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Enhancing oxygen solubility using hemoglobin- and perfluorocarbon-based carriers

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Table 1. Oxygen requirements of some cell types

Cell type	O_2 requirements	References
Hybridoma cells	0.2 mM/L/Hr[1]	195
Human foreskin fibroblasts	>10 mm Hg	196
Rat retinal cells	10-15 mmHg	197, 198
Rat myocytes from hearts	2-50 mmHg	199
Stromal osteoblasts	Within 150-200 μm from O_2 source	14
Hepatocytes	Within 240 μm from O_2 source	200

[1]Henry's law conversion: $p = \kappa c$, where ***p*** is the partial pressure of the solute above the solution, **κ** is the Henry's law constant. For O_2 dissolved in water at 25°C, **κ** is 769.2 L*atm*mol^{-1}, and **c** is the concentration of the solute in the solution.

1. ABSTRACT

The objective of this paper is to review the properties of fluids capable to enhance oxygen transport and transfer and recent developments in this field. This paper reviews and compares two different approaches, which could be used to solve the important problem of oxygen supply in some cell and tissue cultures: 1) hemoglobin-based and 2) perfluorocarbon-based (PFC) oxygen carriers. Several types of modified hemoglobin-based oxygen carriers (HBOCs) and their preparation are reviewed, as well as advances in the field of PFC oxygen carriers. Finally, we highlight the advantages and pitfalls, when reported in the literature, associated with each technique.

2. INTRODUCTION

The use of oxygen carriers has been identified as a possible solution in blood replacement therapy (1-5). Although not well considered in the literature, oxygen carriers can also be seen as a solution to supply oxygen (and even to remove CO_2, when needed) in high-density cell culture systems (6,7). In fact, oxygen is required by many organisms. When mammalian cells are used in technical processes, the supply of oxygen is often a limiting factor (8). Fermentation processes are good examples in which oxygen availability is often a limiting step in achieving high-cell density while limiting cell damage by mechanical means. For example, oxygen supply can be modified by increasing the flow rate of the medium. However, such a method would give a very dilute solution of the desired soluble products, which is undesirable in down-stream processing. Problems with oxygen supply are especially pronounced when high-cell densities are used, such as in immobilized preparations.

This paper aims to review properties of oxygen carriers as part of a larger project leaded by our group in which we wish to examine whether oxygen carriers can be used to support oxygen supply in the culture of large tissue mass. In growing large tissue mass, such as in tissue growth, one must be able to provide sufficient oxygen, which is required for cell growth or tissue formation. Such an optimal oxygen delivery should facilitate tissue growth. The lack of sufficient (i.e., optimal) oxygen leads inevitably to the necrosis of the tissue.

Both for nutrient needs and waste elimination, mass transfer to and from tissues is a critical issue in many culture systems (9). *In vivo*, cells beneficiate from the proximity of blood capillaries for their mass transfer requirements; in most tissues, cells are located no more than 100 μm from these capillaries (10). Also, the small diameter of capillaries (between 6-8 μm) ensures a residence time long enough in tissues to permit the radial diffusion of chemical species (11). As tissues gain on size, cell proliferation increases the mass transfer requirements, limiting the final size of the grown tissues. For example, *in vitro*-grown chondrocyte-poly (glycolic acid) (PGA) constructs were reported to lose their diffusional permeability to up to 97% of its initial value after four weeks, proportionally to the quantity of extracellular matrix (ECM) deposited on the construct (12,13). For hepatocyte culture, it has been shown that when a reactor design relies solely on diffusion for the mass transfer of oxygen, cells must be within 150 to 200 μm of an oxygen source to survive and proliferate (14). One last example is a study reporting that the deposition of mineralized matrix by stromal osteoblasts cultured into poly (DL-lactic-co-glycolic acid) foams reached a maximum penetration depth of 240 μm from the top surface of the foam (15).

Oxygen is one of the most important nutrients for cells, being a major actor in all aerobic metabolic cycles. See Table 1 for a review of the oxygen requirements of some cell lines and cell types. However, it is often the limiting nutrient in successful tissue growth *in vitro*. The reason for this arises from the difficulty of bringing sufficient oxygen concentration to the surface of the cells mainly because of the poor solubility of oxygen in culture media. The oxygen solubility in a typical culture medium is limited to ca. 0.2 mmolO_2/L when atmospheric oxygen is used, twice its solubility in pure water (16). Oxygen is typically consumed approximately at the same rate as glucose (on a molar basis), but oxygen solubility is lower than the availability of glucose (e.g., 20 mM). As a result, medium must be continually circulated and re-oxygenated by passing through an in-line gas-exchanger. Moreover, an excess of oxygen in the medium surrounding the cells without an appropriate carrier such as hemoglobin, achieved by using pure oxygen instead of air or increasing gas pressure, induces the production of free radicals, which are cytotoxic (17). Indeed, hypo- and hyperoxic stress have been implicated as causes of programmed cell death or apoptosis, which appears to be the main mode of cell death in many cultured cell lines (18). Low oxygen tension (~ 40 mmHg) and low pH (~ 6.7) were associated with anaerobic cartilage cell metabolism while higher oxygen tension (~ 80 mmHg) and higher pH (~ 7.0) were associated with more aerobic cell metabolism (19). More aerobic conditions resulted in larger constructs containing higher

amounts of cartilaginous tissue components, while anaerobic conditions suppressed chondrogenesis in 3-D tissues. Thus, in cell culture systems, oxygen concentrations are usually maintained at between 20-100% air saturation to maintain a certain balance between oxygen needs and tolerance to free radicals of the cells (17). The need for an oxygen feeding strategy to allow large tissue mass formation seems greatly required.

The importance of cell culture in a solid 3-D environment is clearly demonstrated by the increasing market size of new therapeutic products derived from mammalian cells. Therefore, one must consider two options:

1) Bringing the optimal oxygen concentration in gaseous phase to the growing tissue;
2) Modifying the environment so it has the ability to dissolve more gas.

In the first option, one must consider the fact that the liquid environment in which mammalian cells are normally grown has a very low solubility for most gases, resulting in extremely low oxygen concentration available to the cells. In addition, oxygen has the possibility to reach and nourish the cells located around the core of cell aggregates, but leave the ones in the center to die. The challenge is therefore to target these cells in the center of the aggregates and allow their optimal growth and differentiation by delivering and transferring, closely and effectively sufficient oxygen molecules. Also, direct exposure of cells to a gaseous phase can be problematic because it has been shown that fragile cells can be damaged by shear stress caused upon contact of these cells with gas bubbles (20-22).

The other idea brought forward to improve cell and tissue growth is to modify the environment in which the cells are cultured. This could be achieved by adding nano- and micro-droplets made of perfluorochemicals to the media to enhance the O_2 delivery to the growing cells. These compounds, as it will be discussed in this review, have the properties to dissolve large concentration of oxygen.

This review encloses a two-section bibliographic study in which we review the work and progress that has been done in the last 40 years in the field of oxygen carriers. The first section will deal with a molecule instinctively capable of carrying oxygen in nature i.e., the hemoglobin protein. In the same line of thought, the second section will examine the outputs and accomplishments done in the field of perfluorocarbons (PFCs), in which synthetic substances are added to an aqueous medium to modify and increase its capacity to dissolve oxygen, which could be used in cell and tissue cultures.

3. THE HEMOGLOBIN PROTEIN

Hemoglobin (Hb) is a tetrameric spheroid protein of 64 kDa composed of four sub-units: each sub-unit is a combination of a polypeptide chain and a heme moiety. Each heme contains an iron ion Fe (II) at its center that can combine reversibly with one O_2 molecule. It is responsible for the red coloring of blood and is found, trapped in the red blood cells. Its essential utility stems from its ability to unite in loose combination with atmospheric oxygen to form oxyhemoglobin (HbO_2). Hemoglobin accounts for approximately 97% of the dry content of a red blood cell.

3.1. Porphyrin structure

The heme moiety is an iron porphyrin i.e., the union of an iron with four pyrrole groups. It is the functional unit or active site to which oxygen can bind. The porphyrin structure is planar, although it is significantly ruffled and can be deformed, to some extent, normal to the main plane. It has the ability to resist to radial expansion and contraction. The iron atom occupies the central position of the porphyrin ring and is coordinated to the four pyrrole nitrogens. The two other positions, perpendicular to the porphyrin plane, are available for binding of other groups to form octahedral complexes.

The central hole of the porphyrin has a radius of approximately 2 Å. Low-spin iron can fit into this space and is therefore strictly in plane with the porphyrin ring. High-spin iron, though, is too large to be accommodated into the central hole and is therefore out of plane of the four pyrrole nitrogens by approximately 0.5 Å (23). The iron binding group is the imidazole of a histidine residue, F8 (proximal histidine). Another histidine is present on the other side of the heme plane, E7 (distal histidine).

Structural changes occurring on the ligand binding at the C-terminal ends are regarded as of primary importance for the mechanism of the interaction effects in hemoglobin (24). The deoxygenated structure, stabilized by six salt bridges, would correspond to the “T” (for tensed) or low-affinity for oxygen form in the allosteric models, the oxy structure to the “R” (for relaxed) or high-affinity for oxygen form.

3.2. Oxygen affinity

Purified Hb (i.e., free) has a greater oxygen affinity than hemoglobin entrapped in red blood cells (25). This phenomenon suggests the presence of a molecule complexed with Hb in red blood cells that reduces its affinity for O_2: D-2,3-diphosphoglycerate or 2,3-DPG. 2,3-DPG bounds strongly with deoxygenated Hb and loosely with oxyHb. The presence of this molecule reduces the oxygen affinity of Hb by keeping it in the deoxy conformation. It is important to note that purified hemoglobin stripped from its erythrocyte environment, for further use in an oxygen carrying system, will no longer have its affinity be governed by 2,3-DPG (unless this molecule is added in the carrier formulation).

