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Poly(Ethylene Glycol)–branched Polyethylenimine–poly(L-phenylalanine) Block Copolymer Synthesized by Multi-initiation Method for Formation of More Stable Polyelectrolyte Complex with Biotherapeutic Drugs

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ABSTRACT – An amphiphilic cationic branched methoxy poly (ethylene glycol)–branched polyethylenimine – poly(L-phenylalanine) (mPEG–bPEI–pPhe) block copolymer was successfully synthesized by ring-opening polymerization (ROP) of N-carboxyanhydride of L-phenylalanine (Phe–NCA) with mPEG–bPEI for the preparation of more stable polyelectrolyte complex (PEC) included a hydrophobic interaction. mPEG–bPEI was firstly prepared by the coupling of mPEG and bPEI using hexamethylene diisocyanate (HMDI). The structural properties of mPEG–bPEI–pPhe copolymers were confirmed by ^{1}H NMR. The copolymers exhibited a self-assemble behavior in water above critical aggregate concentration (CAC) in the range of 0.01–0.14 g/L. The CAC of copolymers obviously depended on the hydrophobic block content in the copolymers (the value decreased with the increase of the pPhe block content). The cationic copolymers have the ability to form multi-interaction complex (MIC) with bovine serum albumin (BSA) and plasmid DNA through multi-interaction (electrostatic and hydrophobic interaction). The physicochemical characterization of the complex was carried out by the measurement of zeta potential and particle size. Their zeta-potentials were positive (approximately +10 mV) and their sizes decreased with increasing pPhe contents in the copolymers (PPF/BSA wt% ratio = 2). The complex showed good stability at high ionic strength. Therefore, mPEG–bPEI–pPhe block copolymer was considered as a potential material to enhance the stability of complex including biotherapuetic drugs.

Key words – Polyelectrolyte complex, Multi-interaction complex, Amphiphilic copolymer, Hydrophobic interaction, Drug delivery

Over the past decade, amphiphilic block copolymers have been investigated due to their unique properties such as self-assembly which forms a polymeric micelle. The micelle has a good thermodynamic stability in physiological conditions because of their low critical aggregate concentration (CAC) (Hagan et al., 1996; Kataoka et al., 2001; Park and Na, 2010; Yin et al., 2008). More recently, some groups have reported a new class of polymeric micelles, a polyelectrolyte complex (PEC) induced by an electrostatic interaction between a pair of oppositely charged polymer and biotherapeutic drug (protein and plasmid DNA) (Chelushkin et al., 2008; Harada and Kataoka, 1995; Kabanov et al., 1996; Lee et al., 2009). The PEC is a spherical particle with < 200 nm (Kataoka et al., 1996). Because of the size and the coreshell structure, the potential of PEC have been reported as a biotherapeutic drug carrier for human therapy (Katayose and Kataoka, 1997).

Its stability which is very important factor to use as a drug carrier in clinical try is influenced by various factors such as concentration, temperature, and chemical structure of the polymer. PEC as well as micelles composed of amphiphilic block copolymers dissociate upon dilution when the concentration reaches the CAC. In addition, the salt concentration is a key parameter for the dissociation of the PEC because the Coulombic interactions between charged segments are screened by the added salt (Kakizawa et al., 1999). Therefore, the destabilization of PEC in salt condition (under physiological condition) limits their applications as a drug carrier. Several approaches have been reported to enhance the stability of PEC by chemical cross-linking of the core or shell (Hu et al., 2008; Yuan et al., 2005; Zhang et al., 2008). However, the approach has disadvantages in terms of the biocompatibility and biodegradability of the material for medical applications. Moreover, the chemical reactions in the cores or shells may induce side effects because the reaction change in the properties of the micelles and/or encapsulated drugs by undesirable intermicellar cross-linking (Akagi et al., 2009).

In this study, in order to overcome the problems, the multi-branched copolymer was developed for the stabilization of

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ionic complex added with hydrophobic interactions. The cationic branched methoxy poly(ethylene glycol)–branched polyethylenimine–poly(Phenylalanine) (mPEG–bPEI–pPhe) copolymer was synthesized by two-step procedures. The cationic mPEG–bPEI as a hydrophilic skeleton was firstly synthesized, and then used as a multi-initiator to start the ring opening polymerization (ROP) of Phe–NCA to prepare mPEG–bPEI–pPhe. L-phenylalanine (phe), a hydrophobic amino acid, was introduced to give another interaction (hydrophobic interaction) to PEC.

