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PAPER

Dual-vectors of anti-cancer drugs and genes based on pH-sensitive micelles self-assembled from hybrid polypeptide copolymers

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A series of amphiphilic pH-sensitive hybrid polypeptide copolymers, poly(ethylene glycol)-*b*-poly(L-lysine)-*b*-poly(L-phenylalanine) (PEG-PLL-PLP) were synthesized. The copolymers could self-assemble into micelles with PLP as the hydrophobic core and PEG-PLL as the hydrophilic shell, as evidenced by ^{1}HNMR and TEM. These micelles exhibited obvious pH response in hydrodynamic diameter and pH-dependent drug release behavior, attributed to the protonation/deprotonation of amino groups in PLL segments. The copolymers could further condense plasmid DNA efficiently. Importantly, the polymer/DNA complexes showed high transfection efficiency in 293T cells under optimized conditions. This study suggested the copolymers may have great potential in both drug and gene delivery.

Introduction

Over the past decade, abundant attention has been paid to investigating the properties and applications of various self-assemblies in aqueous solution.[1–4] Micelles, self-assembled from amphiphilic block copolymers, are certainly one of the most promising nanostructures, for their biomedical applications as drug delivery systems (DDSs).[5,6] Generally, hydrophobic anti-cancer drugs could be loaded in the hydrophobic cores of the micelles, while plasmid DNA (pDNA) could be condensed by the cationic micelles in gene delivery systems (GDSs). However, polymer-based carriers in DDSs and GDSs have been extensively studied separately.[7,8] To our knowledge, there were limited reports on the carriers that could be used in both DDSs and GDSs,[9–11] which may overcome the limitations of many mono-therapies by attacking the disease system on multiple fronts, due to that multi-target therapeutics can be more efficient and less vulnerable to adaptive resistance because the biological system is less able to compensate for the action of two drugs simultaneously.[12]

As for DDSs and GDSs, the biocompatibility and biodegradability of the polymer-based carriers are always crucial issues, with regard to the risks brought by the accumulation of non-degradable polymers in the body and the interaction with the host cells and tissues, which may cause a long-term toxicity.[13] In the mid 1970s, Gallot and coworkers first reported the synthesis of polypeptide hybrid block copolymers.[14] These copolymers have potential to be biocompatible and biodegradable polymer-based carriers used in DDSs and GDSs because of their chemical and structural similarity to natural biomolecules such as proteins. Several kinds of polypeptides used in DDSs or GDSs have been reported, like poly(L-lysine),[15] poly(L-glutamate),[9] poly(L-aspartate)[16] and poly(L-leucine).[17] Since Laemmli[18] demonstrated the exceptional capability of poly(L-lysine) (PLL) to condense DNA in 1975, PLL was widely used as *in vitro* and *in vivo* GDSs,[19–22] due to the abundant amino groups on the side chains of PLL. In GDSs, to achieve a high efficiency of condensing DNA, the molecular weight of PLL is usually above 3000 Da. However, high molecular weight PLL exhibits relatively high toxicity,[23] as well as the penalty of the high tendency of PLL/DNA complexes to aggregate or even precipitate depending on the ionic strength of the solution.[24] To overcome these defects of high molecular weight PLL, poly(ethylene glycol) (PEG), a widely used polymer with good biocompatibility, non-toxicity as well as ease of excretion from living organisms, is commonly used to modify the PLL based vectors. For example, Kim and coworkers synthesized biodegradable multiblock copolymers for gene delivery based on PEG and PLL.[25] Their results showed improved transfection efficiency without adversely affecting the cell viability.

In our research, we designed and synthesized a new type of hybrid polypeptide micelle based on poly(ethylene glycol)-*b*-poly(L-lysine)-*b*-poly(L-phenylalanine) (PEG-PLL-PLP) for both drug and gene delivery applications. All of the polymer blocks in this system have good biocompatibility. The *in vitro* drug release of drug loaded PEG-PLL-PLP micelles was studied under different pHs, and the ability of the copolymer to condense pDNA and to mediate gene expression was evaluated. The

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results showed these cationic micelles were able to deliver both drug and gene.

Experimental

Materials

O-(2-aminoethyl)-O′-methylpolyethylene glycol (PEG-NH_2, Mw = 2000) was purchased from Sigma Chemical Co. and used without further purification. ε-Benzyloxycarbonyl-L-lysine (LL(Z)) and L-phenylalanine (LP) were purchased from GL Biochem (Shanghai) Ltd. Hydrogen bromide 33 wt% solution in glacial acetic acid was purchased from ACROS Organics. Tetrahydrofuran (THF) and n-hexane were of analytical grade and dried with sodium to remove water before use. N,N′-Dimethyformamide (DMF) was obtained from Shanghai Chemical Reagent Co., China and used after distillation under reduced pressure. Triphosgene was recrystallized from diethyl ether before use. All other solvents were of analytical grade and used without further purification.