As discussed in Section 3.1, hemoglobin exists in two quaternary forms: oxy (or R) and deoxy (or T). When hemoglobin presents itself in the oxy form, it is linked to the oxygen molecule, while in the deoxy form, the position once occupied by the O_2 molecule is now empty. The T structure has a relatively low affinity for heme ligands such as oxygen and carbon monoxide. Environmental factors

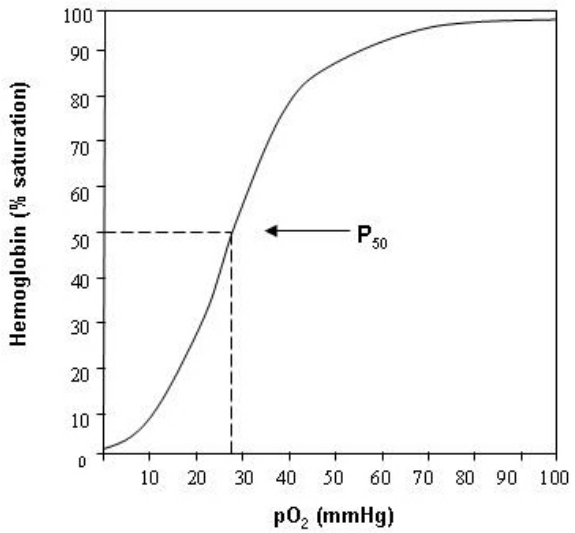

Figure 1. Oxyhemoglobin dissociation curve.

such as protons and organic phosphates, which stabilize the T structure, will lower oxygen affinity. Conversely, many chemical and genetic modifications of Hb impair its ability to assume a stable deoxy conformation. As a result, these modified hemoglobins often have increased oxygen affinity.

3.3. Hill coefficient and P_{50}

The ability of Hb to shift between conformations is reflected by the Hill coefficient (*n*). Archibald Hill was the first to attempt to formulate the dissociation curves of oxyHb (25). This analysis is used to characterize not only the cooperative conduct of hemoglobin, but also other oligomeric enzymes behaving in the same way. Hill suggested the use of a similar approach to that of Michaelis-Menten;

Suppose a hemoglobin protein, Hb, constituted of *n* subunits that can each bind to an oxygen (O_2) molecule. Suppose that O_2 binds to the hemoglobin protein with an infinite cooperativity, yielding:

$$Hb + nO_2 \rightleftharpoons Hb(O_2)_n \quad (1)$$

where all or none of the binding sites of the protein Hb are occupied by an O_2 molecule, and no intermediates are present. The equilibrium dissociation constant of the reaction is then

$$K = \frac{[Hb]\cdot[O_2]^n}{[Hb(O_2)_n]} \quad (2)$$

Its partial saturation is expressed by:

$$Y_{O_2} = \frac{n\,[HbO_2]}{n([Hb] + [HbO_2])} \quad (3)$$

Where Y_{O2} is the partial saturation of hemoglobin expressed as the fraction of the binding sites of oxygen occupied by O_2 molecules. By combining and rearranging equations 2 and 3, it becomes the Hill equation:

$$Y_{O_2} = \frac{[O_2]^n}{K + [O_2]^n} \quad (4)$$

which expresses the degree or percentage of saturation of an oligomeric protein in function of the O_2 concentration.

An infinite cooperative binding of the oxygen molecule (*n* equal to the number of subunits), is physically impossible. Nevertheless, *n* can be considered as a parameter depending on the degree of cooperativity between the binding sites of O_2 in interaction, rather than the number of subunits of the hemoglobin protein. The Hill equation then becomes a useful empirical relation in accordance with Figure 1 rather than an indication on a particular model of oxygen binding mechanism.

The *n* value, known as the Hill coefficient, increases with the cooperativity degree of a reaction, which is a simplistic way to characterize a reaction of a binding ligand. If $n = 1$, the equation is said to be non-cooperative and the equation is that of a hyperbole. It indicates that Hb is locked in either the T or R conformation. A reaction where $n>1$ is said to be positively cooperative: the binding of the hemoglobin molecule increases the affinity of oxygen for further binding of other oxygen molecules. On the other hand, if $n < 1$, the reaction is said to be negatively cooperative: the binding of oxygen molecules decreases the affinity of hemoglobin for additional oxygen molecules. In the case of normal human hemoglobin and oxygen, the Hill coefficient is between 2.8 and 3.0 (25).

The affinity of hemoglobin for oxygen can also be conveniently expressed by the oxygen tension at which hemoglobin is half saturated, P_{50} (Figure 1). The higher the affinity of hemoglobin for oxygen, the lower the P_{50}, and vice versa. The P_{50} for human blood at physiological pH (7.4) and temperature (37°C) is approximately 27 mmHg (25). However, a number of genetic and environmental factors can alter the oxygen affinity of human blood. The variability in P_{50} is due in part to differences in the intracellular milieu. The oxygen affinity of Hb can be markedly influenced by alterations in solvent conditions: it can vary over a 100-fold range owing to changes in pH, ionic strength, and, in particular, organic phosphates. For example, oxygen affinity is inversely related to temperature.

3.4. CO_2 transport

In the bloodstream, CO_2 is transported in 3 forms: 1) in the form of carbonate ions, 2) dissolved in the plasma and in red blood cells, and 3) as $HbCO_2$. Since no clear and detailed information is available on how CO_2 is transported in a modified HBOC, one can speculate that, like it is the case in human blood, a large part of the carbon dioxide is

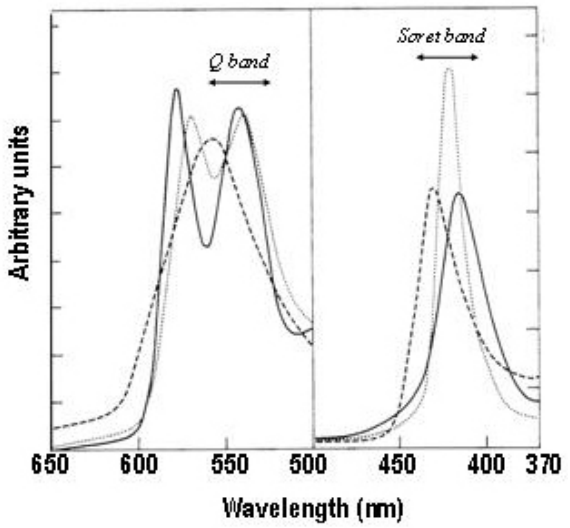

Figure 2. Absorption spectra of the oxy (___), deoxy (---), and carbonmonoxy (…) forms of human hemoglobin.

transformed in HCO_3^- ions. The formation of carbonate ions is catalyzed by the presence of carbonic anhydrase which is found in the red blood cells. This enzyme, which speeds up the ion formation process, is denaturated following the lysis of red blood cells and the subsequent purification/heating process. One can also speculate that part of the carbon dioxide is transported in the form of $HbCO_2$. Indeed, the CO_2 molecule is able to bind to the multiple amine groups present on the hemoglobin protein, allowing CO_2 transport in the modified HBOC (25,26).

The formation of $HbCO_2$ is highly influenced by the pCO_2. In tissue culture, oxygen carrier could be used to remove CO_2 produced by cell metabolism. Depending on pCO_2 and pO_2, the hemoglobin molecule can load or unload the gas molecule (25,27).

A delicate balance in CO_2 concentration is needed. Often in cell and tissue cultures, carbonate-based buffers are used (17,28). Therefore, in many mammalian cell culture processes, CO_2 is needed to maintain pH at a given value. However, it is possible that some CO_2 would need to be removed from large tissue mass aggregates. But, the necessity and/or the capacity of removing CO_2 using Hb-based carriers need to be tested for cell and tissue cultures. Often, in cell and tissue cultures, change in culture medium colour is used as an indication of a pH change – it is corrected by replenishing the culture medium. Although this strategy has been successfully used in standard cell culture systems, it would be economically less viable in large-scale tissue cultures owing to the high cost of many culture media, which often necessitate the addition of fetal bovine serum (FBS).

4. HEMOGLOBIN DERIVATIVES

The physiologically relevant derivatives of hemoglobin are the deoxygenated and oxygenated forms. However, other derivatives can exist *in vivo*, and some of them, such as the carbon monoxide (CO) or the ferric forms, are of importance and must be taken in account in the conception of oxygen carriers using Hb.

The hemoglobin visible absorption spectrum consists in 2 specific regions known as the Soret band (around 430 nm) and the Q band (around 550 nm) (Figure 2). These bands are due to the electronic cloud of the porphyrin that emits energy when excited at a certain wavelength in the visible light. It should be kept in mind that small differences in absorption maxima and extinction coefficients may be found with hemoglobins of different animal species (29,30). One should also note that the nature of the anticoagulant has no effect on the visible absorption spectrum.

4.1. Reduced hemoglobin

Reduced hemoglobin, also known as deoxygenated hemoglobin, represents in many respects the derivative with unique properties relatively to the structure of the heme site as well as of the protein as a whole. It may be prepared by simply removing the ligand from the corresponding ferrous ligand-bound form. For example, starting from oxyhemoglobin, the deoxy derivative can be obtained by removing the O_2 from the gas phase by vacuum or by equilibration with nitrogen (N_2) or argon (Ar) (29). Alternatively, it can be obtained by the use of reducing agents, such as NADH, NADH-cytochrome *c* reductase, methylene blue, dithionite and Na_2SO_4 (29,31). This last option should be employed with some precautions owing to possible undesirable side effects caused by the formation of bi-products. Solutions of deoxyHb are typically red violet in color. DeoxyHb absorption spectrum is characterized by one asymmetrical absorption band centered at 555 nm, and one band (typically at 430 nm) in the Soret region (Figure 2). The deoxygenated derivative of hemoglobin is very stable, and can be stored in the cold for very long times.

4.2. Oxyhemoglobin

Oxyhemoglobin or HbO_2 is the form obtained by classical preparation procedures. It can also be obtained from the deoxy form (ferrous derivative) by exposure to O_2 after reduction of the metal to the ferrous form. For example, oxy-Hb can be obtained from ferrous Hb by treatment with an excess of hydrogen peroxide (H_2O_2) (32).

Oxyhemoglobin has a characteristic bright-red color, typical of arterial blood. It is characterized by two absorption bands in the visible spectral region (at 541 and 577 nm) and one band in the Soret region at 415 nm (Figure 2). The spectra of deoxyHb and oxyHb are pH and temperature dependent.

