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SECOND-GENERATION PERFLUOROCARBON EMULSION BLOOD SUBSTITUTES

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ABSTRACT

A novel series of perfluorocarbon (PFC) emulsions, based on perfluorodecalin ($C_{10}F_{18}$) and stabilised with up to 2.5% (w/v) of lecithin have been produced for evaluation as injectable, temporary respiratory gas-carrying blood substitutes. Some formulations contained 1.0% (w/v) of perfluorodimorpholinopropane ($C_{11}F_{22}N_2O_2$) to retard droplet growth through molecular diffusion (Ostwald Ripening). Other emulsions contained novel, amphiphilic fluorinated surfactants, such as, for example, the monocarbamate, $C_8F_{17}C_2H_4NHC(O)(CH_2CH_2O)_2Me$ (designated compound P6), at 0.1% (w/v) to enhance stability. Emulsions were prepared by homogenisation, were steam sterilisable and were stable for >300 days (25°C). Injection of rats (7.5 ml kg^{-1} b.w.) with emulsions produced significant ($P < 0.05$), transient increases in liver and spleen weights. One emulsion inhibited phorbol 12-myristate 13-acetate (PMA)-stimulated, *Luminol*®-enhanced, chemiluminescence of human polymorphonuclear leucocytes (PMNL) *in vitro*, suggesting possible applications in ischaemic tissues for suppressing PMNL-mediated inflammation. The P6 fluoro-surfactant inhibited spontaneous platelet aggregation in hirudin-anticoagulated human blood *in vitro*, suggesting possible applications as an anti-thrombotic agent.

INTRODUCTION

Perfluorochemicals (PFCs) are highly fluorinated, linear, cyclic or bicyclic organic compounds that can dissolve large volumes of respiratory gases. PFC liquids are immiscible with aqueous systems, including blood and other body fluids, but can be injected safely into the vascular system in an emulsified form. Emulsions of PFCs have been evaluated clinically as, for example, temporary respiratory gas-carrying

fluids for intravascular administration (i.e. so-called 'blood substitutes') and as diagnostic contrast imaging agents (1-5). Neat PFC liquids are also being assessed as respiratory tract infiltrates, in both babies and adults, for the treatment of acute respiratory failure through liquid ventilation (1,5). PFCs are chemically inert, biologically unreactive and excreted from the body primarily as a vapour by exhalation.

This paper gives an overview on PFC emulsions as intravascular respiratory gas carriers, focusing particularly on improved, 'second-generation' formulations that have been developed in recent years. Whilst the title of the paper utilises the commonly-used phrase, blood substitute, it is recognised that PFC emulsions can only replace the *respiratory gas-carrying* functions of whole blood. However, recent research indicates that the emulsions and their components may additionally have novel, and clinically relevant, applications, as immunomodulating and anti-thrombotic agents, and discussion of these aspects is a particular focus of the paper.

FIRST-GENERATION PFC EMULSION-BASED BLOOD SUBSTITUTES

The first injectable PFC emulsion that was produced commercially was *Fluosol*® (Green Cross, Japan), which contained 14.0% (w/v) of perfluorodecalin ($C_{10}F_{18}$), and 6.0% (w/v) of perfluorotripropylamine [$(C_3F_7)_3N$] (Table I). *Fluosol*® had an oxygen-carrying capacity of approximately 40% of that of red blood cells at 37°C and received regulatory approval in the USA and Europe for clinical use as an adjunct to transluminal coronary balloon angioplasty in 1989-90 (1,3). However, *Fluosol*® was not widely adopted by cardiologists and the emulsion was subsequently withdrawn in 1993. The main drawbacks of the *Fluosol*® formulation were (i) its relatively low oxygen-carrying capacity, due to its low PFC content, (ii) poor stability, and the need for the stem emulsion component to be stored frozen, (iii) a relatively short intravascular dwell time in humans (*ca.* 7.5 hours at 10 ml kg^{-1}), which was the principal reason why the emulsion was not approved for use as a treatment for anaemia (3), (iv) the occurrence, in some patients, of transient, adverse cardiorespiratory and anaphylactoid-like reactions, thought to be due to the uptake of emulsion droplets by phagocytic cells of the monocyte-macrophage system followed by the release of cytokines, and (v) problems associated with the purity and bioreactivity of its main surfactant constituent, the polyoxyethylene-polyoxypropylene co-polymer, *Pluronic*® F-68 (poloxamer 188).

