Engineering considerations on the use of liquid/liquid two phase systems as a cell culture platform

Halina Murasiewicz[1*], Alvin W. Nienow[1, 2], Mariana P. Hanga[2], Karen Coopman[2], Christopher J. Hewitt[3] and Andrzej W. Pacek[1]

1 School of Chemical Engineering, University of Birmingham, Birmingham, Edgbaston, B15 2TT, UK

2 Centre for Biological Engineering, Dept. Chemical Engineering, Loughborough University, Loughborough, Leicestershire, LE11 3TU, UK

3 Aston Medical Research Institute, School of Life and Health Sciences, Aston University, Aston Triangle, Birmingham, B4 7ET, UK

*Corresponding author: h.murasiewicz@bham.ac.uk, phone number: +44(0)121 414 5081

Abstract

BACKGROUND: Application of perfluorocarbon based liquid/liquid two phase systems for cell culture expansion has been investigated at small scale for more than 30 years and it has been established that such systems are able to support the survival of a variety of cell lines. Application of drops in liquid/liquid dispersions as temporary microcarriers is an exciting prospect as it enables adherent cells to be grown in stirred bioreactors, without the need to use enzymatic dissociation methods to harvest the cells.

RESULTS: Two aspects of scaling up of perfluorocarbon/cell culture medium dispersions were investigated: (i) the effect of processing conditions on drop size/interfacial area and (ii) the kinetics of separation of a stagnant dispersion. The processing conditions to produce the stable

"liquid microcarriers" with the average drop size between 150 - 220 μm have been established. Separation of dispersion into two continuous systems requires complete removal of proteins from the perfluorocarbon/cell culture media interface.

CONCLUSIONS: The correlation relating average drop size to the energy input and physical properties of both phases was developed and the method of separation of stable perfluorocarbon/cell culture medium dispersion was established. As the perfluorocarbon does not deteriorate during cell expansion and subsequent separation followed by sterilization, it could be re-used, making application of such systems at a large scale very attractive and economical.

Key words*: "Liquid microcarriers", Cell culture, Emulsification/dispersion, Perfluorocarbon, Bioreactors*

Introduction

Human stem cells have proven very difficult to expand in culture because of the absolute need for adherent culture conditions. Since it is the stem cells themselves that form the basis of the products, there are further challenges in harvesting these whilst ensuring their survival and the maintenance of their multipotency for specific clinical purposes. In recent small - scale investigations of human mesenchymal stem cells (hMSCs) expansion on microcarriers (Cytodex-3[1] and SoloHill Plastic[2]), the cells were harvested by a process called trypsinisation that uses an active concentration of the proteolytic enzyme, trypsin, to remove cells from the surface of microcarriers. However, attempts to harvest larger quantities of cells using the same method often resulted in a high degree of cell death and loss of multipotency[3].

To solve this problem, two-phase liquid/liquid systems, or more specifically the interface between two immiscible liquids, have previously been used to culture mammalian cells and the

system where perfluorocarbon was dispersed in cell medium, was particularly popular as shown in Table 1. Perfluorocarbons were selected because they are inert, do not have an adverse effect on cell growth and have very high oxygen solubility and the first application of fluorocarbon emulsions in cell culture (drops between 100 and 500 μm) for cell cultivation was reported in the early eighties[4]. Since then many researchers used such systems with different cells and the wide range of perfluorocarbons and the main results are briefly summarised in Table 1.

Table 1 here

Table 1 clearly shows that perfluorocarbon either improves or at least does not have an adverse effect on cell growth and that in all reported cases harvesting is far simpler (via filtration, centrifugation or aspirating at the interface) and more efficient than harvesting from solid surfaces. However, a key limitation of all these studies is that only the biological aspects of the application of perfluorocarbon in cell cultivation have so far been investigated. Whilst the above results can be treated as a proof of concept, there is no information in the open literature on scaling-up of perfluorocarbon/cell culture two-phase systems so they can be used in large, commercial manufacturing systems for cell-based therapeutic applications.

Scale-up of the systems to form a flat interface (*i.e.* an interface between two stationary liquids) is of no interest for manufacturing applications because the specific interfacial area (ratio of the area of the interface to the volume of liquids) is very small. Therefore, here a system where the interface is spherical and similar to the morphology of solid microcarriers *e.g.* dispersion of perfluorocarbon drops in cell culture medium was investigated. There is a large body of literature on emulsification/dispersions of organic liquids in water[17], but the majority of the results are for pure liquids (without surfactant or salts) with the organic phase less dense than the aqueous phase. As fluorocarbons are nearly twice as dense as water and culture media often contain surface active compounds and salts, not all the literature data is applicable for

perfluorocarbon/cell culture medium dispersed systems. Therefore, two aspects of the processing of such systems were investigated here: (i) the emulsification/dispersion of fluorocarbon in a typical cell culture medium aimed at establishing optimal processing parameters enabling formation of the required drop size (interface area similar to the surface area of standard micro-carriers particles) and (ii) the separation of the emulsion/dispersion into two continuous phases. The results of the first part are essential to control drop size/interfacial area during cell growth whilst the results of the second part are necessary during the cell-harvesting step.

Materials and Methods

Materials

The results of preliminary experiments with human mesenchymal stem cells (data not shown) as well as the literature[9,11] indicated that Fluorinert®FC-40 (further referred to as FC-40) is particularly useful for cell growth. Therefore, both freshly supplied and recycled after use in cell culture, FC-40 (3M, Sigma-Aldrich, UK) was used here as the dispersed phase. Fresh FC-40 was used without further treatment. FC-40 was used for culturing bone marrow derived hMSCs (Lonza Biologics, passage 2-8) in an FC-40/supplemented DMEM system. A medium exchange was carried out at day 3 and after a total of 6 days, cells were harvested and the separated FC-40 sterilised in two ways: by filtration at room temperature with a 0.2 μm syringe filter with PES membrane (Millipore) and by autoclaving at 121°C, 2.1 bar for 15 minutes.

DMEM (1 g/L glucose, Lonza, UK) supplemented with 2 mM of ultraglutamine (Lonza, UK) and 10% foetal bovine serum (Life Sciences: GIBCO, ThermoFisher Scientific, UK) and Dulbecco's Phosphate Buffered Saline (D-PBS, Life Sciences: GIBCO, ThermoFisher Scientific, UK) was used as the continuous phase. DMEM supplemented with ultra-glutamine and foetal bovine serum was chosen. The same medium was used in cell growth experiments[18]

and it represents a common cell culture medium used with a variety of cell types. For the detailed chemical composition of DMEM and D-PBS see http://www.thermofisher.com/uk/en/home/technical-resources/media-formulation.183.html [19] and http://www.thermofisher.com/uk/en/home/technical-resources/media-formulation.147.html[20].

