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Synthesis of a novel PEG-*block*-poly(aspartic acid-*stat*-phenylalanine) copolymer shows potential for formation of a micellar drug carrier

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Abstract

A novel functionalised copolymer with three polymeric components, poly(ethylene glycol)-*block*-poly(aspartic acid-*stat*-phenylalanine), PEG-P(asp-phe), was synthesised and investigated for its potential to form micelles via ionic interactions with a model water-soluble drug, diminazene aceturate. Drug-free solutions of structurally related PEG-P(asp-phe) 5:6:4 and PEG-P(asp-phe) 5:4:6 copolymers indicated polymeric aggregation into micellar-type constructs. The size of PEG-P(asp-phe) 5:6:4 micelles was found to be pH and drug content-dependent. The drug-loaded systems existed as discreet units and were fairly uniform in size and shape. More drug could be included in the PEG-P(asp-phe) 5:6:4 micelles as compared to if only interaction with carboxyl groups from aspartic acid units was responsible for micelle formation, indicating the augmentative role of phenylalanine moieties in drug-incorporation. The slower in vitro drug release from PEG-P(asp-phe) 5:6:4 micelles as compared to PEG-Pasp (AB) micelles indicated the role of the phenylalanine moiety in controlling drug release. This study, therefore, confirmed the potential of a novel tri-component copolymer structure, PEG-P(asp-phe), for the formation of polyionic micelles for drug delivery.

Keywords: Drug–polymer interactions; Polyionic micelles; Drug delivery; Block copolymers

1. Introduction

In recent years, polymeric micellar-type constructs have emerged as promising novel nano-sized carriers for drug targeting and gene therapy (Yokoyama, 1998; Kakizawa and Kataoka, 2002; Cheon Lee et al., 2003; Lee et al., 2003; Shuai et al., 2004). These polymeric micelles are characterised by a core–shell structure and are mostly centered on copolymers having an AB diblock structure. In an aqueous (selective) solvent, the hydrophobic A block of the copolymer forms the core, whilst the hydrophilic (B) block forms the

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shell or corona. These core–shell micellar-type constructs offer the following advantages for drug delivery; the hydrophobic core can be used for the solubilisation of hydrophobic drugs and also to prevent drug molecules against possible in vivo degradation (Kwon et al., 1994; Harada and Kataoka, 1998; Yu et al., 1998). The hydrophilic shell (e.g. polyethylene glycol) should suppress uptake by the reticuloendothelial system, and in that way, prolong circulation times and modify biodistribution of the delivery system (Stolnik et al., 1995). The small size of copolymer micelles is also advantageous in that it can promote escape through the permeable vasculature at tumour sites as well as facilitate the delivery of DNA to the nucleus for gene therapy (Kataoka, 1994; Kakizawa and Kataoka, 2002).

Yokoyama et al. (1992) reported the self-assembly of a block copolymer based on PEG-P(asp) and doxorubicin. More recently, Cheon Lee et al. (2003) and Thunemann et al. (2000) prepared polyionic micelles from poly(2-ethyl-2-oxazoline)-*block*-poly(caprolactone) and PEG-poly(lysine), respectively. Most studies in the literature have focused on the incorporation of hydrophobic drugs such as doxorubicin (Kataoka et al., 2000), indomethacin (La et al., 1996; Shin et al., 1998)), amphotericin B (Yu et al., 1998), testosterone (Hagan et al., 1996) and cisplatin (Yokoyama et al., 1996), and further these have been achieved mainly by chemical conjugation and/or physical entrapment of the hydrophobic drugs into the micellar environment. There is clearly a lack of data on the characterisation of self-assembling micellar systems driven by ionic interactions of the polymer with a drug. The advantages of such a system may include the following: (i) as micelle preparation is achieved by simple mixing of the drug and polymeric solutions, this approach will eliminate the need for a synthetic procedure of attaching the drug to the polymer; (ii) nanosystems such as polymeric micelles suffer from poor drug-incorporation efficiencies due to the small size, and this limitation is exacerbated with water-soluble drugs due to its loss in the aqueous phase. The interaction of drug with pendant groups on the polymer may, therefore, lead to improved drug-incorporation efficiencies; (iii) ionic interactions are further advantageous in that the drug would be easily released from the micelles, while with covalent attachment, cleavage of the drug from the polymer would be required. Harada and Kataoka (1998) prepared micelles via ionic interactions between PEG-polyaspartic acid, PEG-P(asp), and protein lysozyme. We have also reported the formation of polymeric micelles driven by ionic interactions from the diblock polymer PEG-P(asp) with a small molecular weight drug, diminazene aceturate (Govender et al., 2001). Molecular association of this copolymer into 20–60 nm-sized micellar structures occurred in the presence of this cationic drug at a pH window of 3.4–7.2; while in the absence of drug, the copolymer formed a unimolecular solution in the buffer. While the level of drug-incorporation was high; the micelles, however, suffered from a rapid and almost immediate drug release. Whilst the majority of studies on polymeric micelles have focused on diblock (AB) copolymers with two polymeric components using hydrophobic drugs, no study to date has been reported on the use of a diblock copolymer that has three polymeric components, A(BC), to facilitate micellar formation via ionic interactions with a water-soluble drug. In the present work, we synthesised a copolymer composed of three monomeric units, poly(ethylene glycol)-*block*-poly(aspartic acid-*stat*-phenylalanine), PEG-P(asp-phe). In the poly(ethylene glycol)-*block*-poly(aspartic acid-*stat*-phenylalanine) copolymer, PEG comprises one block, while the other block is made of aspartic acid and phenylalanine monomeric units in a random/statistical arrangement. The components of the copolymer structure were chosen to perform specified functions. Poly(aspartic acid) was selected as the poly(amino acid) to provide functionality to the copolymer in order to ionically interact with a drug to be incorporated; also it is water-soluble, non-toxic and biodegradable (Kataoka, 1994; Ehtezazi et al., 2000). The incorporation of phenylalanine units into the copolymer was considered in order to enhance the hydrophobic/aromatic interactions within the micellar core; in that way, enhancing construct stability towards dissociation and consequently possibly reducing the drug-release rate. PEG was attached as the hydrophilic moiety to balance the molecular association forces and also to provide steric stabilisation of the micelles. Diminazene aceturate was selected as a model drug due to its cationic nature, water solubility and its confirmed interaction with poly(aspartic acid).

