

# LIQUID PERFLUOROCHEMICALS AS FLEXIBLE AND EFFICIENT GAS CARRIERS APPLIED IN BIOPROCESS ENGINEERING: AN UPDATED OVERVIEW AND FUTURE PROSPECTS

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Fully synthetic, biochemically inert and water-immiscible liquid perfluorochemicals (PFCs) are recognised as flexible liquid carriers/scavengers of gaseous compounds (respiratory gases mainly, i.e. O<sub>2</sub> and CO<sub>2</sub>) and increasingly applied in bioprocess engineering. A range of unmatched physicochemical properties of liquid PFCs, i.e. outstanding chemo- and thermostability, extremely low surface tension, simultaneous hydro- and lipophobicity, which result from carbon chain substitution with fluorine atoms (the most electronegative chemical element) and the presence of intramolecular C–F bonds (the strongest single bond known in organic chemistry) have been described in detail. Exceptional propensity to solubility of respiratory gases in liquid perfluorinated compounds has been widely discussed. Advantages and disadvantages of bioprocess applications of liquid PFCs in the form of a pure PFC as well as in an emulsified form have been pointed out. A liquid PFC-mediated mass transfer intensification in various types of microbial, plant cell and animal cell culture systems: from miniaturised microlitre-scale cultures, via biomaterial-based scaffolds containing culture systems, to litre-scale bioreactors, has been reviewed and elaborated on bearing in mind the benefits of bioprocesses.

**Keywords:** liquid perfluorochemical (fluorocarbon), liquid gas carrier, solubility, cell/tissue culture, hybrid culture system

## 1. INTRODUCTION

The key function of a properly developed culture system (i.e. bioreactor) is to provide a designed and properly controlled environment as well as a predicted concentration of nutrients and culture medium components (dissolved oxygen, mainly) sufficient to achieve optimal growth in bioprocesses focused on cell cultures. The level of dissolved oxygen in the culture medium is the result of a balance between its consumption rate in the cells suspended in the broth (i.e. the oxygen uptake rate), and the rate of oxygen transfer from the gas carrier (sparged air bubbles, typically) to the liquid phase (i.e. oxygen transfer rate). Up to date, a considerable amount of extensive literature focused on mass transfer in variously agitated gas – liquid contacting culture systems has been reported, discussed or reviewed (Galaction et al., 2004; Garcia-Ochoa et al., 2010; Gogate et al., 2000; Martin et al., 2010; Suresh et al. 2009). A sufficient level of oxygen dissolved in the culture medium must be provided as a mandatory issue in every aerobic bioprocess because the concentration of dissolved oxygen becomes the key-factor governing the metabolic pathway complexity in all kinds of aerobic cells (i.e. many microbial, all plant as well as insect, mammalian, and human cells) which are cultured *in vitro*. This is due to the fact

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that in every aerobic metabolism process the energy is generated by substrate (organic carbon source, mainly) oxidation.

Therefore, from the bioprocess point of view, oxygen is often the limiting nutrient in microbial cultures, as well as in plant, insect or animal cell/tissue successful growth *in vitro*. This results directly from the poor solubility of oxygen in water (which is the basic solvent of nutrients in all culture media) which is finally responsible for insufficient transfer of oxygen from gas phase through aqueous medium to cell surface. The oxygen concentration in a typical aqueous-phase culture medium is limited to ca. 0.2 mM (i.e. twice its solubility in pure water) when atmospheric air is used for aeration. Based on equimolar intracellular biochemical reactions of monosaccharides oxidation, oxygen is typically consumed at approximately the same rate as glucose, but oxygen solubility is much lower than the availability of glucose in a typical culture medium (i.e., ca. 20 – 200 mM depending on type of cultured cells) (Centis and Vermette, 2009; Martin and Vermette, 2005).

At the same time, significant carbon dioxide accumulation in culture medium can exist, especially at high cell density conditions observed in a range of bioprocess applications of microbes (bacteria, yeasts, fungi, as well as microalgae), in monolayered mammalian cell cultures and especially during *in vitro* development of 3-D aggregated mammalian and human cell constructs or implants where the value of the cell number to medium volume ratio is much higher than in culture systems containing suspended cells. It results in a significant acidification of culture medium and in increase of its osmolality due to the dissolution of bicarbonate anions (Glazyrina et al., 2012; Zhu et al., 2005).

Difficulties, both in achieving a sufficient level of dissolved oxygen and in reducing a culture system carbon dioxide level to the physiological range motivate investigators to continue search for bioprocess solutions which intensify the mass transfer between the elements of a culture system (i.e. oxygenation agent, culture medium and continuously metabolising cells). Water-immiscible liquid gas carriers such as saturated hydrocarbons (Lai et al., 2012; Li et al., 2012; da Silva et al., 2006), oils (Dumont et al., 2006; Han et al., 2009), haemoglobin derivatives (Davey et al., 2003; Lowe et al., 2001) or perfluorinated compounds (Dumont et al., 2006; Menge et al., 2001; Ntwampe et al., 2010; Pilarek and Szewczyk, 2005) characterised by O<sub>2</sub>/CO<sub>2</sub> solubility much higher than that in water, are alternatives to conventional aeration systems applied in bioreactors and other small (i.e. micro- and milliliter-) scale culture systems. Fully synthetic, biochemically inert and immiscible with aqueous phases, liquid perfluorochemicals (synonym: fluorocarbons; PFCs) are recognised as some of the most flexible liquid carriers/scavengers of gaseous compounds (respiratory gases mainly, i.e. O<sub>2</sub> and CO<sub>2</sub>) in bioprocess engineering applications. Other types of liquid gas carriers may cause negative or even detrimental effects on cultured cells, e.g. hydrocarbons can be toxic and oils can be metabolised by cells (Menge et al., 2001; Pilarek and Szewczyk, 2008). Up to date, PFCs have been frequently applied in bioreactors, as well as small-scale or miniaturised culture systems of various types of cells, i.e. microorganisms, e.g. bacteria (Pilarek et al., 2011; Pilarek et al., 2013a), yeast (Pilarek and Szewczyk, 2008; Pilarek et al., 2006), fungi (Elibol, 2001) or microalgae (Hillig et al., 2013; Hillig et al., 2014), or plant cells (Davey et al. 2005; Lowe et al. 2003; Pilarek and Szewczyk 2008; Sykłowska-Baranek et al. 2014) as wells as animal (Douglas et al. 2014; Pilarek et al., 2013b; Pilarek et al., 2014; Rappaport, 2003; Shiba et al., 1998) to successfully obtain higher cell densities or to identify positive influence on metabolite excretion due to enhanced the mass transfer effects in culture systems, mainly.

The aim of this review is to widely outline and extensively discuss the application effects of liquid perfluorinated gas carriers in various types of microbial, plant cell and animal cell culture systems: from miniaturised microlitre-scale cultures, via biomaterial-based scaffolds containing culture systems, to litre-scale bioreactors. A range of unmatched physicochemical properties of liquid PFCs, i.e. chemo- and thermostability, extremely low surface tension, simultaneous hydro- and lipophobicity, have been described in detail. Exceptional propensity to solubility of respiratory gases in liquid perfluorinated compounds has been widely discussed. The benefits of liquid PFC-mediated mass transfer intensification in bioprocesses have been also looked into.

## 2. CHARACTERISTIC AND PHYSICOCHEMICAL PROPERTIES OF PFCs

PFCs are fluorine substituted analogues of saturated aliphatic hydrocarbons with linear, cyclic or polycyclic molecules. A loose but common definition of perfluorinated compounds encompasses highly fluorinated molecules containing also occasional hydrogen, oxygen, nitrogen atoms or halogens other than fluorine. The general empirical formula of acyclic PFCs fully substituted with fluorides ( $C_nF_{2n+2}$ ) is corresponding to the general empirical formula of fully saturated acyclic alkanes ( $C_nH_{n+2}$ ) (Riess, 2001).