Methods

The physical properties: density, viscosity and surface tension and interfacial tension of fresh and sterilized FC-40 were measured as follows. Density and viscosity of all liquids were measured respectively using a densitometer (DMA 4100 M, Antoor Paar) and Bohlin rheometer (Malvern Instruments Ltd, UK) fitted with 2°/55 mm cone and plate geometry. Static interfacial tension between the fresh FC-40 and the aqueous phases and dynamic interfacial tension between FC-40 and supplemented DMEM were measured by the pendant drop method DSA 25 (Krüss GmbH, UK). The average zeta potential of FC-40 drops was measured by a Nano Zeta Sizer (Malvern Instruments Ltd, UK).

Two sets of experiments were subsequently carried out. In the first set, the effect of energy input (impeller speed) on drop size distribution and mean drop size of FC-40 dispersed in supplemented DMEM, D-PBS and in double distilled water was investigated both during breakage (a step increase of the impeller speed) and during coalescence (a step reduction of impeller speed). In the second set, the kinetics of separation of the resulting dispersions of both fresh as well as recycled FC-40 after stem cell expansion[18] were tested.

Experiments were carried out in the rig schematically shown in Fig. 1. A fully baffled, glass stirred vessel (T= 0.13 m) was connected to a water bath and fitted with a Rushton turbine

impeller (D = 0.05 m) located at 1/3 of the liquid height. Impeller speed was controlled by variable speed motor (IKA® Werke GmbH & Co. KG, Germany) and drop size distributions during emulsification/dispersion and during sedimentation were measured *in-situ* by *video*-microscope-computer system[21]. The captured images were analysed using ImageJ software with a plugin developed for this work.

Figure 1 here

The vessel was filled with 950 ml of continuous phase that was supplemented DMEM, D-PBS or double distilled water (used here as reference), and in all cases 50 ml of FC-40 was added. The minimum speed necessary to completely disperse FC-40 in the whole volume of the vessel was determined by visual observation and the maximum speed was selected to keep surface aeration to a minimum. The effect of the impeller speed (mean specific energy dissipation rate) on drop size distribution and mean drop size was investigated at room temperature both during breakage (a step increase of the impeller speed from 530 rpm to 590 rpm and then to 650 rpm for D-PBS; from 450 rpm to 500 rpm and to 560 rpm for DMEM and from 530 rpm, to 590 rpm and then to 650 rpm for distilled water); and during coalescence (a step reduction of the impeller speed using the same speeds but in the reversed order). In all experiments, the flow was turbulent with *Re* number between: 19000 and 23100 in DMEM, 24700 and 29500 in D-PBS and in 23600 and 29000 in water. For the FC-40/DMEM system, the same methodology was repeated at 37°C to mimic cell culture conditions. At each speed, the liquids were stirred for 30 min to reach the equilibrium drop size and after that time, the drop size distribution was measured. The effect of drop size on separation kinetics for FC-40/DMEM, FC-40/D-PBS and FC-40/double distilled water dispersions was investigated in the same experimental setup and the height of separated phases were measured as a function of time.

Results and discussion

Physical properties of investigated liquid phases

In this study, DMEM supplemented with L-glutamine and foetal bovine serum was chosen as the same composition was used in cell growth experiments[18] and it represents a common cell culture medium used in the culture of a variety of cell types. In terms of physical properties, at room temperature, viscosity and density of the supplemented DMEM and D-PBS were practically the same as that of water at 0.001 Pa s and 1010 - 1020 kg m^{-3} respectively. FC-40 is nearly twice as dense as water (1860 kg m^{-3}) and 4 times more viscous (0.004 Pa s). At the elevated temperature of 37˚C at which the cells are typically cultured, the density of all liquids was marginally lower (by 1 to 2%) and viscosity of FC-40 was equal to 0.003 Pa s.

The static interfacial tension between the fresh FC-40 and water was 52.67 mN m^{-1} and that between FC-40 and D-PBS solution was 64.04 mN m^{-1}. The dynamic interfacial tension between FC-40 and supplemented DMEM gradually decreased from 38.5 mN m^{-1} to reach an equilibrium value (static interfacial tension) of 26.50 mN m^{-1}.

Density, viscosity and surface tension of FC-40 after 3 days in culture in a spinner flask at 37˚C were also measured after cells were harvested from the interface (for details see Hanga et al.[18]). Part of the FC-40 was sterilised by filtration through a 0.2 µm filter at room temperature and the second part by autoclaving at 121^0C for 15 minutes. Density and viscosity were practically un-affected by cell growth and subsequent sterilisation and were equal to 1870 kg m^{-3} and 0.004 Pa s respectively. The interfacial tension of autoclaved FC-40 was practically the same as the interfacial tension when fresh, whereas after filtration, the interfacial tension was reduced by approximately 15% (22.4 mN m^{-1} and 25.3 mN m^{-1} post-filtration and post-autoclaving,

respectively). In culture, adherent cells such as hMSCs excrete a number of proteins, including fibronectin to create an extracellular matrix (ECM) on which they sit. These results might indicate that during filtration traces of the protein (from the ECM) left in FC-40 after cells were harvested are removed from FC-40.

Effect of impeller speed and composition of continuous phase on drop size/size distribution

Typical images of dispersions at different impeller speeds are shown in Fig. 2 and drop size distributions measured from such images are compared in Fig. 3. In stirred liquid/liquid two-phase systems, the average drop size decreases as the impeller speed increases and increases as the interfacial tension increases (see Eq. 1 below).

Figure 2 here

Indeed in both systems, the increase of impeller speed leads to the reduction of drop size (Figs 2a and 2b). However, despite the fact the interfacial tension between FC-40 and supplemented DMEM (26.50 mN m^{-1}) is much lower than the interfacial tension between FC-40 and D-PBS (64.04 mN m^{-1}), at the same impeller speeds the drop size in both systems were rather similar (Figs 2a and 2f). In a stirred system, steady state drop size is determined by the dynamic equilibrium between breakage and coalescence; therefore the above observation could only be explained by the fact that coalescence of the FC-40 drops in supplemented DMEM system is suppressed. Typically, coalescence in liquid/liquid two phase systems can be suppressed by the presence of surface active components or very small particles[22,23]. The presence of surface-

active components also reduces surface tension and promotes incorporation of air through the free surface (so-called surface aeration). In the FC-40/DMEM system, aeration started at a much lower impeller speed than in the FC-40/D-PBS system as shown by air bubbles (black circles) in Fig. 2a - 2c, which confirms that certain components of supplemented DMEM affected the properties of the interface. The reduction of drop size in the FC-40/DMEM system is indeed confirmed by the steady state drop size distribution in both systems, as shown in Fig. 3.