Synthesis of ε-benzyloxycarbonyl-L-lysine N-carboxyanhydride (LL(Z)–NCA) and L-phenylalanine N–carboxyanhydride (LP-NCA)

The following synthesis procedure was adapted from the literature.[26] Typically to a dry THF suspension (90 mL) of LL(Z) (7.6 g, 27 mmol) or LP (4.5 g, 27 mmol), a THF solution (10 mL) of triphosgene (3.4 g, 11 mmol) was dropwise added and the mixture was stirred at 50 °C under N_2 atmosphere. The reaction was stopped when the suspension turned to a clear solution. The solution was poured into excess dried n-hexane to obtain crude crystals of LL(Z)–NCA or LP-NCA, which were further recrystallized from dried THF/n-hexane twice and dried in vacuum. The purified yields of LL(Z)–NCA and LP–NCA were 7.9 g (Yield: 95.7%) and 4.7 g (Yield: 91.5%), respectively. LL(Z)–NCA (NMR): 7–7.5 ppm (m, 5H, $C_6\mathbf{H}_5$), 5.0–5.2 ppm (s, 2H, $C_6H_5C\mathbf{H}_2O$), 4.2–4.5 ppm (t, 2H, $OCC\mathbf{H}_2NH$–), 3.1–3.3 ppm (t, 2H, –$NHC\mathbf{H}_2CH_2$–), 1.0–2.0 ppm (m, 6H, –$CH_2CH_2CH_2$–); LP–NCA (NMR): 7–7.5 ppm (m, 5H, $C_6\mathbf{H}_5$), 4.7–5.0 ppm (t, 2H, $OCC\mathbf{H}_2NH$–), 2.8–3.2 ppm (m, 2H, –$C_6H_5C\mathbf{H}_2CH_2$–).

Synthesis of PEG–PLL(Z) copolymers

PEG–PLL(Z) (**A**) copolymers were prepared by ring opening polymerization of LL(Z)–NCA initiated by PEG–NH_2 in DMF with a feed ratio of 1 : 1.5 (weight ratio of PEG–NH_2 to LL(Z)–NCA). The resultant solution of LL(Z)–NCA and PEG–NH_2 was stirred at 30 °C for 72 h under N_2 atmosphere and then the reaction mixture was poured into a large excess of diethyl ether to precipitate the PEG–PLL(Z) copolymer. The obtained product was purified by repeated precipitation in diethyl ether and dried in a vacuum. The purified yield was 91.6%.

Synthesis of PEG–PLL(Z)–PLP triblock copolymers

Similarly, PEG–PLL(Z)–PLP (**B**) triblock copolymers (P1, P2, P3) were synthesized by ring opening polymerization of LP–NCA initiated by PEG–PLL(Z) in DMF with a feed ratio of 2.5 : 1, 1 : 1, 1 : 2 (weight ratio of PEG–PLL(Z) to LP–NCA), respectively. The resultant solution of LP–NCA and PEG–PLL(Z) was stirred at 30 °C for 72 h under N_2 atmosphere and then the reaction mixture was poured into a large excess of diethyl ether to precipitate the PEG–PLL(Z)-PLP copolymer. The obtained product was purified by repeated precipitation in diethyl ether and dried in a vacuum. The purified yields of P1, P2 and P3 were 86.7%, 75.1% and 53.8%, respectively.

Deprotection of PEG–PLL(Z)–PLP triblock copolymers

PEG–PLL–PLP (**C**) was obtained by the deprotection of PEG–PLL(Z)–PLP. 320 mg PEG–PLL(Z)–PLP was dissolved in 10 mL of trifluoroacetic acid, then 4 equiv of a solution of 33 wt% HBr in HAc with respect to the benzyl carbamate (Z) groups were added and the solution was stirred at 0 °C for 1 h under nitrogen. After precipitation with an excess of diethyl ether, the crude product was dissolved in DMF and the solution was dialyzed against pH 9.0 ammonia solution for 48 h using a dialysis membrane (MWCO = 3500 Da) to remove the HBr attaching on PLL segment. Another exhaustive dialysis against deionized water was conducted for 24 h before the resulting product was collected by freeze-drying. PLL homopolymer was synthesized by using dodecylamine as the initiator under the similar synthesis condition.

FITC Functionalization of PEG–PLL–PLP

PEG–PLL–PLP was fluorescently labeled with fluorescein isothiocyanate (FITC). P1 (20 mg) and FITC (7 mg) were dissolved in 5 mL of DMF. The reaction was performed for 48 h at room temperature (18 °C). For purification, the resultant solution was dialyzed (molecular weight cut-off (MWCO) = 3500 Da) against water (the water was refreshed periodically to remove the excess FITC). Finally, the resulting product was collected by freeze-drying.

Measurements

^{1}HNMR spectra were recorded on a Mercury VX-300 spectrometer at 300M Hz using CF_3COOD and D_2O as the solvents. Fluorescence spectra were recorded on a LS55 luminescence spectrometer (Perkin-Elmer). The morphology of the micelles was observed by a JEM-100CXa TEM at an acceleration voltage of 100 kV. Nano-ZS ZEN3600 was used to determine the size distribution of the self-assembled micelles and the ζ potential of polymer/DNA complexes. The cellular internalization of the micelles was studied by a confocal laser scanning microscope (CLSM, Nikon C1-si, BD Laser) at 488 nm.

