

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

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

10 **Key words:** “*Liquid microcarriers*”, *Cell culture*, *Emulsification/dispersion*, *Perfluorocarbon*,
11 *Bioreactors*

12 **Introduction**

13 Human stem cells have proven very difficult to expand in culture because of the absolute need
14 for adherent culture conditions. Since it is the stem cells themselves that form the basis of the
15 products, there are further challenges in harvesting these whilst ensuring their survival and the
16 maintenance of their multipotency for specific clinical purposes. In recent small - scale
17 investigations of human mesenchymal stem cells (hMSCs) expansion on microcarriers
18 (Cytodex-3¹ and SoloHill Plastic²), the cells were harvested by a process called trypsinisation
19 that uses an active concentration of the proteolytic enzyme, trypsin, to remove cells from the
20 surface of microcarriers. However, attempts to harvest larger quantities of cells using the same
21 method often resulted in a high degree of cell death and loss of multipotency³.

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

1 system where perfluorocarbon was dispersed in cell medium, was particularly popular as shown
2 in Table 1. Perfluorocarbons were selected because they are inert, do not have an adverse effect
3 on cell growth and have very high oxygen solubility and the first application of fluorocarbon
4 emulsions in cell culture (drops between 100 and 500 μm) for cell cultivation was reported in
5 the early eighties⁴. Since then many researchers used such systems with different cells and the
6 wide range of perfluorocarbons and the main results are briefly summarised in Table 1.

7 **Table 1 here**

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

16 Scale-up of the systems to form a flat interface (*i.e.* an interface between two stationary liquids)
17 is of no interest for manufacturing applications because the specific interfacial area (ratio of the
18 area of the interface to the volume of liquids) is very small. Therefore, here a system where the
19 interface is spherical and similar to the morphology of solid microcarriers *e.g.* dispersion of
20 perfluorocarbon drops in cell culture medium was investigated. There is a large body of
21 literature on emulsification/dispersions of organic liquids in water¹⁷, but the majority of the
22 results are for pure liquids (without surfactant or salts) with the organic phase less dense than
23 the aqueous phase. As fluorocarbons are nearly twice as dense as water and culture media often
24 contain surface active compounds and salts, not all the literature data is applicable for

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

9 **Materials and Methods**

10 **Materials**

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

20 DMEM (1 g/L glucose, Lonza, UK) supplemented with 2 mM of ultraglutamine (Lonza, UK)
21 and 10% foetal bovine serum (Life Sciences: GIBCO, ThermoFisher Scientific, UK) and
22 Dulbecco's Phosphate Buffered Saline (D-PBS, Life Sciences: GIBCO, ThermoFisher
23 Scientific, UK) was used as the continuous phase. DMEM supplemented with ultra-glutamine
24 and foetal bovine serum was chosen. The same medium was used in cell growth experiments¹⁸

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

7 **Methods**

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

16 Two sets of experiments were subsequently carried out. In the first set, the effect of energy
17 input (impeller speed) on drop size distribution and mean drop size of FC-40 dispersed in
18 supplemented DMEM, D-PBS and in double distilled water was investigated both during
19 breakage (a step increase of the impeller speed) and during coalescence (a step reduction of
20 impeller speed). In the second set, the kinetics of separation of the resulting dispersions of both
21 fresh as well as recycled FC-40 after stem cell expansion¹⁸ were tested.

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

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

6

7 ***Figure 1 here***

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

1

2 **Results and discussion**

3 **Physical properties of investigated liquid phases**

4 In this study, DMEM supplemented with L-glutamine and foetal bovine serum was chosen as
5 the same composition was used in cell growth experiments¹⁸ and it represents a common cell
6 culture medium used in the culture of a variety of cell types. In terms of physical properties, at
7 room temperature, viscosity and density of the supplemented DMEM and D-PBS were
8 practically the same as that of water at 0.001 Pa s and 1010 - 1020 kg m⁻³ respectively. FC-40
9 is nearly twice as dense as water (1860 kg m⁻³) and 4 times more viscous (0.004 Pa s). At the
10 elevated temperature of 37°C at which the cells are typically cultured, the density of all liquids
11 was marginally lower (by 1 to 2%) and viscosity of FC-40 was equal to 0.003 Pa s.

