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ARTICLE TYPE

Multi-Responsive Protein Nanocarriers from Anionic Dynamic Covalent Copolymer

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On the basis of an active ester monomer methacryloylacetone oxime (MAO), a well-defined hydrazide-containing block copolymer poly(poly(ethylene glycol methacrylate))-*b*-poly(methylacryloylhydrazide) (P(PEGMA)-*b*-PMAH) was synthesized by RAFT polymerization and subsequent aminolysis in the presence of hydrazine hydrate. The dynamic covalent copolymer was generated by the bioconjugation of pyridoxal phosphate (PLP) to the pendant hydrazide groups through reversible acylhydrazone linkages. An *in vitro* study confirmed that the block copolymer and the PLP-conjugated dynamer were nontoxic to HeLa cells. The PLP-conjugated dynamer was negatively charged at physicological pH. Polyion complex (PIC) micelles were formed through the electrostatic interaction between lysozyme and PLP-conjugated dynamer. These PIC micelles demonstrated pH-, salt-, and enzyme-responsive features. The enzymatic activity of PIC micelles toward the hydrolysis of the bacterial substrate *Micrococcus luteus* cells was evaluated. A reduced activity was observed after lysozyme was entrapped into the PIC micelles because of the shielding effect of P(PEGMA) corona. However, the dissociation of micelles, triggered by the increase in ionic strength of the milieu, led to the recovery of lysozyme activity. These PIC micelles formed by PLP-conjugated dynamer and protein have potential applications in biomedical and bioengineering areas.

Introduction

The successful implementation of dynamic covalent chemistry in polymer chemistry leads to the generation of dynamic covalent polymers, termed as dynamers, which are constructed via reversible covalent interactions between building blocks.[1,2] The reversible nature of the dynamic covalent linkage enables dynamers to modify their constitutions by exchanging and reshuffling their building blocks triggered by physical or chemical stimuli. Dynamers provide a versatile approach to the development of novel intelligent materials with responsive and adaptive properties on the basis of various reversible covalent bonds including thermally activated alkoxyamine bonds, Diels–Alder adducts, chemosensitive imines, boronic esters and disulfide bonds.[3]

Dynamers have intrigued great research interest in the areas of advanced materials, biotechnology and biomedicine. Lehn's group has pioneered the preparation of main chain-type dynamers with tunable mechanical, optical and thermoresponsive properties.[4] The incorporation of biomolecules extended their research to biodynamers that combined the functional properties of biorelevant moieties with the adaptive behavior of constitutional dynamic systems. Dynamic nucleic acid analogues DyNAs,[5] glycodynamers[6] and polypeptide-type dynamic biopolymers[7] have been prepared by polycondensation via hydrazone or imine connection of biologically relevant components. Dynamers with dynamic covalent bonds in the side chains were also developed. Otsuka and Takahara reported the synthesis of alkoxyamine-based dynamic covalent polymers with refined structures such as reactive polymer brushes,[8] graft copolymers[9] and star polymers.[10] Fulton and coworkers constructed polymer-scaffolds dynamic combinatorials libraries (PS-DCLs) on the basis of a synthetic polymer scaffold where functionalized residues were grafted onto the scaffold through dynamic covalent linkages.[11] Responsive nanoparticles and hydrogel were also prepared by the cross-linking of linear copolymers containing pendant aldehyde, amine or pyridyl diasulfide groups via dynamic covalent bonds.[12] Sumerlin's group synthesized a series of core-cross-linked dynamic-covalent stars containing pendant Diels-Alder, boronic ester and disulfide linkages capable of reversibly reconfiguring their composition/structures in response to external stimuli.[13]

In this paper, a well-defined hydrazide-containing diblock copolymer P(PEGMA)-*b*-PMAH was synthesized by RAFT polymerization. As an active form of vitamin B_6, pyridoxal phosphate (PLP) contains an aldehyde group and a phosphate group with negative charges. PLP was chosen to attach to the side chains of block copolymer through reversible acylhydrazone formation between aldehyde and hydrazide. The PLP-conjugated dynamic covalent copolymer was negatively charged because the 5'-phosphate group of PLP existed in the dianionic form at

physicological pH.[14] This anionic dynamer was employed for the preparation of polyion complex (PIC) micelles through the electrostatic interaction with positively charged protein lysozyme. Because of high entrapment efficiency and self-assembly in aqueous medium, PIC micelles have been utilized in drug delivery system as nanocarriers of nucleic acid,[15-19] protein,[20,21] enzyme[22-25] and antibody.[26] The enzyme-loaded PIC micelles are of a particular interest, because they may be used as nanosized biochemical devices for therapy, diagnosis, and production of specialty chemicals.[24a] Herein, the PIC micelles formed by PLP-conjugated dynamer and lysozyme demonstrated pH- and salt-responsive features. Because the phosphoester bond of PLP is enzyme-reactive and can be hydrolyzed by phosphatase, these PIC micelles are also sensitive to enzymatic stimulus. Although lysozyme in the PIC micelles showed a reduced enzymatic activity, an increase in the ionic strength of the milieu led to the dissociation of PIC micelles and the recovery of bioactivity. It is anticipated that these PIC micelles on the basis of the PLP-conjugated dynamer and protein can be used as an intelligent nanoreactor that may have various applications in biomedical and bioengineering areas.