The PEC will be prepared by mixing mPEG-bPEI-pPhe with model protein drug (Bovine serum albumin, BSA) and plasmid DNA in doubly distilled water (DDW). mPEG–bPEI–pPhe copolymer spontaneously forms PEC with BSA and plasmid DNA as a result of cooperative electrostatic interactions between the amine groups of the multibranched structure and the carboxylic group of the BSA at upper *p*I value (4.6). After the preparation of complex between the polymer and bio-agents, hydrophobic interaction between pPhes and other hydrophobic segments adds to the PEC. In this point, mPEG–bPEI–pPhe copolymer is capable of forming multi-interaction complex (MIC) with biotherapuetic drugs at physiological condition because the phenyl groups in the phenylalanine can be interacted with protein/other phenyl groups by hydrophobic interaction in the physiological condition (pH7.4) (Scheme 1).

Herein, mPEG-bPEI-pPhe copolymers with different phe contents were synthesized by a multi-initiation method and analyzed by ^{1}H-NMR. The self-assembled property of the copolymers was monitored by fluoresces method using pyrene as a fluoresce indicator. The complexes of the copolymer and biotherapuetic drugs were prepared by electrostatic and hydrophobic interaction, and their physicochemical properties were investigated in the terms of zeta potential, particle size and gel retardation assay.

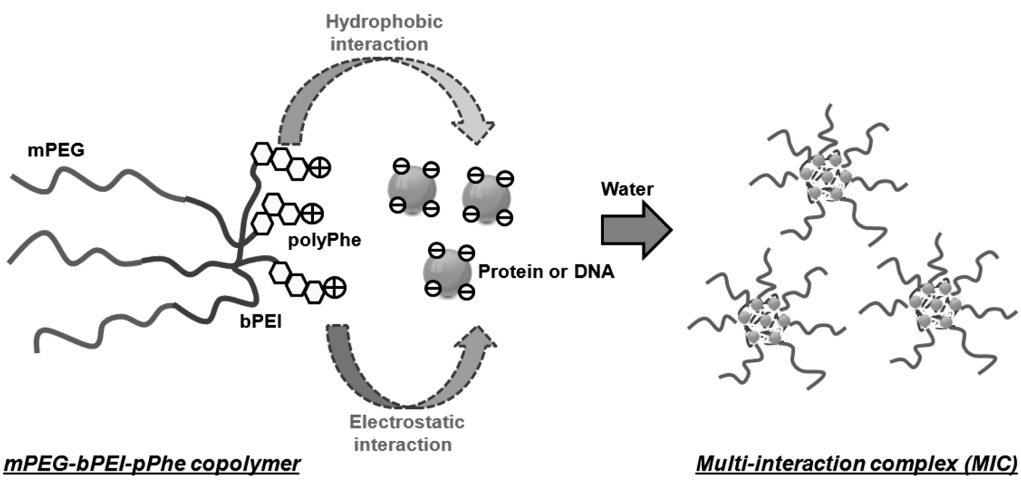
Materials and Methods

Materials

Methoxy polyethylene glycol (mPEG) with molecular weights of 5000 Da (mPEG 5000) and BSA were purchased from Sigma-Aldrich Co. (St. Luis, MO, USA) and first purified by precipitation into hexane from tetrahydrofuran (THF), then the vacuum-dried precipitates were further dried by azeotropic distillation with toluene. Branched polyethyleneimine with molecular weights of 1,800 Da (bPEI 1800) was purchased from Aesar Alfar (Ward Hill, MA, USA) and was dried in vacuo at 40°C for 48 h before use. Hexamethylene diisocyanate (HMDI) (99%) from Fluka (AG, Buchs, Switzerland) was used as received. Toluene, hexane, and tetrahydrofuran (THF) were dried by refluxing over Na metal under argon atmosphere and distilled immediately before use. Chloroform was treated with HMDI for 4 h at 60°C and distilled to remove any traces of water and ethanol.