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

16 Density, viscosity and surface tension of FC-40 after 3 days in culture in a spinner flask at 37°C
17 were also measured after cells were harvested from the interface (for details see Hanga et al.¹⁸).
18 Part of the FC-40 was sterilised by filtration through a 0.2 µm filter at room temperature and
19 the second part by autoclaving at 121°C for 15 minutes. Density and viscosity were practically
20 un-affected by cell growth and subsequent sterilisation and were equal to 1870 kg m⁻³ and
21 0.004 Pa s respectively. The interfacial tension of autoclaved FC-40 was practically the same
22 as the interfacial tension when fresh, whereas after filtration, the interfacial tension was reduced
23 by approximately 15% (22.4 mN m⁻¹ and 25.3 mN m⁻¹ post-filtration and post-autoclaving,

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

5

6 **Effect of impeller speed and composition of continuous phase on drop size/size** 7 **distribution**

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

12

13 **Figure 2** here

14

15 Indeed in both systems, the increase of impeller speed leads to the reduction of drop size (Figs
16 2a and 2b). However, despite the fact the interfacial tension between FC-40 and supplemented
17 DMEM (26.50 mN m^{-1}) is much lower than the interfacial tension between FC-40 and D-PBS
18 (64.04 mN m^{-1}), at the same impeller speeds the drop size in both systems were rather similar
19 (Figs 2a and 2f). In a stirred system, steady state drop size is determined by the dynamic
20 equilibrium between breakage and coalescence; therefore the above observation could only be
21 explained by the fact that coalescence of the FC-40 drops in supplemented DMEM system is
22 suppressed. Typically, coalescence in liquid/liquid two phase systems can be suppressed by the
23 presence of surface active components or very small particles^{22,23}. The presence of surface-

1 active components also reduces surface tension and promotes incorporation of air through the
2 free surface (so-called surface aeration). In the FC-40/DMEM system, aeration started at a
3 much lower impeller speed than in the FC-40/D-PBS system as shown by air bubbles (black
4 circles) in Fig. 2a - 2c, which confirms that certain components of supplemented DMEM
5 affected the properties of the interface. The reduction of drop size in the FC-40/DMEM system
6 is indeed confirmed by the steady state drop size distribution in both systems, as shown in Fig.
7 3.

8

9 **Figure 3.** here

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

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

17

18 **Figure 4 here**

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

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

2 $d_{32} = B \bar{\epsilon}_T^\beta$ (1a)

3 Under these conditions, the theoretical values of exponent α and β are -0.6 and -0.4 respectively
4 and experimental constants A depends on the type of impeller as does the constant B (plus, in
5 this case, the physical properties which are contained in the Weber no.)²⁴. For the impeller used
6 in this work, experiments suggest $A = 0.056$ ^{17, 25}. Here, fitting Eq 1 to the experimental data
7 gave an A value of 0.11, approximately two times larger. However, with increasing volume
8 fraction of dispersed phase, A generally increases at quite different rates in pure coalescing
9 systems; and even with non-coalescing systems as the dispersed phase is considered to damp
10 out the turbulence¹⁷. Here, the results discussed above clearly indicate the presence of surface
11 active components in supplemented DMEM, so both the volume fraction of the dispersed phase
12 and its composition impact on the extent of coalescence and thus the constant A . These different
13 effects on the experimental constant A in Equations 1 for the different continuous phases are
14 summarised in Table 2.

