Journal of Fluorine Chemistry 129 (2008) 1096–1103

Contents lists available at ScienceDirect

Journal of Fluorine Chemistry

journal homepage: www.elsevier.com/locate/fluor

Fluorinated derivatives of a polyaspartamide bearing polyethylene glycol chains as oxygen carriers

Giovanna Pitarresi [a,*], Antonio Palumbo Piccionello [b], Rossella Calabrese [a], Andrea Pace [b], Silvestre Buscemi [b], Gaetano Giammona [a]

[a] Dipartimento di Chimica e Tecnologie Farmaceutiche, Università di Palermo, Via Archirafi 32, 90123 Palermo, Italy
[b] Dipartimento di Chimica Organica "E. Paternò", Università di Palermo, Viale delle Scienze-Parco D'Orleans II, Ed. 17, 90128 Palermo, Italy

ARTICLE INFO

Article history:
Received 14 May 2008
Received in revised form 22 July 2008
Accepted 24 July 2008
Available online 8 August 2008

Keywords:
Artificial oxygen (O_2) carrier
α,β-Poly(*N*-2-hydroxyethyl)-DL-aspartamide
Polyethylene glycol
1,2,4-Oxadiazole
Fluorinated polymeric micelles

ABSTRACT

In this paper the synthesis and characterization of new fluorinated polymers based on a polyaspartamide bearing polyethylene glycol (PEG) chains, are reported. The starting material was the α,β-poly(*N*-2-hydroxyethyl)-DL-aspartamide (PHEA), a water soluble and biocompatible polymer, that has been derivatized with both polyethylene glycol (with a molecular weight of 2000 Da) and 5-pentafluorophenyl-3-perfluoroheptyl-1,2,4-oxadiazole. By varying the amount of the fluorinated oxadiazole, three samples have been prepared and characterized by FT-IR, ^{1}H NMR, ^{19}F NMR and UV–VIS spectroscopy. Size exclusion chromatography analysis of these copolymers revealed the occurrence of a self-association process in aqueous medium. The value of critical aggregation concentration has been evaluated by performing a tensiometric study, whereas the size of these aggregates has been determined by photon correlation spectroscopy. Oxygen solubility studies in aqueous solutions of these fluoropolymers showed their ability to maintain high oxygen levels in solution. The biocompatibility of these fluoropolymers has been evaluated by performing an *in vitro* viability assay on human chronic myelogenous leukaemia cells (K-562), chosen as a model cell line, and haemolysis experiments on human red blood cells. All these properties suggest the potential use of these fluoropolymers as artificial oxygen carriers.

1. Introduction

Artificial oxygen carriers are an alternative to donor blood transfusions during surgery and can be used to improve tissue oxygenation and functions of organs with marginal oxygen supply. They are currently grouped into two classes: (1) modified haemoglobin solutions (e.g. outdated human blood, bovine haemoglobin, etc.); (2) perfluorochemical emulsions (e.g. Fluosol®, Perftoran®, Oxygent®, etc.) [1–4]. However, even if both classes are effective for improving tissue oxygenation and as blood substitutes, they may present some disadvantages. For instance, vasoconstriction which causes an increase in systemic and pulmonary artery pressure as well as a reduction in cardiac output, have been observed with modified haemoglobin. On the other hand, fluorocarbons are virtually immiscible in water, therefore they must be administered as emulsions that cause side effects dependent on the size distribution of the droplets and the presence of emulsifiers not tolerated by patients [5–7].

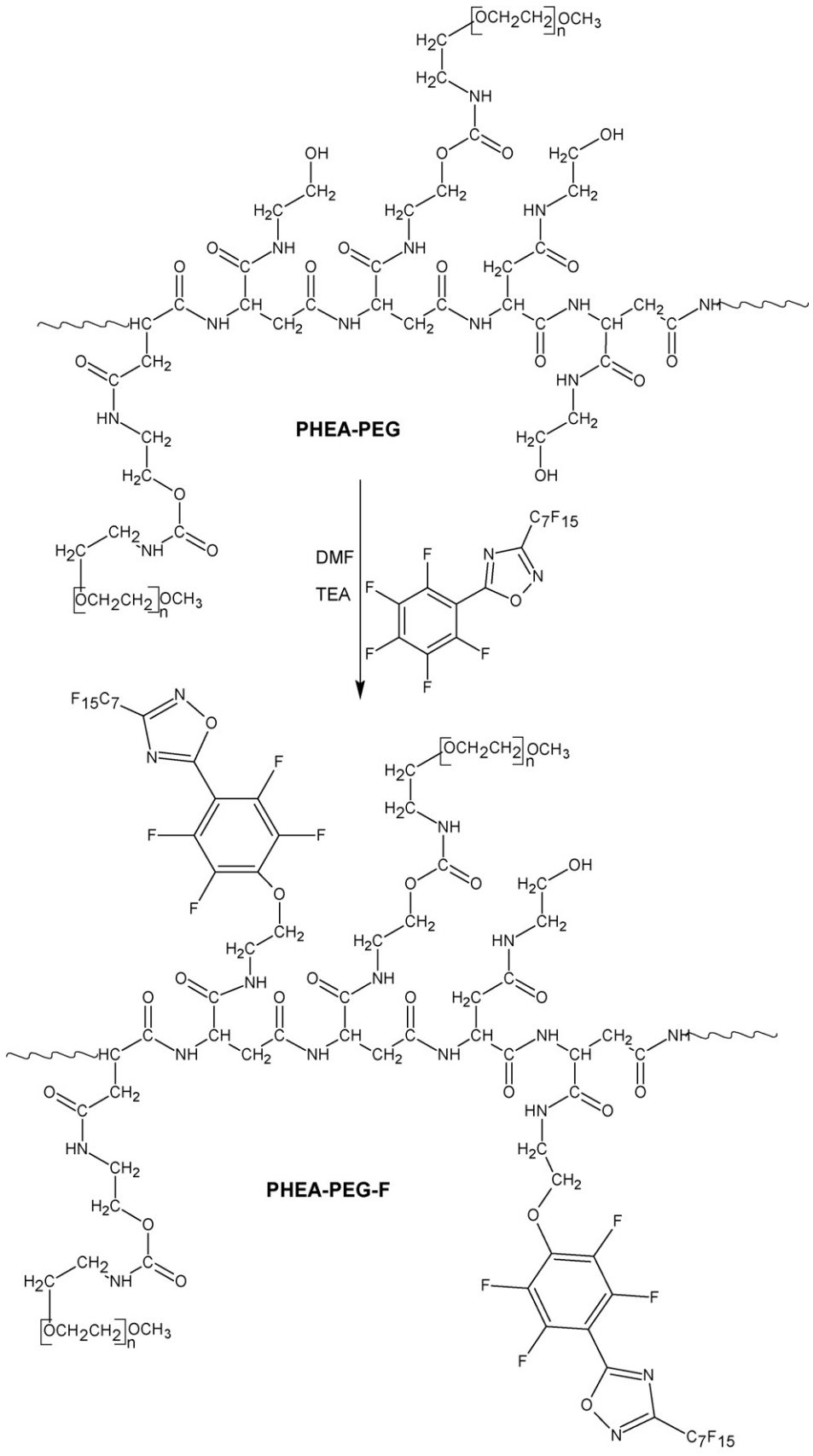
In principle, polymers containing fluorine atoms could be used as artificial oxygen carriers, however, the extremely low water solubility limits their application in this field.

