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pH-Sensitive Block Copolymer Vesicles with Variable Trigger Points for Drug Delivery

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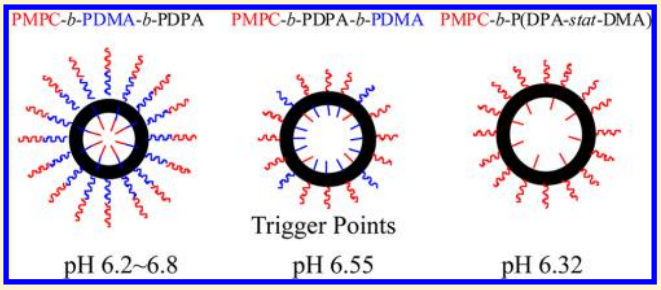
Supporting Information

ABSTRACT: We have previously reported the preparation of a novel pH-sensitive and biocompatible polymer vesicle in pure water based on the spontaneous self-assembly of a diblock copolymer, PMPC-*b*-PDPA, where PMPC is poly[2-(methacryloyloxy)ethyl phosphorylcholine] and PDPA is poly[2-(diisopropylamino)ethyl methacrylate] (*J. Am. Chem. Soc.* **2005**, *127*, 17982). Herein, we intend to report the strategy for controlling the pH trigger points of association/dissociation of pH-responsive polymer vesicles for anticancer drug delivery. We introduced a reactive block, poly[2-(dimethylamino)ethyl methacrylate] (PDMA) into the above diblock copolymer to form reactive PMPC-*b*-PDMA-*b*-PDPA and PMPC-*b*-PDPA-*b*-PDMA triblock copolymers, as well as PMPC-*b*-P(DMA-*stat*-DPA) block-statistical copolymer by atom transfer radical polymerization (ATRP) in methanol at room temperature. As a result of different block length of PDPA, the introduction of PDMA chain at different positions, and different initial copolymer concentrations, those block copolymer vesicles showed tunable pH trigger points and various isoelectric points (IEPs) in aqueous solution. Transmission electron microscopy (TEM) and dynamic light scattering (DLS) studies confirmed that the block copolymers with relatively long PDPA block form polymer vesicles by simply tuning the solution pH in pure water. Above pH 6.2, the PDPA block becomes hydrophobic so it forms the vesicle membrane. In all cases, the hydrophilic PMPC chains form the vesicle coronas. The PDMA chains are designed in three different positions. In PMPC-*b*-PDMA-*b*-PDPA vesicles, the PDMA chains form the middle shell between the PDPA vesicle membrane and the PMPC vesicle corona. In PMPC-*b*-PDPA-*b*-PDMA vesicles, the PDMA can mix with PMPC to serve as mixed coronas. In PMPC-*b*-P(DMA-*stat*-DPA) vesicles, the reactive PDMA chains can be incorporated into the vesicle membrane, which provides an effective strategy regarding the immobilization of vesicles by selective quaternization of PDMA with a bifunctional cross-linker, such as 1,2-bis(2-iodoethoxy)ethane (BIEE). The degree of cross-linking can be tuned by varying the molar ratio of PDMA to BIEE, which was further investigated by ^{1}H NMR, DLS, and TEM, suggesting tunable permeability of vesicle membrane. The triblock copolymer vesicles were able to encapsulate anticancer drugs such as DOX, exhibiting obviously retarded release profile at physiological conditions.



1. INTRODUCTION

Recently, much attention has been paid to the macromolecular self-assembly to form polymer micelles, cylinders, vesicles and other morphologies.[1−15] Smart and functional polymer vesicles which respond to pH, temperature, light, or electrical potential have shown promising potential applications in a wide range of fields.[16−28] Given the wide range of pH gradients present in biological and physiological systems the application of pH-responsive polymer vesicles for controlled release/encapsulation is of great interest.[17] In fact, pH tuning or response has been proposed to be one of most effective ways to control the encapsulation/release of small molecules *in vivo*.[17] However, how to control the trigger point of association/dissociation of pH-responsive polymer vesicles is still remaining challenging.

We have previously reported a new diblock copolymer vesicle based on a highly biocompatible monomer, 2-(methacryloyloxy)ethyl phosphorylcholine (MPC) and a second monomer, 2-(diisopropylamino)ethyl methacrylate (DPA), which confers pH-sensitivity to the membrane wall.[29] These PMPC-*b*-PDPA vesicles were prepared directly in purely aqueous solution without any organic cosolvents and are colloidally stable at physiological pH. Moreover, the dissociation of polymer vesicles into free polymer chains can be triggered at acidic conditions, which suggests the possibility of intracellular delivery of water-soluble drugs and proteins, especially in acidic tumor cells. Since these new vesicles contain the biomimetic phosphorylcholine motif they could be considered as very close polymeric analogues of conventional liposomes. Thus, they are expected to have a number of biomedical applications as nanosized delivery vehicles.[30−30] For example, these vesicles have been widely used in DNA encapsulation and delivery,[31,32] cell uptake control,[33] prognosis