The starting hypothesis in this study was that micelle formation between PEG-P(asp-phe) and diminazene

aceturate would occur as follows: when these two solutions are mixed at pH conditions, where both species are ionised, the cationic drug would interact ionically with the pendant carboxylic acid groups of aspartic acid units making that portion of the copolymer molecule relatively more hydrophobic. The phenylalanine groups of the copolymer would be drawn closer and hydrophobic/aromatic forces would act to stabilise the assembly (possibly also interact with drug molecules); since PEG is hydrophilic, this will lead to the drug/polymer complex self-assembling to form micellar-type constructs. In this paper, the synthesis of the novel tri-component polymer structure, PEG-P(asp-phe), is, therefore, presented. The formation of micelles from this polymer strucutres and physicochemical characterisation in terms of scattering intensity, micellar size, morphology and drug release are also reported to assess the potential for drug delivery.

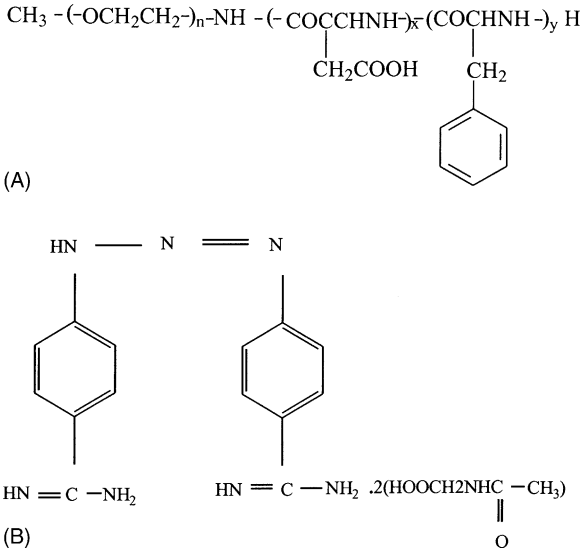


Fig. 1. (A) Chemical structure of poly(ethylene glycol)-*block*-poly(aspartic acid-*stat*-phenylalanine), PEG-P(asp-phe). (B) Chemical structure of diminazene aceturate.

2. Materials and methods

2.1. Materials

Poly(ethylene glycol)-*block*-poly(aspartic acid-*stat*-phenylalanine), PEG-P(asp-phe) copolymers with two different ratios of aspartic acid and phenylalanine units (5:6:4 and 5:4:6, total M_w = 15,000 Da) were synthesised as described below. The chemical structure of the polymer is shown in Fig. 1. Diminazene aceturate, sodium phosphate monobasic (NaH_2PO_4), sodium phosphate biphasic (Na_2HPO_4) and *N*,*N*-dimethylacetamide were purchased from Sigma Chemical Co. (St. Louis, USA). Polystyrene standard (0.06 μm, S.D. 0.005) for Photon Correlation Spectroscopy measurement was obtained from Interfacial Dynamics Co. (Oragon, USA). Dialysis tubing membranes were purchased from Perbio Science UK, Ltd. (Chester, UK). All other chemicals used were of pharmaceutical grade.

2.2. Methods

2.2.1. Synthesis of PEG-P(asp-phe) 5:6:4 and PEG-P(asp-phe) 5:4:6

The synthesis and characterisation of the PEG-P(asp-phe) polymers are described hereunder. The level of mPEG, aspartic acid and phenylalanine in the polymers was assessed by comparing the integral intensities of the resonances at $\delta = 7.13$ [(C_6H_5–CH_2–) of phenylalanine with those the resonances at $\delta = 3.75$ [(–CH_2–CH_2–O–)of MPEG] and $\delta = 2.63$ [(–CH_2–COOH) of aspartic acid] in the ^{1}H NMR.

2.2.1.1. α-Amino-ω-methoxypoly(ethylene glycol) 5000 (MPEG-NH_2). The amine of PEG was prepared as reported earlier (Kugo et al., 1987; Yuan et al., 1998).

^{1}H NMR of: *MPEG-NH_2:* $\delta = 2.78$ (–CH_2–C$\underline{H_2}$–NH_2), $\delta = 3.38$ (C$\underline{H_3}$–O–), $\delta = 3.70$ (–CH_2–C$\underline{H_2}$–O–).

