 4.2 and Figure 4.42), using a Mastersizer (Malvern Instruments, Malvern UK) and on test tube 2 (Table 4.2) and test tube 3 (Table 4.2 and Figure 4.42) using a Zetasizer 1000 (Malvern Instruments, Malvern UK). Figure 4.39 shows a typical Mastersizer volume histogram obtained in determining size distribution of a preparation of liposomes with encapsulated glucose synthesised using the reverse phase evaporation vesicle method. Figure 4.41 shows the typical Zetasizer 1000 intensity and volume histograms obtained in determining size distribution of a preparation of liposomes with encapsulated glucose synthesised using the reverse phase evaporation vesicle method and exposed to extrusion.

4.1.5.1.2: Determination of whether washing liposome suspensions to remove free glucose or the encapsulated agent affects liposome size results.

It was thought that washing liposomes to remove free glucose might have resulted in liposome aggregation. This would result in the obtained mean diameter being higher than the true mean diameter. To determine whether aggregation was occurring, 1 ml of liposomes with encapsulated 2 M glucose in DPBS were synthesised in triplicate, using reverse phase evaporation as described in Section 4.1.5.1.1. However, no centrifugation process was applied in order to remove free glucose. Liposome size analysis was conducted using the Malvern Mastersizer (Table 4.3).

It was also thought encapsulating buffer instead of glucose could affect the size of liposomes. To evaluate this, in triplicate, 1 ml volumes of liposomes with encapsulated DPBS were synthesised using reverse phase evaporation. However, an aqueous phase of DPBS was used instead of glucose in DPBS. No centrifugation process was applied to these preparations. Size analysis was performed using a Malvern Mastersizer (Table 4.3).

4.1.5.1.3: Effect of increasing sonication time on liposome preparations.

The investigation conducted in Section 4.1.5.1.1 was used to determine whether sonication of liposome preparations for a period of 30 minutes reduced their polydispersity index for PCS analysis. However, it was shown that all preparations had a polydispersity greater than 0.7, thus, were not suitable for PCS analysis. Therefore, the following investigation was undertaken in order to determine whether exposing liposome preparations to sonication for longer periods of time would reduce their polydispersity. Firstly, 1 ml volumes of liposomes with encapsulated DPBS were synthesised using both beaker and reverse phase evaporation methods, as described previously. The liposomes prepared were made up to 8 ml using DPBS. From the diluted samples 1 ml volumes were transferred to separate vials and stored at 4° C. The remainder of the samples were exposed to sonication in a bath type sonicator. Volumes of 1 ml were transferred to vials after 2, 4 and 6 hours of sonication. These vials were stored at 4° C until size analysis was performed using Mastersizer and Zetasizer 1000 instruments (Malvern Instruments, Malvern UK). Figures 4.43 to 4.46 show volume distribution curves for liposomes with encapsulated DPBS synthesised using a beaker method and exposed to sonication for zero, two, four or six hours. Figure 4.51 shows the mean volume diameter of liposomes with encapsulated DPBS synthesised using a beaker method and exposed to sonication for an increasing period of time. Figure 4.47 to 4.50 show volume distribution curves for liposomes with encapsulated DPBS synthesised using reverse phase evaporation and exposed to sonication for zero, two, four or six hours. Figure 4.52 shows the mean volume diameter of liposomes with encapsulated DPBS synthesised using reverse phase evaporation and exposed to sonication for an increasing period of time.

4.1.5.2: Lyophilisation.

MLVs with encapsulated calcein were shown to degrade with time when stored at 4° C (Figure 4.11 and 4.12). Extending the storage time of liposomes prepared in this study would be useful if they are to have applications such as pharmaceutics in the future. Vemuri *et al.*, (1995) explain that such content leakage is often a limiting factor in the commercial development of liposome products. They also describe how lyophilising pharmaceuticals yield high quality products with increased shelf life. A number of researchers have shown liposomes containing drug molecules can be lyophilised and reconstituted with significant drug retention and without significant change in the mean vesicle size (Ward *et al.*, 1996; Crowe *et al.*, 1985; Miyajima, 1997; Zhang *et al.*, 1997). However, cryoprotectants such as sucrose, glucose, lactose and trehalose were required during lyophilisation to achieve this. During rehydration water molecules replace the sugars and liposomes appear to reseal before significant leakage occurs.

To determine the release of calcein from anionic and cationic liposomes that have been pre-incubated with polymer or buffer followed by lyophilisation the following studies were undertaken.

4.1.5.2.1: Preparation of anionic MLVs.

A 10 ml volume of 100 mM calcein in HEPES solution adjusted to pH 7.0 using NaOH was prepared. 10 mg of egg PC (FW 762.10) was transferred to a round-bottomed flask containing 25 ml of diethyl ether. The organic solvent was removed by rotary evaporation to obtain a thin lipid film. A 2 ml volume of 100 mM calcein in HEPES solution adjusted to pH 7 using NaOH was transferred to the flask and vortexed overnight. A 1 ml volume was removed from the flask and passed through a gel filtration column using HEPES buffer as eluent. All orange fractions that are indicative of MLVs with encapsulated calcein were collected.

4.1.5.2.2: Preparation of cationic MLVs.

A 10 ml volume of aqueous phase was prepared. That is, 100 mM calcein in HEPES solution adjusted to pH 6.0 using NaOH. Per ml of aqueous phase prepared, 19.2 mg of egg PC (FW 762.1), 1.94 mg of stearylamine (FW 269.5) and 1.36 mg cholesterol (FW 386.7) were transferred to a round-bottomed flask containing 25 ml of chloroform. The organic solvent was removed by rotary evaporation to obtain a thin lipid film. 2 ml of the prepared calcein solution was transferred to a flask and vortexed overnight. A 1 ml volume was removed from the flask and passed through a gel filtration column using HEPES buffer as eluent. All orange fractions indicative of MLVs with encapsulated calcein were collected.

4.1.5.2.3: Measurement of the pH of HEPES buffers before and after freezing.

To check freezing did not alter the pH of the pH 7 and pH 6 adjusted HEPES solution, samples were frozen at minus 80° C and then allowed to thaw and stabilised to 20°C. The pH values were then checked and it was noted that freezing did not alter the pH of the buffers.

4.1.5.2.4: Determination of calcein release from anionic and cationic MLVs before and following lyophilisation.

The fluorescence of anionic and cationic MLVs with encapsulated calcein prior to lyophilisation was determined by transferring ten 100 µl volumes to wells of a black micro titre plate each containing 10 µl volumes of pH 7 or pH 6 adjusted HEPES solution for anionic and cationic MLVs, respectively. The fluorescence of MLVs prior to lyophilisation when completely lysed was determined by transferring ten 100 µl volumes of MLVs to wells containing 10 µl of 20% Triton X100. From these fluorescence values, the percentage of total fluorescence due to released calcein could be calculated using the following calculation. Percentage of total fluorescence due to released calcein = (A - B)/A, where A is the mean fluorescence of 100 µl volumes of MLVs with encapsulated calcein with 10 µl of 20% Triton X100 added and B is the

mean fluorescence of 100 µl volumes of MLVs with encapsulated calcein with 10 µl of HEPES solution added. The results of these calculations are shown in Table 4.4.

The release of calcein from both cationic and anionic MLVs after lyophilising was determined. Twenty 200 µl volumes of anionic and cationic MLVs were frozen to minus 80° C using a deep freeze, then lyophilised. Lyophilised MLVs were re-suspended with 200 µl volumes of distilled water. The fluorescence of re-suspended MLVs was determined by transferring ten 100 µl volumes to wells of a black micro titre plate containing 10 µl of pH 7 or pH 6 adjusted HEPES solution for anionic and cationic MLVs, respectively. The fluorescence of lyophilised MLVs when completely lysed was determined by transferring ten 100 µl volumes of re-suspended MLVs to wells containing 10 µl of 20% Triton X100. From these fluorescence values, the percentage of total fluorescence due to released calcein could be calculated, using the following calculation. Percentage of total fluorescence due to released calcein = (A - B)/A, where A is the mean fluorescence of 100 µl volumes of MLVs with encapsulated calcein with 10 µl of 20% Triton X100 added and B is the mean fluorescence of 100 µl volumes of MLVs with encapsulated calcein with 10 µl of HEPES solution added. The results for these calculations are also displayed in Table 4.4.

4.1.5.2.5: Determination of calcein release from anionic MLVs pre-incubated with poly(lysine dodecandiamide) followed by pH change using MLVs that have not been exposed to lyophilisation.

In triplicate, 1.5 ml volumes of anionic MLVs were transferred to 1.5 ml volumes of a 2 mg ml^{-1} poly(lysine dodecandiamide) in pH 7 adjusted HEPES solution. These solutions were incubated for 10 minutes at 20° C. Then, 500 µl volumes were transferred to 4.5 ml volumes of pH 7, 6, 5 and 4 HEPES adjusted solutions. In duplicate, 200 µl volumes of each pH adjusted solution was transferred to a black micro titre plate. To one set of wells 20 µl of HEPES solution was transferred. To the other set 20 µl of 10% Triton X100 was transferred. The fluorescence of wells was measured. From these fluorescence values the percentage of total calcein release from MLVs due to polymer could be calculated and are displayed in Table 4.5.

The above procedures were repeated but instead of initially transferring 1.5 ml of MLVs to 1.5 ml of polymer, 1.5 ml of MLVs were transferred to 1.5 ml of pH 7 HEPES solution. The obtained results were used to calculate total calcein release from MLVs in the absence of polymer (Table 4.5).

4.1.5.2.6: Determination of calcein release with pH change from MLVs which are lyophilised in the presence of poly(lysine dodecandiamide).

To determine calcein release with pH from MLVs which are lyophilised in the presence of poly(lysine dodecandiamide) the following methodology was performed. In triplicate, a 1.5 ml volume of anionic MLVs was transferred to a 1.5 ml volume of a 2 mg ml^{-1} of poly(lysine dodecandiamide) solution. This solution was lyophilised then re-suspended using 3 ml of distilled water. 500 µl volumes were then transferred to pH 7, 6, 5 and 4 adjusted HEPES solution. In duplicate, 200 µl volumes of the pH adjusted solutions were transferred to a black micro titre plate. To one set of wells 20 µl of HEPES solution was transferred and to the other set 20 µl of 10% Triton X100 was transferred. The fluorescence of these wells was measured. From these fluorescence readings the percentage of total calcein release from MLVs due to polymer could be calculated.

This experiment was then repeated but instead of initially transferring 1.5 ml of MLVs to 1.5 ml of polymer, 1.5 ml of MLVs were transferred to 1.5 ml of pH 7 buffer solution. The obtained results were used to calculate total calcein release from MLVs in the absence of polymer (Table 4.5).

4.1.5.2.7: Determination of calcein release from cationic MLVs pre-incubated with N,N-diethylamine modified GMP followed by pH change using MLVs that have not been exposed to lyophilisation.

In triplicate, 1.5 ml volumes of cationic MLVs were transferred to 1.5 ml volumes of a 2 mg ml^{-1} *N,N*-diethylamine modified GMP in pH 6 adjusted HEPES solution. These solutions were incubated for 10 minutes at 20° C. 500 µl volumes were

transferred to 4.5 ml volumes of pH 6, 7, 8 and 9 adjusted HEPES solutions. In duplicate, 200 µl volumes of each pH adjusted solution was transferred to a black micro titre plate. To one set of wells 20 µl of buffer was transferred. To the other 20 µl of 10% Triton X100. The fluorescence of wells was measured. From these fluorescence values the percentage of total calcein release from MLVs due to polymer could be calculated.

This experiment was repeated but instead of initially transferring 1.5 ml of MLVs to 1.5 ml of polymer, 1.5 ml of MLVs were transferred to 1.5 ml of pH 6 adjusted buffer. These results are displayed in Table 4.6.

4.1.5.2.8: Determination of calcein release with pH change from MLVs which are lyophilised in the presence of N,N-diethylamine modified GMP.

In triplicate, 1.5 ml volumes of cationic MLVs were transferred to 1.5 ml volumes of a 2 mg ml^{-1} *N,N*-diethylamine modified GMP solution. These solutions were lyophilised and re-suspended using 3 ml of distilled water and volumes of 500 µl volumes were transferred to pH 6, 7, 8 and 9 HEPES buffers. In duplicate, 200 µl volumes of each of the latter pH adjusted solution were transferred to a black microtitre plate, with one receiving 20 µl of pH 6 adjusted HEPES solution and the other 20 µl of 10% Triton X100. The fluorescence was measured and from these values the percentage of total calcein release from MLVs due to polymer could be calculated.

This experiment was repeated but instead of initially transferring 1.5 ml of MLVs to 1.5 ml of polymer, 1.5 ml of MLVs were transferred to 1.5 ml of pH 6 HEPES solution. These results are displayed in Table 4.6.

4.2: Results.

Liposomes change from appearing opaque to clear when they are solubilised. The liposome solubilisation properties of a range of polymers as a function of pH was assessed. This was assessed by pre-incubating liposomes in both the presence and

absence of polymer and observing these liposome suspensions before and following pH adjustment. Plates 4.1 to 4.6 show the observations that were made. A summary of the observations made is also presented in Table 4.1.

To quantify the pH-responsive membrane disruption properties of polymers, liposomes with encapsulated calcein were prepared so that released calcein could be used as an indicator of membrane disruption. Before this was performed preliminary studies were performed to determine the optimal excitation and emission wavelengths of calcein. Also, studies were performed to determine the effect of pH and Triton X100 on calcein fluorescence. The optimal excitation wavelength of calcein was determined and is presented in Figure 4.2. Similarly, the optimal emission wavelength of calcein was determined and is presented in both Figure 4.3 and Figure 4.4. The detection limits of a Spectra Max Gemini XS fluorescent plate reader for calcein was determined. This was achieved by preparing a calcein solution that was increasingly diluted. The fluorescence readings that were obtained for each dilution are presented in Figure 4.5.

The effect of pH on fluorescence of calcein solutions was determined by preparing pH adjusted calcein solutions and measuring their fluorescence. The fluorescence readings obtained are presented in Figure 4.6. Similarly the fluorescence readings of calcein solutions treated with Triton X100 are presented in Figure 4.7 to show the effect Triton X100 has on calcein fluorescence.

Liposomes with encapsulated calcein were prepared and separated from free calcein by passing the liposomes with encapsulated calcein plus free calcein through gel filtration columns. The fluorescence of the fractions collected from gel filtration columns was determined to assess which fractions contained liposomes. The fluorescence readings of the collected fractions are presented in Figures 4.8 to 4.15. Liposomes with encapsulated calcein were pre-incubated with polymer and buffer solutions. The pH of these solutions was adjusted and their fluorescence determined. The fluorescence of these pH adjusted solutions are presented for comparison in Figures 4.16 to 4.34.

The charge on pH adjusted liposome suspensions was determined by taking Zeta potential measurements. The measurements obtained are presented in Figure 4.35 and Figure 4.36.

To determine whether polymer binds to liposome solutions, polymer and liposome solutions were incubated together. Any free polymer was removed from this solution by washing liposomes using centrifugation and re-suspension with buffer. Washed liposomes were re-suspended, their pH was adjusted and the fluorescence of released calcein was measured and is presented in Figure 4.37. For comparison, the fluorescence of released calcein from liposomes due to pH effects alone was determined and is also presented in Figure 4.37. This was performed by repeating the same procedures but with liposomes that were initially incubated with buffer.

The size of liposomes is an important factor for *in vivo* applications. The sizes of liposomes that were prepared and exposed to different conditions are presented in Table 4.2, Table 4.3 and Figures 4.38 to 4.52.

It is also useful if liposomes prepared for *in vivo* applications can be lyophilised. The fluorescence of calcein released from liposomes was used to indicate the extent of membrane disruption caused by the lyophilisation process when liposomes are pre-incubated in the presence or absence of polymer. These fluorescence readings are presented in Table 4.4 to 4.6.

Plate 4.1: Observations made following the indicated pH adjustment, for MLVs incubated with poly(lysine dodecandiamide) (A); MLVs incubated with buffer (B); and poly(lysine dodecandiamide) incubated with buffer (C).

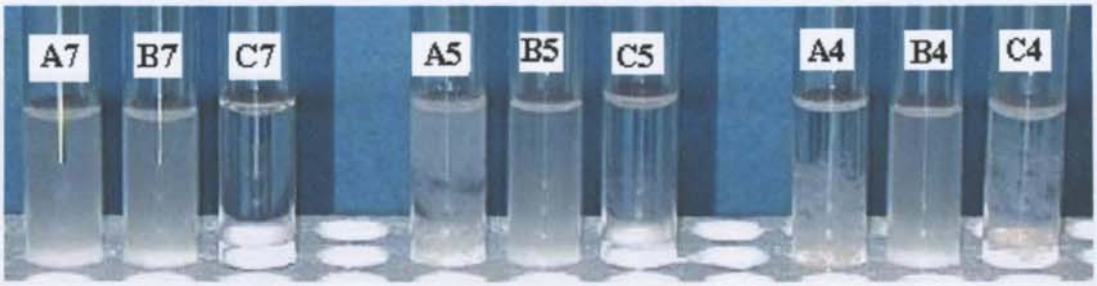


Plate 4.2: Observations made following the indicated pH adjustment, for MLVs incubated with poly(lysine *iso*-phthalamide) (A); MLVs incubated with buffer (B); and poly(lysine *iso*-phthalamide) incubated with buffer (C).

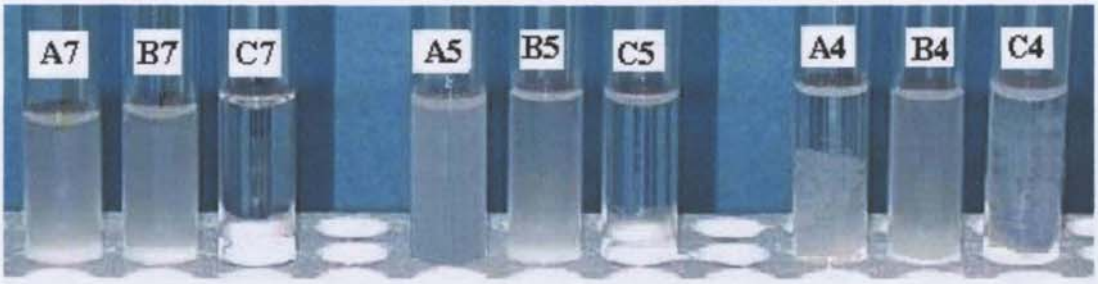


Plate 4.3: Observations made following the indicated pH adjustment, for MLVs incubated with poly(lysine butyl malonamide) (A); MLVs incubated with buffer (B); and poly(lysine butyl malonamide) incubated with buffer (C).

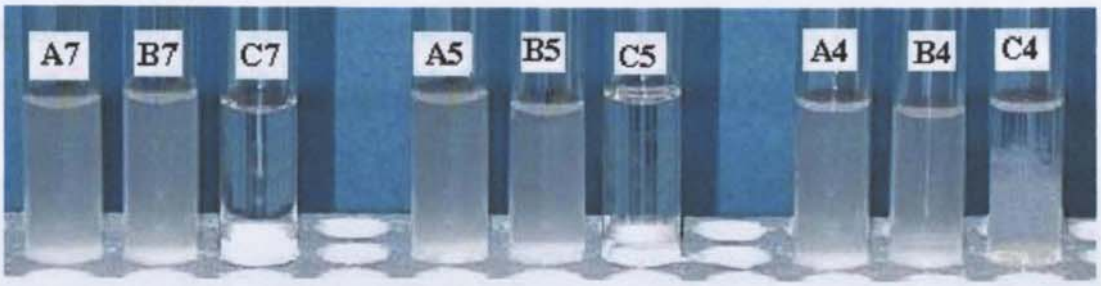


Plate 4.4: Observations made following the indicated pH adjustment, for MLVs incubated with *N,N*-diethylamine modified glycidyl methacrylate polymer (A); MLVs incubated with buffer (B); and *N,N*-diethylamine modified glycidyl methacrylate polymer incubated with buffer (C).

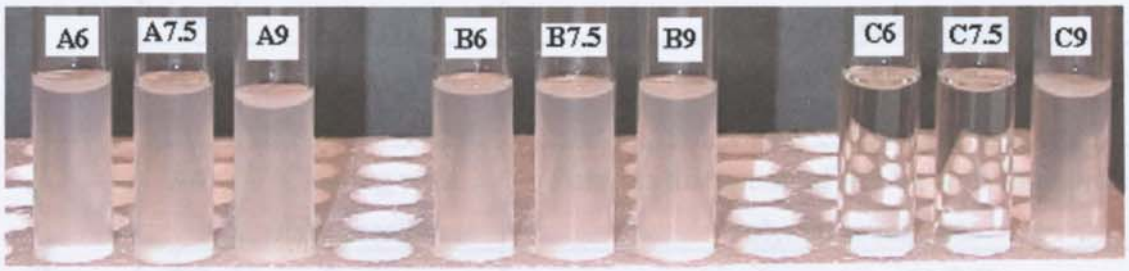


Plate 4.5: Observations made following the indicated pH adjustment, for MLVs incubated with *N,N*-diethyl ethylene diamine modified glycidyl methacrylate polymer (A); MLVs incubated with buffer (B); and *N,N*-diethyl ethylene diamine modified glycidyl methacrylate polymer incubated with buffer (C).

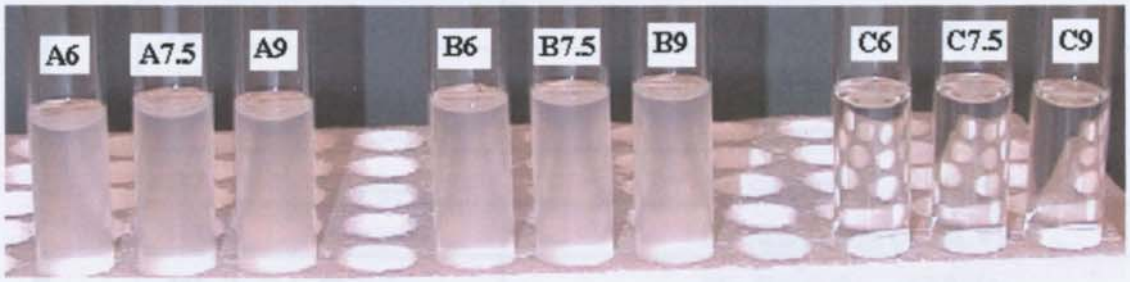


Plate 4.6: Observations made following the indicated pH adjustment, for MLVs incubated with 3-(diethylamino)propylamine modified glycidyl methacrylate polymer (A); MLVs incubated with buffer (B); and 3-(diethylamino)propylamine modified glycidyl methacrylate polymer incubated with buffer (C).

Table 4.1: Summary of the observations made following pH adjustment of: polyamides incubated with MLVs; MLVs incubated with buffer and polyamides incubated with buffer.

Polymer	Tube	Observed result	Description of observed result
Poly(lysine dodecandiamide)	A7	Opaque	No disruption of MLVs
	A5	Clearing with white precipitate	Possible disruption of MLVs with formation of polymer precipitate
	A4	Clearing with white precipitate	Possible disruption of MLVs with formation of polymer precipitate
	B7	Opaque	No disruption of MLVs
	B5	Opaque	No disruption of MLVs
	B4	Opaque	No disruption of MLVs
	C7	Clear	No formation of polymer precipitate
	C5	Cloudiness	Formation of some polymer precipitate
	C4	White precipitate	Formation of polymer precipitate
Poly(lysine *iso*-phthalamide)	A7	Opaque	No disruption of MLVs
	A5	Opaque	No disruption of MLVs
	A4	Clearing with white precipitate	Possible disruption of MLVs with formation of polymer precipitate
	B7	Opaque	No disruption of MLVs
	B5	Opaque	No disruption of MLVs
	B4	Opaque	No disruption of MLVs
	C7	Clear	No formation of polymer precipitate
	C5	Clear	No formation of polymer precipitate
	C4	White precipitate	Formation of polymer precipitate
Poly(lysine butyl malonamide)	A7	Opaque	No disruption of MLVs
	A5	Opaque	No disruption of MLVs
	A4	Opaque	No disruption of MLVs
	B7	Opaque	No disruption of MLVs
	B5	Opaque	No disruption of MLVs
	B4	Opaque	No disruption of MLVs
	C7	Clear	No formation of polymer precipitate
	C5	Clear	No formation of polymer precipitate
	C4	White precipitate	Formation of polymer precipitate

Figure 4.2: Relative fluorescence of a 1 μM calcein solution over a range of excitation wavelengths and using an emission wavelength of 520 nm.

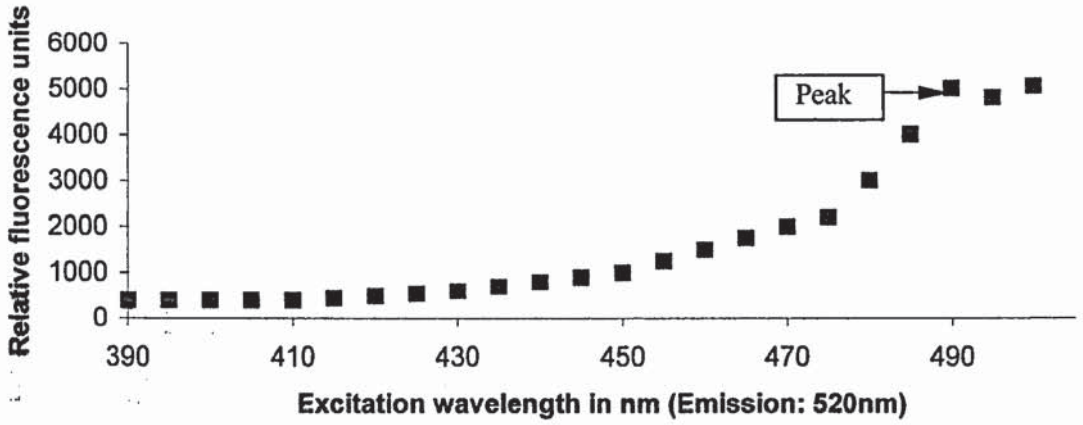


Figure 4.3: Relative fluorescence of a 1 μM calcein solution over a range of emission wavelengths and using an excitation wavelength of 490 nm.

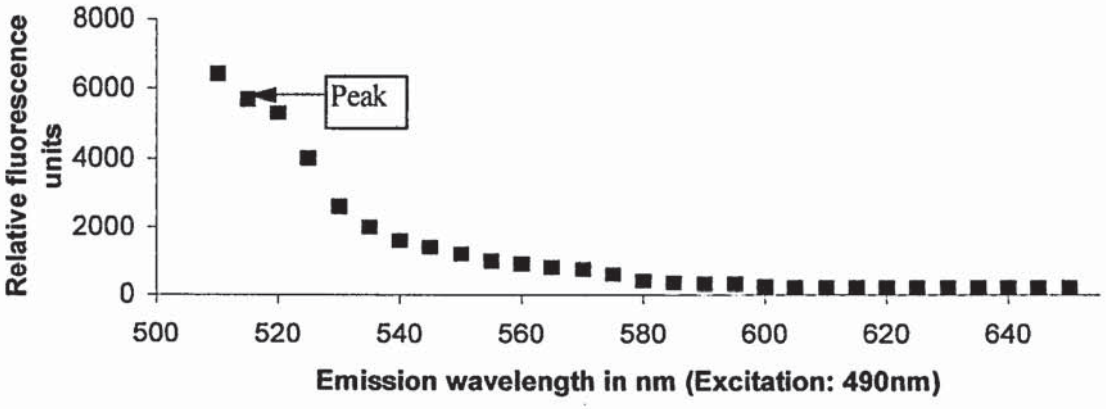


Figure 4.4: Relative fluorescence of a 1 μM calcein solution over a range of emission wavelengths, using an excitation wavelength of 490 nm, with a 515 nm excitation 'cut off' filter introduced.

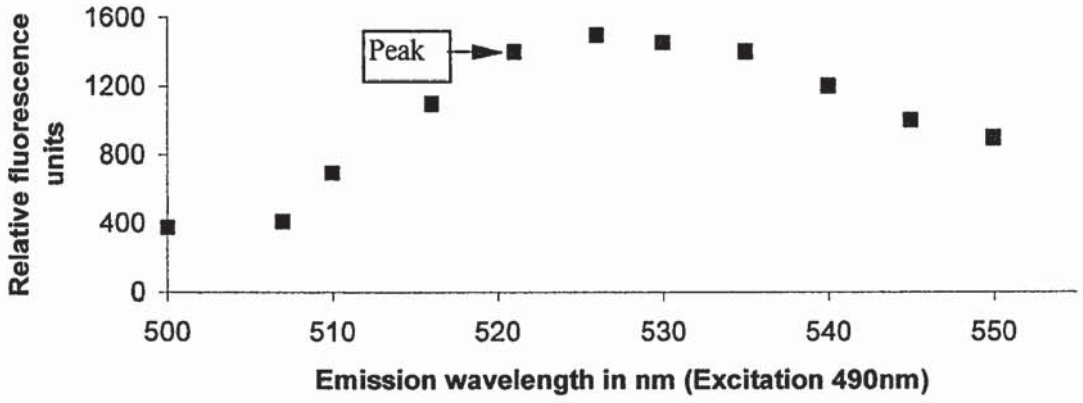


Figure 4.5: Mean relative fluorescence of a calcein solution over a range of concentrations using excitation and emission wavelengths of 490 nm and 520 nm respectively, with a 515 nm emission 'cut off' filter introduced (error bars indicating standard error).

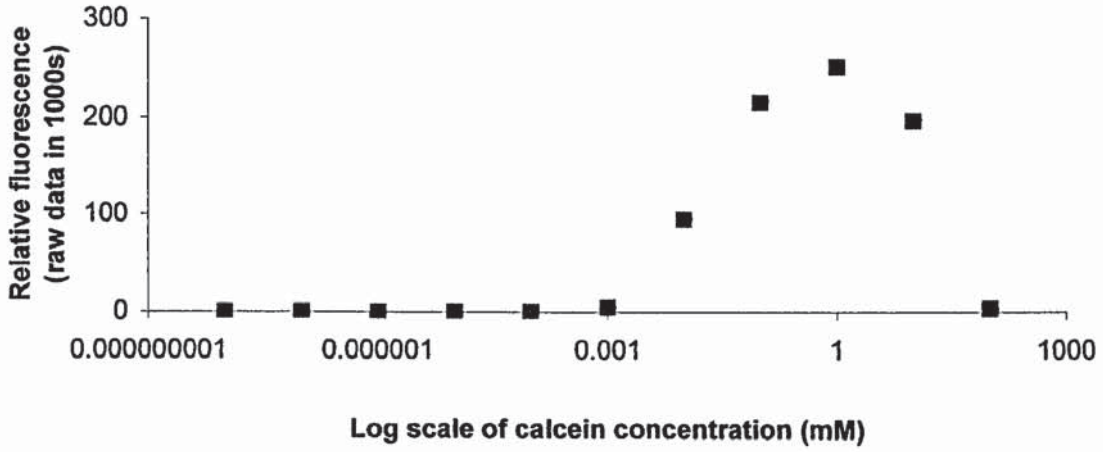


Figure 4.6: Mean fluorescence of 0.01 mM calcein solutions over a range of pH values.

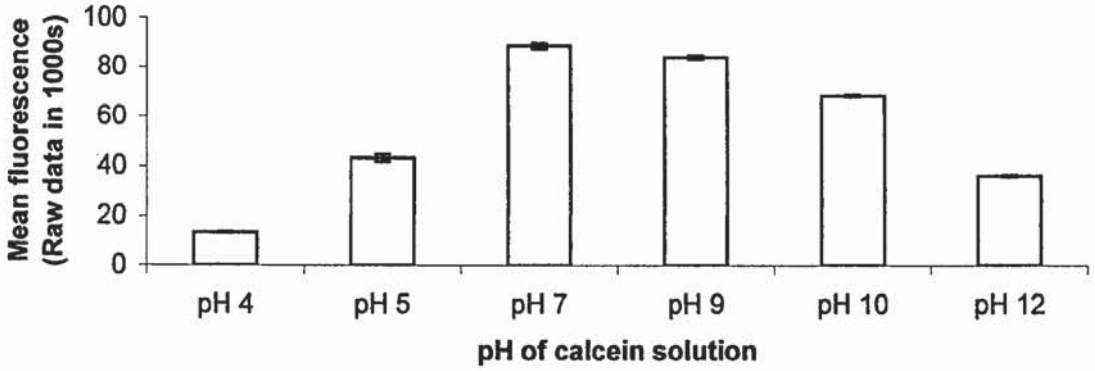


Figure 4.7: Percentage difference in fluorescence of a 0.1 mM calcein solution when treated with buffer and Triton X100.

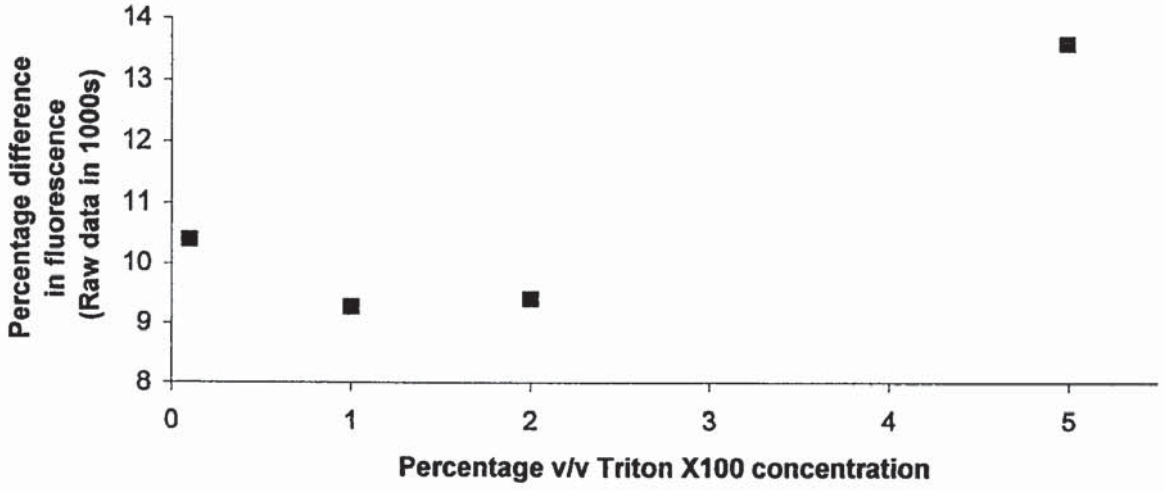


Figure 4.8: Relative fluorescence of fractions collected from a gel filtration column which are not diluted ■, not diluted and treated with Triton X100 □, diluted by a factor of 2▲ and diluted by a factor of 2 and treated with Triton X100 Δ.

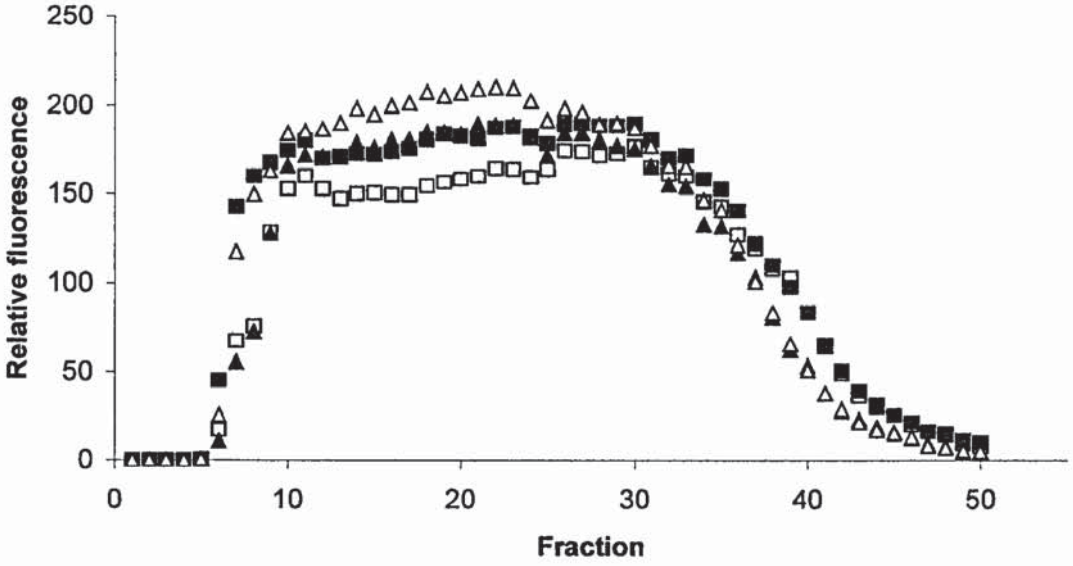


Figure 4.9: Relative fluorescence of fractions collected from a gel filtration column which are diluted by a factor of 4 ■, diluted by a factor of 4 and treated with Triton X100 □, diluted by a factor of 8 ▲ and diluted by a factor of 8 and treated with Triton X100 Δ.