Experimental

Materials

Poly(ethylene glycol) methyl ether methacrylate (PEGMA, M_n=475 g/mol, Aldrich) was purified by passing through a basic alumina column. Acetone oxime (Alfa Aesar, 98%) was recrystallized from petroleum ether. 4, 4'-Azobis (4-cyanopentanoic acid) (ACPA, Acros, 97%) was dried under vacuum before used. Picrylsulfonic acid solution (TNBS, Aldrich), pyridoxal phosphate monohydrate (PLP, Sangon Biotech (Shanghai) Co., 98%), 2-formylbenzenesulfonic acid sodium salt (J&K Chemical, 90%), lysozyme (Sigma), and *Micrococcus luteus* cell (Aldrich) were used as received. Calf intestine alkaline phosphatase (CIAP) was purchase from Takara (Dalian, China). Cell counting kit-8 (CCK-8) was purchased from Dojindo Laboratories (Japan). Dulbecco's Modified Eagle medium (DMEM) and fetal bovine serum (FBS) were obtained from Thermo Scientific. Penicillin and streptomycin were bought from Solarbio Technology Co., Ltd. 4-Cyanopentanoic acid dithiobenzoate (CPADB) was synthesized according to the methods reported in the literatures.[27] Methacryloylacetone oxime (MAO) was prepared by the reaction of acetone oxime and methacyloyl chloride.[28] ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 6.10 (1H, m), 5.57 (1H, m), 2.02 (3H, s), 1.98 (3H, s), 1.95 (3H, s). All solvents were redistilled before use.

Synthesis of P(PEGMA) homopolymer by RAFT Polymerization

PEGMA (2.58 g, 5.42 mmol), CPADB (0.1512 g, 0.542 mmol) and ACPA (0.0253 g, 0.0903 mmol) were dissolved in DMF (7 mL) in a flask. The solution was degassed by three freeze-vacuum-thaw cycles. The polymerization was performed at 70 °C for 24 h, and stopped by cooling the solution in ice water and then exposed to air. The resulting polymer was precipitated into ethyl ether, centrifuged, and dried overnight under vacuum.

Synthesis of P(PEGMA)-*b*-PMAO block copolymer

P(PEGMA) (0.70 g, 0.0838 mmol), MAO (0.24 g, 1.68 mmol) and ACPA (0.0039 g, 0.014 mmol) were dissolved in DMF (3.5 mL), and the solution was degassed by three freeze-vacuum-thaw cycles. The block copolymerization was carried out at 70 °C for 24 h, and stopped by cooling the solution in ice water and then exposed to air. The copolymer was precipitated into petroleum ether, centrifuged, and dried overnight under vacuum.

Synthesis of P(PEGMA)-*b*-Poly(methylacryloylhydrazide) (P(PEGMA)-*b*-PMAH)

Hydrazine hydrate (1.32 mL, 0.0271 mol) was added to a solution of P(PEGMA)-*b*-PMAO (0.30 g) in DMF (3.7 mL) under nitrogen atmosphere. The reaction was stirred for 48 h at room temperature. The block copolymer P(PEGMA)-*b*-PMAH was purified by extensively dialysis against DI water (MWCO 1000) and recovered by lyophilization. The content of hydrazide in copolymer was determined by a modified TNBS assay.[29]

Conjugation of PLP to P(PEGMA)-*b*-PMAH via Acylhydrazone Linkage

P(PEGMA)-*b*-PMAH (0.036 g, 0.0595 mmol of hydrazide) and pyridoxal phosphate (0.0237 g, 0.0893 mmol) was dissolved in DI water (12 mL), and the solution was purged with nitrogen for 10 min. The reaction was carried out at 50 °C for 24 h. The solution was dialyzed against DI water for 3 days (MWCO 1000). Lyophilization of an aliquot of the solution gave a yellow solid for characterization.

pH-Triggered Cleavage of Acylhydrazone Bond in PLP-Conjugated block copolymer

The pH-cleavage reaction was conducted in a NMR tube. Typically, freeze-dried PLP-conjugated block copolymer (6 mg) was dissolved in D_2O (0.4 mL, pH 3.5). The solution was heated at 60 °C for 4 days. The cleavage of acylhydrazone bond was monitored by ^{1}H NMR at specified intervals.

Preparation of Polyion Complex (PIC) Micelles

The PIC micelles were prepared in Tris-HCl buffer (10 mM) or DI water. Typically, lysozyme was dissolved in Tris-HCl buffer (10 mM, pH 7.4) at a concentration of 3 mg/mL, 0.65 mg/mL, 0.25 mg/mL, 0.125 mg/mL, and 0.083 mg/mL. The stock solution of PLP-conjugated P(PEGMA)-*b*-PMAH (2.24 mg/mL) in water was diluted to 0.5 mg/mL in Tris-HCl buffer. After each solution was filtered through 0.22 μm syringe filter, the PIC micelles were prepared by mixing equal volumes (1 mL) of two solutions at various mass ratios (*r*) of lysozyme to polymer.

