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Micellization and Reversible pH-Sensitive Phase Transfer of the Hyperbranched Multiarm PEI–PBLG Copolymer

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Abstract: A novel, hyperbranched, amphiphilic multiarm biodegradable polyethylenimine-poly(γ-benzyl-L-glutamate) (PEI–PBLG) copolymer was prepared by the ring-opening polymerization of γ-benzyl-L-glutamate–*N*-carboxyanhydride (BLG–NCA) with hyperbranched PEI as a macroinitiator. The copolymer could self-assemble into core-shell micelles in aqueous solution with highly hydrophobic micelle cores. As the PBLG content was increased, the size of the micelles increased and the critical micelle concentration (CMC) decreased. The surface of the micelles had a positive ζ potential. The cationic micelles were capable of complexing with plasmid DNA (pDNA), which could be released subsequently by treatment with polyanions. The PEI–PBLG copolymer formed unimolecular micelles in chloroform solution. The pH-sensitive phase-transfer behavior exhibited two critical pH points for triggering the encapsulation and release of guest molecules. Both the encapsulation and release processes were rapid and reversible. Under strong acidic or alkaline conditions, the release process became partially or completely irreversible. Thus, this copolymer system should be an attractive candidate for a gene- or drug-delivery system in aqueous media and could provide the phase-transfer carriers between water and organic media.

Keywords: amphiphiles · drug delivery · micelles · phase transfer · polymers

Introduction

Polymer micelles have attracted much recent attention because of their unique characteristics, such as their nanosize, core-shell architecture, and good thermodynamic stability under physiological conditions. They show great potential for applications in drug and gene delivery.[1–4] Polymer micelles in aqueous solution usually consist of linear amphiphilic copolymers and several amphiphilic dendrimers have been reported.[5–8] Nevertheless, very few aqueous micelles resulting from amphiphilic hyperbranched copolymers have been reported. Amphiphilic hyperbranched copolymers show special assembling behavior and have been the subject of a number of studies.[9,10] Micelles assembled from amphiphilic hyperbranched copolymers could be viewed as micelles with partially cross-linked shells and, hence, have good shape stability.[5,11,12] Furthermore, biocompatibility and biodegradability are of great importance in micelle systems with applications in vivo.[13] The assembly in aqueous solution of micelles from biodegradable amphiphilic hyperbranched copolymers has not yet been reported, except for the linear hyperbranched amphiphilic poly[(ethylene glycol)-polyethylenimine-poly(γ-benzyl-L-glutamate) (PEG–PEI–PBLG) copolymer reported by us.[14] Here, we present a facile synthesis of a novel biodegradable amphiphilic multiarm hyperbranched PEI–PBLG copolymer and its self-assembly in aqueous solution.

Many hyperbranched amphiphilic copolymers form unimolecular micelles. They can be used as effective tools to encapsulate and solubilize guest molecules in solvents with various polarities.[15–18] This process is considered as a phase-transfer phenomenon. Haag et al.[17] reported pH-sensitive hyperbranched amphiphilic copolymers for which one criti-

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cal pH value triggers the release of guest molecules from the unimolecular micelles. An interesting and exciting system would be one that has two trigger pH points to control the release. Here, we report for the first time that the phase-transfer behavior of the PEI–PBLG copolymer exhibits two pH points that trigger the encapsulation and release of the guest molecules.

Results and Discussion

Micelles: The PEI–PBLG copolymer (PP) (Scheme 1A) in a good solvent for PBLG could be considered as a unimolecular micelle, in which hydrophilic PEI is the core and hydrophobic PBLG is the shell. Such a structure is illustrated in Scheme 1B. In an aqueous solution, this copolymer may assemble into an inversed core-shell structure, with PEI as the positively charged shell and PBLG as the hydrophobic core (Scheme 1C). The latter micelle has several advantages[14] over common micelle systems (such as PEG–PLA (PLA = polylactide) or PEG–PCL (PCL = polycaprolactone) micelles) for use in a drug- or gene-delivery system. First, the interactions between positive charges carried by the PEI segment and negative charges on cell surfaces might result in a high cell-uptake efficiency for the cationic micelle system.[1] Second, not only hydrophobic drugs, but also negatively charged water-soluble proteins or DNA could be carried by this cationic micelle. Finally, the PBLG segment would confer biodegradability and biocompatibility to this micelle system.

The micelle formation of the PEI–PBLG copolymer in aqueous solution was monitored by fluorescence spectroscopy using pyrene as a hydrophobic probe. The excitation spectra of pyrene in PP3 solutions of various concentrations are shown in Figure 1A. A red-shift from 333 to 338 nm was observed as PP3 concentration increased, indicating the formation of micelles. The intensity ratio (I_{338}/I_{333}) of pyrene excitation spectra versus the logarithm of copolymer concentration is shown in Figure 1B. The critical micelle concentration (CMC) was obtained from the intersection of the baseline and the tangent of the rapidly rising I_{338}/I_{333} curve in Figure 1B.

