Synthesis of Peptide Nucleic Acid (PNA) and Entrapment of PNA into Liposomes

PENG HE

Master of Philosophy

ASTON UNIVERSITY

September 2004

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Thesis Summary

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Peptide Nucleic Acid (PNA) is a synthetic pseudopeptide nucleic acid analogue in which the deoxyribose phosphodiester backbone of DNA is replaced by a pseudo-peptide composed of N-(2-aminoethyl)glycine units, resulting in an achiral and uncharged DNA mimic. PNA is chemically stable and resistant to enzymatic cleavage and with high binding affinity and specificity to nucleic acids. Therefore PNAs are very strong candidates as effective antisense or antigene agents. They have shown both antigene and antisense activities against target genes and transcripts, respectively. PNAs are also very useful biomolecular tools.

Unfortunately, for certain applications of PNA, there are some severe drawbacks such as its poor water solubility and poor cellular uptake. Fast progress in the exploration of PNA as an experimental and therapeutic regulator of gene expression has been hampered by these drawbacks.

Several transfection protocols for PNA have now been studied, such as microinjection, electroporation and co-transfection with DNA. Among the different delivery strategies, liposomes are a potential way to delivery PNA into cells since liposomes can protect various molecules from the external medium and can deliver molecules directly into cells. Moreover, it is also possible to specifically target liposomes by coupling proteins or antibodies to their surface.

In this project PNA oligomers were synthesized and labeled with fluorescein. These oligomers were entrapped into the liposome and from the results, we can draw a conclusion that the method of dehydration-rehydration is encouraging method for the preparation of PNA-liposomal delivery systems which can offer high PNA loading, mild preparation methods and the potential to optimise PNA release.

Key words: peptide nucleic acid, antisense, solid phase synthesis, liposomes, entrapment, dehydration-rehydration.

Dedication

I would like to dedicate this thesis to my family, especially my parents and my girl friend, with love and thanks for all their support, guidance and encouragement.

Acknowledgements

I would like to offer sincere thanks to my supervisors, Dr Qinguo Zheng and Dr Yvonne Perrie, who have provided me with valuable advice, guidance and encouragement for the completion of this project.

I would like to express my appreciation to Mr. Michael L Davis, for his advice and help on the use of equipment in the medicinal chemistry research lab and also to Mrs. Karen C Farrow for her role in teaching me how to use apparatus in the central equipment lab.

Abbreviations

A/Sichuan: A/Sichuan influenza virus hemagglutinin and neuraminidase
AA: Antimyosin Antibodies
Ac_2O: Acetic anhydride
AmB: Amphotericin B
Ara-C: Cytosine Arabinoside
BBB: Blood-Brain Barrier
Boc: tert-butyloxycarbonyl
Boc-PNA-A(Cbz)-monomer:
N-((N^6-Benzyloxycarbonyl)adenin-9-yl-acetyl)-N-(2-Boc-aminoethyl)glycine
Boc-PNA-C(Cbz)-monomer:
N-((N^4-Benzyloxycarbonyl)cytosin-1-yl-acetyl)-N-(2-Boc-aminoethyl)glycine
Boc-PNA-G(Cbz)-monomer:
N-((N^2-Benzyloxycarbonyl)guanine-9-yl-acetyl)-N-(2-Boc-aminoethyl) glycine
Boc-PNA-T-monomer: N-(2-Boc-aminoethyl)-N-(thymin-1-yl-acetyl)glycine
CF: Carboxyfluorescein
CHOL:Cholesterol
CL: Conventional Liposomes
DCC: Dicyclohexylcarbodiimide
DCM: Methylenechloride
DCP: Diacetyl Phosphate
DIEA: Diisopropylethylamine
DLS: Dynamic Light Scattering
DMF: Dimethylformamide
DMPC: Dimyristorylphophatidycholine
DMS: Dimethylsulphide
DNA: Deoxyribonucleic Acid
DOTAP: 1,2- Dioleoyl-3-Trimethylammonium Propane
DRV: Dehydration-Rehydration Vesicle
dsDNA: Double Stranded DNA
DSPC: Distearoyl Phosphatidylcholine
DSPG: Distearoylphosphatidylglycerol
DT: Diphtheria Toxin
EPR: Electron Paramagnetic Resonance
5(6)-FAM-NHS: 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester
FFEM: Freeze-Fracture Electron Microscopy
FISH: Fluorescence *in situ* Hybridization
HATU: O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HBTU: 2-(1-H-Benzotriazol-1-yl) -1,1,3,3-tetramethyluronium hexafluorophosphate
HPVs: Human Papillomaviruses
LCL: Long-Circulating Liposomes
LCI: Long-Circulating Immunoliposomes

LDV: Laser Doppler Velocimetry
LUV: Large Unilamellar Vesicles
MBHA resin: 4-Methylbenzhydryl Amine resin
MLV: Multilamellar Vesicles
NLS: Nuclear Localization Signal
NMM: Methylmorpholine
NMP: N-methylpyrrolidone
NMR: Nuclear Magnetic Resonance
NNLS: Non-Negatively Constrained Least Squares
ODNs: Oligodoexynucleotides
ONs: Oligonucleotides
PALA: N-(phosphonacetyl)-L-aspartate
PC: Egg phosphatidylcholine
PCR: Polymerase Chain Reaction
PEG: Polyethylene Glycol
PG: Phosphatidyglycerol
PNA: Peptide Nucleic Acid
PO: Phosphodiester
RES: Reticuloendothelial System
REV: Reverse-phase Evaporation
RP-HPLC: Reverse Phase - High Performance Liquid Chromatography
rHBsAg: recombinant Hepatitis B surface Antigen
RIVE: Reconstituted influenza virus envelopes
SA: Stearylamine
SUV: Small Unilamellar Vesicles
$\mathbf{T_a}$: Ambient Temperature
$\mathbf{T_c}$: Crystalline Transaction Temperature
TFA: Trifluoroacetic Acid
TFMSA: Trifluoromethanesulfonic Acid
THF: Tetrahydrofuran
TI: Therapeutic Index
TLC: Thin-Layer-Chromatography
TTP: Triphenylphosphonium
Z: Benzyloxycarbonyl

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Chapter 1 Introduction

1.1 Background of PNA (Peptide Nucleic Acid)

1.1.1 The structure of PNA

PNA (Peptide Nucleic Acid) originated in the early 1990s in a laboratory in Copenhagen led by organic chemist Prof. Ole Buchard together with biochemist Peter Nielsen, in their effort to develop new nucleic acid sequence-specific reagents [1].

PNA is a DNA (deoxyribonucleic acid) mimic with a pseudopeptide backbone composed of N-(2-aminoethyl) glycine units to which the nucleobases are linked through methylene carbonyl linkers [2] (**Figure 1.1**). PNA is chemically stable and resistant to enzymatic cleavage and with high binding affinity and specificity to nucleic acids. Therefore PNAs are very strong candidates as effective antisense or antigene agents [2]. They have shown both antigene and antisense activities against target genes and transcripts, respectively [2]. PNAs are also very useful biomolecular tools.

1.1.2 The hybridization properties of PNA

PNA are depicted like peptides, with the N-terminus at the first (left) position and the C-terminus at the right. Besides the obvious structural difference, PNA is set apart from DNA in that the backbone of PNA is acyclic, achiral and neutral. PNAs can bind to complementary nucleic acid in both antiparallel and parallel orientation. However, the antiparallel orientation is strongly preferred because this is the orientation for all antisense and DNA probe type applications. Usually, the N terminal of the PNA

oligomer is equivalent to the 5'-end of an oligonucleotide and is often referred to as the 5'-end of the PNA.

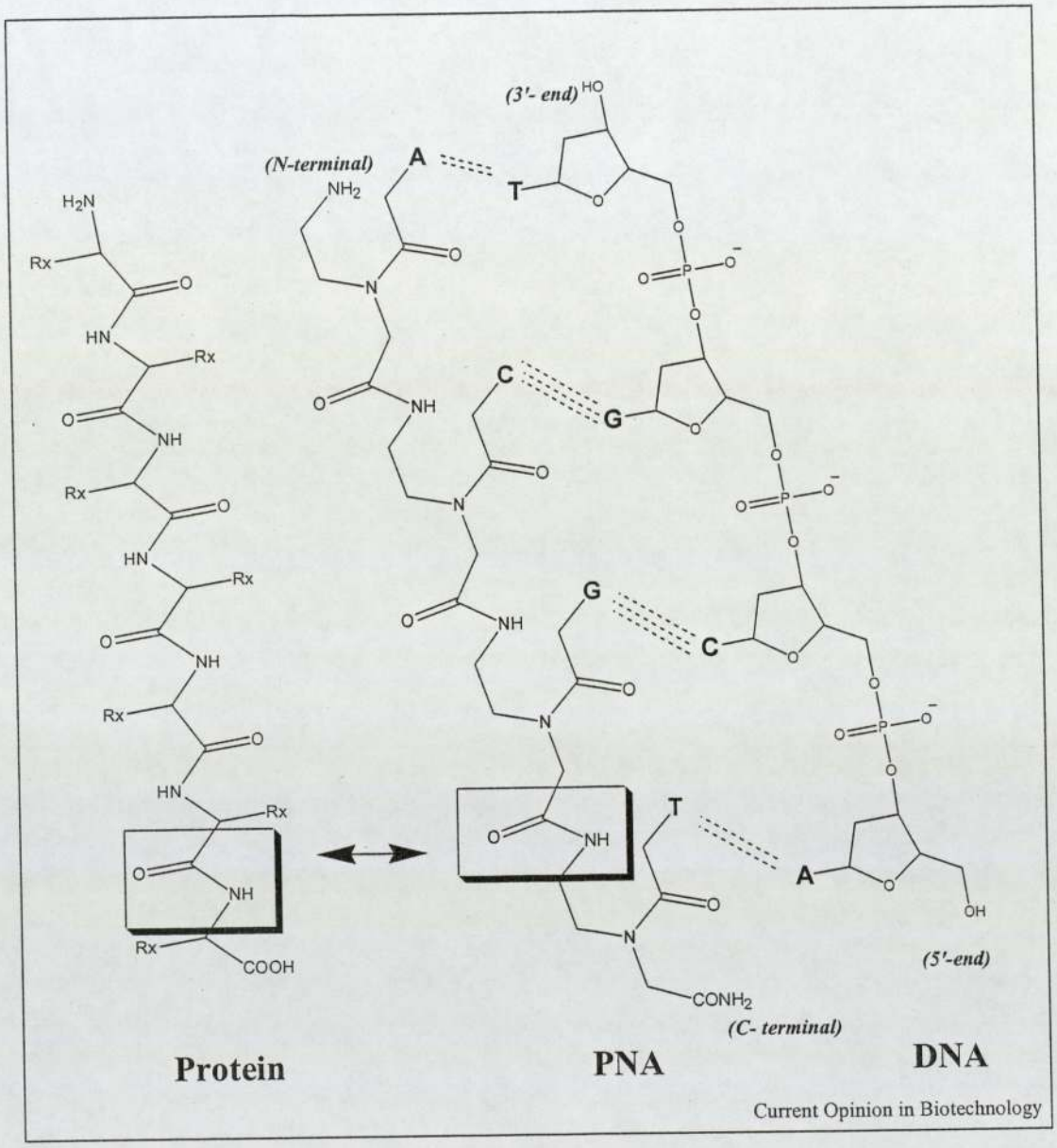


Figure 1.1 Chemical structure of single-stranded PNA compared to DNA and protein (peptide). The design of the PNA originated from a simple computer model of DNA, where the natural backbone was replaced with an achiral one (similar to the protein) that had the same inter-base distances as natural DNA and sufficient flexibility to allow base pairing with DNA. (*Source from:* Peter E Nielsen, Application of Peptide Nucleic Acid, *Current Opinion in Biotechnology* 1999, **10**:72)

1.1.2.1 PNA-DNA duplexes

The neutral character of the PNA backbone is important, which has many consequences. One of the most impressive consequences is that the binding between complementary PNA/DNA strands (**Figure 1.2**) is stronger than the one between complementary DNA/DNA strands at low to medium ionic strength. This oligomer forms a duplex with complementary antiparallel DNA, and the stronger binding between the PNA/DNA strands is mainly due to the lack of charge repulsion between the PNA strand and the DNA strand.

Interestingly, the base-pairing specificity of PNA/DNA duplex was also found to be higher. Generally a mismatch in a PNA/DNA duplex is more destabilizing, compared with a mismatch in a DNA/DNA duplex. A measurement was carried out in a 15-mer PNA with all possible single mismatch combinations to determine its base-pairing specificity. The average $\triangle T_m$ was 15°C in the PNA/DNA duplex, whereas the $\triangle T_m$ for the corresponding DNA/DNA duplex was 11 °C. Beside their average $\triangle T_m$, what is the more important is the lowest measured $\triangle T_m$, for example, it was 4°C for the DNA/DNA duplex compared with 8°C for the PNA/DNA duplex, which means the PNA is 'twice' as discriminating in the least discriminating basepair [3]. Further analogous results for PNA/RNA duplexes (**Figure 1.2**) have been obtained by Jensen and his colleagues [4]. Although a larger selection of sequence contexts has to be contained before the general conclusions are drawn, the increased binding specificity seems to be a significant property of PNA/nucleic acid duplexes, which could have many applications.

Figure 1.2 The structures of a duplex PNA/DNA (RNA). The DNA or RNA sequence is shown as the top strand, while the PNA (bold, italics) is shown in the antiparallel orientation. Base-pairing interactions are denoted by bold bond between the dases.

1.1.2.2 $(PNA)_2$-DNA triplexes

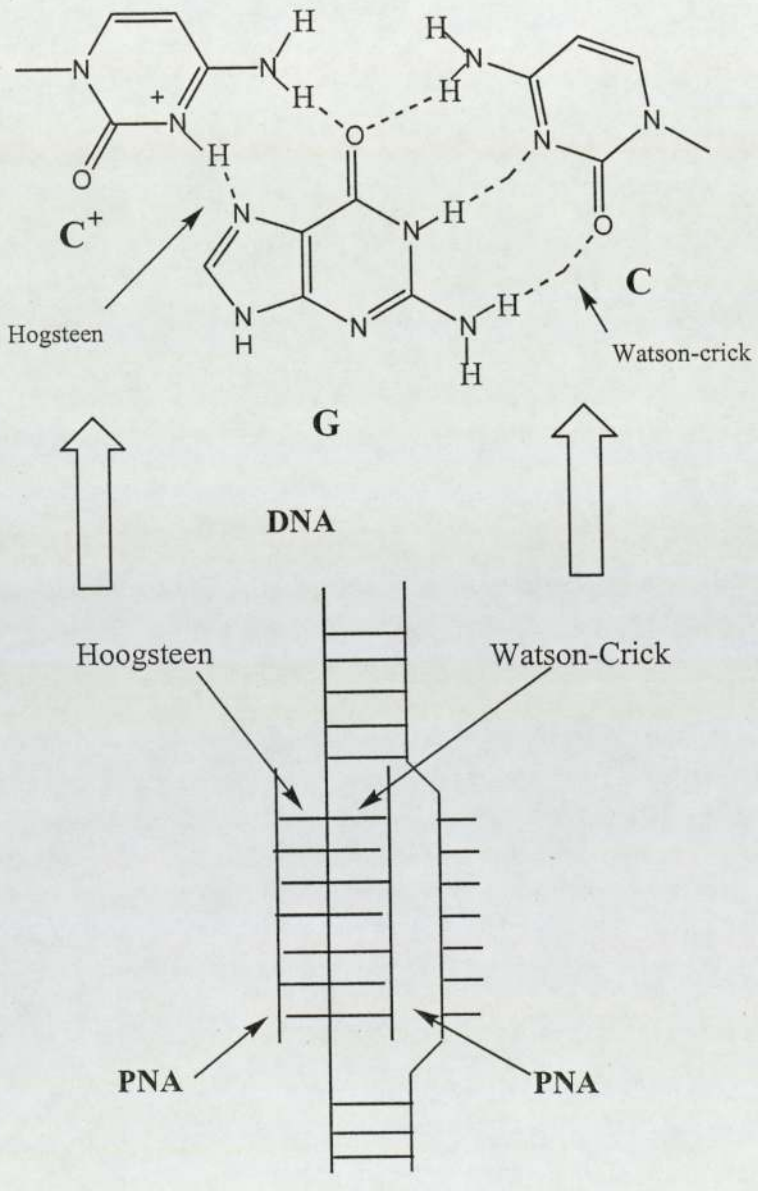


Figure 1.3 Triplex invasion by homopyrimidine PNA oligomers to form $(PNA)_2$-DNA triplexes. One PNA strand binds by Watson–Crick base pairing, which is preferably in the antiparallel orientation, while the other binds via Hoogsteen base pairing which is preferably in the parallel orientation.

$(PNA)_2$/DNA triplex was the very first complex observed with PNA [2], in which homopyrimidine PNA oligomers or PNAs with a high pyrimidine : purine ratio bind to a complementary DNA, forming a $(PNA)_2$-DNA triplex **(Figure 1.3)**. Generally speaking, homopyrimidine PNAs form extremely stable triplexes that have the stability enough to invade intact double stranded DNA. Further studies [5, 6] have revealed that $(PNA)_2$/DNA triplex formation observes the rules of homopyrimidine DNA triplex formation, which is preferably with an antiparallel Watson Crick duplex and a parallel bound Hoogsteen strand. The two PNA strands may be covalently connected by a flexiable linker and are termed bis-PNA.

1.1.2.3 Targeting double strands DNA (ds DNA) by PNA

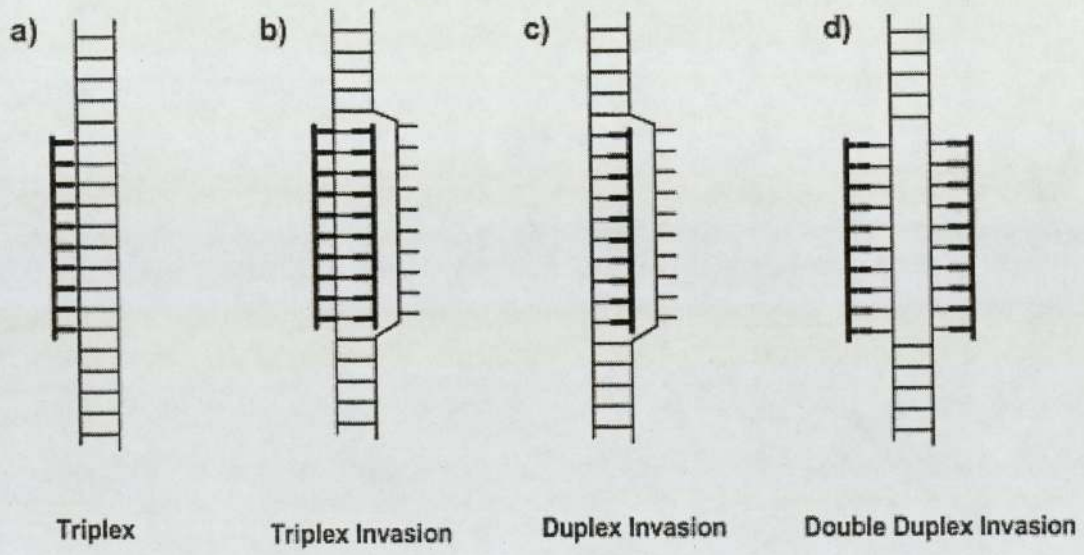


Figure 1.4 Four different types of PNA–dsDNA complexes. DNA is schematically drawn as a ladder, and the PNA oligomers are in bold. (a) A conventional triplex formed with cytosine-rich homopyrimidine PNAs. (b) The triplex invasion complex formed by homopyrimidine PNAs binding to a homopurine DNA target. (c) The duplex invasion complex formed by homopurine PNAs. (d) The double-duplex invasion complex which requires nonstandard nucleobases in the PNAs. [*Source from:* Nielsen, P.E., PNA technology, In *Peptide Nucleic Acid: Methods and Protocols*, edited by Nielsen, P.E. (2002), pp.4].

Figure 1.4 summarises four different types of PNA/dsDNA binding modes. Triplex invasion complex by homopyrimidine PNAs (**Figure 1.4b**) is the preferable strategy for targeting double strands DNA by PNA. This formation has been described in the previous section. Strands invasion based on PNA-DNA formation (**Figure 1.4c**) is that a single PNA strand binds its target via Waston Crick hydrogen bonds and DNA stretch complementary to the target for the PNA is displaced and left in a single stranded conformation. This formation appears to be limited to PNAs that form extremely stable PNA-DNA duplexes, such as PNAs with very rich purines [7]. Classical triplex (**Figure 1.4a**) involving a single strand of a PNA bound via Hoogsteen hydrogen bonds to a DNA duplex possessing a complementary target sequence[8, 9] and this formation appears to be required of a high cytosine content of PNA. However, such $(DNA)_2$ PNA triplexes are much less stable than the corresponding triplex invasion complexes, but are meaningful since this type of complex was the original aim of the PNA design. It should also be mentioned that double stranded invasion complexes (**Figure 1.4d**) can be formed by a set of pseudo-complementary PNAs. These PNAs make strand displacement by the formation of two duplexes of the Waston-Crick type. The formation of PNA-PNA duplexes is prevented by substituting adenine and thymine with diaminopurine and thiourail, respectively [10]. On the other hand, while the rate of formation of invasion complexes is highly sensitive to salt (which stabilises the DNA duplex), triplex-forming bis-PNAs efficiently bind (clamp) single stranded oligonucleotides, even at high concentrations of salt which have almost similar stability as found in triplex invasion complexes.

1.2 Antisense and Antigene Properties of PNA

1.2.1 What is the antisense technology?

ONs (Oligonucleotides, DNA) have sequences which are complementary to specific strands of RNA. Once delivered into a target cell, the oligonucleotide hybridises with its RNA complement and inhibits expression of the corresponding disease-relevant protein. In the 1970s, Paterson and his colleagues first reported that exogenous single-stranded

nucleic acids inhibited translation of RNA in a cell-free system [11]. One year later, Zamecnik and Stephenson did a cell-culture experiment in which it was shown that an oligonucleotide complementary to the 3' end of the Rous sarcoma virus could block viral replication in chicken fibroblasts [12]. With the quick development in the field of

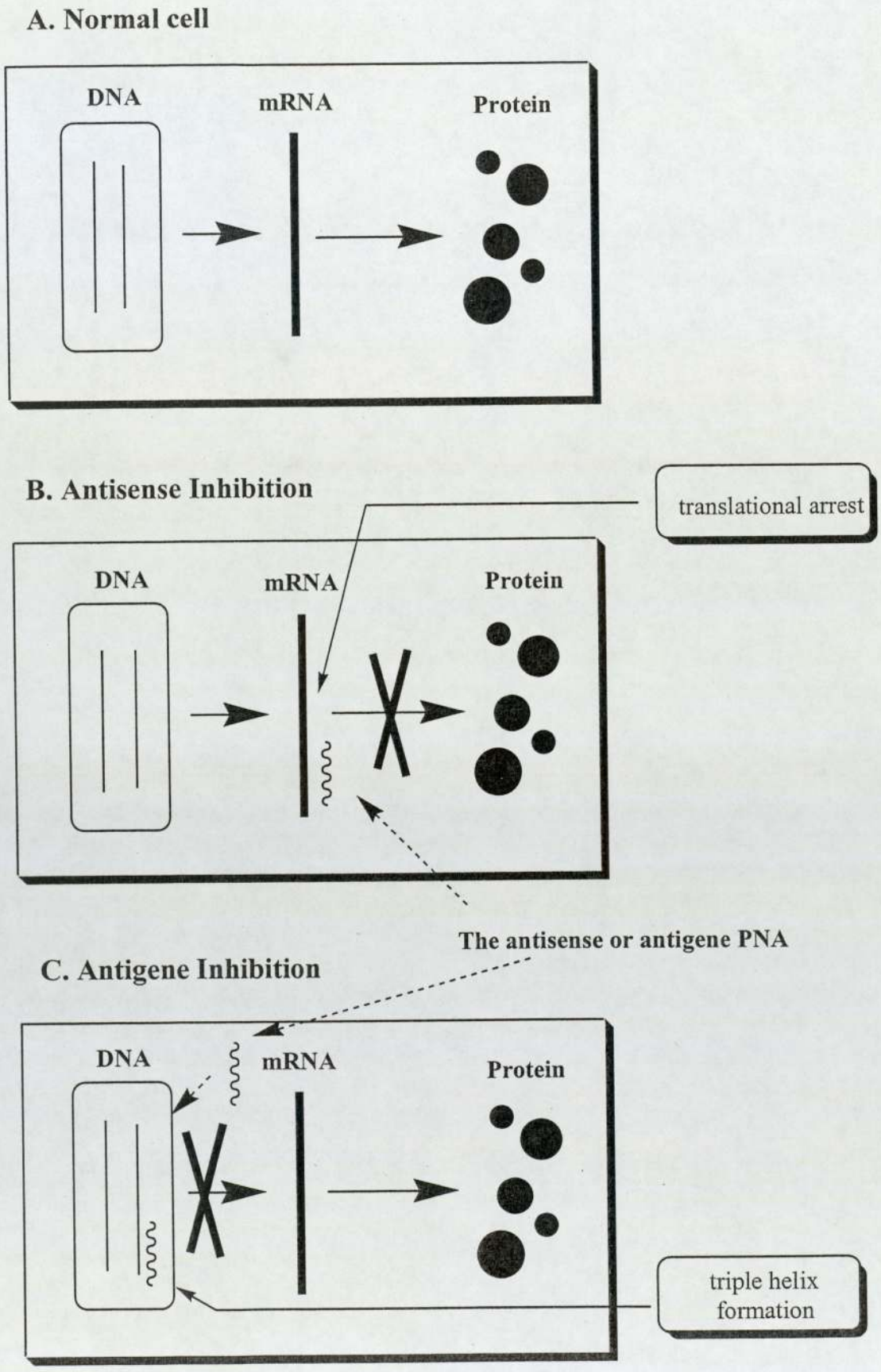


Figure 1.5 A simplified illustration of normal gene expression (A), antisense inhibition (B) and antigene inhibition (C)

nucleic-acid chemistry, the use of this technology for target therapy has made considerable progress.

The aim of antisense strategy is to intervene with gene expression by preventing the translation of proteins from mRNA. Nucleic acid analogs can be designed and created in order to recognize and hybridize to the correspondingly complementary sequences in mRNA and thereby inhibit its translation. Theoretically, an antisense oligonucleotide is a short deoxynucleotide which has a sequence complementary to a part of the targeted mRNA. In terms of Watson-Crick base pairing rules, the antisense oligonucleotides hybridize with the mRNA and sterically block the translation of this transcript into a protein [13] (**Figure 1.5B**). Such mechanism above is called translational arrest.

1.2.2 What is the antigene strategy?

A long term dream in biotechnology and human therapy is selective artificial control of gene expression. Now Oligonucleotides appear to be perfectly suitable for this purpose due to their unique base-base recognition properties. In principle, oligonucleotides or their potential analogs are designed to recognize and hybridize to complementary sequences in a given particular gene, whereby they should interfere with the transcription of such particular gene (Antigene). In this antigene approach, Oligonucleotides selectively bind sequence to genomic double-stranded DNA and interfere with transcription and DNA processing machinery via triple helix formation (**Figure 1.5C**).

There are two intrinsic advantages of the antigene over the antisense principle through the identification. On one hand, as compared with many mRNA copies needed to be targeted in the antisense approach, there are only two target copies of DNA in a diploid cell. This would extremely decrease the amount of oligonucleotide requried for the process. On the other hand, not only transcriptional activation and deactivation but also

targeted mutagenesis involved, targeted recombination and sequence-selective manipulation of genomic DNA can be achieved. However, the progress in this field has been slowed down since the investment of resources has been limited or because of ther practical difficulties. As a result, there is still no antigene oligonucleotide in clinical trials despite the potential benefits in general for biotechnology [14].

1.2.3 Antisense and antigene experiments of PNA

As discussed above PNAs can form a great diversity of complexes by Watson-Crick and Hoogsteen base pairing, which include PNA-DNA and PNA-RNA duplexes and PNA-$(DNA)_2$, $(PNA)_2$-DNA, $(PNA)_2$-RNA, $(PNA)_3$ triplexes and exhibit capacity to hybridise to complementary sequences of single-stranded RNA and DNA, with high affinity [1]. Therefore, PNA is proposed to be an ideal candidate for gene therapeutic drug design. However, well-identified targets and a well-characterized mechanism for their cellular delivery is needed. In the view of chemistry and biology, PNAs are stable molecules and have important effects on replication, transcription, and translation processes, as revealed from their applications. In addition, so far, it has found no sign or evidence of any general toxicity of PNA. Antisense and antigene experiments of PNA will be discussed in the following sections.

1.2.3.1 Inhibition of transcription

PNA should be capable of arresting transcriptional process due to its ability to form a stable triplex structure or a strand-invaded complex with DNA. As such complexes can bring about a structural hindrance to hinder the normal function of RNA polymerase, they are capable of working as antigene agents.

PNA triplex invasion complexes have high enough stability to arrest elongating RNA polymerase, especially when they are positioned on the template of DNA strand [15, 16]. Naturally, DNA recognition by proteins, such as RNA polymerase, is also totally

blocked by PNA binding [17, 18] with the concomitant complete distortion of the DNA helix. Therefore, PNA gene targeting at the DNA level should be highly efficient.

It is most interesting that RNA polymerases recognize PNA triplex invasion loops as transcription initiation sites, most likely because such loops are similar to the loop in a transcription initiation or elongation complex [19]. Therefore, PNA oligomers may function as artificial transcription factors with the PNA target as a "promoter", and it has even been reported that the expected effect takes place in cells that are cultured [20].

There is another encouraging application of PNA which is for antivirus. Reverse transcriptase (such as HIV), as one of the key enzymes in the life cycle of retroviruses, is very sensitive to PNA antisense inhibition. Reverse transcription of the RNA template is effectively arrested by PNA oligomers bound to the template [21, 22, 23]. This finding has brought about hope that PNA antiviral drugs could be developed, and one report has even shown that PNAs targeting the *gag-pol* gene can inhibit HIV from replicating in cell culture [24]. However, it needs to emphasize the requiremnet of an efficient cell-delivery system for PNA because very high PNA concentrations were necessary.

1.2.3.2 Inhibition of translation

The strong antisense effect of commonly used oligonucletides is generally considered to be achieved by the activation of RNase H and the subsequent cleavage of antisense ON-mRNA heteroduplex. The PNA-RNA complex is, however, not recognised by RNases [15] and the PNA mediated antisense effect is probably caused by steric arrest of translation [25]. On the other hand, due to its "unnatural" backbone, PNA is not degraded either by nucleases or peptidases and therefore is extremely stable in biological fluids, although the PNA-RNA duplex is not a substrate for RNase H [26, 27].

The potential of the application of PNA oligomers as antisense reagents was demonstrated in 1992 [15]. Cell free *in vitro* translation experiments have indicated that regions around or upstream the translation initiation (AUG) start site of the mRNA are the most sensitive to inhibition by PNA unless a triplex-forming PNA is used [15, 25, 28]. In addition, the research about nuclear microinjection of PNA showed that antisense efficacy was dependent on the length of the oligonuleotide molecule. Indeed, sensitive RNA targets for PNA oligomers are presumably targets at which the PNA can physically interfere with mRNA function, such as ribosome recognition, scanning, or assembly [25]. Interestingly, but not too surprisingly, it was demonstrated that splicing of pre-mRNA can also be inhibited by PNA bound only by duplex formation at the intro-exon junction [29, 30].