Due to their unique properties like low coefficient of friction, low surface tension, oil and water repellency and non-adhesive nature, fluorinated polymers presently find various applications such as antithermal, lubricant, coating and electric insulating materials [8]. Only recently, we have reported the synthesis and characterization of a new water soluble fluorinated polymer, proposing its use as a potential oxygen carrier [9]. This polymer has been obtained by partial modification of a biocompatible synthetic polyaspartamide, such as α,β-poly(*N*-2-hydroxyethyl)-DL-aspartamide (PHEA) with 5-pentafluorophenyl-3-perfluoroheptyl-1,2,4-oxadiazole **1**. The obtained sample, named PHEA-F showed a dissolving oxygen capacity greater than PHEA. This ability, together with the absence of haemolytic effect, makes this new fluoropolymer a very attractive candidate as oxygen carrier for parenteral administration as aqueous solution, thus overcoming the principal drawbacks of perfluorocarbons administered as oil-in-water emulsions.

In order to increase the water solubility or the degree of fluoroderivatization of this kind of fluoropolymers we decided to

* Corresponding author. Tel.: +39 6236154; fax: +39 6236150.
E-mail address: giopitar@unipa.it (G. Pitarresi).

0022-1139/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jfluchem.2008.07.024



Scheme 1. Synthesis of a typical PHEA-PEG-F copolymer.

prepare novel derivatives, based on PHEA and oxadiazole **1**, in which polyethylene glycol (PEG) chains could be linked as pendants to increase the water affinity. The use of PEG is justified since it is a well-known synthetic polymer, approved by FDA and used for various applications in pharmaceutical fields as co-solvent, excipient in conventional dosage forms, coating material for colloidal carriers with longer blood circulation time [10–12]. The chemical and biological characterization of PHEA-PEG-F copolymers, their ability to dissolve oxygen and their self-association capacity are here discussed.

2. Results and discussion

Due to the presence of hydroxyl groups readily amenable to chemical modification, α,β-poly(*N*-2-hydroxyethyl)-DL-aspartamide has been derivatized with both polyethylene glycol chains (to introduce hydrophilic chains) and fluorinated oxadiazole **1**.

First, hydroxyl groups of PHEA have been activated by bis(4-nitrophenyl) carbonate (PNPC) (added so as to have a ratio *X* = moles of PNPC/moles of PHEA repeating units equal to 0.1) then it was made to react with *O*-(2-aminoethyl)-*O*′-methyl polyethylene glycol 2000 (PEG_{2000}). The reaction between activated PHEA and PEG was performed in anhydrous *N*,*N*-dimethylformamide (DMF), for 2.5 h at 60 °C (see Section 4). The copolymer PHEA-PEG, recovered after purification by washing with organic solvents and dialysis against twice-distilled water, resulted to have a degree of derivatization percentage in PEG chains linked to PHEA, equal to 7.0 ± 0.5 mol%.

PHEA-PEG thus obtained, was reacted in anhydrous DMF with the 5-pentafluorophenyl-3-pentadecafluoroheptyl-1,2,4-oxadiazole **1**, able to undergo aromatic nucleophilic substitution (SN_{Ar}) by the side chain hydroxyl groups of the PHEA (Scheme 1). Three different PHEA-PEG-F copolymers have been obtained, named as PHEA-PEG-F1, PHEA-PEG-F2 and PHEA-PEG-F3 copolymers, for *X* = 0.4, 0.6 and 1, respectively, being *X* the ratio between moles of 5-pentafluorophenyl-3-perfluoroheptyl-1,2,4-oxadiazole **1** and moles of PHEA-PEG repeating unit.

In all the cases, triethylamine (TEA) has been used in an equimolar ratio with oxadiazole **1** (*Y* = 1, see Section 4).

All PHEA-PEG-F copolymers, after purification by washing with diethyl ether and acetone and dialysis against twice-distilled water, have been characterized by spectroscopic analyses.

FT-IR analysis showed the introduction of fluorinated groups in the copolymers PHEA-PEG-F in comparison with the starting PHEA. In particular, the more interesting feature is the presence of the peak at 1240 cm^{-1} (stretching C–F) similar to that observed in the starting oxadiazole **1**, and assignable to fluorinated groups present in the side chain of PHEA-PEG-F.

The introduction in the polymer of covalently bonded fluorinated oxadiazole units was further confirmed by the disappearance of the 4′-fluorine signal in ^{19}F NMR spectra which showed the typical pattern for *para* substitution on the tetrafluorophenyl ring.

^{1}H NMR analysis of PHEA-PEG-F copolymers has been also performed, but it does not allow to determine the derivatization degree in fluoro oxadiazole linked to PHEA-PEG, because the diagnostic peak relative to –NH–CH_2–**CH_2**–O–oxadiazole is overlapped to the broad peak at δ 4.47–4.65 attributable to the signals of –NH–**CH**(CO)–CH_2– of the polymer backbone.

UV absorption spectrum of PHEA-PEG-F copolymers, in the 200–400 nm range, shows an absorption maximum at 267 nm, with a secondary maximum at 325 nm, while PHEA-PEG does not show absorption in the same range. The obtained spectra for PHEA-PEG-F copolymers are also different from that reported for the starting oxadiazole **1** which shows only one maximum at 245 nm [13]. This feature is imputable to the substitution of 4′-fluorine with alkoxy groups on 5-pentafuorophenyl-1,2,4-oxadiazole, that causes a red-shift of the absorption maximum [13].