OxyHb is not infinitely stable and slowly transforms spontaneously into metHb. Autoxidation of oxyHb is greatly accelerated under some conditions; it increases at higher temperatures, at acidic pH and in the presence of salts or metal atoms. In addition, it proceeds more rapidly when the solution is partially deoxygenated (33).

4.3. Carboxyhemoglobin

The derivative of ferrous Hb with carbon monoxide, HbCO, may be easily obtained by exposure of

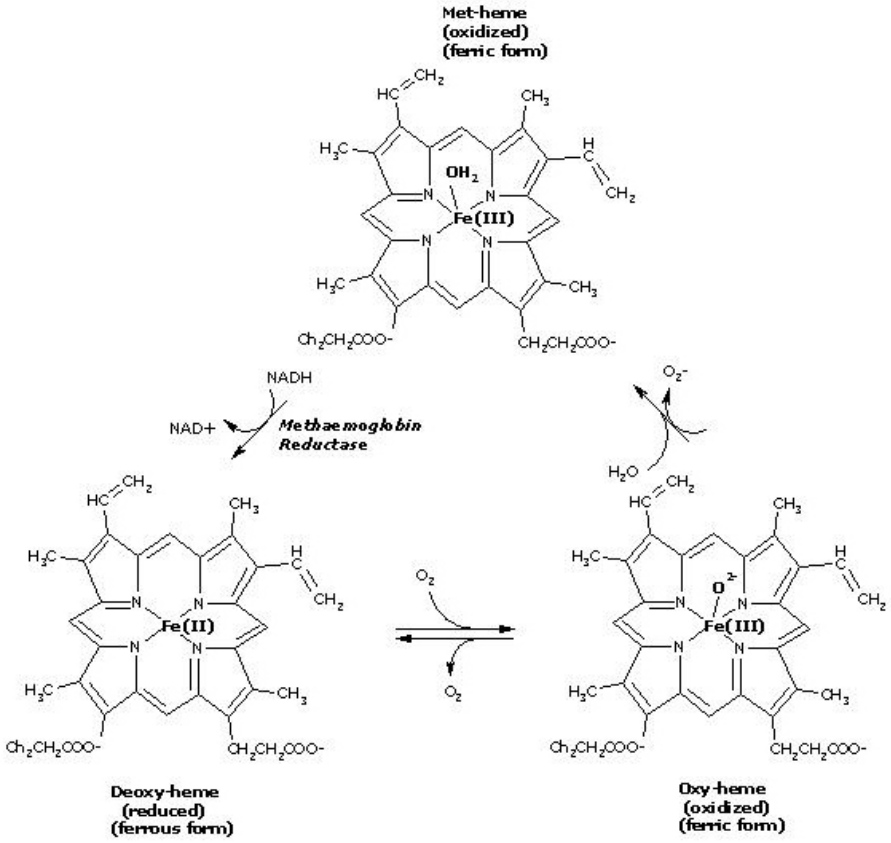

Figure 3. Relationship between the different relevant physiological Hb derivatives.

oxyHb to CO. This ligand easily displaces O_2 due to the difference in affinity which favours carbon monoxide by a factor of approximately 250 (29). For practical work, the CO derivative may be obtained by bubbling coal gas into a solution of oxyHb or ferric Hb, and then adding a few crumbs of dithionite to eliminate free O_2 and reduce the iron atom.

Carboxyhemoglobin is typically pink. In relation to the HbO_2 bands, the absorption bands of HbCO are slightly shifted toward the blue end of the spectrum and are characterized by two bands at 540 and 569 nm in the visible region as well as one band in the Soret region at 419 nm (Figure 2).

4.4. Methemoglobin

Methemoglobin or metHb is the oxidation product of the reaction of potassium ferricyanide with Hb, the divalent iron being oxidized to the trivalent state. In natural erythrocytes or red blood cells (RBCs), hemoglobin is gradually converted to methemoglobin (metHb) by oxidation, and in turn, the methemoglobin is reduced by the action of enzymes in the RBCs, and Hb thereby undergoes a reversible hemoglobin-methemoglobin reaction (Figure 3).

The common problem with Hb-based red cell substitutes is the relatively rapid metHb formation during circulation (34,35) due to the absence of metHb reduction systems originally present in a red blood cell. The reduction systems include NADH-cytochrome *b*5, NADPH-flavin, a direct reduction by glutathione (GSH) and ascorbic acid, and scavengers of active oxygen species such as superoxide dismutase (SOD) for O_2^- and catalase for H_2O_2. Hb in a ferrous state will be autoxidized to the ferric state (metHb) and loses its oxygen binding ability. It is well-known that HbO_2 dissociates into metHb and O_2^- (36-40); however the percentage of metHb in RBC is maintained at less than 0.5 % of the total Hb by systematic reduction (41).

The Hb taken out of the natural erythrocyte is devoided of such metHb reduction system, and the hemoglobin once converted to methemoglobin is not reduced back and thereby loses its oxygen-transportation capacity. Hb autoxidation generates superoxide radicals and hydrogen peroxide. They not only accelerate Hb oxidation but also induce toxicological effects (42).

4.4.1. Limiting methemoglobin formation

Preservation of the activity of methemoglobin reductase in red blood cells has been reported as a method to limit the metHb formation in Hb vesicles (43). However, the enzyme activities would change with the conditions of the outdated red blood cells and the concentration of remaining substances. Virus inactivation using heat treatments is impossible in this case. Moreover, the

mechanism of metHb reduction is complicated and influenced by pH, temperature and many unknown factors.

Reductants can be used to reduce the reaction of metHb to deoxyHb. One example, which limits without completely eliminating metHb formation for acellular modified Hb solutions, is the direct conjugation of superoxide dismutase and catalase to the modified Hb (42). However, lowering of active oxygens by this procedure is not sufficient to maintain the concentration of metHb to an acceptable level. Methemoglobin reduction can also be done by the incorporation of thiol compounds. The thiol group dissociates to a thiolate anion, transferring one electron to metHb therefore resulting in the production of deoxyHb and disulfide. Thiols used for this purpose are L-cysteine (Cys), DL-homocysteine, glutathione (GSH) and *N*-acetyl-L-cysteine (Hcy), which have different dissociation degrees (42). It is generally expected that the thiol with the higher dissociation degree leads to the higher reactivity. However, it is difficult to obtain the dissociation constant of the thiol group, because pH titration is accompanied by the deprotonation of the ammonium group of the thiol (44).

In the absence of a proper reduction system, reductants can also enhance the metHb formation. Indeed, encapsulated reductants generally autooxidize faster than the rate of metHb formation. Furthermore, active oxygens generated from autoxidation of the reductant oxidize the Hb to metHb (45,46). Therefore, a relatively high stability under oxygenated conditions is desired for reductants. Faivre *et al.* (47) reported the co-injection of reductants such as ascorbic acid and methylene blue into modified Hb solutions. These small molecules appeared to be very unstable. GSH, a thiol molecule, has been used to reduce metHb non-enzymatically (12 mM is present in a RBC). Sakai *et al.* (48) have shown that metHb formation was effectively suppressed by the addition of homocysteine (Hcy) or GSH. Hcy has hitherto showed the best suppressive effect as a reductant owing to its relatively slow oxidation rate and high metHb reduction rate (49).

Another interesting way to prevent dissociation of hemoglobin to methemoglobin is to bind carbon monoxide (CO) to the protein to stabilize the in-plane heme moiety. According to Carlson *et al.* (50), in its singlet ground state, the iron ion from the heme moiety is out-of-plane approximately 0.5 Å and O_2 and CO are absent from the hemoglobin molecule. As CO is bonded with the iron through a coordinated-type bound by the carbon atom, it is moved back into the plane. This movement from in-plane to out-of-plane configurations is attributed to the larger size of the iron atom in the high-spin state and the limited space in the rigid porphyrin frame (51). The stabilized state of the hemoglobin molecule bonded to CO enables an efficient manipulation in subsequent manipulations. For example, to purify hemoglobin, Sakai *et al.* used the CO-stabilized Hb to denaturate other proteins by heating the Hb-containing mixtures to 60°C for 10 hours (52). When the HbCO molecule is excited by specific wavelengths, in this case at 470 and 490 nm, the *d*-orbital electrons of the iron reveals a transition into the π^* orbital of the CO. The unfavourable antibonding combination obtained is therefore responsible for the ejection of the CO from the iron ion (50) enabling O_2 to bind to the heme group.

5. HEMOGLOBIN-BASED OXYGEN CARRIERS (HBOC)

Hemoglobin is a biologically highly active molecule, and its presence as a "free" protein is associated with a number of possible undesirable effects such as the dimerization of the molecule leading to a non-functional oxygen-carrying protein. The hemoglobin proteins can therefore be chemically modified and/or encapsulated to limit these potential problems.

In this section, we review different forms of hemoglobin-based oxygen carriers (HBOCs) and, briefly, how these carriers are prepared. We also highlight advantages and pitfalls (when reported in the literature) associated with them.

5.1. Cross-linking of hemoglobin

Cross-linking involves intramolecular binding between the subunits inside the hemoglobin molecule to stabilize its structure. One goal of cross-linking Hb is to optimize its oxygen binding and tissue delivery capacities. Cross-linking agents attempt to stabilize the Hb molecule in the T state (deoxy form). Cross-linking of Hb alters its binding affinity and subsequently Hb ability to deliver oxygen to tissues (53).

There are many possible sites at which cross-linking agents can react on the Hb tetramer (54), although there are essentially 2 sites where cross-linking has been reported to occur. The first cross-linking site is between the beta globin chain lysine 82 residues resulting in beta-beta cross-linked Hb. The second cross-linking site is found to be between the alpha-globin chains at the lysine 99 residues and results in alpha-alpha cross-linked Hb. The cross-linking agents most often reported in the literature include: **salicyl derivatives** ((bis-3,5-dibromosalicyl) fumarate, sebecate, succinate and glutarate) and **pyridoxyphosphate derivatives** (bis-pyridoxyl tetraphosphate (bis-PL P4) and 2-nor-2-formylpyridoxal 5'-phosphate (NFPLP)). Many other cross-linking agents have been reported. Chemicals such as *o*-raffinose and glutaraldehyde are also effective cross-linking agents (53), but they also seem to contribute to polymerize Hb and will therefore be addressed in Section 5.2.