2.2.1.2. L*-Phenylalanine-N-carboxy anhydride (phe-NCA).* L*-Phenylalanine* (28.6 mmol) and THF (100 mL) were added into a dried glass reactor previously flamed and nitrogen-purged for several times. Triphosgene (11.3 mmol) was added under nitrogen protection, and the mixture was reacted at 50 °C under stirring. After the reaction mixture became transparent (about 40 min), the solution was concentrated under vacuum, and was then precipitated from hexane. The product was filtered, washed with hexane and recrystallised from THF/hexane solution

three times (Yuan and Deng, 2000). The purity was a needle crystalline, m.p. of 96–97 °C (yield: 89.3%).

The preparation of β-benzyl L-aspartate-*N*-carboxy anhydride (BLasp-NCA) is similar to the preparation of phe-NCA.

2.2.1.3. Methoxypoly(ethylene glycol)-block-poly(β-benzyl L-aspartate)-stat-poly(L-phenylalanine) copolymer, MPEG-P(BLasp-phe). MPEG-NH_2, BLasp-NCA and phe-NCA were added into a dried 100-mL glass reactor previously nitrogen-purged several times and a mixture of solvents (1,4-dioxane/$CHCl_3$ = 3/2) were injected via a syringe. The reaction temperature was kept at 35 °C for 72 h. The mixture was precipitated with an excess of cold methanol to give a white solid polymer. This method of precipitation allowed the elimination of traces of MPEG-NH_2. The purified product was dried under vacuum at 40 °C for 48 h.

IR of MPEG-P(BLasps-phe) (KBr): 1664 ($\upsilon_{C=O}$), 1550 (υ_{CO-NH}), 1106 (υ_{C-O-C}), 699 and 745 ($\gamma_{=C-H}$).

2.2.1.4. Methoxypoly(ethylene glycol)-block-poly(α, β-L-aspartate-stat-phenylalanine) copolymers (MPEG-P(asp-phe)). MPEG-P(asp-phe) copolymers were prepared by alkali hydrolysis of the side chain benzyl groups of MPEG-P(BLasp-phe). The reaction was performed using 0.5N NaOH at room temperature for 4 h. Composition of the copolymers was determined from ^{1}H NMR measurement.

^{1}H NMR of: *MPEG*-P(asp-phe)*:* δ = 8.3 (–N<u>H</u>–CO), δ = 7.13 ($C_6\underline{H}_5$–CH_2–), δ = 4.5 (CH of amide of polyaspartate), δ = 4.06 (CH of amide of *phenylalanine*), δ = 3.75 (–CH_2–C<u>H</u>$_2$–O–), δ = 3.38 (C<u>H</u>$_3$–O–), δ = 3.07 (–C<u>H</u>$_2$–C_6H_5), δ = 2.63 (–C<u>H</u>$_2$–COOH);

IR of MPEG-P(asps-phe) (KBr): 1660 ($\upsilon_{C=O}$), 1587 (υ_{COONa}), 1550 (υ_{CO-NH}), 1107 (υ_{C-O-C}), 700 and 749 ($\gamma_{=C-H}$).

The reaction scheme for the polymer is indicated below:

2.2.2. Preparation of polyionic micelles

2.2.2.1. PEG-P(asp-phe) 5:6:4. Specified quantities of PEG-P(asp-phe) 5:6:4 were dissolved in phosphate buffer (10 mM, pH 5.3) (5 mL) to obtain drug-free polymeric solutions. For the drug-incorporation studies, specified amounts of diminazene aceturate were added to a polymeric solution containing 5 mg of PEG-P(asp-phe) 5:6:4 to obtain varying drug:monomer molar ratios (+/−, *x*:1). Specified volumes of phosphate buffer solution were added to the polymeric micelle solution to make the final volume up to 5 mL.

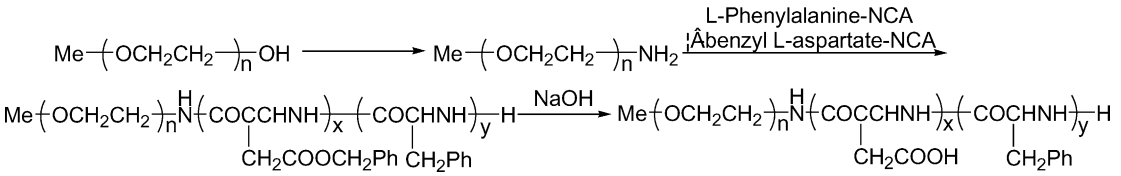
2.2.2.2. PEG-P(asp-phe) 5:4:6. Due to the low water solubility of this polymer, micelles were prepared by a dialysis method. PEG-P(asp-phe) 5:4:6 copolymer (5 mg) was dissolved in *N*,*N*-dimethylacetamide (2 mL) in a scintillation vial and then transferred to dialysis tubing (Spectra/Por 4 molecular weight cut off 12,000–14,000) and dialysed against phosphate buffer (10 mM, pH 5.3) for 24 h. During the first 2 h, the buffer was exchanged two times (every hour) and then three times during the following 22 h. For drug-incorporation studies, the micelles were prepared with a drug:monomer molar ratio of 1:1.

All samples were prepared in duplicate.

2.2.2.3. Effect of pH on micelle size and scattering intensity. PEG-P(asp-phe) 5:6:4 (5 mg/mL)) were transferred to a scintillation vial and the pH was then adjusted to various values ranging from 5.3 to 2.0 using dilute HCl solution. After each pH adjustment, the scattering intensity and micelle size of an aliquot of the sample were determined by photon correlation spectroscopy. For each sample, the mean value ± standard deviation (S.D.) of six determinations were established. Values reported are the mean ± S.D. for two replicate samples.