Liquid PFCs, as well as all perfluorinated materials, are totally synthetic compounds. PFCs are obtained by controlled fluorination (with elementary fluorine) of vaporised alkanes diluted with an auxiliary gaseous agent, e.g. nitrogen, helium, argon, or in a liquid phase, e.g. hydrogen fluoride, liquid PFCs, trichlorofluoromethane (i.e. R-11 freon), in the presence of metal fluorides as a catalyst (cobalt trifluoride or its complex salts, mainly) (Lewandowski et al., 2006; Sanford, 2003). Another way of PFC production is anode electrochemical fluorination and telomerisation of tetrafluoroethylene ( $CF_2=CF_2$ ) in the presence of adequate telogens (e.g. iodine, pentafluoride iodine). Especially telomerisation process has enabled liquid PFC (e.g. perflubron<sup>®</sup>) production on a multi-ton scale in at least 99 % purity (Riess and Krafft, 2006). Perfluorodecalin ( $C_{10}F_{18}$ ; PFD) is actually produced in at least 98 % purity as an equimolar mixture of *cis*-/*trans*- isomers by controlled fluorination of decalin ( $C_{10}H_{18}$ ) in the presence of  $CoF_3$  (Shine et al., 2005).

### 2.1. Chemical characteristic

The chemical structure of carbon chains substituted with fluorine atoms provides a range of unmatched physicochemical properties of PFCs. Fluorine atom is the most electronegative chemical element (electronegativity 3.98) and its van der Waals radius (1.47 Å) is only slightly larger than the value for hydrogen atom (1.20 Å) (Eaton and Smart, 1990; Kirsch, 2004) which results in the higher dipole moment of C–F bond (1.41 D) compared to C–H bond (0.40 D). Fluorine has a higher ionisation potential than hydrogen, just after the inert gases helium and neon. Another attribute of fluorine is lower polarisability than hydrogen – second only to neon. Such differences are the basic structural reasons for different physicochemical properties revealed by conventional hydrocarbons and their perfluorinated analogues. PFCs are recognised as compounds characterised by outstanding chemo- and thermostability (they were initially developed for handling extremely corrosive uranium fluorides), as well as biochemical inertness due to the presence of the intramolecular C–F bonds, the strongest single bond known in the whole organic chemistry with bond strength of ca. 485 kJ/mol (as compared to ca. 425 kJ/mol for C–H bonds) (Krafft and Riess, 2002). Such effects are additionally enhanced by dense electron sheath which coats the carbon chain in a PFC's molecule and results in its stiffness, as well as protection and repellency from attacking ligands higher than in hydrocarbon analogues (Eaton and Smart, 1990; Lowe 2001). The mean volumes of  $-CF_2-$  and  $-CH_2-$  groups are 38 Å<sup>3</sup> and 27 Å<sup>3</sup>, respectively, while the difference in volumes of  $-CF_3$  and  $-CH_3$  groups (i.e. 92 Å<sup>3</sup> and 54 Å<sup>3</sup>, respectively) is even more significant (Gruen, 1985; Riess, 2002a). Furthermore, structural features of stiff chains of linear PFCs allow them to adopt a helical conformation, rather than the usual planar “zig-zag” arrangement found in hydrocarbon chains, in order to minimise steric effects (Gomes and Gomes, 2007). Owing to their bioengineering applications, PFCs exhibit high resistance to heat sterilisation (e.g. by typical autoclaving at 121°C) and long-time storage potential at room temperature. Fluorinated compounds can resist highly aggressive media, including strong mineral acids, alkalis and oxidants, even in high temperature. To give as an example, poly(tetrafluoroethylene), commonly known as Teflon<sup>®</sup> (PTFE), is one of the most inert organic materials known (Hondred et al., 2013).

## 2.2. Physical characteristic

In contrast to the strength of intramolecular bonds, liquid PFCs are characterised by very low values of the van der Waals forces resulting in the lowest polarisability of fluoride substituents ( $0.56 \times 10^{-24} \text{ cm}^3$ ) of all atoms (Kirsch, 2004). A tight packing of highly electronegative fluorine atoms in liquid PFCs causes very low intermolecular cohesiveness which results in the extremely low surface tension (the lowest surface tension of any organic liquids), and therefore PFCs wet almost any surface (Lewandowski et al. 2006). The PFC-water surface tension is below  $6 \times 10^{-2} \text{ N/m}$  (Riess, 2005). Low cohesiveness also causes very weak dispersion forces between PFC molecules and it is the reason for the simultaneous hydro- and lipophobicity of liquid PFCs (Kirsch, 2004; Krafft and Riess, 2007). Therefore they exhibit maximum water solubility level around  $10^{-8} - 10^{-9}$  mole which is the lowest water solubility of all liquids (Ruiz-Cabello et al., 2011). Some physicochemical properties of PFD and 1-bromoperfluorooctane ( $\text{C}_8\text{F}_{17}\text{Br}$ ; perflubron<sup>®</sup>), which are definitely the most frequently applied liquid PFCs as liquid gas carriers on bioprocess and biomedical engineering field, are compared in Table 1.

Table 1. Comparison of some physicochemical properties of PFD and perflubron<sup>®</sup>

property	water	PFD*	perflubron <sup>®</sup>
molecular formula	H <sub>2</sub> O	C <sub>10</sub> F <sub>18</sub>	C <sub>8</sub> F <sub>17</sub> Br
molecular weight [g mol <sup>-1</sup> ]	18	462	499
melting point [°C]	0	6	-10
boiling point [°C]	100	142	142
vapour pressure at 37°C [kPa]	6.27	1.87	1.40
kinematic viscosity [m <sup>2</sup> s <sup>-1</sup> ]	$8.92 \times 10^{-7}$	$2.90 \times 10^{-6}$	$1.00 \times 10^{-6}$
interfacial tension toward water [N m <sup>-1</sup> ]	-	$5.56 \times 10^{-2}$	$5.50 \times 10^{-2}$
spreading coefficient on water [N m <sup>-1</sup> ]	-	$-1.5 \times 10^{-3}$	$+2.7 \times 10^{-3}$
solubility in water [mol dm <sup>-3</sup> ]	-	$1 \times 10^{-8}$	$5 \times 10^{-9}$

\* equimolar mixture of *cis*-/*trans*- isomers

The occurrence of large miscibility gaps in multi-phase solvent systems composed of hydrocarbons, aqueous media and liquid PFCs results in the development of a third liquid phase, named as “fluorous” vel “perfluorous”, in addition to the “organic” and “aqueous” phases. The perfluorous phase is located at the bottom of the water/PFC system due to the relatively high density of liquid PFCs (about  $1.8 - 1.9 \cdot 10^3 \text{ kg m}^{-3}$ ). Such unique immiscibility of liquid PFCs with other organic and aqueous phases is one of the main properties which promotes the spread of liquid PFCs applicability and range of patent submissions applied to their use in organic synthesis (e.g. US Patent 5463082. 31.10.1995; US Patent 6897331, 24.05.2005; US Patent 7404943. 29.07.2008), bioprocess engineering (e.g. EU Patent 0164813. 18.12.1985; EU Patent 2402433. 04.01.2012; PL Patent P-404726 16.07.2013), and on biomedical field (e.g. US Patent 4105798. 08.08.1978; US Patent 5531219. 02.07.1996; US Patent 20060278224. 22.03.2011). It is also important that liquid PFCs added to a reaction or culture system do not change the concentration of its components dissolved in aqueous phase.