5.1.1. Salicyl derivatives

Salicyl derivatives are cross-linking agents, which bind in the 2,3-DPG binding area of oxy-Hb and at the alpha-1 lysine 99-alpha-2 residues of deoxy Hb (55,56). The most prominent of the salicyl derivatives used is (bis-3,5-dibromosalicyl) fumarate, which links Hb alpha-alpha subunits at the amino groups of the lysine 99 residues in the presence of tripolyphosphate. The tight structure of fumarate cross-linked Hb seems to produce an overall more stable molecule by reducing the flexibility of the protein structure, therefore making it the most commonly used cross-linking agent (53). The resulting product is Hb with a

4-carbon fumaryl bridges between the alpha-alpha subunits and is known as DCLHb or diaspirin cross-linked hemoglobin. DCLHb is commercialized under the HemAssist® trademark (Baxter Healthcare, Deerfield, IL, www.baxter.com). The resulting protein has a reported molecular weight of 64 kDa and its P_{50} and Hill number are 32 mmHg and 2.6-2.8, respectively (57,58).

Other salicyl derivatives can be used but as they are not commonly reported; we will simply mention some of them. (Bis-3,5-dibromosalicyl)sebacate can be used to form a 10-carbon bridge, linking the two beta chains of oxy-Hb at the 2,3-DPG binding site (59). Huang and Olsen also attempted to increase the thermal stability and to alter oxygen binding by linking Hb chains with the succinate and glutarate forms of bis-3,5-dibromosalicylic acid (60).

5.1.2. Pyridoxal phosphate derivatives

Pyridoxal phosphate derivatives are reported to be used to mimic the effects of 2,3-DPG (53). It is important to emphasize that pyridoxal phosphate alone cannot play the role of 2,3-DPG. It must be modified with additional phosphate groups. Pyridoxal phosphate derivatives cross-link the tetrameric Hb via beta-beta globin chain linkage (61). Several of the advantages of the pyridoxal derivatives as cross-linking agents were reported in Haney and Buehler's review (53): 1) the two-step process synthesis can be completed in three days, 2) it involves a very specific reaction unlike benzenecarboxylates which are pH dependent and give linkage at various amino acids on various globin chains (also the case for glutaraldehyde), 3) chemical entities can be linked to the pyridoxyl 5'-phosphates, and finally 4) oxygen affinity is reduced and O_2 unloading is improved resulting in potentially more efficient O_2 delivery.

2-Nor-2 formyl pyridoxyl 5'-phosphate (NFPLP) is reported as another commonly used pyridoxal phosphate derivative for Hb cross-linking (53). It is essentially pyridoxal 5'-phosphate with a formyl group at the pyridoxal rings 2 position. Pyridoxal phosphates are cross-linking agents used in 2 products currently in clinical trials. These products are manufactured under Polyheme® (pyridoxylated and glutaraldehyde polymerized HBOC) and PHP® (pyridoxylated hemoglobin polyoxyethylene). The reported P_{50} for these compounds reached 40-45 Torr (53).

5.2 Polymerisation of hemoglobin

The concept of Hb "polymerisation" (actually oligomerization) presupposes that hemoglobin molecules would be bonded together by chemical reagents. Although chemicals such as ***o*-raffinose** and **glutaraldehyde** have been reported as effective cross-linking agents, they can be used to polymerize Hb molecules. Clinical trials for certain polymerized hemoglobin are discontinued at this time due to safety issues; patients suffered from severe side effects, such as myocardial infarction (62).

5.2.1. Glutaraldehyde

Glutaraldehyde forms inter- and intra-molecular bonds resulting in an increase in the hemoglobin molecular weight and size. It is the most widely used reagent, even though many consider it too reactive and it lacks specificity and leads to uncontrollable cross-linking (63). Nevertheless, the site where the reaction usually seems to take place is the ε-amino group of the lysine residues and the N-terminal amino groups, where Schiff bases form. It is also possible for glutaraldehyde to cross-link hemoglobin not by Schiff base, but by aldol condensation between glutaraldehyde derivatives bonded to different hemoglobin molecules (64). Glutaraldehyde exerts non-specific binding: it can bind with any of the 44 lysine residues on the four terminal valines on the hemoglobin molecule. Furthermore, Marini *et al.* (65) and Hsia (66) reported that the intramolecular cross-linking mechanisms have not been well defined, due to glutaraldehyde ability to react with the imidazole group of histidine, the sulfhydryl group of cysteine, and the phenolic ring of tyrosine (65,67).

Glutaraldehyde polymerization must be preceded by pyridoxylation to decrease the P_{50}, although pyridoxylation can increase the concentration of methemoglobin (53). Also, cooperativity is often lost as a result of pyridoxylation (53). Many methods for the polymerization of hemoglobin exist, each one of them trying to optimize the degree of polymerization and the P_{50} and to minimize the methemoglobin levels. Some of these methods are at odds, and great care must be taken into account when using these techniques.

DeVenuto and Zegna's polymerization method of hemoglobin using glutaraldehyde following pyridoxylation was examined and determined to be successful by many groups (68). Glutaraldehyde polymerization and cross-linking were reported to be pH and temperature insensitive (68). In brief, Hb was pyridoxylated to increase the P_{50} to 24-29 mmHg. Excess glutaraldehyde was mixed with hemoglobin directly or by using dialysis. The reaction was typically quenched with excess lysine to prevent the formation of free aldehyde groups, although this procedure did not completely stabilize the molecule. It was reported by Koethe *et al.* that polymer stabilization was possible by the irreversible reduction of the Schiff bases with sodium borohydride (69); however, this chemical is highly toxic and great care should be taken to remove any residual reactives. Bleeker *et al.* reported toxicity after using sodium borohydride to stop the reaction (70). Moreover, Marini *et al.* (65) found that Hb polymerized with glutaraldehyde and reduced with $NaCNBH_3$ led to a heterogeneous product. Furthermore, this heterogeneous mixture was shown to be unstable. Lee *et al.* (71) examined the effects of glutaraldehyde polymerized HBOC and found that the formation of methemoglobin was significant, accounting for ca. 33% of the plasma hemoglobin following 24 hours.

5.2.2. O-raffinose

Two methods used *o*-raffinose as a cross-linking and polymerizing agent. The first one was reported by Hsia and uses non-specific binding, controlled by time and stoeichiometry (66). The reaction sites for this cross-linking process are reported to be the primary amino groups on the globin chains. The second method, patented by Hemosol Corp. (Hemosol, Mississauga, Canada, www.hemosol.com)

uses a method, involving time- and stoeichiometric-controlled reactions, for the binding of *o*-raffinose to the 2,3-DPG binding site, at the lysine-82 on the beta-chains and the terminal valine group from the beta-chain. This patented method uses Tris buffer, avoiding phosphate interactions with the 2,3-DPG binding (reaction) site (72). An improved method by the same group, uses sodium periodate to open up raffinose, yielding *o*-raffinose, a polyaldehyde. This reaction is not carried out in a Tris buffer like the previous. Reacting *o*-raffinose with Hb yields seven sugar moieties per Hb molecule. Forty percent of the structures obtained where tetrameric Hb units. The cross-linking can be stopped by reduction with similar agents as discussed in the previous section. Borane dimethylamine used in this method, enhances the controllability of the previous method. Polymerized and cross-linked *o*-raffinose Hb has a P_{50} of 32 mmHg and a Hill coefficient of 1.6 measured at 37°C in PBS buffer (pH 7.2) (72). Hemosol Corp. has abandoned its commercial activities related to HemoLink ® (62).

5.3. Conjugation of macromolecules to hemoglobin

Compared to polymerization in which several hemoglobins are linked together by a cross-linking agent, conjugation involves the binding of a macromolecule to Hb. As polymerization does, the conjugation of a macromolecule to Hb increases the molecular weight to a larger molecule, which is reported in the Hb literature to prolong its retention. PEG and dextran are the most widely used molecules, although conjugation of hydroxyethylstarch, inulin and albumin has been used, to a limited extent, to achieve conjugation of Hb. For this reason, only the first two molecules will be discussed.

5.3.1. Conjugation of dextran to hemoglobin

The dextran molecule, by itself, is a plasma expander that can restore blood volume, but does not have the ability to transport oxygen. Dextran has been conjugated to hemoglobin with the rationale to increase its molecular weight (53,73). Free Hb has the disadvantage of high renal excretion rate when injected as a blood substitute, thereby leading to a rapid clearance from the circulation. Therefore, covalent conjugation of polymers to Hb has been applied in order to prevent Hb excretion and to prolong its half-life (47,53).

The benzenecarboxylate derivatives mentioned earlier can serve as an example for the use of dextran in conjugation processes. Human hemoglobin is covalently bounded with benzene tetracarboxylate dextran (Dex-BTC-Hb) (74). It is reported that the effector polymer of benzene tetracarboxylate dextran stabilizes the tetrameric conformation of hemoglobin and lowers the P50 (74). Ten moles of dextran were bounded per mole of hemoglobin resulting in 300-kDa conjugates (75). It is interesting to note that because of the non-specificity of the binding process, the reaction yielded 85% of modified hemoglobin. Another down-side must be considered in using this system: mixtures containing dextran-conjugated hemoglobin are characterized by their high viscosities, which are higher than that of plasma (≥2,0 mPa/s) (76).

5.3.2. Conjugation of poly (ethylene glycol) (PEG) to hemoglobin

Unmodified hemoglobin has a circulation half-time of approximately 1 hour, while that of PEG-conjugated hemoglobin can be extended to more than 40 hours (77). Depending on the PEG used in the conjugation process, the post-modified hemoglobin exerts a larger molecular radius than the native hemoglobin (from ca. 3.2 nm for non-conjugated tetrameric hemoglobins to 14 nm for the subsequently modified hemoglobins) and has a great protein repellency capacity (77). The starting material for PEG-hemoglobin is unmodified hemoglobin derived from red cells (the source could be human, animal or recombinant). PEG polymers are synthetic and readily available materials that can be attached to the surface of hemoglobin using simple chemistries. Solutions made of PEG-conjugated Hb have a viscosity that is similar to that of blood (78). By varying the length and type of PEG used, one can try to change slightly the viscosity and size of the molecule. On the other hand, because the changes are not well understood, it is very hard for one to anticipate the results. Different types of PEG polymers can be attached, and different lengths of PEG polymers can be used to customize the final product (79).