Figure 3. here

In the FC-40/DMEM system, drops size distributions are very narrow and close to normal indicating that coalescence is practically suppressed (or very slow) whereas, in the FC-40/D-PBS dispersions, drop size distribution are much wider and biased towards larger drops indicating a strong coalescence.

The Sauter mean diameters of FC-40 dispersed in D-PBS, in supplemented DMEM and in water calculated from drop size distributions measured over the range of specific energy dissipation rates and *We* numbers are compared in Fig. 4.

Figure 4 here

Dispersing organic liquids in water has been extensively investigated and it is commonly accepted that in fully developed, homogeneous turbulent flow, the Sauter mean diameter of non-viscous dispersed drops at very low concentrations so that coalescence is negligible can be correlated with the *We* number or with the specific energy dissipation rate, $\bar{\varepsilon}_T$:

$$\frac{d_{32}}{D} = A\,We^{\alpha} \quad (1)$$

$$d_{32} = B\bar{\varepsilon}_T{}^{\beta} \quad (1a)$$

Under these conditions, the theoretical values of exponent α and β are -0.6 and -0.4 respectively and experimental constants *A* depends on the type of impeller as does the constant *B* (plus, in this case, the physical properties which are contained in the Weber no.)[24]. For the impeller used in this work, experiments suggest $A = 0.056$[17, 25]. Here, fitting Eq 1 to the experimental data gave an *A* value of 0.11, approximately two times larger. However, with increasing volume fraction of dispersed phase, *A* generally increases at quite different rates in pure coalescing systems; and even with non-coalescing systems as the dispersed phase is considered to damp out the turbulence[17]. Here, the results discussed above clearly indicate the presence of surface active components in supplemented DMEM, so both the volume fraction of the dispersed phase and its composition impact on the extent of coalescence and thus the constant A. These different effects on the experimental constant A in Equations 1 for the different continuous phases are summarised in Table 2.

Table 2 here

The value of *B* obtained by plotting drop size as a function of specific energy dissipation rate fall into three distinctive lines that depend, as expected on the different values of interfacial tension and different properties of the interface. As expected, at the same energy dissipation rate, the smallest drops were formed in the FC-40/DMEM system as a result of the lowest interfacial tension and the suppressed coalescence in this system. The largest drops were formed in FC-40/D-PBS system which can be explained by the highest interfacial tension and

also by the presence of substantial amounts of different salts (it total approximately 0.2 M of KCl, KH_2PO_4, NaCl, Na_2HPO_4-$7H_2O$)[20] in the continuous phase. This explanation is in agreement with the results reported by Deshpande and Kumar[26] who found that the presence of salts drastically changes the dynamics of oil/water dispersions and leads to an increase in the average drop size.

By plotting the $d_{3,2}$ normalised with the impeller diameter as a function of the *We* number, the data for FC-40 dispersions can be practically collapsed into one line as shown in Fig. 4b.

The effect of temperature on the mean drop size is also shown in Fig. 4. As expected, the Sauter mean diameter was marginally lower at 37ºC. This reduction might be caused by the small reduction in viscosity of the dispersed phase at 37ºC and cause an increase in steric repulsion between drops.

Typically, cells are cultured for several days with intermittent medium exchanges to prevent critical nutrients such as glucose and glutamine or other substrates becoming limiting in the system or the build-up of metabolites e.g. lactate becoming inhibitory. There is very little information in the open literature on the effect of very long mixing times on the drop size/size distributions. Here, to mimic a typical cell culture process for hMSC, in which cells would be cultured in FC-40/supplemented DMEM for many days, the system was agitated for a total of 6 days to create recycled FC-40[18]. Following sterilization, the FC-40 was mixed for 3 days at N=450 rpm. Drop size distributions measured after 24, 48 and 72 hours were practically overlapping.

Coalescence in supplemented DMEM

Coalescence was investigated by a step reduction of impeller speed from 560 rpm to 500 rpm and then to 450 rpm. At each speed, the drop size distributions were measured after 30 min of mixing (just before a step reduction) and the results are shown in Fig. 5.

Figure 5. here

The steady state drop size distributions at each speed measured from coalescence practically overlap as shown in Fig. 5. Notice the difference between these drop size distributions and drop size distributions after breakage shown in Figs 3.

The Sauter mean diameters, $d_{3,2}$ after coalescence were practically constant (165 μm, 172 μm, 170 μm at 450 rpm, 500 rpm, 560 rpm, respectively). This similarity confirms that coalescence of FC-40 drops suspended in a supplemented DMEM under dynamic conditions was completely suppressed. The average zeta potential of FC-40 drops were Z = -18.1 mV indicating that electrostatic charges are far too low to prevent coalescence. The only possible explanation is the steric repulsion between drops covered by proteins that can be confirmed by the reduction of the interfacial tension between FC-40 and D-PBS (no protein) from 64.04 mN m^{-1} to 26.50 mN m^{-1} for that between FC-40 and supplemented DMEM (proteins). It is well known that during dispersion, proteins with an amphiphilic structure tend to diffuse and consequently adsorb at the oil–water (O/W) interface, lowering the interfacial tension. Proteins also form a protective interfacial membrane and/or generated repulsive forces between droplets, due to a combination of electrostatic interactions and hydrophobic interactions when pH is not close to the isoelectric point of the protein[27-29]. Such membranes may resist tensile or shearing stress and protect the droplets from coalescence[30].

Separation of perfluorocarbon/DMEM dispersions

Cell harvest from the FC-40/DMEM dispersion can be accomplished in two steps: first the dispersion will have to be separated into two continuous phases with a flat interface; and next cells can be harvested by aspiration, filtration or centrifugation. As discussed above, coalescence under dynamic conditions was completely suppressed, but this might also be due to a very short contact time after the collision between two drops during which the film separating them cannot reach the critical thickness required for inducing coalescence. Therefore, the coalescence under static conditions was investigated by recording the images of dispersion in the bottom part of the vessel just before and after the impeller was stopped (Fig. 6). The separation curves[31] determined from those images are shown in Fig. 7.