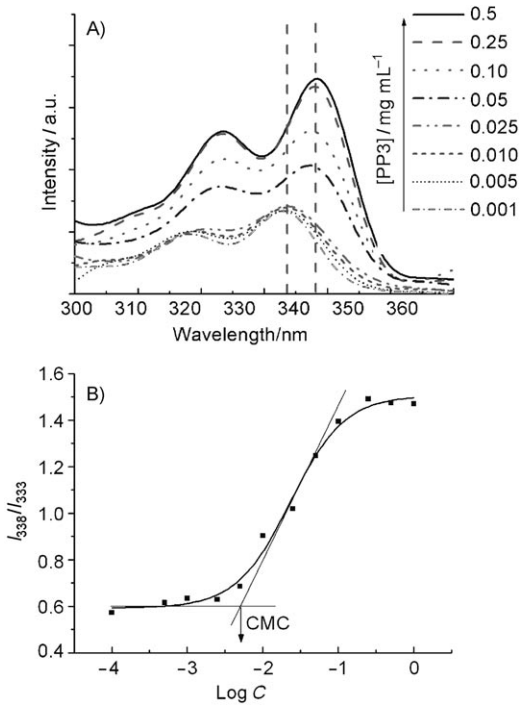
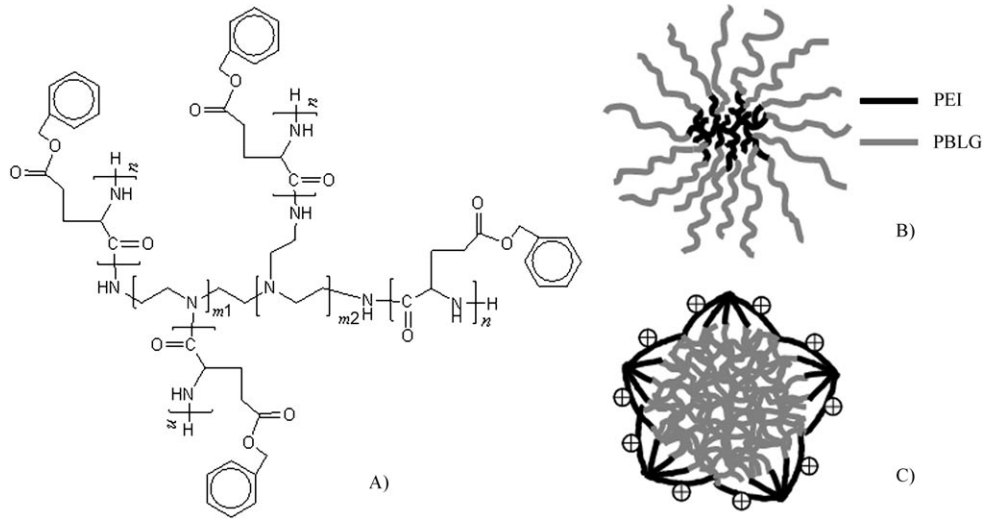


Figure 1. A) Excitation spectra of pyrene as a function of PP3 concentration in water. B) Plot of I_{338}/I_{333} against logarithm of PP3 concentration.

As shown in Figure 1A, the peak wavelength of pyrene in PEI–PBLG micelles (338 nm) is longer than that reported for either the PCL–PEG (336.5 nm) or PLA–PEG (335 nm systems).[19] Because the red-shift is dependent on the relative hydrophobicity of the micelle core, the PEI–PBLG is



Scheme 1. A) Structures of PEI–PBLG. B) PEI–PBLG unimolecular micelle in a good solvent. C) Micelle in aqueous solution.

likely to have more-hydrophobic micelle cores than the other two systems. Thus, this PEI–PBLG system should have enhanced thermodynamic stability and improved encapsulation efficiency towards hydrophobic drugs.

Dynamic light scattering (DLS) was then used to determine the characteristic size of the micelles. The size-distribution histogram of the PP3 solution (Figure 2) showed a unimodal distribution with a mean micelle diameter of 71 nm. The micelle morphology was investigated by atomic force microscopy (AFM). The AFM image of sample PP3 is shown in Figure 3A. Spherical micelles were observed on the substrate. The lateral size was about 60 nm, which was somewhat smaller than that determined by DLS analysis. The smaller size could be attributed to the micelle shrinkage during the drying process. Because the AFM tip could not penetrate into the micelles, the structural information concerning the micelle cores was not attainable by AFM. Hence, transmission electron microscopy (TEM) was carried out on PP3 micelles that were negatively stained with uranyl acetate on a carbon-coated copper grid. As shown in Figure 3B, the micelle appeared as a bright sphere surrounded by a dark ring, and had a diameter in the range of 40–70 nm. The thickness of the dark layer was approximately 3–5 nm. The ring might be considered as the shell of the micelles consisting of PEI segments, and its darkness was attributed to the negative staining by uranyl acetate. These observations confirmed the core-shell structure of the micelles. The morphology and structure determined from AFM and TEM studies were consistent with the proposed structure for the micelles in aqueous solution shown in Scheme 1C.

