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Light-Responsive Polyion Complex Micelles with Switchable Surface Charge for Efficient Protein Delivery

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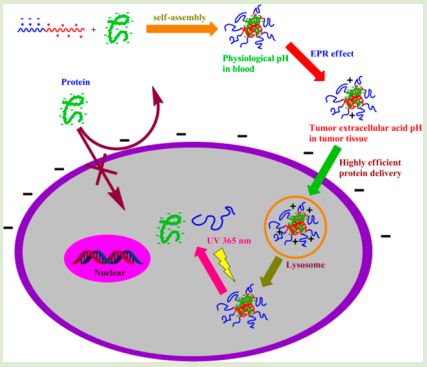
Supporting Information

ABSTRACT: In this letter, light-responsive protein-encapsulated polyion complex (PIC) micelles were prepared by self-assembly of cationic block copolymer poly(*N*,*N*-dimethyl-*N*-(2-(methacryloyloxy)ethyl)-*N*-((2-nitrobenzyl)oxy)-2-oxoethanaminium bromide)-*block*-poly(carboxybetaine methacrylate) (PDMNBMA-*b*-PCBMA) and negatively charged bovine serum albumin (BSA). The PIC micelles were well characterized by dynamic light scattering (DLS) and transmission electron microscopy (TEM). From the zeta potential measurement, the increase of the zeta potential of PIC micelles from ∼10 to ∼20 mV was observed when the solution pH decreased from 7.4 to 6.5, which could enhance the intracellular protein delivery efficiency. Moreover, the positively charged PDMNBMA blocks can be transformed to zwitterionic carboxybetaine units under UV irradiation, which could result in the disassembly of the PIC micelles. The release of BSA can therefore be drastically accelerated in the presence of UV irradiation. Meanwhile, the circular dichroism (CD) spectroscopy confirmed that the secondary structure of BSA was unaffected during the UV irradiation process.



Since the development of the first protein drug (human insulin) more than 30 years ago,[1] protein and peptide-based therapeutics have attracted widespread attention for the effective treatment of human diseases, especially those caused by the temporary or permanent loss of protein functions.[2−4] However, there are a great number of hurdles that have to be overcome before the protein drug can exert its therapeutic activity.[5] These hurdles include (1) structural instability and fast enzymatic degradation in the gastrointestinal tract; (2) potential immune response; (3) inferior cellular uptake because of the charge repulsion of proteins from negatively charged cell membranes and mucosa cells. All these obstacles require suitable protein drug carriers that can (i) protect the protein drug from denaturation; (ii) shield the negatively charged protein and provide an overall positively surface charge to realize efficient cellular uptake; and (iii) release the protein drug rapidly in native forms at the sites of action.[6,7] In order to address these requirements, various nanovehicles have been explored for intracellular protein delivery, such as liposomes,[8] nanogels,[9,10] vesicles,[11] mesoporous silica nanoparticles,[12] nanotubes,[13] and polyion complex micelles.[14−16] The polyion complex micelles are especially promising since the complexes from cationic polymer and anionic protein can maintain a stable state through electrostatic interactions.

Although the drug nanocarriers can accumulate in tumor tissues via EPR effect, the poor cellular internalization as well as insufficient intracellular drug release always hampers the therapeutic efficacy. Since the cellular internalization can often be a stumbling block in drug delivery, positively charged nanocarriers are widely used to enhance the cellular uptake.[17] However, the positively charged nanocarriers might be cleared rapidly from the circulation by the reticuloendothelial system. Stimuli-responsive charge conversional nanocarriers were developed in recently years to address this problem. Typically, the surface charge of the nanocarriers should be neutral or even negatively charged in physiological environment. However, the surface charge of the nanocarriers will become positive in the disease site, such as tumor tissue. Because the extracellular pH of tumor tissue (pH 6.5) is slightly lower than that of normal tissue and blood (pH 7.4), nanocarriers that are capable of changing the surface charge from neutral or negative to positive at pH 6.5 have been designed to achieve efficient cell uptake.[18−21] On the other hand, to maximize the therapeutic outcome, the loaded protein drug should be released rapidly when the desired destination is reached. Therefore, various stimuli-responsive drug carriers were developed for on-demand drug release. In particular, pH and redox-responsive drug carriers are especially attractive because of the existence of pH and redox potential gradient between the extra- and intracellular space.[22,23] For the above-mentioned endogenous pH or redox potential triggered release mechanisms, it is still a considerable challenge to realize accurate controlled release because of the complicated physiological environment. Thus, it would be favorable to develop an exogenous stimuli-triggered release of protein in vitro and in vivo. Light is an especially attractive stimulus since it can be localized in time and space, and it can also be triggered from outside of the system.