In vitro cytotoxicity study

100 μL of cancer cells (HeLa) in DMEM were added to each well of a 96-well plate (concentration = 6×10^7 cells L^{-1}). The number of cells in each well was ≈ 6×10^3. After incubation for 24 h in an incubator (37.0 °C, 5% CO_2), the culture medium was transferred to 100 μL of DMEM containing the PEG–PLL–PLP with varying concentrations, and the mixture was further incubated for 24 h. Then, DMEM with the polymer was replaced by

fresh DMEM and 20 μL of a MTT solution (5 mg mL^{-1}) was added to the cells. After incubation for 4 h, 200 μL of DMSO was added and the mixture was shaken at room temperature. The optical density (OD) was measured at 570 nm with a Microplate Reader Model 550 (BIO-RAD, USA). The viable rate was calculated by the following equation: Cell viability (%) = $(OD_{treated}/OD_{control}) \times 100$, where $OD_{control}$ was obtained in the absence of polymer and $OD_{treated}$ was obtained in the presence of polymer.

Micelle formation

Micelles of PEG–PLL–PLP copolymer in aqueous media were prepared by the membrane-dialysis method at room temperature (18 °C). PEG–PLL–PLP (4 mg) was dissolved in a mixture of DMF (4 mL) with rapid stirring. After that, the solution was subjected to dialysis against deionized water for 24 h using a dialysis membrane (MWCO = 3500 Da). The water was refreshed every 12 h to remove all DMF from the sample.

Fluorescence measurements and determination of CMC

Pyrene was used as a hydrophobic fluorescent probe. Aliquots of pyrene solutions (6×10^{-6} M in acetone, 1 mL) were added to containers, and acetone was allowed to evaporate. Ten-millilitre aqueous polymer solutions at different concentrations were then added to the containers containing the pyrene residue. It should be noted that the concentration of the pyrene in every tube was 6×10^{-7} M, which would be adequate for the critical micelle concentration (CMC) measurements. The solutions were kept at room temperature for 24 h to reach the equilibrated solubilization of pyrene in the aqueous phase. Emission spectra were recorded ranging from 350 to 600 nm with an excitation of 337 nm. Both excitation and emission bandwidth were 5 nm. From the pyrene emission spectra, the intensities at 393 nm were analyzed as a function of the polymer concentrations. A CMC value was determined from the intersection of the tangent to the curve at the inflection with the horizontal tangent through the points at low concentration.

Agarose gel retardation assay

The agarose gel retardation assay was performed as follows. The polymers were dissolved into PBS solution at various concentrations and then added to 1 mL of plasmid EGFP-C1 DNA (100 ng mL^{-1} in 40 mM Tris-HCl buffer solution) to make mixtures of 5 mL. The complexes were vortexed and incubated for 30 min at 37 °C. Then the complexes were loaded onto the 0.7% (W/V) agarose gel containing GelRed™. Electrophoresis was carried out with a current of 80 V for 80 min in tris-acetate (TAE) running buffer. DNA was visualized with a UV lamp using a GelDoc system (Imago).

Drug loading and *in vitro* drug release

PEG–PLL–PLP (2 mg) and doxorubicin (DOX, 1 mg) were dissolved in 2 mL of DMF. The solution was placed into a dialysis tube (MWCO = 3500 Da) and subjected to dialysis against 1000 mL of distilled water for 24 h at 18 °C. The water was refreshed after 12 h to remove the DMF. The dialyzate after drug loading was measured by UV spectroscopy to determine the amount of unloaded drugs. It was found that around 14.8 wt% of DOX was loaded into PEG-PLL-PLP micelles.

After dialysis, the dialysis tube was directly immersed into 400 mL of buffer solution with different pHs. Aliquots of 2 mL were withdrawn from the solution periodically. The volume of solution was held constant by adding 2 mL aqueous milieu after each sampling. The amount of DOX released from micelles was measured by UV absorbance. The cumulative drug release was calculated from the following relationship: Cumulative drug release (%) = $(M_t/M_0) \times 100$, where M_t is the amount of drug released from micelles at time t, and M_0 is the amount of drug loaded into the PEG–PLL–PLP micelles. Here, M_0 was estimated by subtracting the amount of unloaded drug from the feed drug amount (1 mg).

In vitro transfection

The PEG–PLL–PLP micelles were dissolved into PBS solution at various concentrations and then added to PGL-3 plasmid DNA to obtain polymer/DNA complexes with different w/w ratios. The complexes were vortexed and incubated for 30 min at 37 °C before the *in vitro* transfection experiment. Transfection studies were performed with 293T cells, which were seeded at a density of 6×10^4 cells/well in a 24-well plate and cultured 48 h in 1 mL of DMEM. PGL-3 plasmid DNA was used as the reporter gene to evaluate the transfection efficiency. The PEG–PLL–PLP/PGL-3 complexes were prepared at N/P ratios ranging from 20 to 130. The PEG–PLL–PLP/PGL-3 complexes were prepared at each optimal w/w ratio and PEI (25 kDa, branched) was used for comparison. The complexes in serum-free DMEM were added to 293T cells for 4 h at 37 °C, then the serum-free DMEM was replaced by fresh DMEM and the cells were further incubated for 48 h.