In order to determine the derivatization degree of the polymer, we have performed a UV analysis on PHEA-PEG-F copolymers, adopting a procedure elsewhere reported for other PHEA conjugates [14,15]. Because of red-shift on PHEA-PEG-F maximum, we cannot use directly oxadiazole **1** for the calibration curve, so we have accomplished SN_{Ar} on oxadiazole **1** with ethanol, to obtain 5-(2,3,5,6-tetrafluoro-4-ethoxy-phenyl)-3-perfluoroheptyl-1,2,4-oxadiazole (**2**), as a reference compound. By UV analysis, comparing $E^{1\%}_{267}$ of each PHEA-PEG-F copolymer with that of oxadiazole **2**, the content of oxadiazole **1** linked units in PHEA-PEG-F was found to be: 2.9 mol% (8.2 wt%) for PHEA-PEG-F1, 3.8 mol% (10.2 wt%) for PHEA-PEG-F2 and 7.0 mol% (19.1 wt%) for PHEA-PEG-F3.

Considering the low water solubility of PHEA-PEG-F3 (0.04 mg/ml), further characterization concerned only PHEA-PEG-F1 and PHEA-PEG-F2.

In particular, Table 1 shows the results (i.e. the values of weighed average molecular weight, Mw and polydispersity index (PDI)) obtained by SEC analysis performed by using DMF + LiCl 0.01 M or Tris buffer pH 8.0 as medium for sample preparation and mobile phase.

The values of Mw determined in organic medium, confirm that the derivatization of PHEA-PEG with oxadiazole **1** does not cause any degradation in the polymer backbone or significant change in the polydispersity index. In addition, the higher molecular weight found for PHEA-PEG-F2 in comparison with PHEA-PEG-F1 is in agreement with its higher content of fluorinated oxadiazole units. On the other hand, the high values of Mw obtained in Tris buffer pH 8.0 suggest that PHEA-PEG-F copolymers dissolved in aqueous medium, may form micellar aggregates. In fact, PHEA-PEG-F copolymers contain both hydrophilic (due to PHEA backbone and PEG chains) and hydrophobic groups (due to fluorinated oxiadiazole units) that are able to give association colloids at macromolecular unimers in which hydrophobic blocks form the core of the micelle whereas hydrophilic blocks form the corona or outer shell. The capacity for self-assembling (micellization) of amphiphilic derivatives of PHEA has been already showed and reported in our previous work [16]. It is reasonable to suppose that the segregation from the aqueous medium of fluorinated oxadiazole units linked to PHEA-PEG is the driving force for micellization.

Taking into account that SEC measurements were performed by using saturated polymer solutions, we considered interesting to evaluate the value of critical aggregation concentration (CAC) by performing a tensiometric study of PHEA-PEG-F1 and PHEA-PEG-F2 in aqueous solutions. Fig. 1 shows the change in the surface tension (γ) as a function of copolymer concentration.

Table 1
Size exclusion chromatography data of PHEA, PHEA-PEG and PHEA-PEG-F copolymers determined in DMF + LiCl 0.01 M or Tris buffer pH 8.0 as medium for sample preparation (concentration 3 mg/ml) and mobile phase

Sample	Mw (kDa)	Polydispersity index
Medium: DMF + LiCl 0.01 M		
PHEA	40.2	1.82
PHEA-PEG	75.9	1.83
PHEA-PEG-F_1	89.1	1.56
PHEA-PEG-F_2	91.2	1.70
Medium: Tris Buffer pH 8.0		
PHEA	42.4	1.83
PHEA-PEG	82.9	1.85
PHEA-PEG-F_1	195.2	1.80
PHEA-PEG-F_2	252.9	1.95

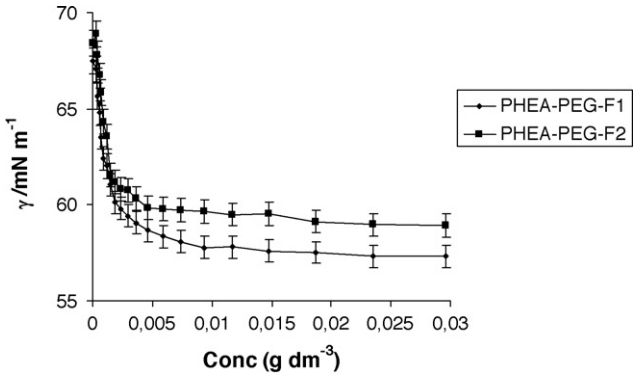


Fig. 1. Plots of surface tension (γ) as a function of the copolymer concentration for aqueous solutions containing PHEA-PEG-F1 or PHEA-PEG-F2.

The values of CAC, determined as reported in Section 4, were 1.8×10^{-4} and 1.1×10^{-4} g dm^{-3} for PHEA-PEG-F1 and PHEA-PEG-F2, respectively.

With the aim of estimating the average diameter and width of distribution (polydispersity index) of aggregates deriving from self-association of PHEA-PEG-F copolymers, a photon correlation spectroscopy (PCS) was performed on samples prepared by dissolving each copolymer in twice-distilled water with a concentration above CAC. The mean diameter was 149.8 ± 1.6 nm (PDI 0.24) and 167.5 ± 5.7 nm (PDI 0.34) for micelles based on PHEA-PEG-F1 and PHEA-PEG-F2, respectively. The obtained size within the nanoscale with a narrow distribution makes these systems suitable for intravenous administration.

The occurrence of micellar aggregates formation, for investigated polymers, could strongly affect their properties as oxygen delivery systems. In order to obtain some information about polymers behaviour as O_2 carriers, oxygen release kinetics, from polymer solutions, were performed by means of a previously reported saturation method [9], at different temperatures (25 and 37 °C) and concentrations (0.04 and 0.4 mg/ml).

Oxygen solubility was determined after saturation of the solutions and monitored, as a function of time, at atmospheric pressure. Accordingly to elsewhere reported *in vivo* kinetics experiments [17], the obtained desaturation curves were approximated, by a single exponential function (Eq. (1))

$$[O_2] = [O_2]_\infty + [O_2]_{load} \exp\left(-\frac{t}{k}\right) \quad (1)$$

where $[O_2]_\infty$ = oxygen solubility at t_∞; $[O_2]_{load} = [O_2]_0 - [O_2]_\infty$ where $[O_2]_0$ = oxygen solubility at t_0; k = clearance constant (min).

The obtained data are reported in Table 2 and in Figs. 2 and 3.

Oxygen solubility maxima ($[O_2]_0$) do not vary significantly with the fluorine content or polymer concentration. This could be ascribed to the generally low concentrations (0.04–0.4 mg/ml) of polymer used, compared to the fluorinated component concentration in commonly used PFC emulsions [2] (see Table 3).

In other words, being the solvent the main component, the electrode will read an oxygen concentration value which is not significantly different from the oxygen solubility in water (see Table 2).

On the other hand the presence of the fluorinated copolymer seems to strongly affect the kinetics of oxygen release, and functionalization with oxadiazole **1** is more effective on increasing k value with respect to unfluorinated polymer. However, the observed behaviour is not linearly correlated to derivatization degree of obtained copolymers.