Synthesis of N-carboxyanhydride of Phenylalanine (phe-NCA)

N-carboxyanhydride of Phenylalanine (phe-NCA) was prepared according to the reported method (Kim et al., 2005). L-Phenylalanine (5.0 g) was suspended in anhydrous 1, 4-dioxane (50 mL), and triphosgene (3.5 g) was added under stirring at room temperature. The temperature was raised to 60°C, and the mixture was stirred until the amino acid was completely dissolved. The solution was condensed at room temperature under reduced pressure, and then the L-phenylalanine NCA was crystallized by addition of hexane.



Scheme 1. Schematic illustration of multi-interaction complex (MIC).

Synthesis of mPEG–bPEI–pPhe

The mPEG–bPEI block copolymer was synthesized according to a similar method (Petersen et al., 2002). Briefly, mPEG 5000 was reacted with large excess HMDI to get mPEG–NCO. Then, the $CHCl_3$ solution of mPEG–NCO was slowly added dropwise into a $CHCl_3$ solution of bPEI 1,800 to obtain mPEG–bPEI. In a dry glass flask, a certain amount of mPEG–bPEI with different ratios of Phe–NCA was dissolved in dried chloroform and stirred for 72 h at 25°C. Then, the mixture was precipitated with an excess of diethyl ether and dried under vacuum. The reaction scheme is shown in Scheme 2. ^1H NMR spectra were recorded in D_2O at 25°C or $CDCl_3$ (TMS as reference) at 25°C on Bruker NMR Spectrometer (Bruker, Germany).

Measurement of critical aggregation concentration (CAC)

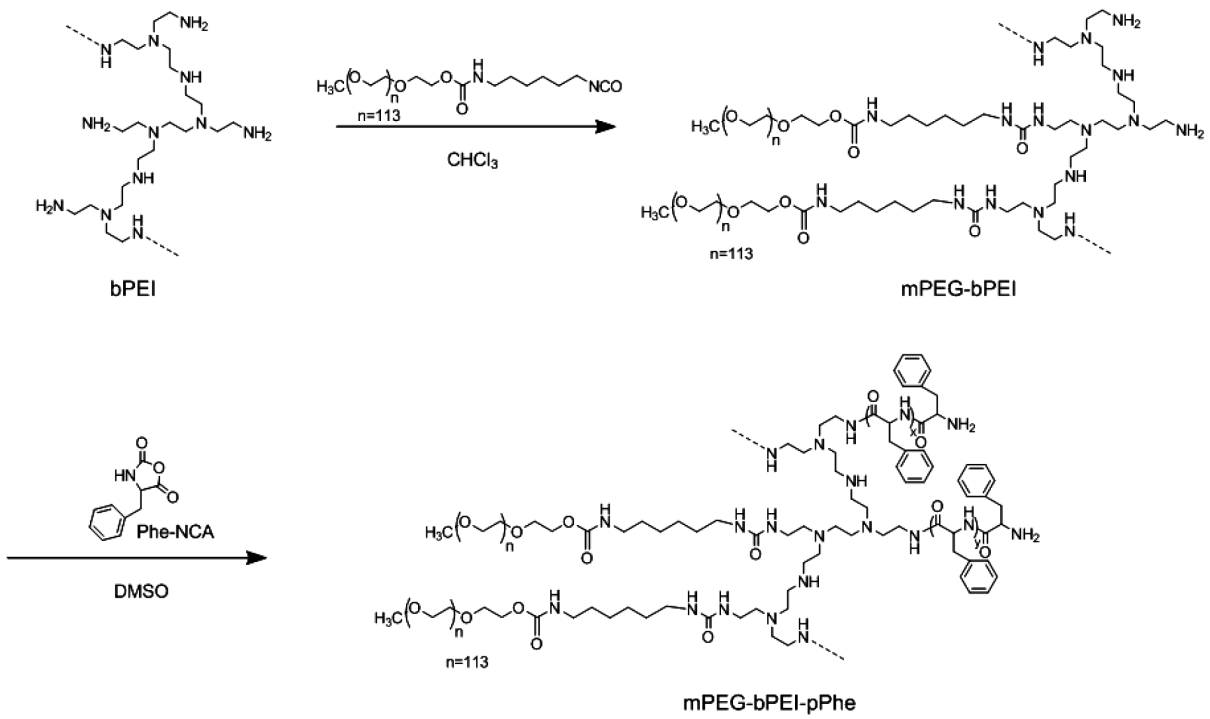
The CAC of the copolymer was calculated via fluorescence spectroscopy (pyrene) (Park et al.). A pyrene stock solution (6.0×10^2M) was prepared in acetone and stored at 5°C until use. For the measurement of steady-state fluorescence spectra, the pyrene solution in acetone was added to distilled water (DW) to yield a pyrene concentration of 12.0×10^7 M. The solution was then distilled in vacuo for 1 hour at 60°C to remove the acetone. The acetone-free pyrene was mixed with solutions of copolymer, the concentrations of which ranged from 1×10^5 to 1.0 mg/mL. The final pyrene concentration in each sample solution was 6.0×10^7 M, which is nearly identical to its solubility in water at 25°C.