A chemiluminometer (Lumat LB9507, EG&G Berthold, Germany) was used to measure relative light units (RLUs). The total protein content measured by the BCA protein assay kit was analyzed to normalize the RLUs and the results were expressed as RLU mg^{-1} protein.

Cell internalization of PEG–PLL–PLP micelles

The behavior of the micelles entering the tumor cells was studied by a confocal laser scanning microscopy (CLSM) at 488 nm. A typical process was as follows: HeLa cells, maintained at 37 °C, 5% CO_2 in DMEM medium, were chosen to assess the cell internalization into the tumor cells. After incubation for 24 h in an incubator, the culture medium was replaced by medium containing P1 (1 mg mL^{-1}) and the mixture was further incubated for 4 h. Cellular internalization was studied after the plates being washed with PBS for six times.

Results and discussion

Synthesis of PEG–PLL–PLP

Hybrid polypeptide block copolymers were synthesized by ring opening polymerization initiated by amino-terminated synthetic polymers, in which the initiator amine underwent a nucleophilic addition to the C-5 carboxy group of the amino acid-NCA,

called the primary amine mechanism, as proposed by Goodman.[27] In this study, PEG–NH_2 was used as the initiator to synthesize the PEG–PLL–PLP hybrid polypeptide copolymer. The synthesis of PEG–PLL–PLP involved three steps as shown in Fig. 1. First, LL(Z)–NCA and LP–NCA were prepared by intramolecular ring closure of LL(Z) and LP, respectively. The triblock copolymer **B**, protected by Z groups, was then synthesized using successive ring opening polymerization. Finally, **C** was obtained after the deprotection of **B** by HBr/HAc.

As shown in Fig. 2, ^{1}HNMR proves the successful synthesis and deprotection of PEG–PLL–PLP. Compared with Fig. 2A, a new peak g appears at $\delta = 2.83$ ppm in Fig. 2B, which is assigned to the protons of the $-CH_2-$ group in the PLP unit, indicating the successful synthesis of the triblock copolymer. This is further confirmed by the increasing peak area ratio of peak f ($\delta = 7.19$ ppm, assigned to the protons of benzene group) to peak a ($\delta = 3.81$ ppm, assigned to the protons of the $-CH_2-$ group in PEG units). In Fig. 2C, peak e at $\delta = 5.0$ ppm, attributed to the $-CH_2-$ in Z group, totally disappears, suggesting a thorough removal of Z groups. Other peaks are also illustrated in Fig. 2. The products were designated P1, P2 and P3 respectively in the order of the increasing feed of LP as shown in Table 1. Since the solubility of P3 in water or organic solvents is poor due to the high content of LP units, we focused our study on P1 and P2.

Cytotoxicity study

Cytotoxicity studies have been carried out in order to investigate the biocompatibility of the PEG–PLL–PLP copolymer. The results show that the PEG–PLL–PLP copolymers do not exhibit apparent cytotoxicity when the copolymer concentration is below 500 mg L^{-1} (Fig. 3), where the cell viability in the whole window is all above 80% although with a little decrease as the concentration increases, whereas PLL homopolymer with the similar length of the PLL block in the copolymer (average 118 units per PLL chain characterized by ^{1}H NMR) displays obvious lower cell viability at higher concentrations, indicating the reduced cytotoxicitity of the block copolymer. Also from the cytotoxicity data of the copolymers (P1 and P2) whose structures differ only in the length of PLP, the copolymer with the longer length shows a slightly higher cytotoxicity, indicating that PLP is also an important factor in optimizing the properties of the copolymer.

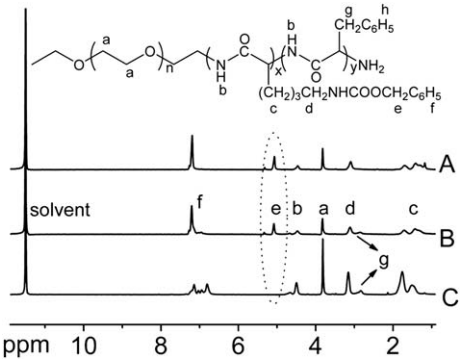

Fig. 2 ^{1}HNMR spectra (II) of PEG–PLL(Z) (A), PEG–PLL(Z)–PLP (B) and PEG–PLL–PLP (C) in CF_3COOD.

Micelle formation and characterization

PEG–PLL–PLP micelles were obtained by a dialysis method. The CMC of the copolymer was analyzed by fluorescent spectroscope using pyrene as a probe. From the plot of fluorescence intensity *versus* copolymer concentration (Fig. 4I and Fig. 4II), the CMC of the P1 and P2 obtained in this study are 375 mg L^{-1} and 139 mg L^{-1}, respectively. Obviously, the values of CMC decrease with the increasing hydrophobic block PLP content in the copolymers, which is in agreement with earlier research.[9]

TEM was engaged to investigate the morphologies of PEG–PLL–PLP self-assemblies. As presented from the insert of Fig. 4(III), well-dispersed PEG–PLL–PLP micelles with a regular spherical shape and a diameter around 25 nm are formed, while from the size distribution of PEG–PLL–PLP micelles (Fig. 4(III)), the mean size of the micelles is around 45 nm. The larger diameter from particle-size analyzer may be due to the micelles with the internal structure of aggregates[28] and the micelles' dehydration induced by water evaporation under the high vacuum condition during TEM measurement.[29]

The micellization behavior of PEG–PLL–PLP in aqueous media was further confirmed by ^{1}HNMR. The micelle suspension was freeze-dried to obtain PEG–PLL–PLP micelles. The ^{1}HNMR spectrum of the micelles in D_2O (Fig. 4(IV)) reveals that the signal of protons in benzene groups of PLP units almost disappears, suggesting a core-shell micellar structure of PEG–PLL–PLP in D_2O with isolated hydrophobic PLP inner cores and hydrophilic PEG–PLL outer shells.