On the other hand, most efforts to develop antisense techniques have been concentrated on prokaryotic and eukaryotic systems, but the potential of antisense methods to highly selectively suppress the growth of specific types of bacteria by applying PNA oligomers should not be underestimated. There are two ways to specifically inhibit translation by binding PNA oligomers to RNA: either targeting ribosomal or mRNA. In particular a bis-PNA that targets the peptidyl-transferase center inhibited translation in cell free system at nanomolar concentration and the growth of *E.coli* permeable AS19 strain was inhibited at micromolar concentration, albeit with low potency, which was ascribed to poor uptake of PNA by the bacteria [31]. Microbe has also been targets for antisense PNA. The binding sites of antibiotics often map to the ribosomal RNA. In an effort to mimic the action of such antibiotics, PNA oligomers were targeted to functionally essential regions of the 23S *Escherichia coli* ribosomal RNA [32]. It appears that PNA oligomers can sequence-specifically target microbial ribosomal RNA and thereby efficiently inhibit translation. Antisense PNA oligomers have also recently shown to downregulate targeted genes in an amoeba (*Entamoeba histolytica*) [33], which suggests that PNA may be a feasible gene-therapeutic agent against amebiasis.

Although it is generally believed that PNA oligomers are poorly taken up by all cell types, somewhat surprisingly, Tyler and colleagues [34] discovered that unmodified PNA not only can be taken up by neuronal cells, but exerted an antisense effect *in vivo*. Later it became evident, that by using very high PNA concentrations and long incubation times, it was possible to demonstrate the uptake of biotinylated PNA by myoblasts, fibroblasts and other cell types and fluorescein-tagged PNA by lymphocytes [24]. In one recent study, scientists at Mayo Clinic discovered that PNA can across the BBB (blood-brain barrier) and target DNA in the brain [35].

1.2.3.3 Inhibition of replication

It is also possible to apply PNA to inhibit the elongation of DNA primers by DNA polymerase. Furthermore, it should be likely to inhibit DNA replication, if the DNA duplex is subjected to strand invasion by PNA under physiological conditions or if the DNA is single stranded during the replication process. Moreover, Taylor *et al* proved efficient inhibition of extrachromosomal mitochondrial DNA, which is largely single-stranded in the course of replication [36]. In addition, inhibition of the replication of mutant human mitochondrial DNA, mediated by PNA, is a potential approach toward the treatment of patients who suffer from ailments relevant to the heteroplasmy of mitochondrial DNA. Here, both wild-type and mutated DNA are present in the same cell. Consequently, experiments have proved that PNA has the capability of inhibiting the replication of mutated DNA under physiological conditions without affecting the wild-type DNA in mitochondria [36].

1.3 Other Applications of PNA

As mentioned previously, high *in vivo* stability of peptide nucleic acid, strong and specific hybridisation to RNA and DNA, as well as lack of toxicity at even relatively high concentrations have made PNA a highly promising tool for many medical and biological studies [15]. Apart from its role as lead compounds for the development of gene-targeted drugs applying antigene or antisense strategy there are other three major

groups of applications for this novel compound. First it can be used as a molecular tool in molecular biology and biotechnology, such as gene delivery, antivirus, *etc.* Second is the usage of PNA for diagnostic purposes and development of biosensors, such as biological targeting, PCR (polymerase chain reaction) method, *etc.* Third, PNA is related to the research of the basic chemistry [37, 38, 39, 40] for the improvement of basic architecture, e.g. for supramolecular constructs and to possibly generate a subsequent generation of PNA molecules.

1.3.1 PNA for gene delivery

Delivery of DNA vectors into the nucleus of cells in expected tissues is required in gene therapy. Thus, it has been needed to exploit the specific and strong binding of PNA to double stranded DNA in order to tag such vectors in the targeted cells [41], and more recently with targeting ligands conjugated to the PNA. These were either the NLS (nuclear localization signal) peptide that improves nuclear entry of the vector [42, 43] or ligands for cell specific receptors [44] that target the vector to cells expressing this receptor involved. In addition, a recent paper [45] has showed that the combined E6/E7 (two early viral genes) with PNA mediated a clear morphological change from suspension to adhereing state and the growth of cells was inhibited. These data could demonstrate a promising method for development of new "anti-gene therapeutics" against human cancers which is relative to the HPVs (human papillomaviruses).

1.3.2 PNA for biological targeting

Organ or cellular targeting of drugs *in vivo* can be achieved by cell-specific antibodies. A delivery system may be advantageous in which the antibody is first administered and then the drug with affinity for the antibody is administered (such as biotin-streptavidin system). Indeed, it has been shown by an antibody-PNA conjugate that the specific PNA-PNA recognition may be discovered in this type of targeting [46].

1.3.3 PNA in PCR methods

PNA-mediated PCR clamping is a versatile and sensitive method to identify single nucleotide changes in DNA molecules, even when these changes appear as a small percentage of the total DNA. As first described by Ørum, *et al.*[47], PNA clamping specifically blocks amplification of a given DNA template, while amplification of another template is allowed. This blockage is very efficient, even when the blocked template is in large excess, resulting in detection of a template present at a ratio of 1/20,000 in some reaction conditions. In addition, PNA clamping is faster and easier than most techniques that are presently used to identify point mutations with great sensitivity and specificity.

The tremendous sensitivity of the PNA clamping technique has proven useful in many different applications where the mutation is at a very low level and difficult to identify by standard point mutation detection methods. Low levels of variation detection in cancer [48] and mRNA editing [49] have been evaluated by PNA clamping methods. In addition basic point mutation detection for identitification of genetic disease is also allowed to carry out [50].

1.3.4 PNA in hybridization and probe technologies

A major advantage of using PNA for *in situ* hybridization procedures stems from the use of the low ionic strength hybridization conditions, which can prevent reanealing of complementary genomic DNA strands. Under these conditions, PNA probes can hybridize in a quantitative manner to denatured chromosomal DNA before the competition from renaturation occurs.

In various genetic diagnostic techniques, the excellent hybridization properties of PNA oligomers combined with its unique chemistry have been exploited. For example, PNA

probes for *in situ* hybridization give superior signal to noise ratios and usually allow milder washing procedures which result in morphologically better samples. Thus, PNA-FISH (fluorescence *in situ* hybridization) techniques have been developed for quantitative telomere analysis [51], chromosome painting [52] and viral and bacterial diagnostics in medical as well as environmental samples [53, 54, 55].

The advantage of the unique chemistry of PNA has been exploited in a variety of beacon [56] or light-up probe technologies [57]. For example, PNA oligomers are perfectly suitable for MALDI-TOF mass-spectrometry which yields very high and distinct signals, and this property has elegantly been exploited in an array hybridization technique in which mass-spectrometry detects the hybridized DNA or RNA by secondary hybridization of a PNA tag. Such tags are simply made with individual molecular weights, and thereby the presence of a specific PNA tag in the MALLDI identifies the presence of a specific hybridization and thus the gene variant. Moreover, it is the most important that many PNA tags can be analyzed in the same experiment [58]. Finally, PNA oligomers can be used as capture probes for DNA or RNA purification as well as sample preparation [59].

The examples given here illustrate the width of PNA applications, and thus it can be expected that further use of this versatile DNA mimic in these established techniques as well as in the development of novel applications will be further discovered.

1.4 Cellular Delivery of PNA

Despite many fantastic characters and initial rapid success, progress in the use of PNA as an antisense or antigene reagent was hampered by the observation that PNA is poorly taken up by cells in culture. However, several effective approaches for intracellular PNA delivery and subcellular trafficking and the enhancement of PNA solubility have been successfully demonstrated. Moreover, several transfection protocols for PNA have

now been established [60], such as microinjection, electroporation, transfected with DNA and direct delivery. These protocols are mainly for the cellular delivery of unmodified PNAs. On the other hand, as to the cellular delivery of modified PNAs, the method of entrapment in lipophilic carriers, combination with peptides or ligands has been developed. Among these different delivery strategies, the use of liposomes is a potential way to delivery PNA since liposomes can protect various molecules from the external medium and can deliver molecules directly into cells. Moreover, it is also possible to specifically target liposomes by coupling proteins or antibodies on their surface.

1.5 The Reasons of Choosing Liposomes as Carriers of PNA

1.5.1 Liposomes used as carriers of peptide and protein

The application of bioactive peptides and proteins as therapeutic agents is strongly limited in many cases by a lack of oral bioavailability and rapid clearance from the blood. Liposomes have been proposed to improve their delivery by functioning as a "microreservoir" for sustained release and as target-specific carriers that may also help to protect entrapped therapeutic agents from degradation.

Under current investigation liposomes are advantageous as particulate carrier systems because of (a) their relatively low toxicity, (b) their structural versatility allowing manipulation of their fate *in vitro* and *in vivo*, (c) their aqueous core decrease the risk of irreversible conformational changes of proteins induced by dehydration, and (d) liposomes are the most extensively investigated colloidal drug carrier system by far. So many novel explorations on liposomes have been carried out in combination to the peptides and proteins [61].

1.5.2 Liposomes used as carriers of antisense oligonucleotides

The development of gene therapy protocols has been rapidly growing since the first trials in the early 1990s. Since then, different gene therapy approaches have been proposed for the treatment of genetic diseases, cancer and viral infection using gene therapy [62]. For the practical administration of gene medicines, many delivery systems have been proposed, including both viral and non-viral vectors [63].

Liposomes have gained great interest as nonviral vectors as they are able to ionically bind oligonucletides for efficiently delivery to cells. The simplicity and the versatility of this technology have made liposomes important delivery systems for human gene therapy [64, 65, 66]. In addition to those previously mentioned, liposome possesses other general advantages as carriers of antisense oligonucleotides or as a delivery system for gene medicine. They are able to protect nucleic acid from degradative processes, they have a large loading capacity, accommodating also very large genes, potentially including chromosomes, and finally liposomes can be targeted to specific cells or tissue by conjugation with appropriate vector molecules such as monoclonal antibodies [67].

Cationic liposomes are able to interact with different types of negatively charged DNA molecules [68] generating complexes that have high loading efficiency [69]. Corey's group has developed a method that is highly efficient in intracellular delivery of PNA *in vitro* using cationic liposomes. This involves annealing of PNA with a short, biologically inert PO (phosphodiester) ODNs (oligodoexynucleotides) that subsequently serves as a carrier to form complexes with cationic liposomes [70]. From the recent report [71], the oligonucleotide delivery by cationic liposomes has been applied to inhibit the tumorigenicity of Kaposi's sarcoma.

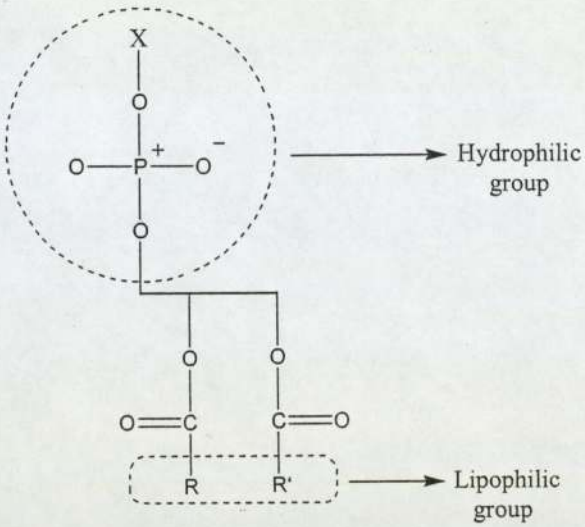
1.6 Liposome Technology

In 1947, when Bernard discussed microscopic studies of myelin figures established by ammonium oleate in water, the possible present of vesicle-shape structures in aqueous systems containing amphipathic molecules was first found [72]. Until 1962, Bangham and his colleague, Home, using an electron microscope, examined phospholipid dispersions in water by negative staining and obtained conclusive experimental evidence that phospholipids could assemble themselves to form "bag-like" structures. After that their colleague Gerald Weissman named them as liposomes. The history of the Babraham's work had been reviewed by Bangham [73]. One of the early papers became a "citation classic" and the work gave rise to a new field which has continued to expand for the last 30 years and has become a major area of research. In the last quarter century over 17,000 papers have been published with liposomes in their title and numerous books have been focused on the subject of liposomes [93, 94]. The widespread use of liposomes in biotechnology and medicine is further addressed in **section 1.9** and **1.10**.

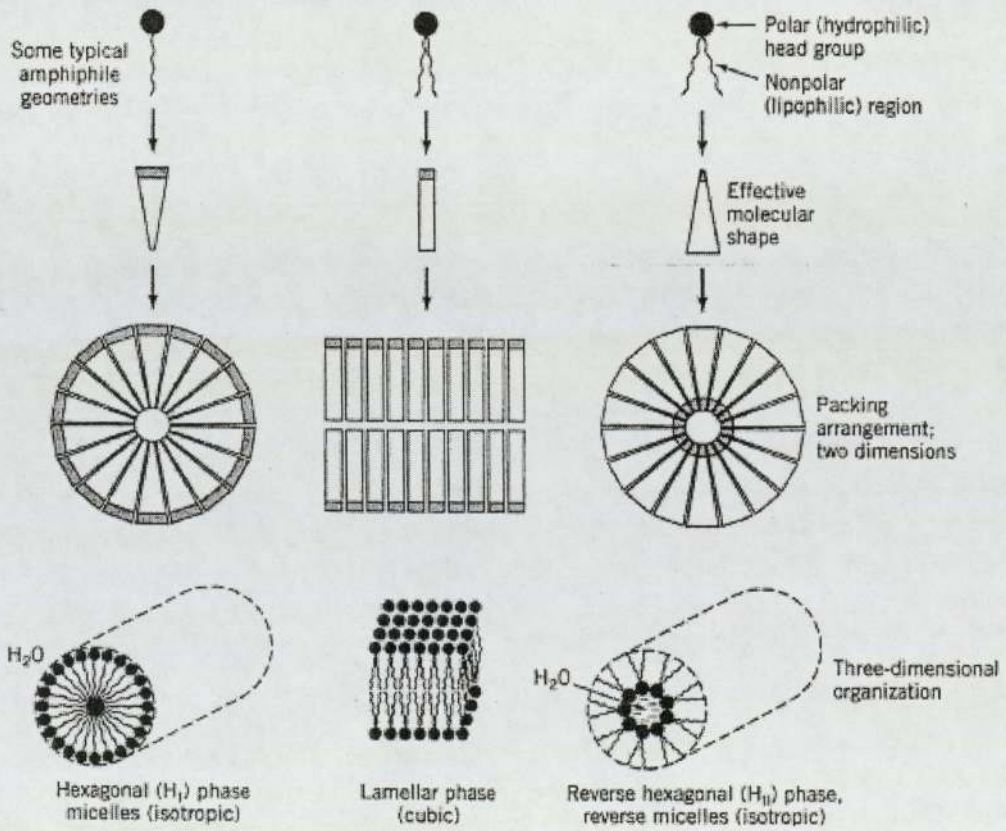
1.6.1 Molecular aspects of liposome structure and formation

1.6.1.1 Self assembly of lipids

Liposomes are microscopic aggregates of highly ordered lipid molecules that are normally dispersed in a hydrophilic solvent. The feature of liposomes, which is distinguished from other types of microparticles, is that their lipid components are in a lamellar assembly. When any molecule is dispersed in a solvent, its fate is depended on the polarity of the molecule, which reflects the distribution of the electron clouds surrounding it. Polar molecules tend to dissolve in polar solvents, whereas nonpolar ones are generally hydrophobic and only dissolve in nonpolar organic solvents. However, it is common to have both polar and nonpolar residues on the same molecule, in which it is described as amphiphilic substance and its solvent distribution characteristics are very complicated [73].



A



B

Figure 1.6(A) Chemical Structure of the Phospholipid. X part can be referred as nitrogen compound which contains hydroxyl group. R and R' can be referred as C_{12}~C_{18}. **(B) Dependence of phase behavior on the molecular geometries of the individual**

amphiphile molecules. [Figure 1.6B: *Source from*: Israelachvili, J.N., *et al.* (1977) Theory of self-assembly of lipid bilayer and vesicles. *Biochim. Biophys. Acta.* **470**, 185-201]

When the cross-sectional areas of the polar and nonpolar regions are approximately equal, the molecule is effectively cylindrical in shape and adjacent cylinders then arrange in a parallel alignment to form a two-dimensional monolayer, with the polar head groups (hydrophilic group) forming one surface of this monolayer and the fatty acid acyl chains (lipophilic group) as the other (**Figure 1.6A**). Two of these monolayers are organized back-to-back to form a bilayered lamella in which the hydrophobic regions are sandwiched between the polar groups and segregated from the water. Such an arrangement is typical of naturally occurring phospholipids such as the phosphatidylcholines, which establish the major component of cell membranes.

On the other hand, if there is a significant difference between the cross-sectional areas of the polar and nonpolar regions, the amphiphile molecules attempt to the shape of a wedge rather than a cylinder and are unable to arrange in a planar arrangement [74]. These differences may be due to steric factors that include head group size, variation in the number of fatty acyl chains, attractive or repulsive forces between adjacent head groups. In these situations, the aligning amphiphiles are forced to form curved structures, the most common of which include hexagonal phases and micelles. Depending on which end of the molecule is widest, each of these structures forms a normal or reverse configuration. Thus, if the polar head group is broader than the nonpolar region, curvature will be "positive," with the polar group forming the outer surface of the curve (e.g. normal hexagonal [H_I] phase, **Figure 1.6B left**). Conversely, if the nonpolar tail is broader than the head group, curvature will be "negative," with the fatty acyl chains forming the outer surface (reverse hexagonal [H_{II}] phase, **Figure 1.6B right**). Another important nonbilayer phase is the cubic phase (**Figure 1.6B middle**), alternative forms of which can resemble close-packed micelles. It is normal to accommodate nonbilayer-forming amphiphiles into lipid bilayers by including

amphiphiles of opposite shape tendency to compensate. Thus mixtures of wedges, reverse micelles, and hexagonal phases can be formulated with planar arrangement.

1.6.1.2 Lipid polymorphism

Polymorphism is the capability of amphiphiles to establish various structures and phases and is utmost important in normal biological processes which include cellular trafficking, digestion, absorption, and fusion. An important aspect is that different phases can interconvert, either by inside or outside migration of particular amphiphiles. For instance, increased temperature can improve the dynamic motion of the hydrocarbon chains, which effectively broadening the nonpolar region. Changes in pH can affect ionization of polar head groups and alter their diameter. Thus, bilayer to nonbilayer transitions can take place, leading to the loss of membrane barrier function or increased fusogenicity. These transitions can be discovered to design lipid-based carrier systems with proposed release properties which are triggered by changes in their local ambient conditions.

1.6.1.3 Aqueous dispersions of liposomes

The liposomal system is composed of phospholipids and additional agents. The common additional agents are CHOL (cholesterol), phosphatidate, *etc.* CHOL (**Figure 1.7A**), like phospholipids, CHOL is an amphiphile. Usually, normal micelles form spontaneously when the appropriate phospholipids are added to water and dilute freely in excess water. So phospholipid and CHOL are mixed with organic solvents when preparing liposomes (due to the struture of CHOL, its lipophilic property is stronger than that of hydrophilic character). When evaporating the solvents, a thin film can be formed which is composed of a bilayer with order arrangement of phospholipid and CHOL molecules. As to the alignment mode (**Figure 1.7B**) of them, the polar group of phospholipid molecule is curve, like a "crook", which is connected with the polar group, so from **Figure 1.7C**, we can see the hydrophilic group is combined with two lipophilic

groups (one is the hydroxyl side shain of phospholipid, the other is the lipophilic group of CHOL)[74].

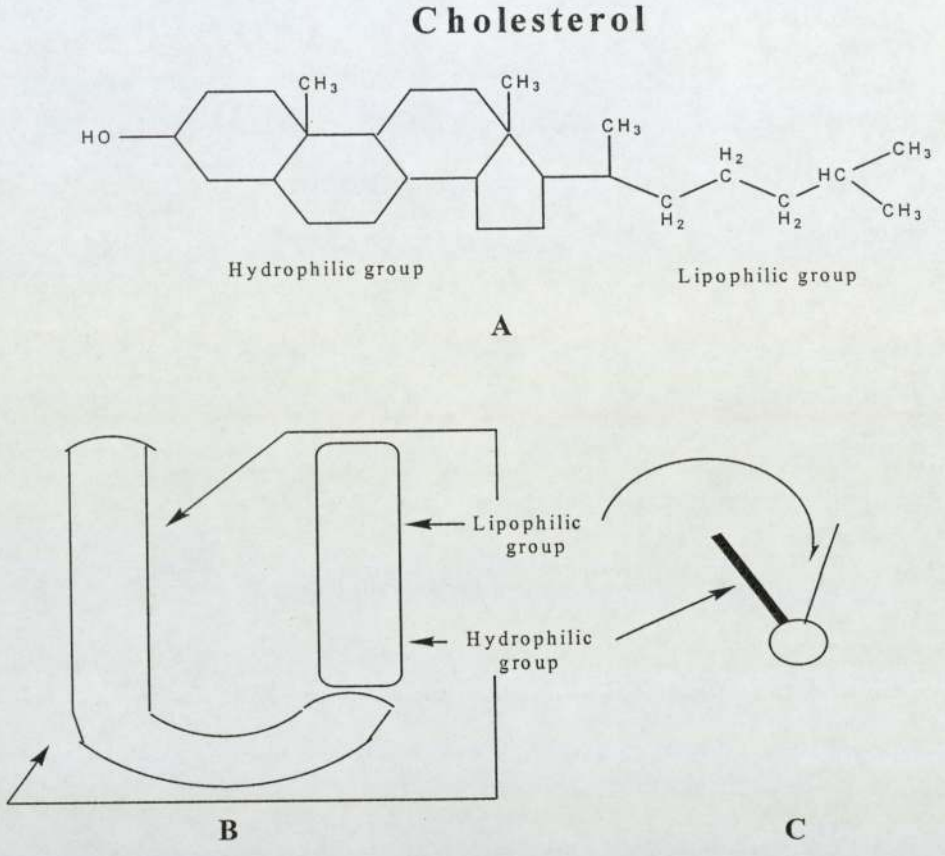


Figure 1.7 The structure of phospholipid and CHOL in bilayer

When lamellar phase lipid is diluted with excess aqueous phase, areas of lamellae are able to detach from the bulk lipid and round off to form sealed spherical liposomes (**Figure 1.8**), thereby ensuring that lipophilic chains at the bilayer "edges" are not exposed to the aqueous phase [73]. So the water-soluable drugs could be taken up in the central region with the surrounding of polar heads. On the other hand, lipid-soluable drugs are connected with lipophilic group of liposomal membrane. Under the microscope, the shape of the liposomes could be several strucures besides that standard round type normally reported [74]. Also from **Figure 1.8**, the position of various type drugs in liposomes is shown and several modifications on the surface as well.

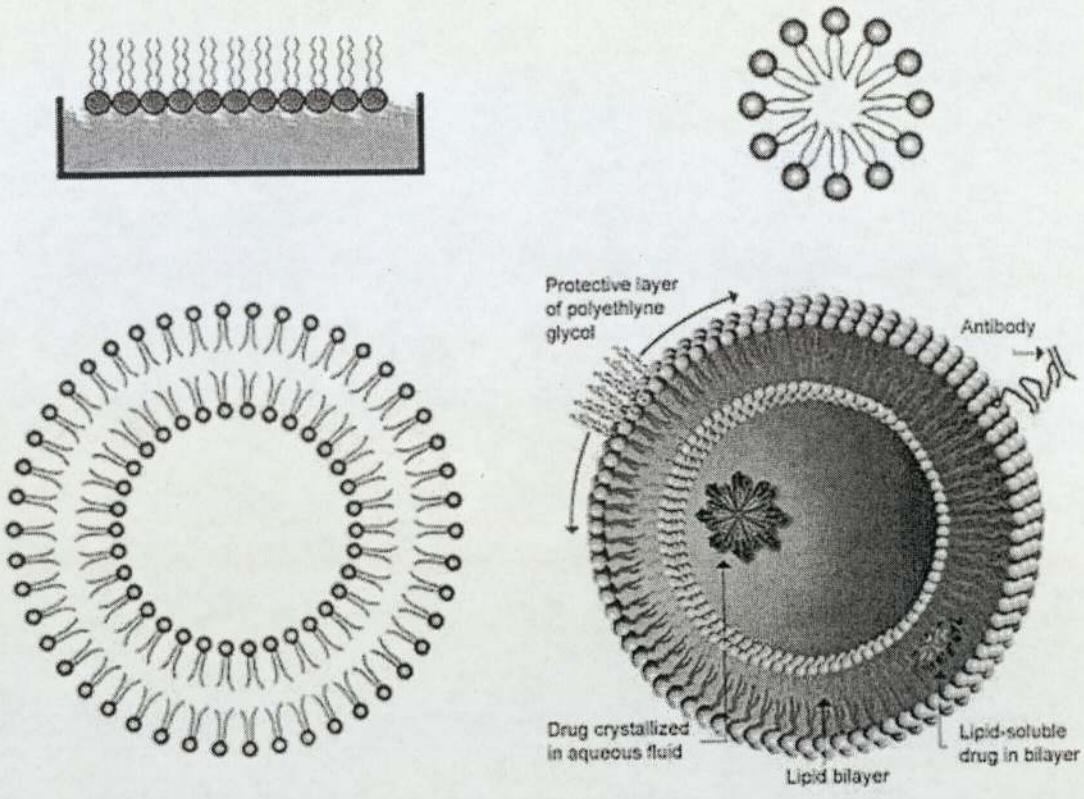


Figure 1.8 The structure of sealed spherical liposomes and its modifications. A diagrammatic representation of a unilamellar liposome showing the molecular structure of the lipid bilayer (left). The diagram at right side shows the positions of water-soluble and lipid-soluable drugs. In addition, some of the modifications can be made in order to tailor liposomes for specific applications, PEG (polyethylene glycol) is the modified phospholipid for preparing sterically stabilized liposomes, whereas antibody with the targeting moiety attached to the terminal region of a PEG chain. (*Source from*: www.cella.cn)

1.6.2 Characterization

There are numerous methods for the characterization of liposomes, based on the aspect of the system under investigation. Such aspects include those that are related to the overall structure of liposomes, such as vesicle size, lamellarity, surface potential, and morphology. Others relate to the stability of liposomes in terms of changes in vesicle size, general characteristics on storage and ability of vesicles to retain entrapped drugs in a given surroundings.

1.6.2.1 Overall structure of liposomes

Vesicle size can be determined either by hydrodynamic methods [75] using instrumentation that is appropriate for the size range of the vesicles. Values are expressed as zeta average mean in "nm"or "μm". A polydispersity index provides information on the range of vesicle size distribution. Size measurements can also be made in a zeta sizer which also determines the zeta potential of vesicles. Zeta potential values range from negative to positive and reflect the surface charge of the vesicles. The lamellarity of liposomes can be determined by small rangle X-ray scattering [76] which can discover whether bilayers are multivesicular structures.

Morphological observations of liposomes can be carried out by light microscopy and electron microscopy. Light microscopy, due to its resolution limits, is only suitable for large and giant liposomes [77]. For electron microscope imaging, liposome samples in suspension are firstly negatively stained and then directly examined by electron microscopy [76]. The technique can discover unilamellar, oligolamellar, or multilamellar structures. In addition, a three-dimensional image of liposomes is obtained by FFEM (freeze-fracture electron microscopy) where samples of liposome suspensions are quickly frozen, fractured at very low temperature and immediately replicated by appropriate means [77].

Other methods for the characterization of liposomes include spectroscopy, which can be used to measure the turbidity of a liposomal suspension [78]. Sets of calibration curves are then employed to devise semiquantitative estimates of vesicle size or concentration. On the other hand, the structure and dynamics of the acyl chains of liposomal phospholipids can be determined by infrared or Raman spectroscopy, and the hydration of phospholipid polar heads by Fourier transformation methods. Liposomal parameters such as T_c (liquid-crystalline transition temperature), or diffusion constants within bilayers can be measured by NMR (nuclear magnetic resonance) and EPR (electron paramagnetic resonance) [79].

1.6.2.2 The stability of liposomes

The stability of liposomes during storage can be monitored by the techniques for morphological analysis. However, these techniques cannot detect structural changes that lead to the leakage of drugs entrapped in the aqueous phase of liposomes. Drug retention by liposomes during storage can be determined by many techniques [79, 80]. Usually, it is appropriate that the stability of the bilayers within a given milieu is monitored when the lipid composition of liposomes for the entrapment of a drug (aqueous phase) is designed. One of the most effective methods is the use of CF (carboxyfluorescein) as marker solutes. CF self-searching at high concentrations acquire a green fluorescent color on excessive dilution. Suspended liposomes containing quenched dye appear as brown. However, destabilization of the vesicles leads to the leakage of some of the dye and its excessive dilution into the medium. Through the measurement by spectrofluorimetry, the result of fluorescence reveals the extent of leakage. For liposomal formulations containing drugs, drug leakage during storage can be detected by techniques that monitor entrapped and leaked content of drugs. This can be achieved by ultracentrifugation, dialysis, molecular size chromatography [80].

1.7 Classification of Liposomes

The liposome size can range from very small (0.02 μm) to large (>5 μm) vesicles. Furthermore, liposomes may have single or multiple bilayer membranes (**Figure 1.9**). The vesicle size is one critical parameter which determines circulation half-life of liposomes, and both size and number of bilayers influence the extent of drug encapsulation in the liposomes.