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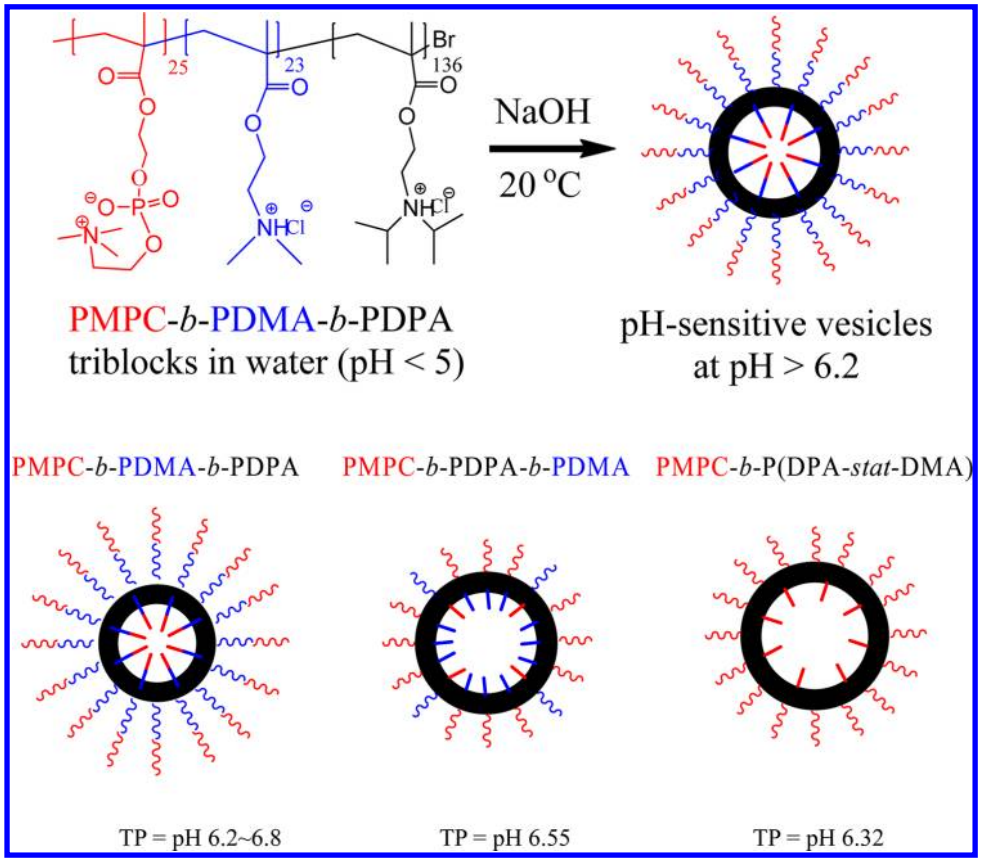
Scheme 1. Synthetic Strategy of Polymer Vesicles with Various Structures and Tunable pH Trigger Points (TP)[a]



[a]Block copolymers were synthesized by ATRP. The pH-sensitive vesicles were obtained by simply adding aqueous NaOH solution to the aqueous copolymer solution at low pH. Red: biocompatible and hydrophilic PMPC. Blue: hydrophilic and reactive PDMA. Black: PDPA (hydrophilic when protonated at lower pH whereas hydrophobic when deprotonated at higher pH). PDMA chains can be cross-linked by 1,2-bis(2-iodoethoxy)ethane (BIEE) in aqueous solution at room temperature.

Table 1. Properties of block copolymers synthesized by ATRP[a]

polymer	composition	$M_{n,NMR}$	$M_{n,GPC}$	M_w/M_n	triggering pH	morphology
1.1	$PMPC_{25}$-*b*-$PDMA_{23}$-*b*-$PDPA_{136}$	40 300	60 300	1.24	6.20	vesicles
1.2	$PMPC_{25}$-*b*-$PDMA_{21}$-*b*-$PDPA_{71}$	26 100	49 000	1.31	6.40	vesicles
1.3	$PMPC_{25}$-*b*-$PDMA_{17}$-*b*-$PDPA_{37}$	18 200	36 000	1.27	6.80	micelles
2	$PMPC_{25}$-*b*-$PDPA_{107}$-*b*-$PDMA_{14}$	32 700	32 700	1.30	6.55	vesicles
3	$PMPC_{25}$-*b*-P(DPA_{115}-*stat*-DMA_{21})	34 500	39 300	1.40	6.32	vesicles

[a]Polymers **1.1**−**1.3** are ABC triblock copolymers; polymer **2** is an ACB triblock copolymer; polymer **3** is a diblock-statistical copolymer with statistically copolymerized B and C. The compositions of copolymers are determined by ^{1}H NMR in D_2O/DCl at pH 2. The triggering pH was recorded when the solution suddenly became blue, accompanying with a plateau of solution pH against added volume of NaOH water. Polymer **1.3** does not form a blue solution at pH 5.0−8.0. DLS revealed that the polymer micelle was formed at pH 6.80. The morphology of self-assembly was revealed by TEM.

for oral squamous cell carcinoma (OSCC),[34] head and neck cancer treatment, etc.[35] However, tunable pH trigger points for these polymer vesicles are demanded for various applications. Therefore, in this paper we try to solve this problem by synthesizing block copolymers with different structures and block length, and preparing vesicles at different initial copolymer concentrations.

Herein we designed a new class of tri- and diblock copolymers by introducing reactive PDMA chains in the previously reported PMPC-*b*-PDPA diblock copolymer. Scheme 1 shows the preparation of polymer vesicles with different structures. In all cases, the hydrophilic PMPC chains form the vesicle coronas. The PDMA chains can be selectively located in three different positions. In PMPC-*b*-PDMA-*b*-PDPA vesicles, the PDMA chains form the middle shell between the PDPA vesicle membrane and the PMPC vesicle corona. In PMPC-*b*-PDPA-*b*-PDMA vesicles, the PDMA can mix with PMPC to serve as mixed coronas. In PMPC-*b*-P(DMA-*stat*-DPA) vesicles, the reactive PDMA chains can be incorporated into the vesicle membrane, which provides an effective strategy regarding the immobilization of vesicles by selective quaternization of PDMA with a bifunctional cross-linker, such as BIEE. The degree of cross-linking can be tuned by varying the molar ratio of PDMA to BIEE, suggesting tunable permeability of vesicle membrane.