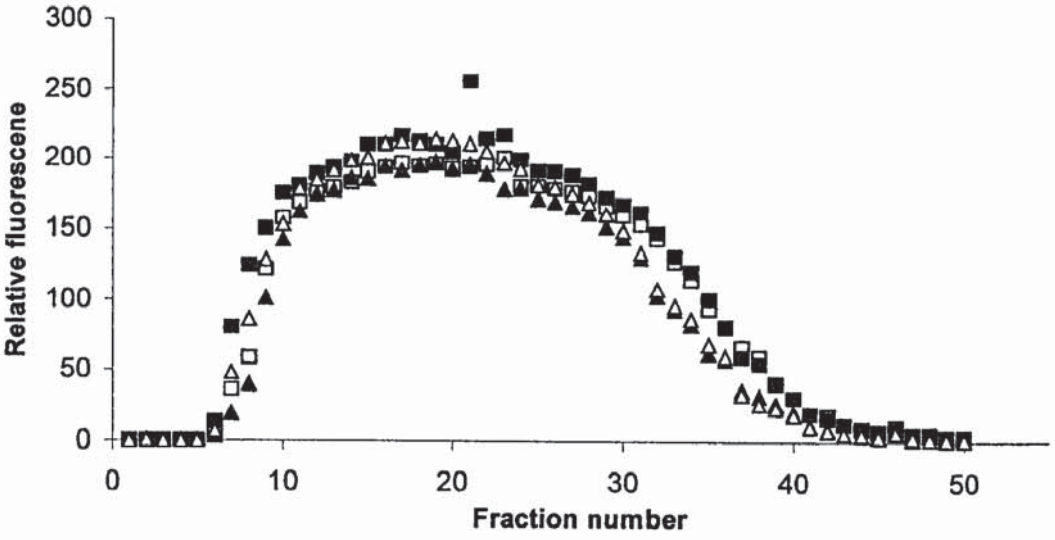


Figure 4.10: Fluorescence of eluate treated and not treated with Triton X100 and in comparison to control solutions, buffer and buffer treated with Triton X100.

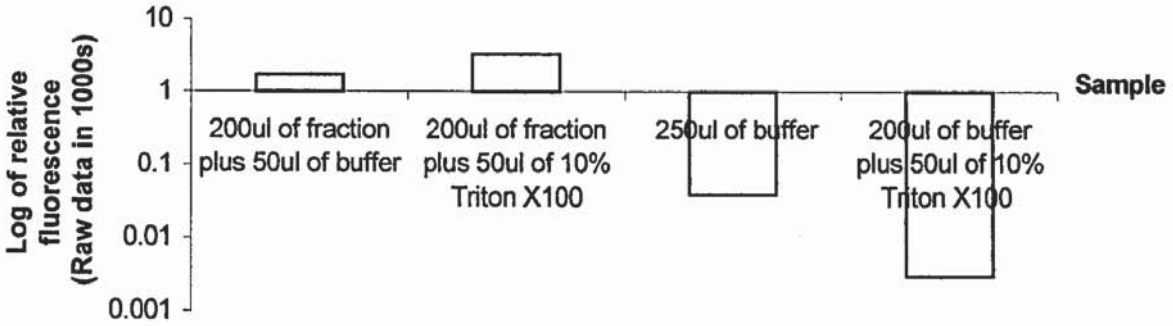


Figure 4.11: Fluorescence of buffer treated ■, 1% Triton X100 □ and 2% Triton X100 treated fractions •, collected from a size exclusion column loaded with anionic MLVs with encapsulated calcein, immediately after synthesis.

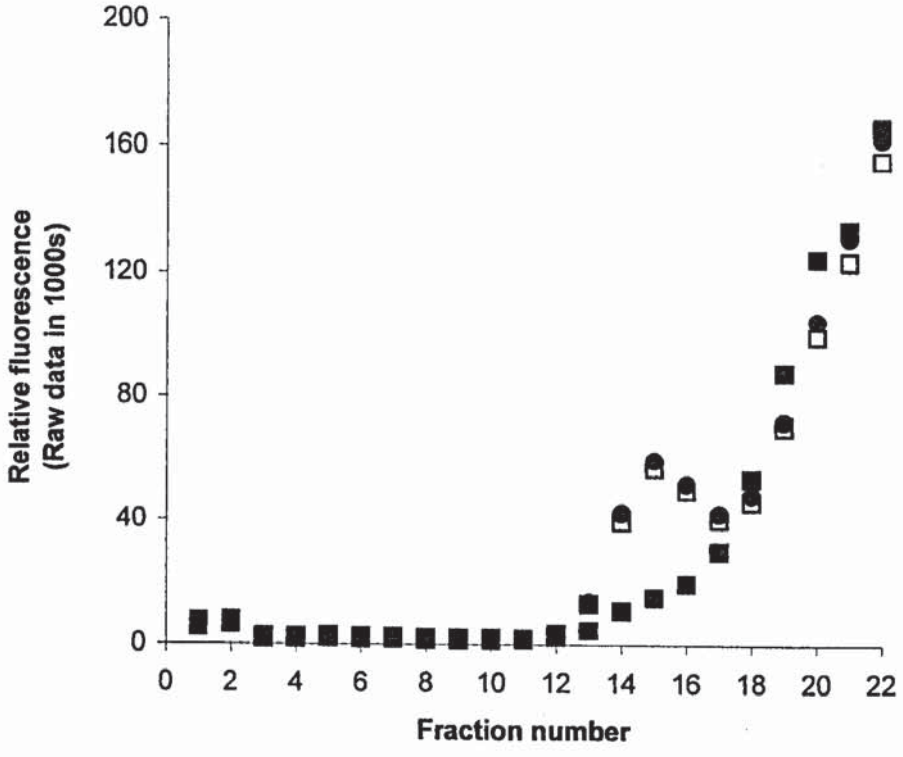


Figure 4.12: Fluorescence of buffer treated ■, 1% Triton X100 □ and 2% Triton X100 treated fractions •, collected from a size exclusion column loaded with anionic MLVs with encapsulated calcein, 24 hours after synthesis.

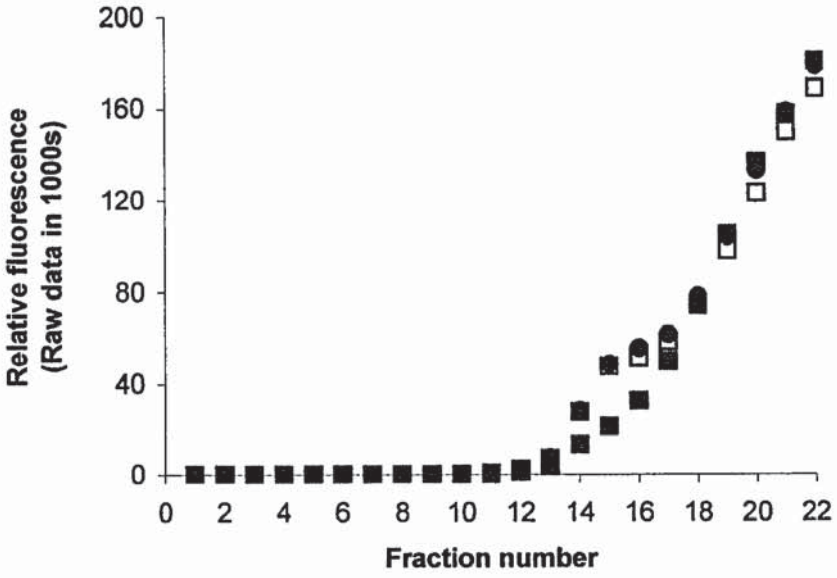


Figure 4.13: Fluorescence of buffer treated ■, 1% Triton X100 □ and 2% Triton X100 treated fractions •, collected from a size exclusion column loaded with anionic MLVs with encapsulated calcein, immediately after synthesis.

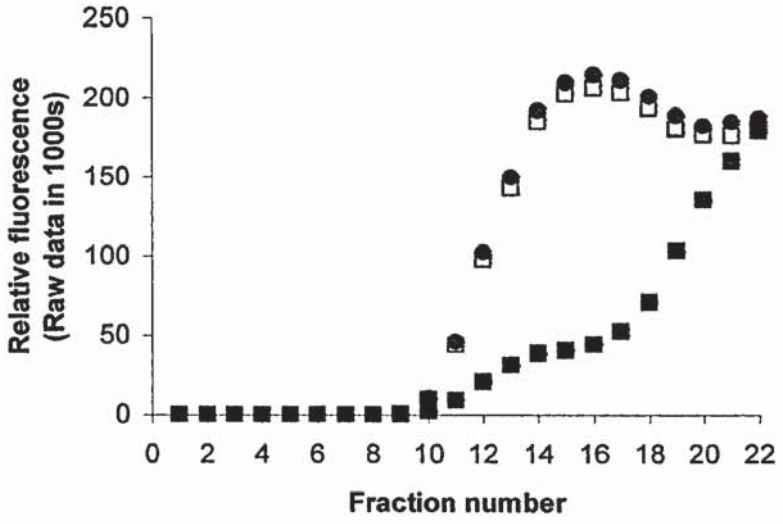


Figure 4.14: Fluorescence of buffer treated ■, 1% Triton X100 □ and 2% Triton X100 treated fractions •, collected from a size exclusion column loaded with anionic MLVs with encapsulated calcein, 24 hours after synthesis.

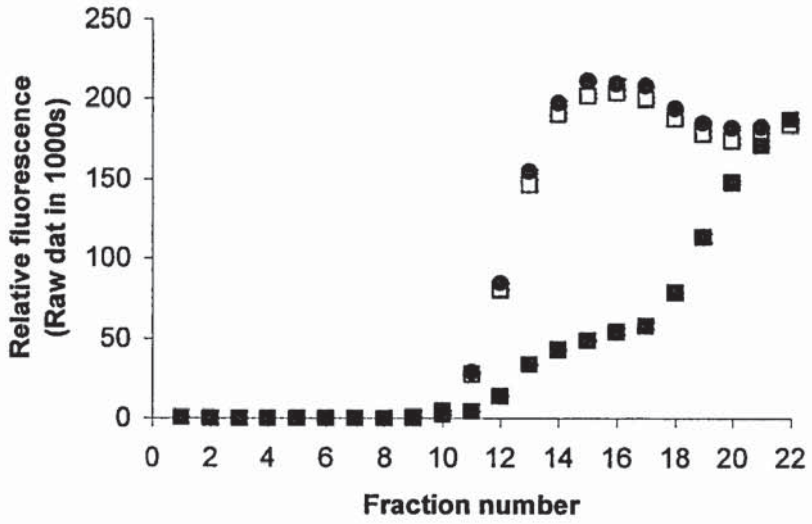


Figure 4.15: Relative fluorescence of a suspension of MLVs with encapsulated calcein decimally diluted and treated with buffer ■ and Triton X100 □.

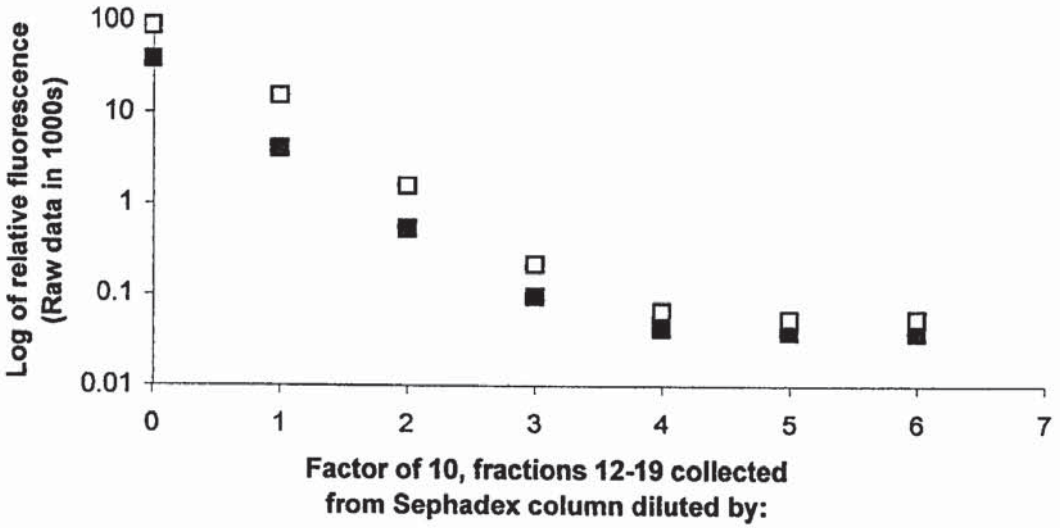


Figure 4.16: Mean fluorescence following pH change of anionic MLVs with encapsulated calcein incubated with poly(lysine dodecandiamide) ■; anionic MLVs with encapsulated calcein incubated with pH 7 HEPES □; anionic MLVs with encapsulated calcein incubated with poly(lysine dodecandiamide) and treated with 1% v/v Triton X100 ▒; anionic MLVs with encapsulated calcein incubated with pH 7 HEPES and treated with 1% v/v Triton X100 ▓.

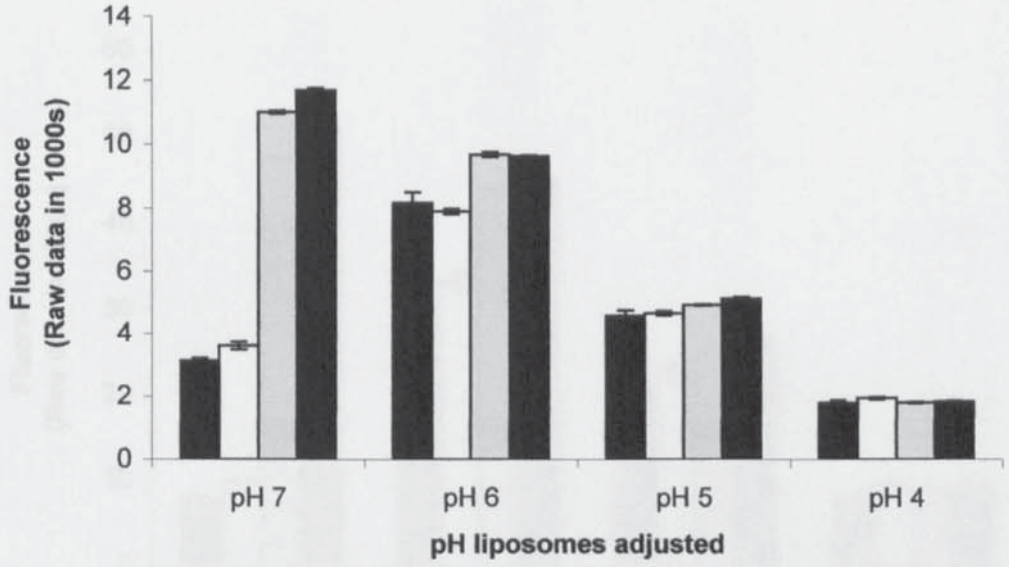


Figure 4.17: Mean fluorescence following pH change of anionic MLVs with encapsulated calcein incubated with poly(lysine dodecandiamide) ■; anionic MLVs with encapsulated calcein incubated with pH 7 HEPES □; anionic MLVs with encapsulated calcein incubated with poly(lysine dodecandiamide) and treated with 1% v/v Triton X100 ▒; anionic MLVs with encapsulated calcein incubated with pH 7 HEPES and treated with 1% v/v Triton X100 ▓.

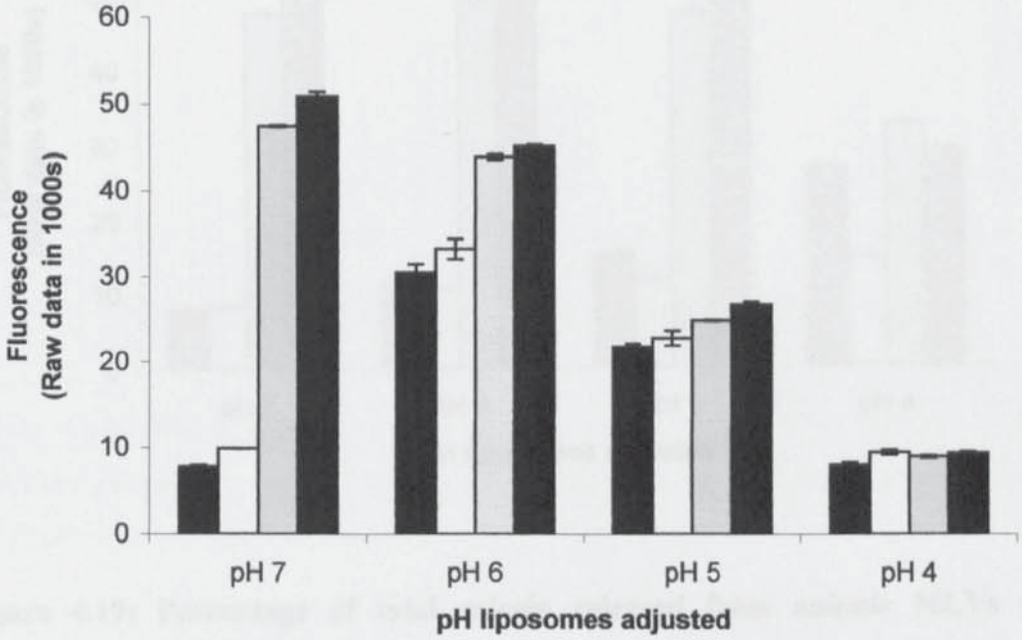


Figure 4.18: Mean fluorescence following pH change of anionic MLVs with encapsulated calcein incubated with poly(lysine dodecandiamide) ■; anionic MLVs with encapsulated calcein incubated with pH 7 HEPES □; anionic MLVs with encapsulated calcein incubated with poly(lysine dodecandiamide) and treated with 1% v/v Triton X100 ▓; anionic MLVs with encapsulated calcein incubated with pH 7 HEPES and treated with 1% v/v Triton X100 ▒.

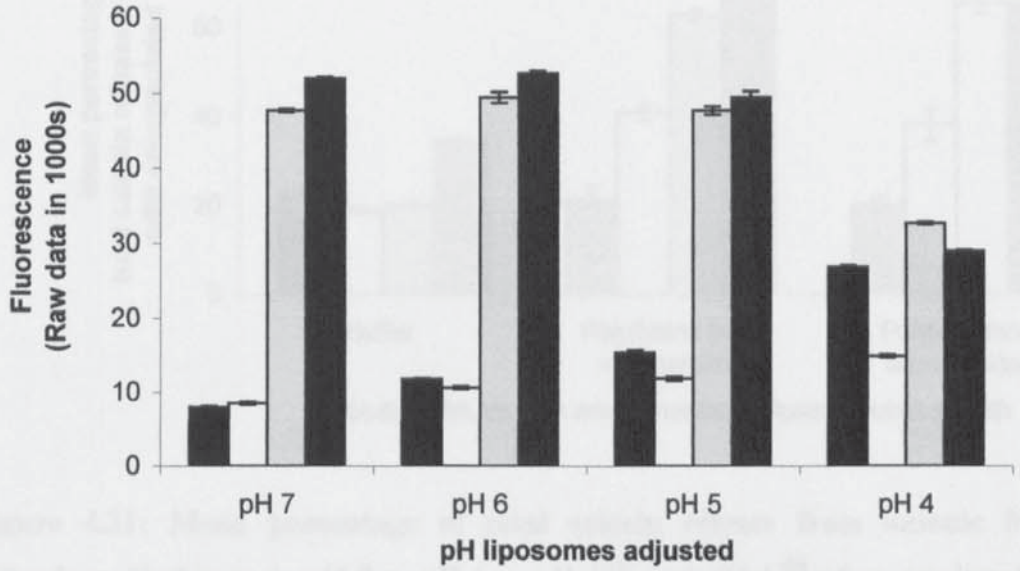


Figure 4.19: Percentage of total calcein released from anionic MLVs with encapsulated calcein with reducing pH following pre-incubation with buffer ■ and 1 mg ml^{-1} poly(lysine dodecandiamide) □.

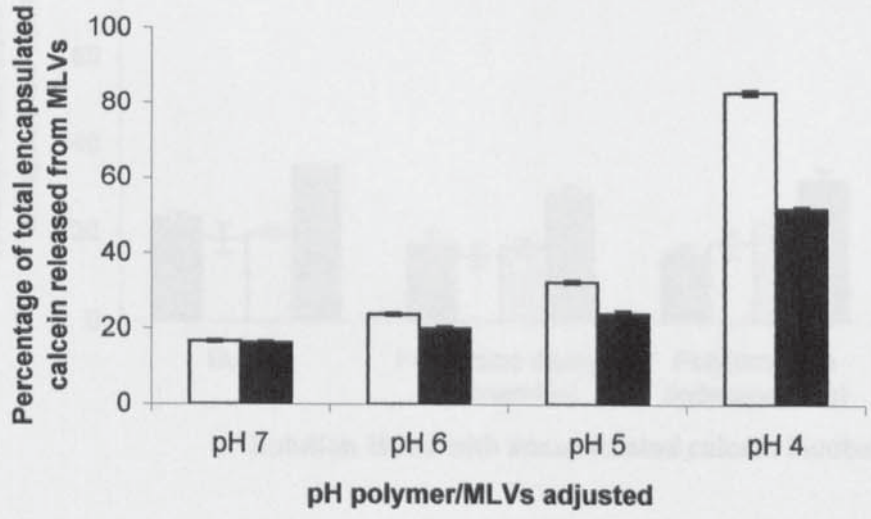


Figure 4.20: Mean percentage of total calcein release from anionic MLVs following pH change to pH 7 ■, pH 6 □, pH 5 ▒ and pH 4 ▓ when pre-incubated with buffer or polymer solutions.

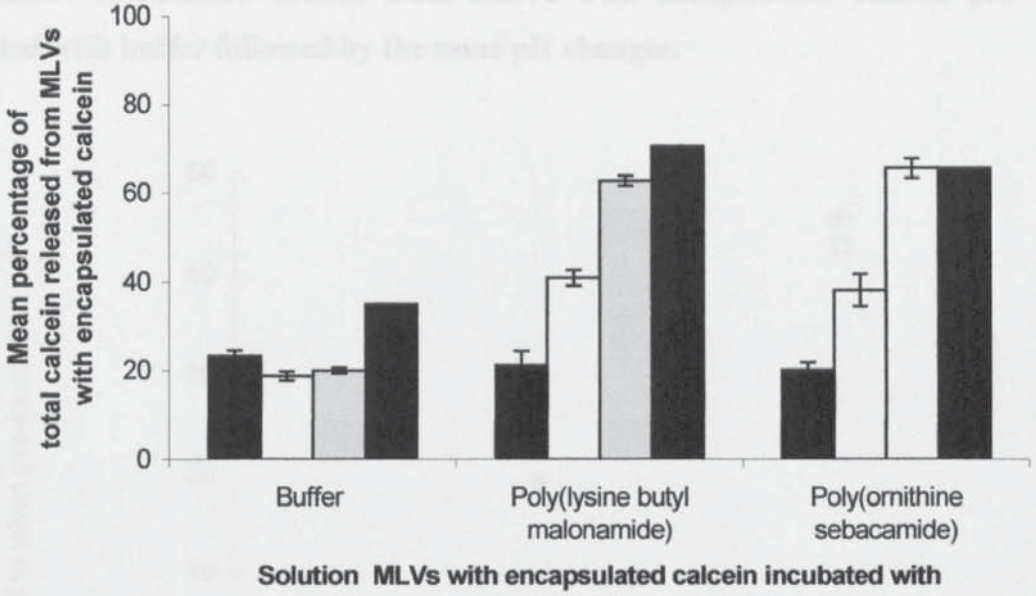


Figure 4.21: Mean percentage of total calcein release from anionic MLVs following pH change to pH 7 ■, pH 6 □, pH 5 ▒ and pH 4 ▓ when pre-incubated with buffer or polymer solutions.

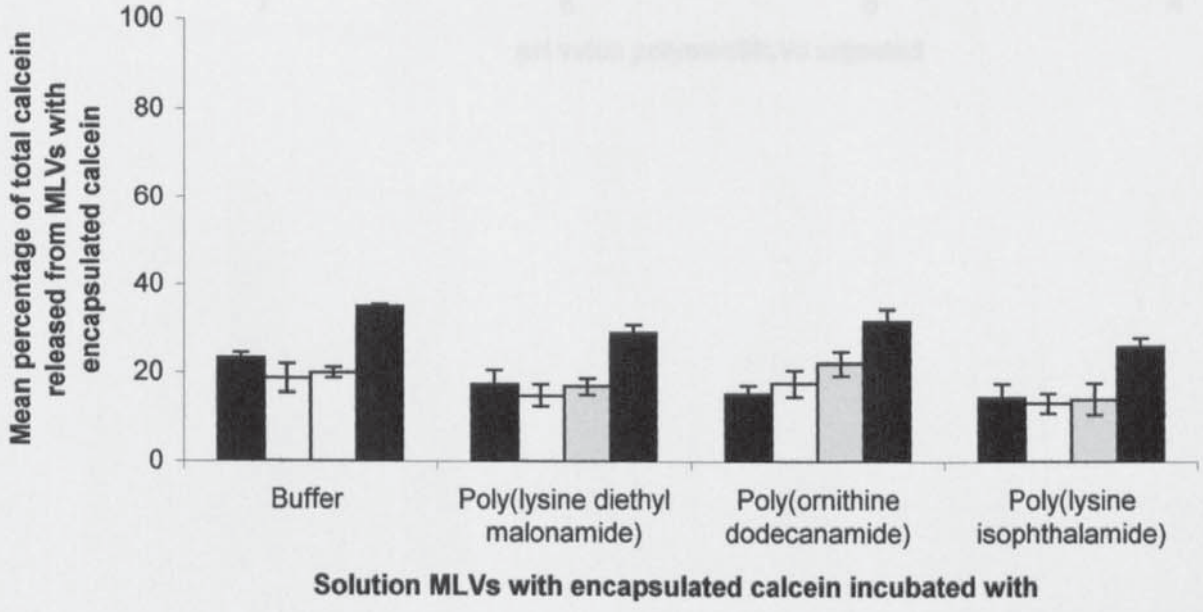


Figure 4.22: Difference between mean percentage fluorescence of released calcein from MLVs with encapsulated calcein pre-incubated in the presence of polymers, poly(lysine *iso*-phthalamide) ■, poly(lysine butyl malonamide) □, poly(ornithine sebacamide) ●, followed by a pH change; and mean percentage fluorescence of released calcein from MLVs with encapsulated calcein pre-incubated with buffer followed by the same pH changes.

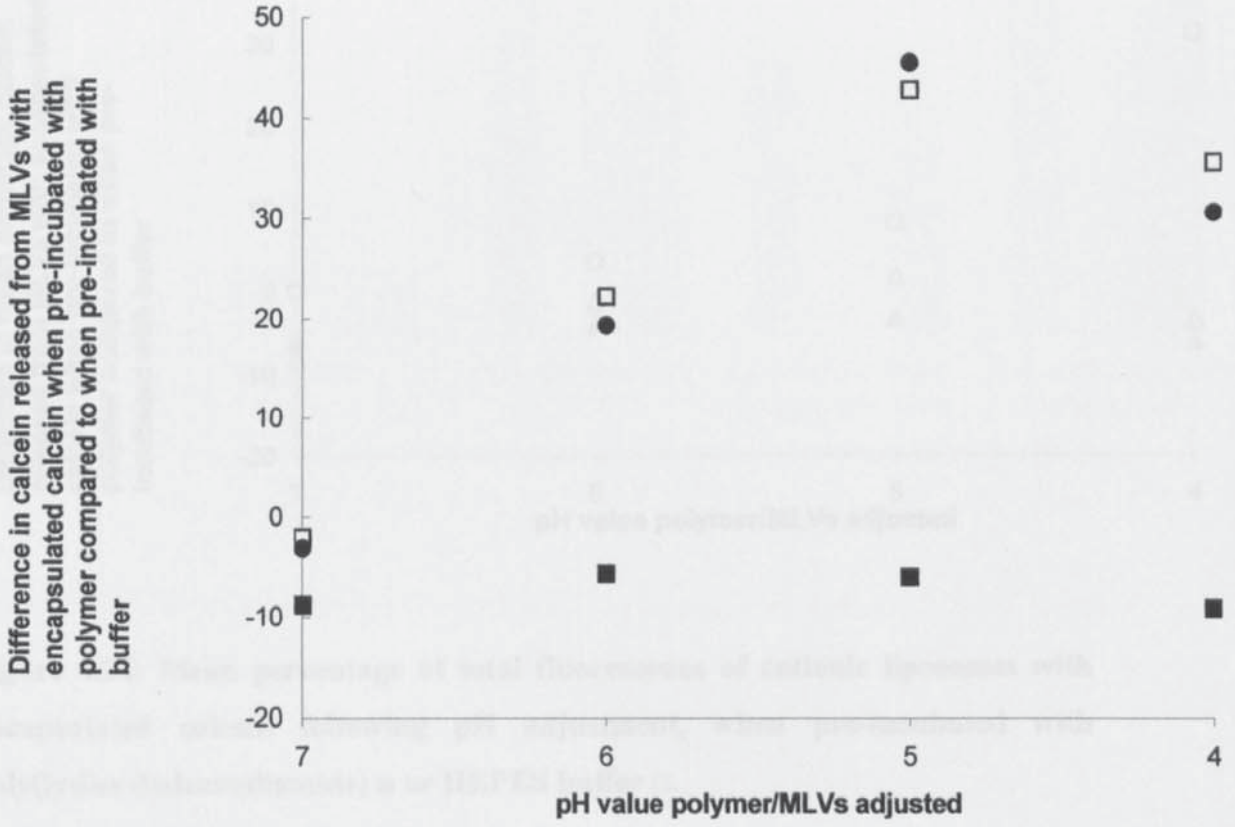


Figure 4.23: Difference between mean percentage fluorescence of released calcein from MLVs with encapsulated calcein pre-incubated in the presence of polymers, poly(lysine dodecandiamide) ○, poly(lysine diethyl malonamide) ▲ or poly(ornithine dodecandiamide) Δ, followed by a pH change; and mean percentage fluorescence of released calcein from MLVs with encapsulated calcein pre-incubated with buffer followed by the same pH changes.

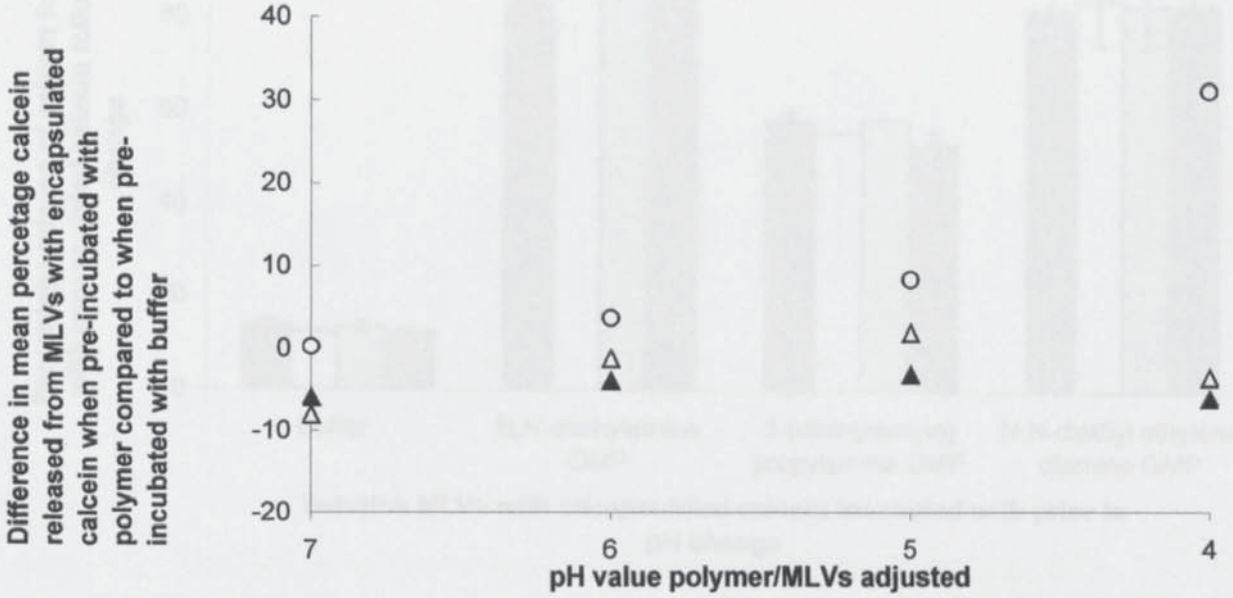


Figure 4.24: Mean percentage of total fluorescence of cationic liposomes with encapsulated calcein following pH adjustment, when pre-incubated with poly(lysine dodecandiamide) ■ or HEPES buffer □.

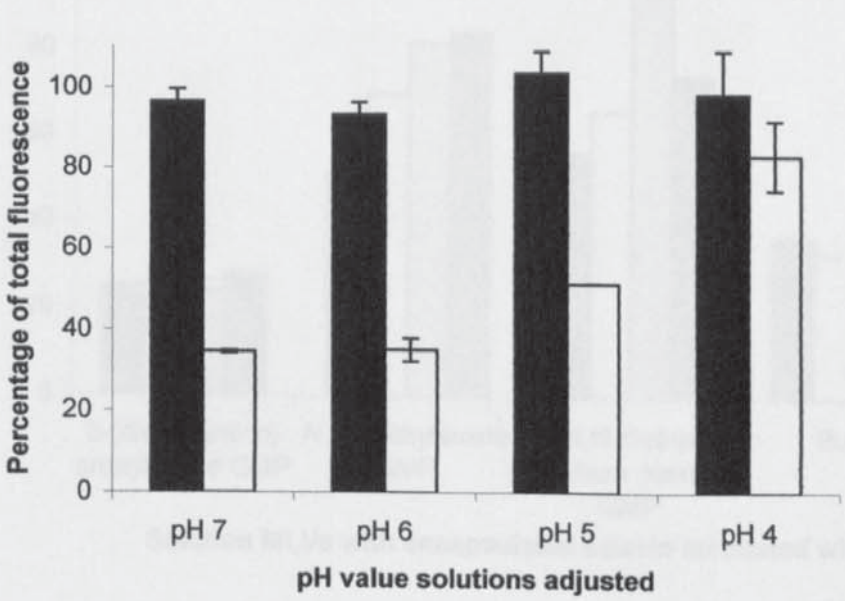


Figure 4.25: Mean percentage of total calcein release from MLVs which are pre-incubated in the presence of polymer or buffer then adjusted to pH 6 ■, pH 7□, pH 8 ▒ and pH 9 ▓.