Figure 6 here

Figure 7 here

Fig. 7 shows that sedimentation of the FC-40 drops was very fast which is not surprising considering the large density difference between FC-40 and DMEM ($\Delta\rho$=800 kg m^{-3}), a very low viscosity of DMEM (0.001 Pa s) and relatively large drops; all those factors accelerate sedimentation.

The exceptional stability of the FC-40 dispersion in supplemented DMEM under static conditions can be explained by the steric stabilisation of drops by proteins present in the foetal bovine serum added to DMEM as a supplement for cell culture. Proteins have amphiphilic properties and are commonly used in the food industry for the stabilisation of oil-in-water emulsions[32]. Once adsorbed on the interface, the proteins unfold enabling the hydrophilic groups to protrude away from the surface into the aqueous phase, thus stabilising the drops by

steric interactions. This effect was confirmed here by dispersing FC-40 at the same volume fraction and impeller speed in: (a) pure DMEM, (b) DMEM supplemented only with ultraglutamine, (c) DMEM supplemented only with foetal bovine serum and (d) DMEM supplemented with both ultraglutamine and foetal bovine serum. In all those systems, the initial drop size was similar and once the impeller was stopped, the dispersions without proteins (systems (a) and (b)) separated into two continuous phases within 1 min. Only in systems containing proteins (system (c) and (d)) were stable FC-40 drops observed at the bottom of the vessel for more than 40s. Notably, although the manufacture of stem cells such as hMSCs for clinical use will occur in xeno-free formulations, alternatives to foetal bovine serum such as human platelet lysate are still protein rich and thus the stability of FC-40 in these systems should not be affected.

An attempt was made to break the FC-40/supplemented DMEM dispersion by centrifugation (Eppendorf, Model 5810). At acceleration up to 10G, the separation was not observed and at 210G, only about 10% of drops coalesced and formed a continuous FC-40 phase at the very bottom of the centrifuge tube. An increase in acceleration up to 1900G lead to the coalescence of approximately 50% of droplets, but a further increase to 3200G did not lead to an increase in the volume of the FC-40 coalescing into a continuous phase. Taking into consideration the fact that excessive forces during centrifugation might have a negative effect on the quality of the cells[33], higher accelerations were not tested.

The above results indicate that in order to break the FC-40/DMEM dispersions, proteins have to be removed from the drops' surface. To accomplish this, supplemented DMEM was replaced with D-PBS and the suspension was gently stirred for 10 min. After the impeller was stopped, the volume of the stable FC-40 drops was reduced by approximately 15%. This procedure was repeated and after the third replacement, sufficient proteins were removed so that when mixing

was stopped, the FC-40/D-PBS system completely separated into two distinct continuous phases.

Re-suspension of FC-40 drops

As already mentioned, the expansion of stem cells in stirred bioreactors can be a very long process often lasting for up to six days or more[34] and during this time, it might be necessary to stop the stirring to replace the DMEM to add fresh nutrients. It might also be necessary to restart at different speeds to alter the mass transfer rate for specific purposes. Also, though the dispersion was exceptionally stable due to the presence of proteins on the drop surfaces, when agitating, there is still the possibility of the coalescence of drops when they are stationary. There are no correlations in the open literature for the minimum impeller speed required for the suspension of liquid drops. Therefore, because of the stability of the drop sizes, the correlation for the minimum speed required to suspend solid particles, N_{js} was tested here[35] (Eq. 2)

$$N_{js} = S d_p^{0.2} \left(\frac{g\Delta\rho}{\rho_c}\right)^{0.45} v^{0.1} X^{0.13} D^{-0.85} \quad (2)$$

where S is a dimensionless parameter dependent on the precise geometry of the vessel and d_p is the size of the particle to be suspended At the impeller speed used in this work after sedimentation, the FC-40 drop size in modified DMEM was in the range of 100 - 300 μm and in D-PBS between 100 - 600 μm. For the configuration used in this work, S = 7[35] and the minimum impeller speeds necessary to re-suspend such drops, N_{js}, calculated from Equation 2 were approximately 840 rpm and 980 rpm respectively.

Therefore, to ascertain what happened to drop sizes if dispersion was followed by settling and then re-dispersion, the following experiments were undertaken. 5% FC-40 in the supplemented DMEM was dispersed by mixing for 30 min to produce drops between 90-320 μm and then

they were allowed to sediment without stirring for 2h. After that time, agitation was started again at 50 rpm and the speed was gradually increased. The images of the system at each speed are shown in Fig. 8.

Figure 9. here

It can be seen that the fluorocarbon drops can be re-suspended in DMEM and the drop size after re-suspension is the same in the initial dispersion. The correlation for N_{js} developed for solid/liquid suspension over-predicts the minimum suspension speed in the FC-40/DMEM system and the complete suspension of the FC-40 droplets was observed from 400 rpm at 25°C. This result suggests that the use of Eq 2 for predicting the impeller speed required for suspending protein stabilised drops may not be appropriate, which is unfortunate as no other correlations are available.

Conclusions

It has been reported in the literature that different anchorage-dependent cells could be successfully expanded on the liquid-liquid interfaces formed between fluorocarbons and culture media. However, only the biological aspect of cell expansion was investigated previously at very small scale ranging from 2 to 40 ml.

In this work, the scaling-up of FC-40/cell culture systems in a 1L stirred vessel was investigated. It has been shown that the stable "perfluorocarbon microcarriers" of required, adjustable size and the interfacial area could be produced in a standard stirred vessel. A

correlation relating the size of such micro-carriers (drops) to the impeller speed and physical properties of the cell culture has been developed.

It has been found that in the FC-40/DMEM dispersion, coalescence whether under dynamic or static conditions is completely suppressed by the proteins adsorbed at the interface. After the impeller was stopped the FC-40 drops sedimented rapidly and remained stable for more than 5 days. They could only be destabilised by removing the adsorbed proteins from the drop surfaces by a gentle washing with water or with D-PBS. After sufficient surface adsorbed proteins were removed, the perfluorocarbon formed a flat interface with the aqueous phase from which cells could be harvested without trypsinisation.

It appears that the expansion of anchorage-dependent cells could be carried out in scalable stirred bioreactors and as sterilisation does not affect the physical properties of the FC-40 it could be recovered and recycled, thereby reducing the cost of the process.

Acknowledgments

This work has been financially supported by BBSRC BRIC (research grant BB/K011066/1 and BB/K01099/1).