Enzyme-Triggered Disassembly of the PIC Micelles

Calf intestine alkaline phosphatase (CIAP, 1 μL, 30 U/μL activity) and Tris-HCl buffer (50 μL, 0.5 M, 10 mM $MgCl_2$, pH 9.0) were added to the solution of the PIC micelles (1 mL) and the mixed solution was kept at 37°C. The variation of PIC micellar size was monitored by DLS at specified intervals.

Lysozyme Bioactivity Measurement

The bioactivity of lysozyme was assayed using *Micrococcus luteus* (*ML*) cells as substrate.[30] After *ML* cells was suspended in PBS buffer (66 mM, pH 6.6) at 4 °C overnight, the stock solution of *ML* cells was prepared so as to have the transmittance at 450

nm (T %) of 20-30 %. The PIC micelles were incubated in Tris-HCl buffer (10 mM, pH 6.5) solutions containing various concentrations of NaCl. The lysozyme bioactivity of each micelles solution was measured at specified intervals. Lysozyme bioactivity was expressed as the relative activity, which was defined as the ratio of the activity in the PIC micellar solution to the activity in the control solution prepared with the same mass of lysozyme as that in the micellar solution. After 0.24 mL of the PIC micellar solution or the control solution was added to 2.74 mL of *ML* cell stock solution at 25 °C, the absorbance at 450 nm was recorded immediately with a UV-vis spectrophotometer. The absorbance was measured every 10s for 60s and plotted as a linear curve. The slope of the curve was used for the calculation of the relative bioactivity according to the following equation:

$$\text{Relative Bioactivity}(\%) = \frac{k_{\text{sample solution}}}{k_{\text{control solution}}} \times 100\ \%$$

Where $k_{\text{sample solution}}$ and $k_{\text{control solution}}$ are the slopes for PIC micelles and free lysozyme, respectively.

Cytotoxicity Assay

Cytotoxicity of the PLP-Conjugated dynamer and the corresponding block copolymer precursor were evaluated using a standard cell-counting kit-8 (CCK-8) assay. The assay was carried out in the following manner. HeLa cells were cultured in DMEM, supplemented with 10 % FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37 °C in an atmosphere containing 5 % CO2. The cells were seeded in a 96-well plate with a density of 6000 cells per well. After 24 h incubation, the culture was replaced with fresh medium containing the block copolymer P(PEGMA)-b-PMAH and the PLP-conjugated dynamer (concentration: 10-200 μg/mL) was added into each well, and the cells were incubated for 48h. Thereafter, the medium was removed, and 10 mL of CCK-8 and 90 mL of fresh medium were added to each well. After 2 h, the plate was gently shaken for 2 min to dissolve formazan crystals. The absorbance was measured using a multifunctional ELISA plate reader (Thermo Varioskan Flash) at 450 nm. Cell viability was calculated by the equation

$$\text{cell viability}\,(\%) = \frac{A_{sample} - A_{blank}}{A_{control} - A_{blank}} \times 100\%$$

where A_{sample} and $A_{control}$ represent the absorbance of CCK-8 reagents determined for cells treated with different samples and for control cells (untreated), respectively. A_{blank} is the absorbance of CCK-8 reagents without cells. Data are presented as average ±SD (n=5).

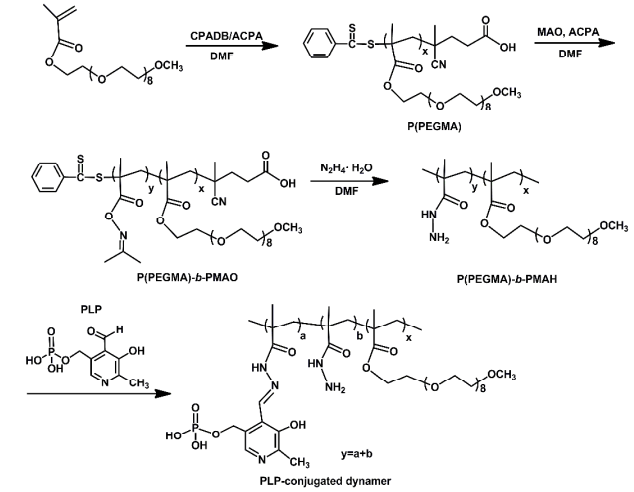
Characterization

All NMR spectra were recorded on a Varian UNITY- plus 400 M nuclear magnetic resonance spectrometer. Molecular weight and polydispersity (M_w/M_n) of the polymers were determined by GPC at 35 °C with a Waters 1525 chromatograph equipped with a Waters 2414 refractive index detector. The eluent was THF at a flow rate of 1.0 mL/min. Molecular weights were calibrated on PS standards. UV-vis spectroscopy was performed on a Shimadzu UV-2450 UV–visible spectrophotometer. Dynamic light scattering (DLS) measurements were conducted on a Zetasizer Nano ZS from Malvern Instruments equipped with a 10 mW HeNe laser at a wavelength of 633 nm. Phosphorus was determined by inductively coupled plasma-atomic emission spectrometry (ICP-9000(N+M), USA Thermo Jarrell-Ash Corp). Transmission electron microscopy (TEM) observations were carried out on a Tecnai G2 20 S-TWIN electron microscope equipped with a Model 794 CCD camera. The solution of PIC micelles was deposited on a carbon-coated copper grid. Water was evaporated in air. To increase the contrast, the sample was stained by OsO_4 vapor.