15

16 **Table 2 here**

17

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

1 also by the presence of substantial amounts of different salts (it total approximately 0.2 M of
2 KCl, KH₂PO₄, NaCl, Na₂HPO₄·7H₂O)²⁰ in the continuous phase. This explanation is in
3 agreement with the results reported by Deshpande and Kumar²⁶ who found that the presence of
4 salts drastically changes the dynamics of oil/water dispersions and leads to an increase in the
5 average drop size.

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

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

12 Typically, cells are cultured for several days with intermittent medium exchanges to prevent
13 critical nutrients such as glucose and glutamine or other substrates becoming limiting in the
14 system or the build-up of metabolites e.g. lactate becoming inhibitory. There is very little
15 information in the open literature on the effect of very long mixing times on the drop size/size
16 distributions. Here, to mimic a typical cell culture process for hMSC, in which cells would be
17 cultured in FC-40/supplemented DMEM for many days, the system was agitated for a total of
18 6 days to create recycled FC-40¹⁸. Following sterilization, the FC-40 was mixed for 3 days at
19 N=450 rpm. Drop size distributions measured after 24, 48 and 72 hours were practically
20 overlapping.

21 **Coalescence in supplemented DMEM**

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

4

5 **Figure 5.** here

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

9

10 The Sauter mean diameters, $d_{3,2}$ after coalescence were practically constant (165 μm , 172 μm ,
11 170 μm at 450 rpm, 500 rpm, 560 rpm, respectively). This similarity confirms that coalescence
12 of FC-40 drops suspended in a supplemented DMEM under dynamic conditions was
13 completely suppressed. The average zeta potential of FC-40 drops were $Z = -18.1$ mV
14 indicating that electrostatic charges are far too low to prevent coalescence. The only possible
15 explanation is the steric repulsion between drops covered by proteins that can be confirmed by
16 the reduction of the interfacial tension between FC-40 and D-PBS (no protein) from 64.04 mN
17 m^{-1} to 26.50 mN m^{-1} for that between FC-40 and supplemented DMEM (proteins). It is well
18 known that during dispersion, proteins with an amphiphilic structure tend to diffuse and
19 consequently adsorb at the oil–water (O/W) interface, lowering the interfacial tension. Proteins
20 also form a protective interfacial membrane and/or generated repulsive forces between
21 droplets, due to a combination of electrostatic interactions and hydrophobic interactions when
22 pH is not close to the isoelectric point of the protein²⁷⁻²⁹. Such membranes may resist tensile or
23 shearing stress and protect the droplets from coalescence³⁰.

1 **Separation of perfluorocarbon/DMEM dispersions**

2 Cell harvest from the FC-40/DMEM dispersion can be accomplished in two steps: first the
3 dispersion will have to be separated into two continuous phases with a flat interface; and next
4 cells can be harvested by aspiration, filtration or centrifugation. As discussed above,
5 coalescence under dynamic conditions was completely suppressed, but this might also be due
6 to a very short contact time after the collision between two drops during which the film
7 separating them cannot reach the critical thickness required for inducing coalescence.
8 Therefore, the coalescence under static conditions was investigated by recording the images of
9 dispersion in the bottom part of the vessel just before and after the impeller was stopped (Fig.
10 6). The separation curves³¹ determined from those images are shown in Fig. 7.

11 **Figure 6** here

12

13 **Figure 7** here

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

18 The exceptional stability of the FC-40 dispersion in supplemented DMEM under static
19 conditions can be explained by the steric stabilisation of drops by proteins present in the foetal
20 bovine serum added to DMEM as a supplement for cell culture. Proteins have amphiphilic
21 properties and are commonly used in the food industry for the stabilisation of oil-in-water
22 emulsions³². Once adsorbed on the interface, the proteins unfold enabling the hydrophilic
23 groups to protrude away from the surface into the aqueous phase, thus stabilising the drops by

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

12 An attempt was made to break the FC-40/supplemented DMEM dispersion by centrifugation
13 (Eppendorf, Model 5810). At acceleration up to 10G, the separation was not observed and at
14 210G, only about 10% of drops coalesced and formed a continuous FC-40 phase at the very
15 bottom of the centrifuge tube. An increase in acceleration up to 1900G lead to the coalescence
16 of approximately 50% of droplets, but a further increase to 3200G did not lead to an increase
17 in the volume of the FC-40 coalescing into a continuous phase. Taking into consideration the
18 fact that excessive forces during centrifugation might have a negative effect on the quality of
19 the cells³³, higher accelerations were not tested.

