Injectable oxygenation therapeutics: evaluating the oxygen delivery efficacy of artificial oxygen carriers and kosmotropes in vitro

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ABSTRACT
The aim of this paper was to utilise an existing in vitro setup to quantify the oxygen offloading capabilities of two different subsets of injectable oxygenation therapeutics: (1) artificial oxygen carriers (AOCs), which bind or dissolve oxygen and act as transport vectors, and (2) kosmotropes, which increase water hydrogen bonding and thereby decrease the resistance to oxygen movement caused by the blood plasma. Dodecafluoropentane emulsion (DDFPe) was chosen to represent the AOC subset while trans sodium crocetinate (TSC) was selected to represent the kosmotrope subset. PEG-Telomer-B (PTB), the surfactant utilised to encapsulate DDFP in emulsion form, was also tested to determine whether it affected the oxygen transport ability of DDFPe. The in vitro set-up was used to simulate a semi closed-loop circulatory system, in which oxygen could be delivered from the lungs to hypoxic tissues. Results of this study showed that (1) 0.5 ml of a PFC outperformed 6.25 ml of a kosmotrope in a controlled, in vitro setting and (2) that PTB and sucrose do not contribute to the overall oxygen transportation efficacy of DDFPe. These results could be therapeutically beneficial to ongoing and future pre-clinical and clinical studies involving various oxygenation agents.

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Introduction
With respect to a variety of acute medical conditions including traumatic brain injury [1–3], haemorrhagic shock [2,4], stroke [5,6], and acute respiratory distress syndrome, intravenous therapeutics that are able to facilitate respiratory gas exchange have become a promising avenue for treatment as post-onset prophylactics. Though these injectable therapeutics serve to restore adequate oxygen delivery after acute hypoxic injury, the mechanism through which they facilitate tissue oxygenation can greatly vary. One subset of these oxygenation therapeutics is known as artificial oxygen carriers (AOCs), which physically carry and transport oxygen (O2) by either binding or dissolving diatomic O2 molecules. AOCs found their origin as haemoglobin based oxygen carrier solutions, due to the stability and efficacy of haemoglobin as an O2 carrier, and the field grew from there [7,8]. These O2 carriers are efficient drug delivery systems in that they are cyclic transport vectors and can repeatedly load and unload O2 for as long as they remain in circulation [9]. Moreover, in lower O2 partial pressure environments, AOCs with high O2 binding affinities may deliver even more O2 than red blood cells (RBCs) [7].

Within this field of AOCs, perfluorocarbons (PFCs) have a long-standing history of use and exploration as RBC substitutes. PFCs are highly fluorinated carbon chains with an ideal gas like chemical inertness, which allows for the dissolution of similarly inert gasses, such as O2, CO2, N2 and NO, into stable CF3 pockets formed by adjacent PFC molecules [9]. The efficacy of PFCs results from their increased O2 delivery capability, which takes priority over O2 absorption ability in vivo [10]. Consequently, although PFCs, which dissolve O2, possess a lower O2 affinity than RBCs, which physically bind O2, the combination of this lower O2 affinity in conjunction with higher diffusion rates, creates a reservoir of O2 that is more readily available for extraction by the tissues [11]. Additionally, PFCs preferentially dissolve CO2 over O2, which could aid in the targeted oxygenation of hypoxic tissues primarily undergoing anaerobic respiration and consequently, producing large quantities of CO2 [11,12]. In order to be administered intravenously as an effective therapeutic, PFCs must be encapsulated as a microbubble or nanodroplet in a lipid monolayer and stabilised in an emulsion—a process that has continuously been refined over decades. When improving the efficacy of PFCs, two main variables must be considered: (1) the PFC itself and (2) the PFC encapsulation surfactant. For this reason, third generation emulsions have focussed on optimising the O2 carrying capacity of PFCs, by increasing the CF3 group density [13] while decreasing the molecular weight and boiling point [14], and utilising surfactants such as PEG-Telomer-B (PTB), which decrease the reactivity of the PFC with surrounding material, allowing for increased shelf life and stability during in vivo circulation.
compounds that increase protein stability, reduce hydrophobic molecule stability, and form strong hydrogen bonds with water [15]. The mechanism of action of kosmotropes, with respect to O₂ delivery, is based on studies that identify blood plasma as accounting for nearly 70% of the resistance to O₂ movement experienced as O₂ diffuses from RBCs to tissue [16,17]. Due to their hydrophobic intermolecular forces, kosmotropes can form strong hydrogen bonds with surrounding water molecules, which make the water more structured on a microscopic scale, thereby decreasing its density. This physical change "opens up" the water phase to facilitate rapid O₂ diffusion and decrease plasma resistivity [18].