mPEG-bPEI-pPhe copolymer and BSA complex characterization

The hydrodynamic size of mPEG-bPEI-pPhe copolymer/BSA complexes (PPF/BSA %wt ratio = 2) was determined by PCS using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). PCS was performed at 25°C in DDW in triplicate with sampling time and analysis set to automatic. Particle size is presented as the % number of three measurements ±SD. To investigate the effect of salt on hydrodynamic size, sodium chloride was added to the particle solution for set time points before remeasurement. Surface charge was measured by determination of zeta-potential using a Zetasizer Nano ZS in DDW.

Gel retardation assay

The gel retardation assay was performed as follows (Kunath et al., 2003). The plasmid was diluted in 1 μg/μl. copolymer solutions were then added to the plasmid solutions with same volume at various concentration ratios ranging from 0 to 10 and vortexed. After 10 min incubation, the complex solutions were mixed with loading buffer (50% (v/v) glycerol 85%, 1 mM EDTA and 40 mM tris-HCl, pH 7.4) and loaded onto an ethidium-bromide (EtBr) containing 1% agarose gel. Elec-



Scheme 2. Synthesis of mPEG-bPEI-pPhe.

trophoresis (DY-4C, Liuyi, Beijing, China) was carried out with a current of 100 V for 1 h in TAE running buffer solution (40 mM tris-HCl, 1% (v/v) acetic acid, 1mM EDTA). DNA was visualized on a UV transilluminator.

Results and Discussion

Synthesis of multi-initiated block copolymer as a drug carrier

An amphiphilic cationic branched mPEG–bPEI–pPhe block copolymer (abbreviated as PPF) was synthesized to enhance the stability of polyelectrolyte complex (PEC) with protein or gene. The complex was prepared by electrostatic and hydrophobic interactions. The cationic mPEG–bPEI as a hydrophilic skeleton was firstly synthesized, and then used as a multi-initiator to initiate the ring opening polymerization (ROP) of phe–NCA to prepare mPEG–bPEI–pPhe. There are two advantages for that branched polyethylenimine (bPEI) which is introduced as a main chain and frame in the amphiphilic copolymer. (1) bPEI has a branched molecular structure that can be inherited by the target copolymer through ROP of phe–NCA initiated by primary amine groups of bPEI. (2) bPEI with positive charges provides the ability to complex a protein or other DNA drugs (Chen et al., 2010; Tian et al., 2005). Poly-phenylalanine (pPhe) was chosen as the hydrophobic segment. pPhe is one of the synthetic polypeptides attracting attention for their application in drug delivery matrices (Cho et al., 2004; Huh et al., 2005; Kim et al., 2005). Poly (ethylene glycol; PEG) was also introduced into the copolymer as hydrophilic segment to enhance the copolymer's amphiphilic properties for its good biocompatibility and water solubility (Jeong et al., 1999). Because of the amphiphilic properties, it can be predicted that the copolymer possesses multi-interaction ability to form multi-interaction complex (MIC) with protein drug or gene. The successful synthesis of the copolymer can be analyzed by ^{1}H NMR spectra of mPEG–bPEI in D_2O and mPEG–bPEI–pPhe in $CDCl_3$ as shown in Figure 1. The component ratio of mPEG/bPEI is the same in mPEG–bPEI–pPhe and its precursor mPEG–bPEI, so mPEG/bPEI in mPEG–bPEI–pPhe can be calculated from ^{1}H NMR spectra of PEG–PEI in D_2O (Figure 2(A)), whose signals of mPEG (3.65 ppm) in a and bPEI (2.3-2.7 ppm) in (g+h) were separated completely. The signals in ^{1}H NMR spectra of bPEI and pPhe could not be separated completely in $CDCl_3$, because of the overlapping of the mixture signals from multi-amino groups around 2.5 ppm, so that it is difficult to calculate the bPEI content by the ^{1}H NMR signals in Figure 1(B). However, the component ratio of mPEG/pPhe can be estimated from peak intensities of the