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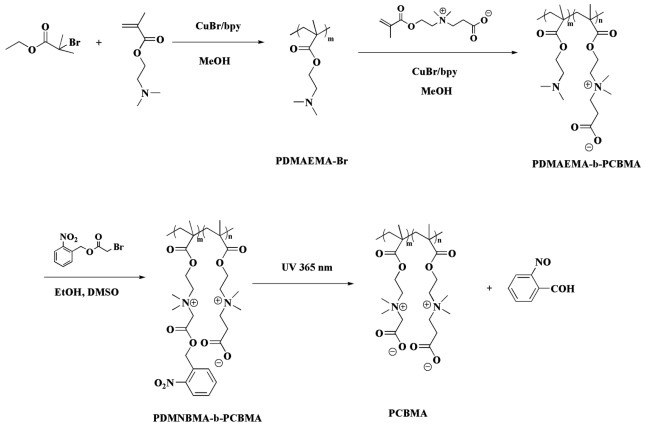
Furthermore, a lot of parameters such as the intensity and the wavelength of light can be easily adjusted during the irradiation time that enables good control over the reaction.[24,25] Various light-responsive drug nanocarriers were therefore reported in the past years.[26−31]

Due to the biomimetic nature and excellent stealth properties, zwitterionic poly(carboxybetaine)-based polymers are well developed for biomedical applications.[32−37] In this research, we designed and prepared pH- and light-responsive block copolymer poly(*N*,*N*-dimethyl-*N*-(2-(methacryloyloxy)-ethyl)-*N*-((2-nitrobenzyl)oxy)-2-oxoethanaminium bromide)-*block*-poly(carboxybetaine methacrylate) (PDMNBMA-*b*-PCBMA) that can form polyion complex (PIC) micelles with negatively charged bovine serum albumin (BSA). The surface charge of the PIC micelles was converted from neutral in physiological environment to positive at tumor acidic pH because of the protonation of the carboxylate groups. The cell uptake of PIC micelles was expected to be enhanced. Moreover, the positively charged PDMNBMA blocks can be transformed to zwitterionic carboxybetaine under UV irradiation which can result in the disassembly of the PIC micelles and BSA can be rapidly released from the PIC micelles.

The carboxybetaine-based photoresponsive cationic block copolymer poly(*N*,*N*-dimethyl-*N*-(2-(methacryloyloxy)ethyl)-*N*-((2-nitrobenzyl)oxy)-2-oxoethanaminium bromide)-*block*-poly(carboxybetaine methacrylate) (PDMNBMA-*b*-PCBMA) was synthesized by sequential atom transfer radical polymerization (ATRP) and subsequent quaternization with *o*-nitrobenzyl 2-bromoacetate, as depicted in Scheme 1. The successful

Scheme 1. Schematic Illustration of the Synthesis and Photolysis of PDMNBMA-*b*-PCBMA



synthesis of PDMNBMA-*b*-PCBMA was approved by ^{1}H NMR. The typical ^{1}H NMR spectra of each step in the synthesis is shown in Figure 1 with the relevant resonance signals labeled. By comparing the well-defined peak integrals of DMAEMA (δ 2.26) with that of the ATRP initiator fragment (δ 1.42), the polymerization degree of DMAEMA block was 37, calculated from Figure 1a. The GPC chromatogram of PDMAEMA-Br indicated that the resulting polymer had relatively low polydispersity (1.25) with M_n of 7300. The synthesis of PDMAEMA-*b*-PCBMA was also verified by ^{1}H NMR. The polymerization degree of CBMA was 8 by comparing the peak integrals of DMAEMA block and CBMA block in Figure 1b. PDMNBMA-*b*-PCBMA was obtained by quaternization of DMAEMA block with o-nitrobenzyl 2-bromoacetate. The PDMAEMA characteristic signals at δ