Nomenclature:

A, B – coefficient in Equation 1

d_p – particle diameter, m

$d_{3,2}$ – mean Sauter diameter, μm

D – impeller diameter, m

N – impeller speed, rpm

N_{js} – minimum impeller speed for suspending particles, s^{-1}

Po – Power number

Re – Reynolds number, dimensionless

S – dimensionless suspension parameter, dimensionless

T – vessel diameter, m

V – volume of liquid, m^3

X – mass of liquid drops/mass of continuous liquid x 100

Z – the average zeta potential, mV

Greek letters

α, β – coefficient in Equation 1

$\Delta\rho$ – density difference between the liquids

$\bar{\varepsilon}_T$ – mean specific energy dissipation rate, $\bar{\varepsilon}_T = \frac{P}{\rho V} = \frac{Po \cdot N^3 \cdot D^5}{V}$, W kg^{-1}

ρ_c – density of continuous phase, kg m^{-3}

ρ_d – density of dispersed phase, kg m^{-3}

v – kinematic viscosity of the liquid, m^2 s^{-1}

σ – interfacial tension, mN m^{-1}

Dimensionless Groups

We – Weber number, dimensionless, $We = \frac{N^2 D^3 \rho_c}{\sigma}$

Re – Reynolds number, $Re = \frac{N D^2}{\nu}$

References:

1. Hewitt ChJ, Lee K, Nienow AW, Thomas RJ, Smith M, Thomas CR, Expansion of human mesenchymal stem cells on microcarriers. *Biotechnol Lett*, **33**(11): 2325-2335 (2011)
2. Kulkarni N, Vaidya A, Rao M, Extractive cultivation of recombinant Escherichia coli using aqueous two phase systems for production and separation of extra acellular xylanase. *Biochem Biophys Res Commun*, **255**: 274-278 (1999)
3. Baksh D, Davies J, Culture of mesenchymal stem/progenitor cells in adhesion-independent conditions, in: *Methods in Cell Biology*, ed. by Mather J. Elsevier. p. 279-293 (2008)
4. Keese ChR, Giaver I, Cell growth on liquid microcarriers. *Sci*, **219**(4591): 1448-1449 (1982)
5. Giaever I, Keese C, Behavior of cells at fluid interfaces. *Cell Biol*, **80**(1): 219-222 (1983)
6. Keese ChR, Giaver I, Cell growth on liquid interface: role of surface active compounds. *Proc Natl Acad Sci USA*, **80**(18): 5622-5626 (1983)
7. Sato M, Shinozawa T, Ueno H, Sadakata M, Cultivation of adherent cells on liquid–liquid interface. *Kagaku Kougaku Ronbun-syuu* **17**: 671–673 (1991) (in Japanese)
8. Terada S, Sato M, Katayama R, Shinozawa T, Recovery of intact membrane proteins from adherent animal cells grown in a liquid-liquid interface. *J, Ferment Bioeng*, **74**: 330–332 (1992)
9. Ju L, Lee JF, Armiger WB, Enhancing Oxygen transfer in Bioreactors by Perfluorocarbon Emulsions. *Biotechnol Prog*, **7**(4): 323-329 (1991)

10. Ando J, Albelda SM, Elliot A, Levine M, Culture of human adult endothelial cells on liquid-liquid interfaces: a new approach to the study of cell-matrix interactions. *In Vitro Cell Dev Biol*, **27A**(7): 525-532 (1991)

11. Shiba Y, Ohushima T, Sato M, Growth and morphology of anchorage-dependent animal cells in a liquid/liquid interface system. *Biotechnol Bioeng*, **57**: 583-589 (1998)

12. Kwon YJ, Yu H, Peng C, Enhanced retroviral transduction of 293 cells cultured on liquid–liquid interfaces. *Biotechnol Bioeng*, **72** (3): 331-338 (2001)

13. Juszczak MT, Elsadig A, Kumar A, Muzyamba M, Pawelec K, Powis SH, Press M, Use of perfluorodecalin for pancreatic islet culture prior to transplantation: a liquid-liquid interface culture system-preliminary report. *Cell Transplant*, **20** (2): 323-332 (2010)

14. Hiroki M, Camelita KM, Kenichi H, Dodecafluoroheptanol: Oxygen reservoir for the culture of mouse melanoma B16 cells. *J Fluorine Chem*, **163**:46-49 (2014)

15. Pilarek M, Grabowska I, Ciemerych MA, Dabkowska K, Szewczyk KW, Morphology and growth of mammalian cells in a liquid/liquid culture system supported with oxygenated perfluorodecalin. *Biotechnol Lett*, **35**(9):387–1394 (2013)

16. Pilarek M, Grabowska I, Senderek I, Wojasinski M, Lanicka J, Janczak-Illach K, Ciach T, Liquid perfluorochemical-supported hybrid cell culture system for proliferation of chondrocytes on fibrous polylactide scaffolds. *Bioprocess Biosyst Eng*, **37**(9): 1707-1715 (2014)

17. Leng DE, Calabrese RV, Immiscible liquid–liquid systems, in: *Handbook of industrial Mixing: Science and Practice*, ed. by Paul WL, Atiemo-Obeng VA, Kresta SM. John Wiley&Sons: New York, p. 639-755 (2004)

18. Hanga MP, Murasiewicz HM, Pacek AW, Nienow AW, Coopman K, Hewitt CJ, Expansion of bone marrow derived human mesenchymal stem/stromal cells (hMSC) using a two phase liquid/liquid system. *Biochem Eng J*, submitted (2016)

19. http://www.thermofisher.com/uk/en/home/technical-resources/media-formulation.183.html. [Accessed 25 August 2016]

20. http://www.thermofisher.com/uk/en/home/technical-resources/media-formulation.147.html. [Accessed 25 August 2016]

21. Pacek AW, Moore IPT, Nienow AW, Calabrese RV, Video technique for measuring dynamics of liquid-liquid dispersion during phase inversion. *AIChe J*, **40**(12):1940-1949 (1994)

22. Whitby C P, Wanless E J, Controlling Pickering emulsion destabilisation: a route to fabricating new materials by phase inversion. *Materials*, 9(8):626 (2016)

23. Binks BP, Particles as surfactants - similarities and differences. *Curr Opin Colloid Interface Sci*, **7**(1-2): 21-41(2002)

24. Pacek AW, Chamsart S, Nienow AW, A. Bakker, The influence of impeller type on mean drop size and drop size distribution in an agitated vessel. *Chem Eng Sci*, **54**(19):4211-4222 (1999)

25. Chen HT, Middleman S, Drop size distribution in agitated liquid-liquid systems. *AIChE J*, **13**(5):989–995 (1967)

26. Deshpande KB, Kumar S, Phase inversion in agitated liquid–liquid dispersions: Anomalous effect of electrolyte. *Chem Eng Sci*, **78**: 33-37 (2012)