## 3. SOLUBILITY OF RESPIRATORY GASES IN LIQUID PFCs

From the bioprocess engineering point of view, liquid PFCs have one special advantage over any other aqueous or organic compounds applied into culture systems – they exhibit an exceptional propensity to dissolve gases, including respiratory gases, i.e. oxygen and carbon dioxide. A very high oxygen carrying capacity ( $O_{2c.c.}$ ) of liquid PFCs combined with their extreme oxidative decomposition

strength make liquid PFCs an efficient oxygen carrier with very wide applicability in biomedical engineering field, as well as in bioprocess engineering. It is assumed that the gas absorption rate in liquid PFCs increases linearly with the partial pressure of a component in the gaseous phase, according to the Henry's Law, in contrast with sigmoidal dissociation curves for biochemical oxygen carriers, i.e. oxygen-binding globular proteins naturally occurring in animal blood (haemoglobin, Hb) or muscle tissue (myoglobin, Mb) (Elliott, 2011; Kraft and Riess, 1998; Lowe, 2001; Wesseler et al., 1977). In general, the basic molecular difference between oxygen transfer by liquid PFCs and Hb is that PFCs passively dissolve and release gases without interacting with them, whereas Hb reversibly binds dioxygen molecules. Gas molecules occupy intermolecular spaces within the liquid phase of PFC (Lamy and Deby-Dupont, 2009; Lowe 2001; Riess 2001). A comparison of oxygen-carrying capacity of PFD and perflubron<sup>®</sup>, i.e. two biomedically utilised perfluorinated oxygen carrier agents, with human blood plasma, Hb and Mb is presented in Fig. 1. Thus, at a given partial pressure of oxygen, Hb or Mb binds more O<sub>2</sub> than can be dissolved in typical liquid PFCs. However oxygen solubility in liquid perfluorinated compounds (with a range of 35 – 44 mM (Deschamps et al., 2007; Riess, 2006a) is approximately 20 times higher than the solubility of oxygen in water (2.2 mM) at standard temperature and pressure. The solubility of carbon dioxide exceeds 200 mM (Lowe, 2002; Riess, 2001). What is also valuable, gas solubility in liquid PFCs decreases in the following order (Lowe, 2001):

$$\text{CO}_2 \gg \text{O}_2 > \text{CO} > \text{N}_2 \quad (1)$$

which makes them suitable in a range of bioprocess applications for oxygen delivery and removal of metabolically generated carbon dioxide from culture systems.

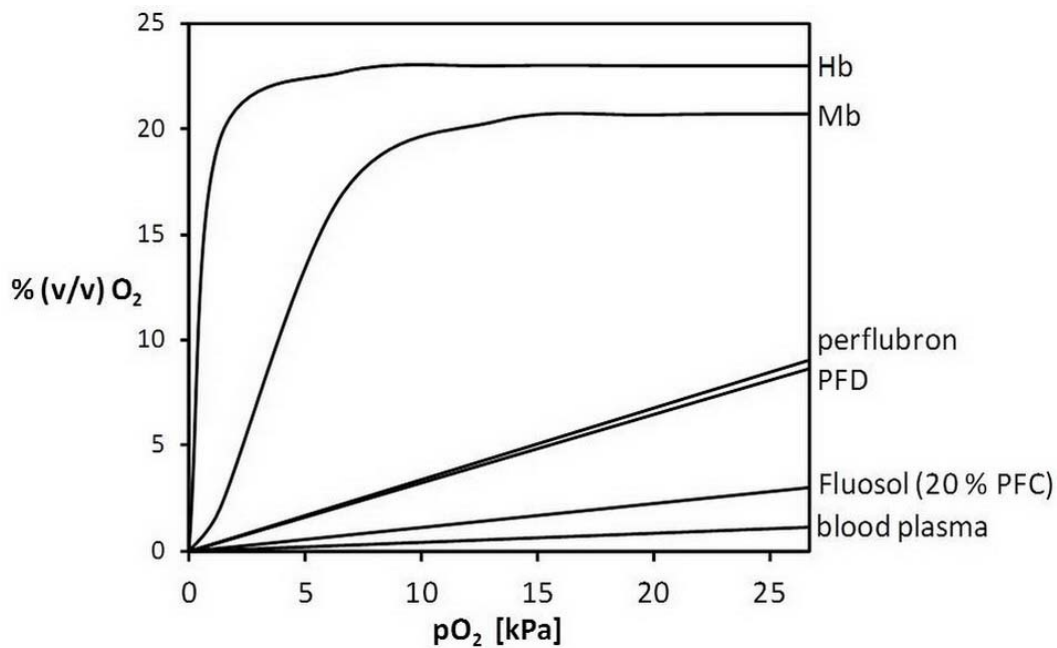


Fig. 1. Comparison of oxygen-carrying capacity of PFD, perflubron<sup>®</sup> and Fluosol-DA (as an example of biomedically utilised liquid PFC-based emulsion) with human blood plasma, Hb and Mb (based on Kirsch, 2004; Lowe 1999 and Riess, 2001)

Previously published data on the solubility of oxygen and carbon dioxide in PFD and perflubron<sup>®</sup> are listed in Table 2. In order to facilitate a comparison of the data, they were expressed (after conversion from the Ostwald coefficient ( $L_{2,1}$ ) if needed) as the Henry's Law constant ( $H$ ) defined as:

$$p_i = Hc_i \quad (2)$$

Table 2. Solubility of oxygen and carbon dioxide in perfluorodecalin and 1-bromoperfluorooctane expressed as the Henry's law constant defined as in Eq. 2

liquid PFC	O <sub>2</sub>	CO <sub>2</sub>	<i>T</i> [K]	<i>p</i> [kPa]	reference
	<i>H</i> [kPa m <sup>3</sup> mol <sup>-1</sup> ]	<i>H</i> [kPa m <sup>3</sup> mol <sup>-1</sup> ]			
perfluorodecalin (C <sub>10</sub> F <sub>18</sub> ; PFD)	5.94 – 6.29	–	288.6 – 311.4	96.1 – 98.4	Deschamps et al., 2007
	–	1.29 – 1.72	288.6 – 312.3	95.0 – 97.2	Costa Gomes et al., 2004
	5.79 – 6.38	–	288.3 – 312.2	101.3	Freire et al., 2004
	–	0.820	293.1	101.3	Sobieszuk and Pilarek, 2012
	2.85	–	293.1	101.3	Pilarek et al., 2013c
1-bromoperfluorooctane (C <sub>8</sub> F <sub>17</sub> Br; perflubron <sup>®</sup> )	5.26 – 5.73	1.17 – 1.55	289.8 – 311.0	93.5 – 97.7	Deschamps et al., 2007
	5.43 – 5.84	–	289.2 – 316.5	95.7 – 97.1	Costa Gomes et al., 2004

It should be emphasised that the data available in the open literature for the solubility of gases in liquid PFCs are still rather limited, with most data widely scattered. The incomplete quantitative characterisation of the used PFC samples or the imprecise descriptions of the employed experimental methods makes it difficult to compare most of previously published data. Moreover, most of the available data on the O<sub>2</sub> and CO<sub>2</sub> solubility in liquid PFCs refers to the values measured in only one temperature, e.g. 20 or 37 °C, as well as measured for perfluorinated compounds in an emulsified form.

#### 4. GAS CONCENTRATION DETERMINATION IN LIQUID PFCs

It is not possible to directly measure the concentration of dissolved oxygen in liquid PFCs. The standard method using the Clark electrode is not applicable due to hydrophobicity of fluorinated phase. Therefore, it is necessary to apply indirect methods. Because of the relatively high costs of liquid PFCs, using a typical apparatus to determine the values of gas solubility, e.g. the laminar-jet (Pohorecki and Moniuk, 1988), is uneconomical and thus problematic. An isobaric saturation method was most frequently employed to quantitatively determine solubility of gases in liquid PFCs (Costa Gomes et al., 2004; Deschamps et al., 2007; Freire et al., 2004) expressed as the Ostwald coefficient ( $L_{2,1}$ ). Furthermore, a modified enzymatic method (Pilarek et al., 2013c), originally developed by Freire (2005) to determine the oxygen solubility in liquid PFCs-based emulsions, was successfully employed to determine oxygen concentration in microliter-scale samples of PFD, as well as volumetric gas-side mass transfer coefficient ( $k_{pa} = 0.051 \text{ mol m}^{-3} \text{ s}^{-1} \text{ kPa}^{-1}$ ) and volumetric liquid-side mass transfer coefficient ( $k_{la} = 3.63 \times 10^{-3} \text{ s}^{-1}$ ) for a PFD-oxygen/air two-phase system. The gas-liquid flow in a microchannel has been proposed by Sobieszuk and Pilarek (2012) for mass transfer investigations in PFD/CO<sub>2</sub> systems. The Henry's law constant has been determined based on the estimated values of the CO<sub>2</sub> absorption rates in the applied microchannel device and using the correlation for mass transfer coefficient in the Taylor flow due to Yue et al. (2007). Such investigation was also the first report of the application of liquid PFC in a microreactor system in general. Summarising, all previously published data on oxygen solubility in PFD and perflubron seems to be consistent (see Table 2) and basically they are sufficient to approve biomedical application of liquid PFCs or to develop bioprocesses utilising liquid PFC-based carriers of respiratory gases.