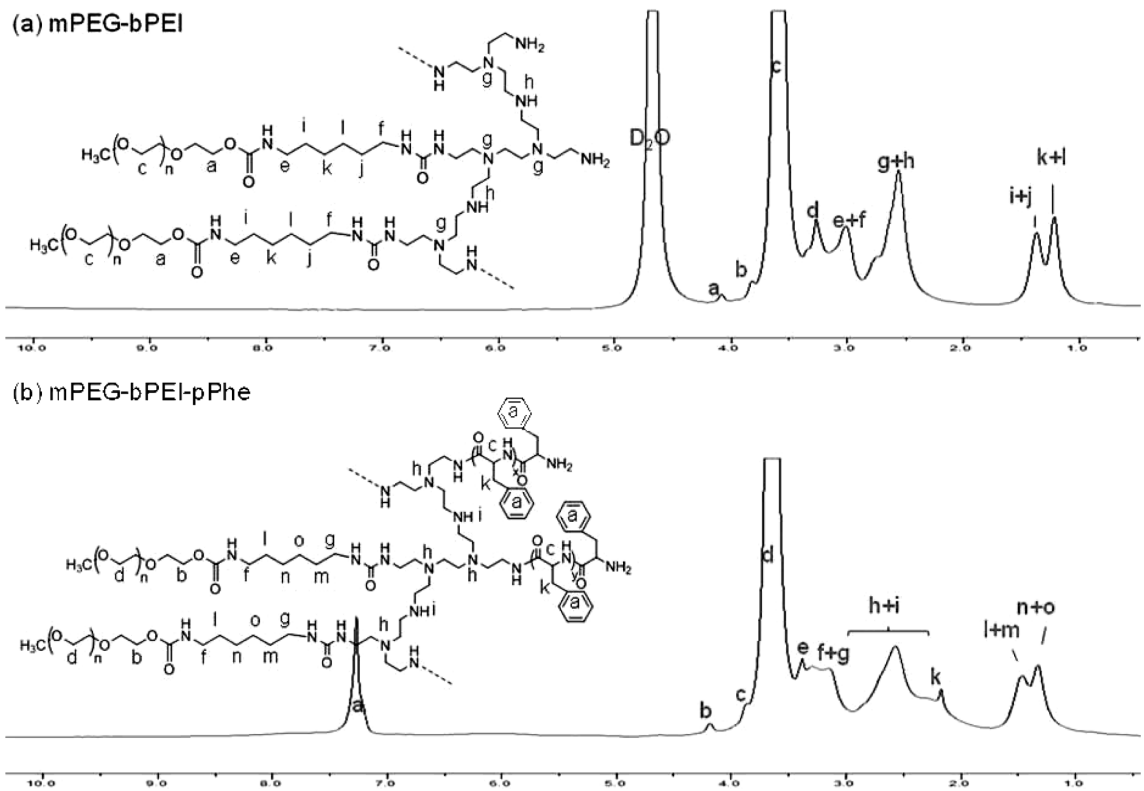


Figure 1. The ^{1}H-NMR spectra of (a) mPEG-bPEI in D_2O, (b) mPEG-bPEI-pPhe in $CDCl_3$.

