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Increased oxygen solubility in aqueous media using PEG-poly 2,2,2-trifluoroethyl methacrylate co-polymer micelles and their potential application as volume expanders and as an artificial blood product.

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ABSTRACT

One of the most important functions of blood is to solubilize and distribute oxygen within the body. As such, it is vital that this property is replicated (safely) by any artificial blood product. In this paper, we describe the facile synthesis of a series of simple diblock polymers capable of self-assembling into micellar structures at concentrations around 3×10^{-3} mg/mL. Using a dissolved oxygen meter, we were able to demonstrate that aqueous solutions of these aggregated structures could retain oxygen and release it (into the aqueous bulk phase). The increased oxygen retention was quantified by measuring the rate of oxygen release and its half-life. These experiments indicated that oxygen retention/binding was dependent on the fluorine concentration. ^{19}F NMR experiments on a micellar solution saturated with oxygen showed small upfield shifts in the fluorine peaks, which provided qualitative evidence that indicated oxygen binding occurred within the fluorine region of the polymer aggregates. Using a modified enzyme/glucose oxidation assay, we were able to establish that the aqueous oxygen concentrations were 33% higher in a solution of polymer.

KEY WORDS

Amphiphilic fluorine co-polymer, micelle, artificial blood, oxygen binding/release.

INTRODUCTION

Oxygen makes up nearly 21% of air and is a useful gaseous reagent within the chemical industry.[1,2] Oxygen is also indispensable to the life of all of human tissues and is transported around the body by blood.[3] Specifically, oxygen is bound to one of four central porphyrin macrocycles held within the globular protein hemoglobin, which is contained within red blood cells. Blood loss can be fatal and rapid replacement and restoration of oxygen transport is often the first thing required in a medical emergency. Traditionally this is achieved using transfusion of donated blood, but this is expensive, has problems with contamination/storage and is not practical or ideal in a challenging trauma situation, *i.e*. the scene of an accident, natural disaster or on the battlefield. In these situations, plasma (an aqueous solution of protein, clotting factors, glucose and electrolytes) is given principally to replace the volume of blood lost. A better solution would be to administer an alternative plasma solution that could act as a volume expander and replace the lost volume of blood, but could also bind deliver and release oxygen. In effect; a *minimal* blood substitute, or artificial blood product. There have been numerous attempts to develop artificial blood; including biological methods that utilize cell-free hemoglobin and synthetic methods that mimic the oxygen binding mechanism and oxygen-carrying capacity of hemoglobin. These include simple porphyrin analogues, as well as macromolecular systems that possess porphyrin moieties within their structures. However, the most successful blood substitutes with respect to clinical trials and application are the perfluorocarbons, which do not mimic Nature's oxygen binding mechanism. Instead, perfluorocarbons trap and bind oxygen in the non-polar spaces within their assembled structures. However, perfluorocarbons are not miscible with water and must be prepared as emulsions before use as a blood substitute. In addition, there are major problems associated with their clinical use. Limitations include instability of the emulsions, complicated regimes for preparation, storage and use, and excessively long half-lives, which can be as high as 1-2 years.[4,5] It has also been observed that around two-thirds of the injected

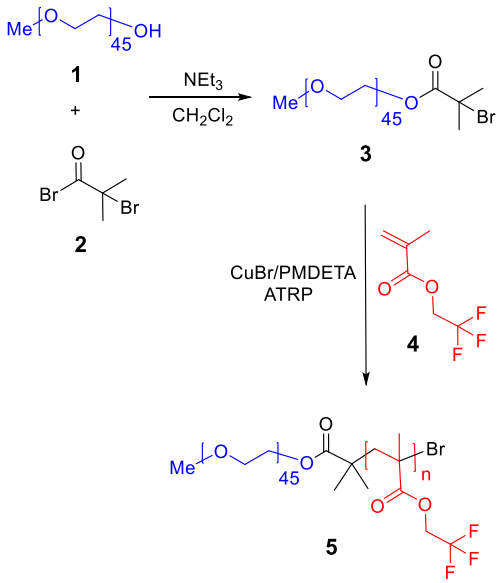
perfluorocarbon becomes trapped within tissue, mainly within the lungs.[6] In addition, the specific surfactants used to solubilize the perfluorocarbons have been associated with severe side effects, including myalgia and fever,[7] a decrease in platelet count [8] and possible anaphylaxis.[9]