## 5. LIQUID PFC-BASED EMULSIONS

As insoluble in water, liquid PFCs can be converted to an emulsified form using different types and concentrations of a range of surfactants. The form of liquid PFC-based emulsions depends on the way of its bioengineering application. Two main lines of research are being actively pursued: (i) PFC-in-water emulsions for *in vivo* intravascular oxygen delivery (known as perfluorinated temporary blood substitutes) (Krafft et al., 2003; Riess, 2006b; Vasquez et al., 2013), as storage-media during organ transplantation procedures (Bezinover et al., 2014; Maillard et al., 2008; Terai et al., 2010), currently under development for treatment of traumatic brain and spinal cord injury (Spiess, 2009) as well as in decompression sickness therapy (Randsoe and Hyldegaard, 2009), and (ii) water-in-PFC reverse emulsions for pulmonary drug delivery (Courrier et al., 2004; Smoła et al., 2008), targeted emulsions for diagnosis and therapy (Kaufmann and Lindner, 2007; Lanza and Wickline, 2001; Martin and Dayton, 2013), as well as valuable research tool used in polymerisation technology (Chen et al., 2010; Zetterlund et al., 2008).

It should be emphasised that from the point of view of bioengineering, PFC-in-water emulsions (particle size of approximately 0.2  $\mu\text{m}$ ) have much greater importance for intravenous administration than the others. Nowadays, they are usually formulated as submicron, i.e. nano- or micro- PFC-in-water emulsions, preferably, to increase their stability (Maevsky and Ivanitsky, 2005; Riess, 2002b) and thus significantly smaller (10 to 100 times) than the size of erythrocytes. Not only smaller droplet size, but also significantly larger relative surface-to-volume ratio of PFC drops create favourable gas exchange conditions. Aside from adequate dispersion, homogeneity and reproducibility of small droplets of liquid PFC, formulation of emulsion with high applicability in bioengineering field requires a surfactant system and manufacturing procedures capable of ensuring stability, biocompatibility and possibility for heat-sterilisation. Poloxamers (e.g. Pluronic and Proxanols, mainly) and natural phospholipids (e.g. from egg yolk, mainly), which are commonly utilised in the formulation of pharmaceuticals, seem to be capable of meeting all of these requirements (Bouchemal, 2004; Kaufman, 1992; Riess and Krafft, 2006). The key idea in developing liquid PFCs-based emulsions is to apply them *in vivo* as intravenously injectable temporary blood substitutes. PFC-in-water emulsions intended for biomedical use are produced at concentrations ranging from 20 % up to 120 % weight/volume. Osmolarity of the suspending media is independent of liquid PFC concentration and is adjusted by the addition of tonicity agents (Cabrera and Intaglietta, 2013). Up to date, several emulsion systems based on a few liquid PFC agents have been commercially developed and they are generally in preclinical/clinical trials, as reported in Table 3.

Three main criteria to differentiate between 1<sup>st</sup> and 2<sup>nd</sup> generations of liquid PFC-based emulsions have been proposed by Kusnetzova (2003):

- concentration of liquid PFC agents in 2<sup>nd</sup> generation emulsions is twice or even four times higher compared to 1<sup>st</sup> generation,
- natural emulsifiers are ingredients of 2<sup>nd</sup> generation emulsions,
- 2<sup>nd</sup> generation emulsions can be stored in room temperature in contrast to 1<sup>st</sup> generation emulsions that need to be frozen.

Simplifying, the 2<sup>nd</sup> generation emulsions have been developed in order to overcome the difficulties in biomedical applications exhibited by the 1<sup>st</sup> generation. They are supplied in an emulsified form and capable to withstand temperature variations during preparation and shipping (e.g. Oxycyte can be stored at 4°C, with a shelf life of 18 – 24 months). Liquid PFCs are used in an emulsified form in order to increase the interfacial exchange surface. Nevertheless, from the bioprocess point of view, most commercially developed liquid PFC-based emulsions are not fully applicable in culture systems containing living cells. The main reason is the content of non-perfluorinated additives which may influence negatively cells (e.g. phospholipids utilised as emulsifiers) or they may be metabolised (e.g.

plant-origin additives,  $\alpha$ -tocopherol). They may negatively affect culture processes conducted in bioreactor systems (e.g. increased foaming caused by surfactants).

Table 3. Liquid PFC-based emulsions developed as injectable temporary blood substitutes

generation	product (manufacturer, country)	PFC [%w/v]	surfactants [%w/v]	additives and buffer	$O_{2c.c.}$ [cm <sup>3</sup> O <sub>2</sub> dm <sup>-3</sup> kPa <sup>-1</sup> ]	reference
1 <sup>st</sup>	Fluosol-DA (Alpha Therapeutic Corp., US; previously: Green Cross Corp., JP)	14 % PFD 6 % PFTPA	2.7 % Pluronic F-68 0.4 % EYP 0.03 % potassium oleate	NaCl, KCl, MgCl <sub>2</sub> , CaCl <sub>2</sub> , Ca(HCO <sub>3</sub> ) <sub>2</sub> , glycerol, dextrose	0.06×10 <sup>-2</sup>	Mitsuno et al., 1982
	Oxypherol (Alpha Therapeutic Corp., US; previously: Green Cross Corp., JP)	20 % PFTBA	Pluronic F-68	Krebs-Ringer bicarbonate solution	0.45×10 <sup>-2</sup>	Ruland et al., 1987
	Perftoran (Perftoran Company, RU)	12 % PFD 3 % PFMCP	6.5 % Proxanol 268	NaCl, KCl, MgCl <sub>2</sub> , NaHCO <sub>3</sub> , NaH <sub>2</sub> PO <sub>4</sub> , glucose, H <sub>2</sub> O	0.06×10 <sup>-2</sup>	Maevsky et al., 2005
2 <sup>nd</sup>	Oxycyte (Oxygen Biotherapeutics, Inc., US; previously Synthetic Blood International Inc., US)	60 % TBPFC	EYP	n/a	1.27×10 <sup>-2</sup>	Yacoub et al., 2014
	Oxyfluor (HemaGen/PFC, US)	78 % PFDCO	EYP, safflower oil	n/a	0.13×10 <sup>-2</sup>	Rémy et al., 1999
	Oxygent (Alliance Pharmaceutical Corp., US)	58 % perflubron <sup>®</sup> , 2 % PFDB	3.6 % EYP	NaCl, EDTA, $\alpha$ -tocopherol, phosphate buffer	2.32×10 <sup>-2</sup>	Hill et al., 2002
currently in progress	“Columbian emulsion”	58 % perflubron <sup>®</sup>	3 % soybean lecithin	NaCl, glucose, glycerol, $\alpha$ -tocopherol, phosphate buffer	n/a	Gardeazabal et al., 2008
	“French emulsion”	90 % perflubron <sup>®</sup>	4 % EYP 2.5 % diblock perfluoro- /hydrocarbon compound	phosphate buffer	n/a	Audonnet- Blaise et al., 2006

$O_{2c.c.}$  - oxygen carrying capacity (cm<sup>3</sup> O<sub>2</sub> dm<sup>-3</sup> kPa<sup>-1</sup>); n/a - not available

liquid PFCs: PFDB - 1-bromo-perfluorodecane; perflubron<sup>®</sup> - 1-bromo-perfluorooctane; PFD - perfluorodecalin; PFDCO - perfluorodichlorooctane, PFMCP - perfluoromethylcyclohexylpiperidin, PFTBA - perfluorotributylamine, PFTPA - perfluorotripropylamine, TBPFC - tributylperfluorocyclohexane; surfactants/emulsifiers: EYP - egg yolk phospholipid;