2.2.3. Physicochemical characterisation

2.2.3.1. Micelle size. Micelle size was determined using Dynamic Light Scattering (Malvern S4700 PCS



System, Malvern Instruments Ltd., Malvern, UK). The analyses were performed at a laser wavelength of 488 nm, scattering angle of 90° and at a bath temperature of 25 °C. For each sample, the mean diameter ± S.D. of six determinations were calculated applying multimodal analyses. Values reported are the mean ± S.D. for two replicate samples.

2.2.3.2. Micelle morphology. Morphological evaluation of drug-free polymeric solutions was performed using transmission electron microscopy (TEM) (Jeol 1010 Electron Microscope, Japan) following negative staining with phosphotungstic acid solution (30%, w/w). Micellar constructs prepared from PEG-P(asp-phe) 5:6:4 and 5:4:6 with diminazene aceturate were prepared as described and the morphological evaluation performed using positive staining with uranyl acetate.

2.2.3.3. In vitro drug release. The in vitro release of diminazene aceturate from the polymeric micelles was determined using the membrane diffusion (dialysis bag) technique.

2.2.3.3.1. PEG-P(asp-phe) 5:6:4 micelles. A 2 mL solution containing diminazene and PEG-P(asp-phe) 5:6:4 in phosphate buffer (10 mM, pH 5.3) at a drug:monomer molar ratio of 1:1 was prepared. The polymeric solution obtained was then added into dialysis tubing (Spectra/Por 6 molecular weight cut off 2000–3000) and dialysed against phosphate buffer solution (pH 5.3, 10 mM, 50 mL, 37 °C). The dialysis solution was agitated by a magnetic stirrer. At given time intervals (0.25, 0.5, 1, 3, 5, 7, 9, 12, 24, 48 h), aliquots of the release medium (1 mL) were taken and replaced with an equal volume of the phosphate buffer solution. The samples were analysed by UV spectroscopy at 257 nm and diminazene aceturate quantified using an appropriate calibration curve (Beckman DU 64 Spectrophotometer, UK). The percentage drug released was calculated by also correcting for sample removal and dilution. A control experiment to determine the release of diminazene aceturate dissolved in phosphate buffer solution (10 mM, pH 5.3) was also undertaken by using an equivalent amount of the drug alone in 2 mL of phosphate buffer solution and the experiment was performed as for the polymer-containing samples. The drug-release data obtained for the above were also compared to the release profile of micelles prepared from a PEG-P(asp) copolymer used in our previous study (Govender et al., 2001).

2.2.3.3.2. PEG-P(asp-phe) 5:4:6 micelles. Diminazene and PEG-P(asp-phe) 5:4:6 were dissolved in *N*,*N*-dimethyl acetamide (2 mL) to obtain a drug:monomer molar ratio of 1:1. The solution was then magnetically stirred for 10 min. The polymeric micelle solution was transferred to dialysis tubing (Spectra/Por 4 molecular weight cut off 12,000–14,000) and dialysed against 10 times excess volume of phosphate buffer solution (pH 5.3, 10 mM) for 24 h with stirring. The dialysis buffer was exchanged five times during the total 24 h of dialysis. The micellar solution was retained within the membrane after dialysis and was thereafter transferred to the dialysis tubing membrane for the drug-release experiment (Spectra/Por 6 molecular weight cut off 2000–3000). The drug-release study was then undertaken as described above.

3. Results and discussion

3.1. Characterisation of drug-free polymeric solutions

Initial studies were undertaken on drug-free polymeric solutions of PEG-P(asp-phe) 5:6:4 and PEG-P(asp-phe) 5:4:6 copolymers. Light scattering, indicative of molecular assembly, was observed for the aqueous buffer solutions of PEG-P(asp-phe) 5:6:4 at pH 5.3. Further, the light-scattering values were also found to increase as the polymeric concentration increased (Fig. 2). Since there was no distinct change in the light-scattering curve profile at the polymeric concentrations

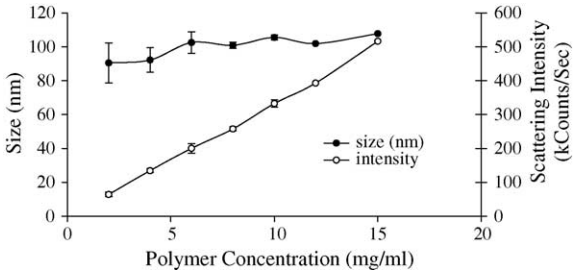


Fig. 2. Scattering intensity and size of drug-free PEG-P(asp-phe) 5:6:4 polymeric solution (in 10 mM phosphate buffer pH 5.3).

investigated, this implied that the cmc for this polymer would be less than 2 mg/mL. The micellar size for the PEG-P(asp-phe) 5:6:4 solutions remained unaffected by the polymeric concentration and was approximately 100 nm (Fig. 2). A similar size trend with polymeric concentration has been previously observed for PLA-PEG micellar association at a low molecular weight of the PLA moiety. A dependence of micellar size on the PLA-PEG copolymer concentration was only observed at higher molecular weights of the hydrophobic PLA moiety (Riley et al., 2001).