Results and Discussion

Synthesis of Hydrazide-Containing Block Copolymer P(PEGMA)-*b*-PMAH

On the basis of an active ester monomer methacryloylacetone oxime (MAO), diblock copolymer of PEGMA and MAO was synthesized by sequential RAFT polymerization. As shown in Scheme 1, the macro-RAFT agent P(PEGMA) (M_n = 6200, PDI=1.18, $DP_{n,NMR}$=17) was synthesized by CPADB-mediated RAFT polymerization with ACPA as initiator in DMF at 70 °C. P(PEGMA) was then chain extended with MAO to obtain the diblock copolymer P(PEGMA)-*b*-PMAO (M_n = 9300, PDI = 1.15), as revealed by the shift of GPC peak to a higher molecular weight relative to that of the P(PEGMA) precursor (ESI, Figure S1). The number-average polymerization degree (DP_n) of MAO was calculated to be 16 by comparison of the integration of peak c at 4.2 ppm to that of peaks (a, e, g) at 1.5~2.5 ppm in the ^{1}H NMR spectrum (Figure 1B).



Scheme 1. Outline for the synthesis of P(PEGMA)-*b*-PMAH block copolymer by RAFT polymerization and conjugation of PLP via acylhydrazone bond.

By treatment with excess hydrazine hydrate, the activated acetoxime esters in P(PEGMA)-*b*-PMAO block copolymer was converted into the corresponding hydrazides, and a hydrophilic hydrazide-containing block copolymer P(PEGMA)-*b*-PMAH was obtained. The transformation of acetoxime ester to hydrazide was confirmed by the dramatically decreasing intensity of signals at δ 2.0 ppm corresponding to methyl protons of oxime units in ^{1}H

NMR spectrum and the appearance of amide band at 1647 cm^{-1} in FTIR (Figure 2). The average number of hydrazide group in the block copolymer was calculated to be 16 by a modified TNBS assay, which was in agreement with the ^{1}H NMR data.

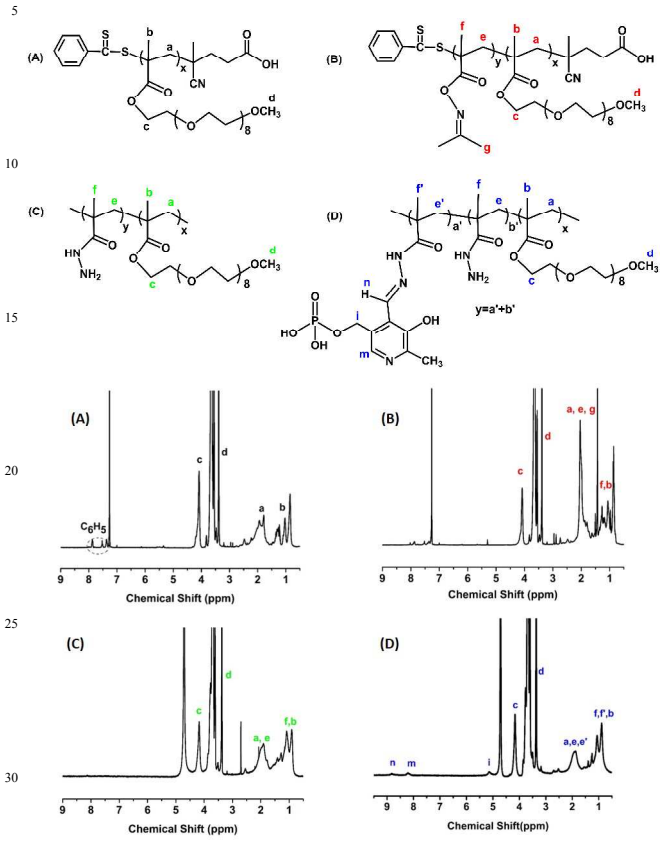


Figure 1. ^{1}H NMR spectra of (A) $P(PEGMA)_{17}$, (B) $P(PEGMA)_{17}$-*b*-$PMAO_{16}$, (C) $P(PEGMA)_{17}$-*b*-$PMAH_{16}$ and (D) PLP-conjugated dynamer.