## 6. LIQUID PFC-BASED GAS CARRIERS IN VARIOUS CELL CULTURE SYSTEMS

Oxygen has a low solubility in water and in aqueous media. Additionally, the concentration of oxygen dissolved in culture media decreases during heating the broth, and the presence of dissociated microelements in the culture medium also influence negatively oxygen solubility. Combinations of spargers and various types of agitators, as well as air-lift systems are typical and commonly used mixing-and-aerating systems applied in a range of bioprocesses related to living cells. However, their application can result in undesired by-product production (e.g. biosynthesis and secretion of stress-derived metabolites as the response to environmental stressors, leak of metabolites through weakened cell membrane) (Enfors et al., 2001; Garcia-Ochoa and Gomez, 2009; Neubauer and Junne, 2010) or even in structural disruption of cells as a result of the hydrodynamic cell stress caused by high shear forces generated during mechanical mixing and/or continuous bubble aeration of culture broth/medium due to turbulence intermittency (Bałdyga and Pohorecki, 1998; Garcia-Ochoa et al., 2013; Pohorecki et al., 2001). All these effects negatively affect biomass performance, and contribute to bioprocess yield decrease.

Water-immiscible carriers of respiratory gases are the alternative to conventional aeration systems applied in a range of bioreactors and miniaturised-scale culture systems. Such agents exhibit one common property – they have greater capacity to solubilise the oxygen than water, i.e. the basic solvent of all culture media. Besides liquid PFCs, a few other organic materials have been applied in bioprocesses as gas (oxygen, mainly) carriers so far: saturated hydrocarbons, oils and modified-haemoglobin-based derivatives. Initially, water-immiscible gas carriers have been utilised in the field of biomedical engineering, e.g. haemoprotein-based derivatives and emulsified liquid PFCs as intravascularly applied temporary blood substitutes (Bucci, 2009; Kim, 2007; Kołtuniewicz, 2014) or pure liquid PFCs as media suitable for liquid ventilation procedures (Kaisers et al., 2003; Tawfic and Kausalya, 2011). Nevertheless, droplets or layer of gas saturated water-immiscible liquid carriers (liquid PFCs, mainly) can be applied to enhance gas supply (e.g. in the case of oxygen, ethene) or removal (e.g. in the case of carbon dioxide) through liquid-liquid (i.e. PFC-medium) interfacial area in culture systems. Examples of bioprocess engineering applications of liquid PFC-based gas carriers in culture systems of various types of cells are reviewed in Table 4.

Water-immiscible liquid PFC-based gas carriers were applied in submerged cultures of all main types of cells utilised in bioprocess engineering, i.e. microorganisms (bacteria, yeasts, microalgae), plant and animal (insects, mammalian, human) cells. To date, depending on the specificity of a particular culture, liquid PFCs were used to intensify the transfer of the following gases: oxygen, carbon dioxide, nitrogen, nitrous oxide and ethene. The main benefits of bioprocesses carried out in the presence of a gas-saturated liquid PFC compared to traditional cultures (without liquid gas carrier) include: (i) providing additional liquid-liquid (i.e. liquid PFC - culture medium) interfacial area for effective mass transfer independent from gas-liquid interface typically occurring in culture vessel filled by aqueous broth/medium and air phase; (ii) enhanced gas absorption rate without the need for supplementary energy input for mixing the culture; (iii) reduction of the detrimental effects of mechanical damage of cells occurring during a conventional intensive mixing and bubble aeration; (iv) easy separation of liquid PFC from culture medium as well as its regeneration (e.g. in typical absorption column) and re-use (v) possibility to use liquid PFCs in an autoclave directly in culture vessel together with broth/medium prepared for culture (independent sterilisation of liquid PFCs via filtration is also possible).

Table 4. Bioprocess engineering applications (in chronological order) of liquid PFC-based gas carriers in culture systems of microorganisms, plant and animal cells

origin of cells	liquid PFC (D - dispersed; E - emulsified; L - layer; P - pure)	gas supplied (source)	scale of culture / operating volume (B - bioreactor; E - Erlenmayer flask; M - miniaturized culture)	effects reported	reference
<b>MICROORGANISMS</b> (A - microalgae; B - bacteria; N - nematode; Y - yeast)					
<i>Gluconobacter oxydans</i> (B)	Fluorinert FC-72 32.4 (v/v) (E; Pluronic F 68)	oxygen (pure O <sub>2</sub> )	column B (7 × 10 <sup>-3</sup> dm <sup>3</sup> ) with cells immobilised in alginate perfused with culture medium and emulsified PFC	5.0 x increase in dihydroacetone yield	Adlercreutz and Mattiasson, 1982
<i>Enterobacter aerogenes</i> (prev.: <i>Aerobacter aerogenes</i> ) (B)	Forane F66E 19 % (v/v) (E; Pluronic F 68)	oxygen (air)	12 dm <sup>3</sup> medium; B with Rushton turbine (500 rpm)	3.5 x <i>k<sub>L</sub>a</i> increase(*)	Rols et al., 1990
<i>Escherichia coli</i> (B)	FlurO <sub>2</sub> emulsion 15 - 25 % (v/v) (E)	oxygen (air)	0.7 dm <sup>3</sup> medium; glass-bottle B with mechanical agitation (100 rpm)	2.55 x increase in biomass yield;	Ju et al., 1991
<i>Clostridium acetobutylicum</i> (B)	Forane F66E 18.5 % (v/v) (E; Pluronic F 68)	carbon dioxide (culture medium)	0.2 dm <sup>3</sup> medium; B with magnetic stirrer (400 rpm)	8.8 % of total generated CO <sub>2</sub> extracted from culture by PFC	Percheron et al., 1995
<i>Escherichia coli</i> (B)	perflubron® 50 % (v/v) (E; Pluronic F 68)	oxygen (pure O <sub>2</sub> )	0.25 dm <sup>3</sup> medium; B with magnetic stirrer (200 - 900 rpm)	PFC-supported cultures provided: 0.17 g O <sub>2</sub> dm <sup>-3</sup> h <sup>-1</sup> supply; 0.23 g CO <sub>2</sub> dm <sup>-3</sup> h <sup>-1</sup> remove	Martin, et al., 1995
<i>Mycobacteria parafortuitum</i> (B)	Fluorinert FC-40 18.2 - 26.0 % (v/v) (P, D)	ethene (pure C <sub>2</sub> H <sub>4</sub> )	1.3 dm <sup>3</sup> medium; B with Rushton turbine (800 rpm)	1.2 - 1.8 x <i>k<sub>L</sub>a</i> increase(*)	Cesario et al., 1996
<i>Pseudomonas denitrificans</i> (B)	Fluorinert FC-77 10 % (v/v) (E; Pluronic F 68)	nitrous oxide (pure N <sub>2</sub> O)	0.05 dm <sup>3</sup> medium; mechanically agitated E (100 rpm)	2.7 x increase in N <sub>2</sub> O reduction rate; 2.1 x increase in biomass yield	Turick and Bulmer, 1998