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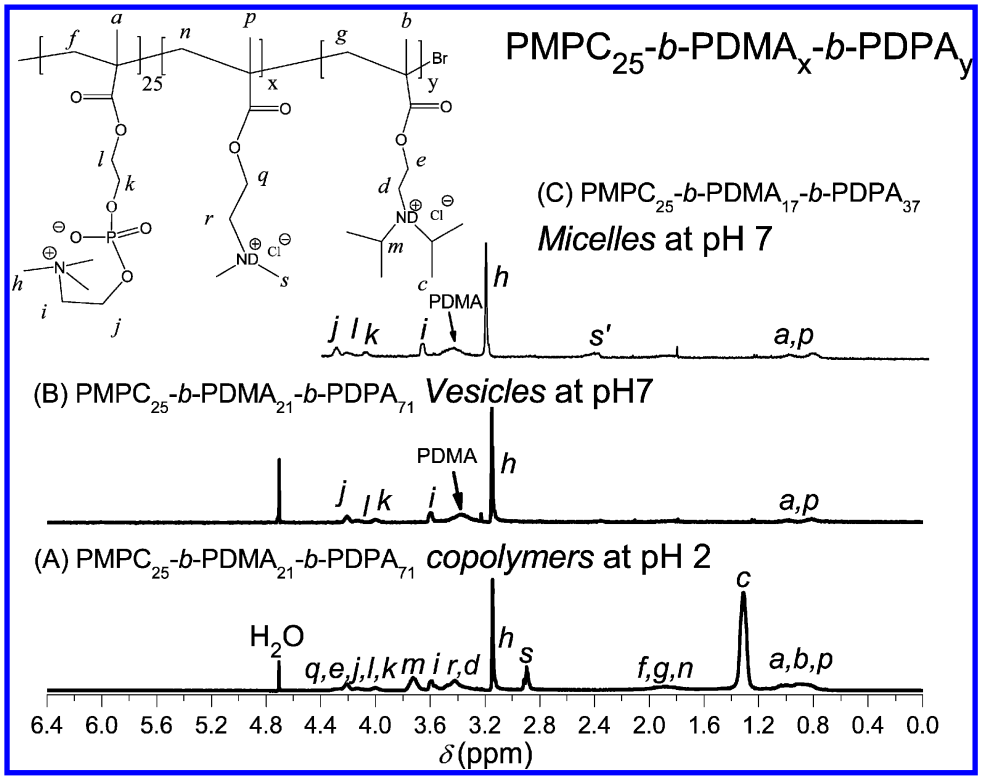

Figure 1. Assigned ^{1}H NMR spectra of PMPC-*b*-PDMA-*b*-PDPA triblock copolymers (in D_2O/DCl at pH 2), vesicles and micelles (in D_2O/H_2O at pH 7). Peak *s*′ is the methyl groups near deprotonated *N* atom in PDMA segment because PDMA is only partially protonated at pH 7, which is undetectable for copolymer vesicles but detectable for copolymer micelles by ^{1}H NMR. Signals from PMPC (typical peak *h*) is visible in the whole pH range. However, signals from PDPA such as peak *c* completely disappeared above pH 7, indicating the formation of nonhydrated PDPA vesicle membrane or micelle core.

2. RESULTS AND DISCUSSION

2.1. Synthesis of Block Copolymers by ATRP in Methanol at Room Temperature. The ABC triblock copolymer, PMPC-*b*-PDMA-*b*-PDPA, was synthesized by sequential ATRP in methanol at room temperature. Starting from PMPC, DMA monomer was added after the conversion of PMPC > 99% (by ^{1}H NMR). DPA monomer was added after the conversion of DMA exceeded > 96% as judged by ^{1}H NMR spectrum. Few residual DMA monomer was statistically copolymerized with the DPA monomer by comparing the ^{1}H NMR spectra of PMPC-*b*-PDMA diblock copolymer and PMPC-*b*-PDMA-*b*-PDPA triblock copolymer.

Three PMPC-*b*-PDMA-*b*-PDPA triblock copolymers with similar PMPC-*b*-PDMA composition but different PDPA length are listed in Table 1 (polymers **1.1**−**1.3**). A typical ^{1}H NMR spectrum of PMPC-*b*-PDMA-*b*-PDPA triblock copolymer is shown in Figure 1A. The compositions of copolymers were determined by ^{1}H NMR spectra in D_2O/DCl at pH 2 by comparing the integration of peaks *h* (PMPC), *s* (protonated PDMA), and *c* (protonated PDPA). Neither residual monomers nor other impurities were detected by ^{1}H NMR, indicating the high purity of triblock copolymers. Also, the M_w/M_n values of triblock copolymers determined by GPC were relatively low.

Using similar protocols but different sequences to add DPA or DMA monomers, ACB type of $PMPC_{25}$-*b*-$PDPA_{107}$-*b*-$PDMA_{14}$ triblock copolymer (polymer **2** in Table 1) and A(C+B) type of *block-statistical* $PMPC_{25}$-*b*-P(DPA_{115}-*stat*-DMA_{21}) copolymer (polymer **3** in Table 1) were synthesized by ATRP to study the influence of block sequence on vesicle formation and the pH trigger points. The ^{1}H NMR spectra of both copolymers are shown in Figure S1A and Figure S2A in the Supporting Information, respectively.

2.2. PMPC-*b*-PDMA-*b*-PDPA Triblock Copolymer Vesicles and Micelles in Pure Water. Triblock copolymer vesicles or micelles were formed by simply adjusting the copolymer solution pH from 2 to around 7. Below pH 5, the triblock copolymers are molecularly dissolved. Above pH 6.2 (depending on the PDPA block length), the PDPA block becomes hydrophobic whereas the PMPC block is still hydrophilic. ∼50% of PDMA segments are protonated at pH 7.2. However, both protonated and deprotonated PDMA chains are water-soluble below pH 8.0.

We found the length of PDPA block significantly affects the morphology of self-assemblies. Triblock copolymers with longer PDPA chains form vesicles whereas shorter PDPA chains lead to simple micelles. As summarized in Table 1, $PMPC_{25}$-*b*-$PDMA_{23}$-*b*-$PDPA_{136}$ and $PMPC_{25}$-*b*-$PDMA_{21}$-*b*-$PDPA_{71}$ form vesicles while $PMPC_{25}$-*b*-$PDMA_{17}$-*b*-$PDPA_{37}$ form micelles in aqueous solution. The solution is colorless when the pH is below 5.0. It then changes to bluish or milky white when the pH increases above 6.2 (the specific triggering pH value depends on the copolymer composition and concentration), indicating the self-assembly of triblock copolymers. This is because the PDPA block is soluble in water at low pH as a weak cationic polyelectrolyte, but becomes insoluble above pH 6.2 due to deprotonation of its tertiary amine groups.