Concurrent with the development of *Fluosol*® was the production of other, first-generation, PFC emulsions, including *Emulsion No. II* and *Perftoran*® from China and Russia, respectively (1-4,6). Both

formulations have similar compositions to *Fluosol*®, except that in *Perftoran*®, perfluoromethylcyclopiperidine ($C_{12}F_{23}N$) was used in place of perfluorotripropylamine to stabilise the core perfluorodecalin emulsion (Table I)(6). For both emulsions, a poloxamer-type, co-polymer compound was incorporated as sole or principal surfactant. *Perftoran*® was approved (in 1995-96) for clinical use in Russia, as a temporary intravascular oxygen carrier and perfusion fluid for human organ preservation and storage. A further commercial emulsion, *Oxypherol*®, containing 20% (w/v) of perfluorotributylamine [$(C_4F_9)_3N$], also produced by the Green Cross Corporation (Table I), was not intended for clinical use because of the prolonged retention half-time of this PFC in the body (>500 days).

SECOND-GENERATION PFC EMULSION BLOOD SUBSTITUTES

The main objectives of the research and development effort to produce superior PFC emulsions to supersede *Fluosol*® and other first-generation emulsions were (i) identification of PFCs with biocompatibility and excretion properties suitable for *in vivo* use, (ii) improvement in stability characteristics, through the use of perfluorinated stabilisers and non-poloxamer surfactants (e.g. lecithins and/or tailor-made 'fluorophilic' compounds), thus producing room temperature-stable products, and (iii) the development of sterilisable emulsions having significantly increased PFC content conferring superior oxygen-carrying capacity. Such physico-chemical developments were linked with a need for further understanding of the behaviour and efficacy of PFCs and other emulsion constituents in biological systems.

Selection of biocompatible PFCs

The two PFCs most widely studied as core constituents of injectable emulsions are the linear molecule, perflubron (perfluoro-octyl bromide; $C_8F_{17}Br$) and the bicyclic compound, perfluorodecalin. The molecular weights of both compounds fall within the range 460-500, which is recognised as that giving acceptable tissue retention times (1-5). It has been emphasised (7), that the excretion rate of PFCs from the body depends primarily on molecular weight, with molecular structure and the presence of cycles or heteroatoms having minimal influence. Perflubron and perfluorodecalin can be synthesised to a very high degree of purity, thereby avoiding unwanted side-effects that have often been attributed to partially-fluorinated contaminants (5).

Other, second-generation, commercial emulsions include *Oxyfluor*™ (formerly *Supercytes*®), consisting of 40% (w/v) perfluoro-dichlorooctane ($C_8F_{16}Cl$) (8), and *TherOx*®, containing 83% (w/v) bis(perfluorobutyl)ethene ($C_6F_{13}CH{=}CHC_6F_{13}$; Table I) (2,9). A range of

TABLE I. First- and second-generation perfluorocarbon emulsion blood substitutes.