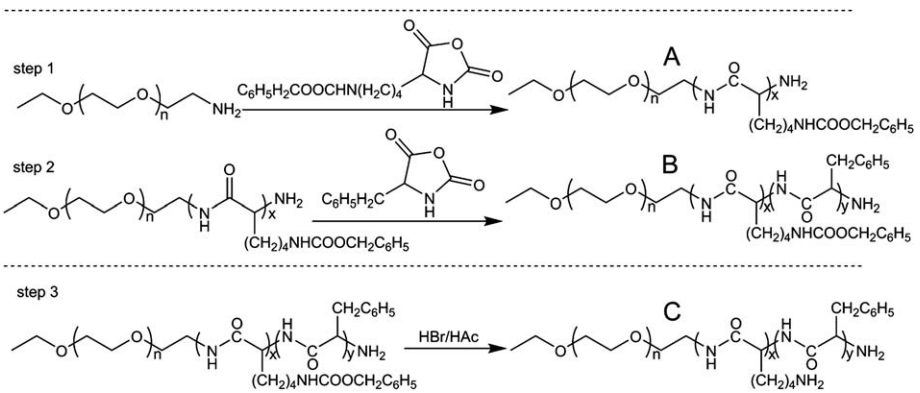

Fig. 1 Synthesis of the triblock polypeptide hybrid copolymer.

Table 1 Characteristics of polymer molecular weight and composition calculated from ^{1}H NMR spectra

	Content of monomeric units in mol%			
Polymer	EG	LL	LP	M_n
P1	45	96	16	15 000
P2	45	96	37	18 000

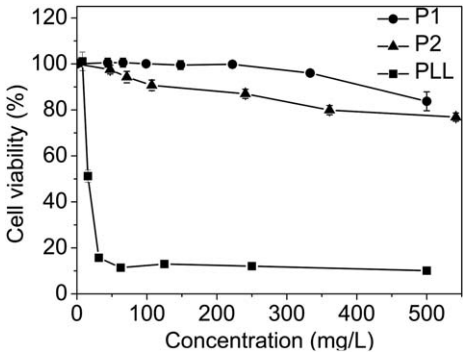

Fig. 3 Cell viability of HeLa cells after 48 h incubation with PEG–PLL–PLP (P1, P2) or PLL homopolymer.

pH response of the PEG–PLL–PLP micelles

Due to the amino groups on the side chains of the PLL segment, PEG–PLL–PLP copolymers are expected to possess pH-sensitive properties. To estimate the effect of environmental pH on the hydrodynamic diameter of PEG-PLL–PLP micelles, micelles under different pHs were prepared. As shown in Fig. 5, by increasing the pH from 4 to 10, the hydrodynamic diameter of PEG–PLL–PLP micelles decreases apparently, *i.e.* around 15 nm at pH 10 and around 60 nm at pH 4. This pH-induced size variation is related to the ionization of amino groups in the PLL segment. As we know, during the micellization of PEG–PLL–PLP, the hydrophobic interactions between PLP blocks induce

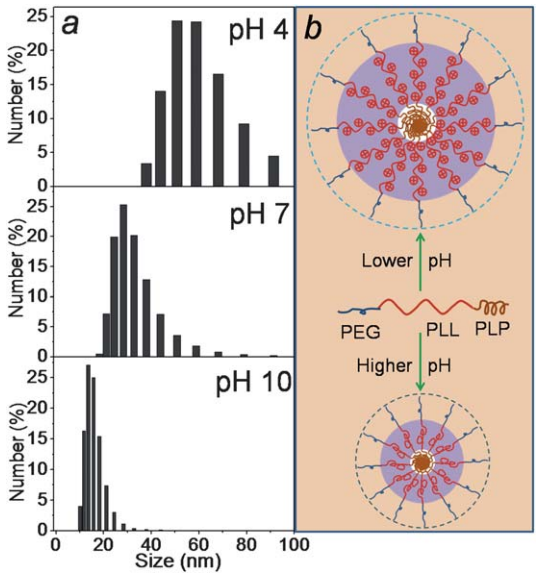

Fig. 5 (a) Size distribution of PEG–PLL–PLP micelles at different pHs. (b) Schematic illustration of the self-assembly of PEG–PLL–PLP copolymers at different pHs.