The aim of this experiment was to utilise an existing in vitro setup [12], capable of evaluating the O₂ offloading ability of a drug product in a hypoxic environment, to assess the relative efficacies of these two subsets of injectable oxygenation therapeutics in vitro. For the purposes of this experiment, dodecafluoropentane emulsion (DDFPe) was chosen to represent the AOC subset while trans sodium crocetinate (TSC) was selected to represent the kosmotrope subset. PTB, the surfactant used in DDFPe, was also tested to determine whether it affected the O₂ transport ability of DDFPe. The utilised setup allows for the simulation of O₂ transfer from the lungs to hypoxic tissues through a semi-closed circulatory system, with 0.9% saline serving as a blood plasma substitute. Testing drug products through such a setup allows for the evaluation of the relative efficacy of O₂ delivery while accounting for the effects of cyclic oxygenation and de-oxygenation of "blood" as seen in biological circulatory systems, as well as the additional pressures exerted by a semi-closed circulatory system [12].

Materials and methods

The in vitro set-up illustrated in Figure 1, established by Jayaraman et al. [12], was designed to simulate gas exchange and O₂ delivery from the lungs to hypoxic tissues. The O₂ off-loading capability of various stabilised solutions including DDFPe, TSC, and PTB was measured through a series of assays (Table 1) utilising the set-up depicted in Figure 1.

Preparation of product

Sodium chloride was purchased from Sigma Aldrich. Sodium sulphite was purchased from J.T. Baker. Purified water (18MΩ cm) from an in-house purification system was used as the diluent. Dissolved O₂ measurements were obtained using an Oakton DO110 metre. Silastic tubing (1.47 mm I.D. × 1.96 mm O.D.) was purchased from VWR. The finished medicinal product, 2% w/v dodecafluoropentane emulsion (DDFPe), manufactured by NuvOx Pharma (Tucson, AZ) was used [19]. Specifically, a 30% sucrose solution was
homogenised along with PTB and DDFP. The emulsion was processed using a semi-sealed, stainless steel containment system attached to an Avestin Emulsiflex-C50 homogeniser keeping the temperature below 8°C. The homogenates were then subject to terminal sterile filtration immediately prior to filling into 10ml vials. Particle sizing by Nycomp showed a mean particle size of approximately 250nm. The PTB + Sucrose solution was prepared by adding 0.779g of purified PTB into 150ml of a buffered sugar solution composed of sodium phosphate (Sigma Aldrich) and sucrose (Emprove Low Endotoxin Sucrose). Lastly, the stabilised TSC solution was prepared in accordance with the referenced United States Patent 6,060,511 by combining TSC with a mixture of 8% cyclodextrin (Sigma Aldrich) and 2.3% mannitol (Sigma Aldrich) [20].

**In vitro performance testing**

**In vitro model**

The *in vitro* oxygenation experimental setup has previously been described in detail by Jayaraman et al. [12]. The setup was designed to simulate the O₂ uptake by the blood in the lungs, the transport of O₂ by the blood to the tissues, and finally, the uptake of O₂ by the tissues from the blood. The fluid reservoir (80ml of 0.9% saline solution) represented O₂ uptake while the gas exchange vessel (900ml of 0.9% saline solution) was purposed for O₂ offloading (see Figure 1). Silastic™ tubing placed in the gas exchange vessel simulated the circulatory system by permitting gas exchange and offloading O₂ to areas of low O₂ concentration. Sparging with nitrogen gas was used to lower the dissolved O₂ concentration of saline in the gas exchange vessel to approximately 1.75mg/l. A continuous supply of nitrogen (2.0 standard ft³/minute) was also pumped into the headspace of the gas exchange vessel throughout the experiment to displace air since the vessel was not completely sealed. Once the sparging process was complete, the solution in the fluid reservoir received a constant influx of O₂ (2.0l/min). Fluid was pumped out of the reservoir through the silastic tubing with a peristaltic pump at a rate of 30ml/min. A galvanic dissolved oxygen (DO) sensing probe (DO 110 m, Oakton Instruments), which utilises a Clark electrode to assess the dissolved O₂ concentration, was used to monitor the O₂ concentration within the gas exchange vessel, which was recalibrated each day. Each assay was conducted over a 45-min duration at ~21°C.