Emulsion	Perfluorocarbon(s)	Perfluorocarbon concentration (% w/v)	Surfactant(s)	Storage conditions
'First-Generation' emulsions				
Fluosol®	Perfluorodecalin	14.0	*Pluronic*® F-68	Frozen*
	Perfluorotripropylamine	6.0	EYP	
			Potassium oleate	
Perftoran®	Perfluorodecalin	14.0	*Proxanol*®	Frozen*
	Perfluoromethylcyclopiperidine	6.0		
Oxypherol®	Perfluorotributylamine	20.0	*Pluronic*® F-68	Refrigeration**
'Second-Generation' emulsions				
Oxyfluor™	Perfluorodichlorooctane	40.0	EYP	Refrigeration
FMIQ	Perfluoromethylisoquinolone	50.0	EYP	Refrigeration
Oxygent™	Perflubron	58.0	EYP	Refrigeration
	Perfluorodecyl bromide	2.0		
TherOx®	Bis(perfluorohexyl)ethene	83.0	EYP	Refrigeration

*Stem emulsion stored frozen; annex solutions stored under refrigeration (*ca.* 4°C); **Emulsion ideally stored at < 10°C; EYP = egg phospolipids.

second-generation emulsions containing increasing concentrations (25-50% w/v) of perfluoromethylisoquinoline ($C_{10}F_{19}N$), together with an emulsion consisting of 54% (w/v) bis(perfluorobutyl)ethene, have also been reported (2,9). It has been necessary to achieve a compromise between producing highly concentrated PFC emulsions with increased potential respiratory gas-carrying capacities, whilst avoiding formulations with viscosity's that may limit their use in the vasculature.

One crucial variable in the selection of PFCs for *in vivo* applications is the critical temperature (°C) of solubility in n-hexane (CTSH) (10). The CTSH of a PFC is a measure of its relative solubility in lipids and characterises the rate of transfer of individual compounds through alveolar membranes. Thus, PFCs with CTSH values of <28°C, such as perfluorodecalin, are considered as lipophilic (10), whereas compounds with CTSH values of >42°C, such as perfluorotributylamine, have prolonged retention times in the body. Both perfluorodecalin and, particularly, perflubron, the latter because of its molecular composition, fall into the former category and are therefore considered suitable for *in vivo* applications.

One recent development in the search for other PFCs suitable for evaluation as constituents of biological gas carriers has been the synthesis of a range of novel perfluoroether derivatives, a class of compounds hitherto considered unacceptable for use *in vivo* because of their generally high vapour pressures (7). One compound, $HC_2F_2OCF_2C(CF_3)_2CF_2OC_2F_4H$, when emulsified with lecithin and safflower oil, had a body retention time in rats of 0.5 days (11), which is considerably lower than the corresponding values for perflubron or perfluorodecalin of *ca.* 4 days and 7 days, respectively (1,2,4,5).

Emulsion stabilisation issues

As emphasised already, a major goal in the production of second-generation injectable emulsions has been to significantly improve stability and, thus extend shelf-life. Emulsions are thermodynamically unstable systems and, in PFC-based formulations, the principal mechanism by which droplets grow is through a process of molecular diffusion known as Ostwald Ripening. During this process, PFC molecules from smaller droplets diffuse through the continuous phase to the larger droplets which progressively increase in size at the expense of the former (12). Ostwald Ripening in emulsions of perfluorodecalin can be retarded by the addition of, for example, a small amount of a perfluorinated, high molecular weight, high boiling point oil (HBPO) additive, such as perfluoroperhydrophenanthrene ($C_{16}F_{26}$) (13). This general approach was that used in the production of both *Fluosol*®, in which emulsified perfluorodecalin was stabilised with perfluorotripropylamine, and, similarly, in *Perftoran*®, where

perfluoromethylcyclopiperidine was used as the HBPO (1,2,4,5). More recently, second-generation emulsions, based on perflubron or perfluorodecalin, have similarly been stabilised against Ostwald Ripening using small quantities of an appropriate HBPO, as described later. Effective stabilisation of PFC emulsions against Ostwald Ripening-mediated ageing has been a major hurdle for researchers to overcome in the development of room temperature-stable formulations for biomedical applications.

Emulsions based on perflubron

Perflubron has one of the highest respiratory gas-dissolving capacities of any of the PFCs commonly-used for biological applications (*ca.* 44.0 mmol l^{-1} at S.T.P. This compound is also attractive for *in vivo* use because of its excellent imaging properties (14). Perflubron can be readily emulsified with egg phospholipids (EYP) and shows exceptionally fast excretion characteristics (1,2,4,5). This latter property arises as a result of its very high lipophilicity due to the presence of a single bromine atom on the terminal carbon (2,5).