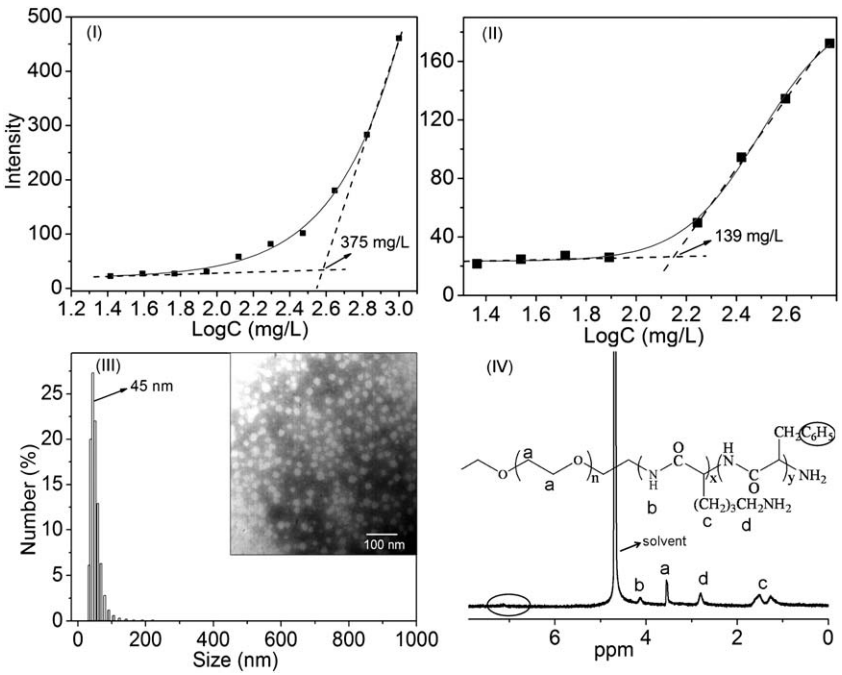

Fig. 4 Intensity of the emission spectra at 393 nm as a function of the logarithm of the concentration of PEG–PLL–PLP (P1) (I) and PEG–PLL–PLP (P2) (II); size distribution (III) and TEM image (the insert) and of PEG–PLL–PLP micelles, the concentration of the copolymer was kept at 500 mg L^{-1}; and ^{1}HNMR spectrum of PEG–PLL–PLP micelles in D_2O (IV).

the microphase separation of intra-polymer chain with PEG–PLL as the hydrophilic outer shell and PLP as the hydrophobic inner core, respectively. When the micelles are prepared at lower pH, more amino groups in PLL units could be protonated, which means more positive charges appear in the hydrophilic shell of the micelle, leading to an increased repulsion between PLL segments and a larger hydrodynamic diameter.

Agarose gel retardation assay

The agarose gel retardation assay was performed to evaluate the ability of PEG–PLL–PLP polymers to condense DNA. As shown in Fig. 6, when PEG–PLL–PLP is bonded to DNA completely, the weight ratios of P1 and P2 to DNA are 2 and 15, respectively. Obviously, it is easier for P1 to condense DNA as compared with P2, due to the higher density of amino groups of P1. At the same weight ratios, there are more amino groups in P1 than P2, because of the higher M_r of P2 and the same number of amino groups on each P1 and P2 chain. Hence, P1 demonstrates a stronger ability to condense DNA.

Particle size and ζ potential measurements

The ability of PEG–PLL–PLP to condense DNA was also evaluated by particle size and ζ potential measurements. As shown in Fig. 7a, P1/DNA and P2/DNA complexes demonstrate a similar trend in the variation of the particle size. With the increasing of w/w ratios, the particle sizes of P1/DNA and P2/DNA decrease, suggesting that DNA is not condensed compactly at lower w/w ratios. The ζ potential of polymer/DNA is presented in Fig. 7b. The ζ potential of P1/DNA keeps constant at 27 mV at w/w ratios higher than 20, while the ζ potential of P2/DNA rapidly increases with the enhancement of w/w ratio from 10 to 30. The particle size and ζ potential measurements indicate that the polymers could bind DNA to form nanoparticles with positive surface charges, suggesting that PEG–PLL–PLP/DNA complexes are suitable candidates for gene transfection.

In vitro drug release

As is well known, hydrophobic drugs could be loaded in the hydrophobic cores of the micelles.[30] In this study, DOX, one of the most common chemotherapeutic drugs, was chosen as the hydrophobic model drug for its poor solubility and easy detection by fluorescence monitors. The applicability of PEG–PLL–PLP micelles in controlled drug release was examined in buffer solutions at different pHs. As shown in Fig. 8, the drug release at pH 4 exhibits two-phase drug release, where burst release occurs

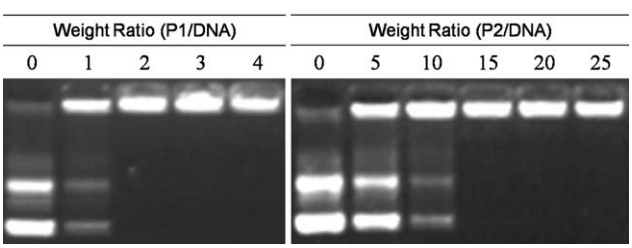

Fig. 6 Gel retardation assay of polymer/DNA complexes.