<i>Saccharomyces cerevisiae</i> (Y)	PFD 5 - 15 % (v/v) (E; Pluronic F 68)	oxygen (air)	0.8 dm <sup>3</sup> medium; B with Rushton turbine (300 - 600 rpm)	2.5 x <i>k<sub>L</sub>a</i> increase(*); 20 % increase in biomass yield	Eilbol, 1999
<i>Caenorhabditis elegans</i> (N)	PFD 35 % (v/v) (P, L)	oxygen (pure O <sub>2</sub> )	5.5×10 <sup>-3</sup> dm <sup>3</sup> medium; M in 6-DWP	12 % increase in worm length; improved larvae development; 3.5 x extended duration of standard toxicology assays; the first case of culture of whole eukaryotic multicellular organism	Jewitt et al., 1999
<i>Chlorella vulgaris</i> (A)	Fluoroinert FC-77 50 % (v/v)	oxygen (60 % O <sub>2</sub> + 40 % N <sub>2</sub> )	0.05 dm <sup>3</sup> medium; B (glass spinner flask) mechanically agitated (25 rpm)	16 % increase in biomass yield; lower dissolved O <sub>2</sub> concentration in cultures supported with PFC	Wasanasathian and Peng, 2001
<i>Streptomyces coelicolor</i> (B)	PFD 10 % (v/v)	oxygen (air)	12 dm <sup>3</sup> medium; B with Rushton turbine (200 rpm)	3.0 x <i>k<sub>L</sub>a</i> increase(*); 2.0 x increase in antibiotic yield	Eilbol, 2001
<i>Saccharomyces cerevisiae</i> (Y)	PFD 20 % (v/v) (P, D)	oxygen (air)	1.0 dm <sup>3</sup> medium; B with circulation of dispersed-and- coalesced droplets of PFC and Rushton turbine (150 - 320 rpm)	1.5 x <i>k<sub>L</sub>a</i> increase(*); 110 % increase in biomass yield	Pilarek et al., 2006; Pilarek and Szezyk, 2008
<i>Azotobacter chroococcum</i> (B)	PFD, PFMD 1.0 - 24 % (v/v) (P, D)	nitrogen (air; 88.5 % N <sub>2</sub> + 11.5 % O <sub>2</sub> )	0.045 dm <sup>3</sup> medium; mechanically agitated E (200 rpm)	5.0 x increase in biomass yield; up to 3.4 x increase in nitrogenase activity; 4.5 x increase in N <sub>2</sub> accumulated in culture medium(mg cm <sup>-3</sup> )	Bakulin et al., 2007
<i>Escherichia coli</i> (B)	PFD 50 % (v/v) (P, D)	oxygen (air; pure O <sub>2</sub> )	3×10 <sup>-3</sup> dm <sup>3</sup> medium; M in mechanically agitated 24-DWP (250 rpm); high-cell density fed-batch culture	40 % increase in biomass yield; significantly higher alcohol dehydrogenase yield; lower level of inclusion bodies (insoluble fraction of protein)	EU Patent 2402433. 04.01.2012; Pilarek et al., 2011
<i>Escherichia coli</i> (B)	PFD 50 % (v/v) (P, D)	oxygen (air; pure O <sub>2</sub> )	5×10 <sup>-5</sup> dm <sup>3</sup> medium; M in mechanically agitated Eppendorf tube (300 rpm); high-cell density fed-batch culture	20 % increase in biomass yield; 25 % increase in plasmid DNA yield; culture method ready to automated high-throughput process	EU Patent 2402433. 04.01.2012; Pilarek et al., 2013a; Grunzel et al., 2014

<i>Cryptocodinium cohnii</i> (A)	PFD 50 % (v/v) (P, D)	oxygen (pure O <sub>2</sub> )	3×10 <sup>-3</sup> dm <sup>3</sup> medium; M in mechanically agitated 24-DWP (270 rpm); high-cell density fed-batch culture	2.0 x increase in ω-3 fatty acid specific yield (% acid cell <sup>-1</sup> ); enhanced CO <sub>2</sub> removing from culture medium	Hillig et al., 2013; Hillig et al., 2014
<i>Nannochloropsis oculata</i> (A)	perflubron <sup>®</sup> , PFD, HFE-7100, HFE-7200; 20 % (v/v) (P, D)	oxygen (60 % O <sub>2</sub> + 40 % N <sub>2</sub> )	0.1 dm <sup>3</sup> medium; B (glass photobioreactor) mechanically agitated (100 rpm)	reduction of O <sub>2</sub> -based inhibition effect in investigated photobioreactor; 3.0 x increase in biomass yield	Lee et al., 2013
PLANT CELLS					
<i>Petunia hybrida</i>	PFD 75 % (v/v) (P, L)	oxygen (pure O <sub>2</sub> )	2×10 <sup>-3</sup> dm <sup>3</sup> medium supplemented with 0.01 % Pluronic F 86); M in glass tubes	52 % increase in cell wall biosynthesis	Anthony et al., 1994
<i>Oryza sativa</i>	PFD 50 % (v/v) (E; Pluronic F 68)	oxygen (pure O <sub>2</sub> )	1-2×10 <sup>-3</sup> dm <sup>3</sup> medium; M in 24-DWP	21 % increase in post-thaw mitotic divisions of cells after cryopreservation	Wardrop et al., 1996
<i>Nicotiana tabacum</i> BY-2	PFD 20 - 60 % (v/v) (P, D)	oxygen (air; pure O <sub>2</sub> )	0.1 dm <sup>3</sup> medium; mechanically agitated E (100 rpm)	2.0 x increase in biomass yield; extension of the exponential growth phase; inhibited growth of cells in cultures with PFD saturated with pure O <sub>2</sub>	Pilarek and Szewczyk, 2007; Pilarek and Szewczyk, 2008
<i>Nicotiana tabacum</i> BY-2	PFD (P)	oxygen (air)	1.8 m <sup>2</sup> hollow-fibre module B	1.1 x <i>k<sub>L</sub>a</i> increase(*); 5.0 x increase in biomass yield	Pilarek et al., 2009
<i>Arneubia euchroma</i>	PFD 28.5 % (v/v) (P, D)	oxygen (air; pure O <sub>2</sub> ); carbon dioxide (pure CO <sub>2</sub> ); ethene (pure C <sub>2</sub> H <sub>4</sub> )	0.05 dm <sup>3</sup> medium; mechanically agitated E (250 rpm)	50 % increase in alkannin/shikonin yield; extracellularly secreted naphthoquinones were dissolved in PFD; an innovative two-phase <i>in situ</i> extraction culture system developed	PL Patent P-404726 16.07.2013; Sykłowska-Baranek et al., 2014

ANIMAL CELLS (F - fish; H - human; I - insect; M - mammalian)						
4C10B6 hybridoma cells (M)	Fluoroinert FC-40 31 % (v/v) (P, D)	oxygen (pure O <sub>2</sub> )	0.225 dm <sup>3</sup> medium; B supported with prototyped aerator and mechanically agitated (150 rpm)	increased yields of biomass and IG <sub>2b</sub> -type mAbs	Hamamoto et al., 1987	
#824 hybridoma cells (M)	PFMD 13 % (v/v) (P, D)	oxygen (air, pure O <sub>2</sub> )	B with low shear agitation system (50 - 250 rpm) and circulation of PFC applied through bar of syringe-needles	5.0 x <i>k<sub>L</sub>a</i> increase(*); cell aggregation at PFC-medium interface	Cho and Wang, 1988	
HeLa cells (H)	PFD 2.3 - 8 % (v/v) (P, L)	oxygen (air)	0.011 dm <sup>3</sup> medium; M in tissue culture test tubes (0.015 dm <sup>3</sup> ) with gelatin-PFD matrix	5.5 x increase in biomass yield; cells formed tissue-like structure of more than 19 layers of cells	Rappaport et al., 1996	
3C11 hybridoma cells (M)	Oxyfluor 0.1 - 2.0 % (v/v) (E)	oxygen (air)	0.14 m <sup>2</sup> hollow-fibre module B	78 % increase in IG <sub>1</sub> -type mAbs yield	Shi et al., 1998	
L-929 cells (M)	FC-series: 40, 70; KPF-series: 9I, 102, 142 44.4 % (v/v) (P, L)	any gas supplied	6×10 <sup>-3</sup> dm <sup>3</sup> medium; two-phase liquid-liquid culture system; M in multi-well plate	cells proliferation and aggregation at PFC-medium interface possible; weak adhesion of cells to liquid-liquid interface	Shiba et al., 1998	
Sf9 cells (I)	Fluoroinert FC-40 (P, D)	oxygen (air)	0.18 dm <sup>3</sup> medium; prototyped column B with PFC-wetted wall	1.6 x <i>k<sub>L</sub>a</i> increase(*); 2.2 x increase in biomass yield; 79 % decrease in lactate yield	Gotoh et al., 2001a; Gotoh et al., 2001b	
<i>Oncorhynchus mykiss</i> (rainbow trout) eggs (F)	Fluoroinert FC-77 50 % (v/v) (P, L)	oxygen (pure O <sub>2</sub> )	0.05 dm <sup>3</sup> medium; M in plastic containers	4.2 x increase in egg hatching	Richardson et al., 2002	
Hep G2 cells (H)	Fluoroinert FC-71 + 3(perfluorooctyl) propanol applied to prepare perfluoroaldehyde matrix	oxygen (air)	6×10 <sup>-3</sup> dm <sup>3</sup> medium; M in prototyped multi-well plate with collagen-coated perfluorocarbon matrix	10 x increase in cell density; 3-D growth of anchorage-dependent cells (more than 10 layers of cells achieved); continuous secretion of albumin on a per-cell basis	Rappaport et al., 2002; US Patent 5702949. 30.12.1997	