As shown in Scheme 1, PDPA block forms vesicle membrane and PDMA shell is in the middle between the PMPC corona and the PDPA membrane. ^{1}H NMR studies of vesicle-forming

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triblock copolymers revealed that the signals from PDPA completely disappeared when the solution pH is 7. A typical ^{1}H NMR spectrum of $PMPC_{25}$-*b*-$PDMA_{21}$-*b*-$PDPA_{71}$ triblock copolymer vesicles in H_2O/D_2O at pH 7 is shown in Figure 1B. The signals assigned to PMPC (typical peak *h*) are still very sharp at pH 7, as a result of solvated vesicle corona. However, the signals from PDPA chains (typical peak *c*) completely disappeared compared with Figure 1A, as a result of the formation of the compact nonhydrated vesicle membrane. The signals from PDMA segments such as peaks *s* and *r* shifted to one peak at 3.4 ppm.

With less hydrophobic fraction, $PMPC_{25}$-*b*-$PDMA_{17}$-*b*-$PDPA_{37}$ triblock copolymers only form simple spherical micelles at pH 7. The signals from PMPC segments are still sharp and the signals from PDPA segments are not visible in ^{1}H NMR spectrum (Figure 1C), as a result of solvated micellar corona and compact micellar core. The only difference between triblock copolymer vesicles and micelles lies in the PDMA block. For vesicles, the characteristic peaks *r* and *s* at pH 2 were shifted to one complex peak at 3.4 ppm above pH 7 (Figure 1B). This is perhaps because the compulsive vesicle membrane formation of PDMA segment due to the polydispersity of copolymers. In other words, part of the PDMA chains is compulsively involved in the formation of vesicle membrane by some copolymers with a shorter PDPA block. In contrast, peak *s* at ~2.85 ppm in the micelles shifted to ~2.4 ppm as a result of deprotonation of a part of PDMA (Figure 1C). This difference is repeatable and verified by a series of PDMA-*b*-PDPA-based diblock or triblock copolymer vesicles and micelles. Thus, this difference may empirically distinguish PDMA-based vesicles and micelles.

DLS studies (Figure 2) revealed that below pH 5.0, the $PMPC_{25}$-*b*-$PDMA_{23}$-*b*-$PDPA_{136}$ copolymer was molecularly

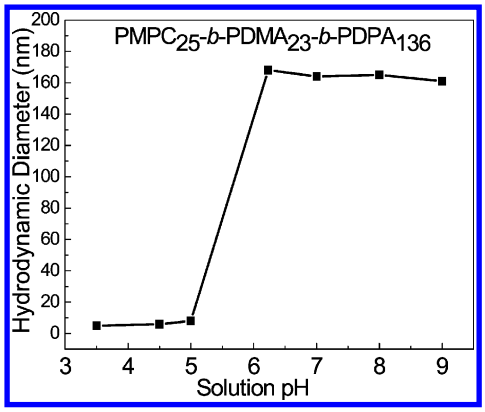

Figure 2. Hydrodynamic diameters of $PMPC_{25}$-*b*-$PDMA_{23}$-*b*-$PDPA_{136}$ self-assemblies as a function of solution pH. The final copolymer concentration in water is 0.031 wt % at pH 8.0. The data point at pH 5.56 is not listed due to very high PDI of particles.

dissolved in water. Ill-defined aggregates were formed between pH 5.0 and 6.2 because the PDI of particles was very high. Vesicles formed when pH was above 6.2. This is consistent with the empirical observation when the solution becomes blue at this pH range.

We found that when the copolymer concentration at pH 2 is more than 0.1 wt %, the vesicle solution is opaque and very cloudy, accompanying with some large compound micelles. Also, the vesicles are sensitive to the shocks of aqueous NaOH solution. For example, it may precipitate if the NaOH solution is too concentrated or the dropping rate is too fast or the stirring rate is too slow. However, it becomes less sensitive to those factors when the initial copolymer concentration is less than 0.05 wt %. Less or no precipitation was found when decreasing the copolymer concentration, gently adding NaOH solution, or using dilute NaOH solution (~0.01 M). A larger preparative scale (e.g., > 40 mg copolymer in one batch) also helps to decrease/eliminate the precipitation due to better amortizing to the shocks of NaOH solution.

Figure 3 shows the typical hydrodynamic diameter distribution of triblock copolymer micelles and vesicles. The

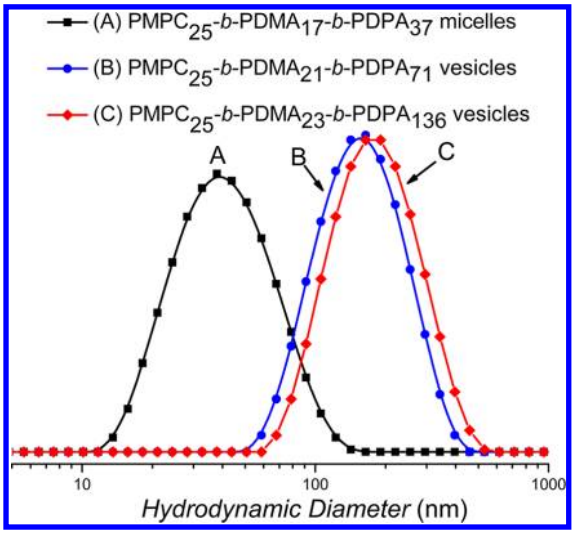

Figure 3. DLS study of triblock copolymer vesicles and micelles (See corresponding correlation functions in Figure S3, Supporting Information) at pH 7. The initial copolymer concentration in water at pH 2 is 0.10 wt %. The final copolymer concentration, the mean intensity-averaged diameter and PDI are as follows: (A) 0.096 wt %, 36.7 nm, 0.178; (B) 0.068 wt %, 143 nm, 0.130; (C) 0.067 wt %, 164 nm, 0.158.

triblock copolymer micelles have an intensity-averaged mean diameter of 36.7 nm. However, the triblock vesicles have larger size than micelles. The mean diameters of $PMPC_{25}$-*b*-$PDMA_{21}$-*b*-$PDPA_{71}$ and $PMPC_{25}$-*b*-$PDMA_{23}$-*b*-$PDPA_{136}$ vesicles are 143 and 164 nm, respectively.