27. Dickinson E, Properties of emulsions stabilized with milk proteins: overview of some recent developments. *J Diary Sci*, **80**(10):2607–2619 (1997)

28. Dickinson E, Adsorbed protein layers at fluid interfaces: interactions, structure and surface rheology. *Colloids Surf B*, **15**: 161-176 (1999)

29. Mc Clements D J, *Food emulsions: Principle, practice and techniques*. CRC Press: London (1999)

30. Velev OD, Nikolov AD, Denkov ND, Doxastakis G, Kiosseoglou V, Stalidis G, Investigation of the mechanisms of stabilization of food emulsions by vegetable proteins. *Food Hydrocolloids*, **7**: 55-71 (1993)

31. Hartland S, Jeelani S, Gravity settler, in: *Liquid–liquid extraction equipment*, ed. by Godfrey JC, Slater MG. John Wiley & Sons: New York (1994)

32. Evans M, Ratcliffe I, Williams PA, Emulsion stabilisation using polysaccharide–protein complexes. *Curr Opin Colloid Interface Sci,* **18**(4): 272–282 (2013)

33. Delahaye M, Lawrence K, Ward SJ, Hoare M, An ultra scale-down analysis of the recovery by dead-end centrifugation of human cells for therapy. *Biotechnol Bioeng*, **112**(5):997-1011 (2015)

34. Nienow AW, Rafiq QA, Coopman K, Hewitt CJ, A potentially scalable method for the harvesting of hMSCs from microcarriers. *Biochem Eng J,* **85**(15): 79–88 (2014)

35. Nienow AW, Suspension of solid particles in turbine agitated baffled vessels. *Chem Eng Sci*, **23:** 1453-1459 (1968)

Table 1. Application of perfluorocarbons based two phase liquid/liquid systems for cell expansion.

System	Cell culture	Volumes of PFC/medium, ml	Shape of interface	Main conclusion
PFCs*/DMEM**[4-5]	murine and human newborn fibroblasts cells	0.1 – PFCs 0.3 - complete DMEM + cells	Droplets/flat surface	-very stable emulsion - after centrifugation cells harvested from the interface without trypsin,
PFCs/DMEM[6]	murine cells and human lung fibroblasts cells	0.1 - PFCs 0.2 - complete DMEM +cell	Droplets/flat surface	- excellent cell growth at the PFC interface combined with F_5BzCl, - perfluorocarbon affects the strength of the protein layer and growth pattern, - cells were harvested without trypsin from interface after mechanical separation of the emulsion.
PFC/Culture medium[7-8]	human skin tumour cells		Flat surface	- cells harvested without trypsin, -behaviour of the cells on interface similar to that on a plastic surface coated with collagen

PFCs/DMEM[9]	bacteria	6 to10 - PFCs ~ 30 medium	Droplets	- very high cell populations at an aerobic state in PFC emulsions, - enhanced oxygen transfer in perfluorocarbon emulsions
PFC/Medium 199[10]	endothelial stem cells	0.4 or 0.6 – PFC 0.7 or 3 - medium + cell	Flat surface	- cells grown on interfaces retain their characteristic, - growth rate the same as at the plastic surfaces, - cells harvested without trypsin
PFCs/MEM medium***[11]	mouse fibroblast cells	4 – PFC 6 - medium + cell	Flat surface	- no effect of perfluorocarbon on cells growth/adhesion, - cells harvested without trypsin,
PFC/DMEM[12]	human embryonic kidney cells	1 – PFC 1 - medium	Flat surface	- cells attach and spread on coated FC-40 interface, - gene transfer efficiency on coated interfaces higher than on polystyrene,
PFD/RPMI-1640 medium[13]	human pancreatic islets cells	5 - PFD 4 - medium	Flat surface	- perfluorocarbon provides optimal culture conditions - the risk of cell damage is reduced
PFC/ DMEM:F12[14]	murine tumour cells	0.2 to 25 - PFC 1 to 25 - DMEM	Flat surface	- cells grow fast at the interface, - solvent serves as an O_2 reservoir,

				- cells are attached to each other forming sheet,
PFD[§]/DMEM:F12[15-16]	Human, mouse and hamster kidney cells	1or 2 - PFD 1 or 2 - medium	Flat surface	- robust growth of aggregated mammalian adherent cells, - cells harvested without enzyme, -simple and scalable system, -enhanced mass transfer of respiratory gases,

*****PFCs – perfluorocarbons; **DMEM - Dulbecco's Modified Eagle Medium; ***MEM - Minimum Essential Media, [§]PDF - Perfluorodecalin

Table 2. Coefficients in the correlations for the Sauter mean diameters (Equation 1 and 1a) of FC-40 drops dispersed in different liquids.

Continuous phase	***A***	α	$\mathbf{R^2}$	***B***	β	$\mathbf{R^2}$
Supplemented DMEM 25°C	0.40	- 0.81	0.999	191	-0.54	0.999
Supplemented DMEM 37°C	0.39	- 0.80	0.998	193	-0.54	0.999
D-PBS	0.21	- 0.69	0.996	371	-0.57	0.991
Distilled water	0.11	- 0.61	0.999	266	-0.41	0.999

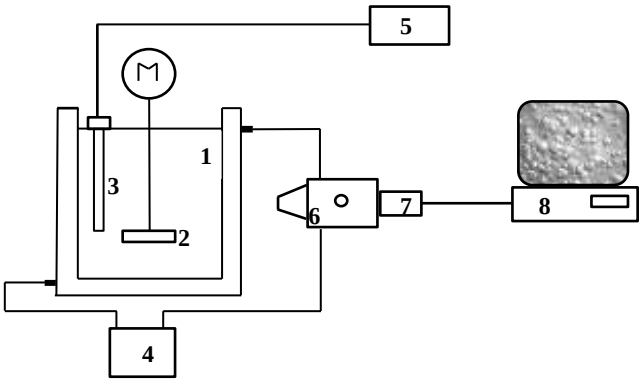
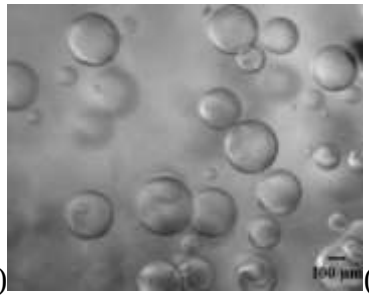
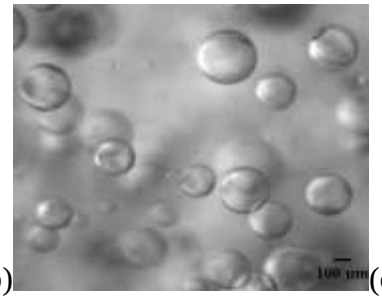
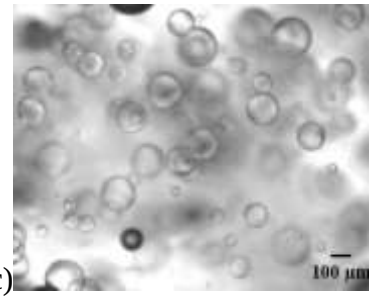