Conjugation of PLP to Hydrazide-Containing Block Copolymer

Hydrazide reaction with carbonyl group to form reversible hydrazone linkages has been implemented in the generation of biologically active substances, self-healing gels, and pH-responsive micelles as drug carriers.[31] The enzyme cofactor pyridoxal-5-phosphate (PLP) is an active form of vitamin B_6 containing an aldehyde group and a phosphate group. As a source of aldehyde, it was involved in hydrazone and Schiff base formation and reacted more efficiently in bioconjugation reaction.[32] At 50 °C in the aqueous solution, the aldehyde group of PLP reacted with the pendant hydrazide groups of the block copolymer to form reversible acylhydrazone bonds and generate PLP-conjugated dynamer (Scheme 1). After 24h, the polymer solution was purified by dialysis. The formation of acylhydrazone bond was confirmed by UV-vis spectroscopy. It was observed that a new absorbance attributed to acylhydrazone bond appeared at 345 nm[33] (ESI, Figure S2). The solid conjugate was recovered by lyophilization and characterized by ^{1}H NMR. The occurrence of new signals at 8.75~9.0 ppm (peak n) and 8.0~8.25 ppm (peak m) corresponding to acylhydrazone bond (C*H*=NNH) and pyridine ring proton of PLP confirmed the successful conjugation of PLP to the hydrazide-containing block copolymer (Figure 1D). The average number of PLP attached to the hydrazide-containing block copolymer was calculated to be 5 on the basis of the integrations of peak n and peak c. The average number of PLP calculated on the basis of inductively couple plasma (ICP) analysis was 5, which was in agreement with the ^{1}H NMR result.

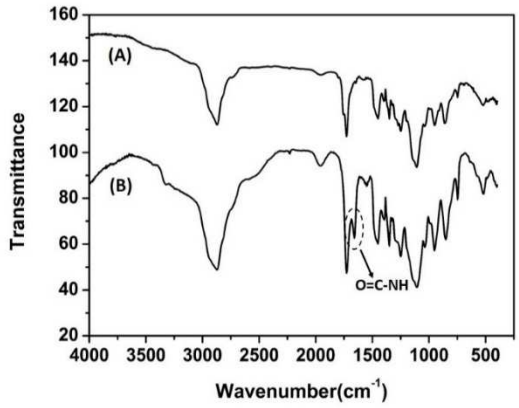


Figure 2. FTIR spectra of (A) $P(PEGMA)_{17}$-*b*-$PMAO_{16}$ and (B) $P(PEGMA)_{17}$-*b*-$PMAH_{16}$.

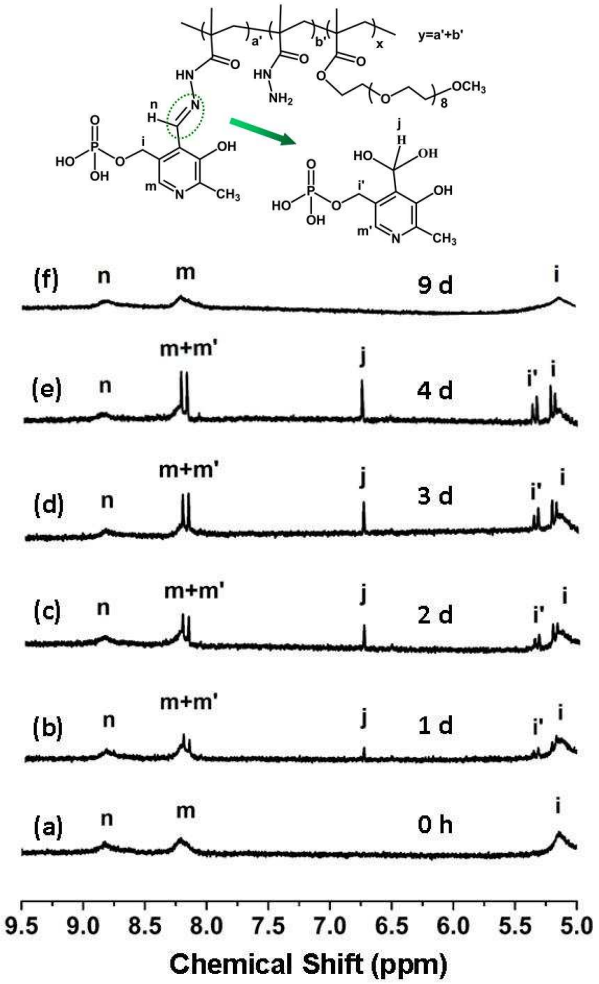


Figure 3. ^{1}H NMR spectra of PLP-conjugated dynamer in D_2O (pH 3.5) recorded at specified intervals. (a)-(e) 60° C for 0, 1, 2, 3 and 4 days and (f) 30 °C for 9 days.