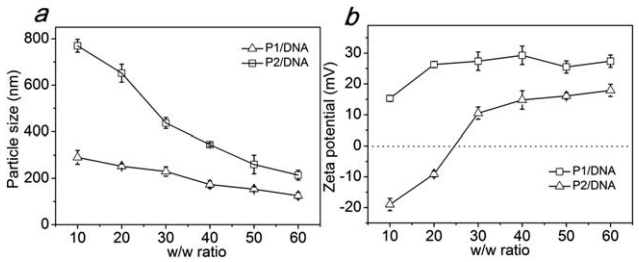

Fig. 7 Particle size (a) and ζ potential (b) of PEG–PLL–PLP/DNA complexes in distilled water at various w/w ratios.

at the initial 20 h and then comes to a slow level. In comparison, at pH 7 and pH 10 it shows nearly zero-order release. The differences in drug release behaviors at different pHs are mainly attributed to the change of the ionization extent of amino groups in the PLL segment as discussed above. Apparently, repulsion between ionized PLL chains at lower pH leads to larger size or looser micelles, as also indicated by pH dependent size alterations, making the drug diffuse out of the polymer matrix more easily. Whereas at pH 7 and pH 10 PLL segment is in low ionization and tends to be more compact which is not favorable for drug diffusion, it thus shows a moderate drug release velocity. As a result, DOX-loaded PEG–PLL–PLP micelles exhibit a pH-sensitive drug release behavior with a higher release rate at a lower pH.

In vitro transfection

Gene transfection mediated by PEG–PLL–PLP/DNA complexes was assessed in 293T cells in the absence of serum. Optimized w/w ratio of 25 kDa PEI/DNA complex was used as the positive control. As presented in Fig. 9, the P1/DNA complexes at N/P ratio of 40 shows the highest transfection of all the ratios, though lower to that of 25 kDa PEI/DNA complex at N/P ratio of 10 (or w/w = 1.3). In addition, the transfection efficiency of P1/DNA is obviously higher than that of P2/DNA at the same N/P ratio, which is probably attributed to the increased units of LP in P2 leading to a lower concentration of amino groups in micelles and thus affecting the ability to condense DNA. As discussed above, the amino density of P1 is higher than that of P2 when at the same weight ratio, resulting in stronger ability to condense DNA and higher transfection efficiency.

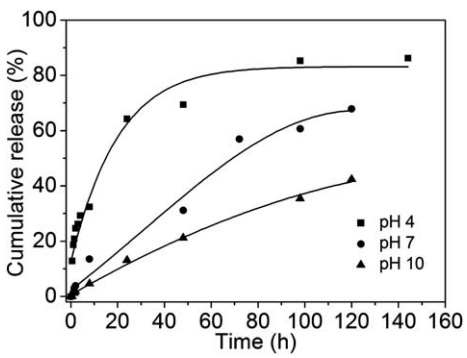

Fig. 8 Release behavior of DOX from DOX-loaded micelles at different pHs at 37 °C.

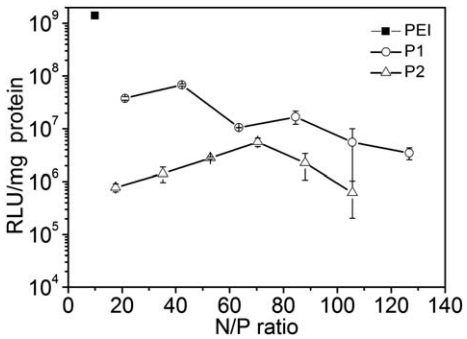

Fig. 9 Luciferase expression in 293T cells transfected by PEG–PLL–PLP/DNA complexes at N/P ratios ranging from 20 to 130.

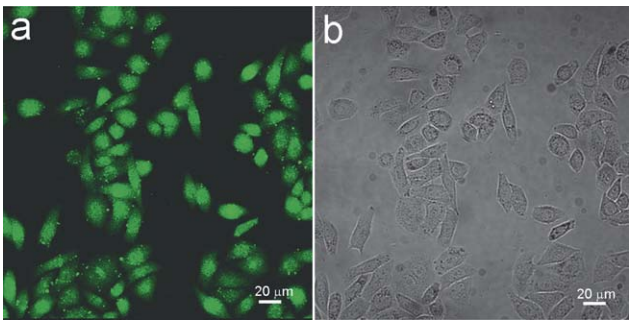

Fig. 10 Confocal laser scanning images of HeLa cells treated with P1 micelles under excitation at 488 nm (a) and under bright field (b). The concentration of the copolymer was 500 mg L^{-1}.

Cell internalization of PEG–PLL–PLP micelles

To examine whether the micelles could enter the tumor cells (HeLa) for potential antitumor therapy, the cellular internalization of FITC functionalized micelles (500 mg L^{-1}) was investigated. After 4 h of incubation with the micelles, the HeLa cells were observed by a CLSM and the result is demonstrated in Fig. 10. It is found that the micelles could be internalized into the HeLa cells because the green fluorescent intensity increases suddenly inside HeLa cells.

Conclusions

In summary, a new type of hybrid polypeptide copolymer (PEG–PLL–PLP) was synthesized. PEG–PLL–PLP copolymers could self-assemble in water to form spherical micelles with a size around 25 nm at neutral condition. A cell internalization experiment exhibited that fluorescent micelles could be internalized into the cells. The micelles showed excellent controlled drug release properties and apparent disparities in drug release rates at different pHs. Cytotoxicity studies showed that the copolymer exhibits good biocompatibility. In vitro transfection showed that PEG–PLL–PLP/DNA complexes demonstrate comparable transfection efficiency in 293T cells with lower cytotoxicity as compared with PEI (25 KDa). This study suggests that PEG–PLL–PLP could be used in both DDSs and GDSs.