Contrary to our previous results with the PEG-P(asp) 5:6 copolymer that did not demonstrate micellar formation in solution in the absence of the drug (Govender et al., 2001), these results with the PEG-P(asp-phe) 5:6:4 copolymer show that an introduction of the phenylalanine monomer resulted in the formation of a micellar system in the absence of drug. For assembly of the copolymer molecules to occur, the attractive hydrophobic interactions should overcome the repulsive forces both between the hydrophilic PEG chains as well as between the ionised moieties of the aspartic acid monomers. The presence of poly(phenylalanine) in this copolymer, therefore, appears to have resulted in hydrophobic/aromatic intermolecular interactions in the aqueous buffer (Yokoyama et al., 1993; Jeong et al., 1999) that could have led to the observed molecular association. A similar role of a hydrophobic moiety has been previously observed for PLA-PEG copolymers. It was shown that PLA 400 Da and PEG of 1800 Da did not form micelles in aqueous media; however, an increase in the PLA molecular weight to 2000 Da resulted in micellisation of the copolymer (Tanodekaew et al., 1997; Govender et al., 2001).

PEG-P(asp-phe) 5:4:6 copolymer was not soluble in water under the same experimental conditions. Hence, the polymer solution in an organic solvent (*N*,*N*-dimethylacetamide) was dialysed against phosphate buffer pH 5.3 solution. The resulting preparation displayed a relatively high scattering intensity (1014.1 ± 10.6) and a hydrodynamic diameter of 59.2 ± 0.8 nm, again indicating molecular association. Interestingly, the PEG-P(asp-phe) 5:4:6 copolymer formed smaller micellar constructs as compared to the PEG-P(asp-phe) 5:6:4 system. However, without further studies, it is not possible to conclude as to whether the size difference is a consequence of different association numbers, an enhanced consolidation of the copolymer molecules in the associates or both phenomena. Moreover, the PEG-P(asp-phe) 5:4:6 micelles were produced by dialysis from an organic solvent, which would affect the association process (Shin et al., 1998), therefore, making the comparisons between these two polymers difficult. Additionally, a higher scattering intensity together with a smaller observed micellar size for a similar amounts of copolymer indicated the less soluble nature of PEG-P(asp-phe) 5:4:6 as compared to PEG-P(asp-phe) 5:6:4.

The molecular association of PEG-P(asp-phe) 5:6:4 and 5:4:6 copolymers is also confirmed and illustrated by transmission electron micrographs shown in Fig. 3. The images clearly indicate the presence of spherical particulates, as shown by lighter entities surrounded by the dark staining. The micellar size obtained from TEM photographs is approximately 74.3 ± 10.4 nm for PEG-P(asp-phe) 5:6:4 and 45.5 ± 15.3 nm for PEG-P(asp-phe) 5:4:6 ($n = 30$) copolymers. The considerably smaller size as compared to the hydrodynamic diameters determined from PCS could be due to material dehydration and collapse of the hydrophilic PEG corona of the micelles during drying and staining of the TEM specimen.

3.2. Effect of pH on the scattering intensity and size of drug-free polymeric micelles

It was assumed that if ionisation of the carboxylic acid groups play an important role in the association of PEG-P(asp-phe) molecules in solution, then the effect of pH and a consequent different degree of ionisation would be reflected in the light scattering and micellar size data. Drug-free polymeric solutions were, therefore, prepared in phosphate buffer pH 5.3 (at which poly(aspartic) acid would be 95.2% ionised if the pK_a value is taken as 4.0). Subsequently, the pH of the solution was gradually altered to pH 2 (poly(aspartic acid) would be only 0.99% ionised) and the size and scattering intensity were determined by PCS. As shown in Fig. 4 for the PEG-P(asp-phe) 5:6:4 copolymer system, a decrease in the buffer pH from 5.3 to 2.0 led to an increased scattering intensity, while the micellar size decreased approximately from 150 to 50 nm. Therefore, a change in the pH of the buffer clearly affected the micellar properties of the PEG-P(asp-phe) 5:6:4 copolymer. These results demonstrate the significance of the phenylalanine groups presence, i.e. in