It is well-known that the acylhydrazone bond is an acid-sensitive covalent bond.[34] The hydrolysis of acylhydrazone bond can be triggered by pH or high temperature. Demonstration of the cleavage of acylhydrazone bonds in PLP-conjugated dynamer was achieved by dissolving copolymer in D_2O at pH 3.5, and monitoring the release of PLP using ^{1}H NMR. The copolymer solution was kept at 60 °C. ^{1}H NMR spectra were recorded at specified intervals (Figure 3). The cleavage of acylhydrazone bonds was confirmed by the appearance of the signals corresponding to free PLP at 8.2 (sharp peak m', pyridine ring proton), 6.5 (peak j, hydrate form of aldehyde proton) and 5.3 ppm (peak i', methylene proton). On the basis of the integration of peak n and peaks (m+m'), it was calculated that 46 % of the acylhydrazone bonds cleaved after 4 days at 60 °C. However, when the hydrolysis was conducted at 30 °C, the acylhydrazone bonds still kept structure intact even after 9 days (Figure 3f). This result indicates that temperature has a pronounced effect on the cleavage of acylhydrazone bonds of the PLP-conjugated dynamer. It is reported that hydrazones formed from aromatic aldehydes are more stable to acidic hydrolysis than those formed from aliphatic ones.[35] The π-bond conjugation of benzene ring with hydrazone increases the stability because of resonance stabilization. A delicate balance of groups adjacent to the carbonyl and hydrazide can modulate the pH-dependent hydrolysis of hydrazone. Wagner and coworkers[36] synthesized a pyridylhydrazone-based PEGylation cholesterol and used this PEG conjugate for shielding targeted lipopolyplexes. It was found that at pH 5.4 the cleavage of hydrazone bond resulted in enhanced transfection efficiency. Hydrazone bonds have been also successfully used to generate a variety of pH-sensitive conjugates of the anticancer drug doxorubicin with various hydrazide-functionalized polymers by reaction with the ketone group of this drug.[37] For the application of the hydrazone-based chemistry in drug delivery system, careful selection of an aldehyde and an acyl hydrazide would be necessary for the development of pH-sensitive nanocarriers with required stabilities at normal and mildly acidic pH values.[38]

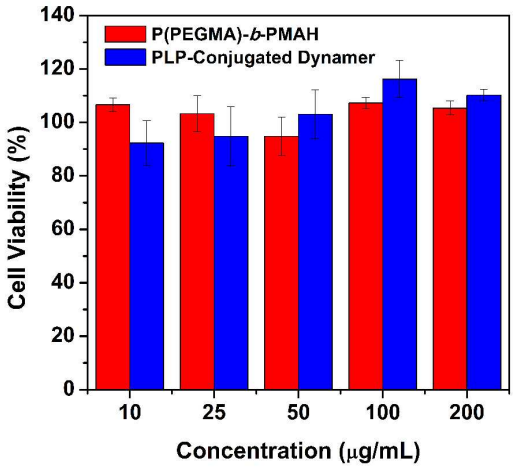


Figure 4. Cytotoxicity of the P(PEGMA)-*b*-PMAH block copolymer and the PLP-conjugated dynamer at different concentrations by CCK-8 assays using Hela cells. Data are presented as the average ±SD (n=5).

In Vitro Cytotoxicity

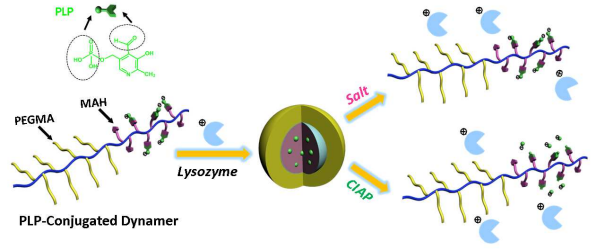
In order to apply the PLP-conjugated dynamer in biologically relevant fields, the relative cytotoxicity of the PLP-conjugated dynamer and the corresponding P(PEGMA)-*b*-PMAH block copolymer was investigated in HeLa cells by CCK-8 assays. The cells were incubated with the polymers for 24 h. As shown in Figure 4, both the block copolymer and the PLP-conjugated dynamer have no noticeable influence on cell viability because the cell viability is greater than 95% up to a tested concentration of 200 μg/mL. This result suggests that the PLP-conjugated dynamer shows a low level of toxicity and thus a potential candidate for biological applications.

Polyion Complex Micelles from PLP-Conjugated dynamer and Protein

It is reported that a pair of oppositedly-charged block copolymers and biomacromolecules such as DNA and proteins can form polyion complex micelles (PIC) in an aqueous medium, driven through electrostatic interaction.[15-21] The core of PIC can serve as a nanoreservoir of charged compounds, e.g. DNA and enzyme, allowing modulation of their inherent properties such as stability, solubility, and reactivity.[22] In this research the conjugation of PLP to PEMGA-*b*-PMAH copolymer generated anionic phosphate containing block copolymer, because the 5'-phosphate group of PLP existed in the form of dianionic ion at physiological pH and accepted a proton only below pH 5.[14] The obtained PLP-conjugated dynamer was expected to form PIC micelles spontaneously upon mixing with positively charged protein (Scheme 2). Lysozyme (pI=11) was chosen as a model protein because it was positively charged over a wide range of pH, and found practical applications in drug delivery as a lytic enzyme.[39] The PIC micelles were prepared by mixing equal volumes of lysozyme solution and PLP-conjugated dynamer solution at various mass ratios (r) of lysozyme to PLP-conjugated dynamer. The final concentration of PLP-conjugated dynamer in the solution was fixed at 0.25 mg/mL. To find an appropriate r value for the formation of PIC micelles, DLS and ζ-potential measurements were utilized to monitor the size and surface charge of the PIC micelles at various r values. As shown in Figure 5A, when r changed from 1:6 to 1:2 at pH 6.5, the average diameter gradually increased and reached maximum at r=1:2. At pH 7.4, the average diameter reached the largest at r=1.3:1. Further increasing of r caused a decrease in the average diameter. When r was below 6:1, the surface charge of the PIC micelles kept negative (ESI, Figure S3). TEM image showed that the PIC micelles prepared at pH 6.5 with r=1:2 were spherical with diameters in the range of 35-55 nm (Figure 5B). The smaller size observed by TEM was attributed to the shrinkage of the PIC micelles in dried state. The results of DLS, zeta potential and TEM studies indicated that lysozyme was entrapped in the core of the PIC micelles.