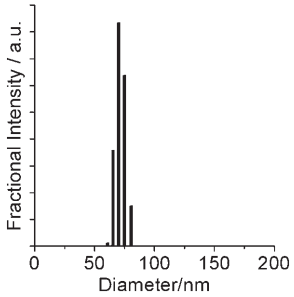


Figure 2. Size distribution of PP3 micelles in aqueous solution.

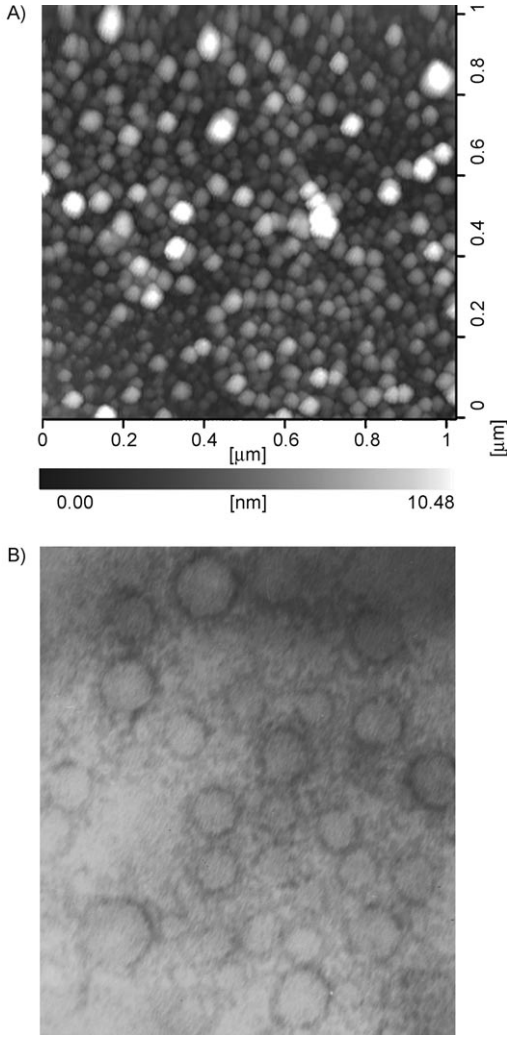


Figure 3. AFM image (height) (A) and TEM image (B) of PP3 micelles (negatively stained with 1 wt % uranyl acetate).

The CMC of various micelles in aqueous solution are summarized in Table 1. In aqueous solutions, PP1 does not form any micelles under our experimental conditions, whereas the other polymers could assemble into micelles in aqueous solution and had CMCs in the range of 3.278 to 0.034×10^{-6} M, which decreased as the PBLG content increased. This indicates that the content of the hydrophobic segment (PBLG) plays an important role in micelle formation. The formation of micelles from an amphiphilic copolymer is accomplished through the balance of two competing functions. The hydrophilic function of the soluble segments keeps the copolymer molecules dispersed stably in water. The hydrophobic function of the insoluble segments induces them to aggregate away from the water phase to form the micelle cores. Consequently, the hydrophilic segments form the shell of the micelle. The copolymer with lower PBLG content has difficulty aggregating from

Table 1. Molecular weights and compositions of the copolymer (as estimated by ^{1}H NMR measurements), and CMC and sizes of PEI–PBLG micelles in aqueous systems.

Sample	Monomeric unit [mol %]		M_n [kDa]	CMC		Size of freshly made micelles [nm]	Size of micelles stored for 3 weeks [nm]
	EI	BLG		[mg mL^{-1}]	[10^{-6} M]		
PP1	91.4	8.6	15	nd[a]	nd[a]	nd[a]	nd[a]
PP2	77.8	22.2	24	0.0786	3.278	56.5	91.5
PP3	67.4	32.6	35	0.0051	0.146	71.6	69.6
PP4	42.6	57.4	79	0.0027	0.034	103.1	113.0

[a] Sample was water soluble within the concentration range used and could not form micelles.

the solution, and it has a higher CMC than the copolymer with higher PBLG content. The PBLG content in PP1 is too low for micelles to form in the concentration range used here. The average sizes of the different micelles are also listed in Table 1. The micelle size increases as PBLG content increases, because the micelle core becomes larger at higher PBLG concentrations.