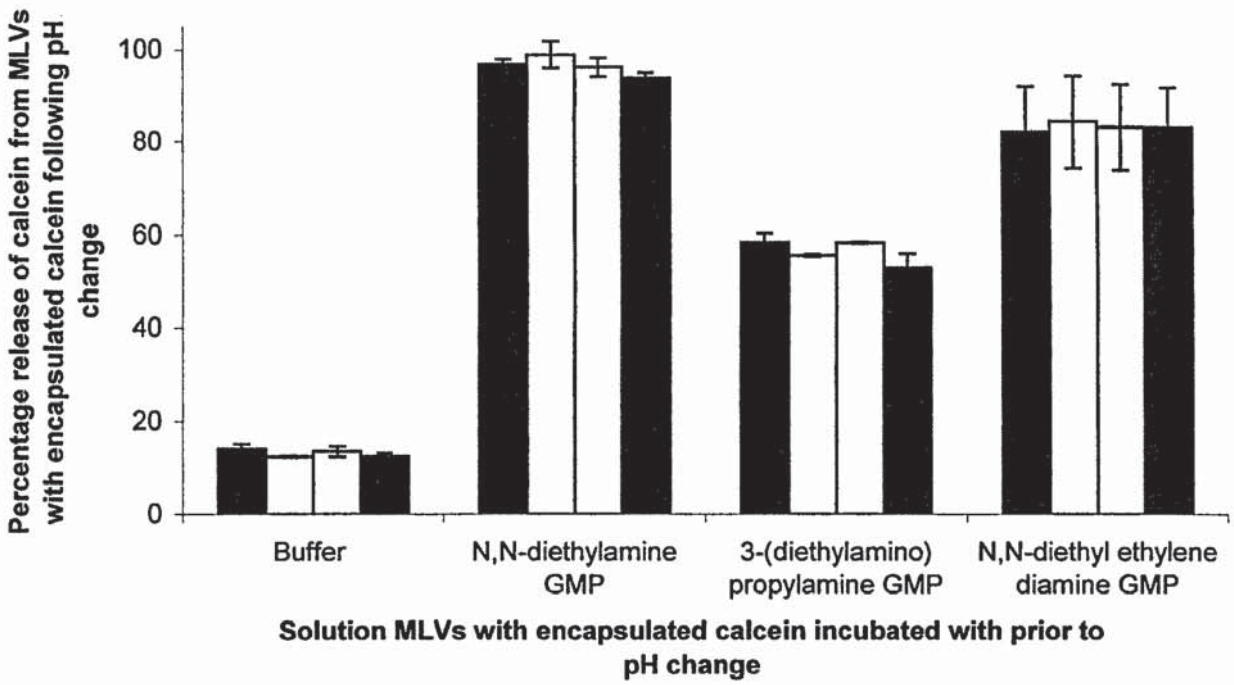


Figure 4.26: Mean percentage of total calcein release from cationic MLVs when pre-incubated with polymer or buffer, then adjusted to pH 6 ■, pH 7 □, pH 8 ▒ and pH 9 ▓.

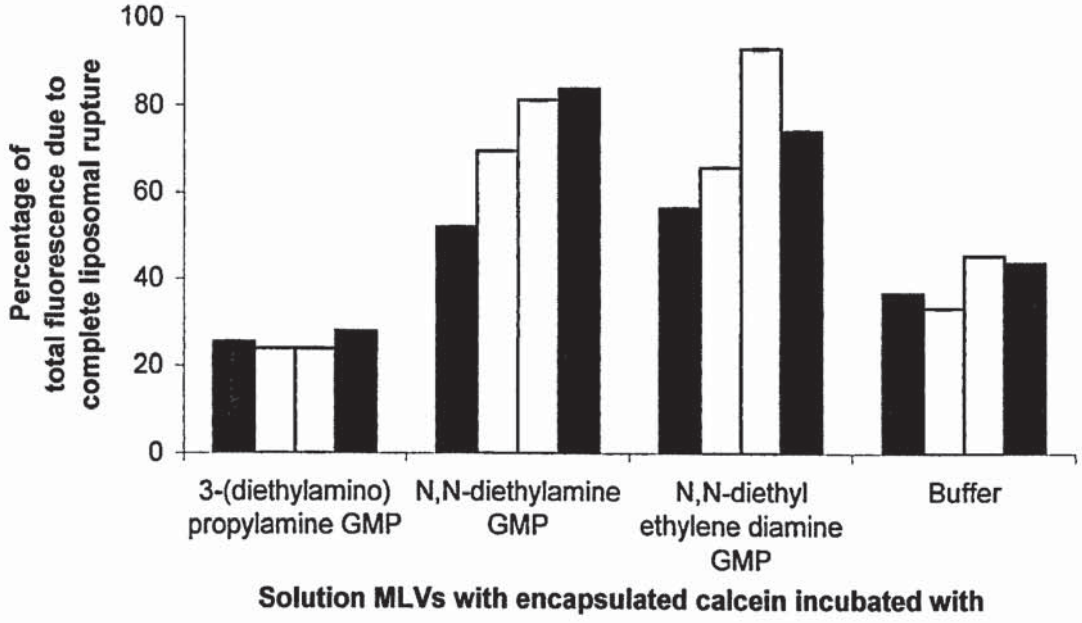


Figure 4.25: Mean percentage of total calcein release from MLVs which are pre-incubated in the presence of polymer or buffer then adjusted to pH 6 ■, pH 7□, pH 8 ▒ and pH 9 ▓.

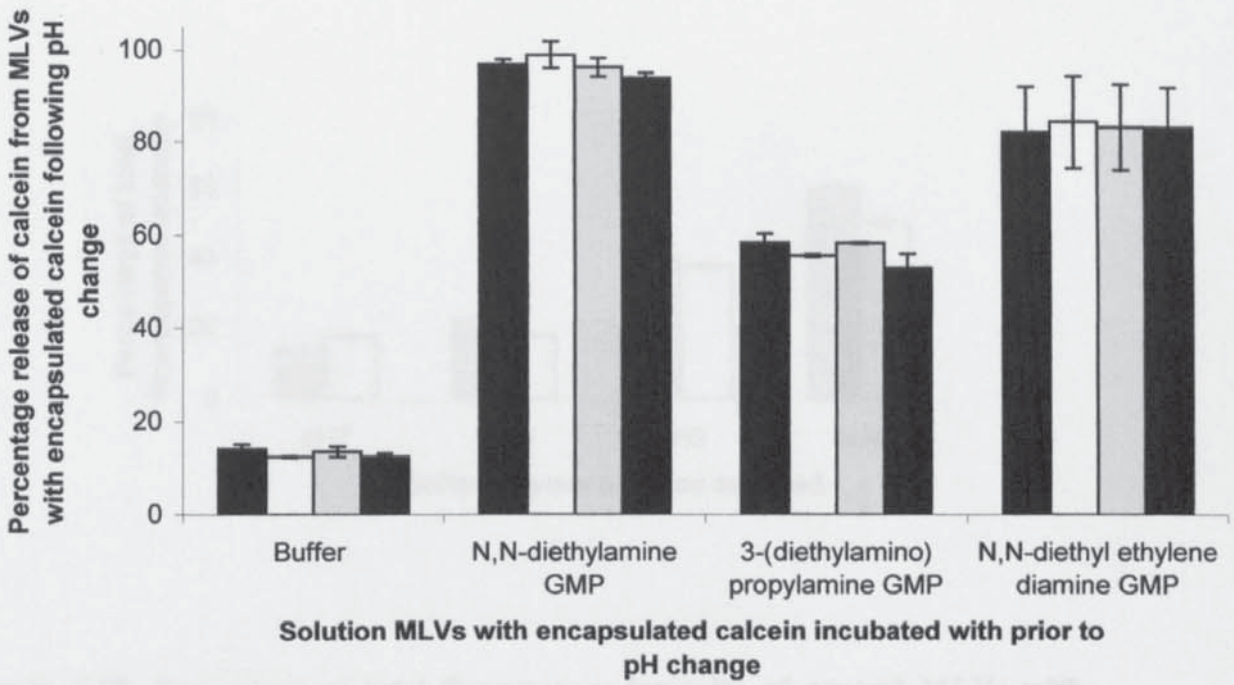


Figure 4.26: Mean percentage of total calcein release from cationic MLVs when pre-incubated with polymer or buffer, then adjusted to pH 6 ■, pH 7 □, pH 8 ▒ and pH 9 ▓.

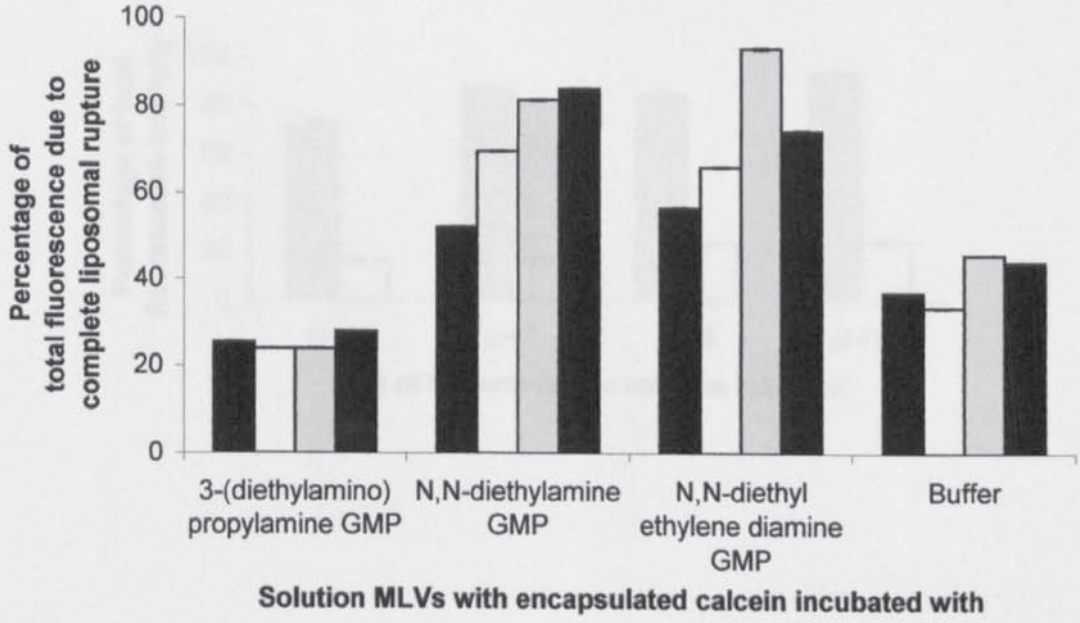


Figure 4.27: Percentage of total fluorescence intensity of neutral MLVs with encapsulated calcein following pH change when pre-incubated with HEPES buffer □ and poly(lysine dodecandiamide) ■.

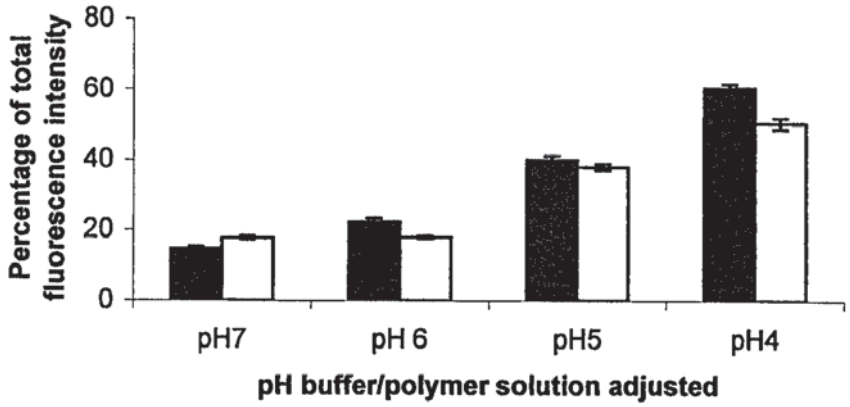


Figure 4.28: Percentage of total fluorescence intensity of neutral MLVs with encapsulated calcein following pH change when pre-incubated with HEPES buffer □ and *N,N*-diethylamine modified glycidyl methacrylate polymer ■.

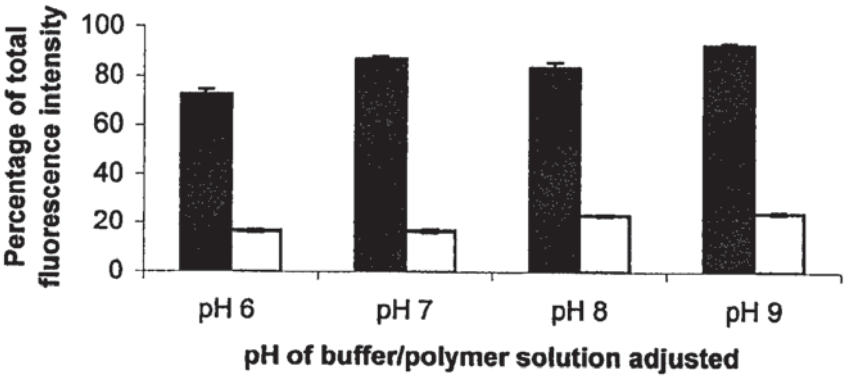


Figure 4.29: Percentage of total fluorescence intensity of small anionic liposomes with encapsulated calcein, following pH change when pre-incubated with HEPES buffer ■ and poly(lysine dodecandiamide) □.

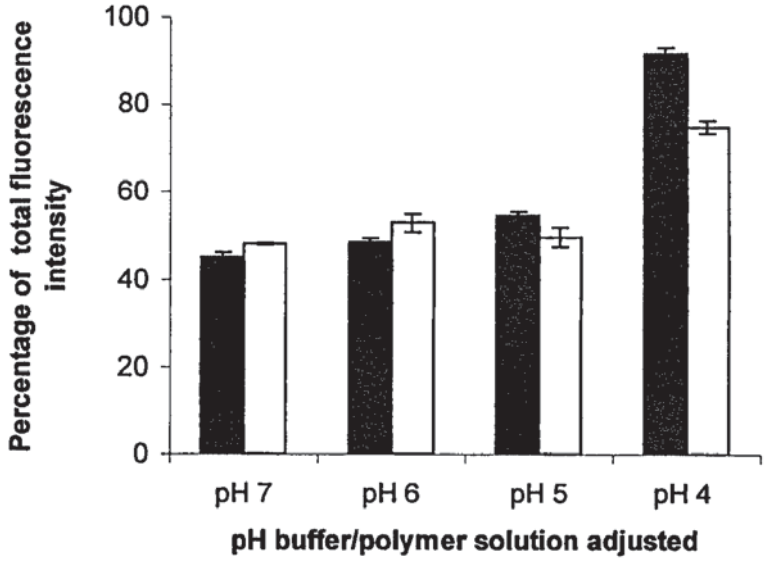


Figure 4.30: Percentage of total fluorescence intensity of small cationic liposomes with encapsulated calcein following pH change when pre-incubated with HEPES buffer ■ and *N,N*-diethylamine modified glycidyl methacrylate polymer □.

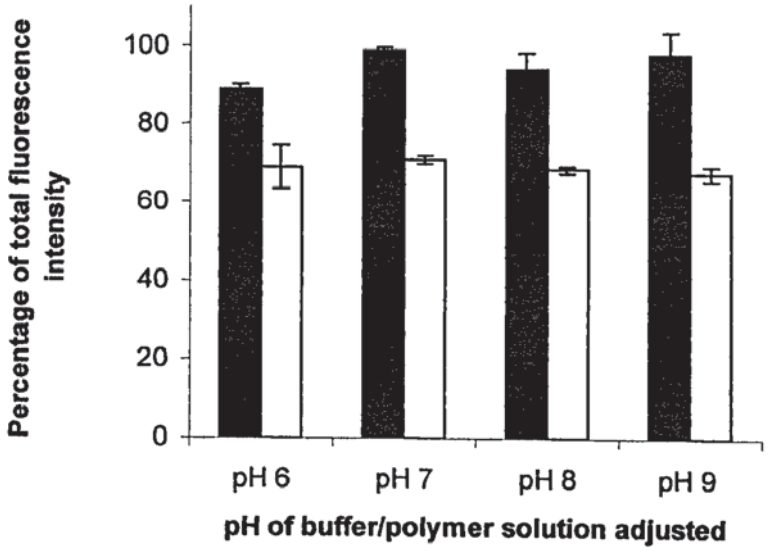


Figure 4.31: Mean percentage of total fluorescence of anionic MLVs following pH change when pre-incubated with poly(lysine dodecandiamide) at concentrations of 2 ■, 1□, 0.5 ▒ and 0 ▓ mg ml^{-1}.

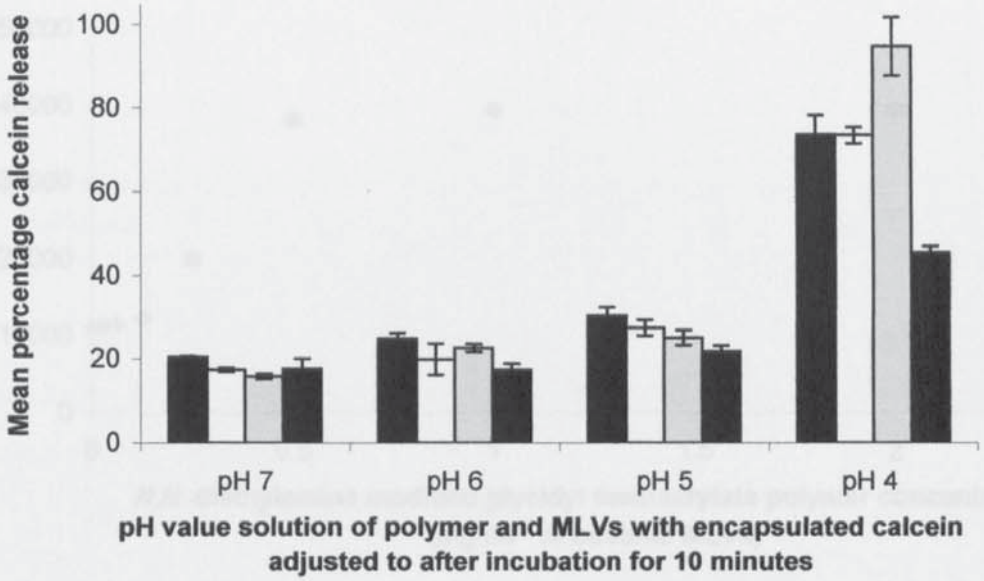


Figure 4.32: Fluorescence of calcein released from MLVs with encapsulated calcein that have been incubated in the presence of varying polymer concentrations followed by pH reduction of the solution of polymer and MLVs to pH 4.

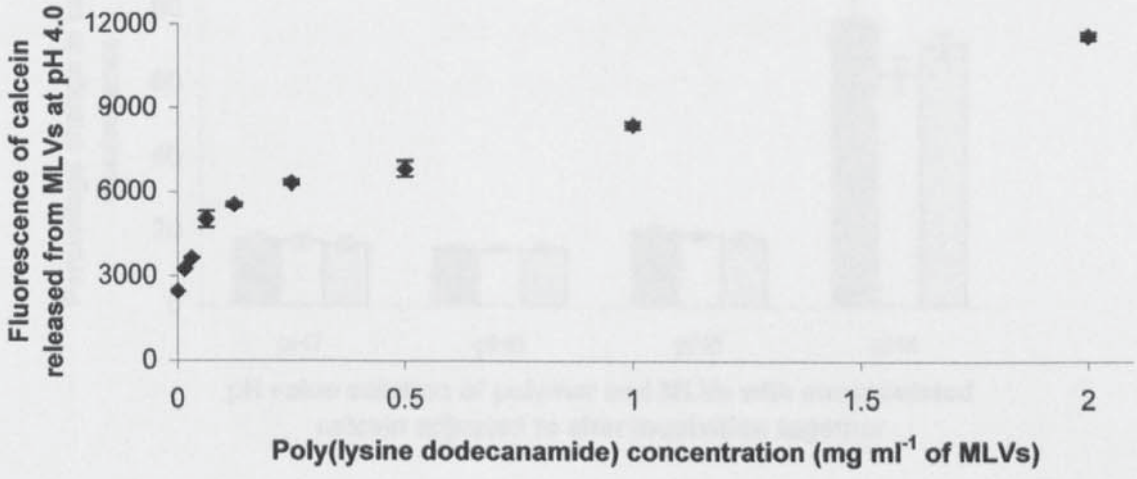


Figure 4.33: Fluorescence of calcein released from MLVs with encapsulated calcein that have been incubated in the presence *N,N*-diethylamine modified glycidyl methacrylate polymer.

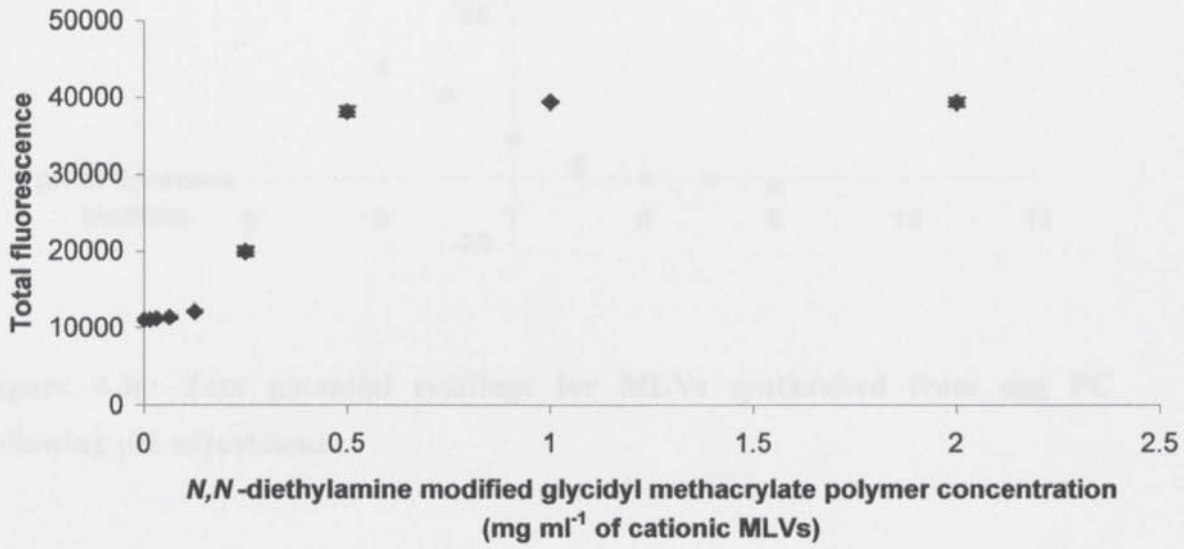


Figure 4.34: Mean percentage change in calcein fluorescence of anionic MLVs following pH change when pre-incubated with poly(lysine dodecandiamide) at a concentration of 1 mg ml^{-1} for 10 minutes ■, 30 minutes □ and 60 minutes ▒.

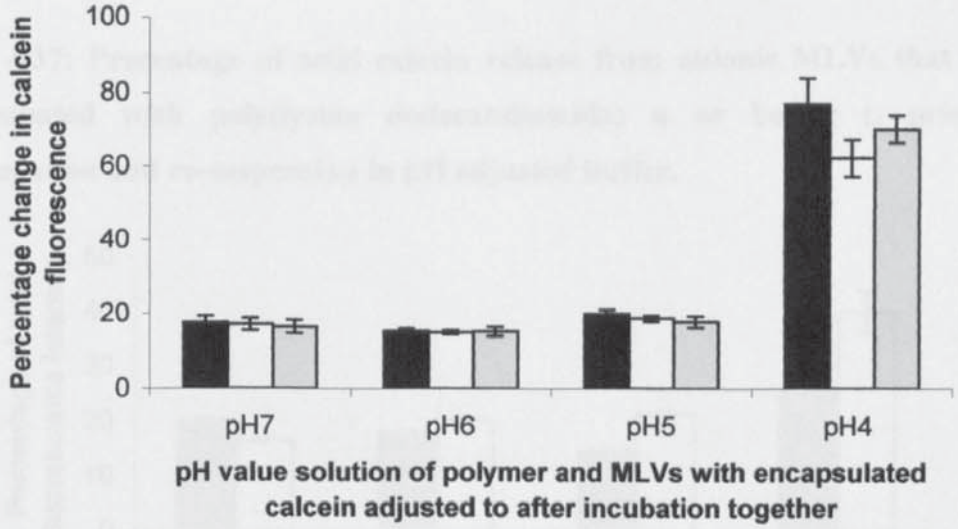


Figure 4.35: Zeta potential readings for MLVs synthesised from egg PC, stearylamine and cholesterol, following pH adjustment.

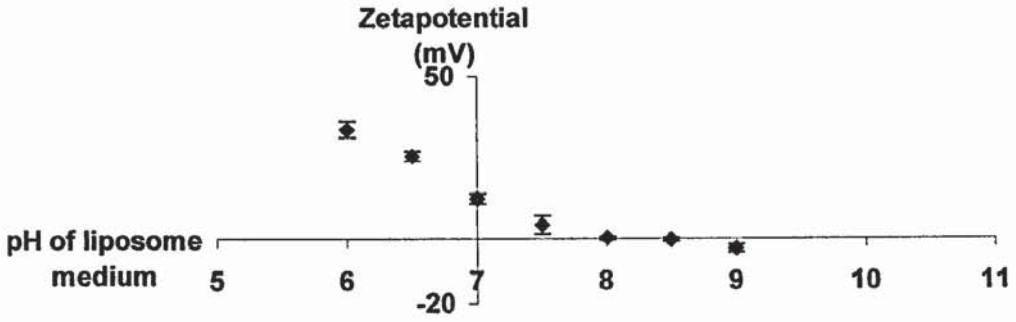


Figure 4.36: Zeta potential readings for MLVs synthesised from egg PC following pH adjustment.

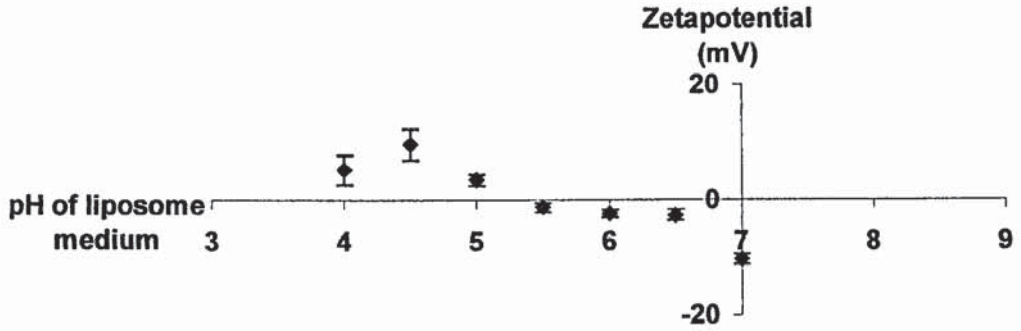


Figure 4.37: Percentage of total calcein release from anionic MLVs that were pre-incubated with poly(lysine dodecandiamide) ■ or buffer □ prior to centrifugation and re-suspension in pH adjusted buffer.

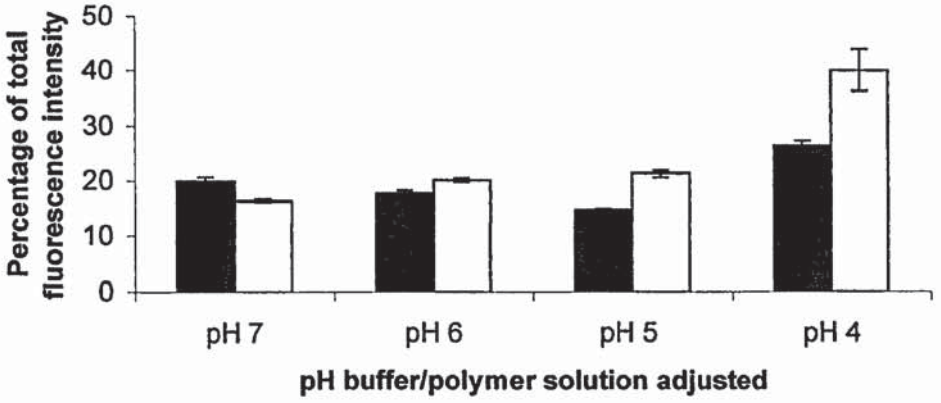


Table 4.2: Size analysis results of liposomes with encapsulated glucose synthesised using the beaker or rotary evaporation technique before and following extrusion or sonication.

Synthesis Method	Treatment	*Mean Diameter (μm)	Standard Error
Beaker	None	7.76	0. 35
	Sonication	—	—
	Extrusion	0.172	0.0123
Reverse phase evaporation	None	7.82	0.60
	Sonication	—	—
	Extrusion	0.437	0.009

*(D3,2) mean diameter given for Mastersizer analysed samples, volume mean diameter given for Zetasizer analysed samples.

— indicates sample was too polydisperse for size analysis.

Figure 4.38: Typical Mastersizer volume histogram obtained in determining size distribution of a preparation of liposomes with encapsulated glucose synthesised using the beaker method.

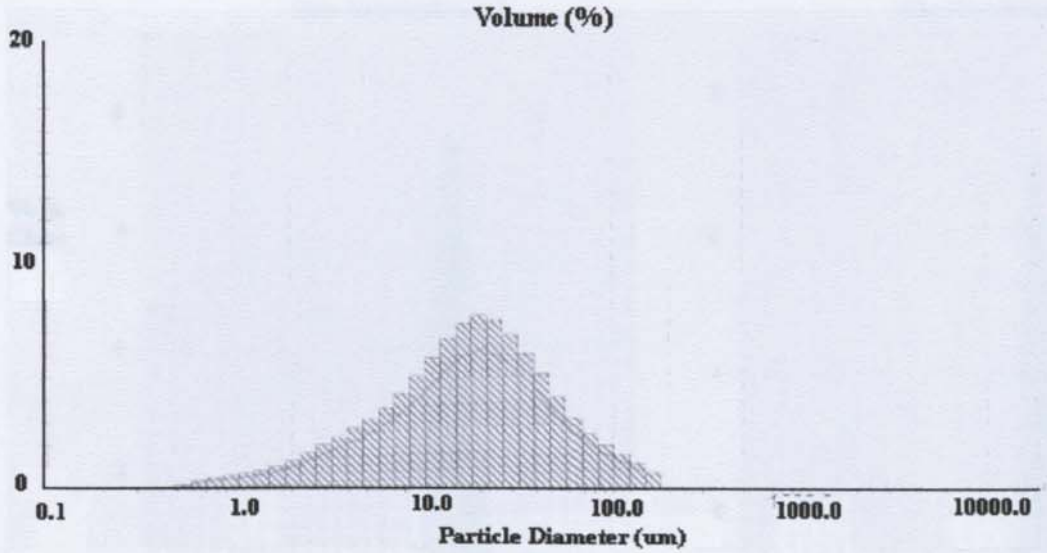


Figure 4.39: Typical Mastersizer volume histogram obtained in determining size distribution of a preparation of liposomes with encapsulated glucose synthesised using reverse phase evaporation vesicle method.

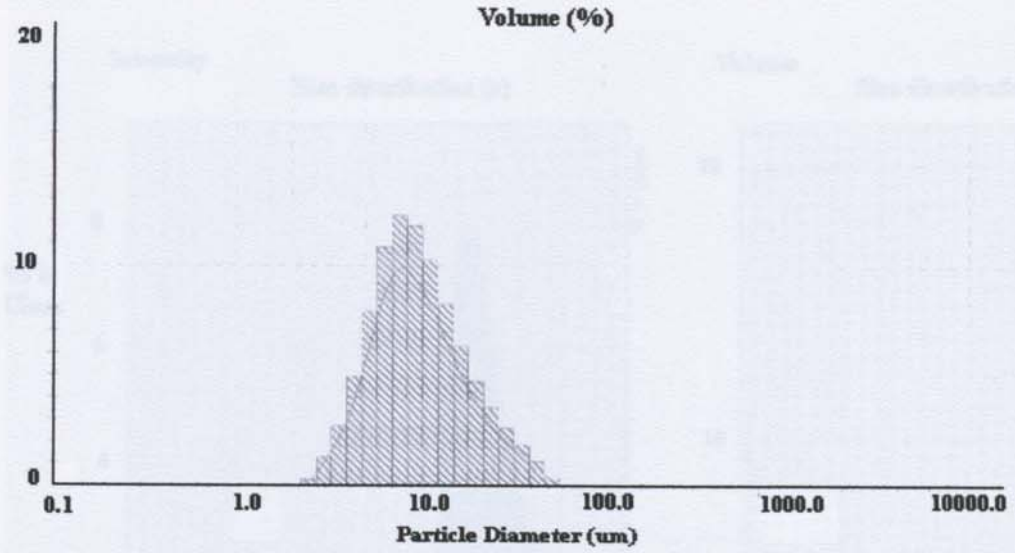


Figure 4.40: Typical Zetasizer 1000 intensity and volume histograms obtained in determining size distribution of a preparation of liposomes with encapsulated glucose synthesised using beaker method and exposed to extrusion.

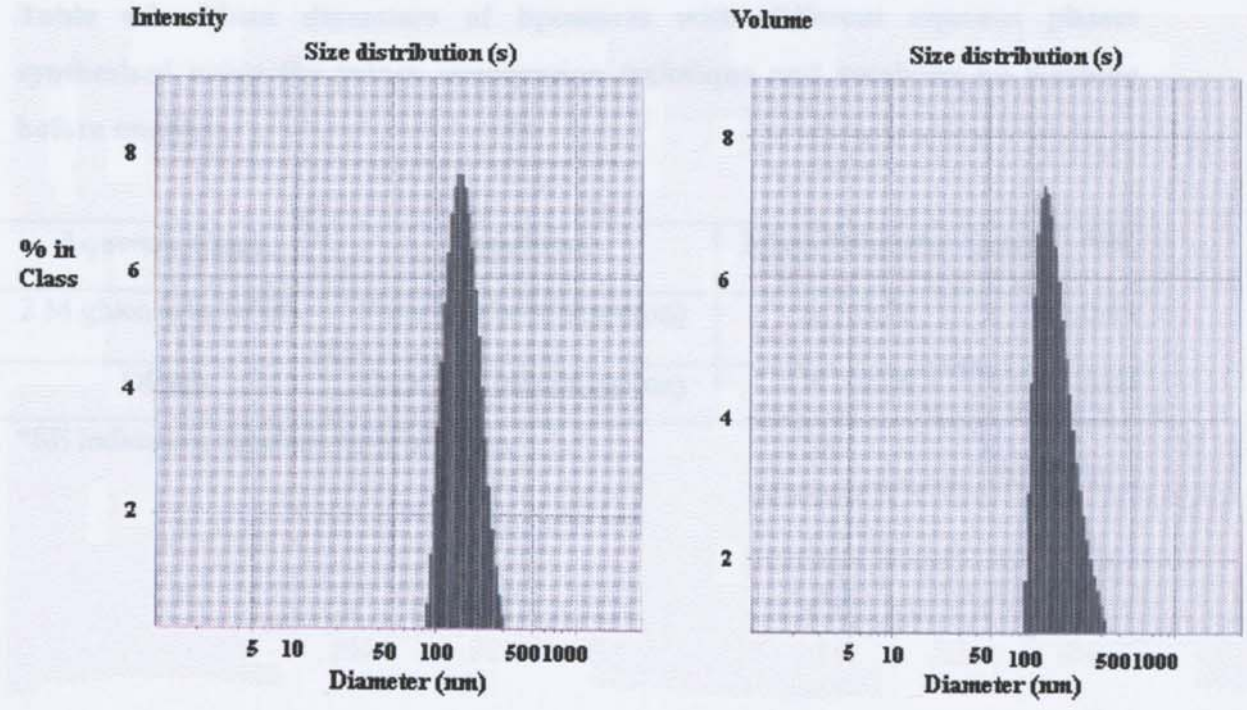


Figure 4.41: Typical Zetasizer 1000 intensity and volume histograms obtained in determining size distribution of a preparation of liposomes with encapsulated glucose synthesised using reverse phase evaporation vesicle method and exposed to extrusion.