Perflubron is the main PFC component in a commercial oxygen-carrying emulsion (*Oxygent*™) developed jointly by the Alliance Pharmaceutical Corporation, San Diego, USA and the Johnson and Johnson subsidiaries, the Robert Wood Johnson Pharmaceutical Research Institute and Ortho Biotech Inc., Raritan, USA (Table II). The current *Oxygent*™ formulation (AF0144) consists of 58% (w/v) perflubron and 2% (w/v) of its higher homologue, perfluorodecyl bromide ($C_{10}F_{21}Br$), to stabilise against Ostwald Ripening (5). The emulsion has a shelf-life at 5-10°C of >1 year.

Oxygent™ is stabilised with EYP (3.6% w/v) which are an obvious choice for emulsifying PFCs since they have been used extensively in injectable lipid emulsions for parenteral nutrition. EYP are excellent stabilisers of fluorocarbon emulsions, as reflected in their ability to reduce the fluorocarbon/water interfacial tension (12,16). EYP are sensitive to slow oxidative degradation but, in some PFC-based emulsions, this has been overcome by the addition of an anti-oxidant, such as α-tocopherol (17). Moreover, α-tocopherol is routinely added (0.1-0.2% w/v) to some commercial phospholipid formulations (e.g. *Lipoid*® E100, Lipoid GmbH, Germany) that have been used in other PFC emulsions, as described later.

Oxygent™ has been studied extensively as an intravascular oxygen carrier in animal experiments (1-5,15), and is currently being evaluated in advanced clinical trials as a temporary oxygenation fluid for use during surgery. The intraoperative use of *Oxygent*™ will reduce the need for transfusion of allogeneic (donor) blood. Multiple-site Phase II efficacy trials with *Oxygent*™ in surgical patients were initiated in the

TABLE II. Some properties of the commercial PFC emulsion, *Oxygent*™ (Alliance Pharmaceutical Corporation, USA).

PFC content (% w/v)	60.20 ± 0.06
Mean droplet diameter (μm)	0.17 ± 0.01
pH	7.10 ± 0.04
Viscosity (cP)	4.0 ± 0.1
Osmolarity (mOsm kg^{-1})	304 ± 6
Heat sterilised, non-pyrogenic	
Stable for >1 year at 5-10°C	

USA and Europe during 1995-96. *Oxygent*™ is currently the front-runner of the PFC-based emulsions being evaluated as intravascular respiratory gas-carrying fluids.

Emulsions based on perfluorodecalin

A further commonly-used PFC in second-generation emulsions is perfluorodecalin. This is because the acceptable tissue retention time of this compound outweigh its fairly poor emulsifying properties (1,2,4,5). Perfluorodecalin can dissolve *ca.* 35.5 mmol l^{-1} of oxygen and *ca.* 125 mmol l^{-1} of carbon dioxide at S.T.P. In addition to being the principal PFC constituent of *Fluosol*®, perfluorodecalin formed the basis of a series of biocompatible emulsions, each containing small (typically 0.5-2.0% w/v) quantities of a perfluorinated HBPO that had been added to retard emulsion breakdown by Ostwald Ripening (13,18). Concentrated emulsions, containing up to 60% (w/v) of perfluorodecalin and stabilised with 4% (w/v) *Pluronic*® F-68 and 2% (w/v) soya oil (19), similarly had good biocompatibility characteristics (20).