But what are the mechanisms involved in the enhancement of the circulation time of PEG-conjugated Hb? In 1971, Napper and Netchey (80) published a study on the principle of steric stabilization of colloid particles by an adsorbed macromolecular or polymer layer on the surface. Later, in 1978, van Oss (81) showed that many pathogenic bacteria possess a surface that consists of a highly hydrophilic hydrated layer of protein, polysaccharide and glycoprotein, which reduces their interaction with blood components and might be involved in inhibiting phagocytosis. At about the same time, Abuchowski *et al.* (82) reported that covalent attachment of PEG to proteins gives conjugates that are less immunogenic and antigenic and have increased serum lifetimes. Since then, PEG coatings have been investigated for many biomedical applications in an attempt to reduce protein adsorption. From our recent review on PEG coatings and their mechanisms of protein repulsion (83), it is clear that PEGs are capable of reducing non-specific protein adsorption. So far, the protein resistance of PEG coatings has been associated with two main mechanisms. These are "steric repulsion" and/or "hydration/water structuring" (83). These two mechanisms are associated directly with the interactions between protein molecules and the PEG surface. However, we would like to point out to the reader that we do not believe that, based on the existing literature, it is possible to draw a clear, absolute picture of the protein resistance of PEG-covered materials.

5.4. Encapsulation of hemoglobin

Cross-linking and polymerization of Hb and conjugation of a macromolecule or a polymer to Hb can all affect its ability to transport and transfer oxygen. Also, although some authors like to establish a "clear" distinction between cross-linking and polymerization techniques, we

submit that some overlapping can exist as some chemicals used in these protocols have the potential to both polymerize and to cross-link Hb molecules. We believe that encapsulation of the protein, in an appropriate carrier, is a more "gentle" alternative than the techniques described in Sections 5.1, 5.2 and 5.3 because it tends to mimic more closely the *in vivo* situation.

Encapsulation of hemoglobins can be divided into three categories:

1. Liposome-encapsulated hemoglobin (LEH)
2. Hemoglobin encapsulated in PEG-bearing liposomes
3. Hemoglobin entrapped in biodegradable-polymer microcapsules

5.4.1. Liposome-encapsulated hemoglobin

The literature of LEH has increased rapidly in the past years, but there still are many obstacles to be further overcomed, such as the relative instability of the lipid bilayer upon storage, as well as the low encapsulation efficiency to name only a few.

Phospholipid vesicles encapsulating concentrated Hb (i.e., between 10-40 g dL^{-1}), also known as Hb-vesicles (HbV) or liposome-encapsulated Hb (LEH) are candidates for oxygen carriers, mimicking to some extent the red blood cell environment, having an excellent capability to concentrate atmospheric oxygen and to release it in response to a lower partial oxygen pressure (4,5,84).

Vesicles with a lipid bilayer membrane have been vigorously studied as a mimetic model of the biological membrane (85-87). These bilayer vesicles also have a high potential for application as carriers of bioactive macromolecules in the medical fields (86,88-91).

The first Hb vesicle was prepared by a film hydration and sonication method in 1980 by Djordjevich *et al.* (92). Several vesicle preparation methods such as reverse phase evaporation (93), detergent removal (94), dehydration and rehydration (95-97), microfluidization (98) or high-speed blending (99), and freeze-thawing (100) have since been used to encapsulate Hb tetramers within vesicles. These preparation methods often do not satisfy the precise size control, high encapsulation efficiency, and high-yield needed for a suitable oxygen carrier to be injected in living organisms. However, in *in vitro* cell and tissue culture conditions, these preparation methods should not be so easily discarded because particle size control would be less of an issue. For example, microfluidization does not seem to be useful for regulating the size of the vesicles. On the other hand, the extrusion method using polycarbonate membrane filters with a regulated pore size is applicable to prepare Hb vesicles having a 0.2-μm pore size - although the production and the encapsulation efficiency are often limited. Extrusion methods to produce liposomes containing proteins can limit the use of lipids with high temperature transitions such as DSPC (distearoyl phophatidylcholine), for which the transition temperature is 55 °C. Furthermore, all of the previously mentioned techniques cause some form of Hb oxidation and/or denaturation as well as rather high methemoglobin formation (1,101).

5.4.2. Hemoglobin encapsulated in PEG-bearing liposomes

Surface modification of phospholipid vesicles with some natural or synthetic glycolipids (90,102-104) or poly (ethylene) glycol (PEG)-conjugated lipids (105-107) is a well-known method to prolong the circulation time of the vesicles *in vivo* for drug delivery systems (105,108). PEG is a physiologically stable water-soluble polymer that prevents the access of plasma proteins by its steric hindrance; it has been extensively used to modify not only the surface of phospholipid vesicles but also those of many biomaterials (83,107,109). The PEG modification and physical properties of PEG-lipid/phospholipid mixed systems have been extensively studied (110-114).

The surface of HbV can be modified with PEG chains to improve the dispersion state of the vesicles in presence of other proteins (99). PEG-modified HbVs were reported to show improved blood circulation and tissue oxygenation due to the absence of HbV aggregation and viscosity increase and prolonged *in vivo* circulation time (105,108). Takeoka *et al.* reported that an ideal structure for a HbV is a large unilamellar vesicle with a ca. 200-nm diameter (115). Such a diameter would allow a relatively high-encapsulation efficiency and subsequent sterilization. The use of saturated phospholipids in the preparation process to decrease lipid peroxidation and metHb formation is reported by Yokohama *et al.* (116) and must be taken into account because of the pro-oxidant behaviour of Hb for vesicles composed of unsaturated phospholipids (117,118).

HbVs are a basic model of the double-layer membrane of red blood cells (RBCs). It is instinctively possible to correlate, to some extent, the oxygen uploading and unloading mechanisms of the HbV and red blood cells. Such mechanisms are governed by the P_{50}. Moreover, Takeoka *et al.* observed that oxygen transporting efficiency was optimized by co-encapsulating allosteric effectors such as pyridoxal 5'-phosphate (PLP) within the vesicles (44). In two different studies, Sakai *et al.* reported P_{50} of approximately 35 Torr for hemoglobin encapsulated in PEG-bearing liposomes (119,120).

5.4.3. Polymer-micro-encapsulated hemoglobin

Publications reporting the micro-encapsulation of Hb within biodegradable polymers (or red cell surrogates) are rather limited. Chang was the first to introduce the concept of an "artificial" cell and polymer encapsulation (121). He used the idea of forming a membrane of completely synthetic polymers. Such a structure would enclose all of the enzymes and products normally present in a red blood cell. Because of its "synthetic" nature, the polymer-microencapsulated hemoglobin may serve as an alternative for red blood cells.

Different types of synthetic polymers can be used and variations in configuration are also possible. The use of a single ultrathin polymer membrane is the most common

approach reported (122). Hb micro-encapsulated within polymer membranes like nylon, polystyrene, Silastic ® rubber, ethyl cellulose, poly (lactic) acid and dextran stearate (123) were tried and were shown to combine reversibly with oxygen (124). Many methods are available for the preparation of such artificial cells. Artificial cells of small dimensions are prepared by emulsification procedures (122). A fine emulsion of aqueous microdroplets is dispersed in an organic phase. The organic phase containing the fine aqueous microdroplets is then dispersed in the form of larger droplets in an aqueous phase. The final product is thus an aqueous suspension of organic phase droplets, each droplet containing finer aqueous microdroplets (122). However, the structure of this colloidal system would be extremely difficult to characterize given its complexity and its dynamics. Smaller artificial cells of nanoscopic dimensions are formed based on the same principles except that the initial emulsion is of much smaller dimensions. Larger artificial cells, especially those in the millimeter-range, are prepared based on modifications of the previously stated methods (4,121-123,125).

One of the main drawbacks for this approach would be the difficulty to obtain uniform droplet size (122). The preparative procedure also results in the formation of a much higher proportion of methemoglobin (125).

6. PERFLUOROCHEMICALS (PFC)

The literature relative to perfluorochemical-based oxygen-carriers is often confusing. Apparent contradictions and lack of fully disclosed information can be found. Some discrepancies in the literature may perhaps be attributable to a great number of filed patents and to the manufacturing of a potentially very lucrative "blood substitute" formulations from perfluorocarbon emulsions.

The features upon which perfluorocarbon-based blood substitute emulsions were originally, and continue to be promoted, are their capacity to dissolve large concentrations of oxygen, their ability to be produced in small particle sizes, and their characteristic low-viscosity suspensions (126-129). Perfluorocarbons, as oxygen carriers, were first proposed in 1966, when Clark and Gollan (130) demonstrated the capacity of perflurocarbon liquids to support life of rats submerged in PFC liquids. It is also interesting to note that, to our knowledge, (22) in 1982, Mattiasson and Adlercreutz were one of the firsts to report the use of perfluorochemicals to increase the oxygen supply to living cells (microorganisms). In this section, we will attempt to overview the characteristics and progress made around perfluorochemical emulsions as oxygen-carriers in general, not only as "blood substitutes".

6.1. General characteristics

Perfluorocarbons, or fluorocarbons, or perfluorochemicals (PFCs) are a class of chemicals essentially composed of carbons and fluorine. The fluorine atoms contribute to their high chemical and thermal stability. Normally, every organic compound has its perfluorinated analog, but in practice, the number of commercially available perfluorochemicals is limited. They are constituted primarily of perfluoroalkanes, halides, ethers, amines and acids. Mixed compounds composed of fluorocarbon-hydrocarbon are as well available. Also, a certain number of compounds having one fluorocarbon chain grafted onto an otherwise "normal" organic molecule are available; these compounds are reported to often display strong amphiphilic character (131).