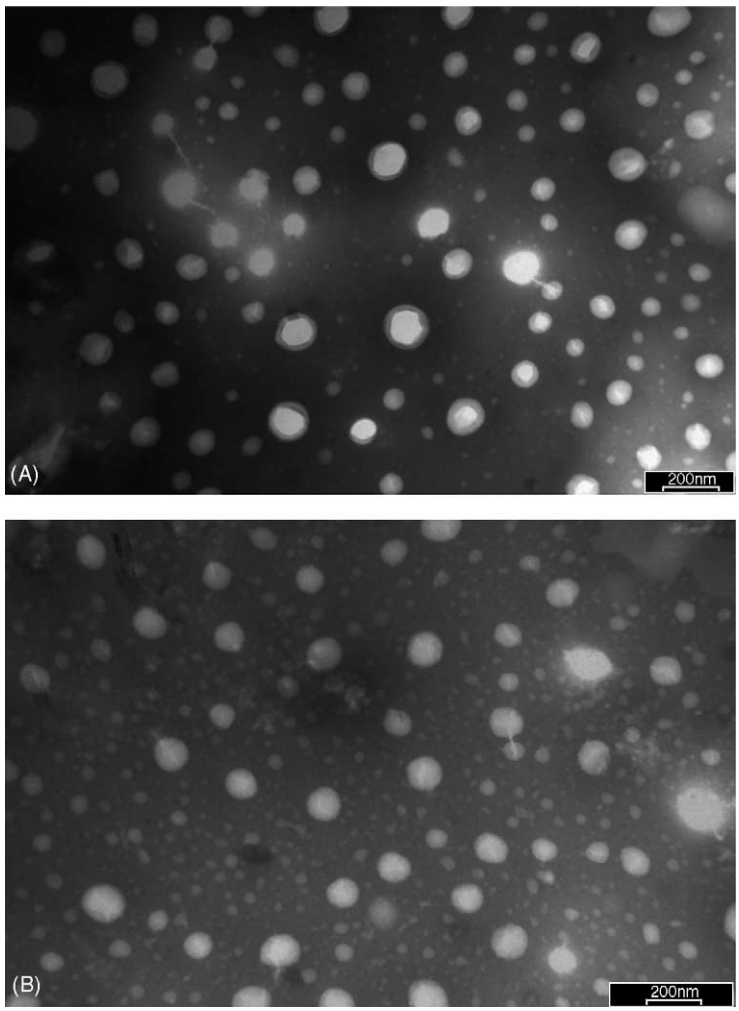


Fig. 3. (A) Transmission electron micrograph of drug-free PEG-P(asp-phe) 5:6:4 polymeric solution (in 10 mM phosphate buffer pH 5.3). (B) Transmission electron micrograph of drug-free PEG-P(asp-phe) 5:4:6 polymeric solution (in 10 mM phosphate buffer pH 5.3).

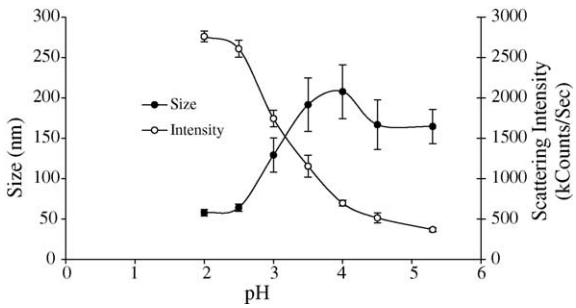


Fig. 4. Effect of pH on size and scattering intensity of PEG-P(asp-phe) 5:6:4 polymeric solution (in 10 mM phosphate buffer pH 5.3).

our previous work with the PEG-P(asp) 5:6 copolymer, molecules did not show micellar association in an aqueous medium when the pH was changed in the same range as used in the present study (Govender et al., 2001). At lower pH values, the reduced ionisation of the carboxylic acid groups of the PEG-P(asp-phe) polymer would be expected to result in reduced repulsive forces between the ionised portions, which consequently leads to a dominance of attractive forces between the non-ionisable and phenylalanine moieties. This may have moved the repulsive-attractive force balance to the more hydrophobic/aromatic forces of attraction resulting in the formation of approximate

Table 1
Effect of diminazene aceturate incorporation into PEG-P(asp-phe) 5:6:4 micelles

Drug:monomer molar ratio (+/−); *x*:1	Size ± S.D. (nm) (polydispersity)
0.06	130.7 ± 19.6 (0.402 ± 0.226)
0.12	145.5 ± 38.8 (0.559 ± 0.008)
0.18	161.7 ± 50.3 (0.411 ± 0.192)
0.6	136.6 ± 33.4 (0.784 ± 0.110)
0.84	124.8 ± 28.1 (0.352 ± 0.094)
1.2	83.2 ± 23.0 (0.504 ± 0.093)
2.4	49.8 ± 7.4 (0.441 ± 0.050)
4.8	45.1 ± 8.1 (0.365 ± 0.132)
6.0	47.7 ± 2.6 (0.317 ± 0.080)
8.4	48.5 ± 0.8 (0.250 ± 0.025

58 nm micelles for the PEG-P(asp-phe) 5:6:4 copolymer. Moreover, the light scattering of the samples increased with a decrease in the pH from 5.3 to 2 (despite formation of smaller size particles), indicating the formation of less solvated particulates as the degree of dissociation is reduced.

3.3. Incorporation of diminazene aceturate into functionalised polymeric micelles

The potential of these novel A(BC) copolymers to incorporate drug was subsequently investigated. Images in Fig. 5 confirm that following drug-incorporation, the morphology of the diminazene-loaded PEG-P(asp-phe) 5:6:4 and PEG-P(asp-phe) 5:4:6 micelles was not adversely affected as they existed as discrete and spherical units. The results for the PEG-P(asp-phe) 5:6:4 system (Table 1), however, revealed a dramatic change in the micellar size with the incorporation of drug at varying drug:monomer molar ratios. As shown in Table 1, the micellar size decreased approximately from 130 to 48 nm with an increase of the drug:monomer molar ratio from 0.06:1 to 8.4:1. This introduces an interesting possibility for the design of nano-sized drug carriers, whereby the system would retain, even reduce, the micelle size following the incorporation of drug.

However, the present findings differ from those obtained in our previous studies with the PEG-P(asp) 5:6 copolymer system, where the micellar size increased from approximately 25–49 nm, with an increase in the drug:monomer molar ratio (Govender et al., 2001). It should be noted that with the PEG-P(asp-phe) 5:6:4 system, the presence of cationic drug interacting with the carboxylic acid groups of the aspartic acids units (Ehtezazi et al., 2000) resulted in a similar micellar size reduction effect as with a decrease in the ionisation of the carboxylic acid groups (Fig. 4 and Table 1). The drug-release profiles (shown later) confirm the drug-incorporation into the micelles. Incorporated drug would require space within the micellar core and it would be difficult to attribute the size reduction to better packing of the molecules in the micelles. Another explanation for the decrease in micellar size could be a reduction in the association number when micelles are formed in the presence of drug.