Because the medium pH has an effect on the charge of PLP molecule, it is expected that the PIC micelles formed by lysozyme and PLP-conjugated dynamer exhibit pH-responsive feature. UV-vis spectroscopy and DLS were utilized to monitor the pH-responsive nature of the PIC micelles (Figure 6). The solution of PIC micelles prepared in a pH 7.4 Tris-HCl buffer

with r=1.3:1 was turbid. As pH changed from 7.4 to 4.5, the solution gradually became transparent. This was clearly evidenced by the naked eye. UV-vis measurement showed that the transmittance of the solution increased with the decreasing of pH value. It was found that the hydrodynamic diameter of the PIC micelles became smaller as the solution was more and more acidic as indicated by DLS data. These results demonstrate that the PIC micelles formed through electrostatic interaction between PLP-conjugated dynamer and lysozyme are pH-responsive.



Scheme 2. Illustration of the formation of PIC micelles through the interaction of PLP-conjugated dynamer and lysozyme, and salt- or enzyme-triggered dissociation of PIC micelles.

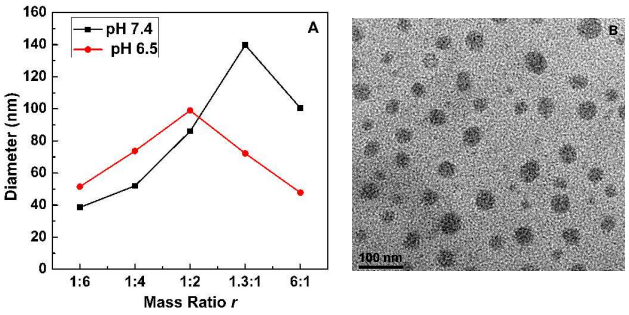


Figure 5. (A) Influence of lysozyme concentration on hydrodynamic diameter of PIC micelles and (B) TEM image of PIC micelles formed in Tris-HCl buffer (pH 6.5) with mass ratio r=1:2.

Since the presence of salts can screen electrostatic interactions between the oppositely charged blocks, the stability of the polyion complex micelles is strong affected by the ionic strength of the milieu.[40] To investigate the effect of the ionic strength on the stability of the PIC micelles, an excess amount of NaCl was added to the micellar solution prepared in Tris-HCl buffer (pH 6.5) with r=1:2. DLS results indicated that the hydrodynamic diameter decreased significantly to 8 nm after the PIC micelles were incubated in Tris-HCl buffer containing 0.15 M NaCl for 30 min (Figure 7A), demonstrating the dissociation of the PIC micelles. This result means that the PIC micelles possess responsive capability to ion strength as well.

The cleavage of phosphoester bond of PLP by phosphatase results in a structural change from a negatively charged molecule into neutral moiety, therefore, the enzyme-triggered disassembly of the PIC micelles was monitored by DLS. When CIAP was added to the solution of PIC micelles, the hydrodynamic diameter decreased remarkably in 1 h and reduced to 14 nm in 8 h (Figure 7B), indicating the dissociation of PIC micelles in response to enzymatic stimulus.

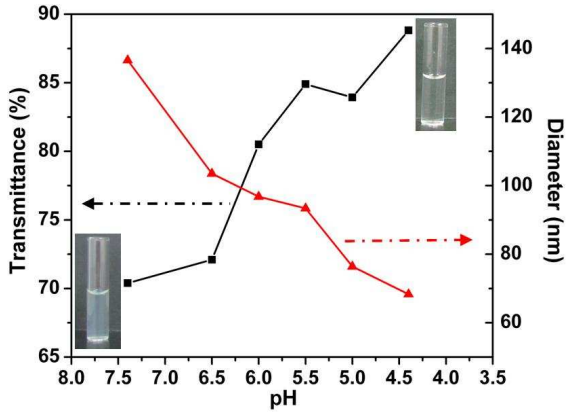


Figure 6. pH-Dependence of the micellar size and the transmittance of the micellar solution. (Inset: photo graphs of PIC micellar solutions at pH 7.4 and 4.25)

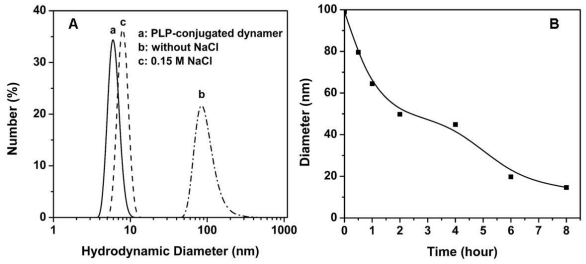


Figure 7. (A) Influence of salt on the size of PIC micelles (Tris buffer, pH 6.5, 0.15 M NaCl). (B) Time-dependence of the hydrodynamic diameter of PIC micelles after the addition of CIAP.