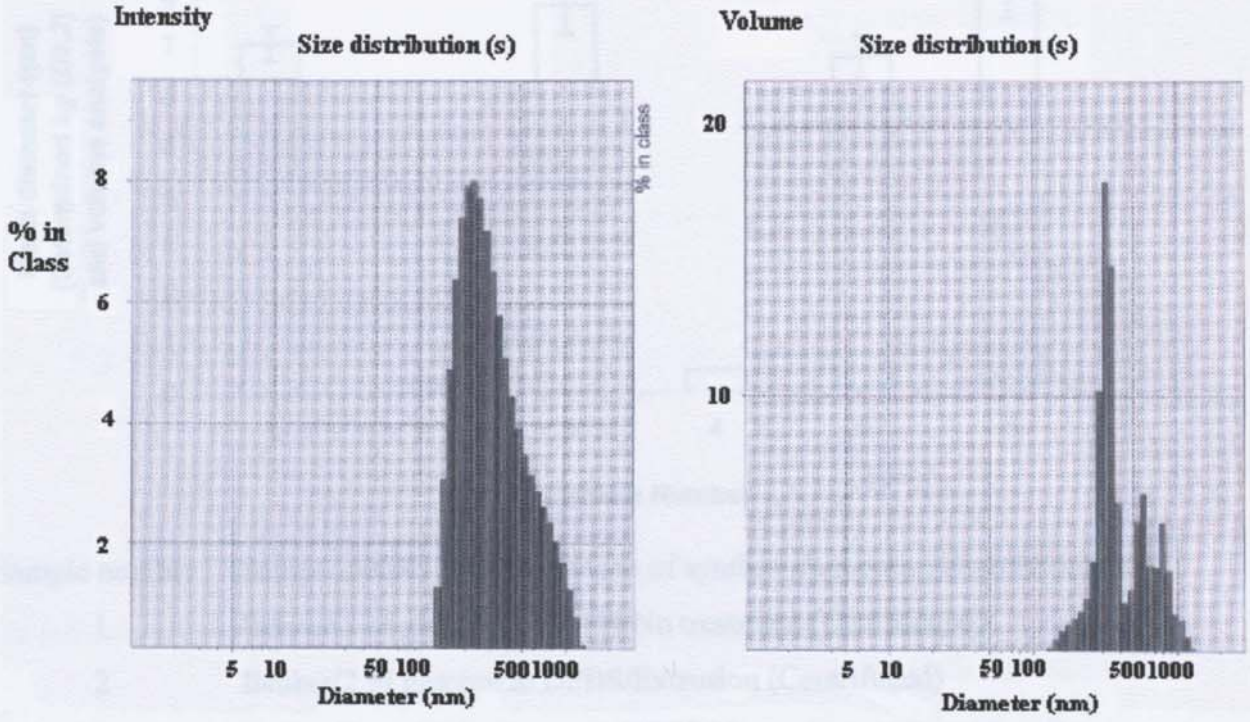
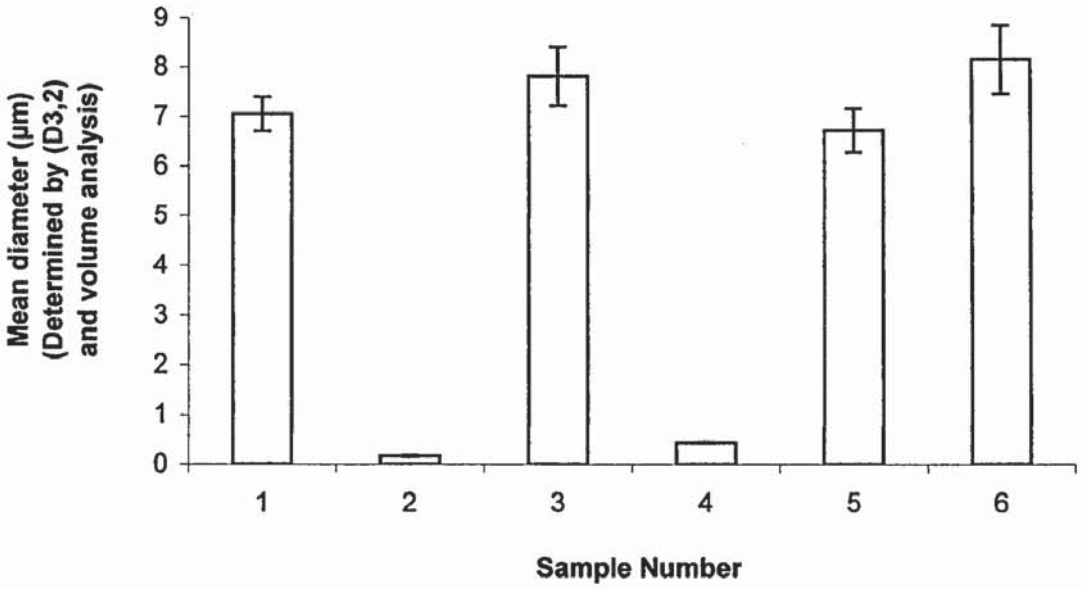


Table 4.3: Mean diameters of liposomes with different aqueous phases synthesised using the rotary evaporation technique and receiving no washing before analysis.

Aqueous phase	Treatment	Mean Diameter (μm)	*SE
2 M glucose in DPBS	None (No centrifugation)	6.72	0.44
DPBS	None (No centrifugation)	8.16	0.69

*SE indicating standard error of mean

Figure 4.42: Mean diameters of various liposome preparations determined using Malvern Mastersizer and Zetasizer 1000 instruments.



Sample number	Sample details including mode of synthesis/aqueous phase/treatment
1	Beaker/2 M glucose in DPBS/No treatment (Centrifuged)
2	Beaker/2 M glucose in DPBS/Extrusion (Centrifuged)
3	Rotary Evap./2 M glucose in DPBS/No treatment (Centrifuged)
4	Rotary Evap./2 M glucose in DPBS/Extrusion (Centrifuged)
5	Rotary Evap./2 M glucose in DPBS/No treatment (Not centrifuged)
6	Rotary Evap./DPBS only/No treatment (Not centrifuged)

Figure 4.43: Volume percentage distribution curve of beaker synthesised liposomes, which have received no sonication.

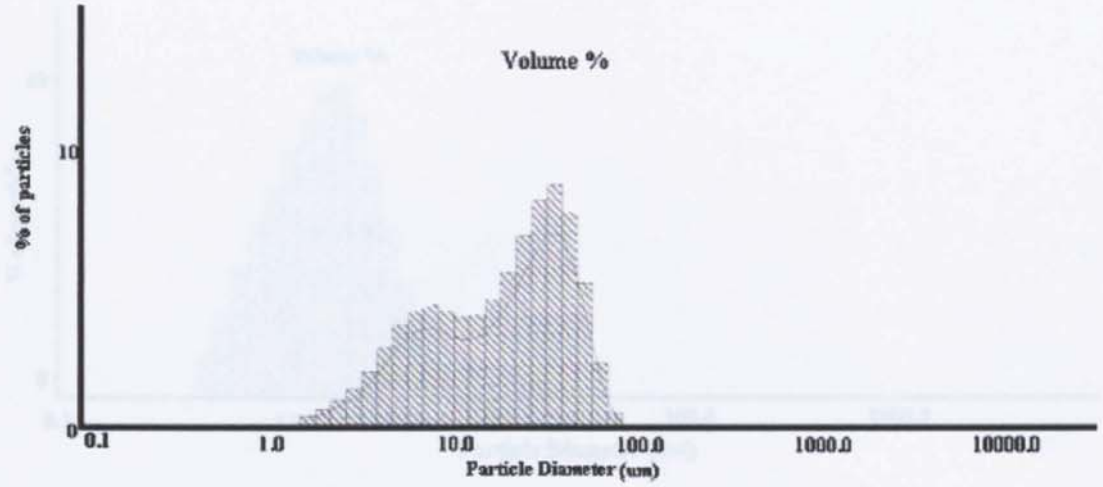


Figure 4.44: Volume percentage distribution curve of beaker synthesised liposomes, which have received sonication for 2 hours.

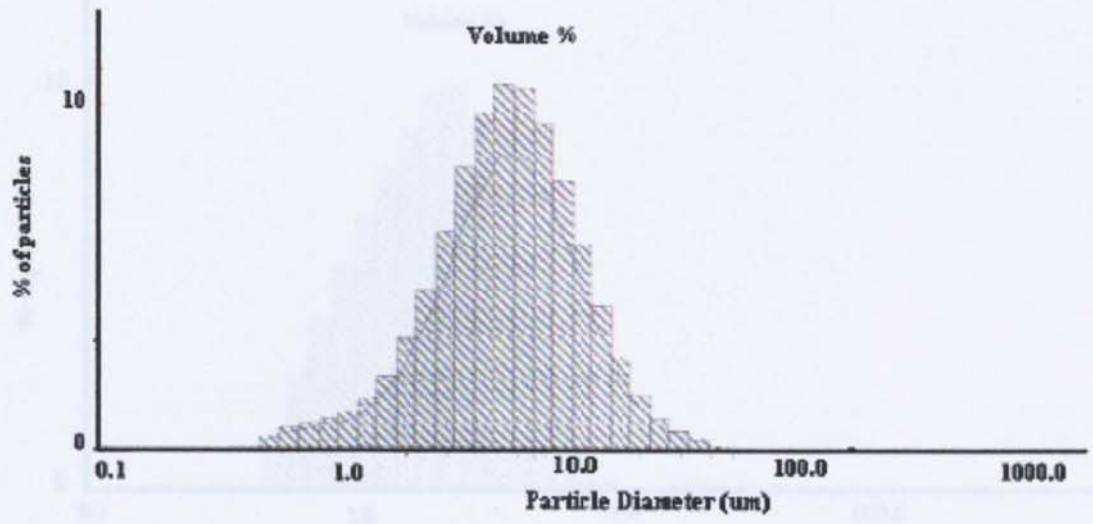


Figure 4.45: Volume percentage distribution curve of beaker synthesised liposomes, which have received sonication for 4 hours.

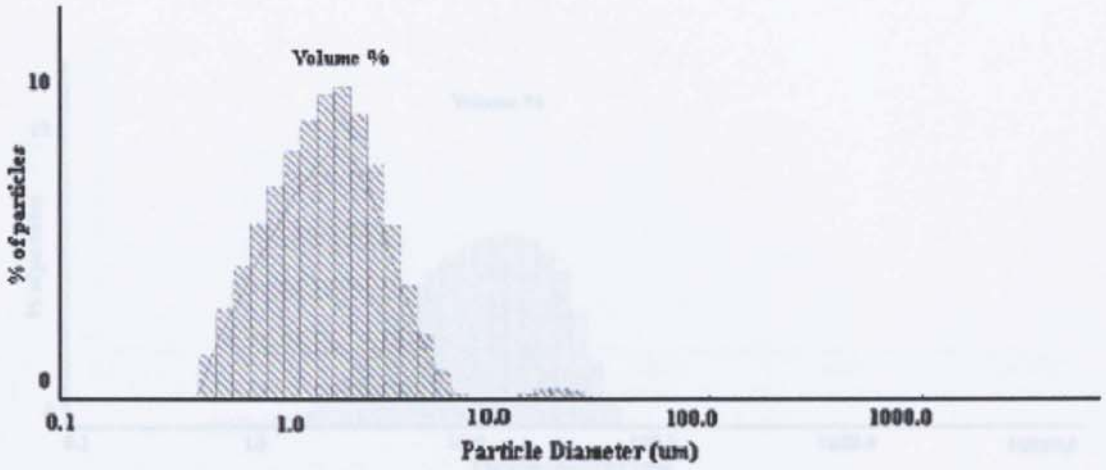


Figure 4.46: Volume percentage distribution curve of beaker synthesised liposomes, which have received sonication for 6 hours.

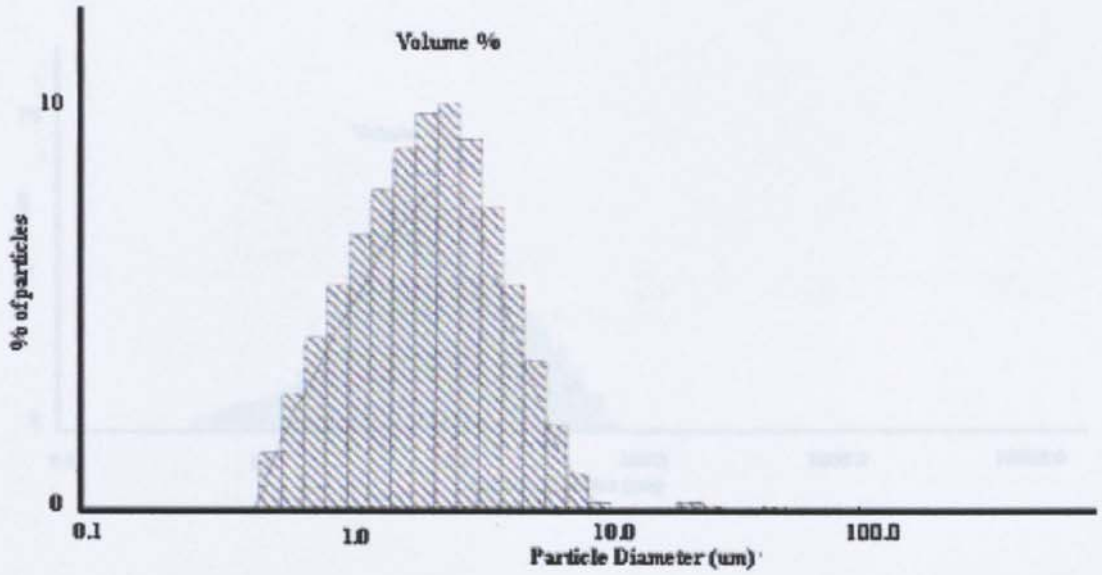


Figure 4.47: Volume percentage distribution curve of reverse phase evaporation liposomes, which have received no sonication.

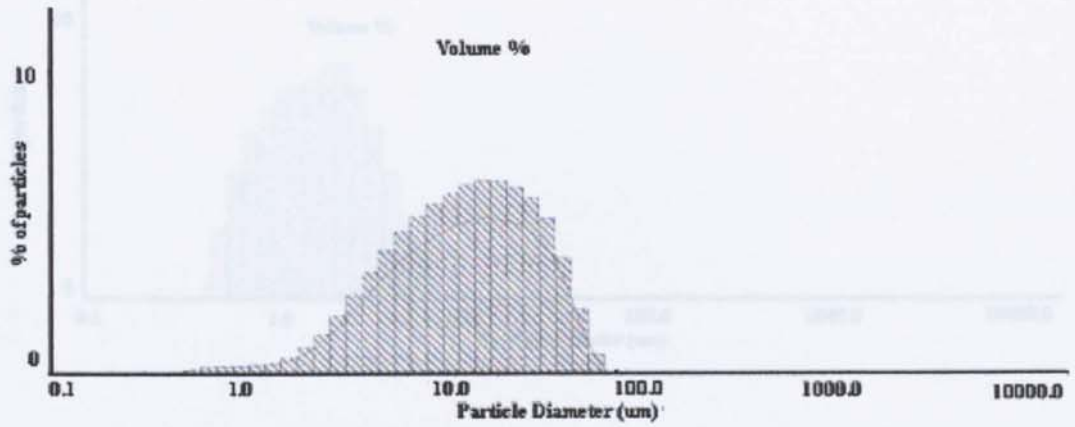


Figure 4.48: Volume percentage distribution curve of reverse phase evaporation liposomes, which have received sonication for 2 hours.

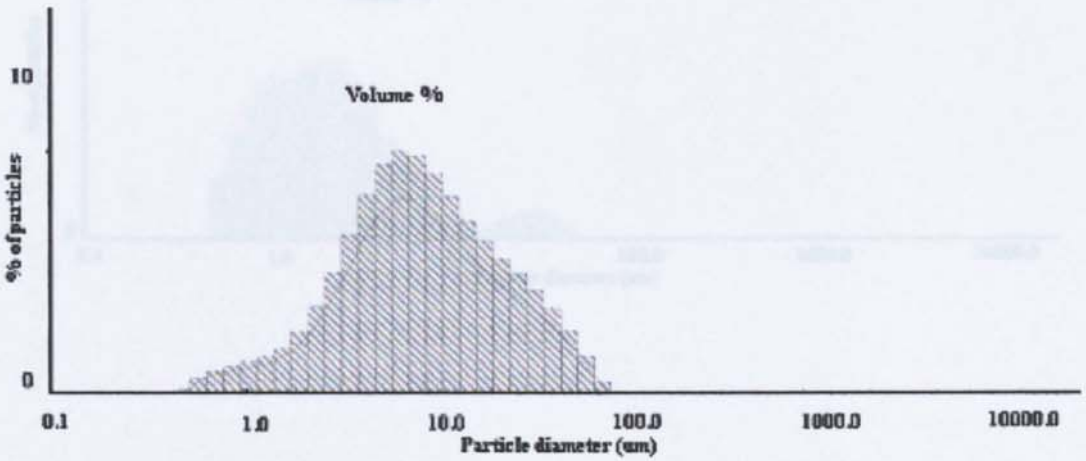


Figure 4.49: Volume percentage distribution curve of reverse phase evaporation liposomes, which have received sonication for 4 hours.

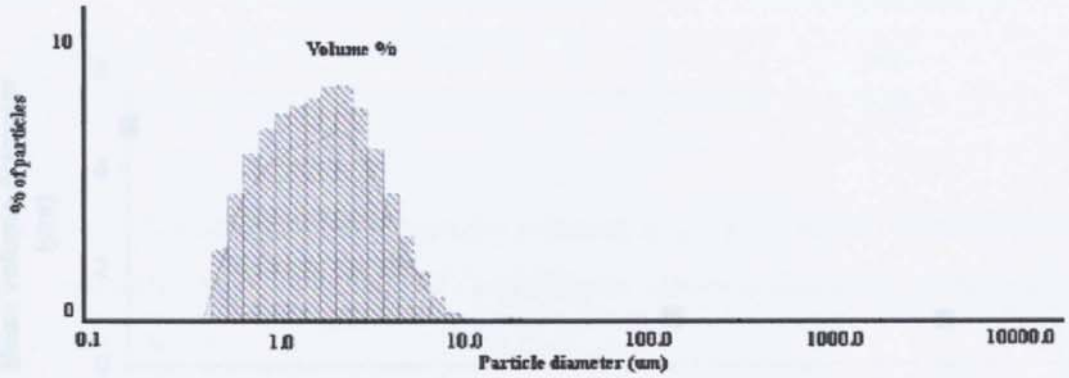


Figure 4.50: Volume percentage distribution curve of reverse phase evaporation liposomes which have received sonication for 6 hours.

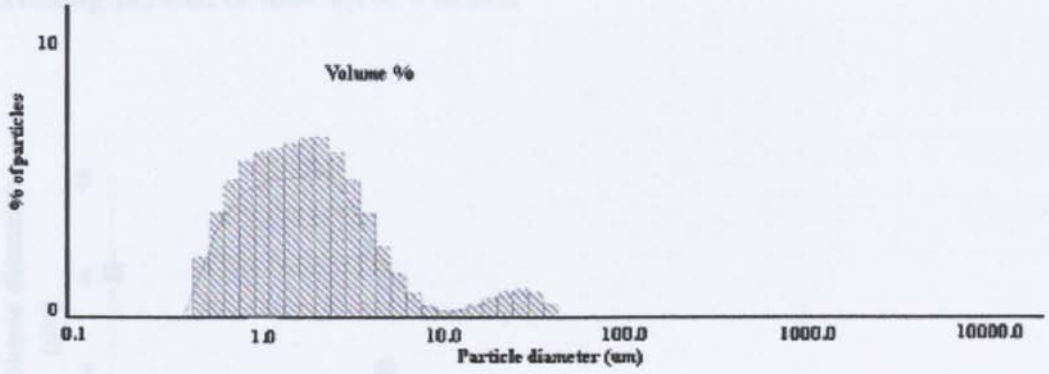


Figure 4.51: Mean volume diameter (μm) of beaker synthesised liposomes with encapsulated DPBS exposed to sonication at 0° C for increasing periods of time up to 6 hours.

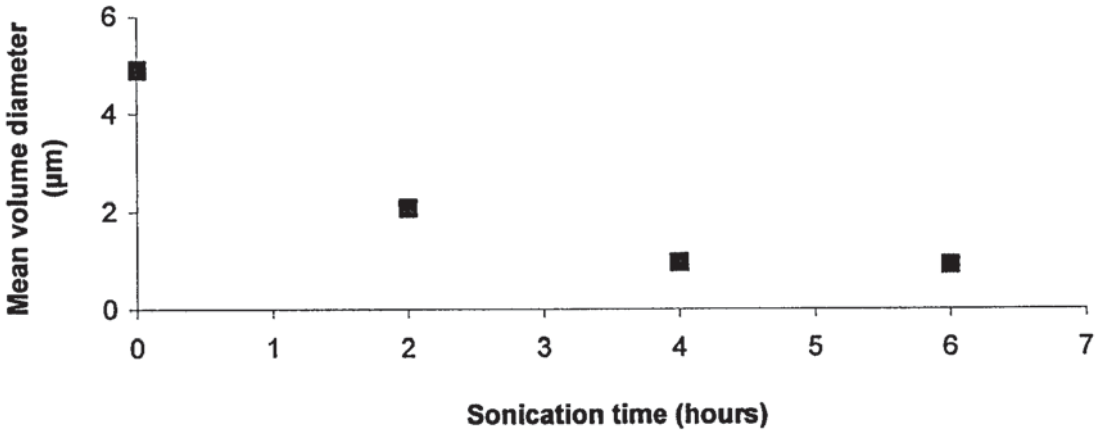


Figure 4.52: Mean volume diameter (μm) of reverse phase evaporation synthesised liposomes with encapsulated DPBS exposed to sonication for increasing periods of time up to 6 hours.

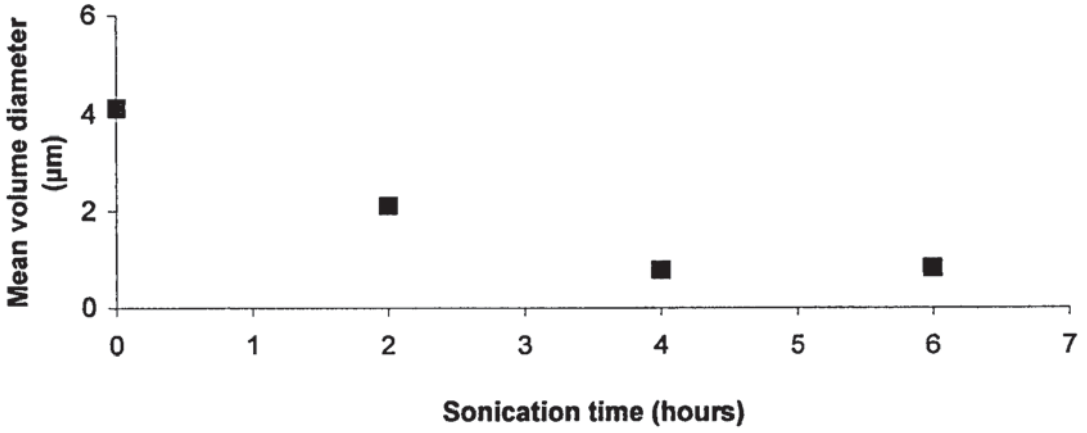


Table 4.4: Percentage of total calcein release from lyophilised and non-lyophilised MLVs.

Charge on MLVs	Percentage of total fluorescence due to released calcein from:	
	Non-lyophilised MLVs	Lyophilised MLVs
Cationic	54.84	-8.90
Anionic	85.80	1.56

Table 4.5: Percentage of total calcein released from anionic MLVs incubated in the presence and absence of poly(lysine dodecandiamide), followed by lyophilisation.

pH of buffer solution liposomes incubated with	Mean percentage of total fluorescence due to calcein release from anionic MLVs in the presence of:		Mean percentage of total fluorescence due to calcein release from anionic MLVs (lyophilised) in the presence of:	
	HEPES buffer	Poly(lysine dodecandiamide)	HEPES buffer	Poly(lysine dodecandiamide)
7	15.13 (0.38)	15.01 (0.13)	102.55 (0.44)	101.185 (0.716)
6	23.77 (1.47)	31.81 (1.59)	92.89 (7.57)	103.89 (0.48)
5	28.40 (1.62)	40.16 (1.45)	114.78 (1.50)	110.02 (0.36)
4	56.79 (3.26)	93.05 (3.05)	106.40 (7.80)	120.82 (4.30)

Value in brackets is standard error of mean.

Table 4.6: Percentage of total calcein released from cationic MLVs incubated in the presence and absence of *N,N*-diethylamine modified glycidyl methacrylate polymer, followed by lyophilisation.

pH of buffer solution liposomes incubated with	Mean percentage of total fluorescence due to calcein release from cationic MLVs in the presence of:		Mean percentage of total fluorescence due to calcein release from cationic MLVs (lyophilised) in the presence of:	
	HEPES buffer	*N,N*-diethylamine modified GMP	HEPES buffer	*N,N*-diethylamine modified GMP
6	62.51 (2.02)	65.06 (1.17)	110.33 (1.39)	112.56 (0.84)
7	64.38 (1.54)	78.21 (0.29)	113.311 (1.87)	106.38 (0.52)
8	66.74 (1.64)	79.45 (3.12)	107.3 (0.68)	109.49 (1.25)
9	68.34 (0.42)	85.18 (2.63)	108.02 (1.19)	112.51 (4.88)

Value in brackets is standard error of mean.

4.3: Discussion.

4.3.1: Determination of liposome solubilisation properties of polymers as a function of pH.

Observing Table 4.1 shows that when MLVs are incubated in the presence of poly(lysine dodecandiamide), then adjusted to pH 7, they are not disrupted since the suspension remains opaque. However, clearing of MLVs at pH values 5 and 4 indicates possible disruption. MLVs incubated without polymer do not show clearing when adjusted to pH values 7, 5 or 4. The cloudiness and white precipitate observed when tubes containing polymer and buffer are adjusted to 5 and 4, respectively indicate the occurrence of conformational changes in polymer. Similar trends are observed for poly(lysine *iso*-phthalamide). That is, when MLVs are incubated in the presence of poly(lysine *iso*-phthalamide), then adjusted to pH values 7 and 5 the suspension remains opaque, indicating no disruption. When MLVs plus poly(lysine *iso*-phthalamide) are adjusted to pH 4 clearing is observed. A white precipitate is observed in the tube containing polymer and buffer adjusted to pH 4 This again

indicates conformational changes in the polymer. Finally, like poly(lysine dodecandiamide) and poly(lysine *iso*-phthalamide), poly(lysine butyl malonamide) forms a white precipitate at low pH. However, when pre-incubated with MLVs, it does not cause clearing following adjustment to pH values 7, 5 or 4. The described observations suggest poly(lysine dodecandiamide) and poly(lysine *iso*-phthalamide) have pH-responsive disruption properties. The precise mechanism by which this disruption occurs is not fully understood. However, it is thought that a likely mechanism is one that has been suggested for pH-sensitive peptides, such as GALA. GALA is a synthetic amphipathic peptide with a repeat unit of glutamic acid-alanine-leucine-alanine, which undergoes a pH-dependent conformational change and induces leakage of contents from large unilamellar phosphatidylcholine vesicles when in a α helical conformation (Parente *et al.*, 1990). The pH at the midpoint of maximal helical conformation corresponds to the pK_a of glutamic acid in poly(glutamic acid). Therefore, signifying the change to α helical conformation is related to the protonation of glutamic side chains. Fattal *et al.*, (1994) suggested a kinetic model for pore-mediated and perturbation-mediated flip-flop to characterise the mechanism of peptide-induced phospholipid flip-flop in bilayers. This model assumed certain peptides bind to and then aggregate within the membrane. When the membrane aggregate reaches a critical size, a channel is created which results in a fast flip-flop of phospholipids. Former studies have suggested that poly(lysine dodecandiamide) and poly(lysine *iso*-phthalamide) exhibit an extended conformation when the majority of their ionisable groups are in a charged state. While, at intermediate degrees of ionisation the polymers show amphiphilic properties existing as unimolecular micelles. With complete loss of charge it is thought they form highly compact tightly coiled structures stabilised by hydrophobic bonding and eventually precipitate from solution (Eccleston, 1995). Therefore, it is believed that either at neutral or with reducing pH, poly(lysine dodecandiamide) and poly(lysine *iso*-phthalamide) bind to the liposomal membranes. When the pH is reduced further it is thought, like GALA, their coiled conformation causes pore formation and eventual disruption of the liposomal bilayer.

It can be observed that with increasing pH, clearing of MLVs does not occur when they are pre-incubated with polymers *N,N*-diethylamine, *N,N*-diethyl ethylene

diamine, 3-(diethylamino)propylamine modified GMPs (Plates 4.4 to 4.6). Therefore, signifying disruption has not occurred. Nevertheless, pore formation may have occurred, however this cannot be determined from the observations alone. No clearing of cationic liposomes was observed when incubated with polyamides and adjusted to pH values 7, 5 and 4. Similarly, no clearing of cationic liposomes was observed when incubated with amine modified GMPs followed by adjustment to pH values 6, 7.5 and 9.

4.3.2: Preparation of liposomes with encapsulated calcein for the application of quantifying membrane disruption properties of polymers.

4.3.2.1: Determination of the optimal excitation and emission wavelengths of calcein.

A full fluorescence spectra of a 1 μM calcein solution was obtained using a Spectra Max Gemini XS fluorescent plate reader (Molecular Devices). The optimal excitation and emission wavelengths were found to be close to 490 nm and 520 nm, respectively, which are the same as those stated in the literature. That is, a plot of relative fluorescence was obtained for excitation wavelengths between 350 nm and 500 nm when using an emission wavelength of 520 nm (Figure 4.2). Similarly, a plot of relative fluorescence was obtained for emission wavelengths between 500 nm and 650 nm, when using an excitation wavelength of 490 nm (Figure 4.3). When the excitation spectra is observed it can be seen there is a clear peak in relative fluorescence at a wavelength of 490 nm. If the emission spectra is observed a 'shoulder' is seen at 520 nm. A distinctive peak is not seen since the excitation and emission wavelengths are very close. Therefore, fluorescence from the excitation spectra overlaps with fluorescence from the emission spectra. To reduce this overlap a 'cut off' filter can be introduced which reduces emission fluorescence above a certain wavelength. Different 'cut off' points were introduced between the excitation and emission wavelengths. The optimal 'cut off' wavelength was found to be 515 nm. A distinctive peak showing this can be observed from the plot of relative fluorescence of calcein over a range wavelengths (Figure 4.4). The same plot without a 515 nm 'cut off' filter does not show such a distinctive peak (Figure 4.3).

It was concluded that optimal excitation and emission wavelengths were found to be 490 nm and 520 nm (with a 515 nm 'cut off' filter), respectively. Therefore, these settings were used to measure calcein in all other experiments in this thesis.

4.3.2.2: Determination of the detection limits of Spectra Max Gemini XS fluorescent plate reader (Molecular Devices).

It can be observed the lowest analysed calcein concentration detectable by the fluorescent plate reader is 0.001 mM (Figure 4.5). A linear relationship is observed between calcein concentration and relative fluorescence, when calcein concentrations are between 0.001 mM and 0.1 mM. This relationship is not observed for calcein concentrations greater than 0.1 mM. The self-quenching effect of calcein at high concentration explains this. Thus, fluorescence of a 10 mM calcein solution is lower than that of a 1 mM calcein solution and similarly, a sharp decline in fluorescence is also seen for 10 mM calcein compared to 100 mM calcein.

4.3.2.3: Determination of pH effect on calcein fluorescence.

It can be observed that calcein fluorescence is dependent upon pH (Figure 4.6). The highest fluorescence is seen at pH 7 with a reduction at flanking pH values. Therefore, controls need to be implemented in future investigations, where experiments are performed as a function of pH.

4.3.2.4: Determination of the effect of Triton X100 concentration on calcein fluorescence.

From Figure 4.7, it can be observed that Triton X100 at a v/v concentration of 1% reduced the fluorescence of a 0.1 mM calcein solution the least. This was closely followed by a concentration of 2%. Triton X100 at a v/v concentration of 5% caused the greatest reduction in fluorescence. Although, not expected a concentration of 0.1% showed a greater decrease in fluorescence of the calcein solution than that caused by concentrations of 1% and 2%. Therefore, it was thought that to minimise the fluorescence effects of Triton X100 a 1% v/v concentration should be used. However,

there was a need to determine whether this concentration was sufficient to cause complete rupture of MLVs, which is described in the next section.

4.3.3: Preparation of multilamellar vesicles (MLVs) with encapsulated calcein and separation from free calcein using various sizes of gel filtration column.

4.3.3.1: Gel filtration column (10 mm width by 100 mm length).

It was expected that the first fractions voided from the column would contain buffer only. This is because in order for the sample to be loaded and pass through the column a certain volume of buffer already in the column must be displaced. As MLVs are relatively large molecules it was expected they would be voided from the column next. Finally, it was expected that free calcein would leave the column becoming increasingly dilute on addition of fresh buffer.

The fractions collected from the column were observed. Fractions 1 to 4 were clear in colour indicating presence of buffer only. This was supported by the fact that there was no obvious change in fluorescence of these fractions before and following Triton X100 addition (Figures 4.8 and 4.9). Fractions 5 to 9 were orange in colour indicating a possible quenching effect and thus presence of MLVs with encapsulated calcein. The fluorescence of these fractions before Triton X100 addition is lower than that following Triton X100 addition. Thus, giving further indication to the presence of MLVs with encapsulated calcein. There is also an increase in fluorescence for fractions 10 to 29 when treated with Triton X100 indicating the presence of MLVs. However, an observed yellow colouring of these fractions indicates the presence of free dye. Fractions following fraction 29 show no obvious change in fluorescence before and following Triton X100 addition. Thus, indicating these samples contain free calcein. This experiment demonstrated MLVs with encapsulated calcein could be separated from free calcein. However, only a low volume of MLVs with encapsulated calcein were collected. It was thought this was due to saturation of the gel filtration column with free calcein. Therefore, causing a certain volume of MLVs with encapsulated calcein to be voided from the column with the free calcein. Thus using a

larger column would increase the volume of free MLVs with encapsulated calcein collected.

4.3.3.2: Gel filtration column (20 mm width by 260 mm length).

The presence of MLVs was indicated by the presence of particles having a mean (D3,2) diameter of 4.37 μm and a standard error (SE) of 0.202. The fluorescence of eluate also showed an increase after treatment with Triton X100 (Figure 4.10). Thus, indicating release of calcein from MLVs in to the surrounding buffer causing a decrease in quenching and thus an increase in fluorescence. Buffer treated with Triton X100 shows a decrease in fluorescence compared to untreated buffer, which is expected as Triton X100 reduces the fluorescence of calcein. This was also indicated in Section 4.3.3.1.

Fluorescence of fractions excluded from a Sephadex column (20 mm width by 260 mm length) loaded with a sample containing free calcein and MLVs with encapsulated calcein were plotted as fraction collected against relative fluorescence (Figure 4.11 and 4.12). It is thought fractions 1-12 (Figures 4.11 and 4.12) make up the void volume. This is because when untreated their fluorescence is only marginally higher than buffer alone. Also, when treated with Triton X100 a slight decrease in fluorescence is seen, which can be accounted for by the actual presence of Triton X100. A fraction containing MLVs with encapsulated calcein should show an increase in fluorescence after treatment with Triton X100 as highly concentrated self-quenching calcein leaks in to the surrounding medium and loses its self-quenching effect. Fractions 13-17 (Figure 4.11) and fractions 14-17 (Figure 4.12) show this trend, indicating the presence of MLVs in these fractions. There was not an obvious difference between the fluorescence of fractions containing MLVs that were treated with either 10% or 20% Triton X100. Therefore, indicating complete rupturing of MLVs can be achieved by using Triton X100 at the lower concentration. It is expected that fractions that have the same relatively high fluorescence before and following treatment with Triton X100 contain only free calcein. This trend was observed for all fractions collected subsequent to fraction 17 (Figures 4.11 and 4.12). If Figure 4.11 is compared to Figure 4.12 it can be see that the increase in fluorescence after treating fractions containing MLVs with Triton X100 is higher on

day 1 compared to that on day 2. This is thought to be due to the loss of total MLVs with time, thus less MLVs are available to lyse. It was thought this could be improved by using a higher concentration of egg PC per ml of calcein solution to obtain a higher concentration of MLVs per sample volume.