The stability of PP micelles in aqueous media was also evaluated by comparing the size of freshly prepared micelles with that of the micelles stored for three weeks (Table 1). The size of PP3 and PP4 micelles did not change much, indicating good stability over three weeks. However, after three weeks, the PP2 micelles were almost twice the size of freshly prepared PP2 micelles. Apparently, aggregation of PP2 micelles occurred during this time. The poor stability of PP2 micelles might be caused by the high hydrophilicity of PP2 molecules with high PEI content. The hydrophilic PEI tended to stretch the PP2 molecules out of the micelle, thereby inducing a loose structure for PP2 micelles. Consequently, aggregation between these loose PP2 micelles was likely, in contrast to the PP3 and PP4 micelles.

Furthermore, the positively charged PEI resulted in cationic micelles. Measurements of ζ potentials of PP3 micelles revealed positive surface charges of around +43 mV. This offered the interesting possibility for the application of PEI–PBLG micelles as gene carriers. We investigated the preparation of complexes between cationic micelles and negatively charged plasmid DNA (pDNA). The preliminary results suggested a successful complexation of PP3 micelles with pDNA, as confirmed by gel retardation assay (Figure 4A). Complete neutralization was achieved at mass ratios of micelles/plasmid of 3–5.

The physical integrity of the DNA after complexation is a prerequisite for maintenance of biological activity and mediation of a successful transfection.[20] Disassembly of DNA from the carrier is a critical step in the final stage of gene expression. The disassembly reaction was believed to occur by interaction of the ionic complex of DNA with other anionically charged macromolecules or cellular components, such as mRNA, sulfated sugars, and nuclear chromatin.[21] Releasing of pDNA was studied by incubating the complexed PP3 micelles with sodium polyacrylate (NaPAA). As shown in Figure 4B, there was no pDNA band in lane 2, indicating that the pDNA was completely complexed with PP3 micelles. The use of 100- and 300-fold excesses of NaPAA relative to pDNA (mass ratio) to release pDNA resulted in the appearance of the pDNA bands. This clearly indicates that pDNA molecules could be readily released from PP micelles/pDNA complexes by treatment with polyanions, such as NaPAA. In addition, the PEI–PBLG copolymer also showed good biocompatibility (see Supporting Information). Thus, the PEI–PBLG micelles in aqueous solution may find applications in the pharmaceutical field, for example, as drug- and gene-delivery systems.

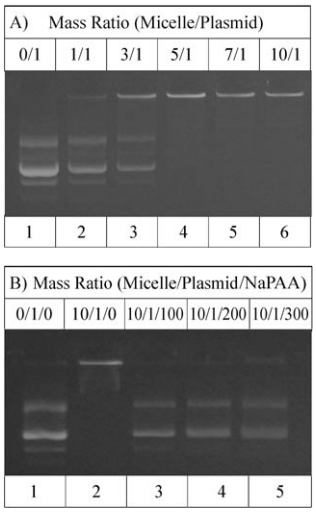


Figure 4. Gel retardation assay: A) Complexation of PP3 micelles with pDNA at different micelle/pDNA mass ratios. B) Release of pDNA from PP3 micelle/pDNA complex upon treatment with NaPAA polyanions.

pH-Sensitive phase-transfer properties of PEI–PBLG: As shown in Scheme 1B, PEI–PBLG has a unimolecular micelle structure with hyperbranched hydrophilic core and hydrophobic shell. This structure offers the ability of transferring guest molecules between two immiscible liquid phases.[15]

DLS measurements of PP in chloroform (0.5 mg mL^{-1}) were recorded at size scale of less than 50 nm (Figure 5). The mean size of PP2, PP3, and PP4 samples ranged from

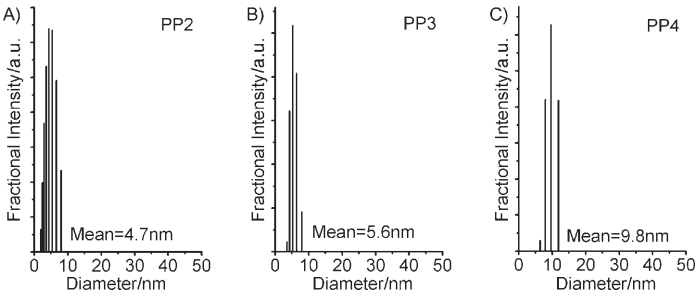


Figure 5. Size distribution of unimolecular micelles for (A) PP2, (B) PP3, and (C) PP4 in chloroform.

4.7–9.8 nm, indicating the existence of unimolecular micelle structures of the PEI–PBLG copolymer in chloroform solution. The increase in size of the unimolecular micelles from PP2 to PP4 might be caused by the increase of PBLG content. The higher PBLG content might produce unimolecular micelles with thicker shells. A small proportion of structures larger than 120 nm were also found for PP samples in chloroform. These large particles were clearly not unimolecular micelles, but most likely aggregations of unimolecular micelles.