Our principle aim was to develop a system that could overcome the problems of using small perfluoro compounds that necessitated the use of emulsions. To achieve this we decided to synthesize and test a series of PEG-polyfluoro diblock copolymers that could form micelles in aqueous media. As such, the self-assembled micellar structures would possess a fluorine rich micellar core that could dissolve oxygen, leading to an increased oxygen concentration in water. A number of fluorine containing polymers and diblock polymers have been reported. These included water-soluble pentafluorophenyl end-capped PEG polymers [10] whose lower critical solution temperatures were sensitive to the gases dissolved within the solution. In other work, fluorine containing polymeric micelles were shown to have distinct CO_2 and O_2 responsiveness in aqueous media.[11,12] However, little work has been directed at investigating and quantifying their oxygen binding and release properties.

RESULTS AND DISCUSSION

To achieve our aims we required a diblock polymer that was biocompatible and contained a core block that was rich in fluorine. Our *prototype* design was relatively simple and would include a solubilizing PEG block as a macroinitiator for an ATRP synthesis [13,14,15] involving a monomer rich in fluorine. The synthesis is shown in Scheme 1 and involves the initial functionalization of Me-PEG-2000 **1** with α-Bromoisobutyryl bromide(BIBB) **2** to generate the macroinitiator **3**. This was then reacted with various amounts of 2,2,2- Trifluoroethyl methacrylate **4** in the presence of CuCl and the ligand N,N,N',N'',N''-Pentamethyldiethylenetriamine (PMDETA), to generate a series of fluorinated polymers (PEG-poly 2,2,2-trifluoroethyl methacrylate/PEG-PTFEMA) **5** with different degrees of

polymerization (DP). GPC using RI detection generated chromatograms that showed a degree of bimodal character and higher than expected polydispersities. However, this is typical of diblock polymers that possess polymer blocks with *very* different refractive indices.[16] For example, Armes *etal* also reported bimodal peaks when studying the GPC properties of a series of fluoro containing diblock polymers.[17] In our case the refractive index of the PTFEMA block is 1.41 and this is much lower than the refractive index of poly(ethyleneglycol), which is 1.47.[18] This leads to a significant underestimation of the fluorinated polymers signal intensity and an exaggeration of the apparent contamination by the PEG macro-initiator.[17]



Scheme 1: *ATRP synthesis of PEG-poly(trifluoroethyl methacrylate) polymers (PEG-PTFEMA).*

The GPC data for our polymers is shown in Table 1. ^{1}H NMR using deuterated chloroform as the solvent confirmed that the targeted DP had been achieved, by comparing the integration value of the PEG peaks at 3.66 ppm with the CH_2 and CH_3 peaks at 4.36 ppm and 3.40 ppm respectively. When the ^{1}H NMR spectrum of the polymers was repeated in deuterated water, a very different series

of spectra was obtained. In these aqueous solutions and at the concentrations studied (1×10^{-3} M), all we could observe were the peaks corresponding to the PEG protons. This is typical behavior for PEG based micellar structures and indicates aggregation of the diblock polymer and congestion within the core. This congestion restricts and slows down any free-motion/rotation of the fluoro monomers, resulting in coalescence (with respect to the NMR timescale).[19,20] A similar result was observed for the ^{19}F NMR, where a strong peak at -73.3 ppm occurred in $CDCl_3$, but only a very weak signal was visible in the D_2O spectrum at -73.1 ppm (1×10^{-3} M in both solvents).

Copolymer	Targeted DP	Actual DP *	Mn (NMR)	Mn(GPC)	PDI	CMC mg/ml
PEG-PTFEMA-17.	15	17	5000	5200	1.42	0.00355
PEG-PTFEMA-25.	25	25	6350	6600	1.55	0.00362
PEG-PTFEMA-33.	35	33	8050	8550	1.53	0.00239
PEG-PTFEMA-45.	45	50	10550	11400	1.59	/

***Table 1**: Molecular weight (GPC/NMR), DP and aggregation data for PEG-PTFEMA polymers.*

Aggregation and micellation were confirmed and quantified through critical micelle concentration (CMC) experiments. These were performed using a fixed concentration of pyrene and measuring changes in emission intensity with respect to increased polymer concentrations.[21] Plots of polymer concentration vs. changes in pyrene emission were used to obtain the CMC values (Table 1), which ranged from 0 .0024 to 0.0036 mg/mL and are typical of those reported for PEG based polymeric micelles.[22,23] Dynamic light scattering experiments were performed above and below the CMC. Whilst no significant peaks could be detected below the CMC, solvated particles with an average size of 80-100 nm were observed when the measurements were taken at a concentration above the CMC (0.1 mg/mL). The formation of micelles was confirmed by microscopy (TEM), which showed spherical particles with diameters around 30-50 nm, Figure 1.