Enzymatic Activity Assay

The activity of lysozyme in the PIC micelles was evaluated using lyophilized *Micrococcus luteus* (*ML*) cells as the substrate.[24] Lysozyme causes the hydrolysis of β-1, 4-glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine at the surface of bacteria cell wall[41] and leads to cell lysis. The activity of lysozyme in the PIC micelles was expressed as the relative activity of free lysozyme. As shown in Figure 8, the lysozyme activity decreased to 11 % of the free lysozyme activity after entrapped into the PIC micelles at pH 6.5. Under this condition, no recovery of activity was observed for a long time, indicating that the PIC micelles were very stable. However, the activity of lysozyme gradually increased and reached 80% of the free lysozyme bioactivity after the PIC micelles were incubated in the buffer solution (pH 6.5) containing 0.15 M NaCl for 5h (Figure 8a). DLS study confirmed that the PIC micelles disassembled in the presence of 0.15 M NaCl and resulted in the release of lysozyme from micelles. These results demonstrate that the P(PEGMA) corona inhibits *ML* cells to interact with lysozyme entrapped in the PIC micelles. The dissociation of PIC micelles with the increase in ionic strength allowed the exposure of lysozyme in the milieu to show lytic activity against *ML* cells. The relative activity increased with NaCl concentration. As the

NaCl concentration increased to 0.3 M, lysozyme recovered 90% of native activity after 30 min. Therefore, salt can modulate the activity of lysozyme in the PIC micelles.

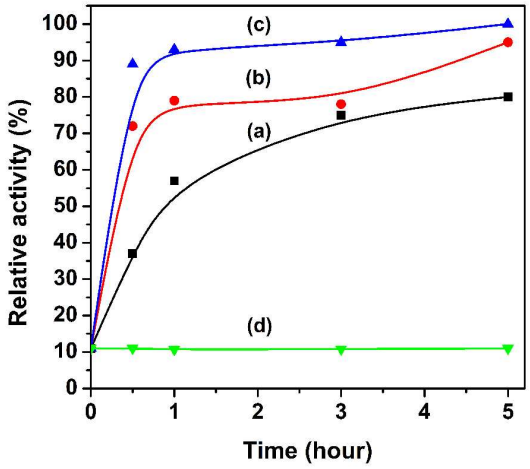


Figure 8. Influence of salt concentration on the relative lysozyme activity in pH 6.5 Tris-HCl beffer solution. (a) 0.15 M NaCl, (b) 0.2 M NaCl, (c) 0.3 M NaCl, and (d) without NaCl.

Conclusions

In summary, a well-defined hydrazide-containing diblock copolymer P(PEGMA)-*b*-PMAH was synthesized by RAFT polymerization. The bioconjugation of pyridoxal phosphate to P(PEGMA)-*b*-PMAH generated anionic dynamer at physicological pH. The hydrolysis of the acylhydrazone linkages between PLP moieties and polymer backbone was temperature-dependent. Half of acylhydrazone bonds were cleaved after 4 days at 60°C under pH 3.5. The PLP-conjugated dynamer has a low level of toxicity and thus a potential candidate for biological applications. Using lysozyme as a model protein, the PIC micelles were formed through electrostatic interaction between the negative-charged dynamer and the positive-charged lysozyme at physicological pH. The PIC micelles exhibited pH-, salt- and enzyme-responsive features. Although the activity of lysozyme in the PIC micelles was inhibited because of the shielding effect of P(PEGMA) corona, an increase in ionic strength of the milieu triggered the dissociation of PIC micelles and the recovery of enzymatic activity against *ML* cells. The activity reached to 80% of free lysozyme activity after 5 h incubation in the presence of physicological ionic strength (pH 6.5, 0.15 M NaCl) and increased to 90% after 30 min in the presence of 0.3 M NaCl. Since the PIC micelles formed by the PLP-conjugated dynamer and protein are responsive to pH-, salt- and enzyme-stimulus, it is anticipated that these multi-responsive PIC micelles can be used as intelligent nanoreactors of enzyme or nanocarriers of protein drugs, which have potential applications in the fields of biotechnology and therapeutics.

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Notes and references

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†Electronic Supplementary Information (ESI) available: GPC rsults, modified TNBS assay, UV-vis spectra, and Zeta potential measurements. See DOI: 10.1039/b000000x/

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Table of Contents

Multi-Responsive Protein Nanocarriers from Anionic Dynamic Covalent Copolymer

Xiaobei Wang, Lin Wang, Shixia Yang, Hanying Zhao, and Li Liu

PIC micelles were formed through the electrostatic interactions between anionic dynamer and lysozyme, and the micelles possessed pH-, salt-, and enzyme-responsive features.