Methyl orange (MO), bromophenol blue, Congo red, and fluorescein were chosen to demonstrate the phase-transfer ability of PEI–PBLG. As shown in Figure 6, the dye/water and chloroform phases were separated clearly in the left vial of each pair. As the copolymer PEI–PBLG was added and

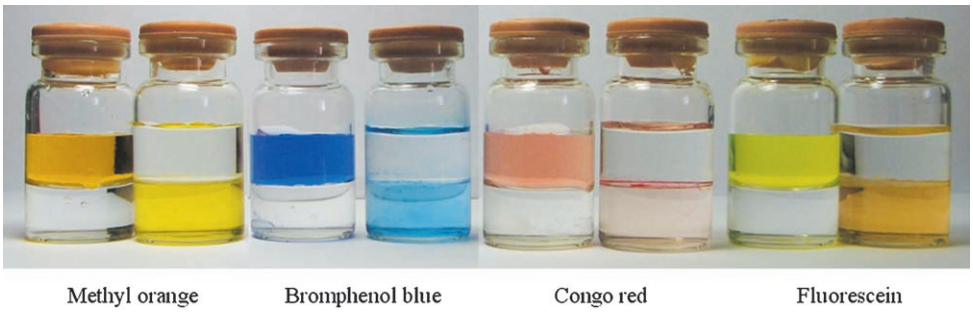


Figure 6. Encapsulation and transfer of various dyes from the aqueous phase (upper) to the chloroform phase (lower) by PP2. In the left picture of each pair, the colorless organic phase indicates insolubility of the dye in the absence of PEI–PBLG, and in the right picture of each pair, the colorless aqueous phase demonstrates complete transfer of the dye upon encapsulation by the unimolecular micelles.

the vial was shaken manually, the colorless chloroform became colored (right vial). This indicated that the dye molecules were encapsulated by PEI–PBLG and transported from the aqueous phase to the chloroform phase completely. The load of dye molecules per polymer was determined by UV-visible spectroscopy. The MO transport capacity for PP3 was 21.4 mol mol^{-1} (MO/PP3) (Supporting information).

The pH-triggered release of MO back into the water phase is shown in Figure 7B and C. Upon addition of acid or alkali solution into the vial, the dye was released from the PEI–PBLG and diffused to the aqueous phase quickly after shaking. The PEI–PBLG nanocapsules appeared to have two trigger pH values, which were investigated further as follows: An aqueous solution of MO (5 mL, 0.01 mg mL^{-1} or 0.0305×10^{-3} M) was mixed with a chloroform solution of PP3 (5 mL, 0.1 mg mL^{-1} or 2.857×10^{-6} M) and shaken manually for several seconds. After phase separation, MO was totally extracted into the chloroform phase. A certain amount of an acid or alkali solution was added to the water phase to adjust the pH. After shaking and phase separation, the pH value and MO concentration (determined by UV-visible spectroscopy) in the water phase were measured. The percentage of MO in the water phase was plotted against the pH value (Figure 8). The trigger pH values were 2.3 and 10.3, which were determined as the values at which the amount of MO in the water phase increased suddenly. The anionic molecules could be encapsulated by PEI–PBLG unimolecular micelles within the pH window (encapsulation window) between these two values. Outside of this window, the dye would be released from PEI–PBLG capsules back into the aqueous solution. To our best knowledge, PEI–PBLG is the first phase-transfer system with two trigger pH points. Haag et al.[17] reported a pH-responsive release caused by hydrolysis of hydrophobic segment. However, in our case, the hydrophobic PBLG shell was not broken during the release of the dye over several hours. The encapsulation and release of the dye were reversible upon appropriate adjustment of the pH of the system, and the reversible process could be completed within one minute. Further studies showed that strongly acidic conditions (pH < 2) and strongly alkaline conditions (pH > 11) caused the release