Two essential features of the PFC are their unique gas-dissolving capacities and their exceptional chemical and biological "inertness". Their great gas-dissolving capacity is a consequence of the weakness of the intermolecular forces that prevail in liquid fluorocarbons, which facilitate the formation of "gaps" that can accommodate gas molecules within the liquid. On the other hand, their inertness reflects the strength of the intramolecular chemical bonds. The C-F bond is the strongest single bond (ca. 485 kJ mol^{-1}, compared to 425 kJ mol^{-1} for a standard C-H bond) (132,133) and its strength is further increased when several fluorine atoms are present on the same carbon atom. Also, the C-C backbone itself is strengthened by fluorine substitution. Fluorine atoms are large in size (with an estimated van der Waals radius of 147 pm) compared to hydrogen (120 pm) (134) and their high-electron density result in a compact electron shield, which provides effective protection of the molecular backbone. As well, when chlorine or bromine atoms are present, it is likely that the electronegativity of the fluorocarbon chain counterbalances that of the heavier halogen, which results in reduced bond polarity, polarizability and reactivity. For all these reasons, fluorocarbons are considered metabolically inert, however, this does not mean that they are not cytotoxic.

The perfluorocarbon liquids have a specific gravity of 1.7-2.0 gm/cm^3, a refractive index of approximately 1.3, and an interfacial tension with water of 52-56 dynes/cm (values provided by the suppliers; www.apolloscientific.co.uk, www.halocarbon.com).

Toxicity of PFCs has been assessed on dogs, rabbits and rats, essentially to test liquid-assisted ventilation, and its clearance route by the kidneys is rather well documented. Millard and McGoron administered 11g of perfluorooctyl bromide (PFOB) per kg body weight from a 90% w/v emulsion over 30 minutes on conscious beagle dogs. The experiment has shown no changes in pulmonary mechanics. Nothing was mentioned on the toxicity of the product on the animals (135). There has also been work suggesting that perfluorochemicals may have some positive effects on the growth of microorganisms (136-139).

What seems astonishing to us is that, to our knowledge, in the last 20 years, no convincing toxicity validation was performed on animals and cells (140). Our group recently published a paper in which we tested the effect of a perfluorocarbon emulsion on cultured human fibroblasts (141). We demonstrated a clear negative effect of the emulsions on the cells, even after short exposure (<6hrs). Transmission electron microscopy also evidenced the presence of *blebs* (i.e. outward bulge of membrane)

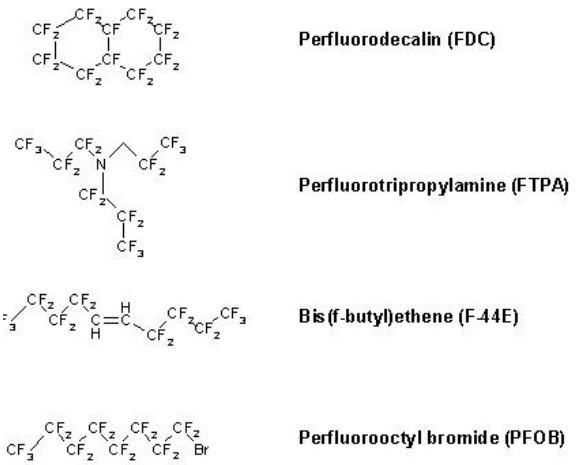

Figure 4. Structures of the most used or investigated fluorocarbons.

which seemed to attach on the cell surface. In our opinion, it is essential that cell toxicity should be addressed before animal testing.

6.2. Synthesis

The procedures used for preparing perfluorocarbons fall into two categories: 1) substitution of hydrogen atoms to fluorine on the hydrocarbon analog, or 2) combination of small, but already fluorinated building blocks. Specific routes for the synthesis of perfluorocarbons will not be extensively treated in this paper, but rather briefly mentioned. For more details, even if not much more is disclosed, the reader can refer to Riess and Le Blanc (142).

The substitution route includes electrochemical fluorination, fluorination by high-valency metal fluorides, and direct fluorination by elemental fluorine. Such methods enable the preparation of cyclic, polycyclic and branched compounds, of amines and of α,ω-di-substituted compounds (126). Substitution processes are reported to be difficult to control because of the energy released by the swapping of an hydrogen molecule for a fluorine one. If the reaction is not well controlled, it can easily lead to bond-breaking, elimination of the hydrogen fluoride, isomerization, and other undesired structural rearrangements. The telomerization route provides most of the linear fluorocarbons. The process is reported to yield well-defined and pure PFCs (126). Structures of the most used or investigated fluorocarbons are available in Figure 4.

6.3. Gas solubilities

Like previously discussed, the gas-dissolving capacity of fluorocarbons comes essentially from the weakness of the intermolecular cohesion forces. No chemical bonding, coordination, or charge-transfer complexes between fluorocarbons and the dissolved gas molecules seem to occur. The enthalpies and entropies of the solutions are reported to be usually small (143). Chadler reports that the behaviour of PFCs is typical of non-polar and non-associated liquids whose gas-dissolving abilities are essentially determined by the shape of the molecule (144). Unlike hemoglobin, oxygen loading and unloading by PFCs is not hindered by a decrease in temperature. As a matter of fact, the oxygen solubility in perfluorocarbons tends to increase as temperature decreases. The oxygen solubility in perfluorocarbon emulsions will be specifically discussed in the next section, because of its potential interest in cell and tissue cultures.

The solubilities of O_2 and CO_2 in a few typical fluorocarbons are reported in Table 2. More data can be found in a paper by Riess and Le Blanc (142). The most commonly methods used to measure the solubilities of gases in perfluorocarbons are manometers (145), chromatography (146,147) or spectroscopy (Nuclear Magnetic Resonance (NMR) in the case of O_2) (148). It is important to note that great inconsistencies in solubility values are obtained. This can come from the lack of standard procedures or the uncertainties about the exact composition of the samples analysed.

For example, at a given molecular weight, differences in structure can translate into values that can reach 20-25% of variation. Linear fluorocarbons, including those which have a double bond, an oxygen atom or a terminal bromine atom, have a clear advantage over cyclic or polycyclic ones.

The reported general dissolving capacity for various gases decreases in the order: $CO_2>>O_2>CO>N_2>H_2>He$, apparently following the decrease in molecular volume of the molecules (126).

6.3.1. Oxygen

One attraction of using PFCs in cell culture systems is their immiscibility with aqueous media; in principle, they could be re-cycled and recovered, an obvious economical advantage (149). It is clear that *in vitro* cellular responses to emulsified PFCs are largely dependent on the cell type and the species from which it originates (149).

Oxygen is soluble in PFCs and oxygen carriage is directly proportional to both the partial pressure of oxygen (Henry's law) and to the concentration of PFC (150). This has important implications since in both basic and applied cell culture studies; gas transport is often a limiting factor.

Applications of PFCs to culture microbial (21,136,151) and mammalian (8,20,152) cells have been reported in the literature. Reviews on this topic are also available (8,22). While some research teams used PFC as liquid micro-carriers (8,22), most researchers took advantage of their high oxygen-carrying capability and circulated the pre- and/or re-oxygenated PFC through the culture system for supplying oxygen to cells (8,20-22,136,151,152). Since oxygen is transferred from the PFC to the aqueous phase, the efficiency of the process is directly related to the PFC-water interfacial area, which is determined by the droplet size of the PFC dispersed or emulsified in the culture media.

Table 2. Gas solubility at 25^0C in some perfluorocarbons

	Solubility O_2	**Solubility CO_2**
	(volume %)	(volume %)
Perfluorodecalin (FDC)	42	142
Perfluorotripropylamine (FTPA)	45	166
Bis (F-butyl) ethene (F-44E)	50	247
Perfluorooctyl bromide (PFOB)	50	210

Source: 126

Oxygen solubility in PFCs is typically of 40-60 mL of gas dissolved per 100 mL of PFC emulsion whereas that for carbon dioxide can be up to 3 times higher (153). Experiments have shown that dropwise addition of an oxygenated PFC oil to high-density suspensions of mouse cells is able to supply sufficient oxygen to allow them to grow normally (154).

The effect of the addition of perfllurochemicals to the medium when immobilized *Gluconobacter oxydans* were used to oxidize glycerol to dihydroxyacetone was very obvious. The productivity increased with the amount of PFC added to the medium. When the medium was saturated with oxygen from the PFC, the productivity was 4-5 times higher than that obtained with air-saturated media. A high portion of perfluorochemicals was beneficial, in the sense that it gave a high-production rate, but solutions containing more than 35% (v/v) PFC were difficult to handle because of their high viscosity (20).

PFC formulations seem to have several advantages compared to hemoglobin carriers if PFC emulsions can be demonstrated not cytotoxic (141). Whereas hemoglobin has a given number of receptor sites and can therefore bind only a finite amount of oxygen, the level of oxygen carried by PFCs is determined by the oxygen partial pressure in the gas with which PFCs are in equilibrium. If necessary, oxygen-enriched gases can be used to further increase the oxygen concentration to cells. In addition, oxygen is not bound to PFCs, as it is to hemoglobin. Perfluorocarbons also have much higher solubilities for CO_2 than ordinary aqueous media. Therefore, PFCs can carry carbon dioxide and handle the concomitant challenge of removing the CO_2 generated by cell metabolism if this would be needed in large tissue mass, for example. One mole of CO_2 is produced per mole of oxygen consumed by the cells. This CO_2 must be carried away from the living organisms and disposed (if not needed to adjust the pH in the culture medium). So far, CO_2 removal has not proved to be a limitation in cell culture because CO_2 can be reacted with components of the culture medium in order to maintain the pH at a desired value and when the buffer capacity is reached, the culture medium is replenished. Nevertheless, CO_2-derived ions raise the osmolality of the medium so that eventually cell functions would be impeded by adverse osmotic pressure across the cell membrane (155,156).

6.4. Surfactants to stabilize PFC emulsions

Surfactants are mainly used to reduce the large tension at the interface between the fluorocarbon and aqueous phase. Surfactants are also used to stabilize the emulsion once it is formed and therefore play a crucial role in emulsion preparation.

Surfactant molecules are amphiphilic molecules that tend to adsorb at interfaces, including on biological membranes. Such surface-active components can hardly be expected to be totally devoided of biological activity. For this reason, only two surfactants have been used to a substantial extent in the formulation of fluorocarbon emulsions: poloxamer 188 (or Pluronic F-68® or Proxanol-188®) and egg-yolk phospholipids (EYP).