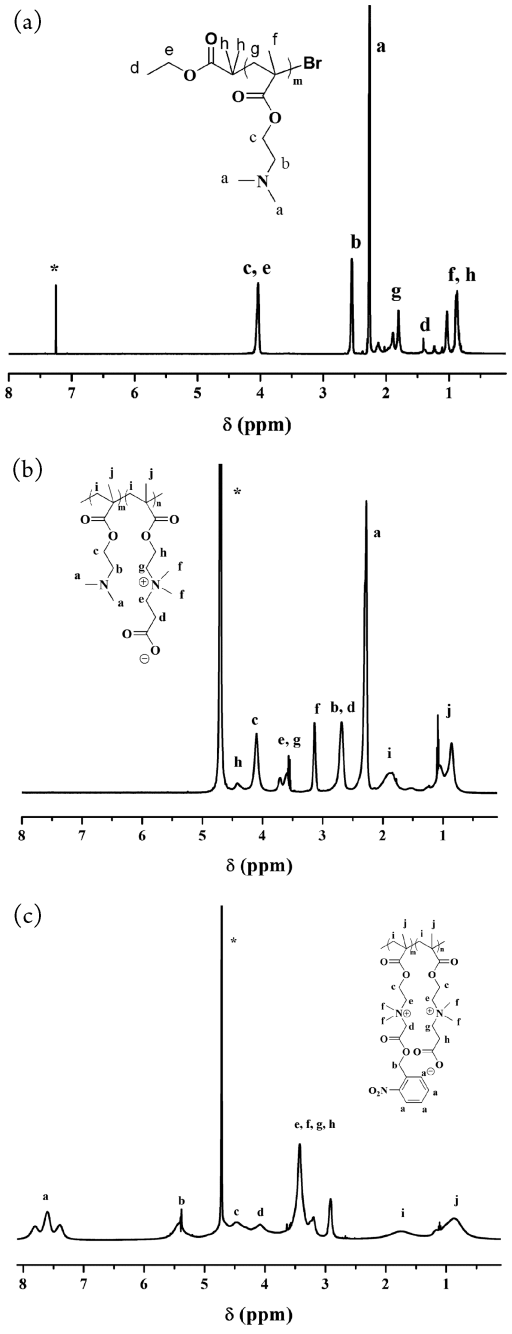


Figure 1. ^{1}H NMR spectra of (a) PDMAEMA-Br in $CDCl_3$, (b) PDMAEMA-*b*-PCBMA in D_2O, and (c) PDMNBMA-*b*-PCBMA in D_2O; *Indicates residual NMR solvent.

2.26 and δ 2.55 disappeared (Figure 1c). At the same time, we can find the characteristic signals of *o*-nitrobenzyl groups at δ 7.61, which showed complete quaternization of DMAEMA. The block copolymer PDMNBMA-*b*-PCBMA was therefore successfully synthesized and denoted as $PDMNBMA_{37}$-*b*-$PCBMA_8$.

PDMNBMA-*b*-PCBMA is present as polycation at physiological environment. Therefore, the electrostatic interaction of PDMNBMA-*b*-PCBMA and negatively charged protein can be expected. Fluorescein isothiocyanate-labeled BSA (FITC-BSA) was used as a model protein drug in this research since BSA has an isoelectric point of 4.7. FITC-BSA loaded PIC micelles were prepared using different polymer/protein weight ratios ranging

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from 5/1 to 20/1. The loading of FITC-BSA and DLS results are shown in Table 1 and Figure S2. The PDMNBMA-*b*-PCBMA copolymers were capable to form nanosized PIC micelles by electrostatic interaction with FITC-BSA. The PIC micelles exhibited very high BSA loading efficiency (typically >85%), which demonstrated that PDMNBMA-*b*-PCBMA was able to bind negatively charged protein with high affinity. The morphology of the PIC micelles was investigated by TEM. Figure S1 showed the typical TEM image of PIC-2 in HEPES buffer solution (10 mM, pH 7.4). The TEM image indicated that the micelles were presumably spherical with mean diameter of about 162 ± 47 nm, which was similar to the hydrodynamic diameter (D_h) measured by DLS (Table 1, 172 nm).

Table 1. BSA Loading Efficiencies and DLS Results of PIC Micelles Prepared Using Different Polymer/Protein Weight Ratios

	polymer/protein weight ratio	D_h[a] (nm)	PDI[a]	FITC-BSA loading efficiency[b] (%)
PIC-1	5:1	229	0.28	85.6
PIC-2	10:1	172	0.32	92.7
PIC-3	20:1	142	0.25	96.1

[a]D_h and PDI were measured by DLS. [b]Percentage of BSA incorporated in the PIC micelles in HEPES buffer solution.