2.3. TEM Study of PMPC-*b*-PDMA-*b*-PDPA Triblock Copolymer Vesicles and Micelles. At approximately pH 7, a drop of $PMPC_{25}$-*b*-$PDMA_{23}$-*b*-$PDPA_{136}$ vesicles solution was dropped onto a cold TEM grid and the dominant vesicular self-assemblies were found by TEM, as shown in Figure 4. The number-averaged mean diameter is 147 ± 43 nm, which is consistent with the hydrodynamic diameter of the same vesicles measured by DLS (164 nm, see Figure 3C). The average PDPA membrane thickness is 20.0 ± 4.0 nm.

TEM images of $PMPC_{25}$-*b*-$PDMA_{17}$-*b*-$PDPA_{37}$ triblock copolymer micelles are shown in Figure 5. No vesicles were found from this image. The number-averaged mean diameter of micelles by TEM is 33.9 ± 5.8 nm, which is reasonably consistent with the intensity-averaged diameter by DLS, 36.7 nm (Figure 3A).

2.4. Controlling Trigger Points of pH-Responsive Block Copolymer Vesicles. First, the length of PDPA block significantly affects the trigger points of association/dissociation. The longer the PDPA block, the lower the triggering pH (Polymers **1.1**−**1.3** in Table 1). Second, the

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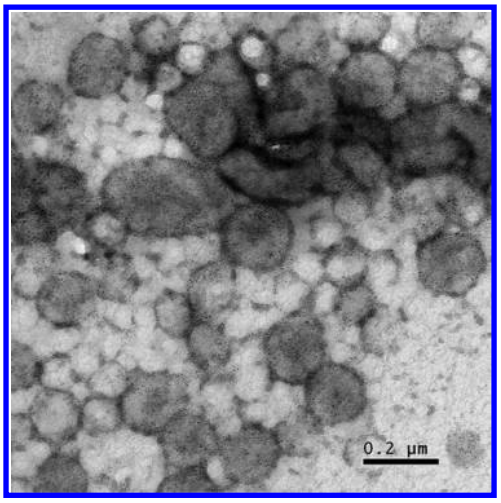

Figure 4. TEM images of $PMPC_{25}$-*b*-$PDMA_{23}$-*b*-$PDPA_{136}$ copolymer vesicles without staining. The number-averaged mean vesicle diameter and membrane thickness are 147 ± 43 nm and 20.0 ± 4.0 nm, respectively.

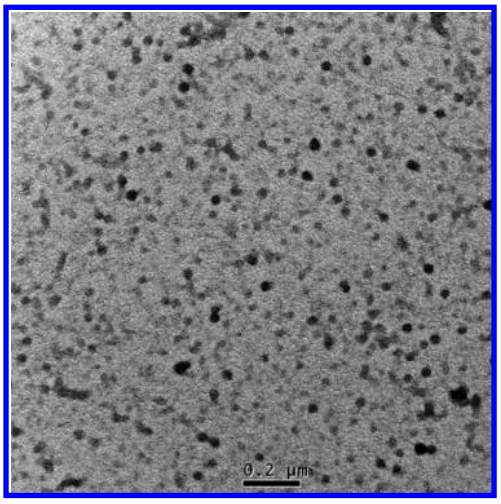

Figure 5. TEM images of $PMPC_{25}$-*b*-$PDMA_{17}$-*b*-$PDPA_{37}$ triblock copolymer micelles stained by ammonium heptamolybdate. The number-averaged mean diameter is 33.9 ± 5.8 nm.

incorporation of PDMA block at different positions also influences the pH trigger points. As shown in Table 1, the trigger points of polymers 2 and 3 are pH 6.55 and pH 6.32, respectively. We also found that the initial concentration affects the trigger points. For polymer **1.1**, when the initial concentrations are 1.0 and 10 mg/mL, the trigger points are pH 6.2 and pH 5.5, respectively. No precipitates and large amount precipitates were found at pH 5.8 at both initial concentrations. This demonstrated that by varying block copolymer composition, block sequence, and initial copolymer concentration, the trigger points can be tuned.

2.5. Cross-linking of PMPC-*b*-PDMA-*b*-PDPA Triblock Copolymer Vesicles. The middle shell of vesicles, PDMA, can be cross-linked at room temperature by adding BIEE into aqueous vesicle solution. Figure S6, Supporting Information, shows TEM images of BIEE-cross-linked vesicles (The molar ratio of BIEE to DMA is 1.2: 1). Theoretically, one BIEE molecules can react with two DMA units. At room temperature, this quaternization reaction is only selectively occurred between BIEE and DMA, rather than DPA or MPC.[36]

To decrease the experimental error, BIEE was first dissolved in methanol to get a 2.00 vol % stock solution. Then different amount of BIEE methanol solution was added into block copolymer vesicle solution. The cross-linked vesicles were then dissolved in D_2O/H_2O at pH 2 to evaluate the degree of cross-linking by ^1H NMR. The results are summarized in Table 2.

Table 2. Cross-Linking of Triblock Copolymer Vesicles by BIEE[a]

entry	molar ratio of BIEE to DMA	reappearance of DPA % at pH 2	reappearance of DMA % at pH 2
1	1.20:1	68	55
2	0.50:1	86	80
3	0.25:1	100	100

[a]Final $PMPC_{25}$-*b*-$PDMA_{23}$-*b*-$PDPA_{136}$ copolymer concentration in water is 0.067%. Reaction time: 4 days.