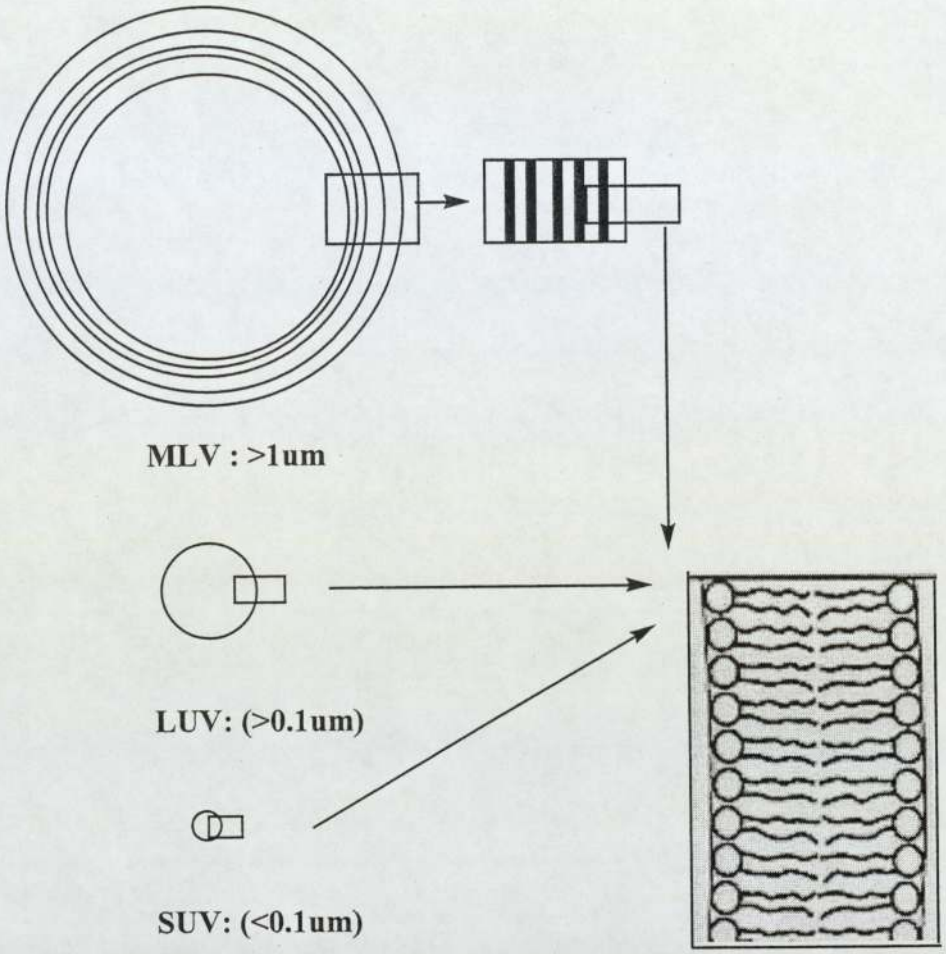


Figure 1.9 Types of liopsomes depending on size

Table 1.1 Lipsome classification based on the size and number of lamellae [a]

Type	size	Characteristics
MLV	> 0.1 μ m	More than one bilayer, moderate aqueous volume to lipid ratio, greater encapsulation of lipophilic drugs, mechanically stable upon long term storage, rapidly cleared by RES (reticuloendothelial system), useful for targeting the cells of RES.
LUV	> 0.1 μ m	Single bilayer high aqueous volume to lipid ratio, useful for hydrophilic drugs, high capture of macromolecules, rapidly cleared by RES.
SUV	<=0.1 μ m	Single bilayer and homogenous in size, thermodynamically unstable, susceptible to aggregation and fusion at low or no charge, limited capture of macromolecules, long circulation half-life.

a *Source from*: Ref. 81

On the basis of their size and number of bilayers, liposomes can be classified into three categories: (a) MLV (multilamellar vesicles), (b) LUV (large unilamellar vesicles) and (c) SUV (small unilamellar vesicles). The size and characteristics of these types of liposomes are listed in **Table 1.1**.

1.8 Method of Preparation of Liposomes

Liposomes usually require different methods of preparation. The most simple and widely used method for preparation of MLV is the thin-film hydration procedure in which a thin film of lipids is hydrated with an aqueous buffer at a temperature above the transition temperature of lipids. The drug to be encapsulated is included either in hydrophilic drugs or in lipophilic drugs. Thin-film hydration method produces a heterogeneous population of MLV which can be sonicated or extruded through polycarbonate filters to produce small and more uniformly sized population of SUV.

Although thin-film hydration is a simple technique, one of the major disadvantages of this method is its relatively poor encapsulation efficiency of hydrophilic drugs. Moreover, decreasing of liposome size further reduces the amount of encapsulated drug. MLV with high entrapment efficiency can be prepared by freeze-drying preformed SUV dispersion in an aqueous solution of the drug to be encapsulated [82]. Several methods have been developed for the preparation of LUV, including solvent injection, detergent dialysis, calcium induced fusion, and REV (reverse-phase evaporation) techniques. SUV can be prepared from MLV or LUV by sonication or extrusion. In addition, double emulsion method, multiple emlusion method and other injection methods also can be applied to prepare the liposomes which are matched to the different requirements.

1.9 Liposomes as Drug Delivery Carriers and Systems

Since discovery of liposomes, liposome carriers appear to have many features of an optimal delivery system and liposomes have been studied extensively *in vitro* and *in*

vivo [83, 84]. A wide variety of drugs have been encapsulated within liposomes. Liposomal drug delivery has been clinically used for the treatment of cancer, infectious disease, rheumatoid arthritis and for the detection of cancer and the preparation of vaccines [85].

Generally, the liposomes can be constructed with widely different physical structures, lipid composition, and surface properties, thus enabling a great deal of control over the formulation of their contents. For these reasons, they have been discoverd very widely as delivery vehicles [74, 86]. The basic aspects of the drug delivery mode are briefly summarized below.

1.9.1 The protection of entrapped drugs and improvement of drug stability

Usually, both the active material and the host are protected by virtue of the membrane barrier function. When enrapping drugs which are not stable and easy-oxidized into the liposomes, the stability of drugs will be improved in the human body due to the protection. This is because the drugs will avoid being degraded by the enzymes before entering the area of the targeting. After entering the area, the liposomes will ineract the cell membrane, such as endocytosis and fusion mechanisms, or are degraded by cellular enzyme to release the drugs.

1.9.2 Long-circulating effect

Liposomes also can be considered as the carriers which has long-circulating properties. Sustained release is dependent on the ability to vary the permeability characteristics of the membrane by control of bilayer composition and lamellarity. Different liposomes have different circulation time/stability in the human body, which can be from several minutes to several days. So the different half-life liposomes can be designed as

long-circulating agents based on the requirement. Controlled release is enabled by exploiting lipid phase transitions in response to external triggers such as changes in temperature or pH. By application of this release, the half-life of the entrapped biopharmaceuticals such as PNA will also be prolonged.

1.9.3 Reduction of drug toxicity

The drugs which are entrapped into the liposomes can be taken up by phagocytic cell systems which mainly focus on the liver and spleen. However, the accumulation of drugs in other sites such as the heart and kidney is limited. Therefore liposomes can decrease the toxicity of the drugs which are harmful and toxic, especially the cellular toxic drugs.

Generally, drugs used to treat diseases such as cancer have a narrow TI (Therapeutic Index) and can cause high toxicity to normal tissues. However, such toxicity may be minimized by decreasing delivery to critical normal organs. Meanwhile, it has been demonstrated that even a small decrease in drugs distributed to critical organs by encapsulation in liposomes can particularly reduce the drug toxicity [87]. Liposomes are taken up poorly by tissues such as heart, kidney, and GI tract, which are main sites for toxic side-effects of a variety of antineoplastic drugs. Therefore, by altering the biodistribution of drug away from normal tissues which are drug sensitive, liposome formulation may improve the TI. For instance, both free amphotericin B and doxorubicin result in severe dose-limiting nephrotoxicity and cardiac toxicity, then reformulation of these drugs in liposomes can reduce toxicity without changing the therapeutic efficacy. Now, it has been approved that liposome formulations of AmB (amphotericin B) and doxorubicin can be used in clinical practice.

1.9.4 Targeted delivery

1.9.4.1 Passive targeting

This is the basic characterization influencing liposome deposition, which is due to the nature targeting when liposomes are intravenously administered into the human body. Such delivery can be achieved by relying on natural attributes such as liposome size and surface charge to affect passive delivery to body organs. Because liposomes mainly naturally target certain cells such as Kupffer cells in the liver and macrophages of RES in the spleen, they will be the ideal carrier for the drugs which treat liver parasite and RES diseases. The CL (conventional liposomes) formulations of drugs and immunostimulators have been successfully used to target the cells of RES, and make significant improvement in the TI of the drugs [88]. In clinical trials, systemic administration of CL which contains muramyl peptide derivatives has led to enhancement in the tumoricidal properties of monocytes in patients with recurrent osteosarcoma [89]. Otherwise, for new vaccine formulations, liposomes have been used to increase the antigenicity of certain molecules and target them to the lymphatic system. Furthermore, CLs have also been used for targeting immunosuppressive drugs to lymphatic tissues (such as the spleen).

1.9.4.2 Active targeting

This targeting occurs when liposomes are modified with antibodies or other ligands (such as saccharide, plantal prothrombin, *etc*) to aid delivery to specific cell types and circumvent passive targeting. At the same time, drugs can be directed away from unintended targets. In a word, the liposomes which combine different ligands will have specific targeting to different targets.

However, active targeting is at a much less advanced stage of development than passive targeting, and so far there are no products on the market related to active targeting. However, active targeting of liposome encapsulated drugs may be achieved by coupling specific antibodies to vesicles (immunoliposomes). Now, it has been shown that immunoliposomes which contain DT (diphtheria toxin) can provide protection against the non-specific toxicity of DT during the period of cancer chemotherapy [90]. Thus,

long-circulating immunoliposomes can be designed to recognize and bind with greater specificity to target cells in systemic administration [86]. Otherwise, LCI (long-circulating immunoliposomes) have been proved to improve therapeutic efficiency of encapsulated doxorubicin in a murine lung tumor model [91]. Moreover, the effect of size on biodistribution of LCI has been studied in a rabbit model of myocardial infarction. Small sized LCI which contains infarct-specific AA (antimyosin antibodies) presented significantly lower accumulation in RES than CL with or without AA did. However, the accumulation of LCI-AA compared with CL-AA was higher in kidneys and lungs. The accumulation of large sized LCI in spleen was 2-fold higher than small sized LCI [88].

Because of non-specific uptake by the cells of RES, one of the major limitations of active targeting in the use of ligand-directed immunoliposomes has been their rapid clearance. Further, since LCL (long-circulating liposomes) is not rapidly cleared by RES, the development of LCL conjugated with ligands has become interested in this field again. However, many problems needed to be overcome still remain. To take an example, foreign immunoglobulin-ligands conjugated with immunoliposomes may induce immunogenicity and enhance clearance on subsequent exposure. The ligand conjugated with liposomes may increase the size of liposome and reduce extravasation, so that the targeting to intravascular targets could be limited [86]. Indeed, it has been proved that the interaction of antibodies with serum components can enlarge the size of LCL in the blood circulation, which in turn can promote their size-dependent uptake by the cells in spleen [92]. Consequently, immunoliposomes enter the cells by endocytosis, and then liposome contents would be degraded in the lysosomes finally, if they are not released from the endosome. Therefore, it would be proved only in drugs sensitive to lysosomal enzymes. Another obstacle [93] is the reduction in biological efficacy, which is related to the possibility that the non-monomeric drug is less efficacious than the monomeric drug and that the dissolution of the aggregated drug to monomers is slow.

1.9.4.3 Physical and chemical drug release

In the design of these liposomes, the permeability of liposomal membrane will be changed by severval physical and chemical factors, such as pH and local temperature changing, resulting in releasing the drugs selectively. The successful design is temperature-sensitive liposomes in the cancer treatment. In this case, the liposomes are prepared using phospholipid mixtures which have transition temperatures a few degrees higher than body temperature [94]. The liposomes which containing cytotoxic drugs was injected into the systemic circulation and then localized heat externally was applied to the target area, the liposomes can be induced to the release of drug in that region.

1.9.5 Cellular affinity and tissue compatibility

Because liposomes are vesicles which can be prepared with compositions similar to biological membrane structure, there is no harmful and stressful effect in the normal cells and tissues. At the same time, liposomes also have cellular affinity and tissue compatibility, which means that liposomes can adhere around the target cells for a long time and lead to increasing drug permeability and release the drugs to the target cells or tissues.

1.10 Applications of Liposomes in Drug delivery

When an existing formulation is not satisfactory (in terms of stability, toxicity, dose etc), new drug delivery systems such as liposomes are developed and such reformulation presents therapeutic efficacy and safety that is superior over the existing formulation. Indeed, compared with those non-liposomal formulations, liposome formulations of some drugs have shown an obviously increase in therapeutic efficacy and therapeutic indices in preclinical models and in humans [94]. Generally speaking, the therapeutic applications of liposomes can be classified into several categories briefly described below.

1.10.1 Formulation aid considerations

Hydrophobic drugs such as cyclosporin and paclitaxel are usually formulated in surfactants and organic co-solvents. These solubilizers may produce toxicity at the doses that are needed in delivering the drugs. In contrast, liposomes can encapsulate a broad range of water-insoluble (lipophilic) drugs, because they are made up of lipids which are relatively non-toxic, non-immunogenic, biocompatible and biodegradable molecules. Now, liposomes or phospholipid mixtures are being used as excipients to prepare better-tolerated preclinical and clinical formulations of several lipophilic, poorly water-soluble drugs such as AmB. Furthermore, liposomes as a vehicle have been used for the delivery of paclitaxel in preclinical studies, and their analogs as an alternative to the cremophor/ethanol vehicle. Thus, paclitaxel liposomes have been shown to deliver the drug systemically and increase the TI of paclitaxel in human ovarian tumor models [95] again deomstrating their applicability for the effectively delivery of a range biopharmaceutics.

1.10.2 Intracellular drug carriers

Drugs with intracellular targets, including PNA, are required to cross the plasma membrane for pharmacological activity. Although certain drugs are normally hard to enter cells, such as PALA [N-(phosphonacetyl)-L-aspartate], liposomes are able to increase cytosolic delivery of such drugs [96, 97]. To illustrate, PALA is taken up into the tumor cells through fluid-phase endocytosis (pinocytosis) and then diffuses out into the cytoplasm as the endosome pH drops, but the efficiency of pinocytosis is very limited. In contrast, liposomal delivery of drugs normally taken up into the cells by pinocytosis can be very effective [98], because liposomes compared with the extracellular fluid can contain higher concentrations of drug and the endocytosis process is more efficient than pinocytosis, that is, negatively charged liposomes are predominantly taken up by the cells in the endocytosis process. Until now, most

encouraging, the development of methods for controlling the intracellular trafficking of macromolecules by liposomes has been considered as one of the most attractive research subjects in the field of drug delivery system in the 21st century [99].

1.10.3 Remote-releasing carriers

Remote release systems are required for drugs such as Ara-C (cytosine arabinoside) that are rapidly eliminated *in vivo* with plasma concentrations at therapeutic levels for a prolonged period as well as for optimum pharmacological effects. Now, it is possible to design remote release liposome formulations with an extended circulation half-life and an optimized drug release rate *in vivo*. Take an example, Ara-C which is encapsulated in LCL is effective as a prolonged release system in treating murine L1210/C2 leukemia [100]. In addition, CL localizes in the cells of RES by phagocytosis and slowly release drugs from RES into the general circulation, so that may act as a sustained release depot. From another effort, in order to induce controlled release of doxorubicin, Zalipsky's group [101] has improved the method for the reversible attachment of methoxyPEG by using an amino-containing anchor and the final product is the natural phosphatidylethanolamine. Such remote release would also provide an effective method of controlling PNA release and action in various treatment regimes including treatment of carinomas.

1.10.4 Gene transfection

Although the starting material is liposomal, electrostatic interaction with DNA results in extensive loss of the original morphology, thus liposome-mediated gene transfection is somewhat confusing. In early studies, encapsulation was supposed to be prerequisite for successful transfection, however, such idea had to be revised because lipid-DNA complexes formed by simple mixing of DNA and cationic SUV were found much more effective. The basis of this approach is that positive charge in the cationic lipid neutralizes the considerably extended, polyanionic nucleic acid chain to form a highly

compact microstructure known as a lipoplex. Each of these particles carries a small net positive charge, which makes it able to approach and interact with the negatively charged cell membranes and perhaps enter by receptor-mediated endocytosis in the end (**Figure 1.10**).

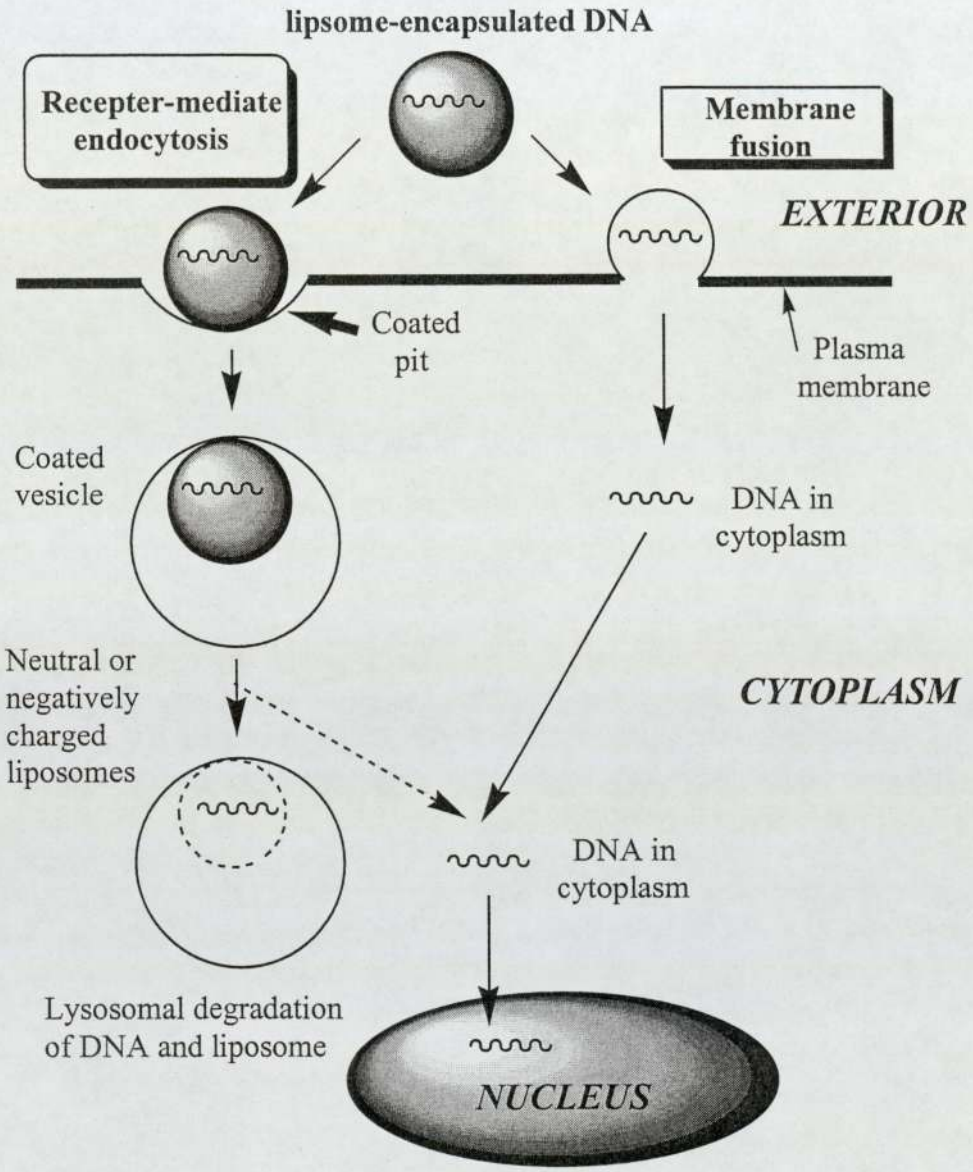


Figure 1.10 Proposed modes of cellular uptake of liposome-associated nucleic acid. (*Source from:* revise from the figure from Kirby, C.J., in R.A. Meyers, ed., *Encyclopaedia of Molecular Biology and Molecular Medicine*, VCH, Weinheim, 1996, pp.415-425.)

Lipoplexes are defined as complexes of cationic liposomes and nucleic acids [102]. Meanwhile, lipoplexes act as delivery systems to introduce the anionic polymers into cells *in vitro* and *in vivo* by adsorptive endocytosis and pore formation and fusion [103]. The main goals in the case of nucleic acids are gene delivery for DNA in antisense therapy. Since 1987, when Felgner's group published the first paper which described the use of cationic liposomes for gene delivery and transfection [104], the lipoplex-mediated delivery of nucleic acids and proteins is very influential in the research and discovery field. Therefore, the lipoplex as a carrier of nucleic acids (nucleic acid lipoplexes) or proteins (peptide lipoplexes) has been accessed and focused on in the future study.

1.11 Limitations of Liposome Technology

As described above, liposomes have a great potential in the aspect of drug delivery, however, it is notable that liposome-based drug formulations have not been applied in the market in large numbers so far. The reason is that there are some problems that have limited the manufacture and development of the liposomal formulations, which are outlined below.

1.11.1 Problems of stability

Stability is one of the major problems limiting the widespread use of liposomes, which is related to physical and chemical aspects. On one hand, chemical instability may be caused by hydrolysis of ester bond and oxidation of unsaturated acyl chains of lipids. Further, physical instability may be caused by drug leakage from the vesicles and aggregation or fusion of vesicles into larger particles. Both of these processes can affect the performance of the drug formulation *in vivo*, and therefore may influence the TI of the drug. For example, RES may rapidly remove large liposomes, which may result in

subtherapeutic plasma concentrations of the drug. However, some of the stability problems may be overcome by lyophilization. The final liposome product is freeze-dried with, in some cases, a cryoprotectant and is reconstituted with vehicle immediately prior to administration thereby increasing the shelf-life of the finished product by keeping it in a relatively more stable dry state. For instance, AmBisome ™, which is the first liposome product and is marketed in several countries, is provided as a lyophilized powder, and can be reconstituted with sterile water for injection [96].

1.11.2 Efficiency of encapsulation

Only if the encapsulation efficiency is appropriately high that therapeutic doses could be delivered in a reasonable amount of lipid, can liposome formulations of a drug be developed, because lipids may be toxic in high doses and also result in saturable pharmacokinetics of liposomal drug formulation. Some new approaches have been developed, which may provide high encapsulation efficiencies for hydrophilic drugs. For instance, it has been shown that active loading of the amphipathic weak acidic or basic drugs in empty liposomes can enhance the encapsulation efficiency [105]. However, because of the low affinity of drug for the lipid bilayers resulting in the low encapsulation efficiency, active loading is not suitable for hydrophobic drugs [96].

1.11.3 The procedure of sterilization

To determine that sterilization of liposome formulations is a suitable method is a major challenge because phospholipids are thermolabile and sensitive to sterilization procedures in which the use of heat, radiation and chemical sterilizing agents is involved. Indeed, the available method for sterilization of liposome formulations after manufacture is filtration through sterile membranes (0.22 μm). However, filtration is not suitable for larger vesicles and also unable to remove viruses. Moreover, because other sterilization approaches such as γ-irradiation and exposure to chemical sterilizing agents

can cause degradation of liposome components and may leave toxic contaminants, they are not recommended [106].

1.11.4 The deficiency of gene therapy

Cationic liposome-mediated transfection could be fully exploited after overcoming a number of technical problems. For example, liposomes are obviously less efficient than viral-vectors in their ability of transfection, and the DNA-lipid complexes are not stable in the size of particle [107] for long periods of time. In addition, there exists insufficient for targeting *in vivo* after systemic administration, and the toxicity of cationic lipids also limits the administered dose of the DNA lipid complex. Nevertheless, plasmid-liposomes complex may be more suitable to deliver genetic material by local administration or for other purposes such as genetic immunization. In addition, in terms of the research about cationic liposomes in DNA delivery, cationic liposomes may be toxic to the phagocytic macrophages, which could result in a great number of adverse effects [108].

Until now liposomology is still confronted with some major deficiencies, which include lack of control over drug release rate, sufficient loading of drugs for which pH and ion gradients do not apply, lack of means to override biological barriers (such as skin, BBB), therapeutically efficient active targeting and cheaper suitable raw materials (lipids). Therefore, the current challenge in research and development of liposome applications is to overcome these deficiencies.

1.12 Applications of Liposomes in the Medical Market

In 1960s, Rahman, *et al* [109] first applied the liposomes as carriers of drugs, and the first drug entrapped into liposome was put into trial on the clinical expreriment twenty years later. Up to now, liposome and lipid-complex formulations of doxorubicin, daunorubicin and AmB have been approved for clinical use in several countries.

However, doxorubicin and daunorubicin are highly effective antineoplastic drugs, but they can produce severe cardiac toxicity in humans. Thus, for free doxorubicin, the major dose-limiting factor is the potential for development of irreversible cardiomyopathy. Otherwise, it has been shown that LCL formulations of anthracyclines can improve the TI of the drugs against a variety of solid tumors by not only decreasing cardiac toxicity but also increasing drug accumulation in tumors [110]. It may be said that doxorubicin is an ideal candidate for encapsulation in liposomes, because it can be encapsulated into liposomes with high efficiency by means of an active loading [111]. In fact, Doxil ™, a LCL formulation of doxorubicin, which was the first liposome product approved for clinical use in USA, has increased circulation half-life compared with free drug [112] and shown a lower incidence of side-effects presumably which is achieved by reducing high serum peak concentrations of the free drug. In addition, mainly by reducing the dose-limiting cardiac toxicity, encapsulation of doxorubicin in liposomes has increased the TI and made possible dose escalation. Meanwhile, it proved that DaunoXome could reduce drug toxicity and improve life quality of the patients, which was as effective as conventional therapies.

AmB is an antifungal agent which is used to treat serious systemic fungal infections. However, AmB therapy may cause high rates of serious side effects which include nephrotoxicity, hypokalemia and anemia [113], which limit the dose levels and are the major reason for failure or discontinuation of therapy. Moreover, it has been demonstrated that liposome-based formulation of AmB has superior TI over the deoxycholate-based formulation (Fungi-zone ™), mainly because of a decrease in the dose which limits nephrotoxicity. In addition, some studies (primarily hypokalemia) have shown that the incidence of other side-effects is also lower [114]. Thus, the reduction in toxicity may be attributed to selective transfer of AmB from lipid bilayers to the fungus, which decreases the interaction of drug with human cell membranes. Further, it has been allowed to increase the AmB doses because of the reduction in the extent and frequency of side effects such as nephrotoxicity.

Liposome-based gene delivery is the focus of several specialized high-technology companies, including Vical (San Diego, CA, USA), Genzyme (Farmington, MA, USA), GeneMedicine (The Woodlands, TX, USA) and Megabios (Burlingame, CA, USA), which have products in clinical trials. Most interestingly, the Swiss Serum Institute (Bern, Switzerland) has successfully launched a liposomal vaccine against hepatitis A [115]. However, currently no liposome-nucleic acid based therapies have completed clinical trials.

In summary, although there are some problems associated with formulation and development of liposomes, an encouraging sign occurs due to the increasing number of clinical trials involving liposome and lipid-based products. With the fresher developments in the field, several companies are actively engaged in expansion and evaluation of liposome products for application in anticancer and antifungal therapy and for vaccines against diseases. Thus, it may be seen that further refinements in the liposome technology will promote the fully-fledged development of liposomes as drug carriers.

1.13 The aim of this project

As mentioned earlier, although PNA has a number of unique properties compared to currently used oligonuceotides, including higher affinity towards DNA or RNA target, resistant to nucleases or proteases, and minimal non-specific interaction with proteins, medical applications are limited by its inefficient intracellular delivery. The application of liposome is a potential method to delivery PNA since liposomes have useful properties, such as protecting various molecules from the external medium and delivering molecules directly into cells. With respect to this issue, we aim to synthesize oligomers of the PNA and to entrap PNA into liposomes and to ascertain liposomal formulation characteristics which influence PNA entrapment within the lipid vesicles.

Chapter 2 Experimental Procedures

Part 1 Synthesis of Peptide Nucleic Acid Oligomers

2.1 Materials and General Procedures

2.1.1 Chemicals

All the chemicals were bought from Aldrich, unless specified otherwise. Solvents and chemicals purchased from commercial suppliers were used without further purification unless indicated otherwise.

Solvents:

DCM (Methylenechloride), DMF (Dimethylformamide), NMP (N-methylpyrrolidone), DIEA (Diisopropylethylamine), NMM (Methylmorpholine), TFA (Trifluoroacetic acid), anisole, TFMSA (Trifluoromethanesulfonic acid), m-cresol, AC_2O (Acetic anhydride), pyridine.

Solutions:

Table 2.1 The solutions used for PNA synthesis

Solutions	Stability	Special Requirement
DIEA in DCM (5/95)	2 months	None
TFA/DCM/Anisole (50/45/5)	2 weeks	Light-sensitive
Pyridine/NMP/AC_2O (50/48/2)	1 weeks	Light-sensitive
Pyridine/NMP/AC_2O (2/2/1)	1 weeks	Light-sensitive
HATU in NMP (0.2mol/L)	2 weeks	None
Boc-PNA-A(Cbz)-monmer (0.26 mol/L)[a]	2 weeks	None
Boc-PNA-C(Cbz)-monmer (0.26 mol/L)[b]	2 weeks	None
Boc-PNA-G(Cbz)-monmer (0.26 mol/L)[c]	2 weeks	None
Boc-PNA-T(Cbz)-monmer (0.26 mol/L)[d]	2 weeks	None

Boc/Z PNA monomers

a. N-((N^6-Benzyloxycarbonyl)adenin-9-yl-acetyl)-N-(2-Boc-aminoethyl)glycine (Boc-PNA-A(Cbz)-monmer)
b. N-((N^4-Benzyloxycarbonyl)cytosin-1-yl-acetyl)-N-(2-Boc-aminoethyl)glycine (Boc-PNA-C(Cbz)-monmer)
c. N-((N^2-Benzyloxycarbonyl)guanine-9-yl-acetyl)-N-(2-Boc-aminoethyl) glycine (Boc-PNA-G(Cbz)-monmer)
d. N-(2-Boc-aminoethyl)-N-(thymin-1-yl-acetyl)glycine (Boc-PNA-T-monmer)

HATU [O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] and MBHA resin (4-methylbenzhydryl amine resin) as well as PNA monomers were purchased from Applied Biosystems. Linker [2- (N-Boc-2-Amino ethoxy) ethoxy acetic acid dicyclohexylamine salt] was from Riedel-de Haen. Fluorescein [5(6)-FAM-NHS, 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester] was from Fluka.

2.1.2 Apparatus

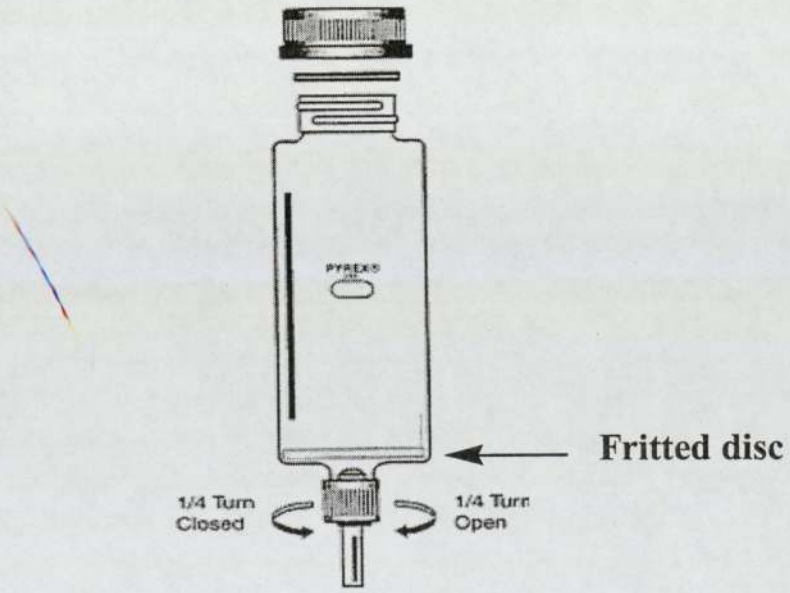


Figure 2.1 Reaction Vessels with cap and fritted disc

TLC (Thin-layer-chromatography) was carried out using aluminium backed Merck Silica Gel 60 F_{254} plates and observed under UV light 254 nm. Proton NMR spectra were recorded at 250 MHz on a Bruker AC-250 instrument. Melting points were

operated on a heated stage microscope melting point apparatus, uncorrected. Analytical RP-HPLC (reverse phase - high performance liquid chromatography) of the PNA oligomers was performed at 25°C using a C_{18} column on a Hewlett-Packard HPLC system (1100). Glass reactor (20 ml) was a vessel with cap and fritted disc, and glass reactor of 6 ml was a fritted filter funnel, both from Aldrich (**Figure 2.1**)

2.2 Chemical Synthesis

2.2.1 Synthesis of N-(2-Boc-Aminoethyl)-N-(thymin-1-ylacetyl)glycine

2.2.1.1 Thymin-1-ylacetic acid

To thymine (**A**) (2 g, 15.87 mmol) and potassium carbonate anhydrous (2.192 g, 15.87 mmol) in dry dimethylformamide (DMF, 48 ml) was added methyl bromoacetate (2.4 g, 15.87 mmol). The mixture was stirred overnight under argon. The mixture was filtered by Hirsh funnel and the filtrate was then evaporated by rotatory evaporator to dryness in *vacuo*, and further dried in a dessicator for two days. The solid residue was cooled to 0 °C in the ice for 10 minutes, then treated with water (15 ml) and 4 M hydrogen chloride (aqueous, 0.64 ml) and stirred for one hour. The precipitate was collected by filtration through Hirsch funnel and washed with water (3 x 10 ml). To the precipitate was added water (16 ml) and aqueous sodium hydroxide (2 M, 8 ml), and the solution was boiled for 20 minutes at 120°C. The mixture was cooled to 0°C or ice for 10 minutes, then treated with 4 M hydrogen chloride (aqueous, 5.4 ml) and stirred for 50 minutes. The title compound was collected by filtration through Hirsch funnel and washed with water (3 x 10 ml). At last, it was dried over P_2O_5 under 60 °C overnight (16 hours), yielding 1.21g (41.4%). Rf: 0.48 (50% methanol and 50% chloroform), m.p. 215-216 (214-217, data of m.p. from Ref. 116); ^{1}H NMR δ 11.33 (S, 1H, COOH), 7.46 (S, 1H, NH), 4.31 (S, 2H, CH_2), 1.72 (S, 3H, CH_3).