It is very important for the therapeutic efficacy of protein drugs whether they can be effectively internalized by cells. In this research, we chose zwitterionic polycarboxybetaine PCBMA as the outer shell of the micelles. PCBMA is pH-responsive because of the carboxylate group of carboxybetaine. The pH-dependence of the surface charges of the PIC micelles was determined by measuring the zeta potential of the micelles in different pHs. As depicted in Table 2, the zeta potential values of the PIC micelles were a little positive (~10 mV) at pH 7.4. Furthermore, if the solution pH decreased from 7.4 (physiological environment) to 6.5 (tumor tissue slightly acid environment), the increase of the zeta potential from ~10 to ~20 mV was observed. It might be attributed to the partial protonation of the carboxylate groups. It is very important since the surface charge of the particles has a great effect on the cellular uptake of the particles. The cellular uptake of FITC-BSA loaded PIC micelles (PIC-2) at different pH (pH 6.5 and 7.4) was evaluated by flow cytometric analysis. This method was demonstrated by quantitative fluorometry of FITC from cell internalization. The A549 cells were incubated with FITC-BSA loaded micelles for the predetermined time intervals. The free FITC-BSA was used as a control. The flow cytometry results indicated the greater uptake of the PIC micelles than free FITC-BSA (Figure 2). The free FITC-BSA was hardly internalized by A549 cells. It is reasonable because the BSA is negatively charged. The charge repulsion occurs between BSA and negatively charged cell membranes. The results indicated that the PIC micelles were very beneficial for the effective internalization of negatively charged proteins. The cellular uptake of the PIC micelles at different pH was also investigated by flow cytometry, as shown in Figure 2. The relative geometrical mean fluorescent intensities of A549 cells pretreated with the PIC micelles increased as the pH decreased from 7.4 to 6.5. The flow cytometry results further confirmed the enhanced internalization of the PIC micelles at pH 6.5, which was evidenced by the remarkably enhanced cellular fluorescence. It might be attributed to the increased positive charge of the micellar surface. It is concluded that the FITC-BSA loaded PIC micelles with switchable surface charge indeed exhibited significantly enhanced cellular internalization at tumor extracellular pH (~6.5).

Table 2. Zeta Potential of the PIC Micelles Prepared Using Different Polymer/Protein Weight Ratios at Different pH Values

	polymer/protein weight ratio	zeta potential (mV) pH 7.4	zeta potential (mV) pH 6.5
PIC-1	5:1	9.5	16.8
PIC-2	10:1	12.3	20.2
PIC-3	20:1	12.5	19.3

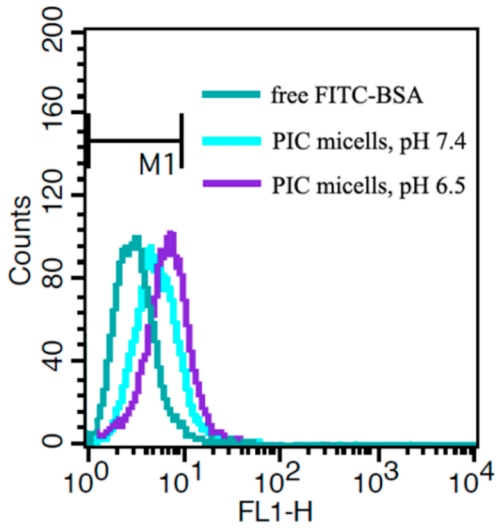

Figure 2. Flow cytometric profiles of A549 cells incubated with FITC-BSA loaded PIC micelles at pH 7.4 and 6.5. M1 refers to the basic fluorescence of negative control. FL1-H refers to the green fluorescent channel.