Data from Table 2 for copolymer concentration equal to 0.04 mg/ml, show that clearance constants are a function of polymer fluorofunctionalization, being $k_{PHEA\text{-}PEG\text{-}F3} > k_{PHEA\text{-}PEG\text{-}F2} > k_{PHEA\text{-}PEG\text{-}F1} > k_{PHEA\text{-}PEG}$.

Furthermore, the increases in clearance constants (Δk) are lower at 37 °C than at 25 °C since the higher temperature favours the oxygen release and tends to level the k values. In summary, at 0.04 mg/ml copolymer concentration, the desaturation curves are strongly affected by temperature and derivatization degree of linked oxadiazole.

Unfortunately a similar comparison is not achievable for higher copolymer concentration (0.4 mg/ml), since PHEA-PEG-F3 copolymer is not soluble in these conditions.

Interestingly, PHEA-PEG-F2 at a concentration of 0.4 mg/ml shows a behaviour which is very similar to that of PHEA-PEG-F3 at 0.04 mg/ml, but with a higher clearance constant (k = 384 min at 25 °C and k = 199 min at 37 °C).

Differences between PHEA-PEG-F series, PHEA-F and unfluorinated PHEA-PEG, in terms of k values, suggest that the formation of polymeric micellar aggregates acts on constitution of a fluorinated reservoir for oxygen, thus explaining the obtained high k values, even at 37 °C. This hypothesis is also supported by oxygen uptake experiments performed at 25 °C and atmospheric pressure versus air (pO_2 = 0.21 atm) (see Fig. 4 and Table 4).

In fact, going from PHEA-PEG to PHEA-F, the rate of oxygen uptake increases due to the higher affinity of oxygen for the fluorinated polymer. On the other hand, increasing the fluorine

Table 2
Parameters of Eq. (1) determined for desaturation curves as a function of time of oxygen saturated aqueous solutions of PHEA-F, PHEA-PEG, PHEA-PEG-F copolymers (concentration 0.04 or 0.4 mg/ml)

Copolymer	C = 0.04 mg/ml		C = 0.4 mg/ml	
	T = 25 °C	T = 37 °C	T = 25 °C	T = 37 °C
PHEA-PEG	$[O_2]_0$ = 39.13 ± 0.04 ppm; $[O_2]_\infty$ = 7.58 ± 0.03 ppm; k = 63 ± 2 min	$[O_2]_0$ = 27.44 ± 0.06 ppm; $[O_2]_\infty$ = 6.68 ± 0.02 ppm; k = 28 ± 1 min	$[O_2]_0$ = 39.18 ± 0.07 ppm; $[O_2]_\infty$ = 7.12 ± 0.03 ppm; k = 43 ± 2 min	$[O_2]_0$ = 23.57 ± 0.05 ppm; $[O_2]_\infty$ = 5.88 ± 0.03 ppm; k = 29 ± 1 min
PHEA-F	–	–	$[O_2]_0$ = 43.42 ± 0.05 ppm; $[O_2]_\infty$ = 7.37 ± 0.04 ppm; k = 60 ± 2 min	$[O_2]_0$ = 28.59 ± 0.04 ppm; $[O_2]_\infty$ = 6.69 ± 0.02 ppm; k = 28 ± 1 min
PHEA-PEG-F1	$[O_2]_0$ = 41.00 ± 0.05 ppm; $[O_2]_\infty$ = 7.81 ± 0.03 ppm; k = 78 ± 1 min	$[O_2]_0$ = 24.24 ± 0.04 ppm; $[O_2]_\infty$ = 6.36 ± 0.02 ppm; k = 58 ± 2 min	$[O_2]_0$ = 44.19 ± 0.05 ppm; $[O_2]_\infty$ = 7.20 ± 0.06 ppm; k = 63 ± 1 min	$[O_2]_0$ = 24.53 ± 0.04 ppm; $[O_2]_\infty$ = 5.83 ± 0.02 ppm; k = 45 ± 2 min
PHEA-PEG-F2	$[O_2]_0$ = 43.82 ± 0.06 ppm; $[O_2]_\infty$ = 8.05 ± 0.02 ppm; k = 146 ± 2 min	$[O_2]_0$ = 24.73 ± 0.05 ppm; $[O_2]_\infty$ = 6.40 ± 0.03 ppm; k = 79 ± 3 min	$[O_2]_0$ = 35.53 ± 0.08 ppm; $[O_2]_\infty$ = 7.16 ± 0.02 ppm; k = 384 ± 3 min	$[O_2]_0$ = 24.22 ± 0.05 ppm; $[O_2]_\infty$ = 5.87 ± 0.02 ppm; k = 199 ± 2 min
PHEA-PEG-F3	$[O_2]_0$ = 38.47 ± 0.06 ppm; $[O_2]_\infty$ = 8.02 ± 0.04 ppm; k = 238 ± 2 min	$[O_2]_0$ = 25.81 ± 0.07 ppm; $[O_2]_\infty$ = 6.58 ± 0.04 ppm; k = 82 ± 2 min	Not soluble	Not soluble

$[O_2]_0$ in pure water: 38.75 ± 0.05 ppm at 25 °C and 30.05 ± 0.04 ppm at 37 °C.

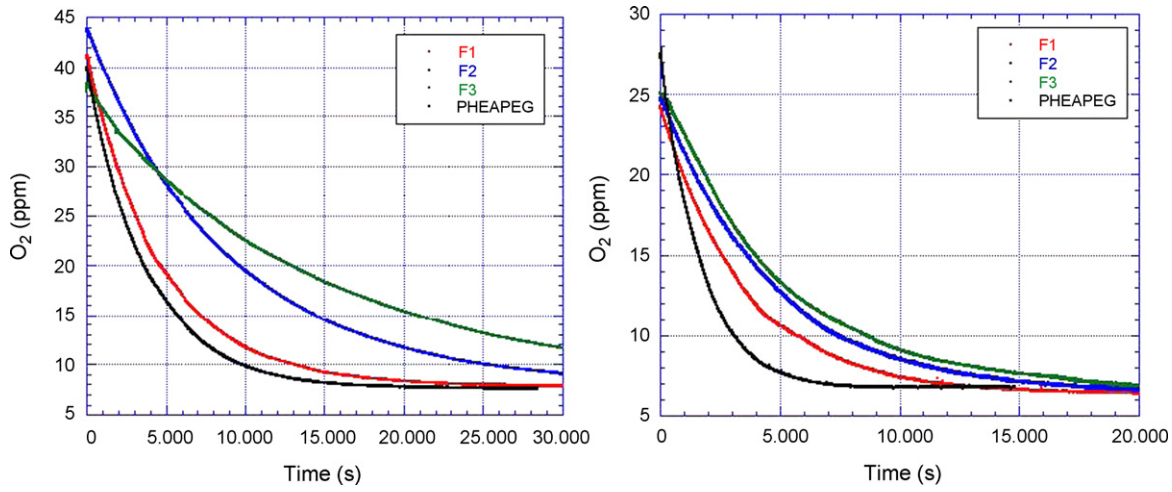


Fig. 2. Oxygen release curves from aqueous solutions containing 0.04 mg/ml of PHEA-PEG-F1, PHEA-PEG-F2, PHEA-PEG-F3 and PHEA-PEG copolymers at 25 °C (left) and 37 °C (right).