To determine whether the results could be improved by using a higher concentration of egg PC per ml of calcein solution, MLVs were prepared using 5 mg as oppose to 1 mg of egg PC per ml of 100 mM calcein solution. The amount of egg PC used was increased to increase the quantity of MLVs per sample volume. Therefore, loss of MLVs with time would not have such a significant effect. The fluorescence of MLVs prepared using a higher concentration of egg PC and treated with buffer or Triton X100 was measured. The measurements were plotted as relative fluorescence of fractions against fraction number (Figures 4.13 and 4.14). It can be observed that Figures 4.13 and 4.14 follow the same trends as those shown in Figures 4.11 and 4.12. However, more of the collected fractions contain MLVs. Also, a higher increase in fluorescence of these fractions is seen after treating with Triton X100. Therefore, indicating as expected the presence of a higher number of MLVs per sample volume. The total increase in fluorescence of fractions 11 to 20 after treating with Triton X100 was determined from the results obtained on day 1 and 2. It was calculated that total relative increase on day 1 was 1171 compared to 1070 on day 2. Thus, there is a decline with time, probably due to loss of available MLVs to rupture. However, a sufficient number of MLVs remain to show a significant increase in fluorescence when treated with Triton X100.

4.3.3.3: MLVs for assay.

The fluorescence of the undiluted sample of MLVs with encapsulated calcein is within the linear range of the spectrophotometer (Figure 4.15). Similarly, samples decimally diluted up to three times are within this linear range. This trend is also seen for decimally diluted MLVs in the presence of Triton X100. The fluorescence of MLVs decimally diluted further than three times is not within this linear range. All dilutions show increased fluorescence when treated with Triton X100 compared to untreated dilutions. Thus, these results indicate equivalent samples of MLVs with

encapsulated calcein are useful for calcein release studies when decimally diluted by a factor of 10 no more than three times.

4.3.4: Determination of calcein release from liposomes in the presence and absence of polymers as a function of pH.

4.3.4.1: Release of calcein from MLVs with encapsulated calcein in the presence and absence of poly(lysine dodecandiamide) with reducing pH.

By observing Figure 4.16 it can be seen that MLVs with encapsulated calcein are almost completely ruptured at pH values of 5 and 4. It was thought that this may have been due to an insufficient amount of MLVs with encapsulated calcein for rupture. To determine whether this could be overcome, the latter experiment was repeated. However, fractions 15-17 collected from the Sephadex column were used instead of fractions 12-19. Fractions 15-17 were used since visually they were the most concentrated fractions of MLVs. As before relative fluorescence of MLVs with encapsulated calcein in the presence and absence of polymer were plotted against pH of the solution (Figure 4.17). However, the same trends can be observed when using a more concentrated suspension of MLVs (Figure 4.17) as what were observed using a less concentrated suspension (Figure 4.16). That is, MLVs are almost completely ruptured by pH adjustment to pH values 5 and 4 when pre-incubated in both the presence and absence of polymer. Therefore, solely increasing the concentration of MLVs did not reduce the rupturing effect. It was thought that an alternative reason for complete rupture of MLVs at low pH was due to a combination of low pH and use of Hydrion buffers, which do not contain any cell protecting EDTA. Therefore, the experiment was repeated, but instead of using Hydrion pH buffers to reduce the pH of solutions of polymer plus MLVs with encapsulated calcein, pH adjusted HEPES buffer (10 mM) containing NaCl (150 mM) and EDTA (1 mM) was used. Relative fluorescence of MLVs with encapsulated calcein in the presence and absence of polymer was plotted against pH of the solution (Figure 4.18). It can be observed at pH 7 calcein released from MLVs in the presence and absence of poly(lysine dodecandiamide) is similar (Figure 4.18). At pH 6, calcein release is slightly higher in the presence of polymer compared to in its absence. At pH values 5 and 4 an obvious

increase in calcein release in the presence of polymer is seen compared to in its absence. Thus, these results show as expected that Hydrion buffer causes an increased rupturing effect on MLVs compared to HEPES buffer at equivalent pH values.

The results displayed in Figure 4.18 were used to calculate the percentage of total calcein released in the presence and absence of polymer. These results were plotted as percentage calcein release values in the presence and absence of polymer against pH of the solution (Figure 4.19). By observing this plot it can be seen that at neutral pH, calcein released from MLVs is similar when MLVs are incubated in the presence or absence of polymer. At pH values 6 and 5, calcein release is slightly higher in the presence of polymer compared to in its absence. At pH 4, approximately thirty-percent more calcein is released in the presence of polymer compared to in its absence. Therefore, indicating poly(lysine dodecandiamide) has a pH-responsive rupturing effect on MLVs.

4.3.4.2: Calcein release from liposomes with encapsulated calcein pre-incubated with poly(lysine diethyl malonamide), poly(ornithine dodecandiamide), poly(lysine *iso*-phthalamide), poly(lysine butyl malonamide) and poly(ornithine sebacamide), following pH change compared to liposomes with encapsulated calcein alone, following the same pH changes.

It can be observed from Figure 4.20 that calcein released from MLVs is similar in the presence and absence of poly(lysine butyl malonamide) at pH 7. Following pH reduction to pH 6, pH 5 and pH 4, released calcein is higher in the presence of polymer compared to in its absence. The highest release of calcein is seen at pH 4. This trend is also seen for MLVs incubated in the presence of poly(ornithine sebacamide) (Figure 4.20).

Released calcein from MLVs is not increased by pre-incubating MLVs with polymers poly(lysine diethyl malonamide), poly(ornithine dodecandiamide) and poly(lysine *iso*-phthalamide) compared to pre-incubating MLVs with buffer alone. Therefore, showing the latter three polymers do not show pH-responsive membrane rupture, unlike poly(lysine dodecandiamide), poly(lysine butyl malonamide) and poly(ornithine sebacamide).

From Figure 4.22 it can be seen that with reducing pH an increased difference in fluorescence is seen for polymers poly(lysine butyl malonamide), poly(ornithine sebacamide). Similarly, this is observed for poly(lysine dodecandiamide) (Figure 4.23). A slight increased difference in fluorescence in the presence of poly(ornithine dodecandiamide) with reducing pH is observed (Figure 4.23). However, no increase in fluorescence is seen for poly(lysine *iso*-phthalamide) (Figure 4.22) and poly(lysine diethyl malonamide) (Figure 4.23).

4.3.4.3: Release of calcein from cationic liposomes incubated with poly(lysine dodecandiamide) followed by decreasing pH.

It can be observed from Figure 4.24 that the release of calcein from cationic MLVs pre-incubated with buffer increases with decreasing pH of buffer in which they are suspended. Thus, showing liposomes show pH-responsive disruption. The release of calcein from MLVs that have been pre-incubated with poly(lysine dodecandiamide) is shown to be 96% of the total release at neutral pH. Minimal further release is seen when suspensions of polymer and MLVs are changed to pH values 6, 5 and 4. It is thought that calcein release is dependent upon charge interactions of polymer and MLVs. That is, at neutral pH cationic MLVs and poly(lysine dodecandiamide) have opposite charges so that they immediately interact and cause MLVs to rupture. This is discussed in more detail in Section 4.3.4.4.

4.3.4.4: Release of calcein from anionic liposomes with encapsulated calcein in the presence and absence of amine modified glycidyl methacrylate pre-polymer with increasing pH.

There is little difference in released calcein from MLVs with encapsulated calcein in the presence of buffer when the pH of the suspension is changed from pH 6 to pH values 7, 8 and 9 (Figure 4.25). Thus, indicating liposomes are relatively stable at these pH values. As explained previously, it was expected that calcein released from liposomes would increase with increasing pH. That is, as polymers change from an extended to coiled conformation they cause MLVs to rupture. However, at pH 6, 96% of total encapsulated calcein is released from MLVs that were pre-incubated with

N,N-diethylamine modified GMP. Changing the pH of the suspension of polymer and MLVs to pH values 7, 8 and 9 does not result in any further release of calcein. Similarly, an increased release of calcein from MLVs with encapsulated calcein was not seen with increasing pH, when MLVs were pre-incubated in the presence of *N,N*-diethyl ethylene diamine or 3-(diethylamino)propylamine modified GMPs. It is thought that calcein release is dependent upon charge interactions of polymer and MLVs. That is, at neutral pH anionic MLVs and hydrophobically modified polyamides have similar charge and thus repel each other. Therefore, since no interaction occurs liposomes are not ruptured. Whereas, cationic polymer and anionic liposomes have opposite charges at pH 6 so that they immediately interact and cause liposomes to rupture. To give further conformation of this explanation the experiment was repeated for cationic liposomes with cationic polymer and for neutral liposomes with both cationic and anionic liposomes.

4.3.4.5: Release of calcein from cationic liposomes with encapsulated calcein in the presence and absence of amine modified glycidyl methacrylate polymers with increasing pH.

Observing Figure 4.26 shows there is no obvious increased release of calcein from MLVs when pre-incubated in the presence of 3-(diethylamino)propylamine modified GMP. However, liposomes pre-incubated in the presence of *N,N*-diethylamine and *N,N*-diethyl ethylene diamine modified GMP show an increased release in calcein with increasing pH compared to liposomes in the presence of buffer alone. The increased release of calcein from cationic liposomes in the presence of *N,N*-diethylamine and *N,N*-diethyl ethylene diamine modified GMPs with increasing pH further illustrates that charge interactions between polymer and liposomes result in liposomal rupture.

4.3.4.6: Release of calcein from neutrally charged MLVs with encapsulated calcein in the presence of anionic and cationic polymers with decreasing and increasing pH respectively.

By observing Figure 4.27 it can be seen that calcein released from neutral liposomes pre-incubated with poly(lysine dodecandiamide) is slightly less than that from

liposomes pre-incubated with buffer when pH of polymer-liposome suspensions are adjusted to pH 7. However, calcein release from neutral liposomes pre-incubated with polymer is higher than that from liposomes pre-incubated with buffer when polymer-liposome suspensions are adjusted to acidic pH values. Thus, the same general trends are observed for neutral liposomes as were for anionic liposomes with reducing pH when pre-incubated with poly(lysine dodecandiamide) and buffer (Figure 4.19).

Similarly, the same general trends are observed for neutral liposomes with increasing pH when pre-incubated with *N,N*-diethylamine modified glycidyl methacrylate polymer as were for anionic liposomes (Figure 4.28 and Figure 4.25 respectively). That is, when pH of polymer-liposome suspension is adjusted to pH 6 near total release of calcein from liposomes is observed compared to a low release from liposomes pre-incubated with buffer alone. Further calcein release from liposome suspensions is only slightly increased by adjusting the pH of suspensions of polymer plus liposomes to more alkaline pH values.

4.3.4.7: Similarities and differences in membrane disruption trends observed for polymers with similar structures.

In Chapter 2 the structural similarities and differences between polymers were discussed. To investigate the conformational trends of these polymers potentiometric titrations were performed. The common trends observed for structurally similar polymers were outlined. In summary, polyamides were synthesised based on an alternating pendant amino acid (either lysine or ornithine) and hydrophobic group structure. The introduction of pendant hydrophobic groups was achieved in 3 ways: 1) by making an alkyl group substitution; 2) enchainment of an aromatic group; 3) by enchainment of a long alkyl group.

4.3.4.7.1: Alkyl group substitution.

Poly(lysine butyl malonamide) and poly(lysine diethyl malonamide) were both synthesised by substituting pendant hydrophobic groups with an alkyl group. As discussed in Chapter 2 these polymers are structurally very similar since they have the same amine group and their alkyl groups have the same number of carbon and

hydrogen atoms, which only vary slightly in their positioning. Potentiometric titrations were used to determine then plot the variation of apparent pK_a of these polymers against their degree of dissociation. This was performed to gain information about their conformational trends. It was found that their plots showed almost exactly the same trends. Therefore, it was expected that when used to disrupt and release liposomal contents as a function of pH the same or very similar trends would be exhibited. However, this was not found, since while poly(lysine butyl malonamide) clearly showed pH-responsive membrane disruption properties (Figure 4.20), poly(lysine diethyl malonamide) did not (Figure 4.21). That is, more calcein was released from liposomes with encapsulated calcein with reducing pH when pre-incubated with poly(lysine butyl malonamide) compared to liposomes pre-incubated with buffer. Whereas, liposomes with encapsulated calcein pre-incubated with poly(lysine diethyl malonamide) showed similar levels of calcein release with reducing pH as for liposomes with encapsulated calcein pre-incubated with buffer.

4.3.4.7.2: Enchainment of an aromatic group.

Compared to the other polyamides studied in Chapter 2 poly(lysine *iso*-phthalamide) is structurally most similar to poly(lysine dodecandiamide), since both polymers have the same amine group. However, they vary in that the hydrophobic groups of poly(lysine dodecandiamide) are long aliphatic groups, whereas, poly(lysine *iso*-phthalamide) has aromatic groups. While structurally they are relatively similar, comparing the plot of variation of apparent pK_a with degree of dissociation for poly(lysine *iso*-phthalamide) (Figure 2.22) with poly(lysine dodecandiamide) (Figure 2.24) shows they have very different conformational trends. The trends observed for poly(lysine *iso*-phthalamide) (Figure 2.22) are more closely matched to those of poly(ornithine dodecandiamide) and poly(ornithine sebacamide). Therefore, on a purely structural basis it would be expected that poly(lysine *iso*-phthalamide) would show similar membrane disruption properties as poly(lysine dodecandiamide). However, this was not found as unlike poly(lysine dodecandiamide) (Figure 4.19), poly(lysine *iso*-phthalamide) (Figure 4.21) shows similar levels of calcein release from liposomes with encapsulated calcein with reducing pH when pre-incubated in either the presence or absence of polymer.

4.3.4.7.3: Enchainment of a long alkyl group.

Poly(ornithine dodecandiamide) and poly(ornithine sebacamide) are very similar in structure since they both have ornithine as their amine group and have long alkyl groups which only vary by two CH_2 groups. In Chapter 2, Figures 2.30 and 2.32 show the variation of apparent pK_a for poly(ornithine dodecandiamide) and poly(ornithine sebacamide) with degree of dissociation, respectively. Almost exactly the same trends can be seen. Therefore, it was expected that polymers poly(ornithine dodecandiamide) and poly(ornithine sebacamide) would show similar pH-responsive membrane disruption properties. However, while poly(ornithine sebacamide) (Figure 4.20) clearly shows pH-responsive membrane disruption properties poly(ornithine dodecandiamide) (Figure 4.21) does not.

Like poly(ornithine dodecandiamide) and poly(ornithine sebacamide), poly(lysine dodecandiamide) has a long alkyl group, however, it has a different amine group. In Chapter 2 the variation of apparent pK_a of poly(lysine dodecandiamide) against degree of dissociation was plotted (Figure 2.24). It was noted that it showed very different trends to those of poly(ornithine dodecandiamide) (Figure 2.30) and poly(ornithine sebacamide) (Figure 2.32).

Figure 4.19 shows the percentage of total calcein released from MLVs with encapsulated calcein with reducing pH following pre-incubation with buffer and poly(lysine dodecandiamide). By observing this plot it can be seen that the same pH-responsive membrane disruption properties are seen for poly(lysine dodecandiamide) as were for poly(ornithine sebacamide) (Figure 4.20).

4.3.4.7.4: Amine modified glycidyl methacrylate polymers.

Glycidyl methacrylate pre-polymer was synthesised then modified using three different amine groups, including *N,N*-diethylamine, 3-(diethylamino)propylamine and *N,N*-diethyl ethylene. As for polyamides the variation of apparent pK_a of these polymers against their degree of dissociation were plotted (Figures 2.34, 2.36, 2.38, respectively), using potentiometric titration data. Since they only varied in their amine group it was expected they would have had very similar conformational trends.

However, this was not observed (Figures 2.34, Figure 2.36 and Figure 2.38). On a purely structural basis it would have been expected they would also have shown similar pH-responsive membrane disruption properties. However, dissimilar properties were observed on Figure 4.26, which can be explained by variations in their conformational trends. That is, the release of calcein from liposomes with encapsulated calcein when pre-incubated with *N,N*-diethylamine and *N,N*-diethyl ethylene modified GMPs is higher with increasing pH when compared to liposomes with encapsulated calcein pre-incubated with buffer. However, 3-(diethylamino)propylamine modified GMP shows similar release to that from liposomes pre-incubated with buffer.

As already explained it was expected that polymers of similar structure would also have shown similar conformational changes and similar pH-responsive membrane disruption properties. However, this was not found and the reasoning for this is unclear. However, it does indicate small structural changes to the polymer can make large differences to the polymers conformational changes and properties they exhibit.

4.3.4.8: Release of calcein from small liposomes with encapsulated calcein in the presence and absence of polymers.

It can be observed that calcein release from small anionic liposomes pre-incubated with HEPES is increased with reducing pH (Figure 4.29). At pH 7 and 6, there is minimal variation in calcein release from liposomes that have been pre-incubated with HEPES or poly(lysine dodecandiamide). When pH is reduced to pH 5, there is a slight increase in released calcein from liposomes pre-incubated with poly(lysine dodecandiamide) compared to those pre-incubated with HEPES. Similar to calcein release from large liposomes (MLVs), reducing the pH to 4 in the presence of poly(lysine dodecandiamide) gives a sharp increase in released calcein.

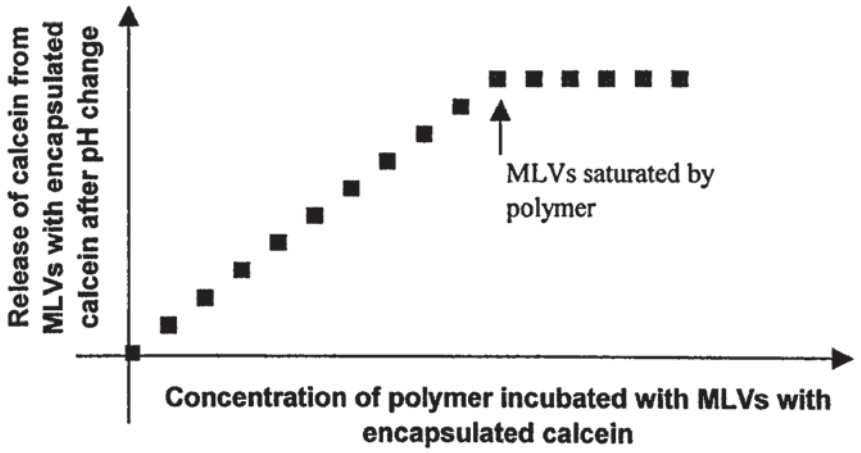
Calcein released from cationic small liposomes when pre-incubated with HEPES shows minimal variation with increasing pH (Figure 4.30). Calcein release from these liposomes at pH 6 when pre-incubated with *N,N*-diethylamine modified GMP is higher than calcein released from liposomes pre-incubated with HEPES. However, with increasing pH, no further release of calcein is seen. It was expected that calcein

release from small cationic liposomes in the presence of *N,N*-diethylamine modified GMP would show a similar increase with increasing pH to that from large liposomes (MLVs). This result might be explained by the mode that *N,N*-diethylamine modified GMP disrupts liposomes. This result suggests it disrupts liposomes layer by layer. Therefore, MLVs that have a number of lipid bilayers require a greater interaction with polymer for complete disruption compared to small liposomes that have less or only a single bilayer.

4.3.4.9: Determination of calcein release from MLVs with encapsulated calcein incubated in the presence of varying concentrations of poly(lysine dodecandiamide) followed by a change in pH.

It would be expected that at pH values where polymer coiling does not take place calcein release would be minimal and independent of polymer concentration. However, at pH values where conformational changes of polymer do take place, it is expected that calcein release from MLVs would increase with increasing polymer concentration. It is thought calcein release would increase with increasing polymer concentration until MLVs become saturated by polymer. At this point it is expected that no further increase would be observed. If these trends were plotted a plot similar to that shown in Figure 4.53 would be the result.

Figure 4.53: Expected plot of calcein release from MLVs with encapsulated calcein incubated with increasing polymer concentrations followed by pH change.



By observing Figure 4.31 it can be seen that at pH 7, the release of calcein is similar for each of the polymer concentrations incubated with MLVs. This is expected as at pH 7, polymer is in an extended form. At pH 6 and 5 the general trend is that calcein release from MLVs is increased following incubation with an increased polymer concentration. However, the difference in calcein release is relatively small indicating a polymer concentration of 2 mg ml^{-1} may be close to the saturation level (Figure 4.53). Following a reduction to pH 4, 73% calcein release from MLVs is seen when incubated with polymer at concentrations of 2 mg ml^{-1} and 1 mg ml^{-1} of MLVs. Since, calcein release is the same at both these polymer concentrations, this suggests that these concentrations are close to or at the saturation level. Polymer at a concentration of 0.5 mg ml^{-1} of MLVs resulted in a higher release of calcein. However, it was thought this result might have been an anomalous value, which is further supported by the wide error bars. Therefore, to investigate this anomalous result it was decided to determine calcein release from MLVs pre-incubated with a wider range of polymer concentrations followed by a reduction in their pH to pH 4. The results of this investigation is shown in Figure 4.32. It can be seen that by increasing the polymer concentration that MLVs are pre-incubated with, increases the amount of calcein released when the solution of polymer and MLVs is reduced to pH 4. The rate of release with increasing polymer concentration follows a similar trend to what was expected (Figure 4.53). However, although the rate of release is reduced at polymer concentrations of greater than 0.25 mg ml^{-1}, the rate of release does plateau by a polymer concentration of 2 mg ml^{-1}. These results further indicate that released calcein from MLVs pre-incubated with a 0.5 mg ml^{-1} polymer concentration was an anomalous value (Figure 4.31). Figure 4.32 shows an increased release of calcein from MLVs when incubated with polymer at a concentration of 2 mg ml^{-1} compared to 1 mg ml^{-1}. Whereas, Figure 4.31 shows the release of calcein from MLVs is the same when incubated with 2 mg ml^{-1} or 1 mg ml^{-1} polymer concentrations. This difference can be explained by the variation in number of MLVs per sample volume. That is, the results shown in Figure 4.31 indicate number of MLVs per sample volume is at a level to become saturated by a polymer concentration of 1 mg ml^{-1} of MLVs. This is thought as no further release is observed when incubated with higher polymer concentrations. However, the results shown in Figure 4.32 indicate a higher number of MLVs per sample volume since incubating with an increased concentration of

polymer results in an increased release of calcein. Therefore, indicating a polymer concentration of 1 mg ml^{-1} does not completely saturate the available MLVs.

These results correspond to those found by Murthy *et al.*, (1999). They found by using percentage haemolysis as an indicator, that membrane disruptive polymers PPAAc and PEAAc are not membrane lytic at pH 7.4 but are at pH 6.1. For both polymers at pH 6.1, percentage haemolysis increases with increasing polymer concentration until a plateau is reached. They also demonstrated that although both are haemolytic at pH 6.1, PPAAc becomes haemolytic one pH unit higher than PEAAc. Thus, a slight modification in polymer structure changes the pH at which haemolysis occurs. Thomas *et al.*, (1994) also illustrated that increasing the concentration of poly(2-ethylacrylic acid) causes a shift in the pH at which liposomes are solubilised.

4.3.4.10: Determination of calcein release from MLVs with encapsulated calcein incubated in the presence of varying concentrations of *N,N*-diethylamine modified glycidyl methacrylate polymer followed by a change in pH.

By observing Figure 4.33 it can be seen pre-incubating MLVs with *N,N*-diethylamine modified GMP at increasing concentrations between 0.015 mg ml^{-1} and 0.5 mg ml^{-1} prior to increasing the pH of suspensions of polymer and MLVs increases total calcein release linearly. Pre-incubating MLVs with polymer at concentrations greater than 0.5 mg ml^{-1} does not cause any further release. Therefore, suggesting MLVs are saturated by polymer at concentrations of 0.5 mg ml^{-1}.

4.3.4.11: Determination of calcein release from MLVs with encapsulated calcein incubated in the presence of poly(lysine dodecandiamide) for increasing incubation times prior to pH reduction.

It can be observed that at pH 7 there is not an obvious difference in fluorescence for each of the incubation times (Figure 4.34). This is expected, as prior to pH reduction conformational changes in polymer to cause membrane disruption do not take place. This trend is also seen at pH 6. Fluorescence at pH 5 is slightly higher than at pH 7 and pH 6, which can be explained by the onset of conformational changes of the

polymer. However, incubating polymer and MLVs for an increasing period of time prior to reducing the pH has no obvious effect. At pH 4, fluorescence is greater than at the higher pH values. However, again increasing the length of time MLVs are incubated with polymer prior to changing the pH does not notably increase the fluorescence. Therefore, indicating an incubation time of 10 minutes is sufficient to allow any interactions between polymer and MLVs to take place. An additional explanation of these results is that the pH of polymer and liposome suspensions needs to be reduced to particular pH values before binding of polymer to MLVs takes place.

4.3.5: Polymer properties which enable the rupturing of lipid membranes.

4.3.5.1: Charge Interactions.

The change in charge that liposomes exhibit when the pH the suspension they are suspended in is adjusted was performed to give further evidence that the interaction between charge of polymer and charge on liposomes affects the point where liposomes are lysed. If Figures 4.35 and 4.36 are observed it can be seen that with changing pH the same general trends in zeta potential for positive and negatively charged liposomes are observed as described by Washington (2001). That is, egg PC liposomes have a negative charge at pH 7. This negative charge becomes weaker with reducing pH and at values tested below pH 5 the surface charge is positive. Liposomes synthesised from a combination of egg PC and stearylamine have a positive charge at pH values between 6 and 8.0. A negative charge is exhibited when these liposomes are adjusted to pH values greater than pH 8. It was also attempted to determine the zeta potential of neutral liposomes for pH values between 4 and 9. However, neutral liposomes were found to be unstable and lysed during analysis. Therefore, the number of available liposomes was too low to determine zeta potential readings.

As discussed earlier lysis is not observed when polymers and liposomes that have the same charge at neutrality are dispersed together. However, lysis of liposomes occurs when the pH of these polymer and liposome suspensions are adjusted to particular pH values. It is thought the point where lysis occurs is when the charge on liposomes and

polymer become opposite. This can be explained by the observation that when polymers which have a negative charge at neutrality are dispersed with liposomes that have a positive charge lysis occurs. Similarly, when polymers that have a positive charge at neutrality are dispersed with negatively charged liposomes lysis occurs.

4.3.5.2: Determination of whether polymer attaches to surface of MLVs.

The fluorescence intensity of MLVs which are pre-incubated with buffer, centrifuged, then re-suspended in pH 7, 6, 5 and 4 adjusted buffer solutions, show the same trends as MLVs which are pre-incubated with buffer then directly transferred to pH adjusted buffers (Figure 4.37). However, MLVs not centrifuged and directly transferred to pH 4 adjusted buffer, have a percentage of total fluorescence intensity of 51%. Whereas, MLVs which are centrifuged then transferred to pH 4 adjusted buffer have a percentage of total fluorescence intensity of 39%. Thus, indicating the centrifugation process lyses a certain amount of liposomes. The percentage of total fluorescence intensity values of MLVs which are pre-incubated with poly(lysine dodecandiamide), then directly transferred to pH adjusted buffers (Figure 4.37) increases with reducing pH of buffer solution, with a value of 82% observed when transferred to pH 4 buffer solution. However, there is minimal difference between the fluorescence intensity values of MLVs pre-incubated with poly(lysine dodecandiamide), centrifuged, then re-suspended with pH 7, 6, 5 and 4 adjusted buffers. This result indicates that centrifugation in the presence of poly(lysine dodecandiamide) lyses the MLVs. Thus, the re-suspended pellet contains mainly lysed MLVs. This can explain why there is little difference between fluorescence intensity values for MLVs re-suspended in each of the pH buffers. Since there is minimal difference between the percentage fluorescence intensity readings of MLVs that are re-suspended in pH 7, 6, 5 and 4 buffers, it is thought that polymer does not attach to the liposomal surface prior to centrifugation. However, this suggestion cannot be concluded from these results since there are only a low number of MLVs available to lyse.

4.3.6: Applicability of liposomes used in latter studies for *in vivo* use.

4.3.6.1: Liposome size studies.

By observing Table 4.5, it can be seen that there is little variation in the mean diameter of untreated liposomes with encapsulated glucose synthesised using the beaker method compared to those synthesised using the reverse phase evaporation method. It can also be seen that sonication of both the liposome preparations described above for a period of 30 minutes at 0-5° C did not reduce their heterogeneity enough for PCS analysis. However, extruding both the liposome preparations caused a reduction in their mean diameter. That is, the mean diameter of liposomes synthesised using the beaker method was reduced from 7.05 μm to 0.172 μm after extrusion. Similarly, the mean diameter of liposomes synthesised using the reverse evaporation vesicle method was reduced from 7.82 μm to 0.437 μm. The heterogeneity of the liposome sizes was also reduced after extrusion. This can be observed by comparing the volume histograms of beaker synthesised liposomes before and after extrusion, which are shown in Figures 4.41 and 4.43, respectively. This trend can also be seen when comparing volume histograms of reverse phase evaporation synthesised liposomes before and after extrusion, shown in Figures 4.42 and 4.44, respectively.

4.3.6.1.1: Determination of whether washing liposome suspensions to remove free glucose affects liposome size results.

From Figure 4.45 it can be seen there is little variation between the mean diameter (D3,2) of liposomes synthesised using the beaker or rotary evaporation methods. Also, washing the liposome preparations by centrifugation and re-suspension does not have an effect on their mean diameter. Extrusion of the liposome preparations results in a significant reduction in their size. That is, the mean diameter of extruded liposome suspensions synthesised using beaker and rotary evaporation methods is 0.172 μm and 0.437 μm, respectively.

4.3.6.1.2: Effect of increasing sonication time on liposome preparations.

Observing the volume histograms of beaker synthesised liposomes with encapsulated DPBS shows the sizes of liposomes which have been exposed to sonication are over a wide range with most being between 10 μm and 100 μm (Figure 4.46). A smaller tail can also be observed with liposomes falling in the size range of approximately 2.0 μm to 10 μm. After sonication for two hours no tail is observed on the histogram (Figure 4.47). A normal distribution curve can be seen with all liposomes falling between the size range of approximately 0.7 μm and 50 μm. Thus, indicating sonication for two hours reduced the size and polydispersity of liposomes. After sonication for four hours, a normal distribution curve can again be observed (Figure 4.48). However, the size range of liposomes is between approximately 0.7 μm and 10 μm. A small tail can also be observed with liposomes falling between the size range of approximately 30 μm and 50 μm. Thus, indicating increasing the sonication time from two to four hours can reduce the size of liposomes. No obvious change in the size of liposomes is observed after sonication for six hours (Figure 4.49) compared to four (Figure 4.48). Therefore, indicating sonication for a time period greater than four hours does not cause any further significant reduction in liposome size. A similar trend is observed for volume histograms of reverse evaporation synthesised liposomes with encapsulated DPBS exposed to sonication. That is, the sizes of liposomes that have received no sonication have a size range between approximately 0.5 μm and 100 μm. After sonication for two hours this size is reduced to between approximately 0.5 μm and 70 μm with an increased number of liposomes being at the lower end of this range. After four hours of sonication a further size reduction is observed with the size of liposomes being between 0.5 μm and 10 μm (Figure 4.52). After sonication for six hours (Figure 4.53), no further reduction in the size range is seen. In fact a small tail is observed with liposomes between the size range of approximately 10 μm and 50 μm. Thus, indicating a possible liposome aggregation effect occurs if sonication time exceeds four hours. Size analysis of all the liposome preparations was undertaken using a Zetasizer (Malvern instruments). Although liposome sizes were reduced by sonication the size reduction was not to a sub-micron level. Therefore, results obtained from the Zetasizer were not reliable for analysis. By observing Figures 4.54

and 4.55 it can be seen the size of liposomes synthesised using either method declines sharply from zero to two hours sonication. The decline in mean volume diameter from two to fours sonication is not so sharp. The beaker synthesised liposomes show only a small decline in mean volume diameter when sonication tme is reduced from six to four hours. No further decline in mean volume diameter is observed for reverse phase evaporation synthesied liposomes. In fact there is a slight increase in mean volume diameter which can be explained by a possible aggregation effect. Gregoriadis (1976) describes a similar method for enzyme entrapment where probe sonication for a time interval greater than 10 minutes produces no further decrease in particle size.

4.3.6.2: Lyophilisation.

From Table 4.4 it can be seen release of calcein from lysed cationic and anionic MLVs is high before lyophilising but low following lyophilisation. Thus, indicating the lyophilisation process lyses liposomes. It can also be observed that liposomes pre-incubated with poly(lysine dodecandiamide) (Table 4.5) and *N,N*-diethylamine modified GMP (Table 4.6) are lysed by lyophilisation similar to MLVs pre-incubated with buffer (Table 4.4). Thus, polymer does not give any protection to liposomes during the lyophilisation process. Without cryoprotection it is thought the probable cause of content leakage from liposomes is a result of liposomes fusing as described by Vermuri *et al.*, (1991). As explained, it would be useful to extend the storage time of liposomes used in this study while retaining pH-responsive content release by pre-incubation with polymer. It would be expected that further insight in to this study might be achieved by using cryoprotectants during the lyophilisation process.

4.4: Conclusions.

There were five main sub-studies to this chapter. The conclusions drawn for each are outlined below:

1) Determination of liposome solubilisation properites of polymer as a function of pH.

Three of the polyamides synthesised in Chapter 2 including, poly(lysine dodecandiamide), poly(lysine *iso*-phthalamide) and poly(lysine butyl malonamide) were assessed for pH-responsive solubilisation properties, Similiarly, the three amine modified glycidyl methacrylate polymers synthesised in Chapter 2 including, *N,N*-diethylamine, *N,N*-diethyl ethylene diamine and 3-(diethylamino)propylamine were assessed.

Solubilisation of anionic MLVs occurred with reducing pH when liposomes were pre-incubated in the presence of poly(lysine dodecandiamide) and poly(lysine *iso*-phthalamide), whereas in the presence of poly(lysine butyl malonamide) or buffer solubilisation did not. Similarly, liposome solubilisation did not occur with increasing pH when cationic MLVs were pre-incubated with amine modifed GMPs.

A result where polymers show pH-responsive liposome solubilisation is indicative of potential pH-responsive membrane disruption properties. Polymers which do not show pH-responsive membrane solubilisaton are not necessarily futile for pH-responsive membrane disruption. That is, liposome solubilisation only indicates whether liposomes are completely lysed, but does not indicate whether pore formation has occurred. Therefore, this led to further studies to quantify pH-responsive membrane disruption of liposomes.

2) Preparation of liposomes with encapsulated calcein for the application of quantifying membrane disruption properties of polymers.

It was concluded optimal excitation and emission wavelengths were found to be 490 nm and 520 nm with a 515 nm 'cut off' filter, respectively. Thus, these settings were used for all the further calcein release measurement studies in this thesis.

The lowest and highest detection limits of the Spectra Max Gemini XS fluorescent plate reader were found to be 0.001 mM and 0.1 mM, respectively. Both pH and Triton X100 affected fluorescence of calcein. Therefore, controls were implemented to overcome pH and Triton X100 effects, when calcein was measured in future investigations. It was found a sufficient volume of anionic MLVs encapsulated with calcein were eluted from a gel filtration column for membrane disruption studies when MLVs were synthesised with 5 mg egg PC per ml of 100 mM calcein. Volumes that were too low were eluted when anionic MLVs were synthesised from a lower concentration of egg PC. It was also noted that MLVs synthesised from 5 mg egg PC per ml of 100 mM calcein should not be diluted by a factor of 10 more than three times when used for assay purposes, otherwise release of calcein would be too low to be detected by the Spectra Max Gemini XS fluorescent plate reader.