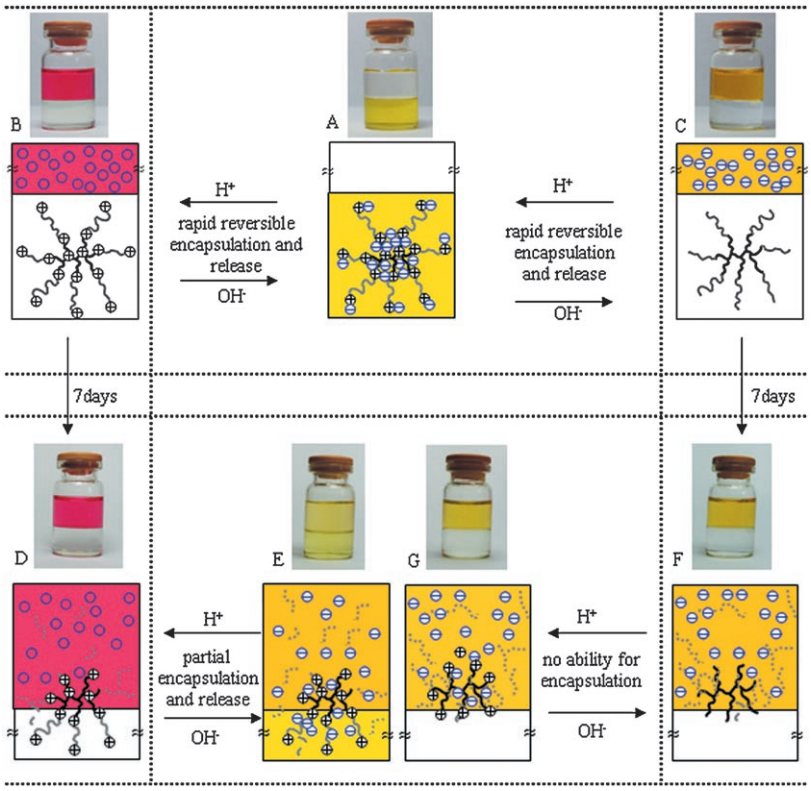


Figure 7. Mechanism for pH-sensitive encapsulation and release of methyl orange (MO) by PEI–PBLG. Blue circles represent the MO color group. Black circles represent the PEI and the amine groups of the PBLG charged state. In each vial or rectangle, the upper phase is water, the lower phase is chloroform. A) Encapsulation of MO by PEI–PBLG unimolecular micelles. B) Acid-triggered release of MO. C) Alkali-triggered release of MO. D) Maintained in acid (pH 1) for one week. E) Partial re-encapsulation of MO. F) Maintained under alkaline conditions (pH 12) for one week. G) No phase transfer.

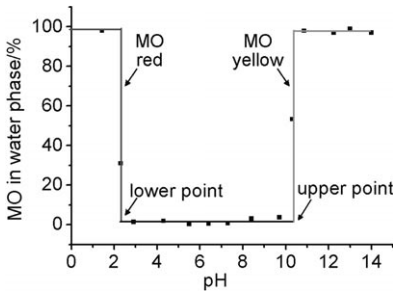


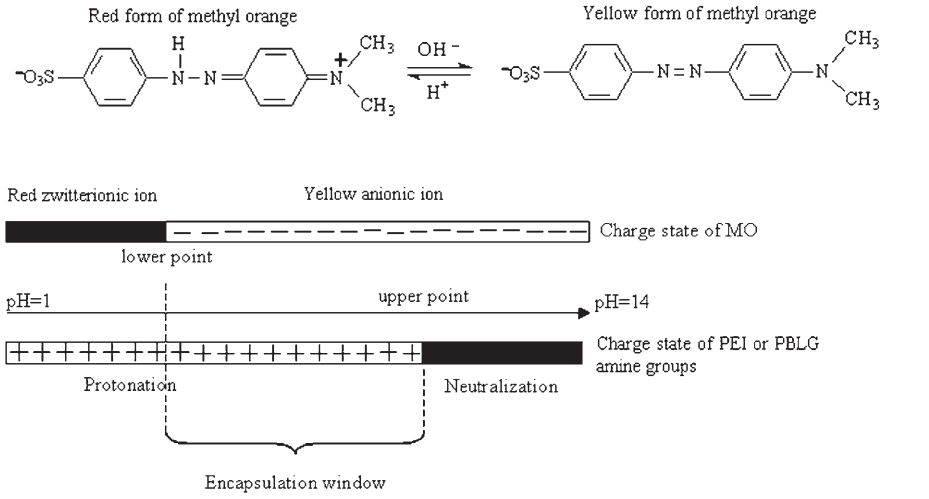
Figure 8. Determination of pH points triggering phase transfer by PP3.

process to be partially irreversible or totally irreversible, depending on the time. As shown in Figure 7D and E, after one week of incubation in the strong acid, the pH was adjusted back to within the "encapsulation window", but the MO could not be transferred totally back into the chloroform phase by PEI–PBLG. By contrast, after one week of incubation in the strong alkali (pH > 11), the release process was totally irreversible (Figure 7F and G).

As reported,[15–18] two criteria are essential for the phase-transfer system: 1) formation of amphiphilic core-shell micelle-like structures in a good solvent (i.e. one in which PEI-PBLG is highly soluble), and 2) hydrogen-bonding or electrostatic interactions between hydrophilic cores of the unimolecular micelles and the anionic dyes. Thus, in our system, hydrogen-bonding or electrostatic attraction at the interface between the two immiscible liquids forced the dye molecules to diffuse into the hyperbranched hydrophilic core of the unimolecular micelles. Consequently, the unimolecular micelles with encapsulated dyes could be dispersed into the oil phase. The encapsulated dyes could be released from the chloroform phase back into the water phase if either of these two criteria was not met.