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

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

3 **Re-suspension of FC-40 drops**

4 As already mentioned, the expansion of stem cells in stirred bioreactors can be a very long
5 process often lasting for up to six days or more³⁴ and during this time, it might be necessary to
6 stop the stirring to replace the DMEM to add fresh nutrients. It might also be necessary to
7 restart at different speeds to alter the mass transfer rate for specific purposes. Also, though the
8 dispersion was exceptionally stable due to the presence of proteins on the drop surfaces, when
9 agitating, there is still the possibility of the coalescence of drops when they are stationary.
10 There are no correlations in the open literature for the minimum impeller speed required for
11 the suspension of liquid drops. Therefore, because of the stability of the drop sizes, the
12 correlation for the minimum speed required to suspend solid particles, N_{js} was tested here³⁵
13 (Eq. 2)

$$14 \quad N_{js} = S d_p^{0.2} \left(\frac{g \Delta \rho}{\rho_c} \right)^{0.45} \nu^{0.1} X^{0.13} D^{-0.85} \quad (2)$$

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

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

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

4

5

6 **Figure 9.** here

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

14

15 **Conclusions**

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

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

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

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

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

13

14 **Acknowledgments**

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16 BB/K01099/1).

17

18 **Nomenclature:**

19 A, B – coefficient in Equation 1

20 d_p – particle diameter, m

21 $d_{3,2}$ – mean Sauter diameter, μm

- 1 D – impeller diameter, m
- 2 N – impeller speed, rpm
- 3 N_{js} – minimum impeller speed for suspending particles, s^{-1}
- 4 Po – Power number
- 5 Re – Reynolds number, dimensionless
- 6 S – dimensionless suspension parameter, dimensionless
- 7 T – vessel diameter, m
- 8 V – volume of liquid, m^3
- 9 X – mass of liquid drops/mass of continuous liquid x 100
- 10 Z – the average zeta potential, mV
- 11 Greek letters
- 12 α, β – coefficient in Equation 1
- 13 $\Delta\rho$ – density difference between the liquids
- 14 $\bar{\epsilon}_T$ – mean specific energy dissipation rate, $\bar{\epsilon}_T = \frac{P}{\rho V} = \frac{Po \cdot N^3 \cdot D^5}{V}$, $W\ kg^{-1}$
- 15 ρ_c – density of continuous phase, $kg\ m^{-3}$
- 16 ρ_d – density of dispersed phase, $kg\ m^{-3}$
- 17 ν – kinematic viscosity of the liquid, $m^2\ s^{-1}$
- 18 σ – interfacial tension, $mN\ m^{-1}$

1 Dimensionless Groups

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

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

4

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10

- 1 **Table 1.** Application of perfluorocarbons based two phase liquid/liquid systems for cell
 2 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 ₅ BzCl, - 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	1 or 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,

1 *PFCs – perfluorocarbons; **DMEM - Dulbecco's Modified Eagle Medium; ***MEM -

2 Minimum Essential Media, §PDF - Perfluorodecalin

3

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

Continuous phase	<i>A</i>	<i>α</i>	<i>R</i>²	<i>B</i>	<i>β</i>	<i>R</i>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