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References

1 G. Riess, *Prog. Polym. Sci.*, 2003, **28**, 1107.
2 Z. Zhang, Z. Wei and M. Wan, *Macromolecules*, 2002, **35**, 5937.
3 H. Kukula, H. Schlaad, M. Antonietti and S. Forster, *J. Am. Chem. Soc.*, 2002, **124**, 1658.
4 H. Wei, C. Y. Yu, C. Chang, C. Y. Quan, S. B. Mo, S. X. Cheng, X. Z. Zhang and R. X. Zhuo, *Chem. Commun.*, 2008, **38**, 4598.
5 K. Kataoka, A. Harada and Y. Nagasaki, *Adv. Drug Delivery Rev.*, 2001, **47**, 113.
6 A. Lavasanifar, J. Samuel and G. S. Kwon, *Adv. Drug Delivery Rev.*, 2002, 169.
7 J. Li, X. Ni, X. Li, N. K. Tan, G. T. Lim, S. Ramakrishna and K. W. Leong, *Langmuir*, 2005, 8681.
8 T. G. Park, J. H. Jeong and S. W. Kim, *Adv. Drug Delivery Rev.*, 2006, **58**, 467.
9 H. Y. Tian, C. Deng, H. Lin, J. Sun, M. Deng, X. Chen and X. Jing, *Biomaterials*, 2005, **26**, 4209.
10 Y. Wang, S. Gao, W. H. Ye, H. S. Yoon and Y. Y. Yang, *Nat. Mater.*, 2006, **5**, 791.
11 J. L. Zhu, H. Cheng, Y. Jin, S. X. Cheng, X. Z. Zhang and R. X. Zhuo, *J. Mater. Chem.*, 2008, **18**, 4433.
12 G. R. Zimmermann, J. L. Lehar and C. T. Keith, *Drug Discovery Today*, 2007, **12**, 34.
13 D. Fischer, T. Bieber, Y. Li, H. P. Elsasser and T. Kissel, *Pharm. Res.*, 1999, **16**, 1273.
14 B. Perly, A. Douy and B. Gallot, *CR Acad Sci. Paris*, 1974, **279C**, 1109.
15 P. Midoux and M. Monsigny, *Bioconjugate Chem.*, 1999, **10**, 406.
16 K. Kataoka, T. Matsumoto, M. Yokoyama, T. Okano, Y. Sakurai, S. Fukushima, K. Okamoto and G. S. Kwon, *J. Controlled Release*, 2000, **64**, 143.
17 S. H. Hua, Y. Y. Li, Y. Liu, W. Xiao, C. Li, F. W. Huang, X. Z. Zhang and R. X. Zhuo, *Macromol. Rapid Commun.*, 2010, **31**, 81.
18 U. K. Laemmli, *Proc. Natl. Acad. Sci. U. S. A.*, 1975, **72**, 4288–4292.
19 M. Ohsaki, T. Okuda, A. Wada, T. Hirayama, T. Niidome and H. Aoyagi, *Bioconjugate Chem.*, 2002, **13**, 510.
20 J. M. Benns, J. S. Choi, R. I. Mahato, J. S. Park and S. W. Kim, *Bioconjugate Chem.*, 2000, **11**, 637.
21 C. H. Ahn, S. Y. Chae, Y. H. Bae and S. W. Kim, *J. Controlled Release*, 2004, **97**, 567.
22 V. Stanic, Y. Arntz, D. Richard, C. Affolter, I. Nguyen, C. Crucifix, P. Schultz, C. Baehr, B. Frisch and J. Ogier, *Biomacromolecules*, 2008, **9**, 2048.
23 Y. H. Choi, F. Liu, J. S. Kim, Y. K. Choi, J. S. Park and S. W. Kim, *J. Controlled Release*, 1998, **54**, 39.
24 G. Liu, M. Molas, G. A. Grossmann, M. Pasumarthy, J. C. Perales, M. J. Cooper and R. W. Hanson, *J. Biol. Chem.*, 2001, **276**, 34379.
25 M. Bikram, C. H. Ahn, S. Y. Chae, M. Lee, J. W. Yockman and S. W. Kim, *Macromolecules*, 2004, **37**, 1903.
26 W. H. Daly and D. Poche, *Tetrahedron Lett.*, 1988, **29**, 5859.
27 M. Goodman and J. Hutchison, *J. Am. Chem. Soc.*, 1966, **88**, 3627.
28 L. Zhang and A. Eisenberg, *J. Am. Chem. Soc.*, 1996, **118**, 3168.
29 Y. Hu, L. Zhang, Y. Cao, H. Ge, X. Jiang and C. Yang, *Biomacromolecules*, 2004, **5**, 1756.
30 K. Kataoka, G. S. Kwon, M. Yokoyama, T. Okano and Y. Sakurai, *J. Controlled Release*, 1993, **24**, 119.