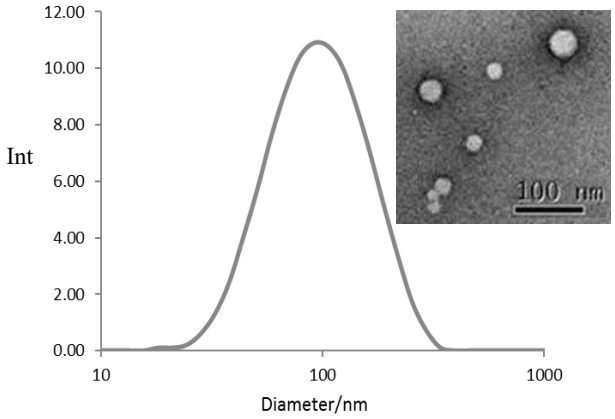


***Figure 1**: DLS trace for PEG-PTFEMA-25 and TEM (insert).*

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Oxygen incorporation was initially studied using ^{19}F NMR, which can be used to probe where oxygen binds within the micelle.[24] Specifically, ^{19}F NMR was used to investigate any effect on the chemical shift of the fluorine groups (of **PEG-PTFEMA-25)**, in the presence and absence of oxygen. To some extent, this was difficult, as the highly restricted mobility within the aggregated micelle results in strong dipolar coupling, leading to a significant attenuation of the ^{19}F NMR signal in D_2O (discussed above).[19] Nevertheless, it was still possible to observe a small peak at -73.1 ppm in the degassed sample. After bubbling oxygen through the same solution, a small but recordable shift of the fluorine peak, to -72.1 ppm, was observed. This represents a shift of 1 ppm, which is similar to the shifts observed for perfluoro emulsions under similar aqueous conditions.[24] Although this method was qualitative, it did provide some initial encouragement that the oxygen can be encapsulated/bind within the fluorine core/block of the polymeric micelle.

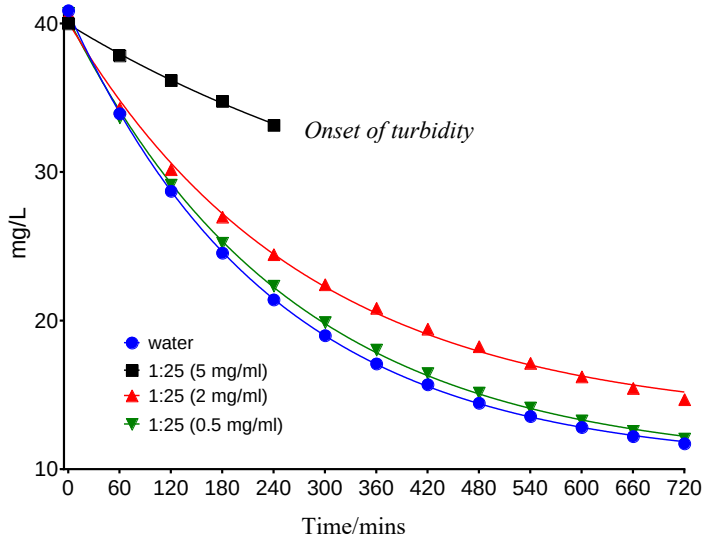

***Figure 2**: Oxygen release curves at various concentrations for PEG-PTFEMA-25.*

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To obtain support that is more conclusive for oxygen binding, we used a commercial dissolved oxygen meter to determine the concentration of oxygen dissolved in an aqueous solution. This is an electrochemical technique and can only measure the amount of oxygen dissolved within bulk water.[25] A dissolved oxygen probe/meter cannot directly measure or detect any oxygen dissolved within other species, such as a micelle. However, if we saturate a micellar solution with pure oxygen, we can measure the rate that oxygen in the solution equilibrates with the air, where the concentration of oxygen is much lower (around 20% in air). This rate can then be compared with a simple aqueous solution that does not contain micelle. Any differences in rate will be related to the oxygen bound within the micelle, which can only be released when the oxygen dissolved in the saturated aqueous solution returns to the atmosphere. This is an established method and has previously been used to measure oxygen content and release from perfluoro systems [26] and fluoro-polymers.[10, 27] The measurements were carried out using solutions of the micelle at various concentrations. These were