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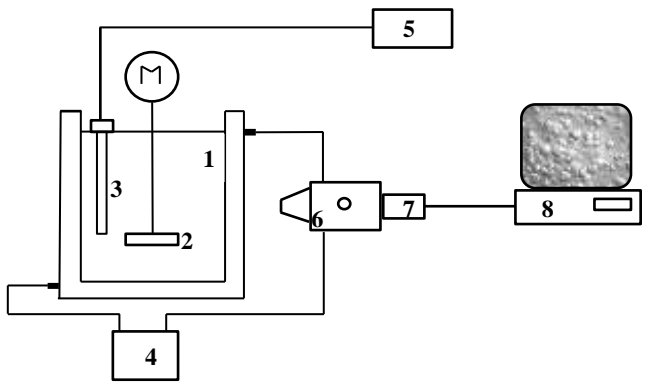
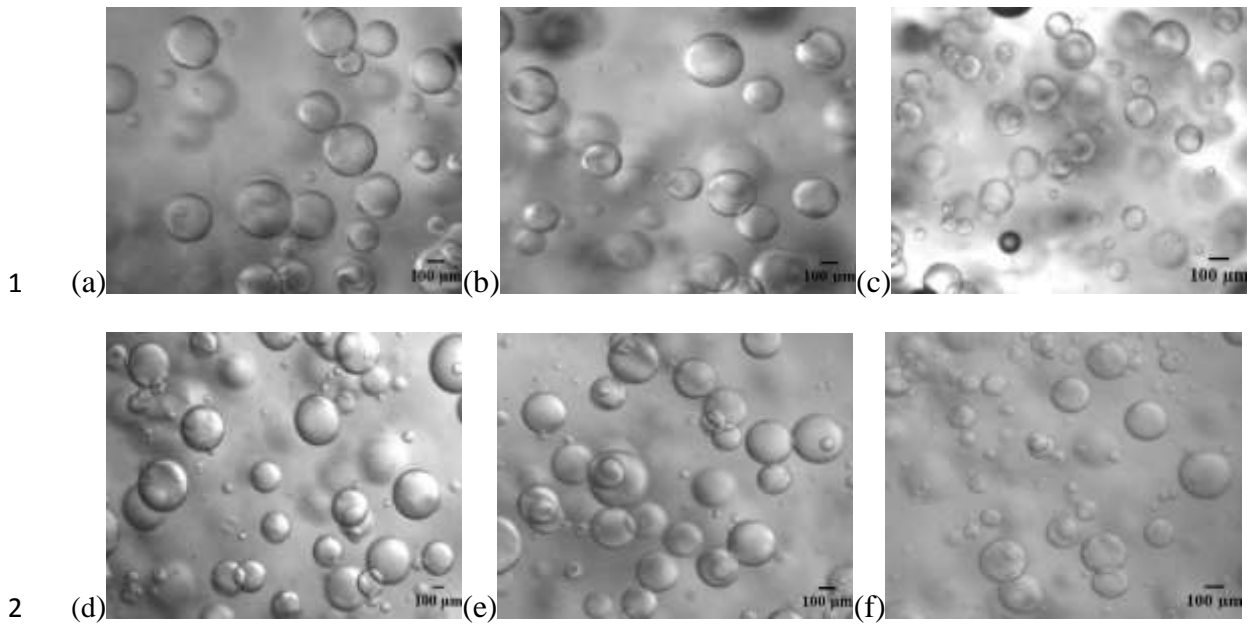
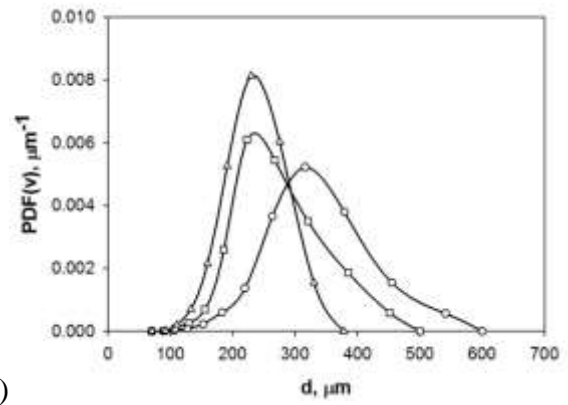
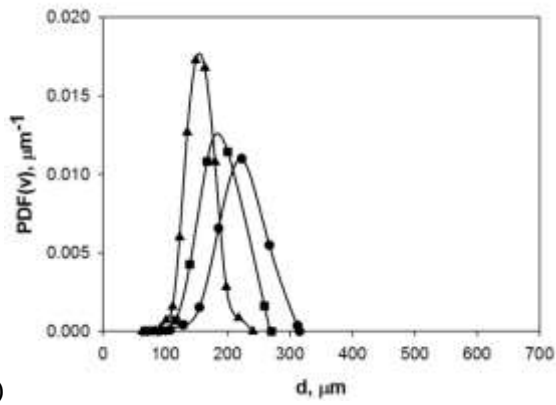