As discussed above, FITC-BSA could be readily loaded into PIC micelles by mixing PDMNBMA-*b*-PCBMA and FITC-BSA. The unloaded protein could be removed by extensive dialysis. The *o*-nitrobenzyl ester groups of the PDMNBMA blocks can be cleaved under UV irradiation (365 nm), as shown in Scheme 1. This photoreaction could be investigated by UV–vis spectra. PDMNBMA-*b*-PCBMA block copolymer in aqueous solution was irradiated under UV light (365 nm). The UV absorption over a wide range of wavelengths between 300 and 500 nm increased during light irradiation as shown in Figure S3, which indicated the light-cleavage of *o*-nitrobenzyl ester moieties.[38] As a result, the positively charged PDMNBMA can be transformed to zwitterionic carboxybetaine under UV irradiation, which can result in the disassembly of the PIC micelles. FITC-BSA might be rapidly released from the PIC micelles. The in vitro release of FITC-BSA from PIC micelles was further investigated in the presence or absence of light irradiation using HEPES buffer solution (10 mM, pH 7.4) at 37 °C. Before the experiment of light-triggered protein release was carried out, we investigated if the secondary structure of BSA can be altered after UV irradiation. The BSA buffer solution before and after UV irradiation (365 nm) was monitored by far-UV circular dichroism (CD). BSA solution was exposed to UV light (365 nm, 8 W) for 30 min. Only negligible changes in the CD spectra of BSA were observed before and after UV

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irradiation, as shown in Figure S4, indicating that the secondary structure of BSA was not altered under UV irradiation. The hydrodynamic diameters (D_h) of PIC-2 micelles before and after light irradiation were measured by DLS. As shown in Figure S5, two peaks were observed after light irradiation. One was small than 10 nm, which might be ascribed to the released BSA. The other one was larger than 600 nm, which might be ascribed to the swell and dissociation of the PIC micelles. The cumulative release percentages of FITC-BSA released from the micelles versus time were presented in Figure 3. FITC-BSA was

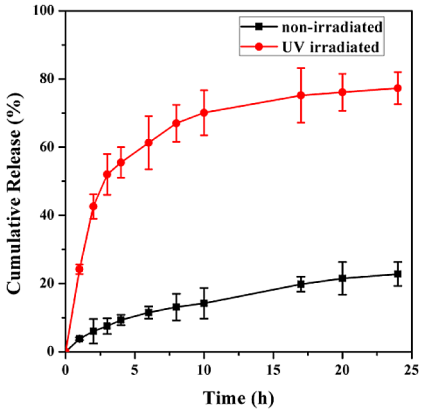

Figure 3. In vitro release of FITC−BSA from PIC micelles in the presence or absence of UV irradiation (365 nm).

rapidly released from PIC micelles in the presence of UV irradiation, in which 55.5 and 77.3% of FITC-BSA was released in 4 and 24 h, respectively. The fast protein release is likely due to the disruption of PIC micelles under UV irradiation. In contrast, low FITC-BSA release (~20%) was observed in 24 h in the absence of UV irradiation and no burst release of protein was observed. These results indicated that protein release from the PIC micelles proceeded in a controlled manner and can be triggered by UV irradiation (365 nm). Compared to longer wavelength lights, the UV light cannot be used to release the drug in vivo due to the short penetration depth, which greatly limits the application of UV light in vivo. With a combination of light and modern fiber optics, site-specific drug release might be realized in vivo. As an alternative strategy, the upconverting nanoparticles-assistant NIR responsive drug nanocarriers were developed.[39] Taking advantage of upconverting nanoparticles, which are able to absorb NIR light and convert it into high-energy photons in a very broad range from the UV to the NIR region, the UV responsive systems can be converted to NIR responsive systems. A detailed investigation of NIR responsive drug release for this effect is in progress in our research group.

In summary, we have illustrated photoresponsive polyion complex micelles with switchable surface charge as protein drug carriers for efficient intracellular protein delivery. The positively charged block copolymers PDMNBMA-*b*-PCBMA can form PIC micelles with negatively charged FITC-BSA. Compared to the free FICT-BSA, FICT-BSA-loaded PIC micelles were much easier to be internalized by A549 cells. When the solution pH decreased from physiological pH (7.4) to tumor tissue slightly acid pH (6.5), the zeta potential of the FICT-BSA-loaded PIC micelles increased from ~10 to ~20 mV, which resulted in the significantly enhanced cellular internalization. Furthermore, in vitro drug release showed that the release of FITC-BSA can be drastically accelerated in the presence of UV irradiation. These results indicated that the novel PIC micelles can be served as promising platform for efficient intracellular protein delivery.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Materials, experimental details, TEM UV−vis spectra, and CD spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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