**Mechanism of action study.** About 0.5ml of DDFPe was tested against 0.5ml of 0.9% Saline and 6.25ml of TSC (Table 1) to determine what effect the mechanism of action of the injected material has on its respective O₂ transport abilities. The doses of DDFPe and TSC tested were determined from clinical literature [18,21] for an assumed 80kg individual with 5l of blood, which translated to 80ml of saline in the fluid reservoir (Figure 1). These assays were conducted under the same experimental conditions as mentioned above. It is important to note, however, that the TSC solution experimentation was performed in a dark environment to minimise potential photodegradation.

**Artificial oxygen carrier (DDFPe) vs kosmotrope (TSC) equivalent dosage calculations:**

**Assumptions:**

- Average human blood volume = 5l
- Weight of patient = 80kg

DDFPe clinical dose: 0.10ml/kg (2% w/v DDFP) [21]

\[
\frac{2 \text{ mg DDFP (active ingredient)}}{\text{kg}} = \frac{2000 \text{ mg DDFP}}{100 \text{ ml DDFPe}} \times \frac{0.10 \text{ ml DDFPe}}{\text{kg}}
\]

Assuming 80 kg patient: \( \frac{0.10 \text{ ml}}{\text{kg}} \times 80 \text{ kg} = 8 \text{ ml DDFPe} \)

Clinical dilution for 1 l: \( \frac{8 \text{ ml DDFPe}}{5 \text{ l blood}} = \frac{1.6 \text{ ml DDFPe}}{1 \text{l blood}} \)

Plan to use 0.5ml DDFPe in the *in vitro* set up:

\[
\frac{0.5 \text{ ml DDFPe}}{80 \text{ ml saline}} = \frac{6.25 \text{ ml DDFPe}}{1 \text{l saline}}
\]

Scaling factor (ratio of *in vitro* to clinical dilution for 1 l): \( \frac{6.25}{80} = 3.906 \)

**TSC clinical dose: 25 mg TSC/kg [18]**

\[
\frac{25 \text{ mg TSC}}{\text{kg}} \times \frac{1 \text{ ml solution}}{20 \text{ mg TSC}} = \frac{1.25 \text{ ml TSC solution}}{\text{kg}}
\]

Assuming 80 kg patient: \( \frac{1.25 \text{ ml}}{\text{kg}} \times 80 \text{ kg} = 100 \text{ ml TSC solution} \)

Clinical dilution: \( \frac{100 \text{ ml TSC}}{5 \text{l blood}} = \frac{20 \text{ ml TSC}}{1 \text{l blood}} \)

Calculate amount of TSC for *in vitro* set up based on 3.906 scaling factor above:

\[
\text{In vitro dilution: } \frac{x \text{ ml TSC}}{80 \text{ ml saline}}
\]

Solve for \( x \) using TSC clinical dilution for 1 l:

\[
\frac{20 \text{ ml TSC}}{1 \text{l blood}} \times 3.906 = \frac{78.12 \text{ ml TSC}}{1 \text{l blood}}
\]

\[
\frac{78.12 \text{ ml TSC}}{1 \text{l blood}} \times \frac{1 \text{l blood}}{80 \text{ ml blood}} = \frac{6.25 \text{ ml TSC}}{80 \text{ ml blood}}
\]
Clinical and *in vitro* DDFPe and TSC dose comparison [22]:

**DDFPe clinical dose**:

\[
\frac{8 \text{ ml DDFPe}}{5 \text{ l blood}} = \frac{160 \text{ mg DDFP (active ingredient)}}{5 \text{ l blood}}
\]

**TSC clinical dose**:

\[
\frac{100 \text{ ml TSC solution}}{5 \text{ l blood}} = \frac{2000 \text{ mg TSC (active ingredient)}}{5 \text{ l blood}}
\]

**Scaled DDFPe in vitro dose**:

\[
\frac{0.5 \text{ ml DDFPe}}{80 \text{ ml saline}} = \frac{10 \text{ mg DDFP (active ingredient)}}{80 \text{ ml saline}}
\]

**Scaled TSC in vitro dose**:

\[
\frac{6.25 \text{ ml TSC solution}}{80 \text{ ml saline}} = \frac{125 \text{ mg TSC (active ingredient)}}{80 \text{ ml saline}}
\]

To account for most effective usage of material, a 0.5 ml dose of DDFPe was selected for experimentation. The scaling factor of the clinical dilution to the *in vitro* dilution of DDFPe was calculated to be 3.906. This scaling factor of 3.906 was then used to determine the equivalent *in vitro* dilution of TSC based on the current clinical dilution of TSC (20 ml TSC/11 l blood). This equivalent *in vitro* dilution of TSC was determined to be 6.25 ml TSC for experimentation. The clinical and *in vitro* dosages of DDFPe and TSC listed under the heading “Clinical and *In Vitro* DDFPe and TSC Dose Comparison” are meant to depict the differences in the magnitude of active ingredient administered with respect to each AOC.