Figure 1. Experimental rig: 1 – jacketed stirred vessel, 2 – Rushton turbine, 3 - strobe lamp, 4 – water bath, 5 – strobe flash, 6 – stereo microscope, 7 – video camera, 8 – computer with image acquisition software

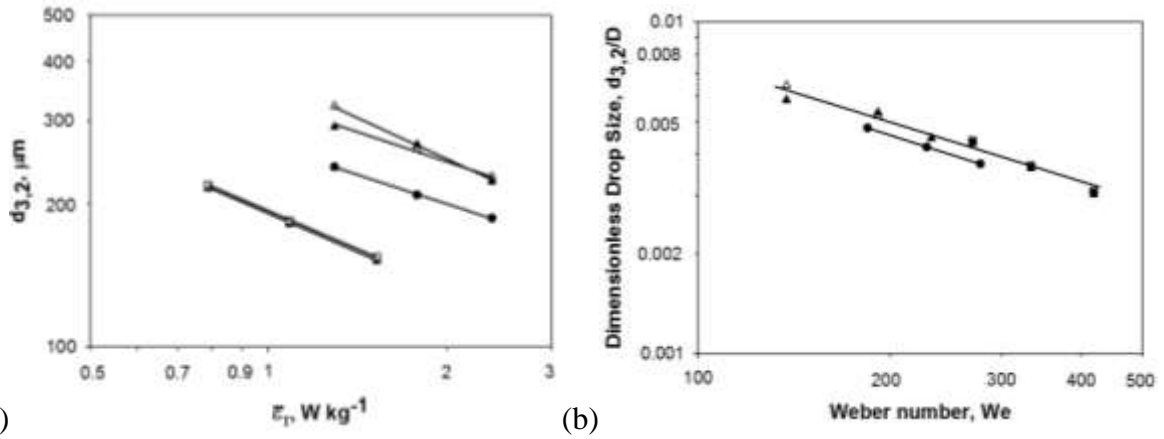


1 (a) (b) (c)
2 (d) (e) (f)
3 **Figure 2.** Dispersion of 5% FC-40 in: (a) supplemented DMEM at N= 450 rpm, (b)
4 supplemented DMEM at N=530 rpm, (c) supplemented DMEM at N=560 rpm, (d) D-PBS at
5 N=530 rpm, (e) D-PBS at N=590 rpm and (f) D-PBS at N=650 rpm

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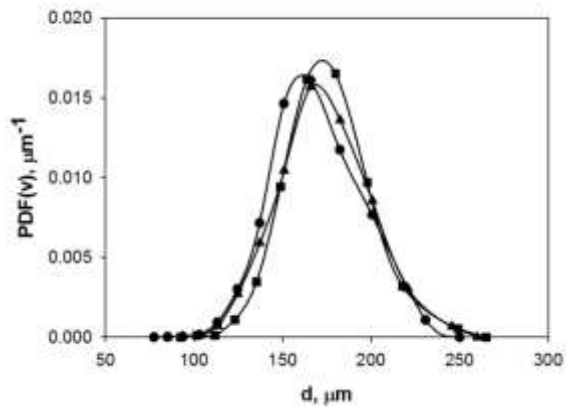
- 1 (a)
- 2 **Figure 3.** Steady state drop size distributions in a breakage mode: (a) FC-40/ DMEM system:
- 3 (●) 450, (■) 500, (▲) 560 rpm; (b) FC-40/D-PBS system: (○) 530, (□) 590 and (Δ) 650 rpm.
- 4 PDF – probability density function