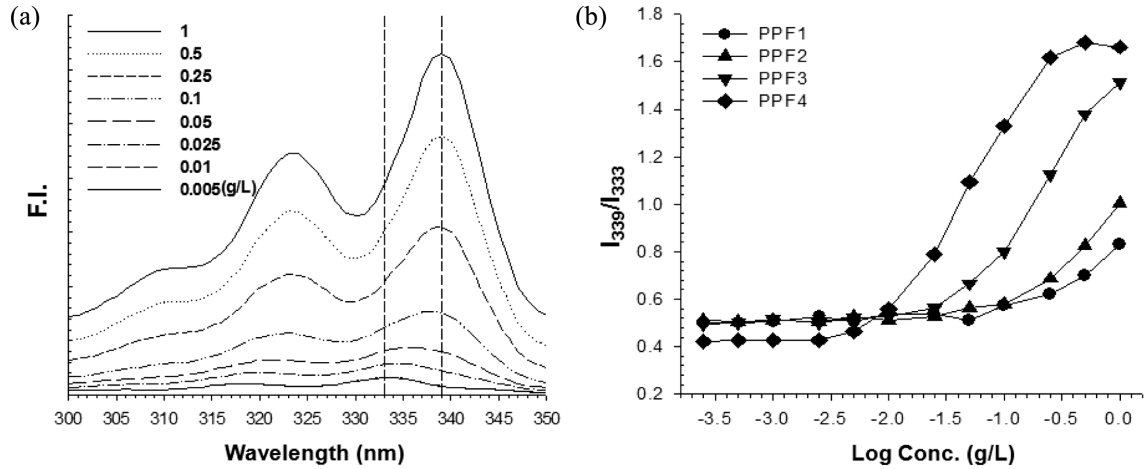


Figure 2. (a) Excitation spectra of pyrene as a function of PPF4 concentration in water and (b) plots of I_{339}/I_{333} versus logarithm of MIC concentration.

Table I. *Characterization of Multi-initiated copolymer (mPEG-bPEI-pPhe)*

Code.	Copolymer	mPEG M_n	bPEI M_n	Content of monomeric units %[a]			M_n[a] (kDa)	CAC[b] (g/L)
				PEG	PEI	Phe		
PPF0	mPEG-bPEI	5,000	1,800	64.9	35.1	0	7.7	N.D
PPF1	mPEG-bPEI-pPhe	5,000	1,800	61.2	33.1	5.7	8.2	0.14
PPF2	mPEG-bPEI-pPhe	5,000	1,800	59.9	32.4	7.7	8.3	0.09
PPF3	mPEG-bPEI-pPhe	5,000	1,800	53.4	28.9	17.7	9.4	0.03
PPF4	mPEG-bPEI-pPhe	5,000	1,800	47.8	25.8	26.4	10.5	0.01

[a]As determined by ^{1}H-NMR
[b]fluorescence technique (pyrene)

methylene proton signal (5.05 ppm) in p (–C_6H_6) of the pPhe block and the ethylene proton signal (3.65 ppm) in a (–CH_2–CH_2–) of the mPEG block in ^{1}H NMR spectra of mPEG–bPEI–pPhe (Figure 1(B)). Then the number–average molecular weights M_n of the copolymer could be calculated from the ratio of monomeric units in the copolymer because M_n of bPEI was available. In Table I are listed the content of monomeric units and the molecular weights of the copolymers calculated from ^{1}H NMR spectra.

Critical aggregation concentration (CAC)

The amphiphilicity of the copolymers provides an opportunity to enhance stability of PEC after interaction with protein drug and gene in water. The nonionic water-soluble mPEG chains served as a hydrophilic shell stabilizing the nanoparticles. bPEI constituted as the positively charged crown and pPhe formed as a hydrophobic core. This copolymer system has several possible advantages when used as biotherapuetic drug delivery system compared with the common polyelectrolyte system (PEI and Polylysine). (1) This PPF copolymer is capable of forming MIC with proteins at physiological condition because the phenyl groups in the phenylalanine can be interacted with protein/other phenyl groups by hydrophobic interaction in the physiological condition (pH 7.4). (2) Not only a protein drug, but also negatively charged DNA can be encapsulated in this cationic copolymer.