**PTB + sucrose control study.** About 0.5 ml of a PTB + Sucrose solution was tested against 0.5 ml of DDFPe and 0.5 ml of 0.9% Saline (Table 1) to observe whether the O₂ offloading behaviour of DDFPe was affected by the presence of PTB and Sucrose in the emulsion during testing. These assays were conducted under the same experimental conditions as mentioned above.

**Statistical analysis**

A statistical analysis to determine the significance of experimental results was conducted on Excel *via* a two-tailed, two-sample unequal variance (heteroscedastic) t-test. A heteroscedastic t-test was chosen since the compared samples had different variances and sample sizes, and were observed and analysed from independent experimental runs. Data are expressed as means ± standard error of mean (SEM). Significance was defined as \( p \leq 0.05 \).

**Results**

**Mechanism of action study**

Figure 2 displays the O₂ offloading data for assays 1–4 (Table 1). Assay 1, the saline control run, exhibited the lowest net O₂ increase, while assay 2, the DDFPe experimental run, resulted in the greatest net O₂ increase within the gas exchange vessel. The “net” increase or decrease refers to the total change in the O₂ concentration as observed from the beginning to the end of the experimental run. In comparing the magnitudes of O₂ transfer of DDFPe and TSC over the course of a 45-min run, an injection of 0.5 ml of DDFPe resulted in a net O₂ concentration increase of 5.95 mg/l ± 0.283 mg/l, while an injection of 6.25 ml of TSC solution resulted in a net increase of 4.59 mg/l ± 0.187 mg/l. A standard t-test was used to verify the statistical significance of these results (\( p = 0.03798 \)).

When assessing the mechanism of oxygenation observed in assays 1–3, Figure 3 displays the average oxygenation curves for the tested compounds over the course of 45-min. Based on Figure 3, it appears that both DDFPe and TSC display a logarithmic oxygenation behaviour, with neither compound having reached its maxima within the 45-min period. However, the relative slopes of the curves imply that, given sufficient time, the maximal oxygenation potential of TSC is most likely lower than that of DDFPe.

**PTB + sucrose control study**

Figure 2 displays the O₂ offloading data for assays 1, 2, 3, and 4 (Table 1). Assay 2, the DDFPe experimental run, resulted in the greatest net O₂ increase within the gas exchange vessel, while assay 1, the saline control run, exhibited the lowest net O₂ increase. The PTB + Sucrose solution (Assay 4) exhibited no significant change in oxygenation when compared to saline over the course of 45 min. The net increase in O₂ concentration resulting from administration of DDFPe, PTB + Sucrose, and Saline were 5.95 ± 0.283 mg/l mg/l, 3.67 mg/l ± 0.103 mg/l, and 3.59 mg/l ± 0.088 mg/l, respectively.

**Discussion**

The results shown in Figure 3 demonstrate that, within the context of this *in vitro* simulation of hypoxia, AOCs appear to facilitate greater magnitudes of O₂ transfer at lower doses than kosmotropes. However, both compounds display statistically significant improvements in oxygenation when compared to the 0.9% saline control. Furthermore, the results in Figure 2 suggest that PTB and sucrose do not contribute to the overall O₂ transportation efficacy of DDFPe.

The diffusion of O₂ *in vivo* follows Fick’s Law, which explains that the rate of O₂ diffusion is dictated by three variables: (1) the plasma thickness, which refers to the distance that O₂ must diffuse over in order to reach the target cells, (2) the O₂ concentration gradient, and (3) the diffusion coefficient or diffusivity of O₂ [23]. However, plasma thickness is physiologically determined by the anatomy of the vasculature. Therefore, injectable oxygenation therapeutics seek to either increase the O₂ concentration gradient or increase the diffusion coefficient *in vivo* to encourage greater magnitudes of O₂ diffusion. PFCs fit into the former mechanism, while kosmotropes fit into the latter [24]. Figure 4 further illustrates...
this concept of how PFCs and kosmotropes theoretically affect normal O$_2$ transport \textit{in vivo}.