2.2.1.2 N-(2-Boc-Aminoethyl)-N-(thymin-1-ylacetyl)glycine

Ethyl N-(2-Boc-aminoethyl)glycinate (**D**) (0.6 g, 2.40 mmol), DhbtOH (3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine,0.437 g, 2.68 mmol) and thymin-1-ylacetic acid (**C**) (0.49 g, 2.68 mmol) were dissolved in DMF (9.3 ml) and DCM (9.3 ml) was added to the above. The solution was cooled to 0°C in an ice bath, and DCC (dicyclohexylcarbodiimide) (0.60 g, 2.90 mmol) was added. The ice bath was removed after 12 hours and stirring was continued for another 2 hours at room temperature. The precipitate was removed by filtration and washed with DCM (2 X 3.3 ml). To the combined filtrates was added more DCM (29 ml) and the solution was washed successively with dilute aqueous $NaHCO_3$ (3 X 22 ml), dilute aqueous $KHSO_4$ (2 X 22 ml), and finally brine (1 X 22 ml). The precipitate in the organic phase was removed by filtration, whereupon the organic phase was dried by Na_2SO_4 and evaporated to dryness, in *vacuo*. The oily residue was dissolved in DCM (7 ml) and filtered, and the title compound was precipitated by the addition of petroleum ether (5 ml) at 0°C. The precipitate was redissolved in DCM and precipitated once more with petroleum ether. This afforded T monomer ethyl ester (**E**), which was suspended in THF (tetrahydrofuran, 8 ml) and 1M LiOH (aqueous, 5 ml) was added. The mixture was stirred for 45 min at rt and then filtered to remove residue. Water (1.8 ml) was added to the solution and it was washed with DCM (13 ml). Additional water (1.3 ml) was added and the solution was washed once more with DCM (7 ml). The aqueous solution was cooled to 0°C and the PH was adjusted to about 2 by dropwise addition of 1 M HCl. The title compound (**F**) was extracted with EtOAc (9 X 10 ml). The combined extracts were dried in Na_2SO_4 and then evaporated to dryness, in *vacuo*. The residue was evaporated once from MeOH and afforded Boc-T-OH **(F)** after drying overnight (0.4 g, 43%). ^1H NMR: 11.33 (m, 1H), 7.29 (ma.) and 7.24 (mi.) (s, 1H), 6.90 (ma.) and 6.68 (mi.) (m, 1H), 4.62 (ma.) and 4.46 (mi.) (s. 2H), 4.18 (mi.) and 3.97 (ma.) (s, 2H), 3.39 (ma.) and 3.30 (mi.) (m, 2H), 3.16 (ma.) and 3.04 (mi.) (m, 2H), 1.78 (s, 3H), 1.37 (ma.) and 1.39 (mi.) (s. 9H).

2.2.2 Solid phase synthesis of PNA oligomers

2.2.2.1 Downloading MBHA resin

MBHA resin (1 g, 0.124 mmol) was weighed into a 20 ml glass rector (**Figure 2.1**), fitted with a sintered glass filter. The resin was swelled in excess DCM for 3 hours with agitation. DCM was removed by suction, and the resin was washed with DCM (2 x 3 min), DIEA/DCM (5/95, 2 x 3 min) and finally DCM (2 x 3 min) under shaking. The resin was dried by suction for 1 min. Two solutions were prepared as follows: Solution 1: 1.20 ml Monomer (120 mg Boc-PNA-T-monomer in 1.2 ml NMP), 2.64 ml DIEA (0.5 M DIEA in NMP), 0.16 ml NMP; Solution 2: 1.08 ml HATU (83 mg HATU in 1.08 ml NMP), 0.92 ml NMP. The two solutions were mixed and allowed to preactivate for 30 sec. The preactivated solution was added to the resin and coupling proceeded for 75 min under shaking. The resin was washed with DMF (2 x 1 min), DCM (4 x 1 min), DIEA/DCM (2 x 3 min) and DCM (4 x 1 min). Cap reagent (Ac_2O) was added in excess and capping proceeded for 2 hours. The resin was washed with DCM (7 x 1 min) and dried by suction for 15 min and qualitative Kaiser test was negative at this stage. (see **section 2.2.2.2**)

2.2.2.2 Performing qualitative Kaiser test

Reagent A was prepared as follow: Solution 1: 40 g phenol in10 ml water and heated gently until all solid was dissolved. Solution 2: 65 mg KCN was dissolved in 100 ml water and 2 ml KCN solution was diluted with pyridine to a total volume of 100 ml. Reagent A was obtained by mixing Solution 1 and Solution 2.

Reagent B was prepared as follow: dissolving 2.5 g ninhydrin in 50 ml ethanol (99%). When performing qualitative Kiaser test, 1 drop reagent A and 2 drops reagent B were added to 2~5 mg lightly dried resin and heat to 90°C for 2 min. The blue colour means positive Kiaser test, yellow colour means negative.

2.2.2.3 Estimation of level of downloading

50 mg of downloaded PNA resin was swelled in DCM overnight, and then deprotected using TFA/DCM/anisole (50/45/5, 2 x 3 min). The resin was washed with DCM (2 x 1 min), DMF (2 x 1 min), DIEA in DCM (2 x 3 min, 5/95) and DMF (2 x1 min) under shaking. The following solutions were prepared: Solution 1: Fmoc-Gly-OH (90 mg dissolved in 0.6 ml NMP). Solution 2: HATU (108 mg dissolved in 0.6mL NMP). Solution 3: NMM (0.06 ml). These solutions were mixed and allowed to preactivate for 1 min. The preactivated mixture was added to the resin and allowed to couple for 60 min under gentle shaking. The resin was washed using DMF (2 x 1 min), DCM (2 x 1 min) under shaking. The Kaiser test was carried out and the result was negative. The dry Fmoc-Gly resin, about 1 μmol (1.9 mg) with respect to Fmoc, was put into one of the match silica UV cells (10 mm). Freshly prepared piperidine was dispensed in DMF (1/4, 3 ml) into two UV cells. The resin mixture was shaken for 2-3 min. The resin beads were allowed to settle on the bottom of the UV cells. The cells were placed in a UV spectrophotometer using the cell containing piperidine only as the reference cell. The absorbance at 290 nm was measured and recorded (**Table 3.1**).

2.2.2.4 Oligomerization

The procedure is seen as **Figure 2.2**.

2.2.2.4(i) Deprotection

1. 120 mg of downloaded PNA resin was transferred into the reaction vessel (20 ml). The dry resin was swelled in excess DCM and shaken for 10 min. DCM was removed by suction.
2. TFA/m-cresol (95/5) was added to cover the resin and the mixture was shaken (4 ml, 2 x 3 min). TFA/m-cresol (95/5) was removed by suction.
3. The resin was washed with DCM (4 ml, 3 x 1 min), followed by DMF (3 ml, 3 x 1 min).

4. The resin was then washed with DIEA/DCM (5/95, 3 ml, 2 x 1 min), DCM (4 ml, 3 x 1 min).
5. A Kaiser test was then carried out. If the result was negative, step 2 to 4 was repeated. If positive, the experiment was continued as follows.

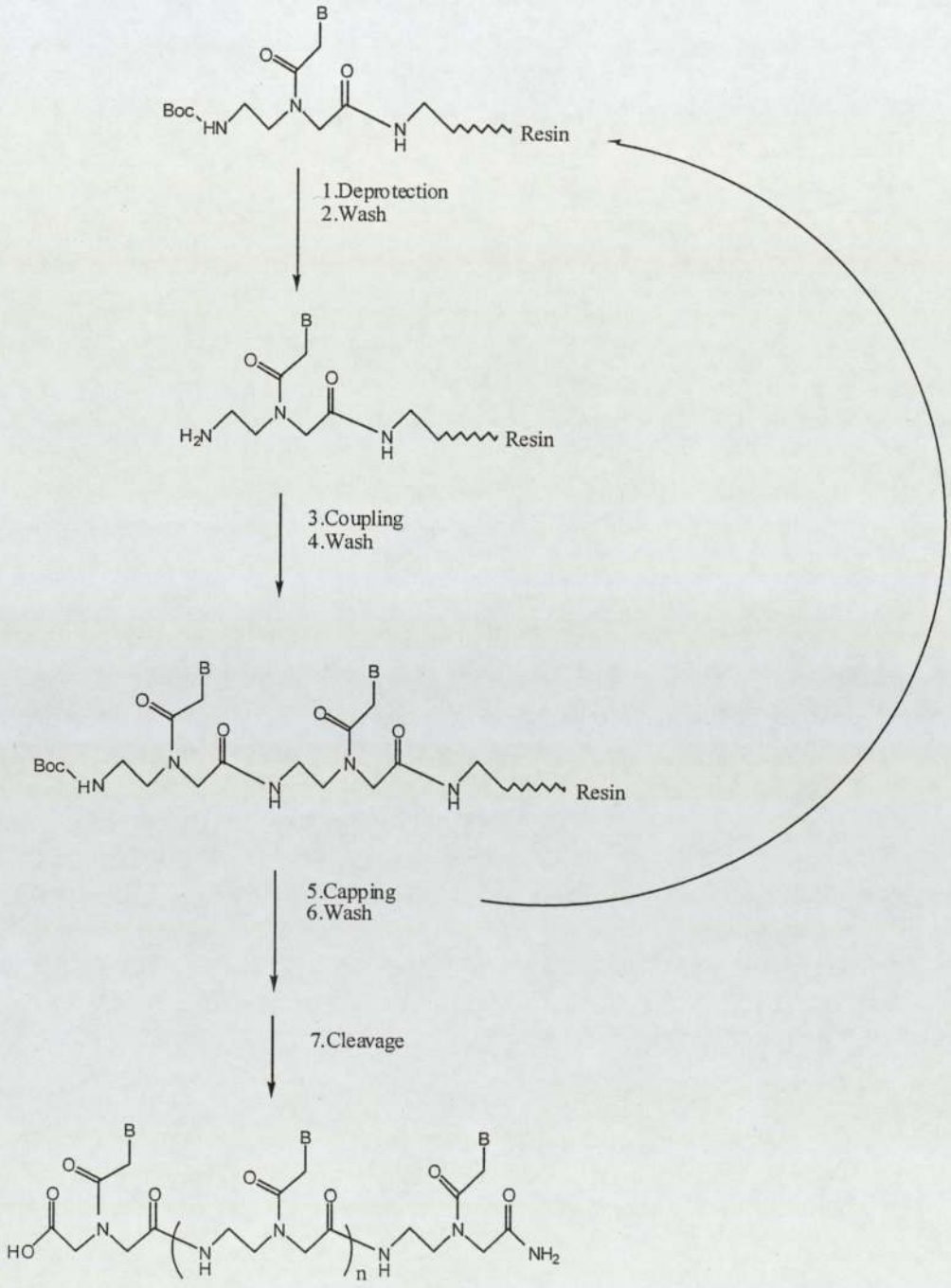


Figure 2.2 Schematic representation of the solid phase PNA synthesis [*Source from*: Koch, T. PNA oligomer synthesis by Boc chemistry. In Peptide Nucleic Acid: Methods and Protocols, edited by Nielsen, P.E. and Egholm, M. (1999), pp.23]

2.2.2.4(ii) Coupling and capping

1. A monomer in NMP (0.26 M, 0.5 ml) and HATU in NMP (0.202 M, 0.5 ml) were mixed, and DIEA in pyridine (0.5 M, 0.5 ml) was then added. The monomer was allowed to preactivate for 1 min.
2. The activated monomer solution was added to the resin and allowed to couple for 15 min while shaking the reaction vessel gently. The reaction mixture was removed by suction.
3. The resin was washed with DMF (3 ml, 3 x 1 min), followed by DCM (4 ml, 3 x 1 min).
4. A Kaiser test was then carried out. If the result was negative, procedure should be repeated from 2.2.2.4 (i) step 4. If positive, the experiment was continued as follows.
5. The oligomer was capped (Ac_2O/NMP/pyridine, 2/50/48, 4 ml) for 1 min under shaking.
6. The resin was then washed with piperidine /NMP (10/90) for 1 min.
7. The resin was washed with DMF (3 ml, 3 x 1 min), followed by DCM (4 ml, 3 x 1 min).
8. The oligomer was elongated by repeating the procedures from step 2 of the section 2.2.2.4 (i) until a desired sequence was obtained and the final procedure was ended at the step 3 of 2.2.2.4 (ii).

2.2.2.5 Labelling of PNA

The PNA-bound resin (63 mg) was weighed into a 6 ml glass rector. TFA/m-cresol (95/5) was added to cover the resin and the mixture was shaken (2 ml, 2 x 6 min). The resin was washed with DCM (2 ml, 3 x 1 min), DMF (1.5 ml, 3 x 1 min), DIEA/DCM (5/95, 1.5 ml, 2 x1 min) and DCM (2 ml, 3 x 1 min). The Kaiser test was then carried out. If the result was negative, deprotection should be repeated. If positive, the experiment was continued as follows. Linker [(2- (N-Boc-2-Amino ethoxy) ethoxy

acetic acid- dicyclohexylamine salt, 0.26 M, 0.4 ml)] and HATU in NMP (0.202 M, 0.4 ml) were mixed, and DIEA in pyridine (0.5 M, 0.4 ml) was added and allowed to preactivate for 2 min. The activated linker solution was added to the resin and allowed to couple 60 min while shaking the reaction vessel gently. The reaction mixture was removed by suction and the resin was washed with DMF (2 ml, 3 x 1 min), followed by DCM (2 ml, 3 x 1 min). The result of the Kaiser test was negative at this stage.

Labeling with Fluorescein was done on-column while the PNA was still immobilized on the resin. Labeling solutions were prepared according the **Table 2.2** for 2-μmol scale.

Table 2.2 Preparation of the labeling solutions

Lable	**Labeling Reagent**	**Weight/mg**	**DMF/μL**	**DIEA/μL**	**Reaction duration (time)**
Fluorescein	5(6)-FAM-NHS	35	900	33	90

TFA/m-cresol (95/5) was added to cover the resin (28.3 mg) containing the linker and the mixture was shaken (2 ml, 2 x 10 min). The resin was washed with DCM (2 ml, 3 x 1 min), DMF (1.5 ml, 3 x 1 min), DIEA/DCM (5/95, 1.5 ml, 2 x 1 min) and DCM (2 ml, 3 x 1 min). The Kaiser test was then carried out. If the result was negative, the above deprotection procedure was repeated. If positive, the experiment was continued as follows.

The labeling solution was transferred into the glass reactor containing the resin and the labeling was proceeded for 90 min. The labeling solution was discarded. The resin washed with DMF (3 x 1 ml), followed by DCM (2 x 1 ml).

2.2.2.6 Cleavage of PNA from the resin

The resin (25 mg) was swelled in TFA (1 x 30 sec). The TFA was removed by suction. The equal amounts of the following two solutions were prepared and added into the resin. [Solution 1: TFA/DMS (dimethylsulphide)/m-cresol (1/3/1, 0.5 ml) and Solution 2: TFA/TFMSA (9/1, 0.5 ml), both freshly prepared], and the mixture was shaken for 1 hour. The reaction mixture was removed by suction. The resin was washed using TFA (1 x 30 sec). The cleaving mixture [TFMSA/TFA/m-cresol (2/8/1, 1.1 ml)] was added and shaken for 110 min. The cleaving mixture was transferred to a glass tube containing cold ether. The resin was washed using TFA (1 ml, 2 x 30 sec). The combined TFA solution was transferred to the glass tube. The PNA was precipitated by centrifugation (20 min, 3,000rpm, -4°C). The ether was discarded, the PNA oligomer was left at the bottom of the glass tube. Fresh cold ether was added and the PNA oligomer was resuspended in the ether and precipitated by centrifugation. The ether was decanted off and the white precipitate was dried. The crude PNA was stored at -18°C.

2.2.2.7 HPLC identification

Analytical RP-HPLC of the PNA oligomers was performed at 25°C using a C_{18} column on a Hewlett-Packard HPLC system (1100) and monitored at 260 nm. Eluent A (0.1% TFA in water) and B (0.1% TFA in acetonitrile) were used. Flow rate was 1 ml/min. The following gradient was used (**Table 2.3**).

Table 2.3 HPLC Program

Time (min)	Eluent B(%)
0	0
2	0
30	35
40	100
50	100
60	0

Part 2 Entrapment of PNA into Liposomes

2.3 Materials and Instruments

2.3.1 Materials

PC (Egg phosphatidylcholine) and DSPC (distearoyl phosphatidylcholine) were purchased from Lipid Products, Nutfield, Surrey, UK. CHOL, SA (Stearylamine) and DCP (Dicetyl Phosphate) were from Sigma, Poole, Dorset, UK. All other reagents were of analytical grade.

2.3.2 Instruments

The z-average diameter of liposomes was measured on the Brookhaven ZetaPlus Particle Sizing Analyser (Brookhaven Instruments Corporation, New York, USA) at 25°C by diluting 20μl of the dispersion to the appropriate volume with doubly-filtered (0.22μm pore size) distilled water. The zeta-potential, which is an indirect measurement of the vesicle surface charge, was measured in 0.001M PBS at 25°C on the Brookhaven Zeta Potential Analyser (Brookhaven Instruments Corporation, New York, USA). The Freeze drier was from AdVantage. Centrifugation was achieved using a Beckman J2-21M/E centrifuge. Fluorescein data of liposome-entrapped or liposome surface-complexed fluoresent PNA were recorded using spectrofluorimeter (Spectra MAX GEMINXS) from Molecular Devices Ltd (Germany) on a 486-514 nm.

2.4 Entrapment of PNA into liposomes

2.4.1 Entrapment of PNA

2.4.1.1 Preparation of the Lipid film

The chloroform/methanol (9:1) solution of lipids (Neutral liposomes were composed of 16 μmol PC and 8 μmol CHOL; Cationic liposomes was composed of 16 μmol PC, 8

μmol CHOL and 4 μmol SA; Anionic liposomes was composed of 16 μmol PC, 8 μmol CHOL and 4 μmol DCP) were placed in three 100-mL round-bottomed spherical Quick-fit flasks respectively and the solvent, using a rotary evaporator at about 37 °C, was removed to leave a thin lipid film on the walls of the flask. The film was flushed for about 1 min with oxygen-free nitrogen (N_2) to ensure complete solvent removal and to replace the air.

2.4.1.2 Preparation of MLV

2 ml of warm distilled water was added to hydrate the dry film in the flask, the stopper was replaced and the flask was shaken vigorously by hand or mechanically by vortexing while maintaining the temperature above the liquid crystalline transition temperature (T_c) until the lipid film has been transformed into a milky suspension. The suspension was allowed to stand at > T_c for about 1 hour whereupon multilamellar liposomes of diverse sizes were formed.

2.4.1.3 Preparation of SUV

The milky suspension at >T_c was sonicated, using a titanium probe slightly immersed into the suspension. This step produced a slightly opaque to clear suspension of SUV of up to 90 nm~100 nm in diameter. The SUV suspension was mixed with the labeled PNA solution (1OD) and the non-labeled PNA solution (1OD) and rapidly frozen before Freeze-drying overnight under vacuum.

2.4.1.4 Rehydration of the powder

Water (0.1 ml), pre-warmed at >T_c, was added and the mixture was agitated vigorously at > T_c. The sample was kept at > T_c for about 30 min. The process was repeated with a further 0.1 ml water. After 30 min at >T_c, 0.8 ml PBS (pre-warmed at >T_c) was also repeated and the sample was allowed to stand for 30 min at >T_c. It now contained multilamellar Dehydration-Rehydration Vesicle (DRV) (size range about 0.5-5.0 μm in diameter) with entrapped and non-entrapped labeled and non-labeled PNA.

2.4.1.5 Separation of DRV liposome-entrapped from non-entrapped PNA

The suspension was centrifuged at 18,000 rpm for 50 min at 4 °C and the pellet was resuspended (PNA-containing DRV) in water (or PBS). The process was repeated at least once to remove the remainder of the non-entrapped material and the final pellet was resuspended in an appropriate volume (e.g. 2 ml) of water or PBS.

2.4.2 Mixture of PNA (labeled and non-labeled) with empty DRV liposomes

The procedure was similar to **2.4.1**. The main difference was that the DRV was mixed with the labeled PNA solution (1OD) and the non-labeled PNA solution (1OD) after rehydration of the powder.

2.4.3 Determination of the release of liposomes after entrapped the PNA

The fresh DRV liposomes (neutral, cationic and anionic liposome) were prepared which contained the labeled and non-labeled PNA. The free PNA was removed by centrifugation. The entrapment of PNA was measured. The PNA release was measured over time in various conditions: PBS at 4 °C and 37 °C for up to 10 days. The PBS (1 ml) was added into the pellet (DRV). The pellet was dissolved and divided into two parts (0.5 ml). And then the solution was incubated in PBS (diluted to10 ml) at either 4 °C or 37 °C. 1 ml solution (dilute to 5ml using the PBS) was taken to centrifuge and used to measure the released PNA under the following time intervals: 0, 1, 4, 24, 48, 72, 120, 240 hours.

2.5 Particle sizing and zeta potential measurements

2.5.1 Particle Size determination – Theoretical considerations

2.5.1.1 Particle sizing

When a beam of light passes through a colloidal dispersion, the particles scatter some of the light in all directions. When the particles are very small compared with the wavelength of the light, the intensity of the scattered light is single form in all directions (Rayleigh scattering), as to larger particles (>250nm diameter), the intensity is depended on the angle (Mie scattering) [117].

Particles suspended randomly in a liquid are subject to Brownian motion. Brownian motion and the size of the particles could be measured DLS (dynamic light scattering), or photon correlation spectroscopy. Brownian motion, the random movement of particles, is due to the impinging by the solvent molecules that surround them. Usually, the velocity of Brownian motion is relative to the size of the particle. Small particles diffuse faster and large particles are slower. The size of the particle is calculated from the diffusion coefficient, which is defined as a measurement of the velocity of movement of particles, using the Stokes – Einstein equation shown below. The diameter that is measured by DLS is a value that refers to how a particle diffuses within a fluid so it is referred to as a hydrodynamic diameter.

$$d(H) = \frac{kT}{3\pi\eta D}$$

The Stokes – Einstein equation

[$d(H)$ = hydrodynamic diameter; D = diffusion coefficient; k = Boltzmann's constant; T = absolute temperature; η = viscosity].

Because Brownian motion is affected by viscosity, this requires to be accounted for. In addition, as the viscosity of a liquid is related to its temperature, accurate result of the

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temperature is important and needs to be stable. The higher size limit of accurate measurement of particle size using this method is determined by the beginning of sedimentation which is in turn dependent upon the density of the sample. The lower size limit relies upon sample concentration, refractive index and the power of the laser.

Another consideration is about the interaction of light with the particles. The magnitude of the intensity of the scattered light is proportional both to particle diameter and inversely concerned with laser wavelength (Rayleigh approximation). From the view of diameter, mixtures of different sizes of particles are difficult to measure because the larger particles will scatter so much more light compared to smaller particles. On the other hand, as to the relationship with wavelength, lasers with shorter wavelengths will give a much greater intensity of scattered light and Mie theory expatiate that when particles are larger than around 50nm, they exhibit variable light scattering according to the angle at which the scattered light is detected. Thus, measurement of particle size using this method is not direct forward and requires care for correct use and interpretation.

2.5.1.2 The principle of determining particle sizing in Brookhaven zeta Plus particle sizing analyzer

The time dependence of the intensity fluctuation is most commonly analysed using a digital correlator. Such an apparatus investigates the intensity autocorrelation function (**Figure 2.3A**) and the autocorrelation function is an exponential decay (**Figure 2.3B**). Each monodisperse population of particle sizes produces its own respective autocorrelation function, which is a single exponential decay. Mixtures of more than one size population produce sums of exponentials. NNLS (Non-Negatively Constrained Least Squares) algorithms can be used to extract true size distributions. As to the results of samples, please see **Appendix 1, 3, 5, 7, 9.**

$$C(\tau) = Ae^{-2\Gamma t} + B$$

where $C(\tau)$ is the autocorrelation function and A and B are instrumental constants

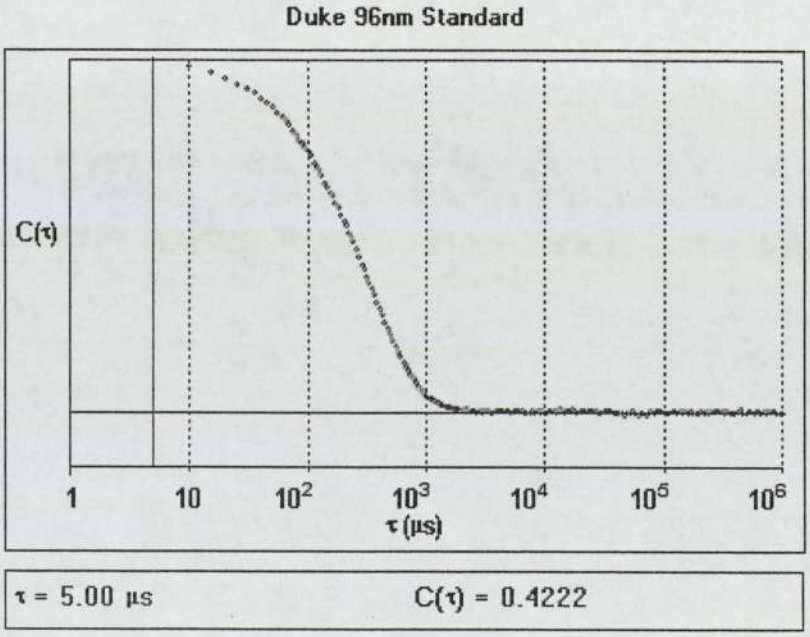
$$\Gamma = q^2 D$$

where q is the scattering vector and D the diffusion coefficient

$$q = \frac{4\pi n \sin\left(\frac{\theta}{2}\right)}{\lambda} \quad and \quad D = \frac{k_B T}{3\pi\eta d}$$

where n is the refractive index, θ is the scattering angle λ is the laser wavelength, k_B is Boltzmann's constant T is the temperature in Kelvin, η is the liquid viscosity and d is the particle diameter

A



B

Figure 2.3 The calculation of the autocorrelation function (A) and an exponential decay of the autocorrelation function (B)

(*Source from*: www.bic.com)

2.5.2 Zeta potential - Theoretical and practical considerations

Generally, the liposomes which contain acidic phospholipid, such as DCP, are negative charge. On the other hand, the positive liposomal formulation includes basic phospholipid, such as SA. The liposomes which have no ions are neutral. The charge of the liposomes is important in the aspects about entrapment percent, liposomal stability, target tissue distributions and effect of target cells.

2.5.2.1 What is Zeta Potential?

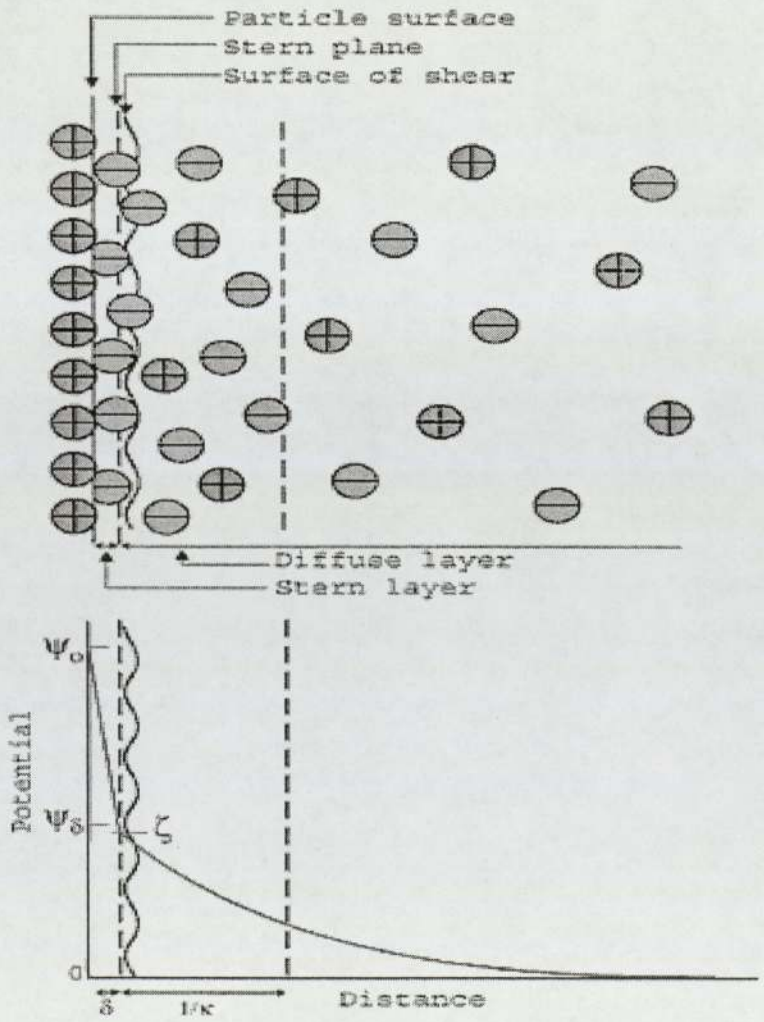


Figure 2.4 Zeta potential (ζ), (*Source from*: www.bio.com)

Zeta potential is the charge that develops at the interface between a solid surface and its liquid medium [118]. The charge on the particle surface affects the distribution of ions in the region directly surrounding the particle. The result of this is that there is an increased concentration of counter ions, which are the ions of opposite charge to that of the surface of the particle, in the nearby interfacial region. This increased concentration of counter ions close to the charged surface of a particle is referred to as the electrical double layer, which is formed in the region of the particle-liquid interface.