stirred vigorously in vials and pure oxygen bubbled through the solution for one minute (until the dissolved oxygen concentration had reached the saturation level of 40 mg/L). The vials were then opened and the oxygen content continuously monitored over time. All experiments were carried out using the same volume of water and at the same temperature (20 °C). The oxygen release from water, along with a representative plot for **PEG-PTFEMA-25**, are shown in Figure 2.

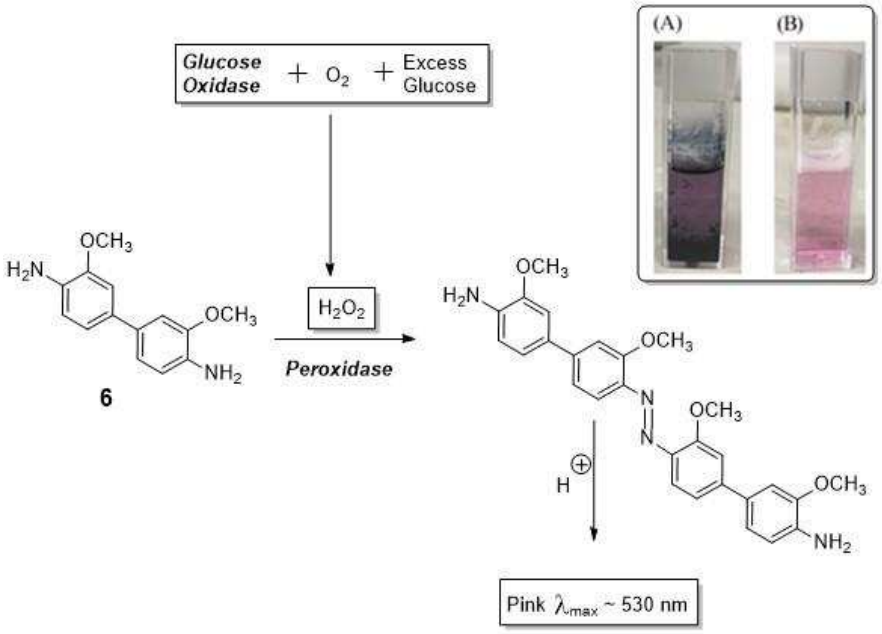
Polymer System	Concentration-mg/mL	Rate of O_2 release – $\times 10^{-3}$ mg/mL/min	Half-life-mins
Water (no polymer)	/	4.2	160
PEG-PTFEMA-33	0.5	2.9	240
PEG-PTFEMA-25	0.5	3.9	180
PEG-PTFEMA-17	0.5	4.2	165
PEG-PTFEMA-25	2.0	3.6	195
PEG-PTFEMA-17	2.0	3.8	182
PEG-PTFEMA-17	5.0	2.1	325

***Table 2**: Oxygen release rates and half-lives for the PEG-PTFEMA polymers.*

Initially, the release of oxygen from a saturated aqueous solution was studied (no polymer) at 20 °C. The rate of release and the half-life was calculated from a plot of oxygen concentration *vs* time, which could be fitted to a first order decay, Figure 2. A second control was also carried out using just the PEG component in water. The release of oxygen from the PEG solution was indistinguishable to the aqueous control, producing an identical plot and kinetic data. This confirmed that at the concentration studied (5 mg/mL), the PEG component had no positive effect on the solubilization of oxygen, which is consistent with previous studies on the oxygen solubilization within PEG solutions.[28] Having established the controls and base line levels, the experiment was repeated using the PEG-PTFEMA diblock copolymers at various concentrations. The solubility of the polymers was relatively poor and dependent on the length of the fluoro-block, which limited the amount of polymer