3) Determination of calcein release from liposomes in the presence and absence of polymers as a function of pH.

Polyamides.

Problems were encountered with using Hydrion buffers when used to adjust the pH of liposome suspensions, since at low pH liposomes were completely lysed. This was overcome by the use of pH adjusted HEPES buffer containing cell protecting EDTA. The pH-responsive membrane disruption properties of polymers studied in Chapter 2 could then be assessed. It was expected that polymers with similar structural properties would show similar membrane disruption characteristics. However, this was not always observed. Poly(lysine butyl malonamide) and poly(lysine diethyl malonamide) are structurally similar. However, when calcein release from liposomes with encapsulated calcein was used as an indicator of membrane disruption poly(lysine butyl malonamide) showed membrane disruption properties, whereas

poly(lysine diethyl malonamide) did not. In studies where liposome solubilisation was used as an indicator of membrane disruption poly(lysine butyl malonamide) had not shown membrane disruption properties. This demonstrates solely observing liposome solubilisation to determine membrane disruption properties is not sufficient.

Poly(ornithine dodecandiamide), poly(lysine dodecandiamide) and poly(ornithine sebacamide) are structurally similar to each other. However, only the latter two polymers show pH-responsive membrane disruption properties when calcein release from liposomes with encapsulated calcein is used as an indicator of membrane disruption. The pH-responsive membrane disruption properties exhibited by poly(lysine dodecandiamide) correspond to those observed in the liposome solubilisation study. That is, poly(lysine dodecandiamide) solubilised liposomes at reduced pH values.

As discussed earlier poly(lysine *iso*-phthalamide) is structurally most similar to poly(lysine dodecandiamide). That is, the enchained aliphatic hydrophobic groups of poly(lysine dodecandiamide), which behave as if pendant, are are replaced by aromatic groups. Poly(lysine *iso*-phthalamide) similar to poly(lysine dodecandiamide) solubilised liposomes with reducing pH. Therefore, it was expected that poly(lysine *iso*-phthalamide) would also show pH-responsive properties when calcein release from liposomes with encapsulated calcein was used as an indicator of membrane disruption. However, this was not observed since when the pH of liposomes was adjusted to pH values 7, 6, 5 and 4 the amount of calcein released from liposomes that were pre-incubated with poly(lysine *iso*-phthalamide) was similar to the amount of calcein released from liposomes that were pre-incubated with buffer. Although not investigated further in this thesis, an increased release of calcein from liposomes adjusted to acidic pH values might have been seen if liposomes with encapsulated calcein had been pre-incubated with higher concentrations of poly(lysine *iso*-phthalamide).

Amine modified glycidyl methacrylate polymers.

Anionic liposomes with encapsulated calcein are immediately lysed by amine modified GMPs when their solution pH is adjusted to pH 6. Whereas, the release of

calcein from cationic liposomes with encapsulated calcein can be controlled by pH when incubated with *N,N*-diethylamine and *N,N*-diethyl ethylene diamine GMPs. That is, release of calcein from liposomes was shown to increase with increasing pH values between pH 6 and pH 9. The release of calcein from cationic liposomes with encapsulated calcein was also shown to increase with increasing pH when pre-incubated with buffer. However, calcein release from liposomes pre-incubated with polymer was greater than that from liposomes pre-incubated with buffer. The difference between calcein release from liposomes with encapsulated calcein pre-incubated with buffer and calcein release from liposomes pre-incubated with *N,N*-diethylamine and *N,N*-diethyl ethylene diamine GMPs was widened at the higher pH values. Cationic liposomes with encapsulated calcein pre-incubated with 3-(diethylamino)propylamine GMP and adjusted to pH values between pH 6 and pH 9 showed a similar release of calcein as that from cationic liposomes with encapsulated calcein pre-incubated with buffer. Thus, these results indicated that by changing the amine group that glycidyl methacrylate pre-polymer is modified with can hinder the polymers ability to cause pH-responsive disruption of lipid membranes.

4) Ascertain the polymer properties which enable the rupturing of lipid membranes.

It was thought rupturing of liposomes may be a consequence of charge interactions between poylmer and liposomes. The charge on liposomal surfaces as a funtion of pH was assessed in order to determine if charge on liposomal surface was related to the point where lysis of liposomes occurs. The charge on liposomes with pH change showed trends similar to those found in the literature as discussed earlier.

Observations were made which indicated charge interactions between liposomes and polymer caused lysis of liposomes. That is, poly(lysine dodecandiamide), which exhibits an anionic charge at neutrality lyses liposomes that exhibit a cationic charge at neutrality when incubated together at pH 7. Similarly, *N,N*-diethylamine which exhibits a cationic charge at neutrality lyses liposomes that exhibit an anionic charge at neutrality when incubated together at pH 6. Therefore, it was thought the latter observations were simply due to the opposite charges on the polymer and liposome surface causing them to attract and lyse liposomal membranes.

Secondly, poly(lysine dodecandiamide), which exhibits an anionic charge at neutrality does not lyse liposomes that exhibit an anionic charge at neutrality when incubated together at pH 7. However, when incubated together and adjusted to lower pH values liposomes are lysed. Similarly, *N,N*-diethylamine, which exhibits a cationic charge at neutrality does not lyse liposomes that exhibit a cationic charge at neutrality when incubated together at pH 6. However, when incubated together and adjusted to higher pH values liposomes are lysed.

These observations can be explained by the fact that reducing the pH of a suspension of anionic liposomes and poly(lysine dodecandiamide) not only changes the charge on the liposome surface but also causes the poly(lysine dodecandiamide) to lose charge. It is thought that this loss of charge reduces charge repulsion effects between the polymer and liposomes at pH values 6, 5 and 4. Thus, allowing polymer and liposomes to interact and cause liposome lysis. Similarly, increasing the pH of a suspension of cationic liposomes and *N,N*-diethylamine not only changes the charge on the liposome surface but also causes the *N,N*-diethylamine to lose charge. Again it thought that this loss of charge reduces charge repulsion effects between the polymer and liposomes at pH values 7, 8 and 9. Thus, allowing polymer and liposomes to interact and cause liposome lysis. Therefore, the latter observations give further indication that rupturing of liposomes is at least partly due to charge interactions.

Studies to determine whether polymer permanently attaches to liposome surface were inconclusive since liposomes were lysed by the centrifugation process used when in the presence of polymer.

5) Applicability of liposomes used in latter studies for in vivo use.

As discussed in Section 1.6.5 in Chapter 1 it is important to be able to produce homogenous sized liposome suspensions for *in vivo* applications. Liposomes that were prepared by both reverse phase evaporation and the beaker method produced heterogeneous sized liposome suspensions. Therefore, without further intervention they would not be useful for *in vivo* use. Extrusion of these liposome suspensions resulted in suspensions that were more homogenous in size and the the mean diameter size was reduced to below 0.2 μm. Sonicating the suspensions for a period of 30

minutes did not reduce the heterogeneity enough for PCS analysis. However, the mean diameter size was increasingly reduced with increasing sonication time for sonication times up to four hours. Sonicating suspensions for times more than four hours did not reduce their mean diameter size any further. Sonication did not produce suspensions with diameter sizes as small as suspensions that were exposed to extrusion. Also, washing liposomes with encapsulated glucose to remove free glucose was shown not to affect liposome sizes.

Suspensions of cationic MLVs with *N,N*-diethylamine modified glycidyl methacrylate polymer and anionic MLVs with poly(lysine dodecandiamide) were lysed by the lyophilisation process. Lyophilising the latter suspensions with the use of cryoprotectants may see a system where MLVs and the latter polymers can be stored by use of lyophilisation.

Chapter 5

Release of solid particles from within polymer coatings as a function of pH

5.0: Aims and Overview.

Aim: To coat solid particles with polymers studied in Chapter 2, then uncoat the polymer coated particles as a function of pH.

In Chapter 4 it was shown that by pre-incubating liposomes with encapsulated calcein with hydrophobically modified polyamides or amine modified GMPs the release of calcein is increased at specific pH values compared to the release of calcein from liposomes with encapsulated calcein pre-incubated with buffer. Thus, indicating polymers give a pH-responsive release of calcein. Thus, it was thought that if these polymers are used to coat solid particles and placed in different pH buffers, particles could be uncoated depending on the buffer pH. The applications of such a system were discussed in more detail in Chapter 1.

To achieve the aim of coating solid particles with polymer, then uncoating the coated solid particles as a function of pH the following procedures were performed:

1) Firstly, the dissolution rate of polymers in different pH buffer solutions needed to be determined so the length of time required to uncoat polymer coated particles when placed in specific pH buffer solutions was known.

In summary, this was assessed using two ways: 1) by producing polymer films by dissolving polymer in water then evaporating off the water. 2) by producing polymer films by dissolving polymer in organic solvent then evaporating off the organic solvent. These films were agitated in different pH buffer solutions and the time required for them to dissolve was measured.

2) The second stage involves coating solid particles with polymer. The solid particles that were coated with polymer were Azocoll and sodium chloride.

3) The final stage involves transferring the polymer coated solid particles in to different pH buffers and quantifying the uncoating of solid particles.

To achieve the second and third stages a solid was required that would not dissolve during polymer coating process but uncoating of the solid could be quantified. Two different solids were used, including Azocoll and sodium chloride. The reasoning for using these will be explained in turn.

Coating Azocoll with polymer.

It was thought Azocoll would be an appropriate solid since it is an insoluble ground collagen that is impregnated with a bright red Azo dye. The dye is released from the Azocoll when Azocoll comes in to contact with subtilisin, a proteolytic enzyme. Therefore, it was thought if Azocoll was coated with polymer and agitated in aqueous buffer solutions, then on addition of subtilisin to the solutions dye release would only be observed for Azocoll that had lost its polymer coating.

The studies that were performed to achieve the latter included:

1) Determining time course of azo dye release from Azocoll in pH 9 buffer.

Since Azo dye release would be used as an indicator of polymer uncoating the time required to give detectable levels of dye release from Azocoll when incubated with subtilisin needed to firstly be determined. To achieve this known quantities of Azocoll and subtilisin were incubated together and Azo release was measured with increasing time.

2) Determining rate of dissolution of calcein from solid polymer.

It was thought Azo dye leaches in to the polymer coating during the aqueous polymer coating process which is described in point 4, thus, when polymer dissolves in to solution so does the dye. To confirm this was happening, a known amount of calcein dye was firstly dissolved with polymer solution and the solution was evaporated off to

produce a film. Calcein release from the film was determined with increasing stirring time in pH 7 and pH 9 buffers.

3) Determining whether subtilisin degrades polymer.

An experiment was set up to check subtilisin does not degrade polymer or cause more rapid dissolution of polymer.

4) Coating of Azocoll with polymer.

Coating of Azocoll was achieved in two ways: 1) by placing Azocoll in to an aqueous polymer solution, then evaporating off the water leaving a polymer coating over the Azocoll. 2) by placing Azocoll in to an organic solvent with dissolved polymer, then evaporating off the organic solvent leaving a polymer coating over Azocoll.
Release of azo dye from polymer coated Azocoll was then determined.

Coating sodium chloride with polymer.

The Azo release from polymer coated Azocoll when stirred in buffer solution required six 15 mg amounts of Azocoll for each stirring time tested. Also Azocoll needed to be separated from the buffer solution. It was thought sodium chloride would be a useful alternative. This was because sodium chloride release in to the buffer solution could be measured using a probe placed directly into the buffer, which was used to stir polymer coated sodium chloride. This allowed measurements to be recorded over a broader time range. Also, it eliminated the need to separate remaining polymer coated sodium chloride from the buffer solution as was required to measure azo dye release from Azocoll.

To determine the release of sodium chloride from within a polymer coating the following studies were performed:

1) Conductivity of buffers that polymer coated sodium chloride were stirred.

Sodium chloride was to be measured using a conductivity meter. Therefore, it was important conductivity of pH adjusted buffer used to stir polymer coated sodium chloride had a low conductivity. Therefore, conductivity of different buffers was assessed.

2) Detection limits of conductivity meter.

The detection limits of the conductivity meter for sodium chloride needed to be determined to make sure any sodium chloride released from polymer coating would be at a level sufficient for detection.

3) Effect of surfactant on release of sodium chloride from within polymer coating.

Surfactant was added to the buffer that polymer coated sodium chloride was stirred in order to assess whether total release or release rate of sodium chloride from within this polymer coating is altered.

4) Coating of sodium chloride and measuring release of this sodium chloride from within the polymer coating.

Sodium chloride was coated with polymer using increasing amounts of polymer. Release of sodium chloride from within these polymer coatings was then measured when stirred in pH 7 and pH 9 buffers.

5.1: Methods.

5.1.1: Determination of dissolution rate of polymer films over a range of pH values.

5.1.1.1: Using water to dissolve polymer.

A film of *N,N*-diethylamine modified glycidyl methacrylate polymer was prepared by dissolving 300 mg in a 30 ml volume of distilled water. This polymer solution was transferred to a flat-bottomed glass vessel with a diameter of 40 mm. The water was completely removed by evaporation in a 30° C incubator leaving a thin polymer film over the entire base of the glass vessel. Various concentrations of the polymer were used to produce films, but a 30 ml polymer solution at a concentration of 10 mg ml^{-1} was the lowest concentration that produced a film, which could be easily handled. Lower concentrations produced films that were too thin to dissect and weigh accurately. The dissolving rate of polymer film as a function of pH was determined by first preparing 10 mM HEPES buffer solutions adjusted to pH values 6, 7, 8, 9 and 10. A 5 ml volume of each pH buffer solution was transferred to test tubes in duplicate. The prepared polymer films were dissected using a scalpel to obtain 15 mg pieces. One 15 mg piece was placed in to each test tube. The tubes were then placed on a shaker (New Brunswick Scientific Company, USA) at a speed of 150rpm. The tubes were observed for polymer dissolution at 5-minute intervals for a total time period of 15 minutes (Table 5.1). Similarly, a film of poly(lysine dodecandiamide) was prepared by first using a 10 ml volume of 0.1 M NaOH to dissolve 200 mg of poly(lysine dodecandiamide). As before, the polymer solution was transferred to a flat-bottomed glass vessel with a diameter of 40 mm. The water was completely removed by evaporation in a 30° C incubator leaving a thin polymer film over the entire base of the glass vessel. The dissolving rate of polymer films as a function of pH was determined by first preparing 10 mM HEPES buffer solutions adjusted to pH values 7, 6, 5 and 4. A 5 ml volume of each pH buffer solution was transferred to test tubes in duplicate. The prepared polymer films were dissected using a scalpel to obtain 15 mg pieces. One 15 mg piece was placed in to each test tube. The tubes were then placed on a shaker (New Brunswick Scientific Company, USA) at a speed of 150

rpm. The tubes were observed for polymer dissolution at minute intervals for 15 minutes (Table 5.2).

5.1.1.2: Using organic solvent to dissolve polymer.

Coating Azocoll with a polymer film by evaporation of water took a period of 3 days. This extended time period allowed leaching of azo dye from Azocoll. Therefore, an alternative method was sought. It was attempted to form polymer films using 300 mg of polymer using the methods described above but with polymer dispersed in organic solvent instead of water. Organic solvents including THF, diethyl ether and dichloromethane were used, but none resulted in an even coverage of the beaker on evaporation, since the solvents dispersed rather than dissolved polymer. Therefore, it was decided to try and coat Azocoll with polymer by dispersing polymer and Azocoll in an organic solvent, then remove the organic solvent by means of rotary evaporation. Thus, leaving a polymer coating over the surface of the Azocoll. The dissolving time for polymer films was previously determined in different pH buffers. However, the dissolving time for a polymer coatings prepared by rotary evaporation was not known, so the following procedures were performed. That is, 7.0 g of *N,N*-diethylamine modified GMP was transferred to a 100 ml round-bottomed glass flask with 70 ml of dichloromethane. The flask was placed on a rotary evaporator until complete removal of organic solvent had been achieved. In sextuplet, 0.05 g of polymer was transferred from the flask to test tubes. A 5 ml volume of pH 7 adjusted TRIS was transferred to three of the test tubes and to the remaining three, a 5 ml volume of pH 9 adjusted TRIS was transferred giving an overall polymer concentration of 0.01 g ml^{-1}. The tubes were then placed on a shaker at a speed of 150 rpm. The tubes were observed for polymer dissolution at minute intervals for 15 minutes, then at 5-minute intervals for a further 45 minutes. The obtained results are displayed in Table 5.6. The latter procedures were also repeated for 0.1 g, 0.2 g, 0.3 g and 0.4 g of polymer.

5.1.2: Coating Azocoll with polymer.

5.1.2.1: Time course of azo dye release from Azocoll.

Azocoll is an insoluble ground collagen with a bright-red dye attached. It has been extensively used for the assay of proteolytic enzymes (Chavira *et al.* 1984). Using these principles it was decided to coat Azocoll with polymer. Polymer coated Azocoll would be stirred in different pH buffers. Following addition of the proteolytic enzyme subtilisin to each buffer solution, azo release would be measured. It was thought a high azo release would indicate proteolysis of exposed Azocoll as a result of removal of polymer coating at specific pH values.

Firstly, the time required giving a sufficiently measurable dye release from Azocoll when incubated with subtilisin needed to be determined. This was achieved by weighing out ten 15 mg pieces of Azocoll. The weighed Azocoll was then rinsed with distilled water using a Whatman filter paper number 1 to retain the solid. Rinsed Azocoll having a dry weight of 15 mg was then re-suspended with 4 ml of pH 9 buffer stabilised at a temperature of 20° C and transferred to test tubes. A 10 ml volume of pH 9 HEPES buffer was used to dissolve 25 mg of subtilisin. 2 ml volumes of this solution were then transferred to four of the test tubes containing Azocoll. As a control to show any azo release in the absence of subtilisin, 2 ml volumes of pH 9 HEPES buffer were transferred to the remaining four tubes. All tubes were incubated at 20° C. After 5 minutes one test-tube with and one test tube without subtilisin was filtered using Whatman filter paper number 1. This was also performed after 15, 30, 60 and 120 minutes. The filtrate from each tube was retained and the absorbency measured immediately using a wavelength of 516 nm. The described procedure was repeated in triplicate and the mean values determined (Table 5.3).

5.1.2.2: Rate of dissolution of calcein from solid polymer when stirred in different pH buffers.

It was thought that during the polymer coating process azo dye leaches from the Azocoll in to the polymer solution. Therefore, giving the polymer coating a purple

colouration due to Azo dye. Thus, when polymer coated Azocoll is stirred in pH 7 buffer the polymer dissolves in to the pH 7 buffer solution. However, when polymer coated Azocoll is stirred in to pH 9 buffer solution polymer does not dissolve, therefore, the dye does not dissolve in the buffer solution. To demonstrate this was happening known amounts of calcein were dissolved in to polymer solutions. These solutions were used to produce polymer films, which were then stirred in to pH 7 and 9 buffers and calcein release measured. This was performed by first preparing a 20 mM calcein solution by first dissolving solid calcein in a minimal amount of 0.1 M NaOH, adjusting to pH 7 with 0.1 M HCl, then making up to a final volume of 10 ml with distilled water. In triplicate, 1 ml volumes of this solution were transferred to glass beakers with a base of 40 mm and containing 9 ml of distilled water. 300 mg of *N,N*-diethylamine modified GMP was then transferred to each beaker and stirred until completely dissolved. The glass beakers and their contents were then placed in a 30° C incubator to allow the evaporation of water and leave a thin polymer film over the entire base of the glass vessel. Using the entire polymer film from each beaker, equal quantities were transferred to two beakers, which were subsequently made up to 100 ml with either pH 7 or pH 9 adjusted (TRIS[hydroxymethyl] aminomethane). Thus, if calcein were completely dissolved, it would be at a concentration of 0.1 mM. The beakers were placed on a shaker (New Brunswick Scientific Company, USA) and stirred at a speed of 150 rpm for 1 minute. The contents of each beaker were then filtered through a Whatman filter paper number 1 and 200 µl volumes of the filtrate removed to wells of a black micro titre plate. The fluorescence of these wells were determined using a Spectra Max Gemini fluorescent plate reader set at excitation and emission wavelengths of 490 nm and 590 nm, respectively, with a 'cut off' filter introduced at 515 nm. The latter procedures were then repeated for 5, 10, 15, 30, 45 and 60 minutes of stirring. Since calcein fluorescence is affected by pH, control solutions of 0.1 mM calcein were prepared in both pH 7 and pH 9 adjusted TRIS buffer. The fluorescence of these solutions was determined by transferring 200 µl volumes in quadruplicate to wells of a black micro titre plate and read using the fluorescent plate reader. These readings were assumed to be maximal for 0.1 mM calcein solutions at the respective pH value. The mean fluorescence value of released calcein from polymer at each stirring time in pH 7 and pH 9 buffer was divided by the fluorescence of pH 7 and pH 9 adjusted 0.1 mM calcein solutions, respectively. These

values were multiplied by 100 to express released calcein as a percentage of total release (Figure 5.1).

5.1.2.3: Determination of whether subtilisin degrades *N,N*-diethylamine modified glycidyl methacrylate polymer.

To check subtilisin does not degrade polymer the following procedures were performed. That is, a film of *N,N*-diethylamine modified GMP, was prepared as described previously. The polymer film was dissected in to twelve 15 mg pieces, which were transferred to glass vials. In triplicate, 5 ml volumes of pH 9 HEPES buffer, pH 7 HEPES buffer, pH 9 HEPES buffer plus 5 mg of subtilisin and pH 7 HEPES buffer plus 5 mg of subtilisin were transferred to vials. The vials were then placed on a shaker (New Brunswick Scientific Company, USA) at a speed of 150 rpm. The vials were observed for polymer dissolution at 5-minute intervals for a total time period of 20 minutes. The obtained results are displayed in Table 5.5.

5.1.2.4: Released azo dye from Azocoll coated by a polymer film.

It was attempted to determine the release of azo dye from Azocoll coated with polymer when the polymer coated Azocoll was stirred in pH 7 and pH 9 buffers. Azocoll first needed to be coated with polymer. This was achieved in two ways 1) by placing Azocoll in to an aqueous polymer solution, then evaporating off the water leaving a polymer coating over the Azocoll; 2) by placing Azocoll in to an organic solvent with dissolved polymer, then evaporating off the organic solvent leaving a polymer coating over Azocoll. Release of azo dye from polymer coated Azocoll was then determined. The two methods will be described in turn.

5.1.2.5: Release of azo dye from Azocoll coated by polymer achieved by placing Azocoll in to an aqueous polymer solution, then evaporating off the water leaving a polymer coating over the Azocoll.

To coat Azocoll with polymer 15 mg of Azocoll was rinsed with distilled water using a Whatman filter paper number 1 to retain the Azocoll. Rinsed Azocoll was re-suspended using 15 ml of distilled water containing 300 mg of dissolved *N,N*-

diethylamine modified GMP. This solution was then transferred to a vessel with a 40 mm diameter base, which had parafilm evenly spread over the bottom. The solid Azocoll sinks to bottom of vessel, thus when the vessel is placed in a 30° C incubator to allow evaporation of water, a polymer film forms over the solid Azocoll giving a polymer coated Azocoll film. To obtain, five further films of polymer coated Azocoll, the latter procedure was repeated five more times. The parafilm was peeled away from polymer films when all water was evaporated.

To determine the release of Azocoll due to dissolution of polymer off Azocoll when stirred in pH 7 buffer the following procedures were performed. In triplicate, the polymer coated Azocoll films were placed in to a shallow vessel containing 10 ml volumes of pH 7 adjusted buffer. These vessels were agitated on a shaker (New Brunswick Scientific Company, USA) at a speed of 150 rpm for 10 minutes to allow dissolution of polymer. A time of 10 minutes was used since this was shown earlier to be sufficient for polymer dissolution (Table 5.1). The contents of the vessel were filtered using a Whatman filter paper number 1. The filtrate was collected and retained (termed stirring solution). The reasoning for measuring absorbency of this stirring solution will be explained in the Section 5.1.2.6. The retained films were re-suspended with 6 ml of pH 9 buffer solution containing 5 mg of subtilisin and incubated for 15 minutes. A pH of 9 is used since this will prevent further dissolution of polymer off Azocoll since polymer does not dissolve at pH 9. Also, at this stage any Azocoll which is not coated with polymer will be exposed to subtilisin. Therefore, due to proteolysis azo dye will be released in to the surrounding medium. After 15 minutes incubation time the contents of the latter vessels were filtered through a Whatman filter number 1 and the filtrate containing any released azo dye was collected. A 1.5 ml volume of this filtrate (termed enzyme solution) was transferred to a cuvette and the absorbency read at a wavelength of 516 nm using a pH 9 HEPES buffer as blank (Table 5.4).

To determine the release of azo dye from Azocoll due to dissolution of polymer off Azocoll when stirred in pH 9 buffer the latter procedures were repeated except polymer coated Azocoll films were initially agitated with 10 ml volumes of pH 9 buffer instead of pH 7.

5.1.2.6: Absorbency of pH 7 and pH 9 stirring solutions.

As explained earlier to coat Azocoll with solid polymer Azocoll is placed in a to water with dissolved polymer. The water is then evaporated off leaving a polymer coating over the Azocoll. The time taken for water to completely evaporate is 3 days. During this time some dye leaches from the Azocoll, which gives the remaining solution a purple colouration and therefore, the final polymer coating takes on a purple colouration. Thus, the reasoning behind obtaining the absorbency of the stirring solutions was to show that during the process of coating Azocoll with polymer Azo dye leaches in to the polymer coating. To demonstrate this the pH 7 and pH 9 filtrates were adjusted to pH 7, eliminating differences in absorbency due to pH, then made up to twice their volume to allow enough volume for reading their absorbency. 1.5 ml volumes of these pH adjusted solutions were then transferred to cuvettes and their absorbency read using a wavelength of 516 nm. The obtained readings were multiplied by two to account for the doubling dilution and the results presented in Table 5.4.

5.1.2.7: Release of azo dye from Azocoll coated by polymer by placing Azocoll in to an organic solvent with dissolved polymer, then evaporating off the organic solvent leaving a polymer coating over Azocoll.

In sextuplet, 15 mg of Azocoll was rinsed using distilled water and a Whatman filter paper number 1 to retain the solid. The solid Azocoll was then transferred to round-bottomed glass, which was placed in a sealed container with silica gel until the Azocoll was dry. When dry, solid Azocoll was agitated with 20 ml of dichloromethane until the Azocoll was evenly dispersed. 60 mg of *N,N*-diethylamine modified GMP polymer was transferred to this dispersion and agitated until monodisperse. The flask was then placed on the rotary evaporator and dichloromethane removed under vacuum to allow coating of Azocoll with an equal mass of polymer.

In duplicate, a 1.0 ml volume of pH 7 TRIS buffer and pH 9 TRIS buffer were added to the flasks containing polymer coated Azocoll. Therefore, completely dissolved polymer would give a concentration of 0.06 g ml^{-1}. The flasks were placed on a

shaker (New Brunswick Scientific Company, USA) at 150 rpm for five minutes. The solid was then filtered using a Whatman filter paper number 1 and transferred to 6 ml of pH 9 buffer containing 5 mg of subtilisin and incubated for 15 minutes to allow enzyme degradation of any uncoated Azocoll. This solution was then filtered and the filtrate retained. A 1.5 ml volume of the filtrate was transferred to a cuvette and the absorbency read using a wavelength of 516 nm. The described procedures were repeated but flasks containing polymer coated Azocoll were placed on the shaker for longer time periods of 15, 30, 45 and 60 minutes. The obtained absorbency readings for each are displayed in Table 5.7.

5.1.3: Coating sodium chloride with polymer.

An experiment to determine azo release from polymer coated Azocoll with increasing stirring time in pH 7 and pH 9 buffers, followed by addition of subtilisin has been described. For each stirring time tested, that is 5, 15, 30 and 60 minutes, six, 15 mg amounts of Azocoll coated in polymer were required. Therefore, making it difficult to determine release of azo dye at more frequent stirring times and with polymer coatings at a broader range of concentrations. Therefore, it was decided to use conductivity to determine release of sodium chloride from a polymer coating instead. The conductivity of uncoated sodium chloride could be determined without first filtering, as was required to determine the amount of uncoated Azocoll. Thus, using this method, readings could be obtained at more frequent stirring times using polymer coatings at a broader range of concentrations.

5.1.3.1: Conductivity of buffers used to stir polymer coated sodium chloride.

A buffer with a minimal conductivity was required, so that conductivity does not mask the conductivity of released NaCl from polymer coating. To determine this a conductivity meter (AGB 1000), was calibrated to 1.413 mSiemens/cm, using a 0.01 M KCl in de-ionised water, stabilised to 25° C. The conductivity was then determined for Hydrion pH buffers 7 and 9, reconstituted with de-ionised water and stabilised to 25° C. Similarly, the conductivity of 10 mM TRIS buffer (Sigma) solutions adjusted to pH 7 and 9 using 0.1 M HCl, then made up to their final volume with de-ionised water and stabilised to 25° C was determined. The results are presented in Table 5.8.

5.1.3.2: Detection limits of conductivity meter.

To determine the detection limits of the conductivity meter for sodium chloride the following procedures were performed. That is, 1.7 M NaCl solutions were prepared using 10 ml volumes of de-ionised water and the pH 7 and 9 TRIS buffers. De-ionised water was used for comparison to buffer solutions. These three solutions were then diluted by a factor of ten a total of ten times using de-ionised water or the relevant buffer solution, respectively. The conductivity of each solution was determined using both 20 mS and 200 mS conductivity settings of the conductivity meter. The results were then presented in Table 5.9.

5.1.3.3: Surfactant effect on dissolution of sodium chloride and polymer.

To determine if surfactant has an effect on rate of sodium chloride release from within polymer coating when stirred in pH 7 and pH 9 buffers the following procedures were performed. Firstly, solid anionic surfactant sodium dodecyl sulphate (SDS) (M_W 222.38) (Fluka) was dissolved in 100 ml volumes of both pH 7 and pH 9 TRIS buffers at concentrations of 0.01 M. Then, as described in Section 5.1.3.4.3 sodium chloride was distributed with *N,N*-diethylamine modified GMP at a 1: 4 ratio. Again in sextuplet, 0.25 g of polymer coated NaCl was transferred to test tubes. Three of the test tubes were made up to 5 ml with pH 7 adjusted TRIS buffers with SDS and the remaining three were made up to 5 ml with pH 9 adjusted TRIS buffer, containing SDS. All test tubes were then placed on a shaker (New Brunswick Scientific Company, USA) at a speed of 150 rpm. The conductivity of tubes was measured after 1 minute, 5 minutes, then at 5-minute intervals for a further 55 minutes (Figure 5.6).

5.1.3.4: Coating of sodium chloride with polymer and measuring the release of this sodium chloride from within this polymer coating.

The release of sodium chloride from within polymer coatings when stirred in pH 7 and 9 buffers was determined. This was performed using polymer coated sodium chloride prepared using increasing polymer: sodium chloride ratios. That is, polymer: sodium chloride ratios used were 1:1, 2:1, 4:1 and 8:1 ratio as described in turn below.

5.1.3.4.1: Polymer: sodium chloride ratio of 1:1.

To a round-bottomed glass flask 0.4 g of polymer, 0.4 g of NaCl and 20 ml of dichloromethane were transferred. The flask was placed on a rotary evaporator until complete removal of organic solvent had been achieved, leaving a 1:1 ratio of polymer: NaCl. In sextuplet, 0.1 g of polymer coated NaCl was transferred to test tubes. Three of the test tubes were made up to 5 ml with pH 7 adjusted TRIS buffers and the remaining three were made up to 5 ml with pH 9 adjusted TRIS buffer. Thus, if NaCl completely dissolves the solution would have a 0.17 M NaCl concentration. The test tubes were then placed on a shaker (New Brunswick Scientific Company, USA) at a speed of 150 rpm. The conductivity of tubes was measured after 1 minute, 5 minutes, then at 5-minute intervals for a further 55 minutes (Figure 5.2). Also, in triplicate, solutions of 0.01 g ml^{-1} of polymer in pH 7 and pH 9 adjusted TRIS buffer were prepared and their conductivity determined to check it did not mask the conductivity of NaCl (Table 5.10).

5.1.3.4.2: Polymer: sodium chloride ratio of 2:1.

Similar to above 0.8 g of polymer, 0.4 g of NaCl and 20 ml of dichloromethane was transferred to a round-bottomed glass flask. The flask was the placed on a rotary evaporator until complete removal of organic solvent had been achieved, leaving a 2:1 ratio of polymer: NaCl. In sextuplet, 0.15 g of polymer coated NaCl was transferred to test tubes. Three of the test tubes were made up to 5 ml with pH 7 adjusted TRIS buffers and the remaining three were made up to 5 ml with pH 9 adjusted TRIS buffer. All test tubes were then placed on a shaker (New Brunswick Scientific Company, USA) at a speed of 150 rpm. Again, the conductivity of tubes was measured after 1 minute, 5 minutes, then at 5-minute intervals for a further 55 minutes. The results of which are displayed in Figure 5.3. Also, in triplicate, solutions of 0.02 g ml^{-1} of polymer in pH 7 and pH 9 adjusted TRIS buffer were prepared and their conductivity determined to check it did not mask the conductivity of NaCl (Table 5.10).

5.1.3.4.3: Polymer: sodium chloride ratio of 4:1.

To a round-bottomed glass flask 1.6 g of polymer, 0.4 g of NaCl and 20 ml of dichloromethane was transferred. The flask was placed on a rotary evaporator until complete removal of organic solvent had been achieved, leaving a 4:1 ratio of polymer: NaCl. In sextuplet, 0.25 g of polymer coated NaCl was transferred to test tubes. Three of the test tubes were made up to 5 ml with pH 7 adjusted TRIS buffers and the remaining three were made up to 5 ml with pH 9 adjusted TRIS buffer. All test tubes were then placed on a shaker (New Brunswick Scientific Company, USA) at a speed of 150 rpm. The conductivity of tubes was measured after 1 minute, 5 minutes, then at 5-minute intervals for a further 55 minutes. The results of which are displayed in Figure 5.4. Also, in triplicate, solutions of 0.04 g ml^{-1} of polymer in pH 7 and pH 9 adjusted TRIS buffer were prepared and their conductivity determined to check it did not mask the conductivity of NaCl (Table 5.10).

5.1.3.4.4: Polymer: sodium chloride ratio of 8:1.

To a round-bottomed glass flask 3.2 g of polymer, 0.4 g of NaCl and 40 ml of dichloromethane was transferred. The flask was placed on a rotary evaporator until complete removal of organic solvent had been achieved, leaving a 8:1 ratio of polymer: NaCl. In sextuplet, 0.45 g of polymer coated NaCl was transferred to test tubes. Three of the test tubes were made up to 10 ml with pH 7 adjusted TRIS buffers and the remaining three were made up to 10 ml with pH 9 adjusted TRIS buffer. These tubes were made up to 10 ml rather than 5 ml in the latter experiments, as polymer is close to saturation at this concentration. All test tubes were then placed on a shaker (New Brunswick Scientific Company, USA) at a speed of 150 rpm. The conductivity of tubes was measured after 1 minute, 5 minutes, then at 5-minute intervals for a further 55 minutes. The conductivity readings obtained were multiplied by two to account for the doubling dilution and then displayed in Figure 5.5. The conductivity of polymer in pH 7 and pH 9 adjusted TRIS buffer did not need to be determined as it was the same concentration as polymer alone in the latter experiment, that is, 0.04 g ml^{-1}.

5.2: Results.

The aim of Chapter 5 was to coat solid particles with polymer, then uncoat these polymer coated particles as a function of pH.