Upon cross-linking, the vesicles should be robust at acid conditions. DLS studies (Figure 6 and Figure S4, Supporting Information) revealed that the vesicle size at pH 7 did not change before and after BIEE-cross-linking. At pH 2, the uncross-linked vesicles would be dissociated into molecularly dissolved copolymer chains. However, BIEE-cross-linked vesicles are still colloidally stable at pH 2. There are trimodal peaks appeared at pH 2, as a result of partial deprotonation of PDPA vesicle membrane at acid conditions. The appearance of a small peak is due to the dissociation of some bigger vesicles into smaller particles. The middle peak indicated the presence of cross-linked vesicles without obvious change in size. The observation of microsize peak may be some aggregated vesicles and/or ill-defined particles due to the deformation of vesicles at low pH. This is particularly interesting because there are some "nanogates" in the vesicle membrane, which can be opened or closed under acidic or basic conditions. This will be discussed in the following ^1H NMR studies (Figure 7).

For example, $PMPC_{25}$-*b*-$PDMA_{23}$-*b*-$PDPA_{136}$ triblock copolymers are molecularly dissolved in acid water (Figure 7A). After cross-linking at pH 7, PMPC signals are still visible while

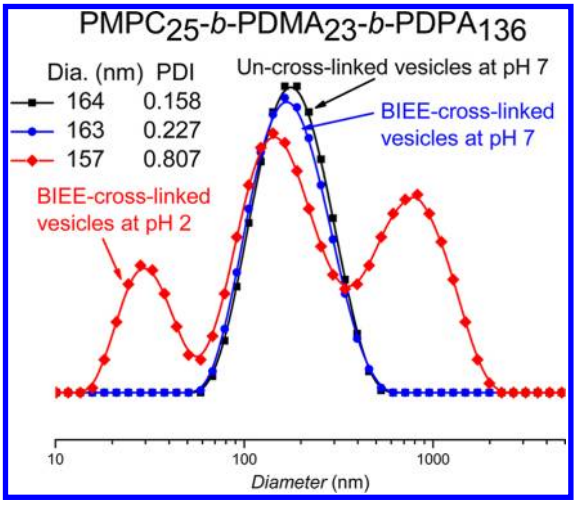

Figure 6. DLS study on BIEE-cross-linked vesicles at acidic and neutral conditions. See Figure S4, Supporting Information, for corresponding correlation functions. The molar ratio of BIEE to DMA is 1.2:1 and the reaction time of BIEE cross-linking is 4 days (entry 1 in Table 2).

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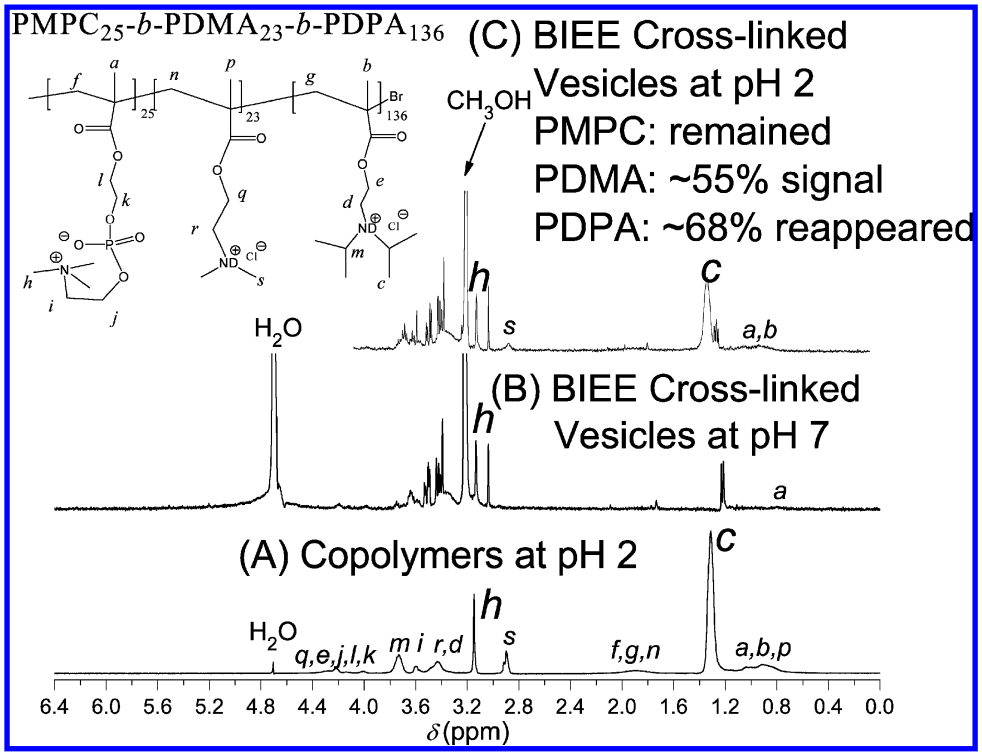

Figure 7. Assigned ^{1}H NMR spectra of $PMPC_{25}$-*b*-$PDMA_{23}$-*b*-$PDPA_{136}$ triblock copolymers at pH 2 and BIEE-cross-linked copolymer vesicles at different pH.

PDPA signals disappeared completely (Figure 7B). The cross-linked vesicles at pH 7 were then switched to pH 2. As shown in Figure 7C, ~55% PDMA (peak *s*, protonation by HCl and quaternization by BIEE) and ~68% PDPA (peak *c*, protonated by HCl) reappeared by comparing with PMPC (peak *h*). This indicated that when the molar ratio of BIEE to PDMA is 1.2: 1, the vesicles were slightly cross-linked. However, the vesicles are not destroyed at acidic condition, as detected by DLS (Figure 6).

As shown in Table 2, when the molar ratio of BIEE to DMA was decreased to 0.5:1 (theoretically corresponding to a 100% quaternization of PDMA), ~86% PDPA and 80% PDMA reappeared at pH 2, indicating a less degree of cross-linking. When the molar ratio was further decreased to 0.25: 1, both PDPA and PDMA were 100% reappeared at pH 2, indicating the unsuccessful cross-linking of vesicles.