Figure 1. Experimental rig: 1 – jacketed stirred vessel, 2 – Rushton turbine, 3 - strobe lamp, 4 – water bath, 5 – strobe flash, 6 – stereo microscope, 7 – video camera, 8 – computer with image acquisition software

(a)  (b)  (c) 

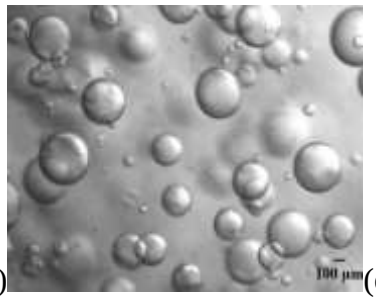
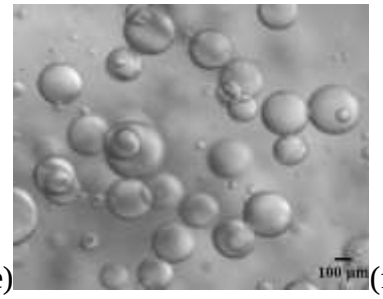
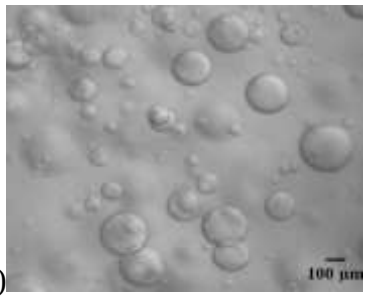
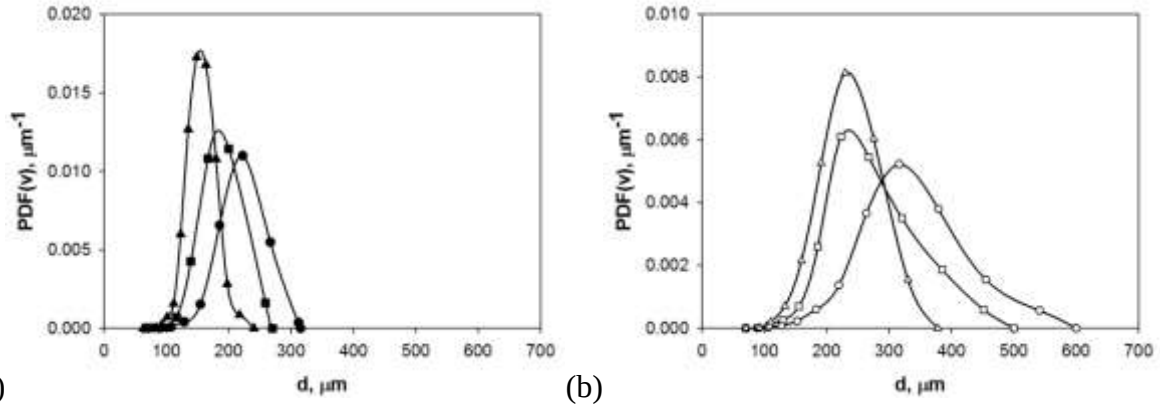
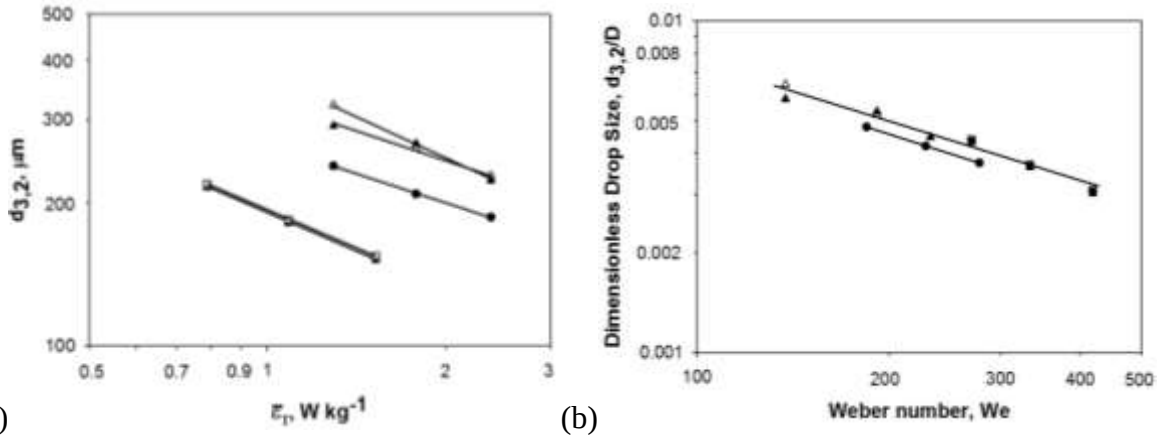
(d)  (e)  (f) 

Figure 2. Dispersion of 5% FC-40 in: (a) supplemented DMEM at N= 450 rpm, (b) supplemented DMEM at N=530 rpm, (c) supplemented DMEM at N=560 rpm, (d) D-PBS at N=530 rpm, (e) D-PBS at N=590 rpm and (f) D-PBS at N=650 rpm



(a) (b)

Figure 3. Steady state drop size distributions in a breakage mode: (a) FC-40/ DMEM system: (●) 450, (■) 500, (▲) 560 rpm; (b) FC-40/D-PBS system: (○) 530, (□) 590 and (Δ) 650 rpm. PDF – probability density function



(a) (b)

Figure 4. The effect of: (a) specific energy dissipation rate and (b) We number on the Sauter mean diameter; points represent experimental data, lines the best fit to Equations 1 and 1a: (■) 25°C DMEM - breakage mode, (□) 37°C DMEM - breakage mode, (Δ) D-PBS –breakage mode, (▲) D-PBS – coalescence mode (●) distilled water