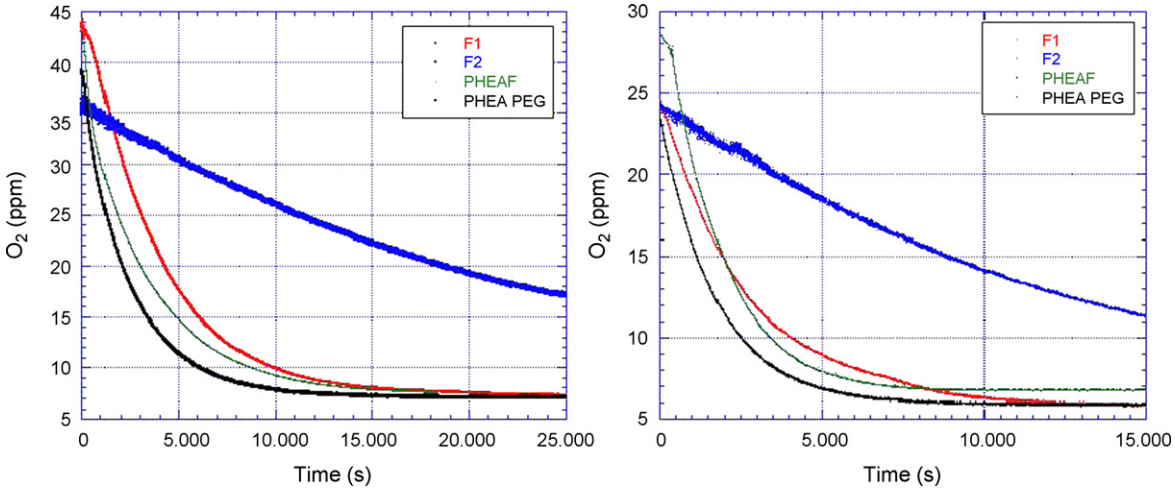


Fig. 3. Oxygen release curves from aqueous solutions containing 0.4 mg/ml of PHEA-PEG-F1, PHEA-PEG-F2, PHEA-PEG and PHEA-F copolymers at 25 °C (left) and 37 °C (right).

derivatization of PHEA-PEG, a decrease in O_2 uptake occurs, although slightly higher O_2 concentrations are reached.

In other words, even if oxygen concentration at the equilibrium does not vary among different polymer solutions, the uptake or release rates seem affected by the $[O_2]_{sol.} \rightleftarrows [O_2]_{Fluorinated\ aggregates}$ equilibrium which compensate the $[O_2]_{sol.} \rightleftarrows [O_2]_{atm.}$ one, accordingly to already observed behaviour for F-self-assemblies which are less dynamic than those made from nonfluorinated polymers and present an ability to exchange components among micelles or vesicles significantly slow [18].

Moreover, while in the case of PHEA-F only the copolymer with a derivatization degree of 2.6 mol% resulted to be water soluble [9], the introduction of PEG side chains allowed to maintain water solubility also for copolymers containing higher amounts of linked fluorinated oxadiazole units. Finally, all PHEA-PEG-F copolymers show value of k higher than those found for perfluorochemicals [18], suggesting their use when longer oxygenation times are required.

In order to investigate the effect of PHEA-PEG-F copolymers on cell viability, each copolymer (final concentration in the cell growth medium equal to 0.04 or 0.4 mg/ml) was kept for 48 h in

Table 3
Oxygen solubility (vol%, under pure oxygen) for PHEA-PEG-F1, PHEA-PEG-F2 and PHEA-PEG-F3 at 25 °C and 37 °C compared to commercial PFC emulsions

Copolymer	Oxygen solubility (vol%)			
	T = 25 °C		T = 37 °C	
	C = 0.04 mg/ml	C = 0.4 mg/ml	C = 0.04 mg/ml	C = 0.4 mg/ml
PHEA-PEG-F1	2.87%	3.12%	1.75%	1.78%
PHEA-PEG-F2	3.07%	2.48%	1.80%	1.76%
PHEA-PEG-F3	2.39%	–	1.88%	–
Oxygent® (F-octylbromide 60%, w/v)	–		16%[a]	
Fluosol® (PFC 20%, w/v)			6%[a]	

[a] From Ref. [2].

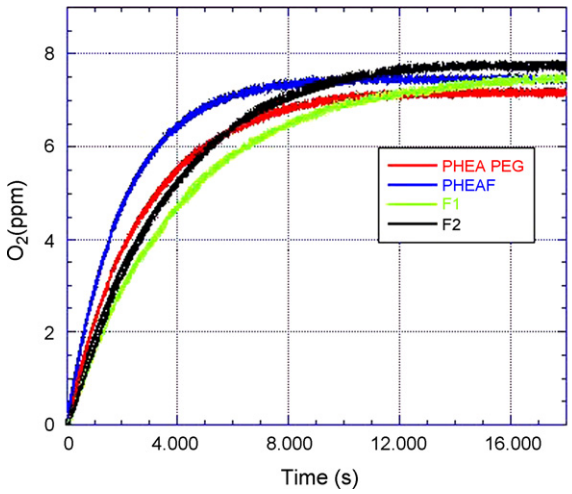


Fig. 4. Oxygen uptake curves from aqueous solutions containing 0.4 mg/ml of PHEA-PEG-F1, PHEA-PEG-F2, PHEA-PEG and PHEA-F copolymers at 25 °C.

Table 4
Parameters determined for saturation curves as a function of time of deoxygenated aqueous solutions of PHEA-F, PHEA-PEG, PHEA-PEG-F copolymers at 25 °C (concentration 0.4 mg/ml)

Copolymer	Oxygen solubility uptake constant
PHEA-PEG	$[O_2]_\infty = 7.19 \pm 0.06$ ppm; $k = 46 \pm 1$ min
PHEA-F	$[O_2]_\infty = 7.47 \pm 0.08$ ppm; $k = 34 \pm 1$ min
PHEA-PEG-F1	$[O_2]_\infty = 7.57 \pm 0.05$ ppm; $k = 69 \pm 1$ min
PHEA-PEG-F2	$[O_2]_\infty = 7.70 \pm 0.08$ ppm; $k = 61 \pm 2$ min

contact with K-562 cells (human chronic myelogenous leukaemia), chosen as a model cell line. In particular, cell viability % was evaluated by the MTS assay [19] (see Section 4), and results are reported in Fig. 5.