Previous work on poly(aspartic acid)–diminazene aceturate interaction using a combination of isothermal titration microcalorimetry and scattering techniques showed that one diminazene aceturate molecule interacts with two monomeric units from poly(aspartic acid) (ratio of 0.5–1) (Ehtezazi et al., 2000). It is, therefore, interesting to note that, in this study, the micellar size of the diminazene aceturate and PEG-P(asp-phe) 5:6:4 system reached its micellar size plateau at an approximate ratio of 2.4:1 (Table 1). It would have been expected that the micelle size would have reached the plateau at a ratio of 0.5:1 (corresponding to one drug molecule interacting with two monomeric aspartic acid units), if there were no further interactions/association of the drug with the micelles. In fact, a lower stoichiometry than 0.5:1 could have rather been expected due to possible steric hindrance to the drug–carboxyl group interaction as a result of the phenylalanine groups as well as the statistical distribution of the carboxylic acid and phenylalanine groups that could reduce the probability of orientation/association of one drug molecule with two consecutive carboxylic acid group containing monomers. Rather, it appears that the stoichiometry is higher. An increased amount of drug associated with this system indicates an augmentative role of the phenylalanine moieties in drug-incorporation, thereby creating a system with an increased drug-incorporation efficiency. Clearly, future studies to determine the thermodynamic parameters for the interaction between diminazene and PEG-P(asp-phe) will be necessary to assess the nature of the phenylalanine role in drug-incorporation.

Incorporation of diminazene aceturate into PEG-P(asp-phe) 5:4:6 was performed by the dialysis method using *N*,*N*-dimethylacetamide. PCS analyses illus-

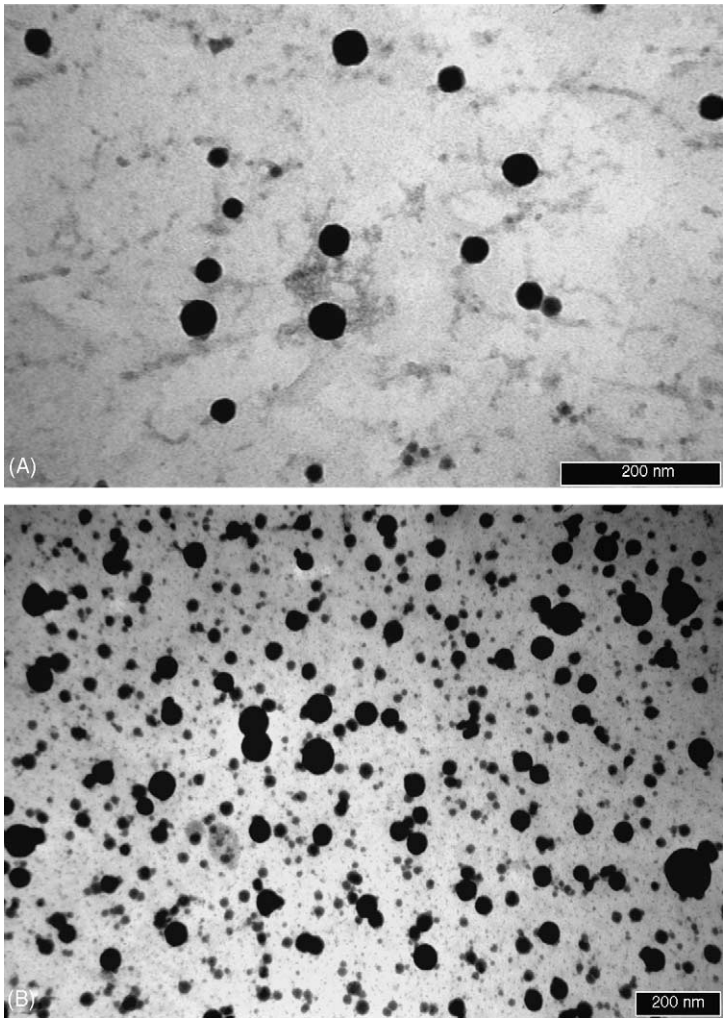


Fig. 5. (A) Transmission electron micrograph of diminazene aceturate-loaded PEG-P(asp-phe) 5:6:4 micelles. (B) Transmission electron micrograph of diminazene aceturate-loaded PEG-P(asp-phe) 5:4:6 micelles.

trated a scattering intensity of 422.4 ± 7.7 kcounts/s and a micellar size of 50.98 ± 2.0 nm. As compared to the diminazene-loaded PEG-P(asp-phe) 5:6:4 system at a similar drug/polymer ratio, the drug-loaded micelles of the PEG-P(asp-phe) 5:4:6 system were smaller. However, as discussed earlier, a different method of preparing the micelles together with the increased proportion of phenylalanine groups may have been responsible for such an effect.

It is worth noting that the size of drug-loaded micelles prepared from this novel A(BC) copolymer is in the sub-200 nanometer size range and may, therefore, be exploited to achieve efficient tissue penetration to target sites in the body.