From the **Figure 2.4**, we can see the number of negative ions increases and the number of positive ions decreases as one moves away from the surface, the electrical potential becoming zero when the concentrations are equal. The liquid layer (**the upper part of Figure 2.4**) surrounding the particle exists as two layers. In the "Stern layer" or inner region, directly surrounding the particle surface, ions are tightly bound and in the "diffuse layer" or outer region, further away from the particle surface, ions are less strongly attached. Within this diffuse layer, there is a notional boundary beyond which ions do not move with the particle when it travels. The potential or charge at the surface of shear which is a little further out from the Stem plane is referred to as the zeta potential (ζ, **the lower part of Figure 2.4**) and the thickness of double layer is given by $1/\kappa$ and the decay of potential with the increasing distance is also shown. Due to the surrounding counter ions, the observed zeta potential is always less than the surface potential (Ψ_O) or the Stern potential (Ψ_δ). Because each particle and its most closely associated ions move through the solution as a unit, however, this impacts upon the electrokinetic measurement of the "surface" charge of the particle and hence analysis of the particle in association with the bound counter ions is by far the most practical measurement. Thus, zeta potential has become the standard function for particle surface charge and is often the key to understanding dispersion and aggregation processes.

2.5.2.2 The principle of determining zeta potential in Brookhaven ZetaPlus zeta potential analyzer

The principle of determining zeta potential by microelectrophoresis is very simple. A controlled electric field is employed by electrodes entered into the sample suspension and this causes the charged particles to move to the electrode of opposite polar. Viscous forces acting on the moving particle tend to oppose this motion and an equilibrium is rapidly established between the effects of the electrostatic attraction and the viscous drag strength. The particles therefore reach a constant speed. This velocity is depended on the electric field strength. As the dielectric constant and viscosity of the liquid calculated from temperature are known, the zeta potential will be calculated. It is usually referred as to the particle mobility that is the velocity under unit field strength. In order to all practical aims, the relationship between mobility, μ, and zeta potential, ζ is quite simple and in water at 25 °C can be expressed as: $\zeta = 12.85\ \mu$. In practice, zeta potentials can lie in the range from -100 to +100 mV. (see **Appendix 2,4,6,8,10**)

2.5.3 Determination of particle size and analysis of Zeta potential

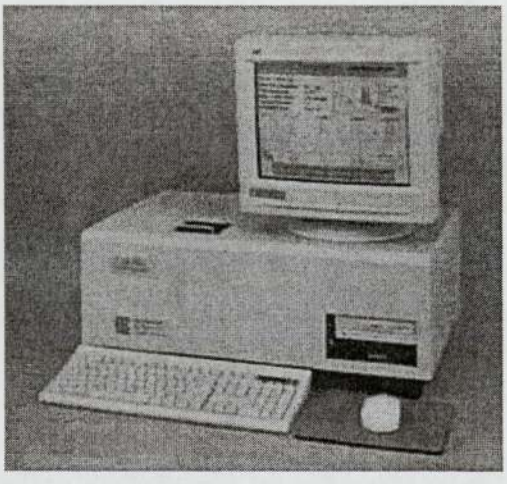


Figure 2.5 ZetaPlus Particle Size and potential Analyzer

(*Source from*: www.bic.com)

ZetaPlus Brookhaven Potential sizing and potential Analyser (Brookhaven Instruments Corporation, New York, USA) was used in my working procedure (**Figure 2.5**), DLS was used in order to determine particle size with a photomultiplier detector fixed at 90°

(wavelength is 659 nm). The sample (temperature is equilibrated to 25°C) diluted in distilled filtered water or PBS, was put into a small ultrasonicator for analysis. 2 or 3 mL of suspension are required to make the measurements. The NNLS algorithm was used and the software supplied by Brookhaven Instruments has automatic quality control analysis of the data giving error messages for samples on which the size interpretation cannot be relied. The parameters for passing the quality control analysis are: average count rate: The detected count rate should be between 10 k and 500k counts *per* second. Polydispersity Index: This needs to be less than 0.6 (but ideally between 0.08 and 0.5). Software is ZetaPlus particle sizing software ver. 3.37.

When samples fail in any aspect of the quality criterion, this will be shown. In all cases, the polydispersity index is also shown, as this is a good guide to the distribution of the size data. The sample concentration should be optimised to provide a sufficient count rate because it is important as this determines the duration of measurement when set to automatic. Samples during my procedure were always analysed under automatic measurement duration in order to ensure that complete data is collected for accurate size determination. The software was designed to give 3 measurements of each sample. Results are shown as average mean with standard error.

Obtaining the results of zeta potential determination was used by adding the facilities of Brookhaven's ZetaPlus to combine zeta potential determination with particle sizing analyzer. It applies the electrophoretic light scattering and the LDV (Laser Doppler Velocimetry) method to detect the particle velocity. By application of an electric current, the intensity of scattered light from interference fringes at the crossing point of two beams of laser fluctuates with a frequency that is relative to the speed of the particle movement and thus the zeta potential can be calculated. Particle mobility is calculated by applying the principle of outlined in the above section. All zeta potential measurements were at neutral pH and wavelength is also 659 nm. The applied current is based on the conductivity of the sample. The sample was prepared the same to that of particle sizing and software is zeta potential analyzer ver. 3.23.

Chapter 3 Results and Discussion

Part 1 Synthesis of Peptide Nucleic Acid Oligomers

3.1 Synthesis of the N-(2-Boc-Aminoethyl)-N-(thymin-1-ylacetyl) glycine (T monomer)

Peptide nucleic acid monomers containing the four natural nucleobases are commercially available. However, they are expensive and of limited availablility (to our knowledge only Applied Biosystems supplies these monomers). To gain some experience on synthetic organic chemistry and, to a less extent, save some expenses on the purchase of the PNA monomers, my project was started with the synthesis of N-(2-Boc-aminoethyl)-N-(thymin-1-ylacetyl) glycine (**F,** T monomer). The synthesis of **F** was essentially followed a reported procedure [116], as shown in **Figure 3.1**.

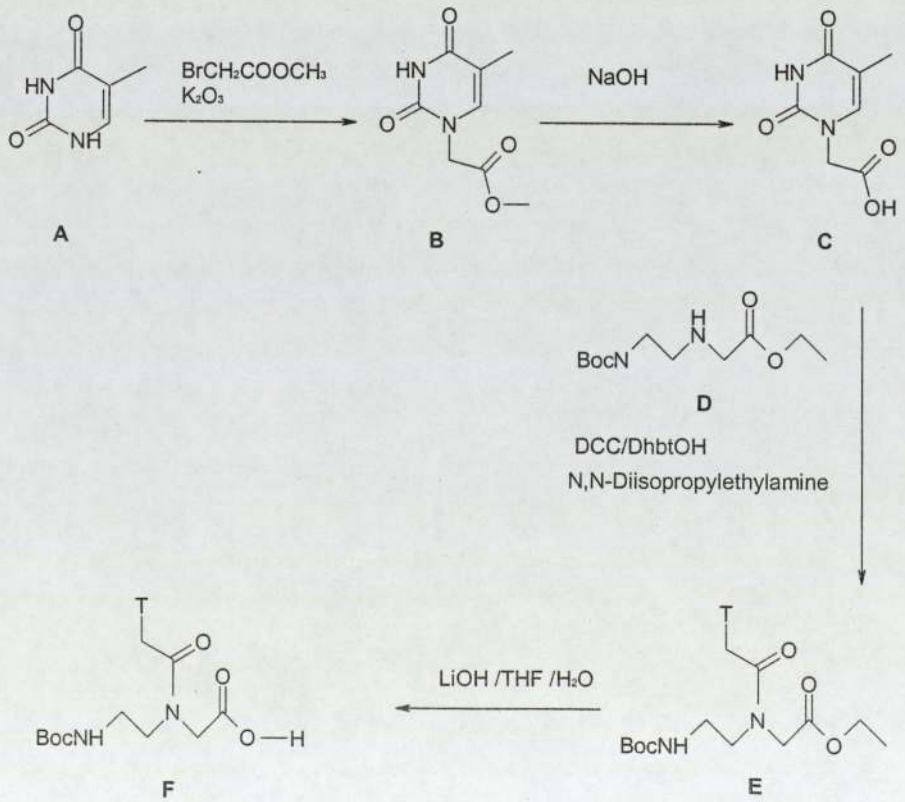


Figure 3.1 Synthesis of N-(2-Boc-aminoethyl)-N-(thymin-1-ylacetyl)glycine

The synthesis of the thymine monomer involved alkylation of thymine (**A**) with methyl bromoacetate followed by hydrolysis of the resultant methyl ester to give thymin-1-ylacetic acid (**C**).

For the attachment to the backbone moiety ethyl N-(2-Boc-aminoethyl) glycinate (**D**) was used. Thymin-1-ylacetic acid was activated with DhbtOH (3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine) and DCC, *in situ*, for the reaction with **D**, and the resulting T monomer ethyl eater (**E**) was hydrolysed with LiOH in water/THF to afford the monomer (**F**). **E** was not isolated prior to the hydrolysis.

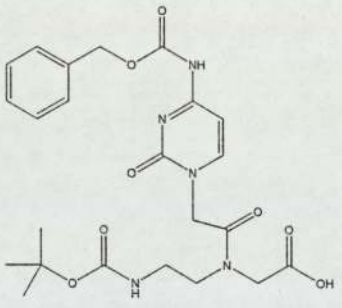
3.2 Synthesis of PNA Oligomers

3.2.1 Solid phase synthesis

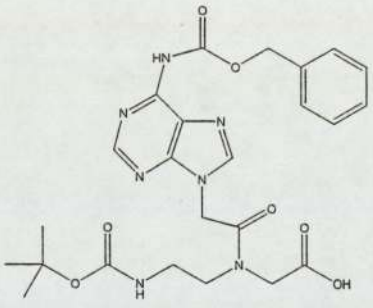
The solid phase PNA synthesis follows the chemistry of solid phase peptide synthesis. The principle of solid phase chemistry was developed by Bruce Merrifield in the fifties and sixties [119]. In his pioneering work, Mrrifield used Z (benzyloxycarbonyl) to protect the α-amino group. However, the more selective protection groups had to be employed since the conditions of Z-deprotection caused side reactions and wrong oligomer removal from the solid support. The Boc (tert-butyloxycarbonyl) protecting group was developed later for α-amino protection in conventional solution phase peptide chemistry [120]. The attempt behind the development of Boc was to increase the deprotection yield under milder conditions with the formation of low boiling products. It was evident that Boc deprotection was nearly quantitative and thus the Boc group was soon preferred in the solid phase procedures. Today the Boc group is still being used widely in combination with different strategies.

3.2.2 Outline of PNA synthesis by Boc/Z chemistry

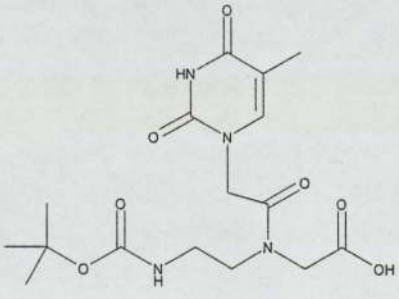
The first reported T_{10} oligomer of PNA was assembled by Boc protected (for α-amino groups) monomers in 1991 [2]. Later, when A, C and G PNA monmers were prepared, the Z-group was used to protect the exocyclic amino groups of the nuclei bases (**Figure 3.2**). In the earlier days, most synthesis of PNAs described in the document use Boc /Z protected monomers with repetitive TFA deprotection during chain elongation and final TFMSA cleavage to cut PNAs from resin. Even today, the Boc/Z chemistry is still the preferred method for PNA synthesis manually. The PNA oligomers in my project were also prepared with this protocol.



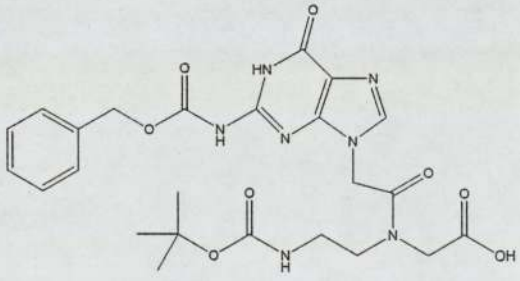
[Boc-PNA-C(Cbz)-monomer]



[Boc-PNA-A(Cbz)-monomer]



[Boc-PNA-T-monomer]



[Boc-PNA-G(Cbz)-monomer]

Figure 3.2 The structure of PNA monomers

Christensen *et al* [121] first described the synthesis of PNA oligomers using the Boc/Z monomers in 1994 (see **Figure 3.3**). They used MBHA- polystyrene as the solid support and coupled the first monomer to the resin using HBTU [2-(1-H-Benzotriazol-1-yl) -1,1,3,3-tetramethyluronium hexafluorophosphate] activation. The Boc group was then

removed by TFA. The next step, the coupling of the next monomer was still preformed with HBTU activation. As outlined in the **Figure 3.3**, DMF and pyridine were used as solvents in the couplings. In this protocol a neutralization step with DIEA was used before the coupling.

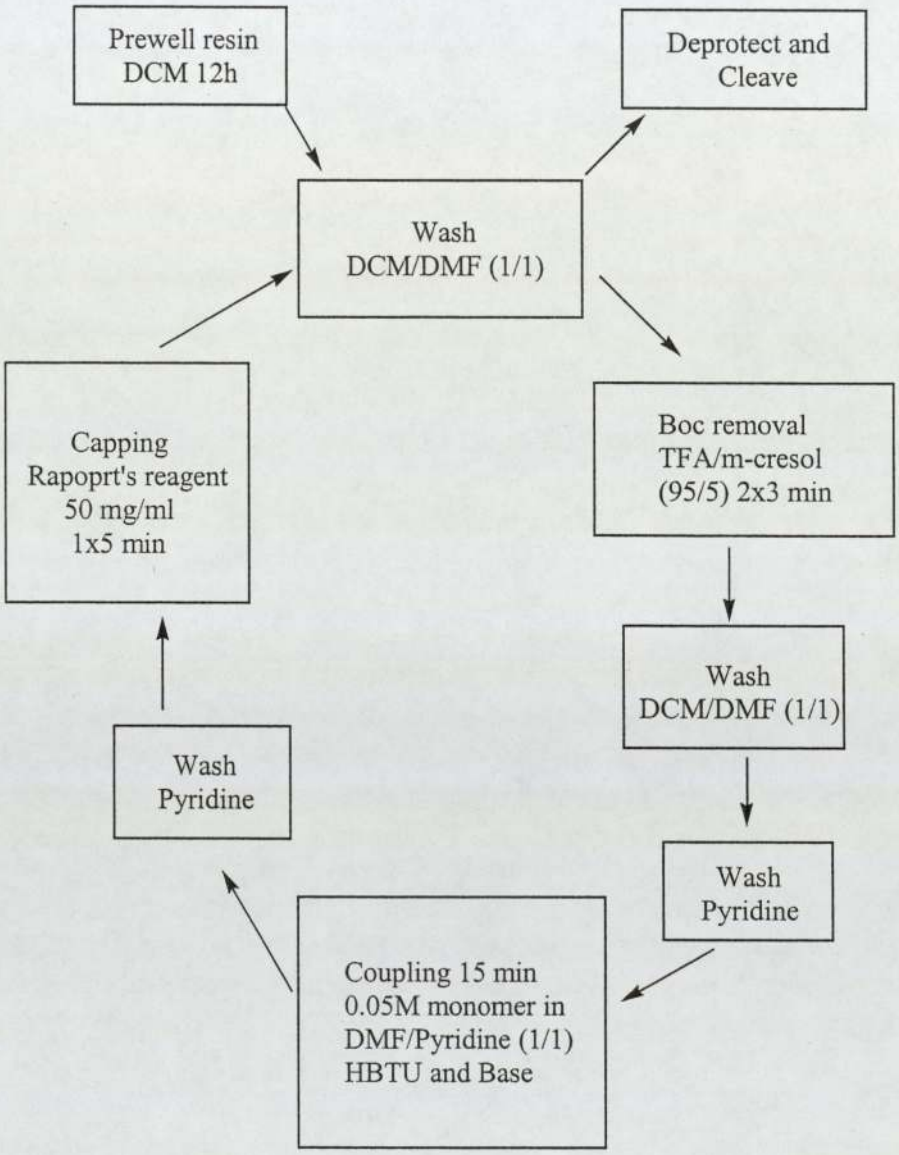


Figure 3.3 Boc/Z PNA oligomer synthesis (*Source from*: Ref.121)

The coupling was performed in DMF/pyridine with N, N-diethylcyclohexylamine as a base. In the capping step, "Rapoports reagent" (N^1-benzyloxycarbonyl-N^3-metliylimidazolium triflate) in DMF was applied to cap unreacted amino groups and the

intact amino groups were capped in 5 min without acylation of other sites on the PNA. After the final synthesis cycle, the oligomer was cleaved from the resin and deprotected using the low/high TFMSA method developed by Tam *et al* [122] which was followed by the precipitation of the deprotected PNA in the diethyl ether. As the time goes by, there have been a number of improvements for the synthesis procedure, including washing, coupling agents, *etc*. I will discuss these in the sections below in details.

3.2.3 Design of PNA sequence

There are more restrictions on the design of PNA sequences than on the design of DNA sequences. However, it is easier to match the requirements for the PNA oligomer because only 12-15 bases long oligomers are needed compared to a length between 25 and 40 units which is the typical length for a DNA oligonucleotide probe. We should obey the the following **Specific Design Rules** [123].

3.2.3.1 Length

Any sequences which are more than 18 bases should be avoided [124, Applied Biosystem's Booklet] as coupling is poor with longer sequences. However, as pointed out above it is not necessary to prepare long PNA oligomers because of the higher affinity of PNA its complemetary RNA/DNA sequence. **The shorter a probe the more specific it is** and **the impact of a mismatch is greater.** For most applications an oligomer length of 12-15 is optimal, and in many cases even shorter probes will work well. Longer PNA oligomers, depending on the sequence, tend to aggregate and are difficult to purify and characterize.

3.2.3.2 Purine content

G-rich oligomers are the worst since purine rich PNA oligomers tend to aggregate and have low solubility.

In order to avoid aggregation, the following specific guidelines should be followed [123]:

(1) There should not be more than 6 purines in the sequence of any stretch of 10 bases

(2) There should be no more than 4-5 purines in a row, specifically no more than 3 G's in a row.

(3) The other strands should be considered if we cannot follow the rules above.

In a word, there should never be more than 6 purines in any stretch of 10 units and no more than 3 purines in a row. If obeying this rule, it will reduce the likelihood that PNA oligomers aggregate.

3.2.3.3 Other issues

Because PNA/PNA interactions are even stronger than PNA/DNA interactions, we should try to avoid self-complementary sequences. Self-complementary sequences such as inverse repeats, hairpin forming and palindromic sequences should be avoided. There is no question for the synthesis but they are difficult to characterize and purify. Yield, purity or proper characterization cannot be guaranteed if a PNA oligomer contains a self-complementary sequence [124].

So based on the above, we designed the sequence of the PNA with the following sequence: **(N)- GCC CGA GAC GTC CTT -(C)**. This sequence is complementary to the 5'-coding region of the **c-erbB1 mRNA**.

3.2.4 Storage and handling of reagents for PNA synthesis

It is essential that the reagents applied for PNA synthesis should be kept as anhydrous as possible because contamination with water will lead to the failure of coupling of monomers during synthesis [125]. To avoid this problem, powdered reagents such as monomers, activator and linker should be placed in a sealed container at freezer

(-20°C). During humid weather, it is always a worthwhile precaution for drying the powdered reagents *in vacuo* overnight prior to their use in order to ensure proper synthesis. In addition, it is most necessary that PNA monomer reagents should be warmed to room temperature before solubilization to prevent water from condensing inside the container. In my project, I added diluent NMP directly to the amber bottle which contained the monomer and allow the mixture to sit undisturbed for 10 min to disolve the monomers.

Usually, monomers A, T, and G are easily to be disolved and do not require vortexing, and a gentle swirling of the bottles contents will be enough. Solubilization of monomer C often requires some assistance. Intermittent heating of monomer C in a shallow water bath at 37°C can assist in its solubilization as can occasional vortexing. As to the use of the solvent, DMF should be used quickly to minimize the contamination of water which will interfere with the synthesis. DMF should be kept as dry as possible to prevent leaving residual water during the procedure.

3.2.5 Solid support and downloading

MBHA resin is most frequently used as solid support in PNA synthesis and has proved to be superior solid support by a comparative study [121, 126]. The amine loading on polystyrene beads is normally too high for PNA synthesis, and it is necessary to reduce the substitution of the solid support (downloading) prior to PNA synthesis and avoid the problems with steric hindrance in the synthesis. If the resin is used without downloading the growing oligomers will aggregate, which is a major reason for synthesis failure. Therefore, the downloading procedure was carried out to reduce the substitution from typically 0.4-0.75mmol/g to 0.1mmol/g. The downloading was carried out and the level of downloading was measured as described in **section 2.2.2.3** and results are summariesed in **Table 3.1**.

Table 3.1 The result of downloading

Absorbance(*Ref.*)	**Absorbance(*Sam.*)**	**PNA Downloading [c]**
0.701	1.038 (*Sample.1*) [a]	0.100mmol/g
0.525	1.049 (*Sample.2*) [b]	0.091mmol/g

a. Fmoc Gly-resin is 20 mg for Sample.1
b. Fmoc Gly-resin is 35 mg for Sample.2
c. Fmoc loading = PNA monomer loading (mmol/g) =

$$\frac{(\text{Abs}_{Sample} - \text{Abs}_{Ref})}{(1.65 \times \text{mg resin})}$$

[*Source from*: Beck, F. Solid phase synthesis of PNA oligomers, In Peptide Nucleic Acid: Methods and Protocols, edited by Nielsen, P.E. (2002), pp.37]

PNA loading (mean) =

[PNA loading (Sample 1) + PNA loading (Sample 2)] ÷ 2 = 0.095mmol/g

3.2.6 Qualitative Kaiser test

Kaiser test is a rapid sensitive method for the qualitative determination of free amino groups during solid phase synthesis of PNA. The technique involves the reaction of the free amine with ninhydin under carefully controlled conditions. The mechanism is as following (**Figure 3.4**).

The qualitative ninhydrin-monitoring method (Kaiser test) is dependent on an understanding of the reactions leading to the colored resin beads that were formed in the previous procedures. Generally, the ninhydrin reaction with a primary amine is believed to result in Ruhemann's purple. On the other hand, there should be no color change in the resin which has been capped due to the Boc group protection (Negative Kaiser test).

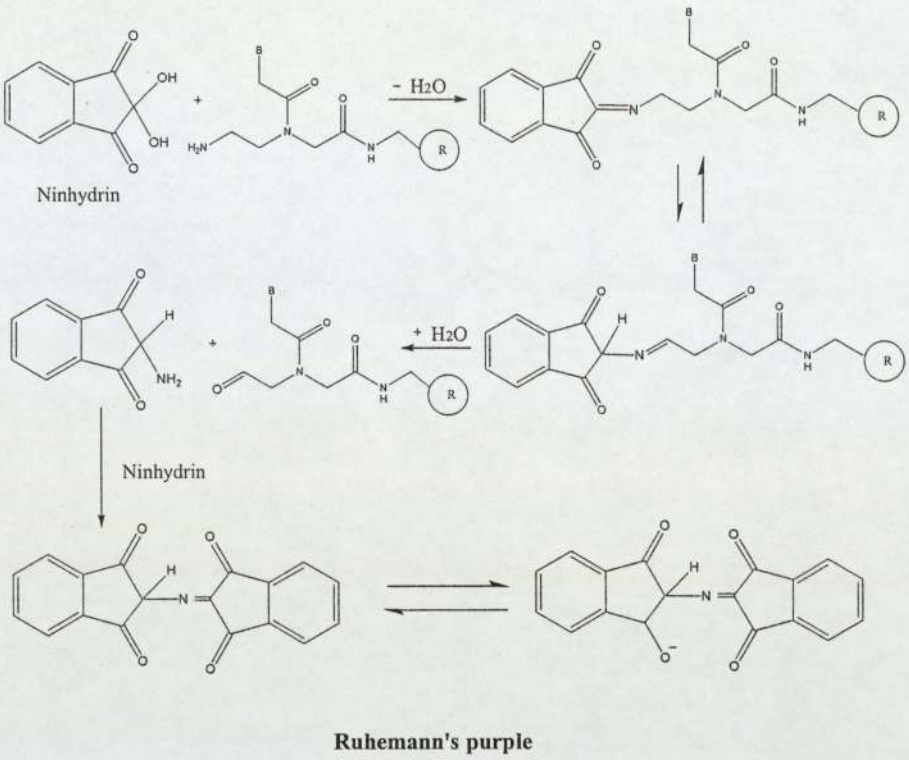


Figure 3.4 Mechanism for the positive Kaiser test (*Source from*: Ref.127)

3.2.7 Neutralization

3.2.7.1 Side reactions during PNA synthesis

Even though the assembly of PNA oligomers is essentially depended on peptide chemistry, the structural differences of the PNA monomers differentiate PNA synthesis from peptide synthesis in several important aspects. One common side reaction is the acyl migration of the nucleobase acetyl moiety to the deprotected primary amine at the N-terminal position during basic conditions which interfere with the synthesis [128].

Another intramolecular side reaction in PNA synthesis is the attack of the primary amine at the N-terminal on the carbonyl group of the inter-unit amide bond [129]. In this case the N-terminal unit is lost and cleaved off in the form of a ketopiperazine derivative (**Figure 3.5**).

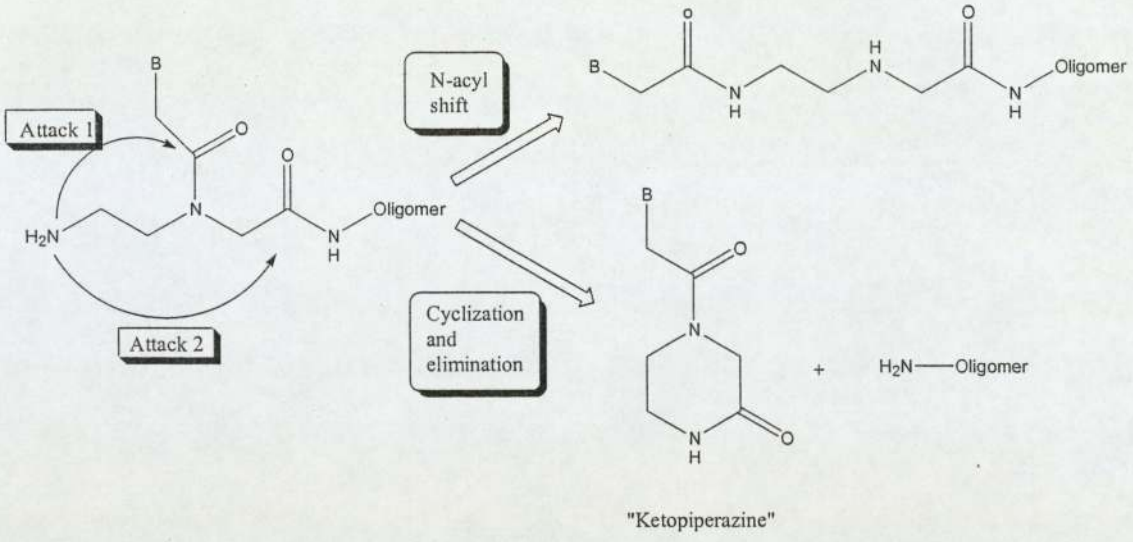


Figure 3.5 Side reactions mediated by attack of N-terminal amino group

3.2.7.2 Resin washing

Until now we can see that the chemical structure of the PNA monomer has certain limits to the choice of optimal oligomerization strategy. Due to the side reactions of PNA oligomers, oligomers must be treated in the shortest possible time with a minimal amount of base. In the PNA synthesis strategies base treatments are only necessary during the neutralization. To give the mildest possible neutralization the resin must be neutralized *in situ* [130].

For the reasons mentioned above, the neutralization step in the protocol employs a minimal excess of base. Such accurate adjustments of base require removal of TFA remainings before the neutralization and coupling step. Sometimes the low coupling yields seem to be due to the insufficient resin washing, which left TFA on the resin. Excess TFA could be removed by two methods: one is to insert a DIEA wash module which was prior to the coupling. The other is to wash the resin completely. In order to reduce whole base treatment of deprotected oligomers, the problem of insufficient coupling should be avoided by the appropriated washing procedures [131].

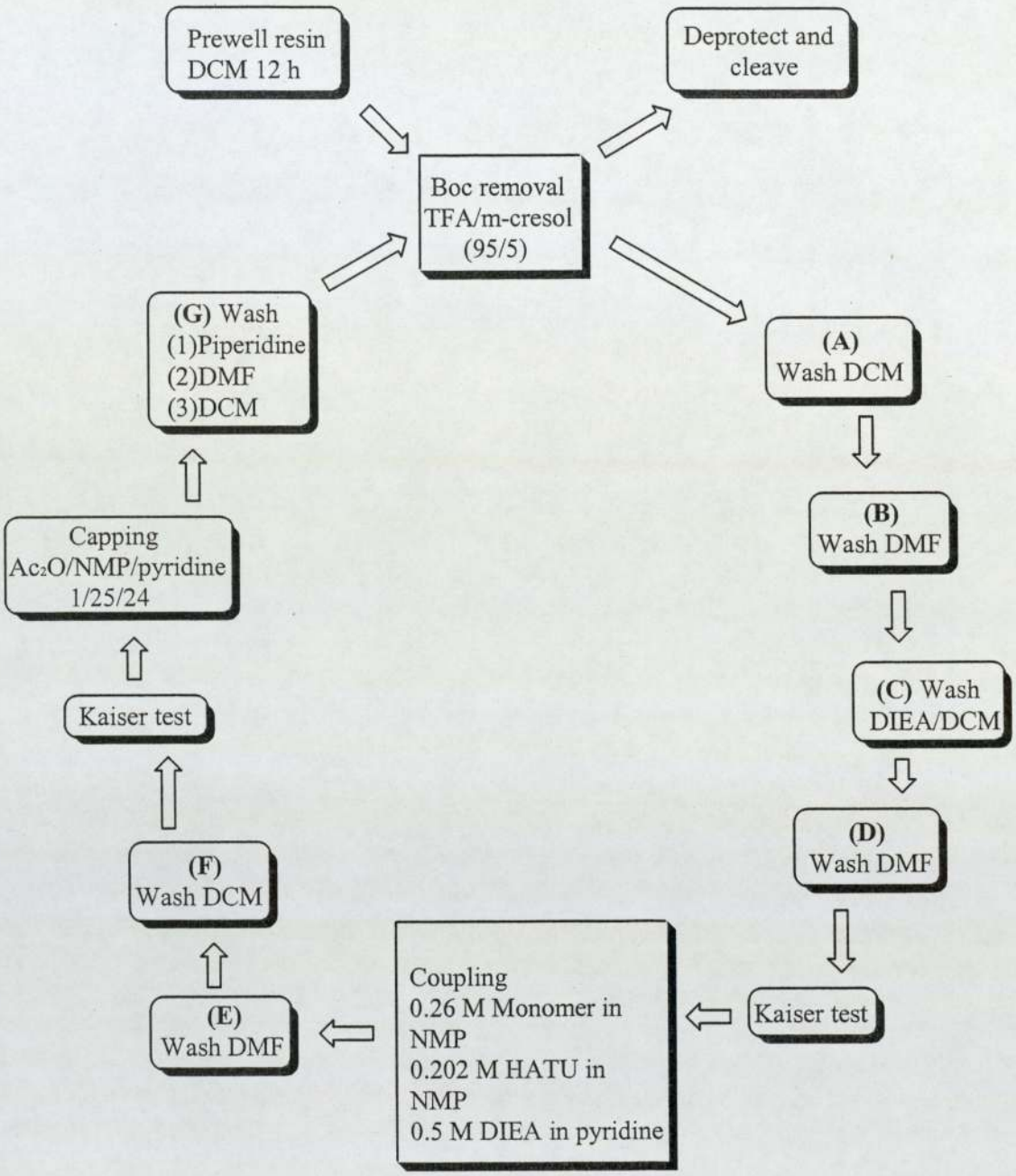


Figure 3.6 Solid phase PNA synthesis and resin washing procedure (A-G)

As shown in **Figure 3.6**, the washing procedure is repeatedly carried out during the process of PNA elongation. Generally, the most efficient wash is obtained when a combination of solvents with different properties is used. Therefore a combination between DCM and a polar aprotic solvent like NMP, DMF, *etc.*, is preferable. DCM

very efficiently swells MBHA resin, and in contrast, the polar aprotic solvent shrinks the resin. This function of washing is to remove fine fragments from crushed resin beads, which can clog the sintered glass filter. Moreover, the reason for changing solvent from DMF/DCM (**Figure 3.3**) to DCM gave better washing as the resin swelled better in pure methylene chloride than in the mixture solvent [131].