Cells showed a high viability % respect to the control when in contact with PHEA-PEG-F copolymers. Cell compatibility has been also confirmed by performing *in vitro* haemolysis assay [9]. Taking into account that a possible interaction of PHEA-PEG-F copolymers with membranes of erythrocytes could cause haemolysis, the release of haemoglobin was used to quantify the membrane-damaging properties of these copolymers. Erythrocytes treated with phosphate buffer solution (PBS) pH 7.4 and 1% Triton X-100 were used as 0 and 100% values, respectively. Solutions of PHEA-PEG-F1 or PHEA-PEG-F2 copolymers in PBS pH 7.4 with a concentration of 0.04 or 0.4 mg/ml were added to the erythrocytes and incubated for 1 h at 37 °C under constant shaking. Under these

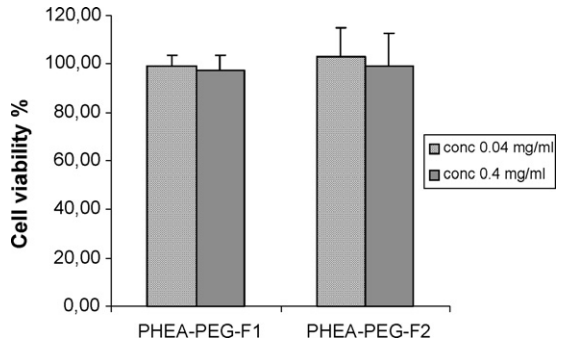


Fig. 5. Viability % of K-562 cells after 48 h of incubation with aqueous solutions containing 0.04 or 0.4 mg/ml of PHEA-PEG-F1 or PHEA-PEG-F2 copolymers.

conditions, PHEA-PEG-F copolymers did not show haemolytic effect, thus indicating no detectable damage on the erythrocyte membranes. In fact, the percentage of haemolysis was always less than 0.5%, data comparable to those of the blank.

Moreover, cell compatibility and haemolysis data obtained for PHEA-PEG-F derivatives result similar to those obtained with nonfluorinated PHEA-PEG copolymers (data not showed), thus indicating the absence of negative effects of the fluorinated chains on the *in vitro* biocompatibility of the produced polymers (see also Ref. [20]).

3. Conclusions

New fluorinated and pegylated copolymers have been prepared starting from a biocompatible polyaspartamide. The prepared samples, having a different derivatization degree in fluorinated moieties undergo a self-association process in aqueous medium. These aggregates have a narrow size distribution in the nanoscale suitable for a potential intravenous administration. They do not cause alteration in cell viability and do not damage erythrocyte membranes. Finally, aggregates of these fluoropolymers are able to maintain high oxygen levels in aqueous medium for a prolonged time. All data suggest the possible employment of the investigated copolymers as oxygen delivery systems with potential use as blood substitutes. In addition taking into account the prolonged release of oxygen, these fluoropolymers could be also advantageous when long oxygenation times are required, e.g. in the ischemic phenomena.

4. Experimental

4.1. Materials

All reagents were of analytical grade, unless otherwise stated. D,L-Aspartic acid, ethanolamine, *O*-(2-aminoethyl)-*O*′-methyl polyethylene glycol 2000, *N*,*N*-dimethylformamide, triethylamine, diethyl ether, THF, acetone, and 2-propanol were from Fluka (Italy). D_2O (isotopic purity 99.9%) was purchased from Aldrich Chemical Co. (Italy). Oxygen is RP 5.0 from ISO (Italy). Oxadiazole **1** was prepared as reported previously [13]. Human chronic myelogenous leukaemia cell line (K-562) was purchased from Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna "Bruno Umbertini" (Italy). Human erythrocytes were from healthy human donors.

4.2. Apparatus

FT-IR spectra were recorded as pellets in KBr in the range 4000–400 cm^{-1} by using a PerkinElmer 1720 Fourier Transform Spectrophotometer with a resolution of 1 cm^{-1}; each spectrum was recorded after 100 scans.

UV–VIS absorption spectra were recorded by using a Jasco 7800 UV/VIS Spectrophotometer.

^1H and ^{19}F NMR spectra were recorded on a Bruker Avance 300 MHz operating at 300 and 282.3 MHz respectively. ^1H NMR spectra were taken with TMS as an internal standard. ^{19}F NMR spectra were taken with $CFCl_3$ as an internal standard.

Centrifugations were performed with an International Equipment Company Centra MP4R equipped with an 854 rotor and temperature control.

Molecular weights of PHEA, PHEA-PEG, PHEA-PEG-F copolymers were determined by a SEC chromatographic system equipped with a pump system, using Phenogel (5 μm particle size, 10^3 Å pore size) as a column, 410 differential refractometer (DRI) as a concentration detector, DMF + LiCl 0.01 M as a mobile phase;

50 °C; 0.6 ml/min, polymer concentration 3 mg/ml (in DMF + LiCl 0.01 M). The molecular weights were estimated based on PEO/PEG standards (range 71000–1500 Da). SEC was also performed by using two columns: Ultrahydrogel (10 μm particle size, 10^3 Å pore size) and Polysep-GCF-P 3000, Tris buffer pH 8.0 as a mobile phase; 37 °C; 0.8 ml/min, saturated polymer solutions (in Tris buffer pH 8.0).

The surface tension measurements have been carried out at 25 °C by means of a KSV-Sigma 70 automatic tensiometer by using the Whilelmy plate method.

The size of PHEA-PEG-F aggregates was measured by a Zetasizer Nano ZS (Malvern Instruments).

Oxygen solubility measurements were performed by using a Delta OHM model HD2109.1 oxymeter. The oxygen solubility probe was a Schott Gerade 120 mm probe having a membrane with an exterior Teflon layer.

4.3. Synthesis of PHEA and PHEA-PEG

α,β-Poly(*N*-2-hydroxyethyl)-DL-aspartamide was prepared by reaction of a polysuccinimide (PSI), obtained by thermal polycondensation of D,L-aspartic acid, with ethanolamine in DMF solution, purified and characterized according to a procedure reported elsewhere [21].