The encapsulation and pH-triggered encapsulation–release behavior of PEI–PBLG towards MO is explained further in Scheme 2 and Figure 7. Scheme 2 shows the MO color-change mechanism triggered by variation in pH. The color-change point for MO is at pH 3.2–4.4. At a pH below 3.2, MO exists as a red zwitterionic ion with no net charge, due to neutralization of the positive and negative charges. At pH > 4.4, MO exists as a yellow anion with net negative charges.

At pH 7, PEI and the amine groups of PBLG were positively charged and MO existed as a yellow anion. As the aqueous solution of MO and the PEI–PBLG/chloroform solution were mixed, the MO anion was absorbed by PEI at the interface of the water and chloroform phases. The unimolecular micelles were then dispersed in the chloroform phase, making the oil phase yellow (Figure 7A). The MO becomes red at pH values below the lower trigger point (i.e., pH 2.3), at which the electrostatic interaction between the positively charged PEI–PBLG and MO was destroyed. MO molecules were released from the unimolecular micelles and returned to the water phase, which became red (Figure 7B). The lower trigger pH value measured (pH 2.3) differed from the MO color-change point (pH 3.2–4.4) because the protonation of PEI consumed some of the protons in the chloroform phase, so that a lower pH was required to trigger the release of MO from the PEI–PBLG unimolecular micelles. On the other hand, PEI and the amine groups of PBLG were deprotonated and became neutral as the pH was increased up to 10.3. The electrostatic interaction between the neutral PEI–PBLG and the anionic MO did not exist, so that the anionic MO was released from the unimolecular micelles into the water phase, which became yellow (Figure 7C). The electrostatic attraction between unimolecular micelles and MO could be restored quickly upon adjustment of the pH back to within the "encapsulation window" (pH 2.3–10.3). Hence, this electrostatically dominated encapsulation–release was a rapidly reversible process. Interestingly, the pH-sensitive phase-transfer behavior reported by Haag et al.[17] was caused by the cleavage of the hydrophobic shell (i.e., shell damage dominated release), and the pH-sensitive release in their work was slow and irreversible. Our PEI–PBLG system also exhibited this shell-damage-dominated release behavior. In strong acid or alkali, hydrolysis of the benzyl ester group in PBLG could occur, followed by gradual degradation of the PBLG backbones. Some degraded PEI–PBLG fragments might be repelled from the chloroform phase into the water phase. This could weaken the phase-transfer ability of PEI–PBLG. After incubation under strong alkaline conditions for one week, PEI–PBLG completely lost its phase-transfer ability, even after the pH was adjusted back into the "encapsulation windows" (Figure 7F and G). After one week in strong acid, PEI–PBLG lost its phase-transfer ability only



Scheme 2. Mechanism for the color change of methyl orange dye (MO) and representation of the "encapsulation window".

partially, however, the weakened encapsulation ability of PEI–PBLG could not transfer all of the MO back into the chloroform solution after adjustment of the pH to 7 (Figure 7D and E). In strongly acidic conditions, the phase-transfer ability will be completely lost over a longer time.

Conclusion

A novel, hyperbranched, amphiphilic multiarm biodegradable copolymer PEI–PBLG with various compositions was synthesized. At high PBLG content, the copolymer forms micelles in aqueous solution, with the highly hydrophobic PBLG as the micelle core and the positively charged hydrophilic PEI as the shell. A greater hydrophobic PBLG content results in a lower CMC and larger PEI–PBLG micelles. The positively charged micelles have the potential to form complexes with DNA or to improve the adhesive properties of cells. The PEI–PBLG copolymers exist in chloroform as unimolecular micelles and have phase-transfer ability for dyes between the chloroform and water phases. There are two trigger pH points for the encapsulation and release of the methyl orange dye at pH 2.3 and 10.3. The electrostatically dominated pH-sensitive phase transfer was rapid and reversible. The encapsulation ability of PEI–PBLG unimolecular micelles can be partially or totally destroyed by strong acidic or alkaline conditions because of the degradation of the PBLG units. This hyperbranched amphiphilic multiarm copolymer system has a variety of potential applications in controlled drug release, gene delivery, and phase transfer.

Experimental Section

Materials: Hyperbranched PEI (Hy-PEI) with molecular weight of 10000 Da (PEI 10000) was purchased from Aesar Alfar and was dried in vacuo at 70 °C for 48 h before use. The *N*-carboxyanhydride used in the polymerization reaction with γ-benzyl-L-glutamate was prepared according to the reported method.[22] Tetrahydrofuran (THF) was dried by refluxing over Na metal under argon atmosphere followed by distillation immediately before use. Chloroform was treated with CaH and distilled. Plasmid DNA (pGFP) was purchased from Clontech (Palo Alto, CA, USA).