DCM removes TFA much more readily than NMP due to the high swelling properties of the solvent, which allow more efficient TFA removal. Even though one DCM wash was enough to remove excess TFA, in this project (**Figure 3.6**), I used three consecutive DCM washes to insure robustness in the TFA removal process. Then two additional DMF washes were used: one is after the coupling process and the other is after the capping process. In both process three consecutive washes were used.

3.2.8 Deprotection

The Boc group was removed by TFA and the m-cresol was added as a scavenger for the tert-butyl cations to avoid alkylation of the nucleobases. The resin was washed after TFA treatment with a combination of DCM and DMF. A solution of 5% DIEA in DCM was also used prior to the coupling in the neutralization step [132].

The treatment of TFA is optimal for PNA synthesis for several reasons [132]: (a) TFA deprotection keeps the primary amine protected until the acylation reaction begins, (b) TFA is an efficient solvent capable of disrupting previously formed secondary structures reducing the risk of intermolecular aggregation of resin-bound oligomers, (c) TFA deprotection is rapid, (d) Boc deprotection with TFA is highly differential and there has not been reported any simultaneous Z removal.

It has been reported that the deprotection of the first PNA monomer linked to the resin is significantly slower than the subsequent deprotections. The data from Koch, *et al.* [131] support this observation. The deprotection rate of a Boc-A-resin was studied as a

function of TFA treatment time. About 1 mL of the deprotection mixture was treateded and the reaction vessel was shaked continuously during the deprotection. In all four base detection, nearly quantitative deprotection was found after 6 minutes TFA treatment, therefore a total deprotection time of 6 minutes is currently used, which was also used in my project (2 x 3 min).

3.2.9 Coupling

3.2.9.1 The Reason for choosing HATU

In the original PNA synthesis protocols, activation of monomers was either performed by carbodiimides or by using pentafluorophenol ester activated PNA-monomer [1]. Later, the uranium salts HBTU and HATU were used as activation agents and give the highest coupling yields in the PNA synthesis [128, 133]. The comparison of the coupling efficiency between HBTU and HATU activated monomer on the resin was performed in the synthesis of a 17-mer PNA. Oligomerization using HBTU resulted in an average coupling efficiency of 98.5, whereas HATU (99.0) was higher than HBTU [128]. It is therefore now generally recommended to use HATU for PNA synthesis.

3.2.9.2 How to avoid the side reaction caused by HATU

The original amine in the PNA backbone is chemically different from those in α-amino acids. The pK_a-value of the 2-ethylamino group in PNA is approximately 10-11, however that of the α-amino group in amino acids is 9-10. The increased basicity in PNAs and the fact that it is less sterically hindered make this amine more reactive compared to the amine in α-amino acids. The higher reactivity will naturally improve the couplings with the activated monomers, but side reactions, such as guanidinium formation with HATU (**Figure 3.7**) [132], will also be triggered. Based on the experiments carried out by Koch *et al*, free HATU not only reacted readily with the primary amine but also competed greatly in this reaction with the activated PNA monomer [131]. These experiments also suggest the importance of pre-activation of the monomers. Furthermore, to be sure that no free HATU is delivered to the resin, it is

important that monomers are present in molar excess to HATU. Therefore, it has been recommended to pre-activate the monomers (1 min) and use a slight excess of monomer over HATU. These recommendations were adopted in my protocols for PNA synthesis.

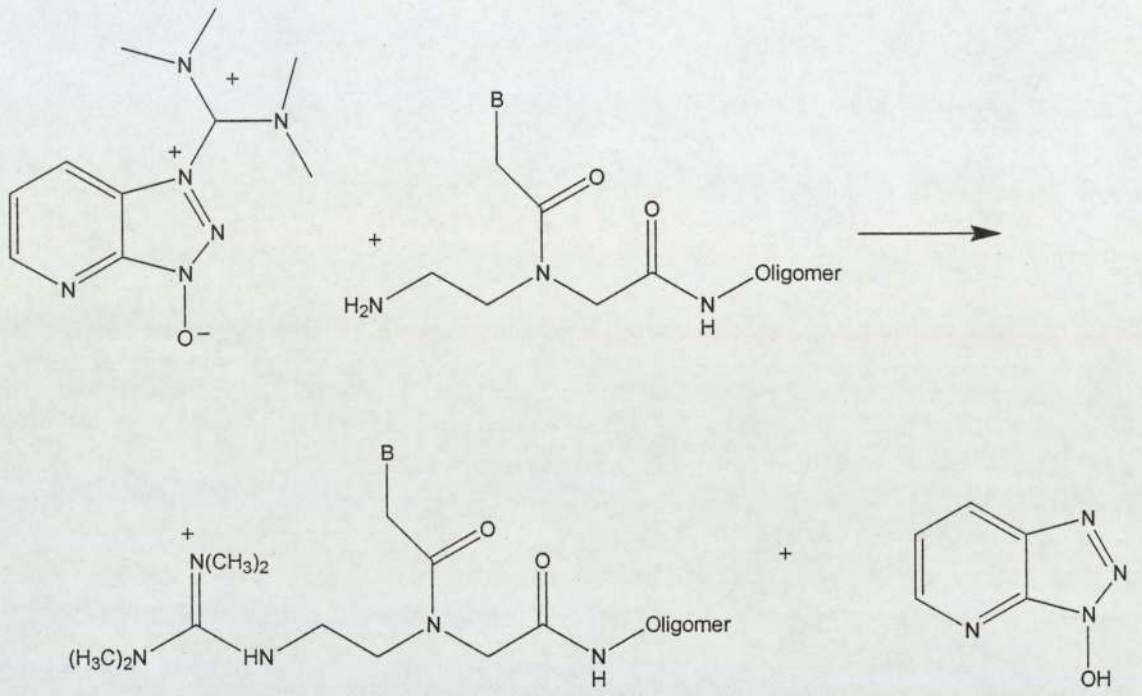


Figure 3.7 N-capping by uranium salts (HATU)

[*Source from:* Koch, T. PNA Oligomer Synthesis by Boc Chemistry. In Peptide Nucleic Acid: Methods and Protocols, edited by Nielsen, P.E. and Egholm, M. (1999), pp.25]

3.2.9.3 How to reduce aggregation

In peptide chemistry intramolecular interactions of the protected oligomers, including polar and nonpolar, are some of the most difficult conditions as they increase the aggregation of the oligomer which might lead to the collapse of the resin and very poor subsequent couplings. These difficult sequences which occur the aggregation have been determined in peptide chemistry [134].

In PNA oligomers, the polar interactions arise from hydrogen bonding between interchain backbones and nucleobases. On the other hand, non-polar interactions arise between interchain lipophilic parts of the nucleobases and base stacking. Based on the Specific Design Rules I mentioned in **section 3.2.3**, a high purine content and high G:A ratio promotes aggregation.

The right solvent mixtures, which must posses high hydrogen bonding and van der Waals interactive properties [135], are the most important factor in reducing aggregation during PNA synthesis. The hydrogen-bonding power of the solvent is mainly determined by the electron donating and accepting ability, whereas the van der Waals interactive properties are induced by dipole-dipole interactions. In peptide chemistry it has been shown that optimal solvation power is obtained by the combination of the two solvation mechanisms [136]. Usually, application of a single solvent is not possible in this combinatory property, which can be obtained by employment of solvent mixtures.

In PNA synthesis polar non-protic solvents are preferred (DMF, NMP, Pyridine) [128]. These solvents have enough electron donor numbers, indicating that the "donor" capacity of the solvent is the important factor for optimal solvation to the protected PNA oligomers. So, solvent interactions with N-H group, other than non-polar interactions with the oligomers, are responsible for a smooth PNA synthesis and aid to delete residuals effectively.

It was reported that [137] NMP/pyridine had the fastest coupling rate because of "acylation enhancement" by pyridine and the best synthesis was obtained when solvent mixtures NMP/pyridine or NMP/DCM were used. The fact that pyridine increases the whole coupling yield is probably a combinatorial effect of acylation enhancement and a high solvation potential. The high solvation potential of pyridine relies on the high donor number of the solvent [135] and on the aromatic nature. DCM increasing the

overall coupling yield is likely a connection of high resin swelling properties by the solvent and the hamper of non-polar interchain interactions. DCM and pyridine was used as a solvent mixture in the coupling which gave average coupling yield higher than 99% [135].

3.2.10 Capping

In my project, the capping step to cap unreacted amino group in the synthesis cycle was carried out with acetic anhydride (Ac_2O/NMP/pyridine, 2/50/48). Capping of the PNA sequences with acetic anhydride was very fast and even a 2% solution gave complete capping in less than 1 minute. In addition, acetic anhydride is a powerful acylation reagent, and in combination with pyridine the reactivity is sufficiently high to react with cyclic nitrogen positions in the nucleobases. With the next step, in order to help to increase the product yield and purity, a piperidine wash was used after the capping and chosen as the acetyl scavenger for remaining acetic anhydride thus preventing nucleobase acetylation [131].

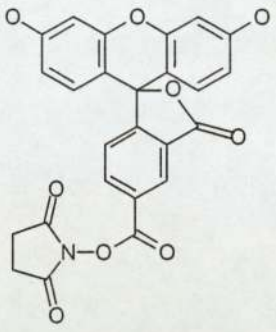
3.2.11 Labeling of the PNA oligomers

Many of the hybridization procedures involving PNA require a label at one of the terminals of PNA. An advantage of PNAs over oligonucleotides is easy attachment with fluorescent labels and other reporter groups. PNA labeling is performed either on resin or in solution. Using which approach is mainly dependent on the stability of the label. The three most common non-radiometric labels for PNA, fluorescein (FAM), rhodamine, and biotin are stable to both Boc and Fmoc cleavage conditions and may be attached to the PNA while still on-resin [138].

In my project, labeling of PNA was accomplished by attaching a fluorescein reporter group (see **Figure 3.8A**) to the PNA oligomer *via* a free amine. In general, there are

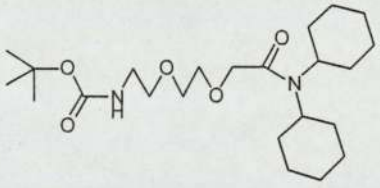
two ways to generate a free amine: one is at the N-terminal of the PNA oligomer, the other is *via* a lysine incorporated into the sequence of a PNA oligomer [139].

A



[label: 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester]

B



[linker: 2- (N-Boc-2-Amino ethoxy)
ethoxy acetic acid- dicyclohexylamine salt]

Figure 3.8 The structure of the label and the linker

Free amines at the N-terminal are generated when the finial Boc group is removed. Labeling of this amine is most easily completed when the PNA oligomer is still attached to the solid support. Particularly, this is the preferred method of labeling using FAM, because coupling of these labels is difficult in solution and results in low yields of the labeled product. However, it is worth bearing in mind, the above procedure cannot be used for acid-sensitive labels [140], which are not stable to the cleavage from the support using a solution of TFMSA/TFA/m-cresol (2/8/1). One more thing need mention, it is important to cool the TFA and TFMSA on dry ice and keep it in the

freezer when a biotynylated PNA is cleavaged, in order to avoid the oxidation of the biotinylated PNA due to the harsh condition required for the cleavage and deprotection.

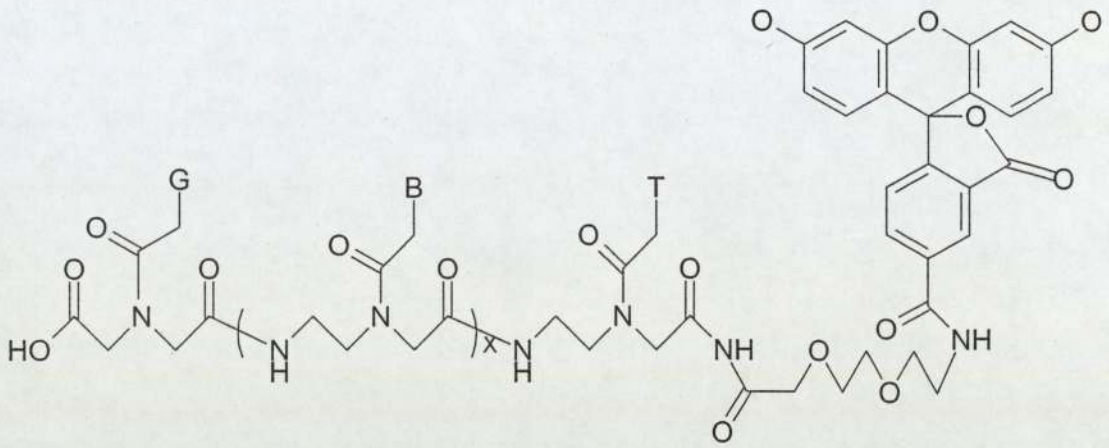


Figure 3.9 The labeled PNA Oligomer with the linker (x = 13)

The labels at the N-terminal require one or two linkers or spacers (see **Figure 3.8B**) to separate the label from the PNA oligomer. This will allows for more efficient attachment of the label, resulting in a higher yield [139]. The procedures of adding the label and linker were both similar to the steps of adding a PNA monomer. The structure of the PNA with the attachment of the spacer and linker is shown in **Figure 3.9**.

3.2.12 Cleaving

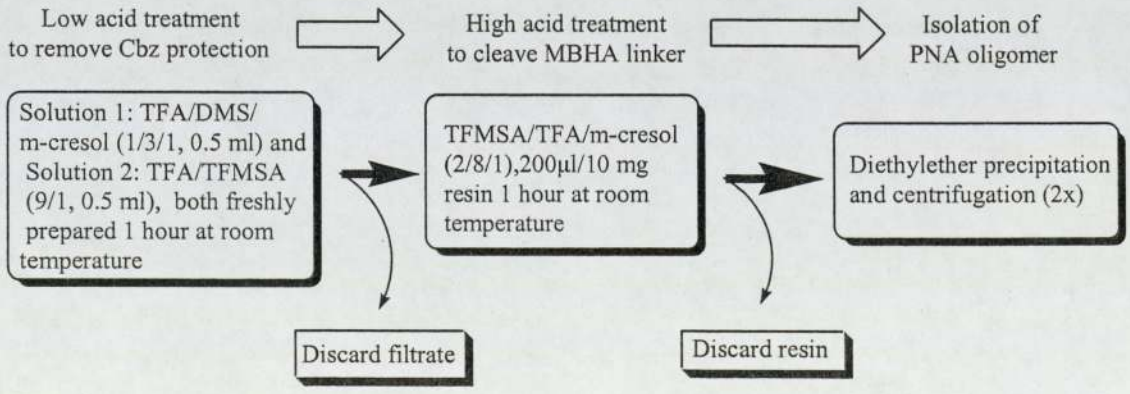


Figure 3.10 Low high cleavage of oligomer from resin

PNA synthesis based on Boc/Z protection relies on differential acid liability between the two groups. The Boc group is removed by TFA, and either HF or more frequently, TFMSA is used to remove the Z-group. The Boc/Z protected oligomer was cleaved from the resin by treatment with strong acid. Although HF (acidity function, H_o = -11) has been the preferred reagent for many years, now TFMSA (H_o = -13) is found to be an appropriate substitution for HF because HF is highly toxic [134,136]. Unlike HF, which is volatile, TFMSA is a viscous liquid that is difficult to handle and is not as readily removable as HF. However, the use of TFMSA does not require special apparatus for its handling in the laboratory.

Following the oligomer assembly, the cleavage of side-chain protecting groups and PNA from the resin was accomplished with the low-high-TFMSA method developed by Tam and Merrifield (see **Figure 3.10**) [122]. Usually, a strong anhydrous acid in the S_N1 mechanism is employed in the final step to remove all the benzyl protecting groups. Because of the strong acidity attendant with the S_N1 condition, peptides in PNA with nucleophilic side chains are strongly susceptible to modifications by the carbocations generated under the S_N1 deprotection condition, which make more side reactions. However, the application of an S_N2 condition will minimize these side reactions due to the acidolytic removal of benzylic protecting groups using a mixture of DMS which is suitable choice as a weak base in a low TFA and TFMSA (hard acid and soft base concept) concentration.

In the procedure, the resin was swelled in TFA first and then the cleaving mixture was added. After the reaction, the cleaving mixture was transferred to a glass tube containing cold ether. The PNA was precipitated by centrifugation. Here one point is worth mentioning, from the comparison between the glass tube and plastic tube in the centrifugation, I found the application of the glass tube will improve the yields, which may be due to the higher affinity of PNA to the surface of the plastic tube. Fresh cold ether was then added to suspend the PNA and the oligomer was precipitated by centrifugation again. The PNA was afforded after the ether was decanted off, and

quantified by measuring the PNA solution at 260 nm. 159.5 OD unlabeled oligomer with the linker and 62.9 OD labeled PNA were obtained. (**Table 3.2**)

Table 3.2 Quantitative analysis of PNA oligomer and labeled PNA oligomer both with a linker

	PNA oligomer with the linker	Labelled PNA oligomer with linker
Procedure	Dissolve the PNA oligomer in the 1 mL 0.1%TFA in water. Dilute 10 μL PNA oligomer solution into 1 mL using 0.1%TFA in water. Run UV. Run HPLC	Dissolve the labelled PNA oligomer after adding the linker in the 0.5 mL 0.1%TFA in water. Dilute 5 μL PNA oligomer solution into 1 mL using 0.1%TFA in water. Run UV. Run HPLC
Result	The result is the following: 10 μL ~ 1.595OD 1 mL ~ 159.5OD ≅7975 μg [* 1 OD ≅ 50 μg] So, total is 159.5OD	The result is the following: 5 μL ~ 0.629OD 0.5 mL ~ 62.9OD ≅ 3145 μg [* 1 OD ≅ 50 μg] So, total is 62.9OD

3.2.13 HPLC Analysis

After the synthesis, deprotection and isolation of the PNAs, they were analysed by HPLC to ascertain that the quality of the synthesized PNAs was good enough for the subsequent experiments.

After analyzing a series of PNA homologs, Hoffmann and his colleagues [141] observed that longer PNA oligomers eluted later. In their study of the retention time of 29 PNA oligomers, it was demonstrated that the length of the PNAs indeed regulated the retention times as expected. However the contribution of every additional monomer to the retention time decreased with the increasing PNA length. Based on their view, the reduction in the contribution of the monomers as the PNA grew could not be explained only on the basis of side-chain hydrophobicity of the additional monomers. In contrast,

it may indicate that the somewhat hydrophobic ethylglycine backbone contributes to the weak column binding at low monomer numbers. Based on the retention time of 29 different PNA oligomers they devised an equation for predicting the retention time of PNA oligomers:

$$\mathbf{Y = 7.373 + 1.883A + 1.655G + 1.398C + 2.338T - 0.055L^2}$$

Y refers to retention time of a PNA, **L** refers to the length of the PNA sequence, **A**, **T**, **C** and **G** refer to the numbers of each base in the PNA sequence.

However, Weia, *et al* [142] suggested that the polymer length is not the only factor determining the retention times of the PNA molecules, they proposed the following equation for the prediction of PNA retention times:

$$\mathbf{Y = 20.76731 - 0.00513M + 1.436339A + 2.555066G + 1.418646C + 2.281181T}$$

Y refers to retention time of a PNA, **M** refers to its molecular mass, and **A**, **T**, **C** and **G** refer to the numbers of each base in the PNA sequence, respectively.

After their determination of 20 different PNAs, it was found that the retention time was positively correlated to molecular mass, which means that when molecular mass increases, the retention time also increases (time range is about 20-30min). And also from this equation, the bases, especially T (2.28) and G (2.55), have some influence on the retention time. When G or T is absent in a PNA sequence, the actual retention time deviates more from the calculated retention time. This is because that G and T are more hydrophilic than A (1.43) and C (1.42). So, when G or T is absent, the interaction between PNA and column stationary phase increases, so does the retention time.

To assess the coupling efficiency during the PNA elongation and to ascertain that its quality is good enough for the liposome experiment, an HPLC analysis was carried out of the fully deprotected non-labelled PNA and the PNA with a linker attached. As

shown in **Figure 3.11**, the retention time of peak **A** was 25.315 min, representing the desired oligomer based on the theory of Hoffmann (the length is the longest) and Weia (the molecule mass is the largest).

Similarly, the peak **C** in **Figure 3.12** with a retention time of 32.448 min was the desired product corresponding to the deprotected labeled PNA oligomer with the linker. As shown in the figure the reaction of the PNA with the linker was not satidfactory, and about half of the PNA was unreacted (**peak B**). Part of the PNA oligomer without the linker with Boc group was deprotected in that procedure and was treated with the label under the coupling conditon.

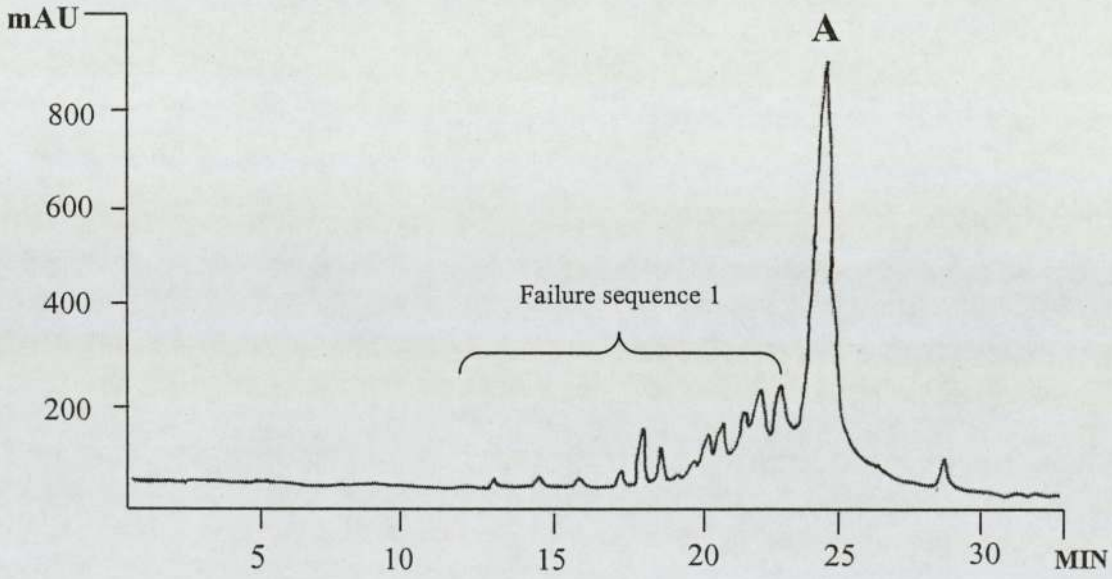


Figure 3.11 HPLC profile of the deprotected non-labeled PNA oligomer.

As shown in **Figure 3.11** and **3.12** there are also a number of minor peaks repersenting the failure sequences. However as the presence of these minor shorter sequencs should not noticeably affect the results of entrapment of PNA into liposomes, the oligomers were used for the liposome experiment without further purification.

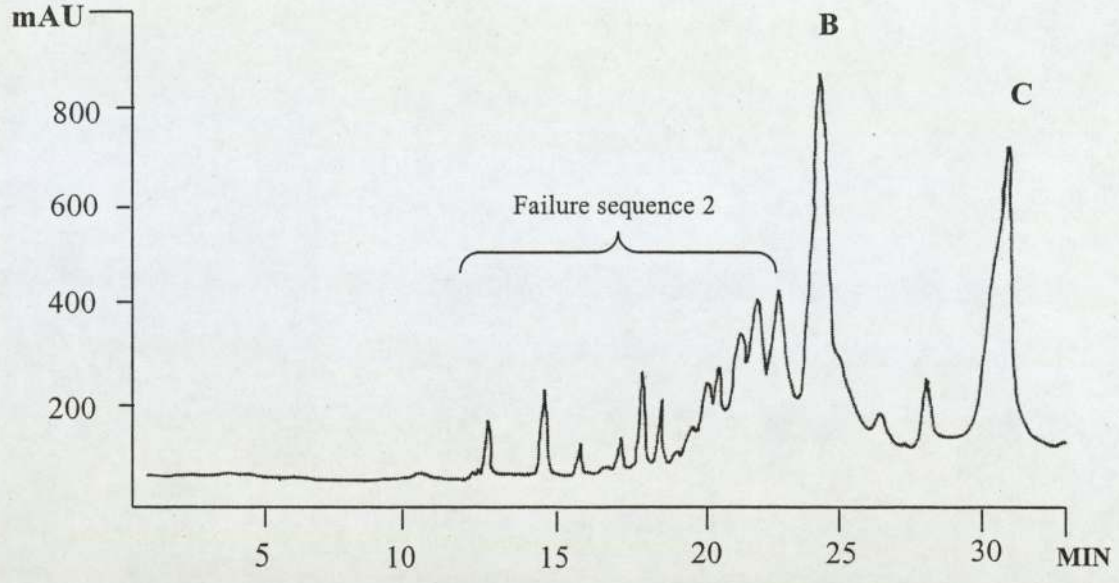


Figure 3.12 HPLC profile of the deprotected labeled PNA oligomer with the linker.

3.3 Handling and Storage of PNA

As recommended by the booklet from Applied Biosystems [143], we usually dissolved PNA in plain water [or occasionally 0.1% aqueous TFA] and divided the solution into aliquots and kept them frozen. Aliquots that are not going to be used for sometime should preferably be dried down, for example using a speed-vac concentrator.

Usually PNA oligomers are purified by RP-HPLC with TFA present and as a result the purified PNA will be protonated at all the basic, amino groups and hence carry trifluoroacetate counter-ions when dissolved in plain water because this will help keep the PNA in solution at the high concentration of the stock solution.

There are two problems which can potentially arise from the TFA. First, addition of a concentrated stock solution of PNA in plain water directly into, for example a cell culture, could lower the pH. Secondly the high concentration of TFA may have an adverse effect on the system tested. However, these problems are easily avoided by using properly buffered solutions.

PNA oligomers have a high affinity for glass surfaces and polystyrene. When working with low concentrations of PNA a majority of the PNA may become bound to the container. Whenever possible, use polypropylene or polyethylene materials during handling and storage of PNA.

Part 2 Entrapment of PNA into Liposomes

3.4 Preparation, formulation and characterisation of liposome systems

3.4.1 Preparation of dehydration-rehydration vesicles

3.4.1.1 Why choose the DRV method?

The mechanism of DRV method had been proposed by Deamer and Barchfield in 1983. However, the procedure was developed by Christopher and Gregoriadis in 1984 [144]. This procedure is capable of encapsulating a wide variety of materials into liposomes of variable lipid composition with high efficiency and using mild and simple conditions, which include two aspects. First, this method can avoid some drastic conditions such as the use of detergents or organic solvents, which are normally employed in other methods to overcome the intermolecular attractive forces of the lipid. Secondly, there are no specialized instruments or complex techniques required by procedure. The DRV method with relatively high entrapment started from hand-shaken, based on the use of dehydration and controlled rehydration, to induce fusion of preformed liposomes, is simple to use and at the same time scaling up is straightforward [145].

Table 3.3 Materials entrapment in liposomes

Entrapment of Peptides and Proteins [a]		Incorporation of Plasmid DNA [a]	
Entrapped material	Entrapment (%)	Entrapped material	Entrapment (%)
Tetanus toxid	40-82	pGL2(encoded luciferase)	44-87
Bovine serum albumin	40-45	pRc/CMV HBS (hepatitis B surface antigen S region)	55-80
RIVE (Reconstituted influenza virus envelopes)	29-31	pRSVGH (human growth hormone)	46-80
A/Sichuan (A/Sichuan influenza virus hemagglutinin and neuraminidase)	38-45	pCMV4.65 (Mycobacterium leprae protein)	29-53
rHBsAg (recombinant hepatitis B surface antigen)	31-33	VR120 (Schistosoma protein)	about 90
LV39(*Leishmania major* antigen)	74-82	Incorporation of other different materials[b]	
Interleukin-2	60-70	CF	40-54
Poliovirus 1-VP2 peptide	74-82	Glucose	about 40
Poliovirus VP2 peptide	62-68	ATP	about 37
HBsAg S peptide*	42-45	5-fluorouracil	about 46
HBsAg preS1 peptide**	46-48	Albumin	about 41

* Synthetic S peptide is the 110-137 amino acid sequence.

** Synthetic preS1 peptide is the 15-48 amino acid sequence.

a *Source from*: Ref.147

b *Source from*: Ref.144

The DRV method was designed to achieve high levels of entrapment, particularly of sensitive biological molecules such as proteins and nucleic acids. From the **Table 3.3** we can conclude that Dehydration - Rehydration Vesicles is a simple method for high yield drug entrapment in liposomes. Also from recent paper, apart from following data, Perrie, *et al* has increased the values of pRc/CMV HBS entrapment about 89%-94% in

DRV of the compositions studied [145]. Most interestingly, from the recent research of Kawano's group, they obtained small-sized liposomes highly entrapping THP (pirarubicin, which is a doxorubicin derivative with higher efficacy against several solid tumors, acute leukemia and malignant lymphoma) by using DRV method in which there is a small modification [146].

3.4.1.2 Characterisation of the DRV process

To effectively formulate and prepare DRV vesicles, initially steps were undertaken to characterise various vesicle constructs prepared at each stage of the DRV method (**Figure 3.13**).