Fluorinated surfactants i.e., surfactants with hydrophobic tails consisting of a fluorocarbon chain, have also been investigated as emulsifiers for PFC emulsions. Fluorinated surfactants tend to display a high affinity for the fluorocarbon phase and very low fluorocarbon/water interfacial tensions. These surfactants will be briefly discussed later.

6.4.1. Poloxamer-based surfactants

Poloxamers are neutral block copolymers. They are composed of two terminal poly (ethylene oxide) (PEO) blocks attached to a central poly (propylene oxide) (PPO) block. When used as an emulsifier in an oil-in-water phase, the hydrophobic segment (PPO) will interact with the surface of the oil droplets, while the two more hydrophilic PEO chains are more likely to extend into the aqueous phase (126). The PEO chains are substantially hydrated due to hydrogen bonding with water molecules.

Poloxamers are low-cost synthetic materials. When analysed by gel permeation chromatography, commercial poloxamer 188 actually shows a bimodal distribution: a major peak (about 95% of the mass) having a molecular weight between 6,000 and 13,000 and a minor peak between 3,000 and 5,000 (157). Impurities can be found in some commercial poloxamer products. The impurities include traces of acetaldehyde, propionaldehyde, formic acid, acetic acid, etc. Purification procedures include treatment with ion exchangers, fractionating crystallization, and other methods (158,159). Standard commercial grade poloxamers with high purities intended for intravascular uses are also available (BASF, www.basf.com).

Many problems arose from the use of poloxamers as surfactants when first introduced in the fabrication of PFC emulsions. First, poloxamers have a relatively poor surface activity and low affinity for perfluorocarbons. For example, it is reported that the interfacial tension between perfluorodecalin and water only reduced from 56 to 30 mN m^{-1} for poloxamer-stabilized PFC emulsions compared to ca. 1-5 mN m^{-1} when phosphatidylcholine lipids were used to stabilize PFC emulsions (126). Another problem can come from the low state of purity and variability of the commercially available products, as noted above. A further

problem reported by many groups reveals the fact that poloxamers tend to form gels at room temperature (160-162). The consequence of this phenomenon is the limitation of the fluorocarbon concentration in size-controlled emulsions, as higher concentrations require larger amounts of surfactant while the volume of the continuous aqueous phase becomes smaller (126). Viscosity increases thus rapidly when the poloxamer-stabilized fluorocarbon content is larger than about 30% (v/v) (126). Furthermore, the Pluronic® F-68-based emulsions cannot tolerate the standard 121°C sterilization temperature usually required, because its cloud point is too low.

6.4.2. Phospholipid-based surfactants

Phospholipids are amphiphilic molecules constituted of a polar (or charged) part, which has a strong affinity for water, and an apolar part made of hydrocarbon chains, which are hydrophobic. This amphipathic structure leads to specific properties including a strong tendency to form multi-layers and even lipid vesicles when dispersed in water and processed appropriately.

For a number of reasons, it is reported that egg yolk phospholipids (EYP) has been selected as an emulsifier replacement for poloxamer in most currently developed fluorocarbon emulsions. Significant improvements in PFC emulsion stability with EYP versus poloxamer were reported (163,164). The good match between the hydrophilic-lipophilic balance of EYP and perfluorooctyl bromide (PFOB) emulsions, in particular, was emphasized (129). It resulted in improved cohesiveness between EYP hydrophobic fatty acid chains and PFOB emulsions, as compared to other PFCs. Another advantage of phospholipid-based emulsion is that phospholipids are well documented and exist in commercial sources of pharmaceutical grade (165,166). It was also shown that phospholipid composition and the level of fatty acid unsaturation may have an effect on the oxidation resistance and particle size stability, and on the viscosity of perfluorocarbon emulsions (167,168).

Advantages of phospholipids are their natural origin (limiting their cost), and the fact that they have been in use for a long time as surfactants for the preparation of commonly used injectable lipid emulsions for parenteral nutrition, liposomes and other drug carriers. Oxidation and chemical hydrolysis of EYP can be of concern, owing to the presence of labile ester linkages and unsaturated fatty acids. Hydrolytic decomposition of EYP occurs primarily via classical acid/base catalyzed reactions. Parameters that are reported to affect hydrolysis include: temperature, pH, ionic strength, phospholipid headgroup, and fatty acid groups (126). Oxidative decomposition of EYP can occur mainly in unsaturated acyl chains and degradation products include fatty acid hydroperoxides, aldehydes, malonic dialdehyde, hydrocarbons, and aldol condensation products.

Tarara *et al.* (169) showed that the phospholipid composition, degree of unsaturation, PFC purity, and the presence of oxygen and trace metals all have significant effect on phospholipid oxidative decomposition. Oxidation in PFC emulsions could be minimized by the inclusion into the formulation of metal chelators (e.g., EDTA), and antioxidants (e.g., alpha-tocopherol).

6.4.3. Fluorinated surfactants

A different way of obtaining stable PFC emulsions is to use fluorinated surfactants, because only rarely does a single surfactant possess all the attributes required to stabilize PFC emulsions. Perfluoroalkylated chains are reported to be more hydrophobic than hydrocarbon chains. Their use in surfactant formulations are reported to increase the amphiphilic character of the surfactants, resulting in significantly enhanced hydrophobic interactions and surface activities (131). Good synergistic activity has also been reported for mixtures of either EYP (170) or poloxamer 188 (171) with fluorinated surfactants. Mixtures of surfactants can combine, for example, the steric stabilizing capacities of poloxamers with the excellent surface activity of the fluorinated surfactants to yield "better" surfactants.

6.4.4. Molecular dowels

In recent literature, fluorocarbon-hydrocarbon diblocks (F_mH_n) were reported to have excellent stabilizing characteristics and high lipophilicity (127,172,173). These diblocks (also termed molecular dowels) are interfacially active compounds (172) that may have increased affinity for the PFC/EYP interface (173). The hydrocarbon group would display good affinity for the acyl chains of the phospholipid monolayer, while the fluorinated group should penetrate through the PFC. The addition of F_mH_n diblocks to PFC emulsions seems to result in the stabilization of particles size ranging from 0.13 to 16 μm (174). Riess' group has used the diblocks as additives to the surfactant phase. In this procedure, the diblock is processed in the initial surfactant dispersion prior to the addition of the PFC. It has also been shown that because these diblocks should act as molecular dowels, the same concentration of the dowel and the surfactant is often used, and seems to have proven effective. It is also important to note, that the exact mechanism of stabilization for F_nH_m diblocks has not been fully elucidated.

6.5. Emulsification procedures

This section attempts to give an overview of the general concepts governing emulsion procedures. Preparation of fluorocarbon emulsions involves the dispersion of the fluorocarbon into submicron droplets in a continuous saline phase in the presence of surfactants. Emulsification is usually achieved by mechanical means. As reported by Krafft *et al.* (126), emulsion stability was observed to be optimal following 15-25 passes through an emulsification device. While numerous passes are necessary to obtain a fine and stable emulsion, significant destabilization was observed when the number of passes was larger than 30-40 (126).

Sonication, high-pressure homogenization or microfluidization are the methods used to obtain PFC emulsions. Details on the processing equipment and operating conditions used to industrially produce fluorocarbon emulsions are not disclosed. Even information originating from academic laboratories is almost always

very scarce.

The impact of various process parameters (temperature, pressure, recycling, preparation, and structure of the phospholipid dispersion, sterilization parameters, etc.) on both initial droplet size and size stability need to be determined and controlled and we therefore "regret" that unfortunately such information is not extensively, if not at all, discussed in the literature.

6.5.1. Sonication

Sonication is based on the cavitation phenomenon, i.e. the rapid and repeated formation and implosion of micro-bubbles in a liquid, resulting in the propagation of microscopic shock waves (175). The advantage sonication presents is to allow the preparation of very small batches, which can be of value for preliminary tests. However, this process tends to yield wide particle size distributions (176), and suffers from poor reproducibility since the results depend on several parameters and their combination. Such parameters include: the size and shape of the ultrasonic probe, the shape and filling of the vessel that contains the emulsion, the position of the sonic probe in the vessel, etc. It also often requires subsequent prolonged low-temperature centrifugation to eliminate large fluorocarbon droplets (176). In addition, this procedure was shown to provoke the release of fluorine as fluoride ions (177,178), meaning that some degradation of the fluorocarbon occurs during this process. However, the formation of fluoride ions was reported by Geyer to be almost suppressed if sonication is performed under an atmosphere of carbon dioxide (179). Finally, sonication has little large-scale feasibility. For all these reasons mechanical procedures such as microfluidization or high-pressure homogenization are considered preferable to emulsify PFCs.

6.5.2. Microfluidization

In this technique, a flow of premix is divided into two separate streams funnelled through precisely defined micro-channels. These streams are forced to impinge on each other under high velocities in an interaction chamber. As an example, Microfluidics' Microfluidizer (Microfluidics, Newton, MA, www.microfluidicscorp.com) operates at pressures up to 20,000 psi using a pump operating with a 100-psi air supply. As the emulsion is prepared, it is circulated through a cooling coil between passes through the interaction chamber to yield the final emulsified preparation.

6.5.3. High-pressure homogenization

High-pressure homogenization seems to be the procedure of choice reported to be used by the major companies manufacturing fluorocarbon emulsions on a larger scale. The advantages of using this technology include GMP compliance design, continuous process and scale-up. Detailed information on emulsification conditions is once again scarce and unfortunately very few reports are available in the literature. High-pressure homogenizers are somewhat easy to control, seem to give narrower particle size distributions and can be operated for large-scale production. Small-size equipment is available (Rannie Minilab, APV Gaulin, Everett, MA) that allows 100-mL and liter-size laboratory sample production. High-pressure homogenizer with small capacity (0.5-20 mL, pressure up to 30,000 psi) is also available (Emulsiflex™ B Class, Avestin, Canada, www.avestin.com). The sample is introduced through the plug seat and placed under high pressure by a pump activated by a 100-psi air supply. A valve is opened and the sample is pushed through the homogenizing gap at a set pressure, which is controlled by a regulator. The reproducibility achieved with this device is much superior that with other emulsification procedures.