3.4. Diminazene aceturate release from polymeric micelles

An in vitro release study was performed on the three polymeric systems of PEG-P(asp) 5:6 (used as a control in this study), PEG-P(asp-phe) 5:6:4 and PEG-P(asp-phe) 5:4:6 with a diminazene aceturate drug:charged monomer molar ratio of 1:1. While the actual incorporated drug was not separated from the free drug and

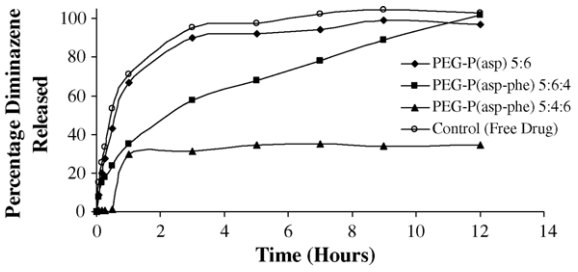


Fig. 6. In vitro drug-release trends of PEG-P(asp) 5:6, PEG-P(asp-phe) 5:6:4 and PEG-P(asp-phe) 5:4:6 micelles.

percentage released calculated as a percentage of the theoretical drug loading, this study nevertheless was useful in identifying the drug-release trends of the three systems relative to free drug in phosphate buffer solution (pH 5.3). Fig. 6 illustrates the release profiles of diminazene aceturate from PEG-P(asp) 5:6, PEG-P(asp-phe) 5:6:4 and PEG-P(asp-phe) 5:4:6 systems in relation to a control experiment. The PEG-P(asp) 5:6 system displayed a rapid drug release with approximately 90% of the drug being released from the micelles within the first 3 h only. Actually, the release profile did not differ significantly from the profile of the control (free drug solution alone).

The release profile for that of the PEG-P(asp-phe) 5:6:4 system differed significantly from that of the phenylalanine-free PEG-P(asp) 5:6 system. The PEG-P(asp-phe) 5:6:4 micellar system displayed a drug release of approximately 35% in the first hour while the PEG-P(asp) system, and the control demonstrated a drug release of approximately 70% within the first hour. Further, following the initial phase the remaining 65% of drug from the PEG-P(asp-phe) 5:6:4 system was gradually released over an extended 12 h period. Therefore, the PEG-P(asp-phe) 5:6:4 system was able to show a more controlled release of diminazene as compared to the phenylalanine-free copolymer, PEG-P(asp).

In micelle preparation for the PEG-P(asp-phe) 5:4:6-diminazene aceturate system, the polymer-drug solution in organic solvent was dialysed against phosphate buffer pH 5.3 for 24 h with five buffer replacements prior to drug-release testing. During this stage, a portion of drug could have diffused into the dialysis medium and the maximum release plateau (Fig. 6) implies that about 35% of total diminazene aceturate was associated with micelles following the dialysis procedure. It is interesting to note that the release profile of diminazene aceturate from PEG-P(asp-phe) 5:4:6 micelles exhibited a lag time in the first 30 min (Fig. 6), which may be a consequence of the high proportion of drug already being extracted from the micelles. However, it is surprising that following the lag time, the entire amount of the drug associated with the micelles was released within the next 30 min. Although the micellar system appeared to still have 35% drug associated with it after 24 h of dialysis, the reason for almost complete release of the remaining associated drug within 1 h during the release study is not clear. It may have been due to the higher temperature (37 °C) during the release experiment as well as manipulations with the sample to set up the release experiment. It could be expected that as an introduction of the phenylalanine group in PEG-P(asp-phe) 5:6:4 copolymer reduced the drug-release rate relative to the PEG-P(asp) 5:6 copolymer, this trend would be followed and an increased proportion of phenylalanine groups in the PEG-P(asp-phe) 5:4:6 copolymer could further retard the drug-release rate (Oh et al., 1999; Allen et al., 2000).

This study has, therefore, shown that the newly synthesised PEG-P(asp-phe) 5:6:4 and PEG-P(asp-phe) 5:4:6 copolymers are capable of incorporating and releasing the drug effectively, and therefore, show potential as a useful drug carrier. Further, the incorporation of phenylalanine moiety played a significant role in decreasing the drug-release rate from the polymeric micelles.

4. Conclusions

The synthesis of a novel block copolymer containing three polymeric components, ethylene glycol, aspartic acid and phenylalanine and its potential for the preparation of functionalised copolymer micelles with a water-soluble drug were investigated in this study. Initial assumption was that the synthesis of the polymer containing a functionalised monomeric unit for electrostatic interaction would allow incorporation of charged water-soluble drugs into the micellar core, while the presence of a relatively more hydrophobic monomer would afford hydrophobic interactions to stabilise the micelles.

Initial studies on the drug-free polymeric solutions of PEG-P(asp-phe) 5:6:4 and PEG-P(asp-phe) 5:4:6 in-

dicated polymeric aggregation into micellar-type constructs in the sub-200 nm size range, whereby the size of the micelles was affected by changes in the pH of the medium, i.e. the degree of aspartic acid carboxyl group ionisation. Interestingly, the polymer interaction with and incorporation of a cationic drug also resulted in a reduction of the micellar size, creating a system that had a clear size-dependence on the pH and drug association.

The drug-loaded micelles were confirmed to be discrete, fairly uniform and spherical with a size appropriate for drug-targeting purposes. The importance of a balanced copolymer composition was also demonstrated as an increased ratio of phenylalanine units resulted in polymer insolubility in water hence necessitating the use of an organic solvent in micelle formation.

The initial hypothesis that incorporation of the relatively more hydrophobic monomer, phenylalanine, would be beneficial, can be confirmed by the slower drug-release profiles of the PEG-P(asp-phe) 5:6:4 system relative to the phenylalanine-free PEG-Pasp 5:6 copolymer.

This study hence demonstrated the advantage of such PEG-P(asp-phe) copolymer micelles as delivery vehicles in that they may be tailor-made (e.g. size, drug loading, hydrophobicity, polarity of core and sensitivity to the environment) to suit a particular application by changing the composition of the copolymer (e.g. block composition, block length, block ratio) and the conditions used in micelle preparation.

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