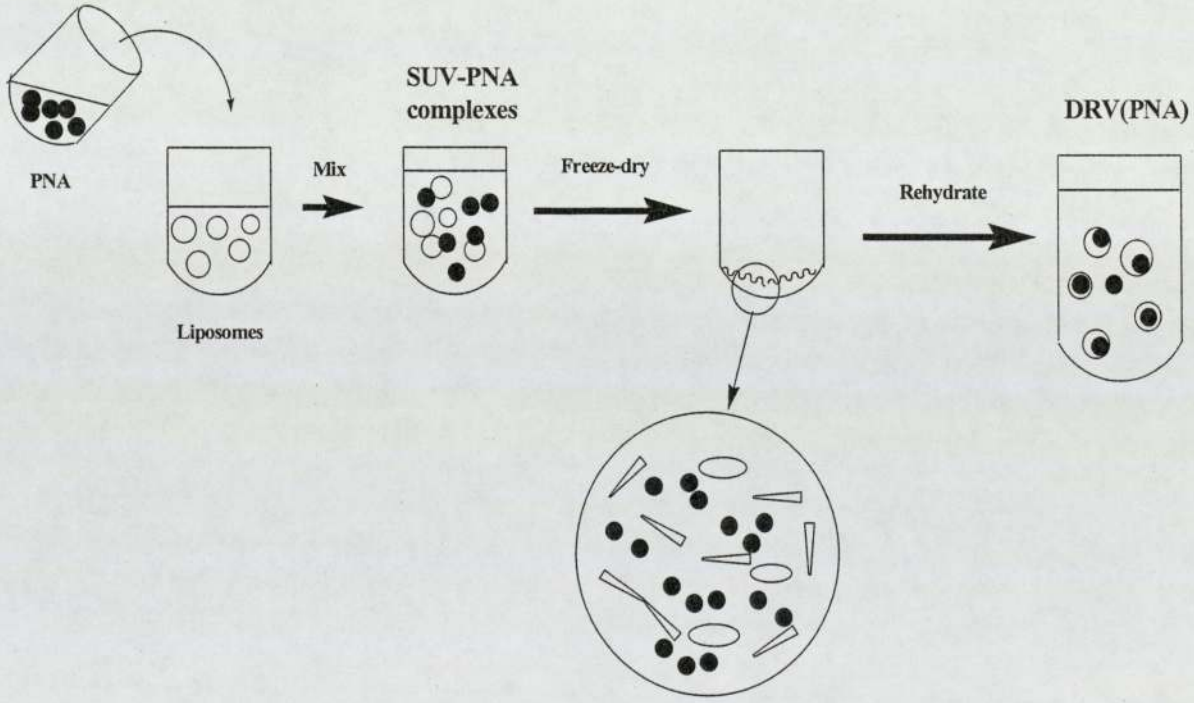


Figure 3.13 Flow diagram of the DRV method (*Source from*: www.lipoxen.com)

Based on the above flow diagram of the DRV method showing we start with large MLV which are then effectively reduced to SUV via sonication and then freeze-dryied to produce larger DRV, the results from all steps were as follow (**Table 3.4**):

Table 3.4 The zeta potential and particle size results of the MLV, SUV and DRV [a]

Liposome formulation	MLV		SUV		DRV	
	Size (nm)	Zeta Potential (mV ± S.D.)	Size (nm)	Zeta Potential (mV ± S.D.)	Size (nm)	Zeta Potential (mV ± S.D.)
PC:Chol	2143 ± 43.4	-0.3 ± 0.5	86.2 ± 7.9	0.0 ± 0.0	1179.5 ± 297.1	-0.5 ± 0.5
PC:Chol:SA	581.1 ± 51.1	60.6 ± 1.2	103.7 ± 6.8	46.1 ± 5.5	570.4 ± 31.1	69.2 ± 9.5
PC:Chol:DCP	658.5 ± 101.4	-55.40 ± 8.8	105.4 ± 7.6	-39.6 ± 5.4	575.6 ± 29.0	-79.0 ± 11.7

a. The zeta potential of the MLV, SUV and DRV was measured in 0.001M PBS at 25 °C using a Brookhaven zeta potential analyzer. Vesicle z-average diameter was determined in a Brookhaven particle sizing analyzer at 25°C. Values denote mean ± S.D. (n = 3)

3.4.1.3 Considerations from practical results of preparing empty DRV

From **Table 3.4**, in terms of size, we can see the order is MLV>DRV>SUV, which is relate to their structures. Compared with MLV, which consists of numerous concentric bilayers in close apposition, altering with layers of water and varies from 0.1-10μm, SUV, which are single layered liposomes, are about 25-100nm in size. Although SUV are the smallest, they are necessary during the step from MLV to DRV. The intention of the DRV method is to maximize exposure of solute to the lipid before its final lamellar configuration has been fixed, so that the liposomes ultimately form around the solute. So based on this mechanism, up to 70% of the total phospholipids will be present in the outer leaflet of the lamella when transferring MLV to SUV. Thus the most of amphiphile is directly exposed to the solute when SUV are mixed with the material to be entrapped.

After that, the water is removed to produce an intimate mixture of the dry components with the phospholipid head groups in close proximity through freeze-drying. The minimum value water is added next step [148]. At the same time, the fusion of the

vesicles surrounding the active and the formation of larger liposomes (DRV) are initiated. The DRV can encapsulate a large proportion of the solute and are bigger than SUV in size.

Also from **Table 3.4**, the size of MLV (581.1 - 2143.1nm), SUV (86.2 -105.4nm) and DRV (575.6 - 1179.5nm) vesicles are similar to previously reported studies [149].

Interestingly, infusion of the charged surfactants SA and DCP resulted in a change in zeta potential and vesicle size of MLV and DRV. The vesicle size of MLV (2143 nm) and DRV (575-658 nm) in neutral are significant larger than the charged MLV (1179 nm) and DRV (570-575 nm) respectively (**Table 3.4**). The effect of vesicle charge on reducing the size of MLV and DRV could be relative to the fusion process present during the procedure. Charged MLV and DRV surfaces repel each other so as to interfere with effective membrane fusion, thus leading to lower number of bilayers and resulting in the smaller MLV and DRV. The neutral MLV can form more bilayer without the effect of the charge resulting in an increased vesicle size [149]. As to the reason for the charged SUV (103-105 nm) are bigger than neutral SUV (86nm), this may be because that in the complexes carried either a net positive or negative charge, charged head-groups in bilayer repel each other and reduce the probability of aggregation resulting in an expanded bilayer and hence formed the bigger single bilayer vesicle [150].

3.4.2 Preparation of PNA loaded DRV

During the procedure, MLV was prepared from single-source natural or synthetic lipids, by suspending the latter, preferably in a finely divided form. After the conversion from MLV to SUV, PNA was mixed with the latter. During the freezing process, some deformation of the SUV is likely to occur as a result of osmotic shrinkage [151], caused by increasing external solute concentration arising from progressive formation of ice crystals. Further destabilization of the bilayer during dehydration facilitates fusion to

form DRV during the subsequent step. Within the water-adding step, the amount of water added at this stage is critical to achieving the optimal level of entrapment. The volume of H_2O added must be kept at a minimum to ensure complete hydration of the powder under vigorous swirling. At the rehydration stage, there is an increase in the overall solute concentration because of the reduced amount of water added. Subsequent dilution with isotonic buffer leads to a large osmotic gradient between the internal and external phases, which results in a redistribution of solute, both within the forming liposome and with the external aqueous phase. This may be due to the increased permeability to solute as a result of bilayer stretching caused by hyperosmotic inflation. At this stage, liposome formation is finished and equilibration obtained. To effectively characterise the entrapment of PNA within DRV and compare this to any surface absorption phenomena which may occur liposome preparation methods were compared.

3.4.2.1 Fluorescence studies

Fluorescence data of liposome-entrapped or liposome surface-complexed fluoresent PNA were recorded using spectrofluorimeter (Spectra MAX GEMINXS) from Molecular Devices Ltd. on a 486-514 nm wavelength band. After the centrifuge and receiving the pellet of the liposomes which contain the labelled PNA, the suspendent was determined by the spectrofluorimeter which indirectly reflect the entrapment of the PNA. The entrapment of PNA (percent) can be calculated from the following equation.

$$\textbf{PNA}\ (\%\ \text{entrapment}) = \frac{\textbf{PNA}\ (\text{total added}) - \textbf{PNA}\ (\text{suspendant})}{\textbf{PNA}\ (\text{total added})} \times 100\%$$

As to the value of the PNA (total added) and PNA (suspendant), they will be available after putting the data from spectrofluorimeter into the PNA standard curve.

3.4.2.2 Incorporation of PNA into liposomes

Table 3.5 Incorporation of PNA into DRV preparations: The effect of vesicle charge [a]

Liposome Formulation	DRV(PNA)			DRV-PNA		
	PNA incorporation (% of used)	Zeta potential (mV± S.E)	Vesicle size (nm ± S.D.)	PNA incorporation (% of used)	Zeta potential (mV± S.E)	Vesicle size (nm ± S.D.)
PC:CHOL (16: 8μmol)	73.8 ± 5.4	-0.1 ± 0.1	1217.6 ± 110.9	5.4 ± 0.9	-0.2 ± 0.1	1327.9 ± 319.0
PC :CHOL:SA (16 : 8 : 4μmol)	65.3 ± 5.7	46.0 ± 5.5	593.8 ± 19.9	3.6 ± 2.2	71.1 ±15.4	625.0 ± 35.8
PC:CHOL:DCP (16 : 8 : 4μmol)	59.2 ± 4.6	-67.9± 6.8	568.3 ± 39.3	4.1 ± 3.2	-81.1 ± 12.9	648.4 ± 51.6

a. Incorporation of PNA into DRV preparations: The effect of vesicle charge. 5(6)-FAM-NHS labeled PNA (50μg) was either complexed with (DRV-PNA) or entrapped in [DRV (PNA)] DRV composed of phosphatidyl choline (PC; 16 μmol); cholesterol (CHOL; 8 μmol) and 4 μmol or either the cationic lipid stearylamine (SA) or the anionic lipid decetyl phosphate (DP). PNA incorporation was measured by the data which is recorded by using spectrofluorimeter (Spectra MAX GEMINXS) from Molecular Devices Ltd. on a 486-514 nm wavelength band. The zeta potential of the DRV was measured in 0.001M PBS at 25 °C using a Brookhaven zeta potential analyzer. Vesicle z-average diameter was determined in a Brookhaven particle sizing analyzer at 25°C. Values denote mean ± S.D. from at least three experiments.

The values of PNA entrapment (% of total used) in DRV of the compositions studied were 60%-70%. As shown in the **Table 3.5**, the order of the entrapment is neutral >cationic>anionic liposomes in the DRV (PNA), presumably because the accessibility of much more lipid (neutral) during the process of dehydration of the SUV-PNA mixture to form, on rehydration, multilamellar vesicles with PNA mostly distributed in the inner bilayers. To confirm this PNA was entrapped with in DRV liposomes and not surface complexed. Preformed 'empty' DRV were incubated with PNA and then subjected to centrifugation to remove non-surface bound PNA. Results (**Table 3.5**) show only a minor amount (3-5%) of the PNA removed associated with the DRV after washing demonstrated that minor surface occur between the PNA and the DRV liposomes and that the PNA incorporation values recorded with DRV (PNA) represents PNA entrapment within the liposomal aqueous core. No significant difference in both vesicle size and zeta potential between DRV-PNA and DRV (PNA). As to the difference between the entrapment of neutral, and anionic vesicles, there is a significant difference in the comparison among neutral, cationic and anionic vesicles, the distance of the entrapment percent is 14.5 ± 5.0 % between neutral and anionic vesicles. As to cationic vesicles, it is about 8.5 ± 5.6 %.

However, compared to other work, neutral liposomes are still beneficial. It was previously shown [152] that entrapment of the plasmid DNA in neutral lioposomes was significant (48.3%) when DRV method was applied. With the development of the technique, efficient encapsulation of DNA plasmids inside small, neutral liposomes was also possible. Through the effort from Bailey *et al.*, they found when they added the ethanol and calcium chloride to an aqueous mixture of SUVs and plasmid the entrapment of DNA will be up to 80% [153]. In addition, based on the basic study on the influence of vesicles and protein size on the entrapment efficiency, large proteins (such as pepsin A, lysozyme, α-amylase, etc) cannot be entrapped effectively inside small vesicles [154]. In **Table 3.5**, the DRV size of neutral liposomes is larger than that of charged liposomes. Also from Walde's work [154], DRV method has shown to be

better than other procedures in the aspect of the high protein and peptide encapsulation efficiencies thereby support the chose of the DRV method for PNA entrapment.

Table 3.6 The comparison between DNA and PNA entrapment in the liposomes

Mode of entrapment	Positive charge	Neutral charge	Negative charge	Mode of entrapment	Positive charge	Neutral charge	Negative charge
DRV (DNA) [a]	96%	48.3%[b]	53.5%	DRV-DNA	73%	9.8%	9.2%
DRV (PNA)	65.4%	73.8%	59.2%	DRV-PNA	5.4%	3.6%	4.1%

a. The composition of the liposome is [PC: DOPE (1:1) in neutral liposomes, PC: DOPE: DOTAP (1,2-dioleoyl-3-trimethylammonium propane)(1:1:1) in cationic liposomes, PC: DOPE: PG (Phosphatidyglycerol)(1:1:1) in anionic liposomes]. *Source from*: Ref.149

b. *Source from*: Ref.152

When comparing the entrapment of DNA with PNA, DNA normally gives high entrapment (96%) in cationic systems because of the electrostatic interaction between DNA which is negative charge and liposome bilayer which contain positive charge, however, lower in neutral or negative liposomes (48.3% and 53.5%) and will be achieved because there is no electrostatic interaction. These entrapment results (see **Table 3.6**) are similar to our reported PNA entrapment studies due to the absence of electrostatic interaction between PNA and any of the liposome systems. Most interestingly, Yuan's group has shown [155] that delivery of PNA with a short PO oligonucleotide to form complexes with cationic liposomes to pulmonary endothelium in intact mice. In this method, DOTAP liposomes were used since a previous study showed that this formulation was highly efficient in delivering ODNs to lung endothelial cells in intact mice. In addition, from the research of Borgatti *et al*, they found that cationic liposomes can be proposed for *in vitro* delivery to target cells of the powerful decoy molecules which are the PNA-DNA chimeras containing NF-kB binding sites [156]. The result from Nastruzzi's group[157] still show that cationic liposomes, when prepared by the REV method, were able to efficiently complexate DNA-PNA hybrid molecules and mediate their binding to target cells although it was

reported that evidence of complexation between cationic liposomes and DNA-PNA hybrid has not yet been documented. So from these these papers, it suggests that if there are some modifications or changes on PNA structure, such as adding a short PO or hybridizing with DNA first, cationic liposomes will be more powerful or beneficial and it is also a motion of development for the liposomes as carrier of PNA.

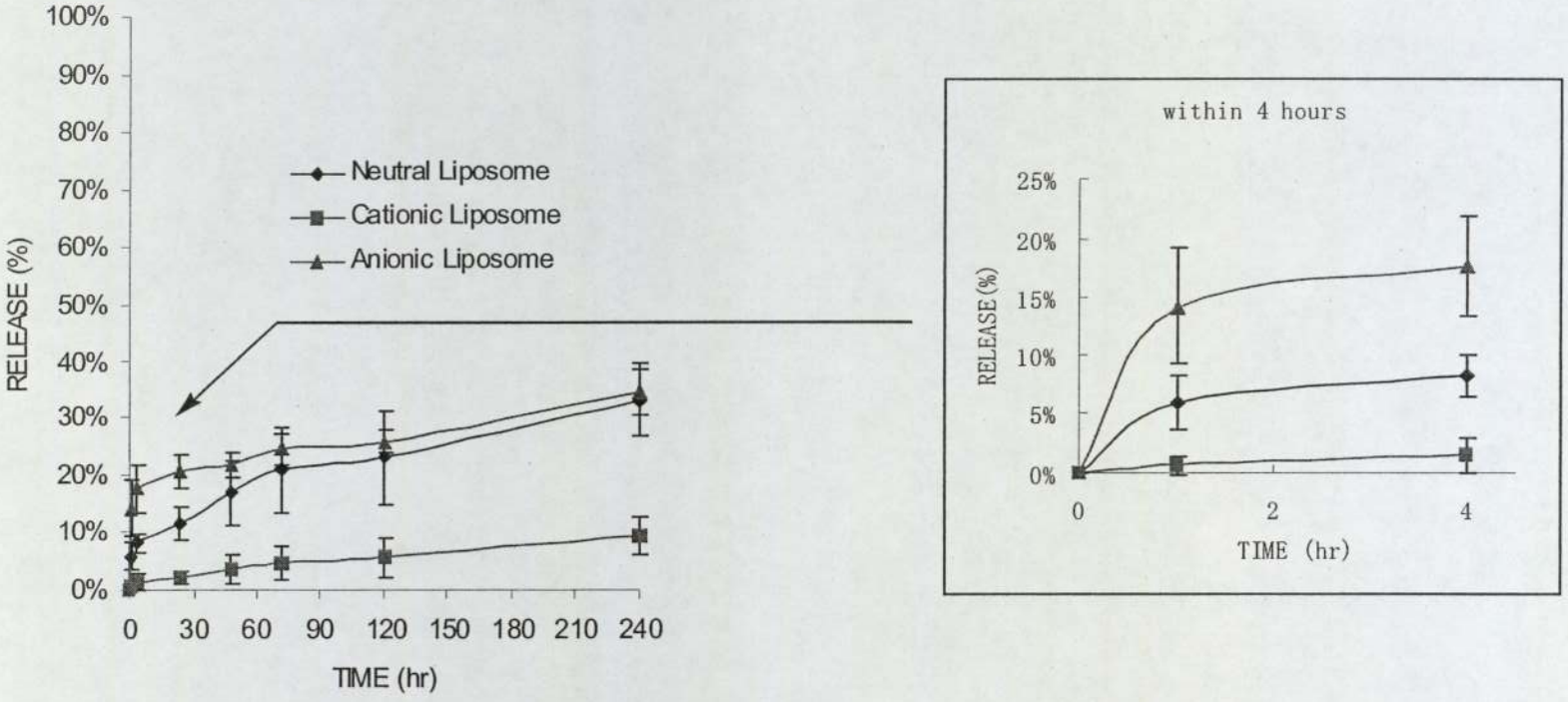
3.4.3 PNA retention within DRV

Changes in the structural characteristics of liposomes on storage were also monitored by the release of PNA at 4°C and 37°C. There are two reasons for determination of PNA retention at 4 °C. One is to see how the stablity of the liposomes are stored in the fridge temperature and thus to observe the basic leakage and important condition of liposomes. The other aspect is to obtain relative data which will be compared with the data from 37 °C.

High-temperature testing (>25 °C) is almost universally used for heterogeneous products, especially for liposomes [158]. In this aspect, increased temperatures may dramatically alter the nature of the interfacial film, especially if the phase-transition temperature is reached.

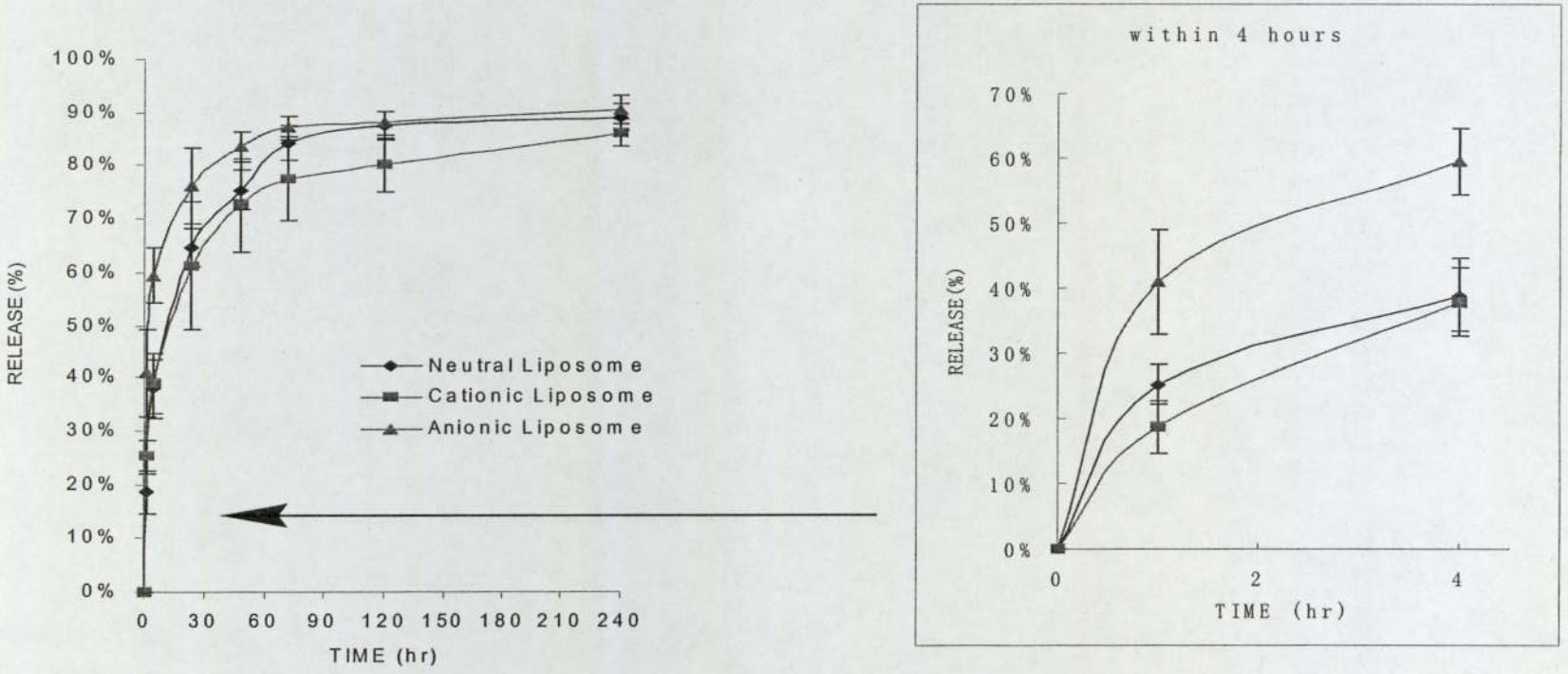
The release of PNA from various compositions of DRV (PNA) incubated at 4°C for up to 10 days is shown in **Figure 3.14**. Results indicate that liposomes composed of PC: CHOL: SA retained more of their entrapped PNA compared to their neutral or anionic counterparts. Indeed cationic DRV (DNA) had released less than 10% of their entrapped PNA after 10 days incubation compared to the neutral and anionic DRV which had released over 30%. Incubation of DRV (PNA) at 37°C resulted in a significantly enhanced PNA release (**Figure 3.15**) with all three formulations releasing 40% of their entrapped PNA after only 4 hours.

Figure 3.14 PNA release from DRV (PNA) stored at 4 °C [a]



a. 5(6)-FAM-NHS labeled PNA (25μg) was incorporated into DRV of various charge. DRV were composed of 16μmol PC and 8μmol CHOL (-♦-); 16μmol PC, 8μmol CHOL and 4μmol SA (-■-); 16μmol PC, 8μmol CHOL and 4μmol DP (-▲-); PNA release was measured at time intervals by 0, 1, 4, 24, 48, 72, 120, 240 hours. Values denote mean ± S.D. from at least three experiments.

Figure 3.15 PNA release from DRV (PNA) incubated at 37 °C [a]



a. 5(6)-FAM-NHS labeled PNA (25μg) was incorporated into DRV of various charge. DRV were composed of 16 μmol PC and 8 μmol CHOL (-♦-); 16μmol PC, 8 μmol CHOL and 4 μmol SA (-■-); 16 μmol PC, 8 μmol CHOL and 4 μmol DP (-▲-); PNA release was measured at time intervals by 0, 1, 4, 24, 48, 72, 120, 240 hours. Values denote mean ± S.D. from at least three experiments.

3.4.4 Formulation of stable DRV

To enhance the stability of these formulations liposomes were prepared substituting PC for DSPC (phosphotidyl choline lipid with a higher transition temperature) and/or incorporating equimolar CHOL - both factors previously shown to increase the rigidity of liposome bilayers thereby reducing drug release [144].

3.4.4.1 Factors influencing liposome stability: T_c and CHOL

Even if the mechanisms behind the leakage of water soluble substances through liposome lipid bilayer are far from fully understood, several factors are well known to influence the release rates. Among these are temperature and liposome associated parameters such as lipid composition.

T_c, the gel liquid crystalline transaction temperature, is the temperature at which the fatty acid chains of phospholipids melt and relative to state of liposomes. Usually, T_c is influenced by the composition of the liposomes. The longer acyl side chain is, the higher the T_c. For exemple, the T_c of DMPC (dimyristorylphophatidycholine, 14:0) is 24 °C and DSPC (18:0) is 56°C [159]. As mentioned, liposomes usually consist of one or more concentric bilayers altering with aqueous compartments and are composed of phospholipids and/or other amphiphiles. Normally, liposomes exist in a densely packed crystalline form when in the dry state. Depending on the nature of the amphiphile, bilayer can be in a "fluid" or "rigid" state at T_a (ambient temperature) [147]. So the fluid state is obtained with amphiphiles that have T_c below T_a, whereas the rigid state requires amphiphiles with a T_c higher than T_a. It is evidenced that liposomes composed of unsaturated lipids with T_c display lower stability towards leakage than saturated analogues with T_c [160]. Generally, if the water is added to the lipid above T_c, the structure of lipid bilayer will change from "rigid" state to "fluid" state. The polar head groups at the surface of the exposed amphiphile become hydrated and begin to reorganize into the lamellar form. The water dispenses through this surface bilayer

resulting in the increasing mobility and fluidity of the underlying lipid, which leads to carry out a similar rearrangement, and the process is repeated until the whole lipid is organized into a series of parallel lamellae, each aparted from the next by a layer of water. Once this liquid crystalline phase is fully filled with and in equilibrium with excess water, mild shaking allows parts of close, multilamellar lipid to break away and leads to the change of membrane permeability [73]. Thus, it is the well known permeability increase at the phase transition temperature can provide a means to trigger release of trapped solute, that is, when the T_c of liposomes is not high enough or less than 37°C, the compound (PNA) which has been entrapped will release quickly when the temperature reaches 37°C. So T_c is the important factor which investigates the stability and permeability of liposomes.

In addition, CHOL is also a notable factor which is relative to the mobility of the membrane of the liposomes. Generally speaking, mobility of the bilayer is important physical properties of the liopsomes and also connected tightly with the stability. The rate of drug releasing will be up to maximum when reaching T_c, which increase the mobility of the liposomal membrane. So CHOL plays an important regulating role in biological membranes, improving the mechanical stability of lipid bilayer, reducing the permeability of water solute substances. CHOL usually is considered as "fluidity buffer" because adding of the CHOL in the lower temperature ($< T_c$) will increase the membrane fluidity and reduce the order arrangement of the bilayer. However when the temperature is higher than T_c, the combination of CHOL will decrease the membrane fluidity and improve the order arrangement of the bilayer. As a result, CHOL content do have a more pronounced effect on the ability of the liposomes to withstand osmotic pressures [144] and the existing of CHOL is also well known to reduce the release rates by an increase in packing order and cohesive strength of the membrane. The reason for choosing PC : CHOL (1:1) is not only because the important function of CHOL, but also from the documents [144]in which there have been shown to be particularly stable on this composition [PC : CHOL (1:1)] when using the DRV method. From the studies

of Dos Santos's group, the results of doxorubicin loading studies indicated that the drug could not be loaded efficiently into cholesterol-free DSPC liposomes [161].

3.4.4.2 High transition tempreture lipids

The use of DSPC in the DRV (PNA) formulation results in the formation of liposomes with more rigid bilayer due to the high transaction temperature (56°C) of DSPC which is higher than 37 °C. These are expected to be less susceptible to breakdown at 37°C compared to DRV (PNA) made of the low melting PC.

As to the combination of equimolar cholesterol and DSPC, there also are many examples for using this composition. In 1998, the research from Dr Mats *et al* showed the decreasing leakage when the composition is changed with DSPC/CHOL (1:1 molar ratio) composition being the most stable, which matched to the theoretical predictions and earlier reported results [162]. Also next year, The effort from Dr Elisabeth *et al* was that they found significantly improved retention could be achieved by substitution of the anionic lipid DSPG (distearoylphosphatidylglycerol) for DSPC in the LUV bilayer when they determined factors influencing retention of amino-containing drugs in LUV [163]. In the paper of Perrie and Gregoriadis, DNA leakage for DSPC DRV (DNA) was shown to be significantly lower than various other lower transition temp lipid formulations [145]. However, as to the relationship between cholesterol and PC or DSPC, the obtained results from very recent paper indicated that the affinity of cholesterol to saturated/ unsaturated lipid does not differ significantly, and at the same time discover strong influence of the kind of a polar group on the cholesterol - phospholipid interactions [164]. Thus, it was interesting to observe whether decrease of vesicle leakage to solutes and resulting in enhanced drug retention by cholesterol-induced bilayer with DSPC, which substitutes PC.

To investigate the effect of employing such liposomes to entrap PNA, DSPC: CHOL (1:1 molar ratio) were prepared, and results in **Table 3.7** show that the substitution of

Table 3.7 Incorporation of PNA into DRV preparations: The effect of hydrophobic bilayer domain [a]

Liposome Formulation	MLV		SUV		DRV(PNA)		
	Zeta potential (mV)	Vesicle size (nm ± S.D.)	Zeta potential (mV)	Vesicle size (nm ± S.D.)	PNA incorporation (% of used)	Zeta potential (mV)	Vesicle size (nm ± S.D.)
PC:CHOL (16: 16μmol)	-5.3 ± 1.7	1067.9 ± 58.2	-4.9 ± 1.5	104.6 ± 7.7	70.9 ± 2.1	-1.4 ± 1.9	1497.5 ± 138.4
DSPC :CHOL (16 : 16μmol)	-7.8 ± 3.5	2021.8 ± 200.0	-2.6 ± 1.8	100.0 ± 5.0	70.6 ± 1.6	-7.0 ± 1.2	2287.4 ± 129.9

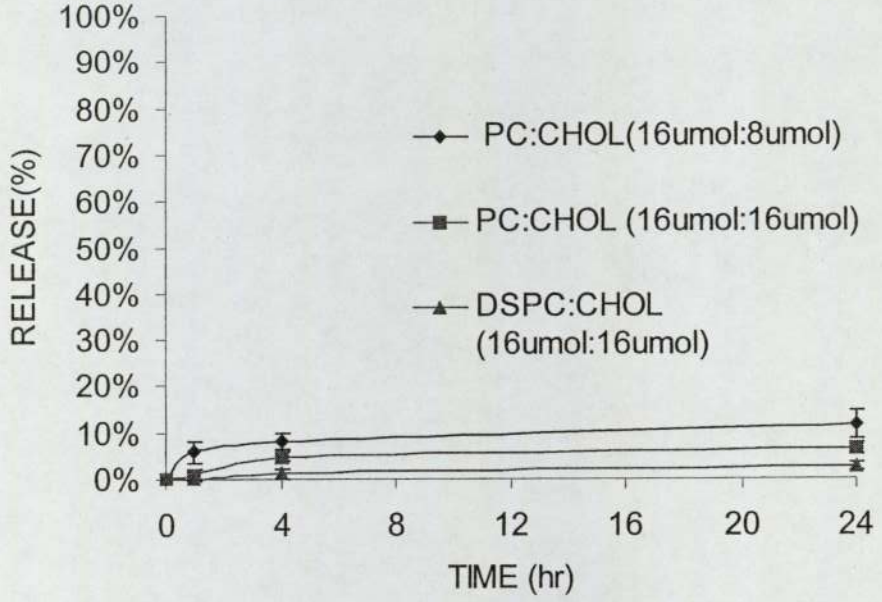
a. Incorporation of PNA into DRV preparations: The effect of hydrophobic bilayer domain. 5(6)-FAM-NHS labelled PNA (50μg) was entrapped in [DRV(PNA)] DRV prepared with either phosphatidyl choline (PC) or distearoyl PC (DSPC). PNA incorporation, zeta potential and vesicle z-average diameter were all measured as outlined in Table 7 legend. Values denote mean ± S.D. from at least three experiments.