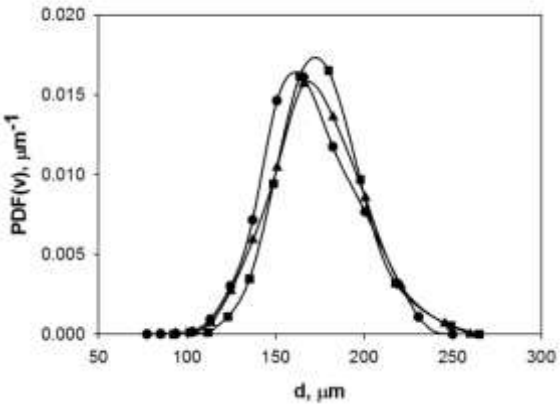
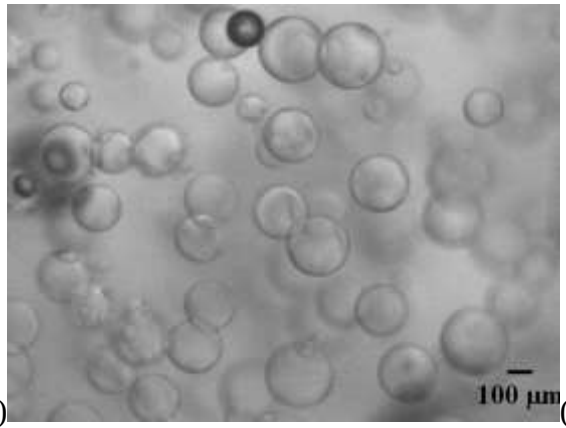
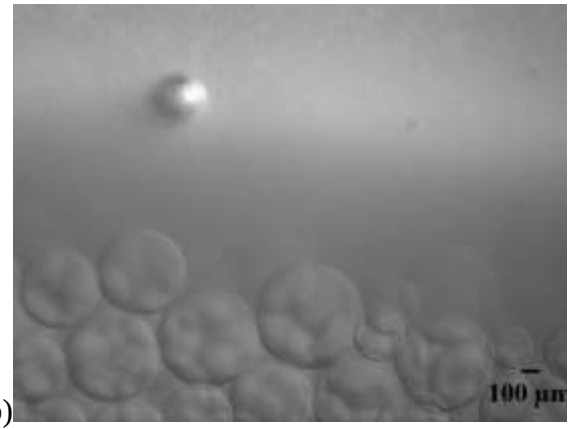
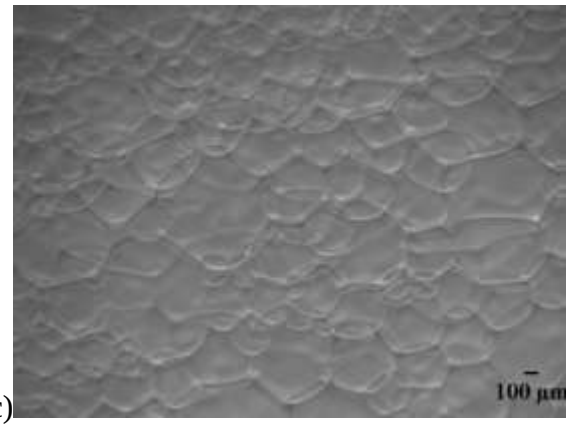


Figure 5. Drop size distributions at the coalescence mode: (●) 450 rpm, (■) 500 rpm and (▲) 560 rpm

(a) 

(b) 

(c) 

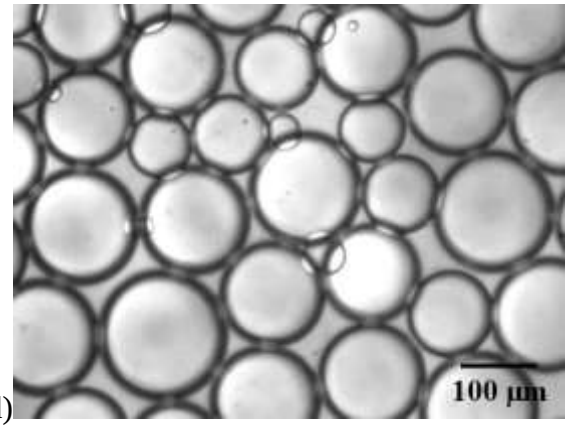
(d) 

Figure 6. FC-40/supplemented DMEM dispersion in a stirred bioreactor: (a) mixing at 450 rpm, (b) bottom phase (drops of perfluorocarbon) and top phase (continuous DMEM) after mixing was stopped; (c) bottom phase (drops) after 24 hours, (d) drops after 24 hours under optical microscope

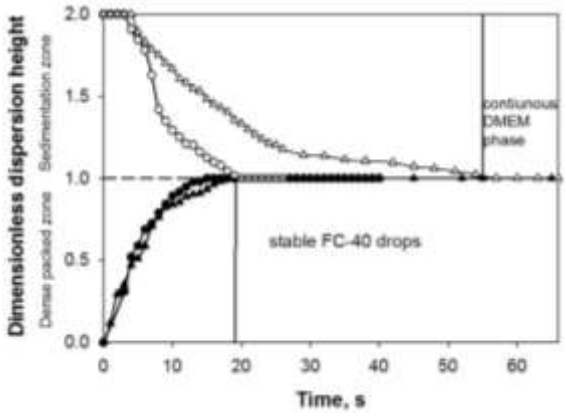
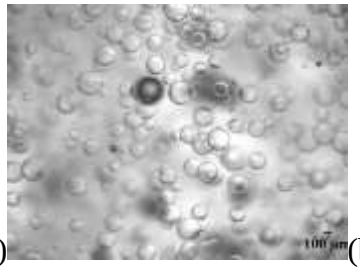


Figure 7. The separation/sedimentation curves of FC-40/DMEM system: (●) (○) after mixing at 450 rpm; (▲) (Δ) after mixing at 560 rpm

(a)  (b)  (c) 

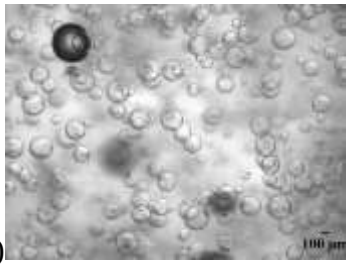
(d)  (e)  (f) 

Figure 8. Dispersion of FC-40 in supplemented DMEM: (a) mixed at 450 rpm for 30 min, (b) separated for 2h at 0 rpm and re-suspended at (c) at 200 rpm, (d) at 300 rpm, (e) at 400 rpm and (f) at 450 rpm