$PMPC_{25}$-*b*-$PDPA_{107}$-*b*-$PDMA_{14}$ and $PMPC_{25}$-*b*-P(DPA_{115}-*stat*-DMA_{21}) block copolymer vesicles were also cross-linked by BIEE at a molar ratio of BIEE to DMA of 1.2:1. The ^{1}H NMR studies were shown in Figure S1 and Figure S2, Supporting Information, respectively. For $PMPC_{25}$-*b*-$PDPA_{107}$-*b*-$PDMA_{14}$ copolymer vesicles, ~45% of PDMA and ~81% PDPA reappeared at pH 2. For $PMPC_{25}$-*b*-P(DPA_{115}-*stat*-DMA_{21}), the percentages were ~62% and ~76%.

The above results revealed that the cross-linking degree of vesicles can be tuned by the feed ratio of BIEE to copolymer vesicles. This also provided a possibility to tune the permeability and stability of vesicle membrane at low pH.

2.6. ζ-Potential Studies on Vesicles. ζ-Potential studies on the vesicles are shown in Figure 8 to roughly confirm the structure of vesicles made from different type of block copolymers.

At lower pH, the vesicle was positively charged because the PDMA residues were protonated. At higher pH, the vesicles are

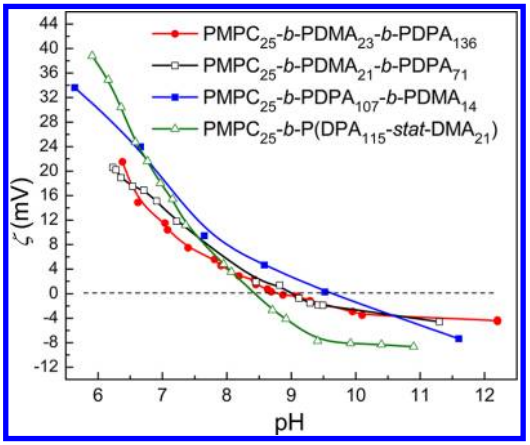

Figure 8. Variation of ζ potential of block copolymer and vesicles in water as a function of pH.

negatively charged, which was considered as absorption of OH^- anions on the particle surface.[37−39]

As shown in Figure 8, $PMPC_{25}$-*b*-P(DPA_{115}-*stat*-DMA_{21}) copolymer vesicles have the lowest isoelectric point (IEP) at around pH 8.5 because the cationic DMA chains are located in the vesicle membrane (see Scheme 1). $PMPC_{25}$-*b*-$PDPA_{107}$-*b*-$PDMA_{14}$ vesicles have the highest IEP at ~9.5 because part of PDMA chains comprises the outside vesicle corona. The IEPs of Two PMPC-*b*-PDMA-*b*-PDPA triblock copolymer vesicles are reasonably at ~pH 9.0 because the PDMA chains comprise the middle corona of vesicles. This also suggested that for $PMPC_{25}$-*b*-$PDPA_{107}$-*b*-$PDMA_{14}$ block copolymer vesicles, at least part of $PDMA_{14}$ chains are expressed outward.

2.7. Drug Delivery Studies. Doxorubicin (DOX) is a water-soluble anticancer drug in its hydrochloride salt form. DOX encapsulation/release profiles obtained for the $PMPC_{25}$-

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b-PDPA$_{107}$-*b*-PDMA$_{14}$ vesicles are reported in Figure 9. The drug loading content was estimated to be 6.7% relative to the

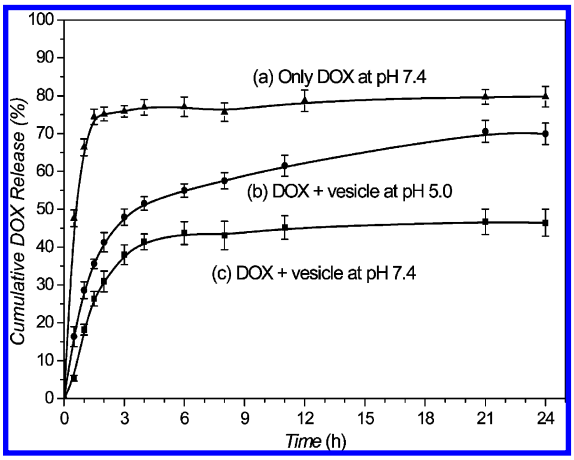

Figure 9. Cumulative release profile of DOX-loaded PMPC$_{25}$-*b*-PDPA$_{107}$-*b*-PDMA$_{14}$ vesicles at 37 °C: (a) only DOX at pH 7.4; (b) DOX-loaded vesicles at pH 5.0; (c) DOX-loaded vesicles at pH 7.4.

polymer vesicles, which is closed to our reported results (6.5%) based on PMPC-*b*-poly(ε-caprolactone) vesicles.[40] This value may be improved by increasing the feeding DOX.[40]

The drug loading efficiency was approximately 33.6%. Data (triangles) obtained for a control experiment utilizing an aqueous solution of 0.035 mg/mL DOX in the absence of any vesicles indicated rapid drug elution, as expected. The kinetic release profiles of drug-loaded PMPC$_{25}$-*b*-PDPA$_{107}$-*b*-PDMA$_{14}$ copolymer vesicle solution were carried out in 0.01 M tris buffer at 37 °C and pH 7.4 and pH 5.0, respectively. As shown in Figure 9, after 24 h, the DOX release content was nearly 25% higher in the surrounding of tris buffer solution at pH 5.0 compared to the same DOX-loaded vesicles but kept at pH 7.4. In the controlled experiment the DOX release percentage was about 75% after 1.5 h. The release rates under those conditions indicated significantly retarded release of the drug at pH 7.4 due to its entrapment within the vesicles (see profiles a and c), suggesting that pH-triggered disassembly of vesicles were dominant drug release mechanism (see profiles b and c).