PC for DSPC did not significantly affect the zeta potential of MLV, SUV or DRV (PNA). This is to be expected since the head group of DSPC is the same as that of PC. However the vesicle of both the MLV and DRV (PNA) was shown to be significantly bigger in DSPC formulation. This maybe due to tha fact that the neutral MLV and DRV can establish more bilayer resulting in an increased vesicle size because of no effect of the charge. But this difference did not influence PNA entrapment with both DRV formulations incorporating 71% of PNA add (50 μg).

As shown in **Figure 3.16**, it is demonstrated that the combined use of cholesterol and DSPC has been able to reduce PNA loss. Decreasing the bilayer cholesterol content from 16 to 8μmols increased PNA release for liposomes incubated at both 4 and 37°C (**Figure 3.16 and 3.17** respectively), especially from **Figure 3.17**, it can be seen that the release percent of PC: CHOL (16:8 μmol) is 21% higher than that of PC: CHOL (16:16 μmol). Further the substitution of PC for DSPC also decreased PNA release under both conditions with only 39% being released from DSPC: CHOL (16: 16 μmol) compared to 65% for PC: CHOL (16:8 μmol).

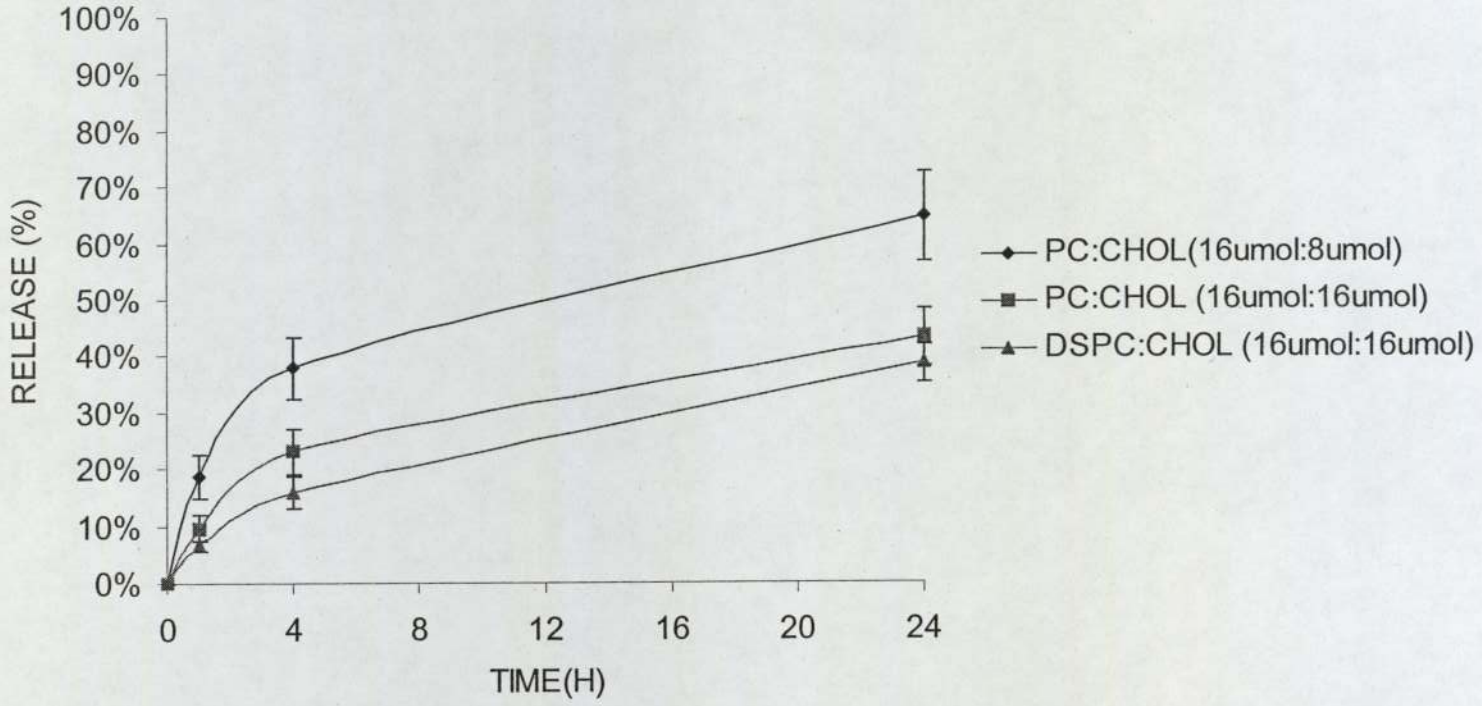
Taken together, these results indicate that neutral liposomes are the highest in the entrapment of the PNA when the dehydration-rehydration method is employed. The substitution of PC for DSPC can make the liposomes stronger and decrease the release of the PNA but not influence the entrapment of the PNA.

Figure 3.16 PNA release at 4 °C from rigid bilayer liposomes [a]



a. 5(6)-FAM-NHS labelled PNA (25μg) was incorporated into DRV of various composition. DRV were composed of 16μmol PC and 8μmol CHOL (-♦-); 16μmol PC, 16μmol CHOL (-■-); 16μmol DSPC, 16μmol CHOL (-▲-); PNA release was measured at time intervals by 0, 1, 4, 24 hours. Values denote mean ± S.D. from at least three experiments.

Figure 3.17 PNA release from rigid bilayer liposomes incubated at 37 °C [a]



a. 5(6)-FAM-NHS labelled PNA (25μg) was incorporated into DRV of various composition. DRV were composed of 16μmol PC and 8μmol CHOL (-♦-); 16μmol PC, 16μmol CHOL (-■-); 16μmol DSPC, 16μmol CHOL (-▲-); PNA release was measured at time intervals by 0, 1, 4, 24 hours. Values denote mean ± S.D. from at least three experiments.

4. Conclusions

PNA is a DNA/PNA mimic in which the PO linkage is replaced by a peptide bond. The unique properties of PNAs such as high affinity and specificity towards DNA/RNA, resistance to the nucleases or proteases, etc. have attacted the interests of many scientists and the use of PNA as synthetic oligomers has proven their potential usefulness in the field of molecular biology procedures, diagnosis assays and antisense therapeutics. The results from the thesis suggested that the high yield of PNA oligomer can be achieved by applying the solid phase synthesis in hand although the procedure is very laborous.

The dehydration-rehydration procedure for the entrapment of PNA into liposomes, as described here, is characterized by the mild preparation technique. The present results also strongly suggest that neutral liposomes offer the highest PNA entrapment when the dehydration-rehydration method is employed and is therefore a potenial PNA delivery system. In addition, DSPC liposomes (neutral liposomes) where shown to offer high PNA retention and exhibit greater stability when incubated at physiological temperature compared to other liposomal formulations tested. In addition, the results demonstrate that variation in the liposome bilayer composition can have implications on PNA retention and release, offering the potential to provide controlled release systems applicable to nucleic acid therapies. Overall, the method of dehydration-rehydration method is encouraging method for the preparation of PNA-liposomal delivery systems which can offer high PNA loading, mild preparation methods and the potential to optimise PNA release.

5. Future work

The clinical applications of PNAs are usually hampered by its inefficent cellular uptake. The results from the thesis strongly suggested that neutral liposomes with entrapped

PNA could be investigated *in vitro* and *in vivo* in future work to become a potential means of increasing the efficiency of cellular uptake of PNA due to the advantages of liposomal targeting which can increase the PNA delivery to the cells and tissues. In addtion, the PNA can also be modified or hybirdized with DNA and incorporated within cationic liposomes, which can further increase the percent of material (modified PNA or PNA-DNA) entrapment into liposomes and thus attempt to improve the PNA cellular delivery by overcoming both cellular and intracellular barriers.

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Appendices

APPENDIX-1 The Size of MLV (neutral liposome)

Brookhaven Instruments Corp.
ZetaPlus Particle Sizing Software Ver. 3.37

Date: Apr 23, 2003
Time: 11:58:14
Batch: 0

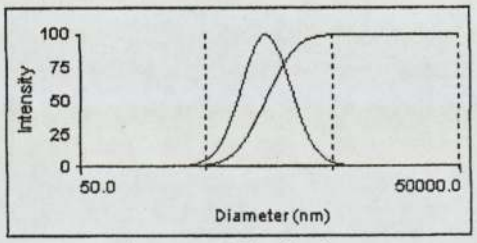
Sample ID **PC:Chol(MLV)-6 (Combined)**
Operator ID **PENG**
Notes **NO CHARGE**

Measurement Parameters:

Temperature	= 25.0 deg. C	Runs Completed	= 3
Liquid	= Aqueous	Run Duration	= 00:02:00
Viscosity	= 0.890 cP	Total Elapsed Time	= 00:06:00
Ref.Index Fluid	= 1.330	Average Count Rate	= 204.3 kcps
Angle	= 90.00	Ref.Index Real	= 1.590
Wavelength	= 659.0 nm	Ref.Index Imag	= 0.000
		Dust Filter	= On

PC:Chol(MLV)-6 (Combined)

Effective Diameter: 1478.8 nm
Polydispersity: 0.215
Sample Quality: 0.0/ 8.75%
Elapsed Time: 00:06:00



Lognormal Distribution

Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Sample Quality
1	1427.8	549.3	0.148	0.0/ 8.47%
2	1749.0	967.4	0.306	0.0/ 6.11%
3	1455.1	751.0	0.266	0.0/ 11.67%
Mean	1544.0	755.9	0.240	0.0/ 8.75%
Std. Error	102.8	120.7	0.047	0.0/ 1.61
Combined	1478.8	685.2	0.215	0.0/ 8.75%

APPENDIX-2 The charge of MLV (positive liposome)

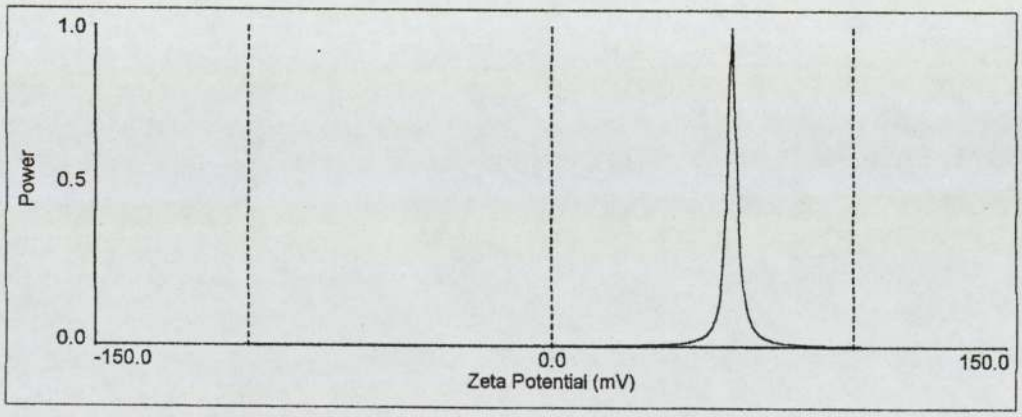
Brookhaven Instruments Corp.
Zeta Potential Analyzer Ver. 3.23

Date: Apr 23, 2003
Time: 12:35:14
Batch: 1

Sample ID **PC:Chol:SA(MLV)-6 (Run 10)**
Operator ID **PENG**
Notes **POSITIVE**

Measurement Parameters:			
Avg. Zeta Potential	= 66.14 mv	Liquid	= Aqueous
Avg. Mobility	= 5.17 (μs) / (V/cm)	Temperature	= 25.0 deg. C
pH	= 7.40	Viscosity	= 0.890 cP
Conductance	= 0 μS	Refractive Index	= 1.330
Concentration	= 1.00 mg/mL	Dielectric Constant	= 78.54
		Particle Size	= 0.0 nm

Instrument Parameters:			
Sample Count Rate	= 1300 kcps	Current	= 0.14 mA
Ref. Count Rate	= 2801 kcps	Electric Field	= 24.02 V/cm
Sampling Time	= 256 μs	User1	= 0.00
Wavelength	= 659.0 nm	User2	= 0.00



Run	Zeta Potential (mV)	Half Width (mV)
1	73.47	1.77
2	67.64	1.67
3	62.85	2.02
4	68.63	2.17
5	64.92	2.87
6	67.68	1.80
7	60.98	4.30
8	68.82	1.88
9	51.35	1.77
10	60.25	1.75
Mean	66.14	2.25
Std. Error	1.42	0.28

APPENDIX-3 The Size of SUV (neutral liposome)

Brookhaven Instruments Corp.
ZetaPlus Particle Sizing Software Ver. 3.37

Date: Apr 15, 2003
Time: 12:40:39
Batch: 0

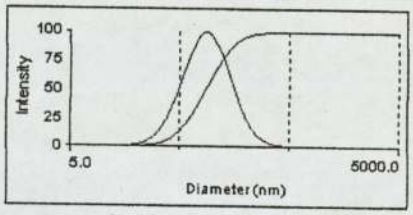
Sample ID **PC:Chol(suv) (Combined)**
Operator ID **PENG**
Notes **NO CHARGE**

Measurement Parameters:

Temperature	= 25.0 deg. C	Runs Completed	= 3
Liquid	= Aqueous	Run Duration	= 00:02:00
Viscosity	= 0.890 cP	Total Elapsed Time	= 00:06:00
Ref.Index Fluid	= 1.330	Average Count Rate	= 27.4 kcps
Angle	= 90.00	Ref.Index Real	= 1.590
Wavelength	= 659.0 nm	Ref.Index Imag	= 0.000
		Dust Filter	= On

PC:Chol(suv) (Combined)

Effective Diameter: 87.1 nm
Polydispersity: 0.279
Sample Quality: 0.0/ 88.43%
Elapsed Time: 00:06:00



Lognormal Distribution

Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Sample Quality
1	81.0	44.0	0.295	0.0/ 79.86%
2	75.5	39.4	0.273	0.0/ 87.22%
3	102.4	53.4	0.272	0.0/ 98.20%
Mean	86.3	45.6	0.280	0.0/ 88.43%
Std. Error	8.2	4.1	0.007	0.0/ 5.33
Combined	87.1	46.0	0.279	0.0/ 88.43%

APPENDIX-4 The Charge of SUV (positive liposome)

Brookhaven Instruments Corp.
Zeta Potential Analyzer Ver. 3.23

Date: Apr 18, 2003
Time: 15:34:52
Batch: 1

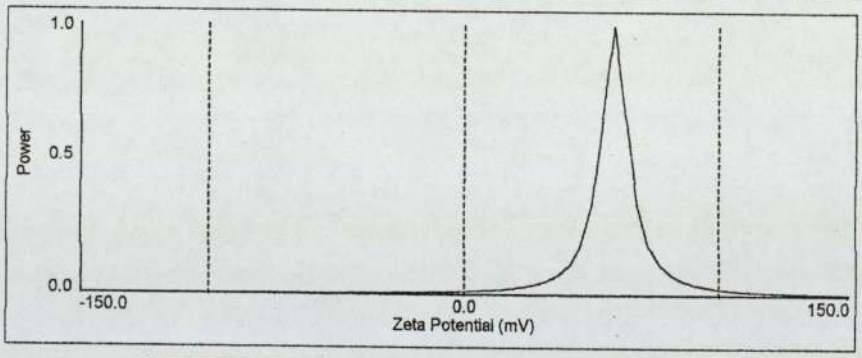
Sample ID **PC:Chol:SA(SUV)-5 (Run 10)**
Operator ID **PENG**
Notes **POSITIVE**

Measurement Parameters:

Avg. Zeta Potential	= 65.11 mv	Liquid	= Aqueous
Avg. Mobility	= 5.09 (μs) / (V/cm)	Temperature	= 25.0 deg. C
pH	= 7.40	Viscosity	= 0.890 cP
Conductance	= 5344 μS	Refractive Index	= 1.330
Concentration	= 1.00 mg/mL	Dielectric Constant	= 78.54
		Particle Size	= 0.0 nm

Instrument Parameters:

Sample Count Rate	= 462 kcps	Current	= 28.97 mA
Ref. Count Rate	= 2777 kcps	Electric Field	= 14.28 V/cm
Sampling Time	= 128 μs	User1	= 0.00
Wavelength	= 659.0 nm	User2	= 0.00



Run	Zeta Potential (mV)	Half Width (mV)
1	71.79	5.87
2	68.04	4.83
3	58.22	5.91
4	69.61	5.37
5	69.61	6.41
6	65.84	4.54
7	56.56	5.91
8	64.69	5.36
9	67.44	7.06
10	59.26	6.24
Mean	65.11	5.75
Std. Error	1.68	0.24

APPENDIX-5 The Size of empty DRV (negative liposome)

Brookhaven Instruments Corp.
ZetaPlus Particle Sizing Software Ver. 3.37

Date: Apr 16, 2003
Time: 11:50:10
Batch: 0

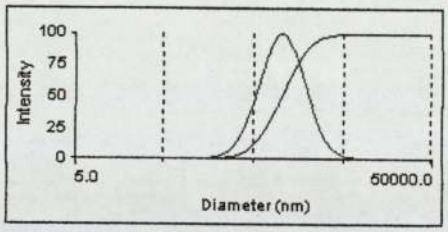
Sample ID **PC:Chol:DP(DRV) (Combined)**
Operator ID **PENG**
Notes **NEGATIVE**

Measurement Parameters:

Temperature	= 25.0 deg. C	Runs Completed	= 3
Liquid	= Aqueous	Run Duration	= 00:02:00
Viscosity	= 0.890 cP	Total Elapsed Time	= 00:06:00
Ref.Index Fluid	= 1.330	Average Count Rate	= 692.0 kcps
Angle	= 90.00	Ref.Index Real	= 1.590
Wavelength	= 659.0 nm	Ref.Index Imag	= 0.000
		Dust Filter	= Off

PC:Chol:DP(DRV) (Combined)

Effective Diameter: 1012.1 nm
Polydispersity: 0.397
Sample Quality: 0.0
Elapsed Time: 00:06:00



Lognormal Distribution

Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Sample Quality
1	1033.7	673.8	0.425	0.0
2	1015.4	625.9	0.380	0.0
3	1018.4	611.3	0.360	0.0
Mean	1022.5	637.0	0.388	0.0
Std. Error	5.7	18.9	0.019	0.0
Combined	1012.1	637.7	0.397	0.0

APPENDIX-6 The Charge of empty DRV (negative liposome)

Brookhaven Instruments Corp.
Zeta Potential Analyzer Ver. 3.23

Date: May 7, 2003
Time: 16:28:18
Batch: 1

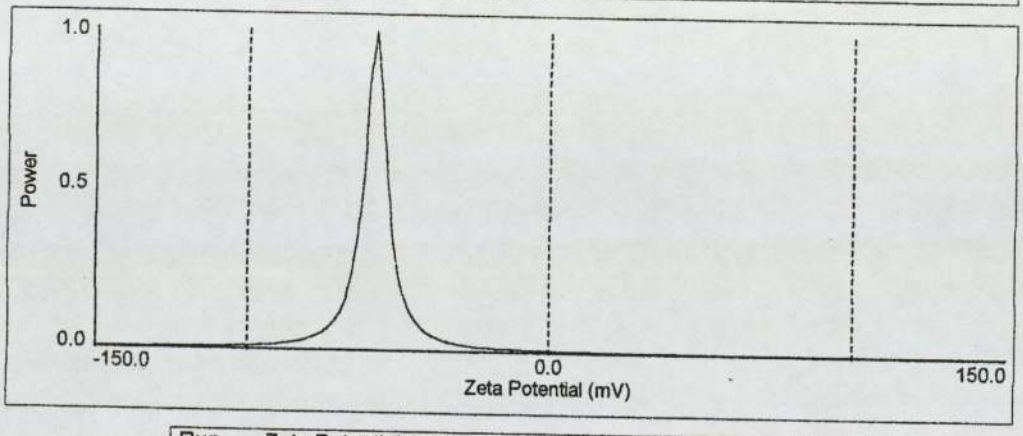
Sample ID **PC:CHOL:DP(DRV)-7 (Run 10)**
Operator ID **PENG**
Notes **NEGATIVE**

Measurement Parameters:

Avg. Zeta Potential	= -63.99 mv	Liquid	= Aqueous
Avg. Mobility	= -5.00 (μ/s) / (V/cm)	Temperature	= 25.0 deg. C
pH	= 3.00	Viscosity	= 0.890 cP
Conductance	= 703 μS	Refractive Index	= 1.330
Concentration	= 1.00 mg/mL	Dielectric Constant	= 78.54
		Particle Size	= 0.0 nm

Instrument Parameters:

Sample Count Rate	= 943 kcps	Current	= 3.91 mA
Ref. Count Rate	= 2566 kcps	Electric Field	= 14.80 V/cm
Sampling Time	= 256 μs	User1	= 0.00
Wavelength	= 659.0 nm	User2	= 0.00



Run	Zeta Potential (mV)	Half Width (mV)
1	-58.89	3.15
2	-66.93	3.69
3	-69.02	4.47
4	-59.69	6.88
5	-66.54	5.36
6	-66.61	2.76
7	-64.23	3.60
8	-66.54	4.49
9	-63.27	4.22
10	-58.13	4.20
Mean	-63.99	4.28
Std. Error	1.22	0.37

APPENDIX-7 The Size of DRV mixed PNA (neutral liposome)

Brookhaven Instruments Corp.
ZetaPlus Particle Sizing Software Ver. 3.37

Date: Apr 24, 2003
Time: 14:46:05
Batch: 0

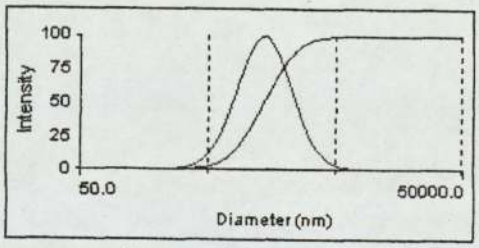
Sample ID **PC:Chol(PNA-DRV)-6 (Combined)**
Operator ID **PENG**
Notes **NO CHARGE**

Measurement Parameters:

Temperature	= 25.0 deg. C	Runs Completed	= 3
Liquid	= Aqueous	Run Duration	= 00:02:00
Viscosity	= 0.890 cP	Total Elapsed Time	= 00:06:00
Ref.Index Fluid	= 1.330	Average Count Rate	= 668.4 kcps
Angle	= 90.00	Ref.Index Real	= 1.590
Wavelength	= 659.0 nm	Ref.Index Imag	= 0.000
		Dust Filter	= Off

PC:Chol(PNA-DRV)-6 (Combined)

Effective Diameter: 1343.6 nm
Polydispersity: 0.265
Sample Quality: 8.5
Elapsed Time: 00:06:00



Lognormal Distribution

Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Sample Quality
1	1263.6	622.3	0.243	6.9
2	1355.6	741.1	0.299	6.6
3	1417.1	754.0	0.283	7.2
Mean	1345.4	705.8	0.275	6.9
Std. Error	44.6	41.9	0.017	0.2
Combined	1343.6	691.2	0.265	8.5

APPENDIX-8 The Charge of DRV mixed PNA (positive liposome)

Brookhaven Instruments Corp.
Zeta Potential Analyzer Ver. 3.23

Date: Apr 24, 2003
Time: 15:23:10
Batch: 1

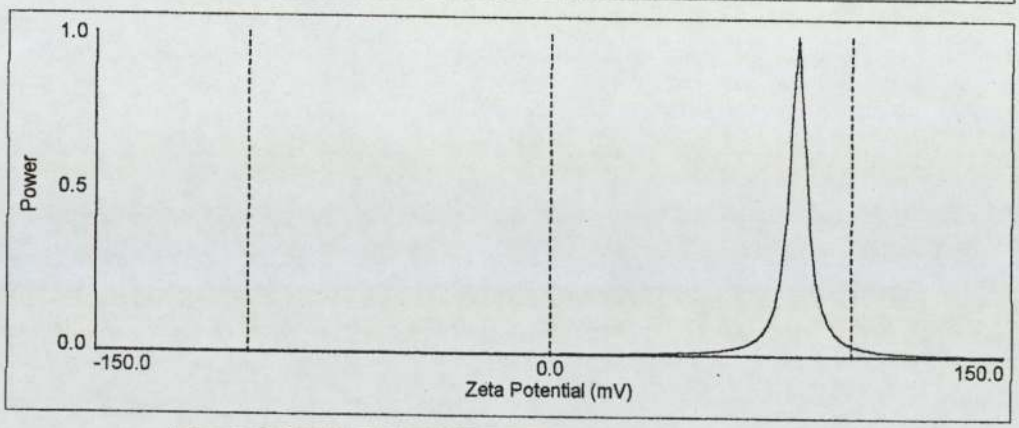
Sample ID **PC:Chol:SA(PNA-DRV)-6 (Run 10)**
Operator ID **PENG**
Notes **POSITIVE**

Measurement Parameters:

Avg. Zeta Potential	= 67.62 mv	Liquid	= Aqueous
Avg. Mobility	= 5.28 (μs) / (V/cm)	Temperature	= 25.0 deg. C
pH	= 7.40	Viscosity	= 0.890 cP
Conductance	= 1097 μS	Refractive Index	= 1.330
Concentration	= 1.00 mg/mL	Dielectric Constant	= 78.54
		Particle Size	= 0.0 nm

Instrument Parameters:

Sample Count Rate	= 1252 kcps	Current	= 6.04 mA
Ref. Count Rate	= 2640 kcps	Electric Field	= 14.39 V/cm
Sampling Time	= 256 μs	User1	= 0.00
Wavelength	= 659.0 nm	User2	= 0.00



Run	Zeta Potential (mV)	Half Width (mV)
1	-56.03	2.79
2	68.92	4.46
3	59.76	3.56
4	65.58	5.32
5	62.30	3.66
6	75.45	3.67
7	68.34	4.70
8	67.48	3.97
9	73.13	4.48
10	-82.34	3.29
Mean	67.62	4.23
Std. Error	1.83	0.22

APPENDIX-9 The Size of DRV entrapped with PNA (neutral liposome)

Brookhaven Instruments Corp.
ZetaPlus Particle Sizing Software Ver. 3.37

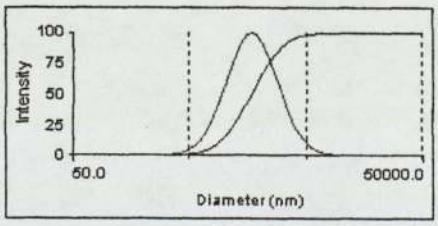
Date: May 7, 2003
Time: 15:39:04
Batch: 0

Sample ID **PC:CHOL[DRV(PNA)]-1 (Combined)**
Operator ID **PENG**
Notes **NO CHARGE**

Measurement Parameters:			
Temperature	= 25.0 deg. C	Runs Completed	= 3
Liquid	= Aqueous	Run Duration	= 00:02:00
Viscosity	= 0.890 cP	Total Elapsed Time	= 00:06:00
Ref.Index Fluid	= 1.330	Average Count Rate	= 341.0 kcps
Angle	= 90.00	Ref.Index Real	= 1.590
Wavelength	= 659.0 nm	Ref.Index Imag	= 0.000
		Dust Filter	= On

PC:CHOL[DRV(PNA)]-1 (Combined)

Effective Diameter: 1701.5 nm
Polydispersity: 0.276
Sample Quality: 1.2/ 32.08%
Elapsed Time: 00:06:00



Lognormal Distribution

Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Sample Quality
1	1451.8	697.2	0.231	2.8/ 25.97%
2	1779.2	955.6	0.288	0.0/ 40.83%
3	1792.6	962.6	0.288	2.8/ 29.44%
Mean	1674.5	871.8	0.269	1.9/ 32.08%
Std. Error	111.5	87.3	0.019	0.9/ 4.49
Combined	1701.5	893.3	0.276	1.2/ 32.08%

APPENDIX-10 The Charge of DRV entrapped with PNA (positive liposome)

Brookhaven Instruments Corp.
Zeta Potential Analyzer Ver. 3.23

Date: May 13,2003
Time: 13:02:17
Batch: 1

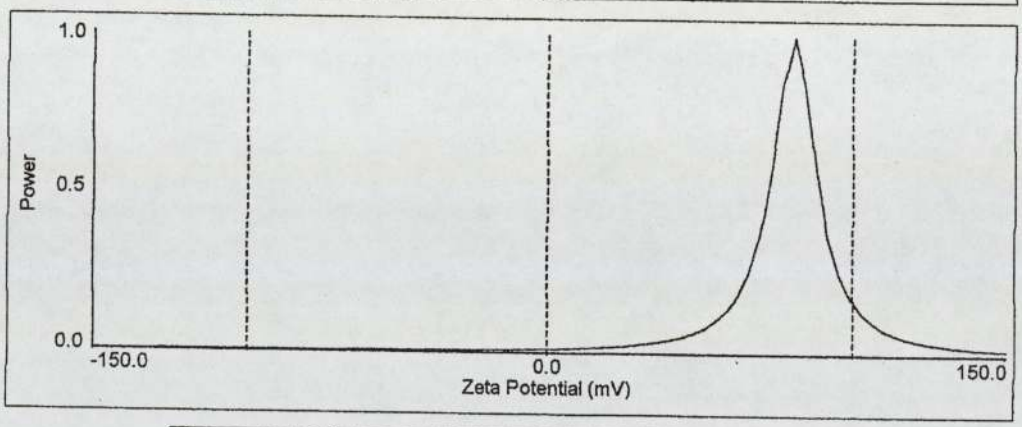
Sample ID **PC:Chol:SA(DRV[PNA])-2 (Run 10)**
Operator ID **PENG**
Notes **POSITIVE**

Measurement Parameters:

Avg. Zeta Potential	= 76.67 mv	Liquid	= Aqueous
Avg. Mobility	= 5.99 (μs) / (V/cm)	Temperature	= 25.0 deg. C
pH	= 7.40	Viscosity	= 0.890 cP
Conductance	= 1969 μS	Refractive Index	= 1.330
Concentration	= 1.00 mg/mL	Dielectric Constant	= 78.54
		Particle Size	= 0.0 nm

Instrument Parameters:

Sample Count Rate	= 1147 kcps	Current	= 10.75 mA
Ref. Count Rate	= 2725 kcps	Electric Field	= 16.62 V/cm
Sampling Time	= 128 μs	User1	= 0.00
Wavelength	= 659.0 nm	User2	= 0.00



Run	Zeta Potential (mV)	Half Width (mV)
1	76.69	2.94
2	65.42	2.99
3	65.42	4.46
4	56.41	4.36
5	62.69	5.31
6	80.89	4.46
7	75.50	4.46
8	84.49	5.10
9	84.22	5.13
10	80.69	8.64
Mean	76.67	4.77
Std. Error	2.69	0.63