A study comparing, for a given emulsion formulation, the results obtained using sonication and high-pressure homogenization was done by Riess' group (180). The study revealed that the finest emulsions and narrowest particle size distributions were obtained by high-pressure homogenization. Sonication, on the other hand, yielded much wider particle size distribution and was poorly reproducible.

6.6. PFC emulsion stability

Stability is a primary requirement for any fluorocarbon emulsions intended to be used as oxygen carriers (127). Stabilization of an emulsion implies minimization of its free energy and creation of an effective barrier to oppose particle growth.

Instability of PFC emulsions appears to involve four mechanisms: 1) Ostwald ripening, 2) coalescence, 3) flocculation, and 4) sedimentation.

6.6.1. Ostwald ripening

The principal mechanism of irreversible droplet growth in PFC emulsions during storage has been reported to be the Ostwald ripening, which was named after Ostwald, which was the first to explain such colloidal behaviour (181-185). Briefly, Ostwald ripening involves the molecular diffusion (or coagulation) of individual PFC molecules through the continuous phase (186). The driving force for the ripening is the small difference in disperse phase chemical potential, which exists between different droplets due to differences in their radii of curvature i.e., the Kelvin effect. Differences in chemical potential are substantial only for submicron droplets.

The aggregates formed in the usual coagulation process are often difficult to re-disperse, but the particles do appear to retain their individuality (at least for times of the order of days and often for much longer). The structure of the aggregates also depends strongly on the way they are formed. If the attraction between the particles is weak or, in other words, the repulsion is significant, the particles are able to coagulate only slowly but when they do, they form very compact structures with little entrapped solvent. On the other hand, when the repulsion is slight and attraction is strong (i.e., in the regime of rapid coagulation) the aggregates formed are usually voluminous with much of the dispersion medium trapped inside the loose open structure (187).

As a result of this effect, the larger particles in a colloidal system (e.g., emulsion or suspension) will grow at

the expense of smaller particles. Accordingly, Ostwald ripening does not lead to phase separation. Emulsion breakage can occur only by coalescence-mediated droplet growth. The reader is referred to the work of Dunning and Smith (188) and Kahlweit (189) for more information concerning the Ostwald ripening mechanism.

6.6.2. Coalescence

Coalescence is the aggregation of micelles. In this process, the micelles fuse together and lose their individuality to form larger size aggregates. When an emulsion coalesces, its size distribution will enlarge.

Emulsion structure and stability is controlled to a large extent by the properties of the monolayer separating the oil and the water domains. Surfactant monolayers are not constrained to a planar geometry, but they can acquire any curvature or topology which is energetically favoured. The hydrophilic portion of the surfactant lies on one side of the surfactant film and the hydrophobic portion on the other (126). Introducing bends within the monolayer causes the hydrophobic portions of surfactant molecules to either squeeze together or be pushed farther apart.

The spontaneous curvature of natural phospholipids (e.g., EYP) is nearly equilibrated at the PFC/water interface i.e., the monolayer does not bend more towards the oil (O/W emulsion) than the aqueous phase (W/O emulsion). Fine adjustment can be achieved by changing the nature of the phospholipid headgroups and the length and degree of saturation of the acyl chains. But, in general, natural EYP mixtures are excellent emulsifiers for PFC emulsions.

PFC emulsions can also be stabilized with respect to droplet coalescence by the addition of polymeric surfactants. This provides a steric barrier preventing droplet contact. Although poloxamers significantly improved the stability of PFC emulsions toward coalescence at or near room temperature, dramatic destabilization resulted at high temperatures such as those used in thermal sterilization (190-191).

6.6.3. Flocculation

The terms flocculation and coagulation are sometimes wrongfully used to express the same phenomenon. Briefly, coagulation describes the phenomenon in which microscopic particles are aggregated together, while flocculation portrays a situation in which particles are aggregated into macroscopic flocs, and if the flocs differ in density from the surrounding medium, they will settle quite rapidly leaving a more or less clear supernatant (187).

Since the particles, if small enough, will be undergoing Brownian motion, they will be continually colliding with one another and they will remain as individual particles only if those collisions do not result in permanent associations. Such systems can remain as individual particles for an appreciable time only if some mechanism prevents aggregation during a collision. This can be done if the particles have similar electric charge, resulting in particles repulsion. However, the charge can be modified by proteins, which can adsorb on the particle surfaces, de-stabilizing the colloidal system. A system is unstable if collisions result in the formation of aggregates. Steric repulsion would appear to be a more efficient method to delay and/or minimize particle aggregation.

6.7. Emulsion rheology

Rheology of PFC emulsions depends on the volume fraction of the dispersed phase, the degree of flocculation, the particle size and the size-distribution of the particles. Viscosity increases as the dispersed PFC phase volume fraction increases. It was noted that below a volume fraction of ca. 0.5, for instance, perfluorooctylbromide emulsions behave as Newtonian fluids i.e., with no dependence of viscosity with shear rate (192). Above a volume fraction of 0.5, the viscosity of the fluid increased dramatically and the fluid became non-Newtonian. Viscosity problems were overcomed by slightly decreasing the volume fraction to values around 0.47 (193). On the other hand, the viscosity of a 60% w/v perfluorooctylbromide emulsion has been reported to be less than that of blood (ca. 3mPas) (194).

7. SUMMARY AND PERSPECTIVE

The main objective of this paper was to review the properties of fluids capable of enhancing oxygen transport and transfer. This paper reviews the work and progress that has been done in the last 40 years in the field of oxygen carriers. A first section deals with hemoglobin, a protein capable to carry oxygen. In the same line of thought, another section examines the outputs and accomplishments done in the field of perfluorocarbon (PFC) emulsions, in which PFCs are added to an aqueous medium to modify and increase its capacity to dissolve oxygen.

Working with a protein like hemoglobin, one must consider its important sensitivity to pH, temperature and gas environment. Moreover, hemoglobin has a tendency to convert to methemoglobin, resulting in a loss of its oxygen-carrying ability. Therefore, to maintain its oxygen solubility capacity, several strategies have been described in the scientific literature and in patents and are reviewed here. Chemically-modified hemoglobin derivatives (produced either by cross-linking, polymerization, or by conjugation of a polymer to the hemoglobin) and colloidal carriers containing hemoglobin are reviewed in this paper. Each system has been shown to have its advantages and pitfalls, but they all try to mitigate the potential problems observed with “free” hemoglobin proteins in solution, such as uncontrollable methemoglobin formation and sensitivity to environmental factors.

Cross-linking involves intramolecular binding between the subunits inside the hemoglobin molecule to stabilize its structure. The concept of hemoglobin polymerization pre-supposes that hemoglobin molecules would be bonded together by chemical reagents. Although many chemicals have been reported as effective cross-linking agents, it appears that they can also polymerize the

hemoglobin molecules, yielding that many hemoglobin proteins could be linked together following the activation procedure. The conjugation of a macromolecule to hemoglobin increases the molecular weight to a larger molecule and is reported in the hemoglobin literature to prolong its retention. Poly (ethylene oxide) (PEO) and dextran are the most used products in hemoglobin conjugation. Their non-specific binding process is often criticized yielding products of high viscosities. We believe that the encapsulation of a protein, in an appropriate carrier, is a more "gentle" alternative than the other techniques in which hemoglobin proteins are chemically modified because it mimics more closely the *in vivo* situation. Thus, encapsulation of hemoglobin proteins within colloidal carriers mimics, to some extent, the red blood cell environment by maintaining the hemoglobin capacity to bind oxygen molecules and to release oxygen in response to a lower partial oxygen pressure. For example, the encapsulation of hemoglobin into liposomes represents a way to transport and to protect the hemoglobin proteins from the aggressive biological fluids in which it is dispersed. The liposome literature is also well documented. It has been shown that the use of PEG-modified lipid vesicles containing hemoglobin were able to improve blood circulation and tissue oxygenation owing to a decrease in aggregation *in vivo.* Although encapsulation of hemoglobin is a promising technique, the difficulty to obtain adequate hemoglobin encapsulation efficiency is a drawback of this technique and this should be further addressed.

Because of the several problems arising from the manipulation of the fragile and sensitive hemoglobin protein, the use of perfluorochemical (PFC) emulsions can be seen as an alternative to carry and deliver oxygen to cells and tissues either *in vitro* or *in vivo*. However, the literature relative to perfluorochemical-based oxygen carriers is often confusing and contradictory.

Perfluorochemical emulsions seem to have certain advantages over hemoglobin-based carriers in the way that the PFC emulsions oxygen loading and unloading is not hindered by as many environmental factors. Oxygen solubility in PFC emulsions actually increases as temperature decreases. Oxygen is soluble in PFC emulsions and oxygen carriage is directly proportional to both the partial pressure of oxygen (Henry's law) and to the concentration of PFCs. The mechanisms governing the instability of PFC emulsions are starting to be identified and are briefly addressed in this review. However, mechanisms involved in the PFC emulsions instability do not seem to be fully understood yet. This lack of knowledge can hinder the development of adequate PFC emulsions to effectively carry oxygen and we think this should be further addressed in the development of these colloidal oxygen carriers. Also, it appears to us that some technical information is missing in many "scientific" papers making it difficult to reproduce many experiments. PFC emulsions are completely synthetic, are believed to be biologically inert and they dissolve higher oxygen concentrations at a given partial pressure, than their hemoglobin oxygen carrier counterparts. Also, our analysis of the scientific literature points out that PFC emulsions can be toxic to human cells. Hemoglobin, on the other hand, is hard to purify, "sensitive" and "fragile" to harsh conditions, and cannot be used unless it has been modified or encapsulated. Nevertheless, hemoglobin-based carriers are well documented and have several modified formulations on trial in humans.

Our interest in hemoglobin-based and PFC-based oxygen carriers is to find a way to increase oxygen transport and transfer to cells in tissue mass culture. But for now, full data was not available to us to clearly determine if these oxygen carriers could successfully be used to deliver oxygen to cells. We do not pretend that we better understand the behaviour of these carriers than the authors from whose works have been used to write this review paper. However, we do hope that we have been critical enough to put in perspective claims concerning these oxygen-carriers that are often found in the literature and, appear to us, not justified well enough.

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