Critical aggregate concentrations (CAC) were estimated to prove the amphiphilicity of PPF copolymers using pyrene as a hydrophobic probe. Figure 2(A) shows the excitation spectrum of pyrene in the various concentrations of PPF4 copolymers. A red shift from 333 to 339 nm was observed with increasing concentration of PPF4 copolymers indicating the formation of aggregates. Figure 2(B) showed the intensity ratios (I_{339}/I_{333}) of pyrene excitation spectra versus the logarithm of the copolymer concentration. The CAC was obtained from the intersection of two straight lines: the base line and the rapidly rising I_{339}/I_{333} line in Figure 2(B). It was found that the wavelength of the maximal peak in the excitation spectrum using pyrene as a probe in mPEG–bPEI–pPhe nanoparticle system (339 nm) is bigger than that in the PCL–PEG system (336.5 nm) (Piao et

al., 2003). It indicated that the mPEG–bPEI–pPhe has a more hydrophobic core. The CAC data in the doubly distilled water (DDW) are listed in Table I. In aqueous solution, PPF0 did not form any aggregate in the experimental condition, while copolymer PPF1, PPF2, PPF3 and PPF4 could self-assemble in water with CAC in the range of 0.01–0.14 g/L, and the CAC of the copolymer decreased with increasing pPhe content. From this it was concluded that the content of hydrophobic segment (pPhe) played an important role in the formation and hydrophobicity of the copolymer aggregate. The PPF4 is water insoluble due to their strong hydrophobicity. Therefore, PPF4 was excluded in complexation study with biotherapeutic drug.

Complex of PPF/biotherapeutic drug (Protein and DNA)

It was reported that the oppositely charged polyelectrolytes could form complexes in aqueous system. The complexes used as drug carriers and gene carriers were extensively studied recently (Lee et al., 2007; Park and Na, 2009a; b). In this paper, preliminary research was carried out to prepare complexes between cationic copolymer and negatively charged biotherapuetic drugs (protein and plasmid DNA). For this study, Bovine Serum Albumin (BSA, p*I* = 4.6 (Brewer et al., 2005)) is selected as a model protein with negative charged protein at physiological condition. The zeta-potential results provide evidence for intermolecular interactions between PPF copolymers and BSA. Figure 3(A) demonstrated that the zeta-potential changed from about 15 mV (native BSA) to about +10 mV (PPF/BSA complexes, %wt ratio = 2) at DDW. This indicates that the addition of PPF copolymers resulted in an increase in the zeta-potential due to the load of negatively charged BSA causing the bonding between amine groups from PPF copolymers and the carboxyl from the BSA. The particle size of PPF1/BSA, PPF2/BSA and PPF3/BSA were 164, 38, and 24 nm, respectively (Figure 3(B)). This result indicates

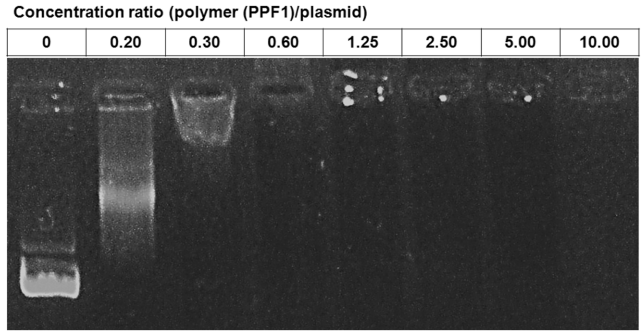


Figure 4. Gel retardation assay of the complexes of PPF1 copolymer and plasmid DNA.

that the particle size of the complex decreased with increasing evidently the increase of the phenylalanine contents (Table I) enhances the chances of hydrophobic interactions between phe and BSA. The PPF copolymers (PPF2) also were employed to prepare copolymer/DNA complexes. PPF2 copolymer solutions were added to the plasmid (DNA) solutions with same volume at a concentration ratio from 0.2 to 10 and vortexed. After 10 min incubation, the complex solutions were taken out for gel retardation assay (Figure 4). Complete neutralization was achieved at the concentration ratios of PPF/plasmid from 0.6 to 10. This suggests that the plasmid was spontaneously absorbed or immobilized in the copolymer through electrostatic and hydrophobic interaction.