To achieve this the dissolution rate of different polymers in pH adjusted buffer solutions needed to be determined. Therefore, polymer films were prepared by dissolving polymer in water then evaporating off the water. These polymer films were stirred in pH adjusted buffers and the time taken for polymer to dissolve measured. The dissolution times for *N,N*-diethylamine modified glycidyl methacrylate films are presented in Table 5.1. The dissolution times for poly(lysine dodecandiamide) films are presented in Table 5.2.

The dissolution time of *N,N*-diethylamine modified glycidyl methacrylate in different pH buffers was also determined when *N,N*-diethylamine modified glycidyl methacrylate had been dissolved in organic solvent and the solvent was removed by evaporation. These dissolution times are presented in Table 5.6.

A method was required to quantify the uncoating of polymer coated solids. Azocoll was primarily chosen to achieve this since it is an insoluble ground collagen with a bright red dye attached. Such that, when Azocoll is placed in a solution containing the proteolytic enzyme, subtilisin, Azocoll is degraded and dye is released in to the surrounding solution. It was thought that when polymer coated Azocoll is placed in to a solution containing subtilisin, enzyme degradation would not occur since the polymer coating would protect Azocoll. Whereas, Azocoll with its polymer coating removed would be exposed to subtilisin and degradation would occur. The time required to cause proteolysis of Azocoll in the presence and absence of subtilisin was determined and is presented in Table 5.3.

To coat Azocoll with polymer, Azocoll was placed in to an aqueous polymer solution. The water was evaporated to leave a polymer coating on Azocoll. During this procedure some dye leached from the Azocoll in to the polymer solution. This was illustrated using the results shown in Table 5.4 and Figure 5.1. An experiment was

also performed to check subtilisin did not degrade polymer. These results are presented in Table 5.5.

Coating Azocoll with polymer by placing Azocoll in an aqueous polymer solution and evaporating the water caused leaching of dye in to the polymer solution. Therefore, an alternative coating method was sought. That is, Azocoll was coated with polymer by placing Azocoll in an organic solvent with dissolved polymer. The organic solvent was removed to leave a polymer coating over the surface of Azocoll. Polymer coated Azocoll prepared using this method was stirred in different pH buffers and the release of dye from Azocoll was determined. These results are presented in Table 5.7.

Sodium chloride was used as an alternative solid to quantify removal of polymer coating from polymer coated solids when stirred in different pH buffers. The release of sodium chloride from within a polymer coating was quantified using conductivity measurements. Again, some preliminary studies were required. That is, the conductivity of pH adjusted buffer solutions used to stir polymer coated sodium chloride were determined. These readings are presented in Table 5.8. The detection limits of the conductivity meter for sodium chloride was determined by taking conductivity measurements of an increasingly diluted solution of sodium chloride. These results are presented in Table 5.9. To check dissolved polymer would not mask the conductivity readings of released sodium chloride, polymer at a range of concentrations was dissolved in buffer solutions and their conductivity was determined. These results are presented in Table 5.10.

The release of sodium chloride from polymer coatings when stirred in different pH adjusted buffers was finally determined. These results are presented in Figure 5.2 to Figure 5.6.

Table 5.1: Dissolving time of *N,N*-diethylamine modified glycidyl methacrylate polymer films at different pH values.

pH value of 10 mM HEPES buffer solution	Time taken for polymer Film to completely dissolve (minutes)
6	5
7	5
8	>15
9	>15
10	>15

Table 5.2: Dissolving time of poly(lysine dodecandiamide) films at different pH values.

pH value of 10 mM HEPES buffer solution	Time taken for polymer film to completely dissolve (minutes)
7	11
6	>15
5	>15
4	>15

Table 5.3: Mean absorbency readings for the filtrate of Azocoll, which has been incubated with and without subtilisin for an increasing time period. Standard error of means in brackets.

Subtilisin added (mg)	Time subtilisin incubated with Azocoll (minutes)				
	5	15	30	60	120
0	0	0	0	0	0
5 mg	0.42 (0.00)	0.63 (0.00)	0.64 (0.01)	0.78 (0.01)	0.78 (0.02)

Table 5.4: Absorbency readings using a wavelength of 516 nm, of azo dye released in to both the stirring solution, indicative of free dye and solutions containing enzyme, indicative of available Azocoll for proteolysis.

pH of HEPES buffer polymer coated Azocoll transferred	Mean absorbency of stirring solution (516 nm) (SE of mean in brackets)	Mean absorbency of enzyme solution (516 nm) (SE of mean in brackets)
9	0.005 (0.001)	0.463 (0.019)
7	0.158 (0.007)	0.379 (0.034)

Figure 5.1: Fluorescence of calcein released from solid polymer when stirred in pH 7 ■ and pH 9 □ TRIS buffers, expressed as a percentage of a 0.1 mM calcein solution buffered in pH 7 and pH 9 TRIS, respectively.

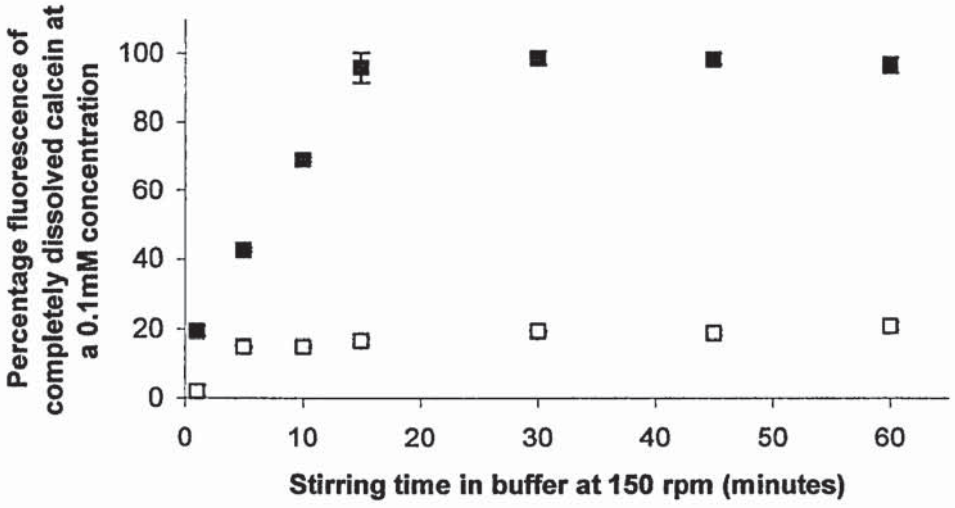


Table 5.5: Dissolving time of *N,N*-diethylamine modified glycidyl methacrylate polymer films at different pH values in the presence and absence of subtilisin.

	Time to dissolve *N,N*-diethylamine polymer film in HEPES buffer:	
	pH 7	**pH 9**
Subtilisin present	5 minutes	>20 minutes
Subtilisin absent	5 minutes	>20 minutes

Table 5.6: Dissolving time of *N,N*-diethylamine modified glycidyl methacrylate polymer.

Polymer concentration (g ml^{-1})	Dissolving time (minutes) in:	
	pH 7 adjusted TRIS	* pH 9 adjusted TRIS
0.01	1	7
0.02	8	12
0.04	10	40
0.06	14	50
0.08	> 60	> 60

* Polymer forms a precipitate at pH 9, therefore the stated dissolving time is time taken for precipitate to become mono-disperse.

Table 5.7: Mean absorbency readings of azo dye release from Azocoll coated polymer when stirred for an increasing time period in pH 7 and pH 9 buffer, with standard errors shown in brackets.

Stirring time of polymer coated Azocoll (minutes)	Mean absorbency of released azo dye by stirring in TRIS adjusted to pH:	
	7	9
5	0.59 (0.01)	0.48 (0.01)
15	0.64 (0.015)	0.56 (0.01)
30	0.63 (0.01)	0.66 (0.005)
60	0.63 (0.025)	0.66 (0.02)

Table 5.8: Conductivity of buffers.

Buffer	pH	Conductivity (mSiemens)
Hydrion	7	6.8
Hydrion	10	11.2
TRIS	7	1.3
TRIS	10	0.112

Table 5.9: Conductivity of sodium chloride at a range of concentrations.

Molar concentration of NaCl	Mean conductivity in:					
	TRIS pH 7 using:		TRIS pH 9 using:		De-ionised water using:	
	200 mS range	20 mS range	200 mS range	20 mS range	200 mS range	20 mS range
1.7 (100 g/L)	138.7	O/R	140.9	O/R	146	O/R
0.17	21	12.2	17.4	12	14	13.82
$1.7x\ 10^{-2}$	3.5	3.17	1.9	1.9	2	2.02
$1.7x\ 10^{-3}$	1.7	1.58	-	0.3	-	0.16
$1.7x\ 10^{-4}$	1.4	1.37			-	-
$1.7x\ 10^{-5}$	1.4	1.37			-	-
$1.7x\ 10^{-6}$	1.4	1.36			-	-
$1.7x\ 10^{-7}$	1.4	1.36			-	-
$1.7x\ 10^{-8}$	1.4	1.36			-	-
$1.7x\ 10^{-9}$	1.3	1.4			-	-
$1.7x\ 10^{-10}$	1.3	1.34			-	-
0	1.3	1.3	-	0.112	0	0

Note (O/R) and (-), depicts over range and not detectable respectively.

Table 5.10: Mean conductivity readings of polymer dissolved in TRIS buffers, read using the 20 mS/cm range.

Polymer Concentration g ml^{-1}	Diluent:	
	PH 7 adjusted TRIS	PH 9 adjusted TRIS
0.01	1.66	1.3
0.02	1.67	1.4
0.04	1.7	1.4

Figure 5.2: Conductivity of a 1:1 ratio of *N,N*-diethylamine modified glycidyl methacrylate polymer to encapsulated sodium chloride in pH 7 ■ and pH 9 □ adjusted TRIS buffers with increasing stirring time.

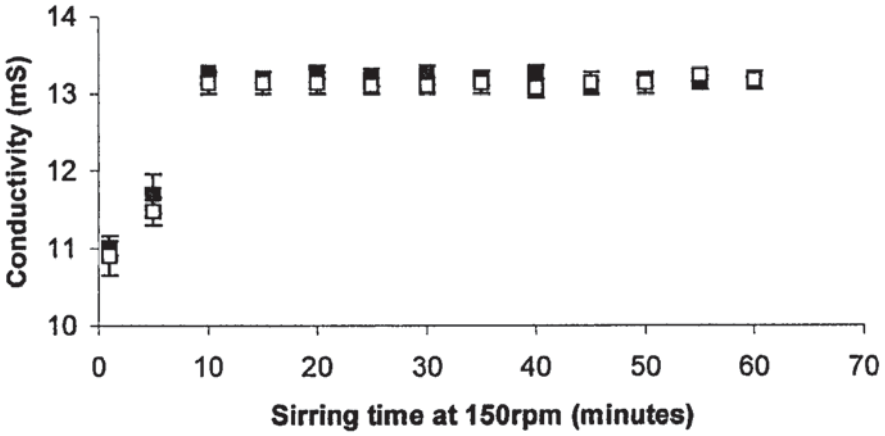


Figure 5.3: Conductivity of a 2:1 ratio of *N,N*-diethylamine modified glycidyl methacrylate polymer to encapsulated sodium chloride in pH 7 ■and pH 9 □ adjusted TRIS buffers with increasing stirring time.

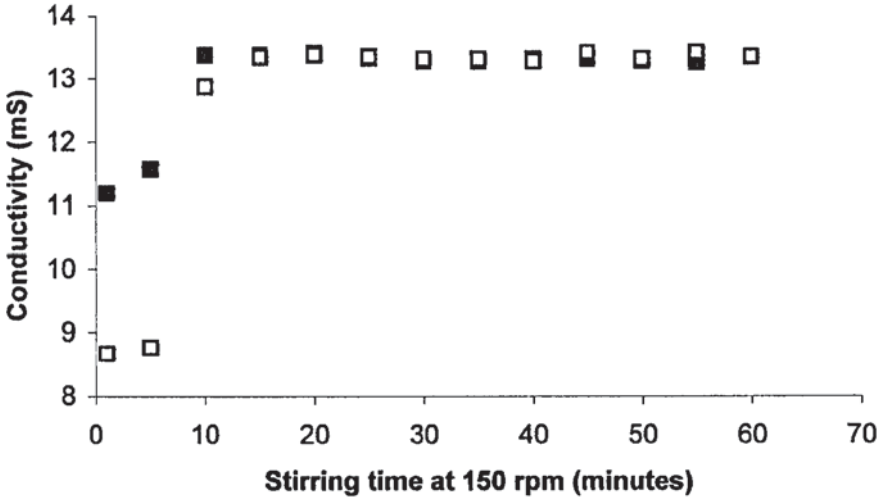


Figure 5.4: Conductivity of a 4:1 ratio of *N,N*-diethylamine modified glycidyl methacrylate polymer to encapsulated sodium chloride in pH 7 ■ and pH 9 □ adjusted TRIS buffers with increasing stirring time.

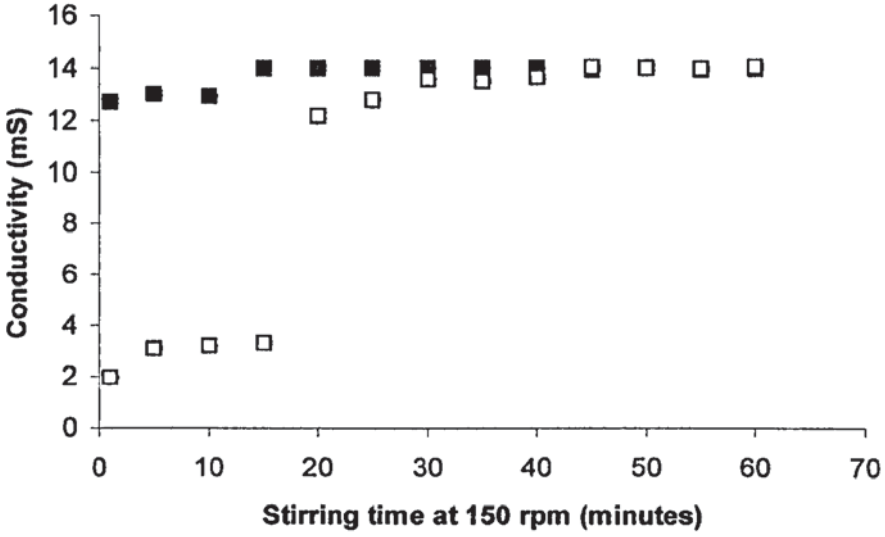


Figure 5.5: Conductivity of a 8:1 ratio of *N,N*-diethylamine modified glycidyl methacrylate polymer to encapsulated sodium chloride in pH 7 ■and pH 9 □ adjusted TRIS buffers with increasing stirring time.

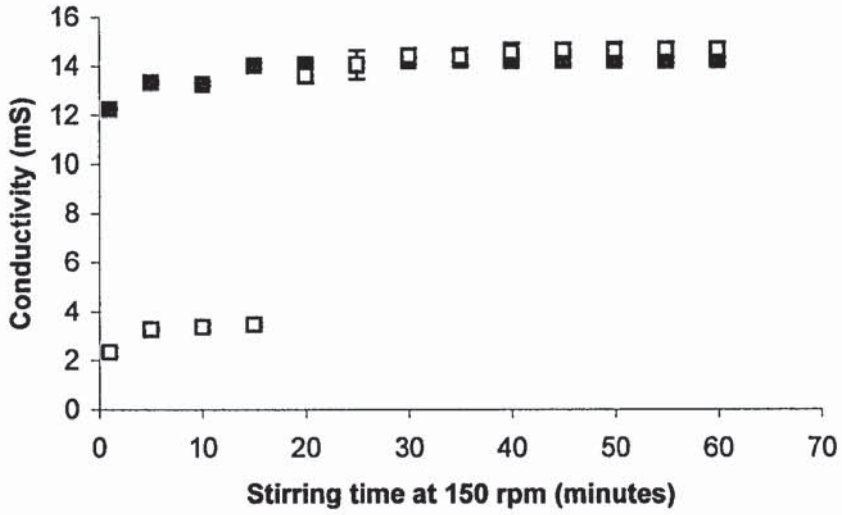
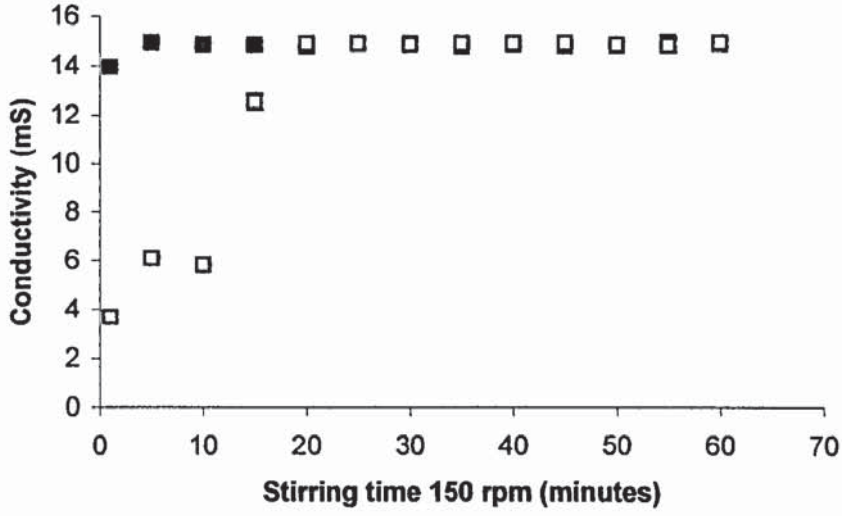


Figure 5.6: Conductivity of a 4:1 ratio of *N,N*-diethylamine modified glycidyl methacrylate polymer to encapsulated sodium chloride in pH 7 ■and pH 9 □ adjusted TRIS buffers containing 0.01 M SDS, with increasing stirring time.



5.3: Discussion.

5.3.1: Determination of dissolution rate of polymer films over a range of pH values.

5.3.1.1: Using water to dissolve polymer.

Table 5.1 shows the time for *N,N*-diethylamine modified GMP to dissolve when stirred in different pH buffer solutions. It can be seen that when *N,N*-diethylamine modified GMP films are stirred in pH 6 and pH 7 buffer solutions dissolution occurs within 5 minutes. Whereas, when the same polymer films are stirred in pH 8, 9 and 10 buffer solutions, dissolution is not seen even after 15 minutes of stirring. Therefore, such properties indicate if these polymers are used coat solid particles, then the solid will be rapidly uncoated when stirred in a pH 6 or 7 buffer but not uncoated when stirred in pH 8, 9 or 10 buffers.

Similar properties are exhibited by poly(lysine dodecandiamide). That is, if Table 5.2 is observed it can be seen that poly(lysine dodecandiamide) dissolves within 11 minutes when stirred in pH 7 buffer but when stirred in buffers with a pH of 6, 5 and 4 dissolution is not seen within 15 minutes of stirring.

5.3.1.2: Using organic solvent to dissolve polymer.

If Table 5.6 is observed it can be seen that the time taken for *N,N*-diethylamine modified GMP to dissolve when stirred in pH 7 buffer is dependent on the polymer concentration. That is, time taken for polymer to dissolve increases with increasing polymer concentration. Although, *N,N*-diethylamine modified GMP is not dissolved when stirred in pH 9 buffer is does become monodispersed. Again the time taken for this to occur depends on the polymer concentration. That is, the time taken for polymer to become monodisperse increases with increasing polymer concentration.

It can be seen that a polymer at a concentration of 0.08 g ml^{-1} does not dissolve or become monodispese when stirred in pH 7 or pH 9 buffers, respectively, after stirring

for 60 minutes, indicating the solution is saturated (Table 5.6). A polymer concentration of 0.06 g ml^{-1} dissolves after 14 minutes when stirred in pH 7 buffer and becomes monodisperse after 50 minutes when stirred in pH 9 buffer. Therefore, indicating polymer used to coat a solid particle must not have a final concentration greater than 0.06 g ml^{-1}, if release is to be achieved.

If Table 5.6 is observed the time taken for polymer to dissolve or become monodisperse when stirred in pH 7 or pH 9 buffers can be compared. It can be observed that at all polymer concentrations, other than 0.08 g ml^{-1}, the time taken for polymer to become monodisperse in pH 9 buffer is greater than the time taken for polymer to dissolve in pH 7 buffer. Therefore, indicating these properties can be used for pH-responsive control. That is, if a solid particle is coated with polymer and stirred in pH 7 and pH 9 buffers, the time taken for the solid particle to be uncoated would be less when stirred in pH 7 buffer compared to when stirred in pH 9 buffer.

5.3.2: Coating Azocoll with polymer.

5.3.2.1: Time course of azo dye release from Azocoll.

If Table 5.3 is observed it can be observed that without the addition of subtilisin, proteolysis of Azocoll does not occur. However, on addition of subtilisin to the suspension containing solid Azocoll, proteolysis of Azocoll is evident after 5 minutes Most proteolysis occurs within 15 minutes and it was thought maximal proteolysis occurred within 1 hour since azo release was the same when measured after 1 hour and after 2 hours.

The proteolysis and thus release of azo dye from Azocoll coated with polymer then stirred in pH 7 buffer needs to be compared with that from Azocoll coated with polymer then stirred in pH 9 buffer. Such that, a high azo release would be thought to be indicative of polymer uncoating, since it allows subtilisin and Azocoll to interact and proteolysis of Azocoll to occur. From the results shown in Table 5.3 it is thought 15 minutes is a sufficient incubation time to incubate subtilisin and polymer coated Azocoll that has been stirred in either pH 7 or pH 9 buffer and compare azo release.

5.3.2.2: Rate of dissolution of calcein from solid polymer when stirred in different pH buffers.

By observing Figure 5.1 it can be seen that calcein completely dissolves out of polymer within 15 minutes when stirred in pH 7 buffer. Whereas, when stirred in pH 9 buffer calcein has still not completely dissolved out of polymer after 60 minutes stirring. Thus, indicating when polymer is stirred in a pH 9 solution, the rate the polymer dissolves is slower than if stirred in a pH 7 solution. Therefore, these results conclude what was indicated earlier. That is, the free azo dye in polymer coating dissolved when stirred in pH 7 buffer but did not when stirred in pH 9 buffer.

5.3.2.3: Determination of whether subtilisin degrades *N,N*-diethylamine modified glycidyl methacrylate polymer.

The results presented in Table 5.5 show that subtilisin does not affect dissolution rate of *N,N*-diethylamine GMP when stirred in pH 7 and 9 buffers. That is, when stirred in pH 7 buffer dissolution rate is 5 minutes in both the presence and absence of subtilisin. Similarly, after stirring for 20 minutes in pH 9 buffer, polymer still does not dissolve in either the presence or absence of subtilisin.

5.3.2.4: Released azo dye from polymer coated Azocoll prepared by placing Azocoll in an aqueous polymer solution, then evaporating off the water leaving a polymer coating over the Azocoll.

It is thought that if polymer coated Azocoll is stirred in pH 7 buffer the polymer would be dissolved off the Azocoll. It was thought during the polymer coating process some Azo dye leaches in to the polymer solution. This is further indicated by observing Table 5.4, since the absorbency for the stirring solution is relatively high. Thus, as polymer coating Azocoll dissolves off Azocoll in to solution so does the dye that has leached in to the polymer coating. As expected polymer and thus leached dye showed low levels of dissolution when stirred in pH 9 buffer since polymer does not dissolve readily at this pH. Therefore explaining why the absorbency for the termed pH 9 stirring solution is close to zero. The leaching effect of calcein dye from solid

polymer when stirred in pH 7 and pH 9 buffer was investigated to confirm these results as explained in the Section 5.1.3.4.3.

It was expected that when polymer coated Azocoll is stirred in pH 9 buffer, then transferred to fresh pH 9 buffer containing subtilisin, the subtilisin would not digest Azocoll since it is protected by a polymer coating. However, a high absorbency for the termed pH 9 enzyme solution is observed. There are two main reasons that could explain this result. Firstly, it was thought subtilisin might be degrading polymer, therefore allowing Azocoll to be exposed to subtilisin and proteolysis. This was assessed and found not be the cause in a separate study described in Section 5.3.2.3. Alternatively, and thought to be the most likely reason is the dissolved subtilisin permeates either through the polymer coating or breaks in the polymer coating, thus, allowing Azocoll proteolysis.

It can be observed that the absorbency of the pH 7 enzyme solution is lower than that of pH 9 enzyme solution. This was not expected since it would be thought polymer coating Azocoll would dissolve away from Azocoll when stirred in pH 7 buffer but not dissolve away from Azocoll when stirred in pH 9 buffer. Therefore, uncoated Azocoll would be readily available to subtilisin and thus proteolysis, showed as a high absorbency reading. The low absorbency reading of the pH 7 enzyme solution can be explained by observing the absorbency readings of the stirring solutions. That is, leached dye is released in to the pH 7 stirring solution, whereas, it is not released in to the pH 9 stirring solution and therefore, more azo dye is available for release later in to the enzyme solution.

5.3.2.5: Released azo dye from polymer coated Azocoll prepared by placing Azocoll in an organic solvent with dissolved polymer, then evaporating off the solvent leaving a polymer coating over the Azocoll.

The results in Table 5.7, show the release of azo dye from polymer coated Azocoll is reduced by stirring in a pH 9 buffer compared to when stirred in a pH 7 buffer. That is, lower Azo release is observed when stirred in pH 9 buffer for 5 minutes compared to when stirred in pH 7 buffer for 5 minutes. Similar results are also seen when polymer coated Azocoll is stirred in pH 7 and pH 9 buffers for 15 minutes. However,

the release when stirred in pH 9 buffer is still relatively high, indicating proteolysis of Azocoll by subtilisin is occurring. Thus, the polymer coating does not give Azocoll complete protection from subtilisin. The release of azo dye from polymer coated Azocoll is similar when stirred in both pH 7 and pH 9 buffers for 30 and 60 minutes. This suggests, although polymer may not be dissolved in to solution, the polymer coating is removed from Azocoll due to stirring for these extended periods of time.

5.3.3: Coating sodium chloride with polymer.

5.3.3.1: Conductivity of buffers used to stir polymer coated sodium chloride.

From Table 5.8 it can be seen that pH 7 and pH 9 Hydrion buffers have relatively high conductivity readings when compared to pH 7 and pH 9 adjusted TRIS buffers. As already mentioned a buffer with minimal conductivity was required so that it does mask conductivity readings of sodium chloride. Thus, from Table 5.7 it can be seen that TRIS buffers are more suitable since they have lower conductivity readings than Hydrion buffers.

5.3.3.2: Detection limits of conductivity meter.

Table 5.9 shows the conductivity readings for sodium chloride when dissolved in water are slightly lower than those for sodium chloride dissolved in buffer solutions. These results are as expected since the higher conductivity of buffer solutions can be explained by the fact that buffer solutions have their own salts which dissolve in to solution. It can also be observed the detection limits of the conductivity meter for sodium chloride when using a 200 mS setting are molar concentrations between 1.7 M and 1.7×10^{-2} M. When using a 20 mS setting, the detection range are molar concentrations between 0.17 M and 1.7×10^{-3} M. A 0.17 M concentration of NaCl is within the detection limits of the conductivity meter for both settings, that is 200 mS and 20 mS. Also this concentration is not masked by the conductivity of buffer solutions. Therefore, it was thought a 0.17 M concentration of NaCl coated with polymer and transferred to buffer solutions would be useful for release studies.

5.3.3.3: Effect of surfactant on release of sodium chloride from within polymer coating.

By observing the conductivity readings of sodium chloride and polymer in pH 7 and pH 9 TRIS with SDS (Figure 5.6), it can be seen that the same general trend is observed as was for conductivity readings for a 4:1 polymer: sodium chloride ratio without SDS. However, the rate of sodium chloride dissolution in the presence of SDS is increased. That is, in pH 7 and pH 9 TRIS with SDS stirring time for maximal dissolution of sodium chloride is 5 and 20 minutes respectively, compared to 15 and 30 minutes without SDS.

5.3.3.4: Coating sodium chloride and measuring release of this sodium chloride from within the polymer coating.

The conductivity readings of buffer solutions and buffer solutions with dissolved polymer were determined to check they were not so high, that they would mask the effect of sodium chloride released from within a polymer coating. The conductivity readings of pH 7 and pH 9 adjusted TRIS[hydroxymethyl] aminomethane) buffer (Sigma, UK) alone, were 1.3 and 0.112, respectively (Table 5.10). Therefore, these readings are low enough so as not to mask the release of sodium chloride. The conductivity reading of pH 7 TRIS buffer when *N,N*-diethylamine modified GMP is dissolved at concentrations of 0.01, 0.02 and 0.04 g ml^{-1} increases the conductivity of pH 7 TRIS buffer by 0.36, 0.37 and 0.4 mS, respectively. Therefore, although the values are increased they still are low enough not to mask the conductivity values of released sodium chloride. For example, the conductivity of a 0.17 M solution of sodium chloride in pH 7 buffer is 21 mS. The conductivity reading of pH 9 TRIS buffer when *N,N*-diethylamine modified GMP is dissolved at a concentration of 0.01 g ml^{-1} is increased by 1.188 mS, while its conductivity is increased by 1.288 mS, when polymer is dissolved at concentrations of 0.02 g ml^{-1} and 0.04 g ml^{-1}. Thus, a greater increase in conductivity is seen when polymer is dissolved in to pH 9 TRIS buffer compared to when dissolved in to pH 7 TRIS buffer. However, the conductivity of pH 7 TRIS buffer is higher prior to dissolving polymer. Therefore, as a consequence of dissolving polymer in pH 9 TRIS, the conductivity increases to values, which are similar to those of pH 7 TRIS buffer with dissolved polymer.

Therefore, any differences between conductivity of polymer coated sodium chloride placed in pH 7 and pH 9 TRIS buffers, discussed below, is a consequence of dissolved sodium chloride, rather than conductivity differences between pH 7 and pH 9 TRIS buffers. Since the conductivity readings of pH 9 TRIS buffer with dissolved polymer are close to those for pH 7 TRIS buffer, they again would not mask the conductivity of a 0.17 M solution of sodium chloride.

It was thought that the sodium chloride would not dissolve in to solution or the rate that it dissolves would be slower when polymer coated sodium chloride is stirred in pH 9 buffer compared to when stirred in pH 7 buffer. This was thought as polymer does not dissolve in pH 9 buffer but does in pH 7. Therefore, at pH 9 sodium chloride would be protected by a polymer coating, whereas at pH 7 this coating would not be present. However, if Figure 5.2 is observed it can be seen that sodium chloride coated in an equal mass of polymer is completely dissolved after 9 minutes when stirred in both pH 7 and pH 9 adjusted TRIS buffers. The rate sodium chloride is dissolved is similar in both buffers, since conductivity readings are similar for both buffers at each time interval tested. It is thought this result could be a consequence of polymer coating being removed from the sodium chloride, buffer permeating through breaks in the polymer coating or polymer coating not being thick enough to prevent sodium chloride being dissolved. If the result is due to the latter then it is thought dissolution of sodium chloride from within a thicker polymer coating would be slowed when stirred in both pH 7 compared to dissolution from within thinner coating. It is also thought when stirred in pH 9 buffer, the rate sodium chloride dissolves from within a thicker polymer coating would be prevented or at least slower. The latter can be seen if Figures 5.3 to 5.5 are observed. That is, sodium chloride coated with twice the mass of polymer and stirred in pH 7 buffer (Figure 5.3) has similar conductivity readings at each time interval tested to sodium chloride coated with an equal mass of polymer (Figure 5.2) and stirred in pH 7 buffer, with complete dissolution of sodium chloride observed after 10 minutes stirring. Sodium chloride coated in twice the mass of polymer and stirred in pH 9 buffer is not completely dissolved after 10 minutes (Figure 5.3), however it is after 15 minutes. The conductivity readings after 1 and 5 minutes stirring are lower for sodium chloride coated in twice the mass of polymer compared to sodium chloride coated in an equal mass of polymer. Therefore, indicating an increase in amount of polymer coating, reduces the rate sodium chloride

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It was thought that the sodium chloride would not dissolve in to solution or the rate that it dissolves would be slower when polymer coated sodium chloride is stirred in pH 9 buffer compared to when stirred in pH 7 buffer. This was thought as polymer does not dissolve in pH 9 buffer but does in pH 7. Therefore, at pH 9 sodium chloride would be protected by a polymer coating, whereas at pH 7 this coating would not be present. However, if Figure 5.2 is observed it can be seen that sodium chloride coated in an equal mass of polymer is completely dissolved after 9 minutes when stirred in both pH 7 and pH 9 adjusted TRIS buffers. The rate sodium chloride is dissolved is similar in both buffers, since conductivity readings are similar for both buffers at each time interval tested. It is thought this result could be a consequence of polymer coating being removed from the sodium chloride, buffer permeating through breaks in the polymer coating or polymer coating not being thick enough to prevent sodium chloride being dissolved. If the result is due to the latter then it is thought dissolution of sodium chloride from within a thicker polymer coating would be slowed when stirred in both pH 7 compared to dissolution from within thinner coating. It is also thought when stirred in pH 9 buffer, the rate sodium chloride dissolves from within a thicker polymer coating would be prevented or at least slower. The latter can be seen if Figures 5.3 to 5.5 are observed. That is, sodium chloride coated with twice the mass of polymer and stirred in pH 7 buffer (Figure 5.3) has similar conductivity readings at each time interval tested to sodium chloride coated with an equal mass of polymer (Figure 5.2) and stirred in pH 7 buffer, with complete dissolution of sodium chloride observed after 10 minutes stirring. Sodium chloride coated in twice the mass of polymer and stirred in pH 9 buffer is not completely dissolved after 10 minutes (Figure 5.3), however it is after 15 minutes. The conductivity readings after 1 and 5 minutes stirring are lower for sodium chloride coated in twice the mass of polymer compared to sodium chloride coated in an equal mass of polymer. Therefore, indicating an increase in amount of polymer coating, reduces the rate sodium chloride

is dissolved in pH 9 TRIS buffer. This is further demonstrated by the conductivity readings of sodium chloride coated in four times the mass of polymer, with complete dissolution not observed until polymer coated sodium chloride is stirred for a period of 30 minutes (Figure 5.4). Complete dissolution of sodium chloride coated in four times the mass of polymer takes 15 minutes when stirred in pH 7 buffer compared to 10 minutes when coated in an equal and mass twice the mass of polymer. Therefore, the rate of dissolution is slightly extended. At all time intervals tested up to 15 minutes, the conductivity is much lower for polymer coated sodium chloride stirred in pH 9 buffer compared to polymer coated sodium chloride stirred in pH 7 buffer. Therefore, indicating the rate of sodium chloride release can be extended by stirring in a pH 9 buffer, rather than stirring in pH 7 buffer.

If Figure 5.5 is observed it can be seen dissolution of sodium chloride from polymer at a 1: 8 ratio respectively, gives a similar trend as was observed for a 1: 4 ratio. However, it would be expected that increasing the ratio of polymer to sodium chloride, would extend the dissolution time of sodium chloride. This result can be explained by the fact that the concentration of polymer in buffers in this experiment is similar to that used in the latter since 10 ml and 5 ml volumes of buffer were used respectively.

5.3.3.5: Comparison of the trends shown for pH-responsive uncoating of *N,N*-diethylamine modified GMP and poly(lysine dodecandiamide) coated solid particles with trends shown for pH-responsive disruption of lipid membranes by *N,N*-diethylamine modified GMP and poly(lysine dodecandiamide).