A novel series of perfluorodecalin-based emulsions, stabilised with up to 2.5% (w/v) of lecithin (*Lipoid*® E100, Lipoid GmbH) have been produced recently by a European consortium (21). Some of the emulsions also contained 1.0% (w/v) of perfluorodimorpholinopropane ($C_{11}F_{22}N_2O_2$) to retard droplet growth through molecular diffusion, analogous with earlier studies (13). Perfluorodimorpholinopropane has a molecular weight of 610, a boiling point of 182°C and can dissolve *ca.* 38.0 mmol l^{-1} of oxygen at S.T.P. It has an estimated body clearance half-time of 55 days (22). This compares favourably with

corresponding values of 60 days for the perfluorotripropylamine and perfluoromethylcyclopiperidine constituents of *Fluosol*® and *Perftoran*®, respectively (4). The novel emulsions were prepared by homogenisation and had a total PFC content of 20-40% (w/v). Emulsions were steam sterilisable (121°C, 2 atm, 20 min), with no significant changes in droplet diameter (*ca.* 0.2-0.3 μm) during 300 days' storage at room temperature.

Injection of male Wistar rats with 7.5 ml kg^{-1} body weight of different emulsions produced significant ($P < 0.05$), transient increases in both mean liver and spleen weights, compared to saline-treated controls, consistent with previous related findings (20). Experiments are in progress to assess the efficacy of the novel emulsions as oxygen-carrying perfusates of animal organs, including the dog heart and pig liver.

Emulsified PFCs as potential immuno-modulating agents

PFC emulsions may be useful for protecting tissues, such as the coronary vasculature, against inflammatory reperfusion damage through transient alterations in blood leucocyte functions, arising as a result of the phagocytic uptake of emulsion droplets (23,24). Exposure of porcine alveolar macrophages to perflubron *in vitro* decreases phorbol 12-myristate 13-acetate (PMA)-induced, *Luminol*®-enhanced chemiluminescence (25) and similar findings have been observed with human neutrophils (26). More recently, a novel emulsion consisting of 18.5% (w/v) perfluorodecalin, 1.5% (w/v) perfluorodimorpholino-propane and 2.5% (w/v) lecithin similarly produced a transient, dose-dependent, decrease in PMA-induced PMNL chemiluminescence in citrated human whole blood *in vitro* (21). The mean chemiluminescence decreased to a maximum of 54% after 12 min ($P < 0.05$), when blood was pre-incubated with 10-40 μl of the novel emulsion, compared to that in saline controls. In contrast, exposure of blood to the PFC emulsion in the absence of PMA did not induce a chemiluminescence response. This suggested that the inhibition of chemiluminescence by the PFC emulsion did not involve pre-activation of PMNL (21). PMA is a diacylglyceol analogue which induces chemiluminescence in PMNL by direct activation of the intracellular protein kinase C (PKC) enzyme (27). Therefore, it is unlikely that novel emulsion droplets inhibited PMA-induced chemiluminescence in PMNL through alterations in receptor-mediated activation pathways. However, other possible mechanisms include coating of PMNL's with emulsion droplets, coupled to changes in membrane function prior to subsequent uptake. This may, in turn, lead to alterations in PKC interaction with membrane-bound proteins, changes in calcium influx into the cells or direct interference of the NADPH-oxidase enzyme which is pivotal to the chemiluminescence response (25).

These findings reinforce previous suggestions (1,4) that, in addition to their use as intravascular oxygen carriers, emulsified PFCs may also be valuable in ischaemic tissues as immuno-modulating agents, acting to temporarily suppress PMNL-mediated inflammation. Clearly, such potential anti-inflammatory effects of PFC emulsions must be balanced against the potential risks of immunosuppression, especially on repeat or prolonged administration. It is known that injection of emulsified PFCs can alter immune system function *in vivo*, with the responses depending on the dose, timing and route of administration relative to immunological challenge (1,28). Future work in this area should determine the extent to which the composition and physical characteristics of PFC emulsions can alter PMNL functions and assess the time course and significance of any immunosuppressive responses in the recipient.