1 (a) (b)

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

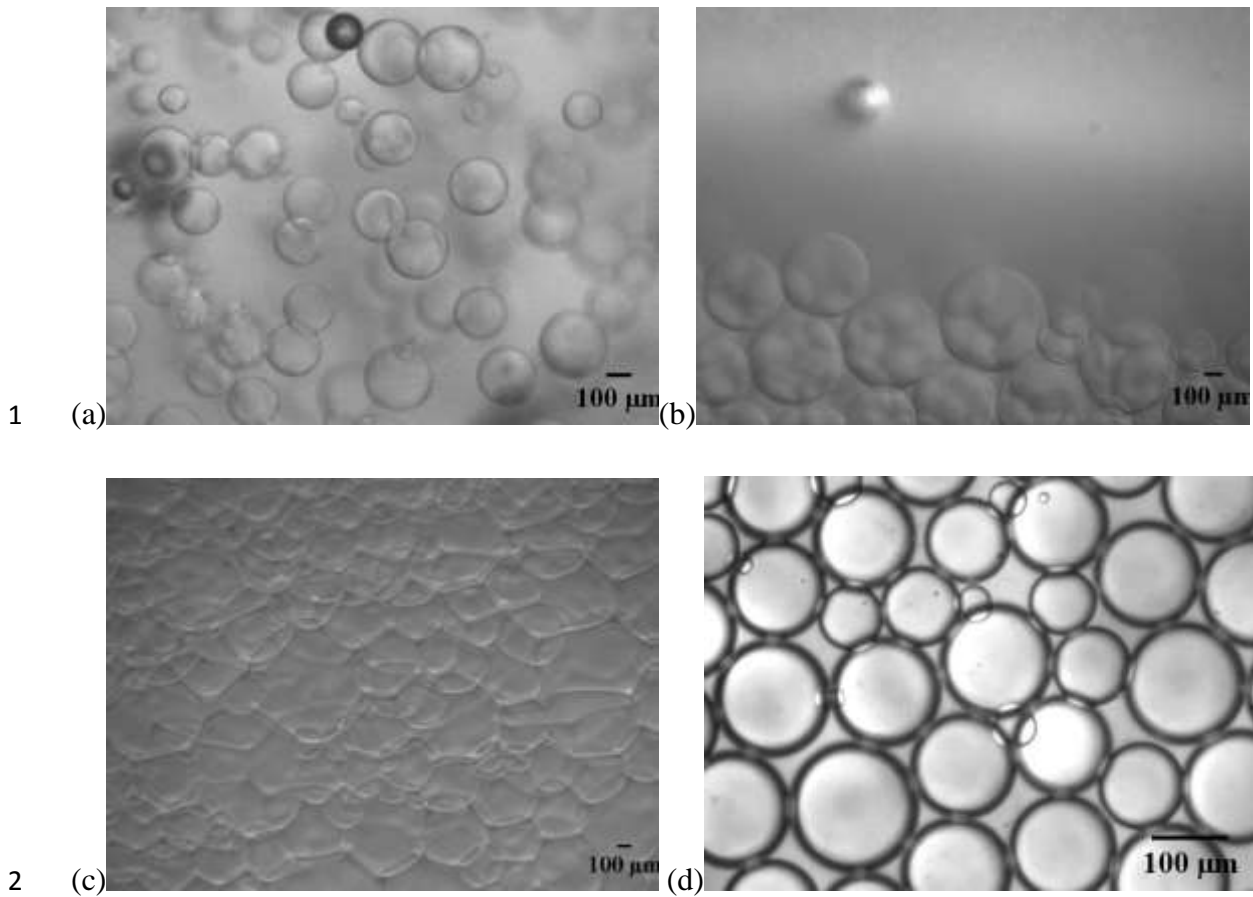
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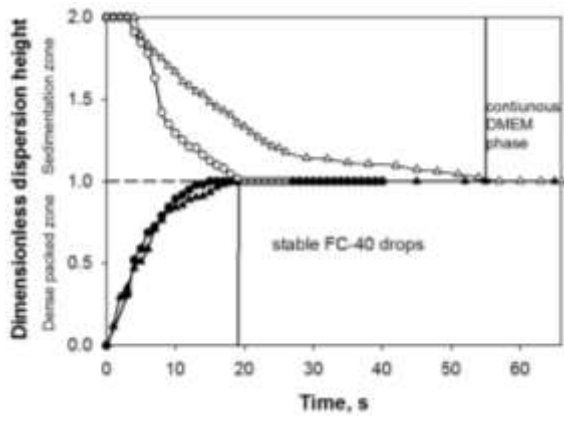
3 **Figure 5.** Drop size distributions at the coalescence mode: (●) 450 rpm, (■) 500 rpm and (▲)