In Chapter 3, it was shown that lysis of erythrocytes can be increased when adjusted to low pH values if erythrocytes are pre-incubated with poly(lysine dodecandiamide) as oppose to buffer. Similar membrane lytic effects were observed in Chapter 4 for this polymer. That is, at low pH values liposomes are solubilised when pre-incubated with poly(lysine dodecandiamide), whereas they are not solubilised when pre-incubated with buffer. Similarly, the release of calcein from liposomes with encapsulated calcein is greater when adjusted to low pH when pre-incubated with poly(lysine dodecandiamide) compared to when pre-incubated with buffer. In this chapter poly(lysine dodecandiamide) was used to coat solid particles. Poly(lysine

dodecandiamide) as a polymer coating is removed from solid particles in a pH-responsive manner. That is, when stirred in pH 7 buffer polymer readily dissolves off solid particles. At this pH it was shown in the earlier chapters that this polymer exerts a minimal membrane lytic effect. When solid particles coated in poly(lysine dodecandiamide) are stirred in buffer solutions adjusted to lower pH values the time to uncoat solid particles is lengthened. In the earlier chapters membrane lytic effects were observed at these pH values for poly(lysine dodecandiamide).

In Chapter 4 liposomes pre-incubated with *N,N*-diethylamine GMP were lysed with greater efficiency when adjusted to pH 9 compared to liposomes pre-incubated with buffer and adjusted to pH 9. In this chapter solid particles coated with *N,N*-diethylamine GMP are not readily uncoated when stirred in pH 9 buffer. However, solid particles coated with *N,N*-diethylamine GMP are readily uncoated when stirred in pH 6 buffer. In Chapter 4, only minimal lysis of liposome is seen when pre-incubated with *N,N*-diethylamine GMP and adjusted to pH 6.

Thus, the results for the latter two polymers indicate that when used to coat solid particles they show uncoating less readily the closer they get to the pH at which they precipitate from solution.

5.4: Conclusions.

The overall aim of this chapter was to coat solid particles with polymer, then uncoat the polymer coated particles as a function of pH. Steps were set out to determine if this could be achieved. These are discussed in turn and the conclusions drawn for each described.

1) Dissolution rate of polymers using polymer films that are prepared by dissolving polymer in water, then evaporating off the water.

N,N-diethylamine GMP was dissolved in water and the water was then evaporated to produce polymer films. These polymer films were stirred in pH 6, 7, 8, 9, and 10 adjusted buffers. Polymer films dissolved in pH 6 and pH 7 buffers within 5 minutes,

whereas, in pH 8, 9 and 10 buffer solutions dissolution was not seen after 15 minutes of stirring.

The reverse properties were seen for poly(lysine dodecandiamide). That is, films of poly(lysine dodecandiamide) dissolved within 11 minutes of stirring in pH 7 buffer but did not dissolve even after 15 minutes in pH 6 , 5 and 4 buffers. It was concluded that these properties could be applied so that solid particles coated with either *N,N*-diethylamine GMP and poly(lysine dodecandiamide) could be released from within the polymer coating as a function of pH.

2) Dissolution rate of polymers using polymer films that are prepared by dissolving polymer in organic solvent, then evaporating off the solvent.

Sodium chloride and *N,N*-diethylamine GMP were pre-dispersed in an organic solvent. On evaporation of the organic solvent sodium chloride was surrounded by *N,N*-diethylamine GMP. As expected, when this polymer coated sodium chloride was stirred in the same buffers as described above in Point 1, the same sodium chloride release trends were observed.

3) *Coating Azocoll with polymer.*

A solid was required that could be coated with polymer and then uncoated as a function of pH. Uncoating needed to be quantified. Therefore, the use of solid Azocoll was thought promising as polymer uncoating could be quantified by measuring azo release, which was thought to be indicative of polymer uncoating since proteolysis of Azocoll can occur due to Azocoll and subtilisin interaction.

Therefore, the aim of this set of experiments was to compare 1) azo release from polymer coated Azocoll that was stirred in pH 7 buffer followed by the addition of subtilisin with 2) azo release from polymer coated Azocoll that was stirred in pH 9 buffer followed by addition of subtilisin.

The latter aims did not prove completely successful. This was firstly because azo dye leached in to the polymer coating when Azocoll was coated with polymer by

suspending in an aqueous polymer suspension and evaporating the water to leave a polymer coating over the Azocoll. This made it difficult to compare azo release due to proteolysis of Azocoll that is coated in polymer then stirred in pH 7 or pH 9 buffer. This leaching effect was overcome by suspending Azocoll in polymer that was dissolved in an organic solvent instead of water. The organic solvent was then evaporated to leave a polymer coating over the Azocoll. The azo release from this polymer coated Azocoll stirred in pH 7 buffer and pH 9 buffer and then exposed to subtilisin was compared. Azo release was less from Azocoll coated in polymer and stirred in pH 7 buffer compared to when stirred in pH 9 buffer. Therefore, concluding release of Azocoll and thus solid particles from within the polymer coating can be controlled by the pH of the suspension the polymer coated Azocoll is stirred. However, although the release of azo dye was reduced by stirring in pH 9 buffer compared to when stirred in pH 7 buffer, azo release for both was still high indicating subtilisin was likely to be permeating the polymer coating causing proteolysis of Azocoll even though it has a polymer coating.

4) Coating sodium chloride with polymer.

An alternative solid to Azocoll was sought since from the latter set of experiments it was thought subtilisin was permeating through the polymer coating and causing proteolysis of the Azocoll. It was thought by using an alternative a more obvious difference between release from within the polymer coating when stirred in different pH buffers would be seen. It was thought sodium chloride could be a possible alternative. Therefore, sodium chloride and polymer was suspended in an organic solvent. The organic solvent was removed to leave a polymer coating over the surface of the sodium chloride. Increasing amounts polymer were used to coat the sodium chloride and release of the sodium chloride from within these polymer coatings was determined when stirred in pH 7 buffer and pH 9 buffers. The release of sodium chloride from within a thin polymer coating was similar when stirred in either pH 7 or 9 buffer solutions. When thicker coatings were used, the rate of sodium chloride release was slower when stirred in pH 9 buffer compared to when stirred in pH 7 buffer. However, the release of sodium chloride similar to azo dye release, from within the polymer coating was not completely prevented by stirring in pH 9 buffer. The surfactant SDS was dissolved in to the pH 7 and pH 9 buffer solutions. Polymer

coated sodium chloride were then stirred in these solutions. The rate of sodium chloride release was again slower when stirred in pH 9 buffer compared to when stirred in pH 7 buffer. However, release of sodium chloride from within polymer coating was faster when stirred in pH 7 and 9 buffers with dissolved SDS, compared to when stirred in pH 7 and 9 buffers with no SDS. Thus, concluding the rate of release of a solid from within the polymer coating can be increased by addition of SDS to the pH buffer.

Thus, in conclusion, the overall aim was achieved as solid particles, that is both Azocoll and sodium chloride coated with *N,N*-diethylamine GMP could be uncoated as a function of pH.

Chapter 6

Conclusions and Future work

Polymers that have alternating charged and hydrophobic pendant groups can show conformational changes with loss of charge from their charged pendant groups. The aim of this thesis was to assess two groups of polymers that were synthesised based on this structure for controlled release applications. The main applications of interest included 1) disruption of liposome membranes by conformational changes of polymers to release liposome contents and 2) cytoplasmic delivery of genes/drugs through exploitation of low endosomal pH to cause endosomal membrane disruption. Additional areas of interest included 3) utilising the membrane disruption properties of polymers to disturb microbial membranes and thus exert an anti-microbial effect. Also, 4) the utilisation of polymers to form solid coatings and release polymer coated agents in response to pH.

The main findings from this study along with future considerations are summarised.

1) In Chapter 2 the molecular weight of poly(lysine *iso*-phthalamide) was analysed relative to poly(ethylene glycol) standards and found to be 18160 Da. This value was used to represent the molecular weight of polyamides. Similarly, the molecular weight of glycidyl methacrylate pre-polymer was analysed and found to be 35411 Da. Both these values were lower than expected. However, this was thought to be a consequence of the mode of analysis. To confirm this further analysis would be required. For example, using alternative standards that are closer in structure to poly(lysine *iso*-phthalamide) and glycidyl methacrylate pre-polymer might provide a better indication of the polymers true molecular weight. With such standards it would be useful to determine the molecular weight of all polymers assessed in this thesis so that any relationship between molecular weight and structural properties can be assessed. With this information adjustments to the molecular weight of particular polymers could be made to improve upon their potential pH-responsive membrane disruption properties.

Also, for Chapter 2 potentiometric titrations were performed to determine each polymers pK_a values and degree of dissociation. These pK_a values were plotted against degree of dissociation to assess the polymers conformational changes. Polyamides that had the same amine groups and also alkyl groups of similar length exhibited the same conformational trends. It was noted that trends indicating more profound conformational changes were exhibited for polyamides with the amine group ornithine, if a short alkyl group was replaced for a longer alkyl group. However, replacing ornithine with lysine in polyamides with a long alkyl group also caused a dramatic difference in conformational trends. That is, transitions between conformational changes were not as obvious for the polyamide with lysine groups. Finally, replacing an alkyl group for an aromatic group in a polyamide with the amine group, lysine, shows conformational changes similar to polyamides with the amine group ornithine and a long alkyl group. However, the degree of dissociation at which these changes occurred varied. The amine modified glycidyl methacrylate polymers only varied in their attached amine group. Nevertheless, when their apparent pK_a was plotted against degree of dissociation they exhibited quite different trends. Thus, it was concluded that for both sets of polymers small structural alterations could cause dramatic changes to the conformational trends the polymers exhibited.

2) While Chapter 2 assessed the pH-responsive conformational changes of a range of polymers, Chapter 3 aimed to determine whether these polymers exhibited pH-responsive membrane disruption properties. The relationship between these membrane disruption properties and pH-responsive conformational changes exhibited in Chapter 2 was assessed. Erythrocyte lysis as a function of pH when pre-incubated in the presence and absence of polymers was used to determine the polymers membrane disruption properties. It was expected that polymers with structural similarities would exhibit similar erythrolysis trends.

The polymers assessed included poly(lysine butyl malonamide), poly(lysine diethyl malonamide), poly(lysine *iso*-phthalamide), poly(lysine dodecandiamide), poly(ornithine dodecandiamide) and poly(ornithine sebacamide). This group of polymers included three main polymer types. That is, poly(lysine butyl malonamide) and poly(lysine diethyl malonamide) were synthesised by making an alkyl group substitution; poly(lysine *iso*-phthalamide) was synthesised by enchainment of an

aromatic group and poly(lysine dodecandiamide), poly(ornithine dodecandiamide) and poly(ornithine sebacamide) were synthesised by enchainment of a long alkyl group.

Erythrocytes pre-incubated with poly(lysine butyl malonamide) and poly(lysine diethyl malonamide) as expected showed very similar pH-responsive erythrolysis properties. That is, erythrolysis was similarly low when erythrocytes were pre-incubated with buffer, poly(lysine butyl malonamide) or poly(lysine diethyl malonamide) then adjusted to neutral and slightly acidic pH values. Lysis of erythrocytes that were pre-incubated with poly(lysine butyl malonamide) or poly(lysine diethyl malonamide) and adjusted to low pH values were similarly greater than the lysis of erythrocytes that was pre-incubated with buffer and adjusted to the same low pH values.

Poly(lysine *iso*-phthalamide) was the only polymer to be synthesised by enchainment of an aromatic group. However, compared to the other polyamides synthesised it is structurally most similar to poly(lysine dodecandiamide), since both polymers have the same amine group. They vary in their hydrophobic groups with poly(lysine dodecandiamide) having long aliphatic groups and poly(lysine *iso*-phthalamide) having aromatic groups. Different pH-responsive erythrolysis trends were exhibited for these two polymers. That is, at all pH values poly(lysine dodecandiamide) causes considerable lysis of erythrocytes and this lysis is increased at low pH values. Whereas, at pH values down to 4.8 lysis of erythrocytes is only marginally higher when pre-incubated with poly(lysine *iso*-phthalamide), compared to when pre-incubated with buffer. Like poly(lysine dodecandiamide), the erythrolysis at lower pH values when erythrocytes are pre-incubated with poly(lysine *iso*-phthalamide) is greater than erythrolysis of erythrocytes pre-incubated with buffer. It was concluded that although poly(lysine dodecandiamide) lysed erythrocytes at all pH values, poly(lysine *iso*-phthalamide) was more pH specific and lysed erythrocytes with greater efficiency at low pH values. It would be interesting to determine whether other polyamides synthesised by enchainment of different aromatic groups would exhibit similar erythrolysis trends as were exhibited by poly(lysine *iso*-phthalamide).

Poly(lysine dodecandiamide), poly(ornithine dodecandiamide) and poly(ornithine sebacamide) were synthesised by enchainment of a long alkyl group. The results for poly(ornithine dodecandiamide) and poly(ornithine sebacamide) were similar. That is, erythrolysis in the presence of poly(ornithine dodecandiamide) and poly(ornithine sebacamide) was greater than erythrolysis in the absence of polymer at all pH values. The difference between relative erythrolysis in the presence and absence of polymer was widened at pH values below pH 5. Poly(ornithine dodecandiamide) and poly(ornithine sebacamide) like poly(lysine *iso*-phthalamide) were more pH specific than poly(lysine dodecandiamide) in causing erythrolysis.

It was concluded that all the polyamides assessed exhibited pH-responsive membrane disruption properties. However, using erythrolysis to assess the membrane disruption properties of polymers gave some anomalous absorbency values. A series of experiments were set up to determine the reasoning for these anomalous values. This included an experiment to determine the effect of pH on absorbency readings of each constituent of the experiment. These constituents included polymer dissolved in a buffer solution, haemoglobin, haemoglobin treated with Triton X100, haemoglobin exposed to freeze-thawing, haemoglobin plus polymer dissolved in a buffer solution, haemoglobin plus polymer dissolved in a buffer solution then treated with Triton X100 and finally haemoglobin plus polymer dissolved in a buffer solution exposed to freeze-thaw. The second experiment assessed whether a wavelength of 492 nm, which was used to measure released haemoglobin was suitable at the full range of pH values used. Finally, the effect of Triton X100 concentration on the absorbency of haemoglobin was determined.

It was concluded that anomalous absorbency readings were not caused by pH effects on polymer alone, polymer exposed to Triton X100 or a freeze-thaw process; haemoglobin alone, polymer plus haemoglobin, haemoglobin exposed to Triton X100 or a freeze-thaw process; or polymer plus haemoglobin exposed to a freeze-thaw process. However, polymer plus haemoglobin exposed to Triton X100 showed unexpected absorbency readings with reducing pH. It was also concluded that a wavelength of 492 nm used to measure released haemoglobin was optimal compared to other wavelengths tested (405 nm, 431 nm, 450 nm). However, an increasing percentage of Triton X100 added to polymer-haemoglobin solution caused an increase

in the absorbency as the pH of the polymer-haemoglobin is reduced. This gives some explanation for anomalous absorbency readings.

Although some anomalous readings were obtained when erythrolysis was used to assess the membrane disruption properties of polymers, the general pH-responsive properties shown for each polymer was thought promising. The work carried out in Chapter 4 was performed to gain further insight in to the membrane disruption properties of poly(ornithine sebacamide), poly(lysine diethyl malonamide), poly(ornithine dodecandiamide), poly(lysine *iso*-phthalamide), poly(lysine dodecandiamide) and poly(lysine butyl malonamide) and to attempt to obtain more reliable results by overcoming the anomalous readings.

Chapter 3 also aimed to determine whether the polymers used in Chapter 2 had anti-microbial properties by disturbing the microbial cell membrane as a function of pH. *Candida albicans* was chosen to represent the yeasts, while *Escherichia coli* and *Staphylococcus aureus* were chosen to represent gram negative and gram positive bacteria, respectively. This study showed poly(lysine dodecandiamide), *N,N*-diethylamine and 3-(diethylamino)propylamine modified glycidyl methacrylate polymers did not exhibit anti-microbial properties. It was thought that anti-microbial properties might have been observed if the polymers could break through the cell wall allowing access to and thus interaction with the plasma membrane. With further work this might be demonstrated. Also further work where polymers could be tested on a wider range of micro-organisms might see that some micro-organisms are sensitive.

3) Chapter 4 aimed to gain further insight in to the membrane disruption properties of polymers using liposomes. A preliminary study was performed to assess whether polymers solubilised liposomes. That is, liposomes that were pre-incubated with or without polymer were visualised before and following pH adjustment. An observation where liposomes change from being opaque to clear is indicative of liposome membrane disruption. Solubilisation of anionic liposomes was observed with reducing pH when liposomes were pre-incubated with poly(lysine dodecandiamide), synthesised by incorporation of a long alkyl group. Similarly, solubilisation with reducing pH was observed when liposomes were pre-incubated with poly(lysine *iso*-phthalamide). However, solubilisation of anionic liposomes was

not observed with reducing pH when liposomes were pre-incubated with buffer or poly(lysine butyl malonamide), which is synthesised by making an alkyl group substitution. While poly(lysine butyl malonamide) did not show pH-responsive solubilisation of liposomes it did show pH-responsive erythrolysis in Chapter 3. Therefore, indicating liposome solubilisation should not be used solely as an indicator of whether polymers do or do not have membrane disruption properties.

Liposome solubilisation was also not observed with increasing pH when cationic liposomes were pre-incubated with *N,N*-diethylamine, *N,N*-diethyl ethylene diamine or 3-(diethylamino)propylamine modified GMP. However, again this result could not rule out that these polymers might have pH-responsive membrane disruption properties.

Calcein release from liposomes with encapsulated calcein was used as an alternative to erythrolysis to quantify membrane disruption properties of polymers. It was expected that that structurally similar polymers would show similar liposome membrane disruption properties. However, this was not always observed.

For example, poly(lysine butyl malonamide) and poly(lysine diethyl malonamide) are structurally similar and showed similar erythrocyte membrane disruption properties in Chapter 3. Whereas in Chapter 4, while poly(lysine butyl malonamide) showed pH-responsive liposome membrane disruption properties, poly(lysine diethyl malonamide) which is structurally similar did not.

Poly(ornithine dodecandiamide), poly(lysine dodecandiamide) and poly(ornithine sebacamide) are structurally similar to each other. However, only the latter two polymers show pH-responsive membrane disruption properties when calcein release from liposomes with encapsulated calcein is used as an indicator of membrane disruption. However, in Chapter 3 all three polymers, that is, poly(ornithine dodecandiamide), poly(lysine dodecandiamide) and poly(ornithine sebacamide) showed pH-responsive erythrocyte membrane disruption properties.

Compared to the other polymers used in this thesis poly(lysine *iso*-phthalamide) is structurally most similar to poly(lysine dodecandiamide). Poly(lysine *iso*-

phthalamide) similar to poly(lysine dodecandiamide) solubilised liposomes with reducing pH. Also, in Chapter 3, poly(lysine *iso*-phthalamide) showed pH- responsive erythrocyte membrane disruption properties Therefore, it was expected that poly(lysine *iso*-phthalamide) would show pH-responsive membrane disruption properties when calcein release from liposomes with encapsulated calcein was used as an indicator of membrane disruption. However, this was not observed since calcein release was similar with reducing pH when liposomes were pre-incubated in the presence of poly(lysine *iso*-phthalamide) or buffer.

The reasoning for the variation between the membrane disruption properties in Chapter 3 and Chapter 4 is unclear. It is thought with further work using a wider range of polymers the reasoning for this variation may become apparent.

Release of calcein from cationic liposomes with encapsulated calcein could be controlled by pH when incubated with *N,N*-diethylamine and *N,N*-diethyl ethylene diamine modified GMPs. That is, release of calcein from liposomes increased with increasing pH values between pH 6 and pH 9. The release of calcein from cationic liposomes with encapsulated calcein was also shown to increase with increasing pH when pre-incubated with buffer. However, calcein release from liposomes pre-incubated with polymer was greater than that from liposomes pre-incubated with buffer. The difference between calcein release from liposomes with encapsulated calcein pre-incubated with buffer and calcein release from liposomes pre-incubated with *N,N*-diethylamine and *N,N*-diethyl ethylene diamine GMPs was widened at the higher pH values. Cationic liposomes with encapsulated calcein pre-incubated with 3-(diethylamino)propylamine modified GMP and adjusted to pH values between pH 6 and pH 9 showed a similar release of calcein as that from cationic liposomes with encapsulated calcein pre-incubated with buffer. Therefore, indicating 3-(diethylamino)propylamine modified GMP does not show pH-responsive disruption properties.

Although not investigated in this thesis it would be interesting to see if pH-responsive disruption of liposomes with encapsulated calcein would be observed if liposomes with encapsulated calcein were pre-incubated with higher concentrations of the polymers that did not show pH-responsive disruption of liposomes. That is,

poly(lysine *iso*-phthalamide), poly(lysine diethyl malonamide), poly(ornithine dodecandiamide) and 3-(diethylamino)propylamine modified GMP.

The polymer properties which enable the rupturing of lipid membranes was also assessed in Chapter 4. It was concluded that rupturing of liposomes was at least in part due to the charge interaction between surface charge of liposomes and the charge that polymers exhibit.

Using poly(lysine dodecandiamide) to represent polyamide polymers it was attempted to determine whether polymers irreversibly adsorbed to the surface of liposomes. This was assessed by measuring release of calcein from liposomes after pH adjustment of liposomes that had been pre-incubated with poly(lysine dodecandiamide) then washed with buffer using centrifugation. The centrifugation process lysed liposomes in the presence of polymer. Therefore, it could not be determined whether polymer was attached to the liposomes surface. Polymer would need to be adsorbed to surface of liposomes to control the release of liposomal contents when delivered *in vivo*. Therefore, it will be necessary to determine whether or not polymer is adsorbed to liposome surface. A possible alternative would be label polymer with the fluorescent dye pyrene. The fluorescence of pyrene eximer and monomer emissions in the presence and absence of liposomes could then be studied. That is, Mizusaki *et al.*, (2001) showed pyrene labelled poly-2-(acrylamido)-2-methylpropane sulfonates in salt solutions present a strong eximer emission centred around 482 nm in addition to the well-resolved emission due to pyrene monomer. The eximer emission is strong since pyrene chromophores are in close proximity to each other within the core of polymeric micelles. Addition of liposomes to a solution of this polymer prompts a sharp increase in pyrene monomer emission intensity at the expense of pyrene eximer emission. The decrease in pyrene eximer emission signals the incorporation of hydrophobic groups in to the liposome bilayer with concomitant separation of the pyrene groups within the lipid bilayer. Thus, the ratio of intensity of pyrene eximer and monomer emissions can be used as a qualitative indicator of the degree of polymer binding to liposomes. If this methodology were successful, the next step would depend on whether polymer was adsorbed to liposome membrane or not. Such that, a result showing polymer was not adsorbed would require further attention to find a means of adsorbing or integrating polymer to liposome membrane. For

example, polymer could be incorporated in to the lipid bilayer of liposomes during their synthesis. On achieving this similar trends in the release of encapsulated markers may be seen with pH change of these liposome suspensions as are seen for liposomes pre-incubated with polymer then exposed to pH change. Alternatively, liposomes could be coated with polymer via a linker that adsorbs to liposome surface. For example, avidin could be used as a linker which has been shown to bind biotinylated liposomes (Liu, 2001; Noppel-Simson *et al.*, 1996; Needham *et al.*, 2000).

For liposomes to be useful for *in vivo* applications it is important to be able to control their size. It was concluded that extrusion of the liposomes synthesised for this thesis produced homogenous suspensions more efficiently than sonication.

It was shown that the lyophilisation process lysed both cationic and anionic MLVs with encapsulated calcein when pre-incubated in the presence or absence of polymers. However, the ability to lyophilise liposomes would be beneficial from a storage viewpoint. It would be possible to prevent this lysis by incorporating sugars in the liposomal lipid bilayer (Ward *et al.* 1996; Crowe *et al.* 2002). However, it is not known whether the pH-responsive release properties exhibited by liposomes pre-incubated with polymer would be retained if sugars were incorporated in the lipid bilayer. Therefore, it would be beneficial to determine whether content release from liposomes could still be controlled by pH change when liposomes have sugars incorporated in their lipid bilayer.

4) Chapter 5 aimed to determine whether solid particles could be coated with polymers studied in Chapter 2. Then determine whether the polymer coating could be removed as a function of pH. Solid particles Azocoll and sodium chloride were used to assess whether this could be achieved.

N,N-diethylamine modified GMP films were stirred in buffer solutions adjusted to pH values 6, 7, 8, 9 and 10. The rate of dissolution of films decreased with increasing pH of the buffer solution. Similarly, poly(lysine dodecandiamide) films were stirred in buffer solutions adjusted to pH 7, 6, 5 and 4. The rate of dissolution of films decreased with decreasing pH. By increasing the amount of *N,N*-diethylamine GMP coating NaCl, the rate of dissolution of NaCl in both pH 7 and 9 buffers is reduced.

When NaCl is coated in more than twice its amount in polymer, the dissolution rate of NaCl in pH 9 buffer is slower than in pH 7 buffer. Thus, showing dissolution rate of NaCl can be extended by coating it in *N,N*-diethylamine modified GMP and dissolving it in an alkaline buffer rather than a neutral buffer. A number of avenues could be taken to give further insight in to the latter study. For example, in the latter study NaCl and polymer were dispersed using an organic solvent. The solvent was removed to leave polymer coated NaCl. The release profiles of NaCl were determined when polymer coated NaCl was stirred in different pH buffer solutions. Additional release profiles could be determined for NaCl when coated with polymer using alternative methods, such as, compression of NaCl within polymer to form a tablet. An alternative coating method may alter the release profile. For example, Wang *et al.*, (1991) describe how by changing the method by which an active agent is coated it alters the release profile. Also, release of NaCl may be more controllable by coating NaCl with polymer in combination with other polymers or non-polymeric materials. For example, Streubel *et al.*, (2000) showed bimodal drug release could be achieved by using multi-layer matrix tablets prepared by compression of drug between multiple layers of drug free polymer.

From Chapter 5, it was concluded that the pH- responsive release of both Azocoll and sodium chloride was shown to be controlled by coating with *N,N*-diethylamine modified GMP. However, it was thought the coating had imperfections since release of solid particles was not completely prevented in any pH adjusted solution.

By performing this study other important considerations became apparent. For example, this study successfully showed certain poly(amides) and amine modified GMPs have pH-responsive membrane lytic properties. However, for biological application, the pH range at which polymers exert membrane lysis may need to be narrower than what has been seen for these polymers. It may be possible to address this by re-examining the structural properties of polymer, since slight changes in polymer architecture can have vast effects. For example, Malik *et al.*, (2000) discuss biological applications of polymers such as drug and gene delivery and how modifications made to polymer structure can improve certain properties, such as allowing easier passage of polymer across biological barriers or decreasing polymer toxicity.

To make *in vivo* use possible, polymers must also be expelled safely from the b Therefore, an important future consideration would be to assess the degradability a degradation products of polymers. Examples of methodologies used to study such properties in the past include degradation rate in plasma (Stoll *et al.*, 2001) and implanting polymers in peritoneal cavity of rats, followed by an assessment of the polymer degradation rate and undesirable reactions to rats (Kawaguchi *et al.*, 1983).

Chapter 7

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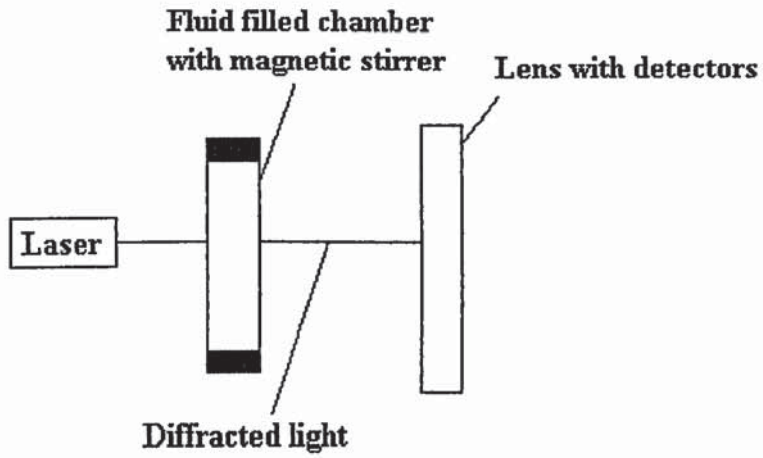
Appendix 1

Operation Principles Of Mastersizer (Malvern instruments, UK).

Below is shown a diagrammatic representation of the internal hardware of the Mastersizer. Before each sample analysis the glass chamber of the Mastersizer was removed and thoroughly cleaned to remove any contaminating particles. It was then flushed and filled with deionised water and placed back in to the Mastersizer. The water filled chamber was used as a blank. The liposome preparation was placed drop-wise in to the chamber. When the obscuration reading was in a suitable range and had stabilised sample measurements were started. During sample measurements the particles pass through the laser light. The light is diffracted, with the diffraction angle being inversely proportional to particle size. The diffraction patterns from all particles that are received by the detectors are averaged over a fixed time period and transformed in to particle size values. These are arranged in to discrete size ranges, given by the size of detectors area. This approach assumes all particles scatter with equal efficiency, are opaque and transmit no light. Such assumptions are not always true. Therefore, the Mie theory is applied which allows the refractive index of the particles to be considered when calculating particle size values. A volume histogram is obtained which shows the percentage of particles between stated size ranges.

Internal hardware of the Mastersizer



Appendix 2

Operation Principles Of Zetasizer 1000 (Malvern instruments, UK).

To analyse a sample of liposomes a 1cm-width clear glass cuvette was washed then filled with fresh deionised water. A drop of the liposome preparation was dispersed in to the water filled cuvette. The cuvette was then placed in to the Zetasizer 1000. A total of three measurements were selected for each sample. Ten repeat scans are automatically taken for each measurement selected. Therefore, for each measurement or run a mean particle diameter (z-average) and standard error between particle diameter readings is given. In addition, an intensity histogram and volume histogram is given which shows the percentage of particles between a particular size range. For a sample to be suitable for PCS the particle sizes should not be too polydisperse. A sample having a polydispersity index greater than 0.7 is not suitable for Photon Correlation Spectroscopy (PCS). In addition for correct analysis the count rate should be between 50 and 200 Kcps (counts per second). Samples having a Kcps of less 50 should be made more concentrated by increasing the amount of dispersed sample. Whereas, samples having a Kcps of greater than 200 should be diluted. In order to determine particle size, the Zetasizer 1000 uses Photon Correlation Spectroscopy (PCS). Also known as, Dynamic Light Scattering (DLS) or Quasi Elastic Light Scattering (QELS). PCS measures Brownian motion, which is directly related to particle size with larger particles having a slower Brownian motion. The Zetasizer 1000 can detect particle sizes between 15nm and 1um. A typical PCS instrument is shown below (Figure 1) and like most use a monochromatic coherent He-Ne laser as a light source that converges in the sample by using a focusing lens. Scattered light due to particle motion is detected by a photomultiplier (Figure 2). The fluctuations in the intensity of scattered light are converted in to electrical pulses, which are passed to a correlator.

Correlators compare the degree of similarity between signals. The Zetasizer compares electrical pulses over time. For larger slow moving particles, the signal changes slowly. Whereas, faster moving small particles show a more rapid signal change. The correlator passes this information to a computer where appropriate data analysis is applied.

Figure 1: A typical PCS instrument (Adapted Malvern Instruments).

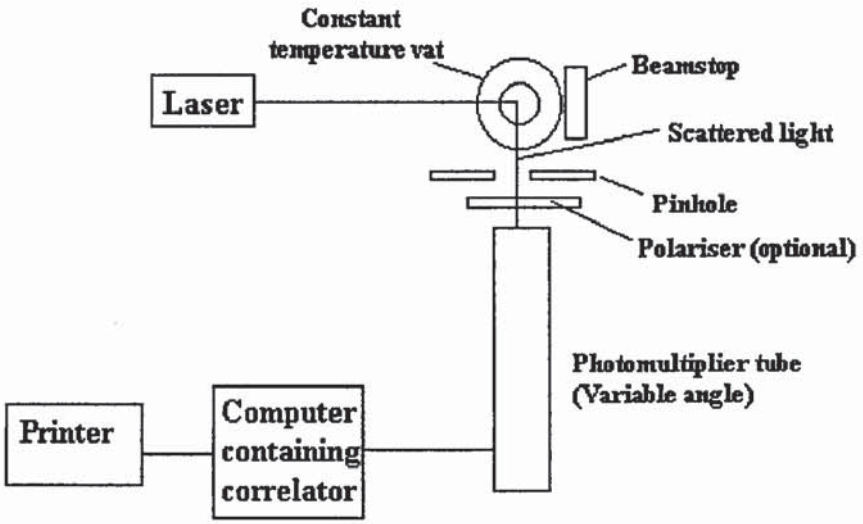
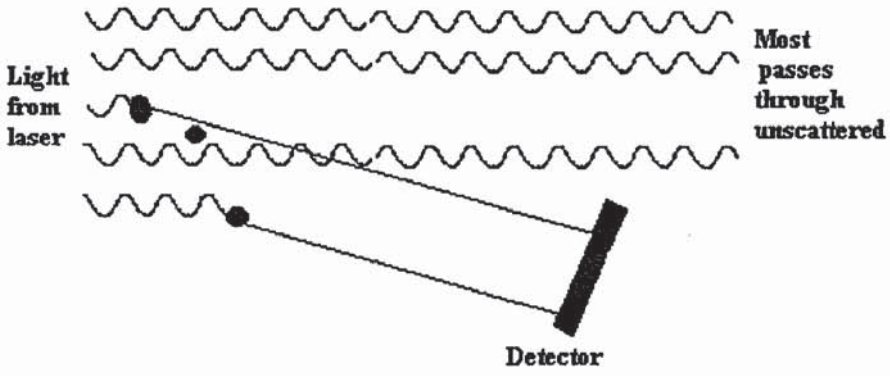


Figure 2: Detection of scattered light.



Data Analysis.

The velocity of particles in the fluid filled cuvette is defined by a term known as diffusion coefficient and is given the symbol D.

The Stokes-Einstein equation states:

$d(H) = (KT)/(3\pi nD)$, where

d(H) = hydrodynamic diameter

T = absolute temperature

D = diffusion coefficient

n = viscosity

K = Boltsmann`s constant

The Rayleigh approximation states:

$I \alpha d^6$ and $I \alpha 1/\lambda^4$, where

I = intensity of light scattered

d = particle diameter

λ = laser wavelength

The results from the Stokes-Einstein equation and Rayleigh approximation give an intensity histogram which show the percentage of particles between the stated size range. The Rayleigh approximation is most useful for small particles (approximately 60nm) since scattered light from vertically illuminated particles is essentially isotropic, that is, equal in all directions. Thus, illuminating the particles from a different angle would not result in any change in light intensity. However, in a suspension containing both large and small particles the scattered light from large particles may swamp that from smaller particles. To overcome this problem the Mie theory can be applied. This explains the maxima and minima in the plot of intensity with angle and gives the correct answer over all wavelengths, sizes and angles. It also transforms the intensity to a volume plot that shows the percentage of particles between the stated size range determined using the Mie theory.

Appendix 3

Nomenclature.

Abbreviation:

DMPC	Dioleoylphosphatidylethanolamine
SUVs	Small unilamellar vesicles
PHC	N-palmitoy-L-homocysteine
PEVP	Poly (N-ethyl-4-vinylpyridium)
PEG	Poly (ethylene glycol)
PEAA	Poly ethyl acrylic acid
PC	Phosphatidylcholine
NMR	Nuclear magnetic resonance
NIPAM	N-isopropylacrylamide
MLVs	Multilamellar vesicles
LUVs	Large unilamellar vesicles
LCST	Lower critical solution temperature
DSPPE	Distearoylphosphatidyl ethanolamine
DPSG	1-2-dipalmitoyl-sn-3-succinylglycerol
DOPE	1,2-miristoyl-sn-3-phosphatidylcholine
DPPC	1,2-palmitoyl-sn-3--phosphatidyl choline
SAS	Supercritical antisolvent
TEM	Transmission electron microscopy
SucPG	Succinylated polyglycidol
GMP	Glycidyl methacrylate polymer
MIC	Minimum inhibitory concentration
DLPC	L- α-dilauroylphosphatidylcholine