Stability of multi-interaction complex in salt condition

The stability of the polyelectrolyte complex (PEC) is influenced by various factors involving their chemical compositions and their surrounding environment. In particular, for PEC, the ionic strength in the solution is a key parameter for stability due to the shielding effect of the ionic species on the electrostatic interactions (Jaturanpinyo et al., 2004). In this study, to overcome these obstructions, MIC prepared from a

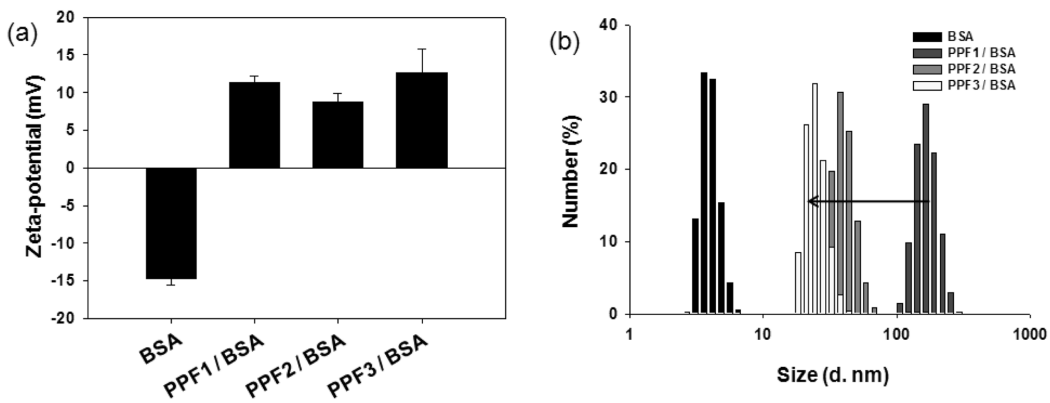


Figure 3. (a) zeta-potential change of PPF copolymers / BSA complexes (wt% ratio = 2) and (b) size distribution of PPF copolymers / BSA complexes (wt% ratio = 2) in water (n=3).

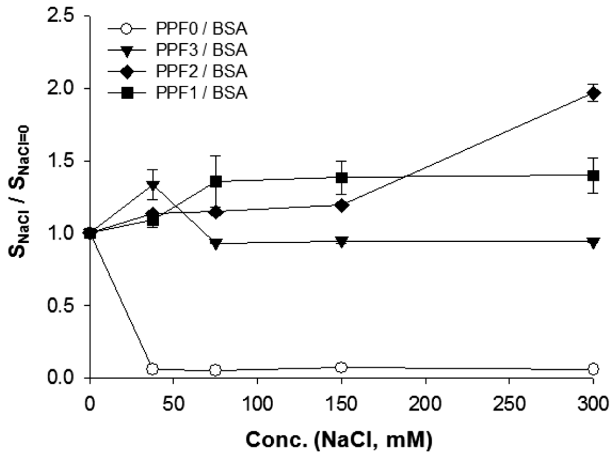


Figure 5. Stability of multi-interaction complex (MIC); variations in $Size_{NaCl}/Size_{NaCl=0}$ with increasing NaCl concentrations for MIC (n=3).

cationic block copolymer and an anionic protein (BSA) was stabilized with phenylalanine through hydrophobic interaction. To examine the stability of PPF/BSA MIC in salt condition, the dependence of the variations of particle size on NaCl concentration was analyzed by using the dynamic light scattering (DLS) measurements (Figure 5). Obviously, the MICs (PPF1/BSA, PPF2/BSA and PPF3/BSA (%wt ratio = 2)) have an improved stability against a high salt concentration. However, PEC group (PPF0/BSA) without hydrophobic interaction was decomplexed from the complex with increasing NaCl concentration. This result indicates that introduction of the polyphenylalanine group's hydrophobicity significantly increases the complex tolerability against increasing NaCl concentrations. These results provide a novel concept in the design of a multi-interaction complex potentially useful as biotherapuetic drug delivery system.

Conclusion

A cationic branched copolymer composing of bPEI (as backbone), mPEG and pPhe (as branched arm) was synthesized to prepare more stable polyelectrolyte complex (PEC) added with hydrophobic interactions. As the content of the hydrophobic block pPhe increased, a CAC value of mPEG–bPEI–pPhe (PPF) copolymer decreased. The cationic copolymers had the ability to form complexes with protein (BSA) and plasmid DNA through multi-interaction (electrostatic and hydrophobic interaction). As ionic strength in the solution increases, the dissociation of PPF/BSA complex did not observe, while the complex of mPEG–bPEI and BSA easily dissociated. This result was led to the introduction of another interaction between hydrophobic chains (pPhe groups) to PPF/BSA complex. Therefore, mPEG–bPEI–pPhe copolymer is a valuable material to improve the stability of electrostatic complex including biotherapuetic drug.

Acknowledgments

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