neonatal rat cardiomyocytes (M)	Oxygent 10 % (v/v) (E)	oxygen (air)	0.03 dm <sup>3</sup> medium; M in perfused channelled scaffolds	60 % increase in biomass yield; 34 % decrease in measured cell death (by lactate dehydrogenase activity assay); significantly extended viability of embryonic stem cell spheres when cultured at PFD-medium interface; extended maintain stem cell spheres under undifferentiated state at PFD-medium interface	Radisic et al., 2006
mouse embryonic stem cell spheres (M)	PFD 50 % (v/v) (P, L)	oxygen (air)	2×10 <sup>-3</sup> dm <sup>3</sup> medium; two-phase liquid-liquid culture system; M in 24-DWP	robust growth of 3-D aggregated cells achieved; fully reversible morphology of cells after passage from flexible liquid-liquid interface (3-D cells) on solid surface (typical monolayer form)	Brzezińska et al., 2012
BHK-21 cells (M)	PFD 50 % (v/v) (P, L)	oxygen (air)	2×10 <sup>-3</sup> dm <sup>3</sup> medium; two-phase liquid-liquid culture system; M in 24-DWP	2.5 x increase in biomass yield (depended on cell type); different 3-D cell constructs achieved: aggregates or sheets; growth of cells was not limited by the available area or confluence effects as in typical solid-surface-based culture systems; proofed applicability of investigated system for animal cell culture studies under hyperoxia	Pilarek and Grabowska, 2012
A431 (H), BHK-21 (M), C2C12 cells (M)	PFD 50 % (v/v) (P, L)	oxygen (air, pure O <sub>2</sub> )	2×10 <sup>-3</sup> dm <sup>3</sup> medium; two-phase liquid-liquid culture system; M in 24-DWP	O <sub>2</sub> concentration inside the hydrogel modified by the type of liquid PFC substitution in the hydrogel network; enhanced proliferation of cells within O <sub>2</sub> -enriched fluorinated hydrogel-based constructs; capability of fluorinated hydrogel-based hydrogels for sequential reloading/releasing O <sub>2</sub> for weeks	Pilarek et al., 2013b
NIH-3T3 cells (M)	fluorinated methacrylamide-chitosan hydrogels	oxygen (pure O <sub>2</sub> )	1.51×10 <sup>-4</sup> dm <sup>3</sup> fluorinated hydrogel-based constructs; M in 48-DWP		Wijekoon et al., 2013

CP-5 cells (M)	PFD 50 % (v/v) (P, L)	oxygen (air)	2×10 <sup>-3</sup> dm <sup>3</sup> medium; hybrid liquid-solid-liquid culture system; M in 24-DWP	robust proliferation of cartilage cells outside and inside the fibrous polylactide-based scaffolds; an innovative, hybrid liquid-solid- liquid self-organizing (in accordance with the buoyancy force affected on used biomaterial) system developed	Pilarek et al., 2014
rat neural stem/progenitor cells (M)	fluorinated methacrylamide- chitosan hydrogels	oxygen (pure O <sub>2</sub> )	2×10 <sup>-4</sup> dm <sup>3</sup> fluorinated hydrogel-based constructs; M in 96-DWP	enhanced local O <sub>2</sub> concentrations within fluorinated hydrogel-based constructs; enhanced O <sub>2</sub> uptake capacity; improved viability and differentiation of cells in 3-D environment of fluorinated hydrogels	Li et al., 2014
L-929 (M), ADSC - adipose tissue- derived stem cells (H)	PFD 7 - 28 % (v/v) (P, D)	oxygen (air)	dispersed PFD droplets in chitosan hydrogels; M in 24-DWP	PFD promotes gelation; PFD-enriched hydrogels improved ADSC viability and reduced cell death	Douglas et al., 2014

(\*) in comparison to gas-liquid systems;

Fluoroimert (e.g. FC-40, FC-72, FC-77) - line of commercially available liquid perfluorotertiary amine-based electronics coolants (detailed composition is not available)  
manufactured by 3M Company (USA); perflubron<sup>®</sup> - 1-bromo-perfluorodecalin; PFMD - perfluoromethylodecalin;  
hydrofluoroethers (HFE; e.g. HFE-7100 - methoxynonafluorobutane, HFE-7200 - ethoxynonafluorobutane) - linear, perfluoroaliphatic ethers;  
6-/24-/48-/96-DWP - 6-/24-/48-/96-deep well plate;  
mAbs - monoclonal antibodies

### 6.1. PFC-mediated oxygen transfer intensification

Regardless of the type of cultured cells, the effects of supplying oxygen into culture media via perfluorinated carriers have been studied frequently. Generally, the application of liquid PFC-based oxygen carriers in culture systems had positive effects on the rate of oxygen absorption and thus enhanced biomass and bioproduct yields. In the case of submerged cultures of microbial cells, the highest increase of volumetric liquid-side mass transfer coefficient ( $k_{LA}$ ) of up to 3.5 times, which been achieved when emulsified Forane F66E has been applied for oxygenation of *Enterobacter aerogenes* (synonym: *Aerobacter aerogenes*) culture performed in 12 dm<sup>3</sup> stirred tank (500 rpm) in comparison to a process with typical bubble aeration (Rols et al., 1994). Liquid PFC-supported bioprocesses resulting in the increase of  $k_{LA}$  from 1.2 to 3.0 times have been reported (see Table 4). In the case of enhancing biomass production, the highest increase in biomass yield (up to 2.22 times) has been reported by Ju et al. (1991) for *Escherichia coli* culture with oxygenation enhanced by FlurO<sub>2</sub>.

Elibol (2001) informed of up to 2.0 fold increase in *Streptomyces coelicolor* secondary metabolite production in culture performed in 12 dm<sup>3</sup> bioreactor with Rushton turbine (200 rpm) and supported with dispersed aerated PFD. More recently it has been proved that water-immiscible liquid PFC-based oxygen carriers are also feasible agents in miniaturised culture systems. A significantly higher yield of genetically modified alcohol dehydrogenase as well as a lower level of inclusion bodies (i.e. insoluble fraction of not-correctly folded protein molecules) achieved in milliliter-scale of dispersed PFD containing cultures of *E. coli* has been reported by Pilarek et al. (2011). The same group has also informed of a 25 % increase in plasmid DNA yield biosynthesised in *E. coli* cells cultured in PFD-supported microliter-scale system (Grunzel et al., 2014; Pilarek et al., 2013). Such a huge range of culture volumes (from 10<sup>-5</sup> to 10<sup>1</sup> dm<sup>3</sup>) which has been reviewed in Table 4 (as well as discussed above) clearly indicates that perfluorinated oxygen carriers are very flexible bioengineering tools for gas transfer intensification in scaling-up or scaling-down of bioprocesses.

Also in the case of *in vitro* cultures of cells isolated from multicellular organisms, liquid PFCs have been applied mainly as carriers of oxygen. The application of the PFD-mediated oxygenation system in *Nicotiana tabacum* BY-2 (*Bright Yellow 2*) cultures performed in a hollow-fibre bioreactor has resulted in 1.1 time  $k_{LA}$  increase, as well as up to 5 times higher biomass yield in comparison to the culture system utilising atmospheric air as oxygenation medium (Pilarek et al., 2009). A hollow-fibre bioreactor has been also utilised for monoclonal antibody (mAb) production in cultures of mouse 3C11 hybridoma cells (Shi et al., 1998). The application of aerated Oxyfluor emulsion as an oxygenation agent has resulted in a 78 % increase in yield of IG<sub>1</sub>-type mAbs.