PHEA-PEG derivative was prepared and purified according to a previously reported procedure [20]. In particular, to a PHEA solution (40 mg/ml) in anhydrous DMF a proper amount of bis(4-nitrophenyl) carbonate (PNPC) was added in a such way to have *X* = moles of PNPC/moles of PHEA repeating units equal to 0.1. The reaction mixture was kept at 40 °C for 2.5 h and then at 60 °C for 30 min. A solution of *O*-(2-aminoethyl)-*O*′-methyl polyethylene glycol 2000 in anhydrous DMF (60.8 mg/ml) was added to the mixture reaction in a such way to have *Y* = moles of PEG_{2000}/moles of PNPC equal to 1.2 and the mixture left at 60 °C for 2.5 h under argon and continuous stirring.

After this time the solution was precipitated in diethyl ether, washed with a mixture of ethyl ether/CH_2Cl_2 (2:1) (3 × 50 ml) and then with acetone (5 × 50 ml). PHEA-PEG copolymer thus obtained was dissolved in twice-distilled water and subjected to an extensive dialysis by using Visking Tubing Dialysis 18/32 in. with a molecular weight cut-off of 12,000–14,000. After dialysis, the solution was dried by freeze-drying. The product was obtained with a yield of 95% (w/w) based on the starting PHEA and characterized by FT-IR and ^{1}H NMR analyses [20]. The molar degree of derivatization percentage in PEG chains linked to PHEA, determined by ^{1}H NMR analysis [20], resulted to be 7.0 ± 0.5 mol%.

4.4. Synthesis of PHEA-PEG-F copolymers

Derivatization of PHEA-PEG with 5-pentafluorophenyl-3-perfluoroheptyl-1,2,4-oxadiazole **1** to obtain PHEA-PEG-F copolymer, was carried out in an organic phase (anhydrous DMF), using TEA as a catalyst, according to the following procedure: 185 mg of PHEA-PEG were dissolved in 3 ml of anhydrous DMF, then suitable amounts of **1** and TEA were added, according to *X* and *Y* defined as:

X = moles of **1**/moles of PHEA-PEG repeating unit
Y = moles of TEA/moles of **1**

In particular, reactions with *X* = 0.4, 0.6 or 1 and *Y* = 1 were performed by keeping the reaction mixture at 25 °C under continuous stirring for 5 days. After this time, each reaction mixture was precipitated in 100 ml of diethyl ether and centrifuged for 15 min at 9800 rpm, at 4 °C. Each product was isolated, washed with diethyl ether (4 × 40 ml) and acetone (2 × 40 ml) and dried under vacuum.

Prepared samples have been named as PHEA-PEG-F1, PHEA-PEG-F2 and PHEA-PEG-F3 copolymers, for *X* = 0.4, 0.6 and 1, respectively.

PHEA-PEG-F derivatives so obtained were dissolved in distilled water and subjected to extensive dialysis against distilled water utilizing Visking Dialysis Tubing (18/32 in.) with a molecular weight cut-off of 12,000–14,000. After dialysis, the solution was lyophilized. Yield varied from 97 to 99% (w/w), based on the starting PHEA-PEG.

FT-IR spectrum (KBr) of a typical PHEA-PEG-F copolymer showed a broad band centred at 3400 cm^{-1} (asymmetric stretching of O–H and N–H groups); bands at 2917 (stretching C–H); 1656 (amide I); 1543 (amide II), 1240 (stretching C–F) and 1110 (stretching C–O) cm^{-1}.

^{1}H NMR spectrum (DMSO-d_6) of a typical PHEA-PEG-F copolymer showed: δ = 2.6 (m, 2H, –CH–**CH_2**–CO–NH), 3.12 (t, 2H, –NH–**CH_2**–CH_2–O–), 3.35 (t, 2H, –NH–CH_2–**CH_2**–O–), 3.50 (t, 176 H, –CH_2–**CH_2**–O–), 4.47–4.65 (m, 1H, –NH–**CH**(CO–)–CH_2–).

^{19}F NMR spectrum (D_2O) of a typical PHEA-PEG-F copolymer showed: δ = −81.5 (bs, CF_3), −112.0 (bs, CF_2), −121.5 (bs, CF_2), −122.4 and −122.6 (overlapped signals, CF_2), −123.2 (bs, CF_2), −126.7 (bs, CF_2), −134.9 (bs, Ar C–F), −155.8 (bs, Ar C–F).

UV (H_2O/EtOH, 20%, v/v): λ_{max} = 267 nm ($E^{1\%}_{267}$ = 19.5, 25.1 and 47.0 for PHEA-PEG-F1, PHEA-PEG-F2 and PHEA-PEG-F3 copolymer, respectively).

4.5. UV determination of the oxadiazole content in PHEA-PEG-F copolymers

The amount of oxadiazole linked to PHEA-PEG-F copolymers was determined by means of UV spectroscopy by comparing the absorbance at 267 nm of each PHEA-PEG-F copolymer in H_2O/EtOH (20%, v/v) solution with a calibration curve of reference compound 5-(2,3,5,6-tetrafluoro-4-ethoxy-phenyl)-3-pentadecafluoroheptyl-1,2,4-oxadiazole, which was obtained as previously reported [9]. The oxadiazole content linked to PHEA-PEG-F copolymers was expressed either as the percent of conjugated units per mass of copolymer or as the percent molar ratio. These values were: 2.9 mol% (8.2 wt%) for PHEA-PEG-F1, 3.8 mol% (10.2 wt%) for PHEA-PEG-F2 and 7.0 mol% (19.1 wt%) for PHEA-PEG-F3.

4.6. Surface activity studies

The surface activity of PHEA-PEG-F1 and PHEA-PEG-F2 copolymers and their ability to self-aggregate have been established by performing a tensiometric study in aqueous solution at 25.0 ± 0.1 °C. The value of critical aggregation concentration of these copolymers in aqueous solution was estimated as the intersection point of the two linear plots, above and below the CAC, of the surface tension versus log of copolymer concentration (investigated range 3×10^{-4} to 3×10^{-2} g dm^{-3}).

4.7. Size of PHEA-PEG-F aggregates measurements

The average diameter and width of distribution (polydispersity index) of aggregates deriving from self-association of PHEA-PEG-F copolymers, were determined by photon correlation spectroscopy at a fixed angle of 90°, at the temperature of 25 ± 0.1 °C. Each copolymer was dissolved in filtered (0.2 μm) twice-distilled water with a concentration above CAC, then it was kept in a cuvette and analyzed by using an algorithm based on the Non-Negative Least Squares (NNLS) method.