Under normal physiological conditions, O$_2$ transport is dictated by the Bohr Effect, which describes how haemoglobin cooperatively binds O$_2$ in higher pH environments and releases O$_2$ in lower pH environments caused by increased CO$_2$ tension [25]. The O$_2$ released by RBCs then passively diffuses through the blood plasma, based on oxygen tension (pO$_2$) gradients, in order to reach the cell membrane [26]—this is the major rate limiting step. At physiological pO$_2$, oxygen possesses an extremely low solubility in plasma. Furthermore, the O$_2$ extraction ratio (OER), which describes the extent to which RBCs release bound O$_2$, is only /C24 25% at rest. This indicates that under normal conditions only 25% of bound O$_2$ is released into the plasma, and even under conditions of hypoxia and exceptional metabolic stress, this ratio rarely, if ever, exceeds 75% [25]. This is in part due to an unstirred, stagnant boundary layer of plasma that forms as a

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**Figure 2.** Net oxygen offloading in mechanism of action comparative study. The net increase in oxygen concentration in the gas exchange vessel for assays 1–4 (Table 1). The error bars displayed reflect the SEM. The statistical analysis conducted was a two-tailed, two-sample unequal variance (heteroscedastic) t-test. p-Values are 0.01520, 0.03798, 0.03208 for Saline vs. DDFPe, DDFPe vs TSC, and Saline vs TSC, respectively. For the PTB$^+$ Sucrose Control study, p-values are 0.01472 and 0.57763 for DDFPe vs PTB$^+$ Sucrose, and Saline vs PTB$^+$ Sucrose, respectively.

**Figure 3.** Effect of administered drug product on oxygen transport curve trend. The increase in oxygen concentration in the gas exchange vessel in assays 1–4 (Table 1), over the course of 45 min. The Logarithmic trend in oxygenation can be observed from the graph below.
result of the turbulent flow through arterioles and capillaries [16,17]. These layers surrounding RBCs create local O₂ concentration gradients of lesser magnitude than that of the overall RBC to tissue O₂ concentration gradient, and therefore (1) limit the extent of O₂ released by RBCs and (2) increase the distance over which O₂ molecules must diffuse in order to reach the cell surface. Figure 4(A) depicts O₂ transport and diffusion under normal physiology, which is limited by low solubility and high resistivity experienced in blood plasma. Consequently, although an OER of 25% is sufficient for normal physiological function, in the event of an acute hypoxic event, even an elevated OER of 75% is often not enough to prevent tissue damage without the aid of molecules to facilitate transport through the plasma.

Figure 4(B) depicts O₂ transport and diffusion in the presence of PFC based artificial O₂ carriers. It was previously theorized that PFCs act as RBC substitutes due to their high affinity for O₂ without the trade-off of reduced bioavailability. However, in terms of mathematical modelling, the effects observed after administration of DDFPe far outweigh what is expected given the small dosage [10]. Consequently, it is more probable that PFCs act in conjunction with circulating RBCs to improve the efficiency of O₂ transfer, as opposed to acting independently as "superior RBCs". As seen in Figure 4(B), PFCs presumably act as an intermediate transport vessel for released O₂ in the vasculature. It is theorised that they improve the efficiency of O₂ transfer by increasing local O₂ gradients caused by unstirred layers, which in turn encourages RBCs to readily release more O₂. Circulating PFCs then dissolve released O₂ and facilitate O₂ transfer through the resistive plasma, and once at the tissue surface, the weak intermolecular forces experienced between PFCs and dissolved gases, and the larger surface-to-volume ratio of PFCs, are more favourable for gas exchange [12]. The results observed using this in vitro setup support that a small dose of PFC can demonstrate a significant increase in oxygenation.