More recent studies on the application of liquid PFCs in animal cell cultures were focused on cultures of 3-D aggregated anchorage-dependent (adherent) mammalian and human cells on a of liquid PFC in hybrid liquid-liquid (PFC-medium) systems. *In vitro* development of 3-D aggregates of cells which typically grow in monolayers (due to growth-inhibition confluence effect) when they are cultured directly on a layer of pure (i.e. non-emulsified and non-dispersed) liquid PFC, has been originally reported by Shiba et al. (1998) and further elaborated by Brzezińska et al. (2012), Pilarek and Grabowska (2012) as well as Pilarek et al. (2013b). Finally, liquid-liquid system has been studied as a new way of a cartilage implant development (Pilarek et al., 2014) and it has been proven that the system is suitable for inoculation and further enhanced growth of CP-5 chondrocytes on biodegradable, fibrous poly-lactide (PLA) scaffolds placed on the interfacial area between immiscible layers of PFD and the medium. From the bioprocess engineering point of view, such a hybrid system simultaneously enables the adhesion of adherent cells to fibers of PLA-based scaffolds, as well as, due to a PFC phase, enhances the mass transfer in the case of supplying/removing of respiratory gases, i.e., O<sub>2</sub> and CO<sub>2</sub>. Another way for liquid PFC-mediated mass transfer intensification in biodegradable scaffolds has been proposed by Douglas et al. (2014). Oxygenated PFD has been successfully applied in the form of dispersed droplets to thermosensitive injectable scaffolds based on chitosan hydrogels in order to



improve human adipose-tissue derived stem cells growth. The incorporation of oxygenated PFD improved the suitability of studied hydrogels as bone regeneration materials.

## **6.2. PFC-mediated transport of other gases**

In the field of water-immiscible liquid gas carriers applied in culture systems, removal of carbon dioxide from broth/medium via liquid PFCs has been much less investigated (e.g. by Percheron et al. (1995) in the case of bacteria culture and by Sykłowska-Baranek et al. (2014) in the case of plant cells) than the problem of oxygen supply. Nevertheless, accumulation of CO<sub>2</sub> which is gas continuously generated by cells and major waste product in culture media may cause detrimental effects in the cells, causing intracellular enzyme inhibition leading to death and further lysis of the cells. Because carbon dioxide is also well soluble in liquid PFCs, one can take advantage of the reversibility of the mechanisms of oxygen-supply and CO<sub>2</sub>-extraction from the culture medium/broth in bioprocesses supported with liquid PFCs. The phenomenon is currently successfully adopted in biomedical applications of liquid PFCs, such as liquid ventilation and temporary blood substitutes. In the case of supply of non-respiratory gases (i.e. gases other than O<sub>2</sub> and CO<sub>2</sub>), liquid PFCs have been applied as water-immiscible carriers of nitrogen, nitrous oxide or ethene (see Table 4). The way of enhancing ethene transfer is important particularly in the development of plant cell cultures because such gaseous compound is one of the biologically active signal molecules which regulate metabolism and growth of plant cells. Ethene has very poor solubility in water and liquid PFC-mediated supply of this compound has great potential of applicability in plant cell cultures. Up to date the effects of ethene-saturated PFD application have been studied only in the case of submerged cultures of *Arneubia euchroma* cells (Syklowska-Baranek et al., 2014) in which 26 x higher value of the ratio between alkanin/shikonin, i.e. extracellularly secreted naphthoquinone-based plant secondary metabolites, has been achieved in comparison to cultures without PFD.

## **7. SUMMARY AND OUTLOOK**

During the past thirty years many studies have shown that various liquid PFCs can be successfully applied as carriers of different kinds of gases (mainly O<sub>2</sub>, but also CO<sub>2</sub>, N<sub>2</sub>O and ethene) to supply them into culture media or as scavengers of gaseous cellular by-products. Data published up to date clearly revealed that the application of a perfluorinated gas carrier can facilitate mass transport in different types and scales of microbial, plant cell and animal cell culture systems. One of the main reasons for this is an increased value of the driving force of the process due to the relatively higher values of gas solubility (e.g. in the case of dissolved oxygen) in perfluorinated compound added to culture system than in aqueous medium. The second reason is the presence of a higher interfacial area in the case of liquid PFCs applied in dispersed forms, which leads to higher  $k_La$  values. On balance, the above findings indicate that liquid PFCs can be recognised as very flexible bioengineering tools for gas transfer intensification in bioprocess engineering.

However, no liquid PFC-based mass transfer intensification method has been implemented in a real scaled-up industry-scale bioprocess installation so far. One of the main reasons why liquid PFCs are still rather rarely applied in the field of bioprocess engineering is the relatively high cost of perfluorinated compounds, which makes their use in large-scale bioprocesses uneconomical. Nevertheless, liquid PFCs could be successfully used in scaled-down miniature-scale cell culture systems to prevent oxygen limitation during grow to high cell densities. In miniaturised-scale culture formats, the costs of perfluorinated compounds are insignificant due to their small volumes. It may be argued that liquid PFC-mediated gas transfer would also offer benefits in the automation of bioprocesses and in microbioreactor cultures of various kinds of cells, especially in high throughput

screening approaches in which the prevention of cells from decrease of dissolved oxygen concentration as well as increase of dissolved carbon dioxide concentration are still unsolved problems.

## SYMBOLS

$c_i$	molar concentration at interfacial area, mol dm <sup>-3</sup>
$H$	Henry's Law constant, kPa m <sup>3</sup> mol <sup>-1</sup>
$k_{l,a}$	volumetric liquid-side mass transfer coefficient, s <sup>-1</sup>
$k_{p,a}$	volumetric gas-side mass transfer coefficient, mol m <sup>-3</sup> s <sup>-1</sup> kPa <sup>-1</sup>
$L_{2,1}$	Ostwald coefficient, -
$M$	molar concentration, mol dm <sup>-3</sup>
$O_{2c.c.}$	oxygen carrying capacity, cm <sup>3</sup> O <sub>2</sub> dm <sup>-3</sup> kPa <sup>-1</sup>
$p$	pressure, kPa
$p_i$	partial pressure at interfacial area, kPa
$T$	temperature, K

*Abbreviations perfluorochemicals*

perflubron <sup>®</sup>	1-bromo-perfluorooctane
PFC	perfluorochemical (synonym: perfluorocarbon)
PFD	perfluorodecalin
PFDB	1-bromo-perfluorodecane
PFDCO	perfluorodichlorooctane
PFMCP	perfluoromethylcyclohexylpiperidin
PFMD	perfluoromethylodecalin
PFTBA	perfluorotributylamine
PFTPA	perfluorotripropylamine
PLA	polylactide
PTFE	poly(tetrafluoroethylene) (Teflon <sup>®</sup> )
TBPFCH	tributylperfluorocyclohexane

*cell lines*

#824	mouse-derived hybridoma cells
3C11	mouse-derived hybridoma cells
4C10B6	mouse-derived hybridoma cells
A431	human epidermoid carcinoma cells
ADSC	adipose tissue-derived stem cells
BHK-21	Syrian hamster kidney normal cells
C2C12	mouse myoblasts
CP-5	bovine articular cartilage progenitor cells
HeLa	human epithelial cervix adenocarcinoma cells
Hep G2	human hepatocellular carcinoma cells
L-929	mouse subcutaneous connective tissue fibroblasts
NIH-3T3	mouse embryogenic fibroblasts
Sf9	<i>Spodoptera frugiperda</i> ovarian cells

*other*

DWP	deep well plate
EYP	egg yolk phospholipid
Hb	haemoglobin
HFE	hydrofluoroether
mAb	monoclonal antibody

Mb myoglobin  
n/a not available

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