that could be used. For example, although **PEG-PTFEMA-17** was the most soluble, it was hard to solubilize at concentrations above 5.0 mg/mL. The larger **PEG-PTFEMA-33** sample was the least soluble and was only soluble at concentrations below 1.0 mg/mL. **PEG-PTFEMA-25** solutions could be made to a maximum solubility of 5.0 mg/mL, but over time, a colorless precipitation was noticed. As a result, the solution became turbid during the oxygen release experiments, which limited the reliable data that could be collected (see Figure 2). Precipitation was also observed by others when studying similar polymers, where phase changes occurred in the presence of oxygen.[11,12] Nevertheless, despite the precipitation of **PEG-PTFEMA-25** at the higher concentration (which limited the amount of data that could be collected), it does indicate good oxygen retention/solubility for these polymers at the highest concentration studied (corresponding to the highest concentration of fluorine). For all solutions, the change in oxygen concentration was plotted with respect to time and the data fitted to a first order decay.[29] The rate of oxygen release, along with the half-lives for all soluble polymers is shown in Table 1. The graphs and kinetic data from the polymer solutions clearly show that oxygen is released more slowly, when compared to the rate of oxygen release from the simple aqueous solution (no polymer). This confirms that oxygen has been retained within the micelle and is only released into the aqueous phase when it can replace the dissolved oxygen as it returns to the atmosphere. The process continues until the equilibrium position is reached, which occurs at an oxygen concertation around 9 mg/L at 20 °C.[30] At the higher concentrations, longer half-lives and slower rates (of oxygen release) were observed. For example, **PEG-PTFEMA-17** at a concentration of 5.0 mg/mL had a half-life that was double the value recorded at 0.5 mg/mL. If we consider *all* polymers at *each* concentration, we observe that half-lives and rates of release are dependent on the length of polyfluoro block. For example, at a concentration of 0.5 mg/mL **PEG-PTFEMA-33** had the longest half-life and slowest rate of release. Overall, it is clear that the ability to dissolve/bind oxygen is directly related to the amount of fluorine in solution.

In a further effort to establish and quantify oxygen binding, we attempted to measure the oxygen concentration using an enzyme-based method. The method was originally developed by Ghosh [31] and subsequently refined for Marrucho.[32] This method is based on the oxidation of glucose by molecular oxygen, and is catalyzed by glucose oxidase. Although developed to measure glucose concentration in the presence of excess oxygen, the method has been adapted to measure the concentration of molecular oxygen when glucose is the excess reactant. The reactions are stoichiometric with respect to glucose and oxygen and can be used to measure the oxygen content in solution. The method is well established and the reagents are available in the form of a glucose assay kit (purchased from Sigma–Aldrich). As with the published procedures,[31,32] air was used instead of oxygen as there is no significant difference in solubility and it simplifies the experimental procedure.



Scheme 2: Enzyme mediated reaction used to estimate oxygen concentrations in water. Reactions performed using excess glucose. Insert shows; (A) the dark precipitate that initially forms within the polymer solution after the oxidation reaction and (B)

The assay were performed at 37 °C and the reactions are shown in Scheme 2. At this temperature, the solubility of oxygen in water is around 6.0 mg/L. As such, a glucose concentration of 55 μg/mL was used (equivalent to 10 mg/L of oxygen) as this was found to be optimum with respect to the experimental conditions.[33] The reaction was initially performed in just water to give a good baseline/control reading. However, when the assay was repeated using a 5 mg/mL aqueous solution of **PEG-PTFEMA-25**, a colored precipitate formed, which precluded an accurate determination of absorption and prevented us from making a comparison with the baseline/control reading. However, filtering or centrifuging the solution resulted in absorptions that were lower than those recorded for water. We assumed that the dye was bound or trapped on the precipitate, reducing its concentration in the water. This assumption proved correct, as over time, the precipitate settled and the solution's color became more intense as the dye was released. Solutions (baseline/control and polymer) were therefore left to settle for 12 hours before filtering (see insert, Scheme 2). The UV spectrum of the two solutions recorded and the absorption intensity at 535 nm compared. The result indicates that the polymer solution has an absorption that is higher than the similarly treated aqueous solution (without polymer), Figure 3. Comparing the intensities of the two peaks, we can estimate a minimum oxygen concentration that is 33% higher than that determined for water alone. This increase in absorption intensity corresponds to an oxygen concentration of around 8-9 mg/L at 37 °C.[34] Although precipitation caused problems, the enzyme method qualitatively supports the results obtained from the NMR and dissolved oxygen experiments. Taking all of the results together, we can conclude that the polyfluoro micelles can increase the concentration of oxygen within water.