4.8. Oxygen solubility measurements

Oxygen solubility measurements were performed, as previously reported [9], on oxygen saturated aqueous solutions (20 ml) containing PHEA-PEG-F copolymers at concentration of 0.04 or 0.4 mg/ml, at atmospheric pressure, accordingly to a method reported elsewhere [22]. In particular, each solution was initially stirred with a magnetic stir bar while pure oxygen was continuously bubbled. The temperature of each solution was adjusted either at 25 or 37 ± 0.1 °C by using a thermostated oil bath. Once the solution reached a stable maximum oxygen concentration (saturated solution), bubbling was stopped and the release of dissolved oxygen was determined by evaluating the change in the oxygen solubility (desaturation) as a function of time.

Oxygen uptake measurements were performed using the same apparatus; copolymer solutions were purged with pure nitrogen until complete deoxygenation. Bubbling was stopped and oxygen uptake from air was monitored as a function of time.

4.9. Cell viability assay

Cell compatibility of PHEA-PEG-F1 or PHEA-PEG-F2 copolymers was tested *in vitro* by using human chronic myelogenous leukaemia (K-562) cells and the MTS assay [19]. Cells were suspended at a density of 1×10^5 cells ml^{-1} in RPMI-1640 medium (supplemented with 10% (v/v) of fetal calf serum, 2 mmol/l L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin) transferred to 96-well plate (0.1 ml for well) and incubated for 48 h at 37 °C in a humidified atmosphere containing 5% of CO_2, in the presence of PHEA-PEG-F1 or PHEA-PEG-F2 aqueous solutions in order to have a final concentration of 0.04 or 0.4 mg/ml. After the incubation time 20 μl per well of MTS reagent were added. After 2 h of incubation, the absorbance at 492 nm was recorded and cell viability data were calculated. Relative cell viability (in percentage) was expressed as (Abs_{492} treated cells/Abs_{492} control cells) × 100.

Cells incubated in RPMI-1640 medium in the absence of PHEA-PEG-F copolymers, were used as a control. Each experiment was performed in triplicate.

4.10. Haemolysis test

Human erythrocytes isolated from fresh citrated-treated blood were collected by centrifugation at 2200 rpm for 10 min at 4 °C and the pellet was washed four times with phosphate buffer solution pH 7.4 by centrifugation at 2200 rpm for 10 min at 4 °C and resuspended in the same buffer.

The erythrocyte pellet was diluted in PBS pH 7.4 to a final concentration of 4% erythrocytes. This suspension of erythrocytes was always freshly prepared and used within 24 h after collection.

Solutions of PHEA-PEG-F1 or PHEA-PEG-F2 in PBS pH 7.4 with a concentration of 0.04 or 0.4 mg/ml were added to the erythrocytes and were incubated for 1 h at 37 °C under constant shaking. The release of haemoglobin was determined after centrifugation (2200 rpm for 10 min at 4 °C) by photometric analysis of the supernatant at 540 nm. Complete haemolysis was achieved using 1% Triton X-100. The experiments were run in triplicate and were repeated twice. The percentage erythrocyte lysis was calculated according to the formula:

$$\%\,\text{lysis} = \left[\frac{(A_{\text{polymer}} - A_{\text{blank}})}{(A_{100\%\,\text{lysis}} - A_{\text{blank}})}\right] \times 100$$

where A_{polymer} is the absorbance value of the haemoglobin released from erythrocytes treated with PHEA-PEG-F polymer solution; A_{blank} is the absorbance value of the haemoglobin released from erythrocytes treated with PBS pH 7.4 and $A_{100\%\ \text{lysis}}$ is the absorbance value of the haemoglobin released from erythrocytes treated with 1% Triton X-100 solution.

Acknowledgements

Authors thank MIUR and University of Palermo for financial support.

References

[1] D.R. Spahn, Adv. Drug Del. Rev. 40 (2000) 143–151.
[2] J.G. Riess, Chem. Rev. 101 (2001) 2797–2919.
[3] J.G. Riess, J. Fluorine Chem. 114 (2002) 119–126.
[4] J.G. Riess,, in: J. Swarbrick, J.C. Boylan (Eds.), 2nd edition, Encyclopedia of Pharmaceutical Technology, vol. 1, Marcel Dekker, Inc., New York, 2002 pp. 263–281.
[5] K.C. Lowe, Blood Rev. 13 (1999) 171–184.
[6] K.C. Lowe, J. Fluorine Chem. 109 (2001) 59–65.
[7] E.D. Shchukin, E.A. Amelina, A.M. Parfenova, Colloids Surf. 176 (2001) 35–51.
[8] M.P. Krafft, Adv. Drug Del. Rev. 47 (2001) 209–228.
[9] D. Mandracchia, A. Palumbo Piccionello, G. Pitarresi, A. Pace, S. Buscemi, G. Giammona, Macromol. Biosci. 7 (2007) 836–845.
[10] M.J. Roberts, M.D. Bentley, J.M. Harris, Adv. Drug Del. Rev. 54 (2002) 459–476.
[11] D.E. Owens, N.A. Peppas, Int. J. Pharm. 307 (2006) 93–102.
[12] H.R. Kim, S. Gil, K. Andrieux, V. Nicolas, M. Appel, H. Chacun, D. Desmaële, F. Taran, D. Georgin, P. Couvreur, Cell. Mol. Life Sci. 64 (2007) 356–364.
[13] S. Buscemi, A. Pace, A. Palumbo Piccionello, N. Vivona, J. Fluorine Chem. 127 (2006) 1601–1605.
[14] G. Cavallaro, M. Licciardi, P. Caliceti, S. Salmaso, G. Giammona, Eur. J. Pharm. Biopharm. 58 (2004) 151–159.
[15] M. Licciardi, Y. Tang, N.C. Billingham, S.P. Armes, A.L. Lewis, Biomacromolecules 6 (2005) 1085–1096.
[16] G. Cavallaro, G. Giammona, R. Lombardo, L. Sciascia, M.L. Turco Liveri, Colloids Surf. 289 (2006) 10–16.
[17] B.A. Berkowitz, C.A. Wilson, D.L. Hatchell, Invest. Ophtal. Vis. Sci. 32 (1991) 2382–2387.
[18] M.P. Krafft, J. Polym. Sci. Part A 44 (2006) 4251–4258.
[19] A.H. Cory, T.C. Owen, J.A. Barltrop, J.G. Cory, Cancer Commun. 3 (1991) 207–212.
[20] E.F. Craparo, G. Cavallaro, M.L. Bondì, D. Mandracchia, G. Giammona, Biomacromolecules 7 (2006) 3083–3092.
[21] G. Giammona, B. Carlisi, S. Palazzo, J. Polym. Sci., Part A: Polym. Chem. 25 (1987) 2813–2818.
[22] P.C. Rooney, D.D. Daniels, Pet. Technol. Quart. 3 (1998) 97–101.