Kosmotropes, contrastingly, facilitate O₂ transport by increasing the diffusion constant, also known as the diffusivity, of O₂ in plasma. They do so by decreasing the entropy of water molecules in the plasma, which thereby reduces the plasma density and decreases the resistance faced by O₂ molecules [18]. As previously mentioned, blood plasma is comprised of nearly 92% water. The intrinsic structure of water, and by extension plasma, is due to hydrogen bonds formed between adjacent H₂O molecules. Theoretically, a single water molecule should be able to form up to four hydrogen bonds simultaneously. However, in reality, this number averages between 2 and 3.6 [24]. Kosmotropes are order inducing molecules. TSC, for example, is a large hydrophobic molecule. Consequently, as illustrated in Figure 4(C), administration of a kosmotrope, such as TSC, decreases plasma entropy by interacting with similarly hydrophobic plasma components and therefore encourages the formation of additional hydrogen bonds between polar water molecules. This brings the average number of hydrogen bonds per water molecule closer to 4 [24], and this physical change in density "opens up" the water phase of the plasma allowing O₂ to diffuse towards the vascular wall more easily [18]. This change in density is illustrated in Figure 4(C) with the lighter plasma regions through which the O₂ is diffusing. This in vitro setup utilised 0.9% saline solution as a plasma substitute in simulating hypoxia. Consequently, the increase in oxygenation observed through these experiments supports that kosmotropes increase the diffusivity of O₂ primarily by affecting interactions between water molecules.

Additionally, these results indicate that the use of PTB as a surfactant to encapsulate PFCs does not beneficially nor detrimentally affect the oxygenation abilities of the PFC. The oxygenation capability of PTB was originally tested due to the similarities between the hydrophilic poly(oxyethylene) (POE) regions of Poloxamer 188 and PTB (see region x, Figure 5(A,B)). Poloxamer 188 is a surfactant that has shown success in mitigating the severity of acute chest syndrome episodes in sickle cell anaemia patients [27,28]. However, the mechanism through which Poloxamer 188 improves micro-vascular blood flow is thought to involve binding between the
hydrophobic core (see region y, Figure 5(A)) of Poloxamer 188 and adjacent hydrophobic RBC and neutrophil cell surface regions, which appears to block extraneous hydrophobic adhesive interactions in the bloodstream and thereby reduce the blood viscosity. This allows the hydrophilic POE chains (see region x and z, Figure 5(A)) free to interact with the surrounding media [28]. PTB shares the same hydrophilic POE chain (see region x, Figure 5(B)) as Poloxamer 188, however, it lacks the hydrophobic core. Instead, PTB’s chemical composition includes a fluorinated tail (see region y, Figure 5(B)). Consequently, based on these in vitro experiments where the hydrophobic silastic tubing is representative of the vasculature, it appears that the chemical similarities between the poly(oxyethylene) hydrophilic units in Poloxamer 188 and PTB, do not amount to the same capabilities of facilitating O₂ transfer in vitro. Rather these in vitro results support the supposition that the success of Poloxamer 188 in vivo is mechanistically more accredited to the hydrophobic core of Poloxamer 188 than its hydrophilic chains.

The results of this study show that 0.5 ml (10 mg DDFP) of a PFC outperformed 6.25 ml (125 mg TSC) of a kosmotrope in a controlled, in vitro setting. It should be stated that the numerical O₂ offloading concentrations measured in this in vitro model cannot be scaled quantitatively to an in vivo situation due to the physiological limitations of an acellular, in vitro model. However, the trends observed in the in vitro model may still be biologically relevant.

With respect to both PFCs and kosmotropes, it would be pertinent to utilise whole blood in this in vitro model as both classes of molecules rely heavily on blood components mechanistically. In terms of PFCs, in these experiments, it is very likely that DDFPe did not display its full oxygenation potential due to the lack of RBCs present. The use of whole blood, therefore, could account for synergistic oxygenation effects of RBCs in tandem with PFCs. Whereas for kosmotropes, the use of whole blood would create a more representative model of the extent to which these molecules can alter the density of plasma and consequent diffusivity of O₂ in vivo. Additionally, the use of whole blood would provide a clearer sense, in general, of how resistive blood plasma is to O₂ movement in a simulation of extreme hypoxia. Furthermore, it would be of interest to include a test group in which DDFPe and TSC were administered together. Future in vitro studies will aim to incorporate such cellular components in order to create a more representative in vivo mechanistic model.

**Disclosure statement**

Meghna Jayaraman is an employee at NuvOx Pharma. Kaitlin Graham is an employee of NuvOx Pharma and owns stock in the company. Dr. Evan Unger is President and CEO of NuvOx Pharma, serves on the Board of Directors, and owns stock in the company. Dr. Unger is also a patent holder of the NuvOx Pharma technology. The authors report no other conflicts of interest in this work.

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**Data availability statement**

All data supporting the results or analyses presented in the paper reside at NuvOx Pharma. For further information please contact the corresponding author.

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