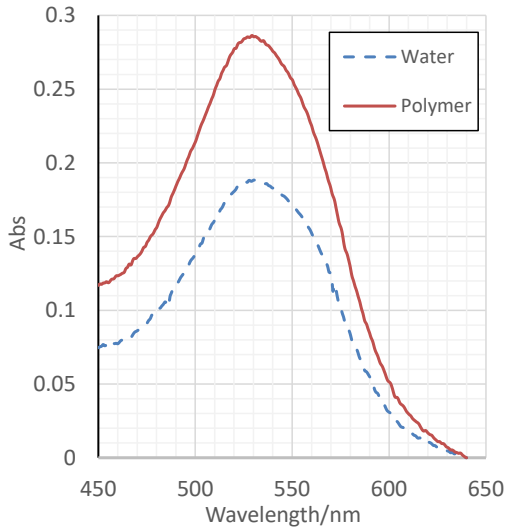


***Figure 3**: UV results from enzyme assay. The data shows a stronger absorption for the PEG-PTFEMA-**25** solution, indicating a higher oxygen concentration.*

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CONCLUSIONS

The aim of this work was to synthesize and test the oxygen binding potential of a polyfluoro amphiphilic micelle. The synthesis involved an ATRP process and was relatively straightforward. Using this method, we were able to obtain a series of polymers possessing a PEG-2000 block and various fluorinated blocks, with degrees of polymerization equal to 17, 25, 33 and 45. With the exception of the polymer with the largest fluoro-block, all polymers were soluble in water. At a given concentration, the solubility was inversely proportional to the length of the fluoro-block. All of the soluble polymers self-assembled into micellar structures with CMC values around 3 x 10^{-3} mg/mL. DLS indicated that the aggregates had a solvated diameter of 90-100 nm. Micellar structures were confirmed by microscopy, which showed that micellar structures, with a diameter of 30-50 nm, had formed. The aggregates formed were able to bind oxygen as demonstrated by oxygen release kinetics, from an oxygen saturated aqueous solution. The rates of oxygen release, and hence oxygen binding,

were dependent on the amount of fluorine present, with higher concentrations and/or polymers possessing more fluorine, retaining oxygen the longest. This was supported by qualitative ^{19}F NMR data in D_2O, which showed an upfield shift of the fluorine resonance in the presence of oxygen. As well as supporting oxygen binding, this experiment also indicates that the oxygen has bound within the polyfluoro core of the micelle. In addition, the use of an enzyme assay indicated that the polymer could dissolve 33% more oxygen than water alone. However, significant loss of dye during the precipitation meant that the concentration of dissolved oxygen could be higher. Taking these results together, we can conclude that aggregates of simple fluorine containing amphiphilic polymers can increase the concentration of oxygen within an aqueous solution. As such, these polymers (or related systems) have the potential to be applied as a simple artificial blood product. Although they will never be able to reproduce all of the functions of blood, they may be able to offer a significant advantage over standard plasma solutions: whose primary function is to replace the *volume* of blood lost during bleeding in an emergency or disaster situation. In addition to replacing lost volume, these (or related) polymers will also be able to transport and deliver oxygen. Work is progressing within our laboratory to exploit the oxygen binding properties of these and related polymers for use in medical and related applications.

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We thank the University of Sheffield for providing funding scholarship (M. F.) and King Saud Bin Abdulaziz University for Health Sciences for a scholarship (T. A).

SUPPORTING INFORMATION

Synthetic details, characterization data, experimental procedures, representative NMR spectra, representative GPC trace, representative CMC plot and additional oxygen release plots

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(33) A glucose concentration of 50-60 μg/mL produced absorption peaks that were identical in profile to those obtained in water (see Figure 3). Polymer experiments using higher glucose concentrations produced broad peaks that were of limited usefulness when comparing to the baseline/control spectra.

(34) The oxygen concentration determined in this experiment was close too, but below the maximum possible oxygen concentration (10 mg/L) that could be recorded for the glucose concentration used (55 μg/mL).

TABLE OF CONTENTS GRAPHIC

Increased oxygen solubility in aqueous media using PEG-poly 2,2,2-trifluoroethyl methacrylate co-polymer micelles and their potential application as volume expanders and as an artificial blood product.

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