Novel fluorophilic surfactants

One strategy for improving the stability of PFC emulsions has been to incorporate specially synthesised, fluorophilic surfactants and/or co-surfactants, most notably compounds derived from sugars, amino acids and lipids (5,29-32). In this respect, a further novel series of fluoro-surfactants, derived from glycosides (monosaccharides; 'S' series) or polyols (ureas or carbamates; 'P' series), have been recently produced for use in PFC emulsions for *in vivo* uses. Compounds were synthesised via simple, but highly selective, routes using highly fluorinated isocyanates with amino alcohols, polyethoxylated alcohols and partially protected sugars at anomeric carbon; the overall yields were 88-95% (33). The resultant compounds were perfluoroalkylated with hydroxylic 'head' groups (Figure 1). An interesting fluoro-surfactant to emerge from this research is an amphiphilic, poly(oxyethylene) monocarbamate with a general formula of $C_8F_{17}C_2H_4NHC(O)(CH_2CH_2O)_2Me$ (designated as compound P6).

The biocompatibility of the novel fluoro-surfactants with human blood *in vitro* was initially assessed using a conventional haemolysis test. Compounds that exhibited insignificant haemolysis at final concentrations of up to 10 g l^{-1} were subsequently evaluated (i) for their effects on PMA-induced human PMNL chemiluminescence, and (ii) in a human blood platelet aggregation bioassay (34).

Fluorophilic surfactants as potential anti-thrombotic agents

Some of the novel fluoro-surfactants, most notably the polyol compounds, inhibited spontaneous platelet aggregation, in human blood anti-coagulated with hirudin, at concentrations of 0.01% (w/v). This suggests possible applications for these compounds as anti-thrombotic agents. In general, the effects of the fluoro-surfactants on platelet

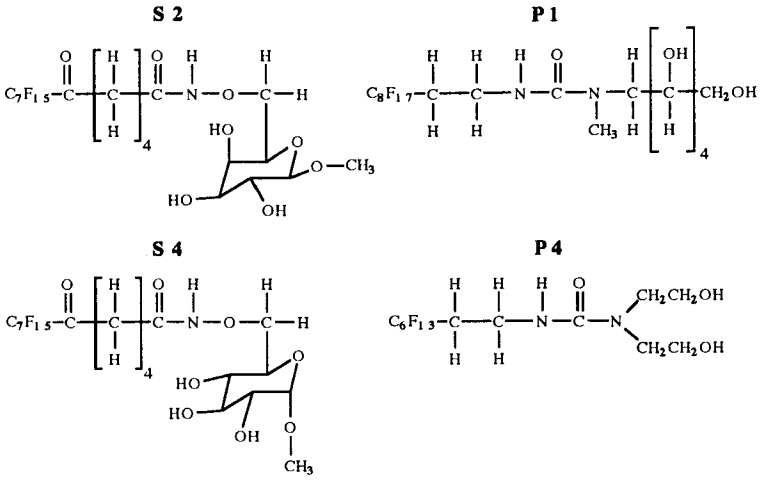

FIGURE 1. Chemical structures of novel fluoro-surfactants; 'S' compounds = glycoside derivatives; 'P' compounds = polyol derivatives. (from Ref. 34).

aggregation were comparable to those described recently for *Pluronic*® F-68 (35-37). The polyol fluoro-surfactants (e.g. compounds P1, P4 and P6), inhibited spontaneous human platelet aggregation and more closely mimicked the anti-thrombotic effects of *Pluronic*® F-68 than did their glycosidic-derived counterparts (e.g. compounds S2 and S4).

It has been proposed that the beneficial effects of *Pluronic*® F-68 in ischaemic injury (38) may be due, at least in part, to the inhibitory effects of this compound on platelet aggregation in the microvasculature. Thus, the beneficial effects of tissue perfusion with oxygen-carrying PFC emulsions containing *Pluronic*® F-68 (1,4) may also involve direct effects of the surfactant on platelets. Since some of the novel fluoro-surfactants, discussed above, may similarly have clinically-relevant anti-thrombotic effects, either alone or as constituents of PFC emulsions, this aspect should be a particular focus of future research effort in this area.

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