4 560 rpm



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

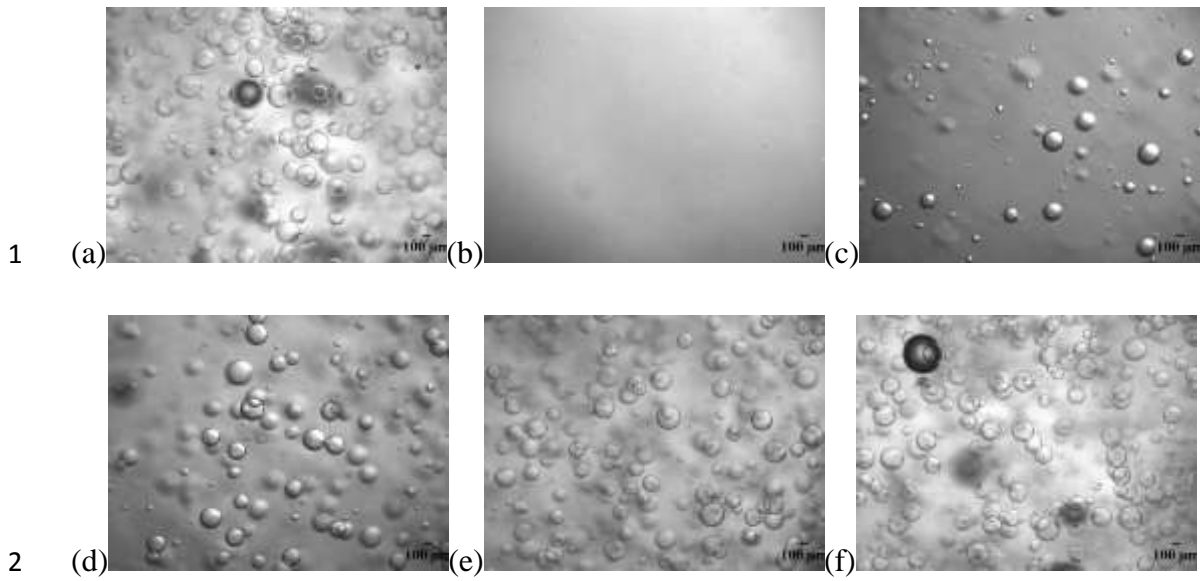
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2 **Figure 7.** The separation/sedimentation curves of FC-40/DMEM system: (●) (○) after mixing
 3 at 450 rpm; (▲) (Δ) after mixing at 560 rpm

4



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