Synthesis of PEI–PBLG: A series of novel, hyperbranched, amphiphilic multiarm PEI–PBLG copolymers, as summarized in Table 1, were synthesized by a simple one-step process, in which hyperbranched PEI was used as a macroinitiator of the ring-opening polymerization of BLG–NCA according to a modified literature procedure.[14] In thoroughly dried glass flasks, mixtures of PEI and BLG–NCA of different ratios in dried chloroform were stirred for 72 h at 25 °C. The solutions were then condensed and dialyzed (molecular weight cut-off 10000–14000 Da) against chloroform (500 mL, with four changes over 48 h). The dialysates were finally dried under a vacuum. ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ (TMS as reference) at 25 °C by using a Unity-400 NMR spectrometer.

Copolymer analysis: The ^{1}H NMR spectrum of PP4 in $CDCl_3$ is shown in the Supporting Information (Figure S1). The component ratio of EI/BLG could be estimated from peak intensities of the methylene proton signal at 5.05 ppm ($-CH_2-C_6H_5$) of the PBLG segment and the ethylene proton signal[23] at 2.3–3.4 ppm ($-CH_2-CH_2-$) of the PEI segment. The molecular weight of the copolymer could be estimated from the ratio of monomeric units in the copolymer, as the molecular weight of PEI was available. The content of monomeric units and the molecular weights of the copolymers calculated from ^{1}H NMR spectra are listed in Table 1. The ^{13}C NMR spectra of PEI and PP3 in $CDCl_3$ are shown in the Supporting Information (Figure S2) and the results are in agreement with proposed structures.

Micelle preparation in aqueous solution: The micelles were prepared by following literature procedures:[24] PEI–PBLG copolymer (0.1 g) was first dissolved in THF (10 mL), and doubly distilled water (40 mL) was added dropwise with gentle agitation in a 100 mL volumetric flask, then THF was removed by using a water aspirator at 25 °C for 4 h. The micellar solution was then diluted to obtain a concentration in the range of 10^{-5} to 1.0 mg mL^{-1}.

Micelle characterization: The formation of micellar structures was confirmed by fluorescence spectroscopy using pyrene as a probe. Steady-state fluorescence-emission spectra were recorded by using a Perkin–Elmer LS50B luminescence spectrometer at a detection wavelength λ_{em} of 391 nm with a scan rate of 500 nm min^{-1}. The size distribution of the micelles was measured by employing dynamic light scattering (DLS) with a vertically polarized He–Ne laser (DAWN EOS, Wyatt Technology). The scattering angle was fixed at 90° and the measurements were recorded at a constant temperature of 25 °C. Each sample was filtrated through a 0.45 μm filter directly into a precleaned cylindrical cell of 10 mm diameter. The sample concentration was maintained at 0.1 mg mL^{-1} for micelles in aqueous solution. The sample concentration was maintained at 0.5 mg mL^{-1} for unimolecular micelles in chloroform solution. The morphology of micelles was examined by atomic force microscopy (AFM SPI 3800/SPA 300HV, Seiko Instruments) in tapping mode. A drop of micelle solution was deposited on a silicon wafer. The samples were then air-dried before measurement. Structures of the micelles was also studied by transmission electron microscopy (TEM JEM-2010 electron microscope, JEOL, Japan). A drop of the sample solution was placed onto a 100 mesh carbon-coated copper grid. About 1 min after deposition, the grid was tapped with a filter paper to remove surface water and then negatively stained with a 1% uranyl acetate solution. The samples were air-dried before measurement. Zeta-potential measurements were recorded at 25 °C by using a Zetasizer 3000 HS from Malvern Instruments.

Gel retardation assay of cationic micelles and pDNA: The gel retardation assay was performed by following literature procedures.[20] Plasmid samples were diluted to a concentration of 0.05 mg mL^{-1}. Micelle solutions were then added to the plasmid solutions with the same volume as plasmid solution, at various concentration ratios ranging from 0 to 10 and vortexed. After 10 min incubation, the solutions were analyzed by 1% agarose gel electrophoresis.

Release of pDNA from complexes:[20] The reversible nature of the micelle/pDNA complexes was investigated through the addition of a polyanion, sodium polyacrylate (NaPAA). The complexes were incubated at RT with NaPAA at a NaPAA/pDNA molar ratio ranging from 100 to 300, and the released pDNA was analyzed by 1% agarose gel electrophoresis.

Phase-transfer studies: To evaluate the transport ability and pH-sensitive behavior of PEI–PBLG, methyl orange (MO), bromophenol blue, Congo red, and fluorescein were used as anionic model compounds, and chloroform was used as an organic phase in which the dyes are not soluble.[17] In a typical experiment, aqueous dye solution (5 mL) was mixed with a chloroform solution of PEI–PBLG (5 mL) and manually shaken for 10 s. After phase separation, the organic layer (3.5 mL) was transferred into a UV/Vis cuvette for measurement.

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