3. EXPERIMENTAL SECTION

3.1. Materials. MPC (>99%) was kindly donated by Biocompatibles, UK. DPA was purchased from Scientific Polymer Products (USA). DMA (98%), copper(I) bromide (CuBr; 99.999%), 2,2-bipyridine (bpy), methanol and 2-propanol were purchased from Aldrich and were used as received. The silica used for removal of the ATRP copper catalyst was column chromatography grade silica gel 60 (0.063−0.200 mm) purchased from E. Merck (Darmstadt, Germany). Doxorubicin hydrochloride (DOX·HCl; 98%) was purchased from Xingcheng Chempharm Co., Ltd., China. (Hydroxymethyl)amino methane was purchased from Aladdin Chemistry Co., Ltd., China. 2-(*N*-Morpholino)ethyl 2-bromo-2-methylpropanoate (ME-Br) initiator was synthesized according to a previously reported procedure.[41]

3.2. Characterization. The M_n and M_w/M_n values of the three PMPC-*b*-PDMA-*b*-PDPA triblock copolymers were assessed by gel permeation chromatography (GPC). The GPC setup comprised a Polymer Laboratories PLgel 5 μm Mixed 'C' column operating at 40 °C in combination with a refractive index detector. The eluent was a 3:1 chloroform: methanol mixture at a flow rate of 1.0 mL min^{-1} and calibration was carried out using five near-monodisperse poly(methyl methacrylate) standards. The data were processed by Cirrus GPC offline GPC/SEC software.

^{1}H NMR spectra were recorded on a Bruker AV400 (400 MHz) spectrometers at ambient temperature, using either D_2O/DCl, D_2O/H_2O/DCl or D_2O/NaOD as solvents. The water suppression software option was selected when D_2O/H_2O/DCl was used as solvent.

TEM images were obtained by using Philips CM100 electron microscope operated at 100 kV. It is equipped with a LaB6 gun and a Gatan 1K × 1K digital camera. To prepare TEM samples, 10 μL of vesicle solution was dropped onto a carbon-coated copper grid and then absorb the residual solution by filter paper, with the water film evaporating in air.

DLS studies were carried out on a ZETA SIZER Nano series instrument (Malvern Instruments). The data were processed by cumulants analysis of the experimental correlation function and vesicle diameters were calculated from the computed diffusion coefficients using the Stokes−Einstein equation.

ζ-Potential studies were done at 25 °C on a ZETA SIZER Nano series instrument (Malvern Instruments) equipped with a multi-purpose titrator (MPT-2). The software is Dispersion Technology Software (version 5.03).

3.3. Synthesis of PMPC-*b*-PDMA-*b*-PDPA Triblock Copolymer by ATRP. In a typical ATRP procedure, a Schlenk flask with a magnetic stir bar and a rubber septum was charged with MPC monomer (2.085 g, 7.050 mmol), ME-Br initiator (0.0790 g, 0.2820 mmol), bpy ligand (0.0881 g, 0.5640 mmol), and methanol (3.0 mL). This solution was deoxygenated by bubbling N_2 for 30 min before adding Cu^{I}Br catalyst (0.0405 g, 0.2820 mmol) into the flask. The [MPC]:[ME-Br]:[CuBr]:[bpy] relative molar ratios were 25:1:1:2. The reaction was carried out under a nitrogen atmosphere at 20 °C. After 40 min, deoxygenated DMA (1.357 g, 8.460 mmol) and methanol (2.0 mL) mixture was injected into the flask and reacted for 3 h. Then deoxygenated DPA (7.820 g, 36.70 mmol), and methanol (9.0 mL) mixture were injected into the flask and reacted for 48 h. Finally, the reaction solution was diluted by addition of 2-propanol (about 200 mL) and then passed through a silica column to remove the catalyst. The solvent was removed by rotatory evaporator. The copolymer was then washed by acetonitrile for at least 6 times within 3 days and dried in a vacuum oven at 50 °C for 2 days. ^{1}H NMR study confirmed all the residue monomers were removed.

3.4. Synthesis of PMPC-*b*-P(DMA-*stat*-DPA) Block-Statistic Copolymer by ATRP. In a typical ATRP procedure, a Schlenk flask with a magnetic stir bar and a rubber septum was charged with MPC monomer (1.317 g, 4.460 mmol), ME-Br initiator (0.0500 g, 0.1780 mmol), bpy ligand (0.0558 g, 0.3570 mmol), and methanol (2.5 mL). This solution was deoxygenated by bubbling N_2 for 30 min before adding Cu^{I}Br catalyst (0.0256 g, 0.1780 mmol) into the flask. The [MPC]:[ME-Br]:[CuBr]:[bpy] relative molar ratios were 25:1:1:2. The reaction was carried out under a nitrogen atmosphere at 20 °C. After 100 min, deoxygenated DPA (4.569 g, 21.40 mmol), DMA (0.8589 g, 5.35 mmol) and methanol (6 mL) mixture were injected into the flask. After 4 days, the reaction solution was diluted by addition of 2-propanol (about 200 mL) and then passed through a silica column to remove the catalyst, removed the solvent by rotatory evaporator, washed the copolymer by acetonitrile for 5 times, dried in a vacuum oven at 50 °C for 2 days.

3.5. Synthesis of PMPC-*b*-PDPA-*b*-PDMA Triblock Copolymer by ATRP. In a typical ATRP procedure, a Schlenk flask with a magnetic stir bar and a rubber septum was charged with MPC monomer (1.317 g, 4.460 mmol), ME-Br initiator (0.0500 g, 0.1780 mmol), bpy ligand (0.0558 g, 0.3570 mmol), and methanol (2.5 mL). This solution was deoxygenated by bubbling N_2 for 30 min before adding Cu^{I}Br catalyst (0.0256 g, 0.1780 mmol) into the flask. The [MPC]:[ME-Br]:[CuBr]:[bpy] relative molar ratios were 25:1:1:2. The reaction was carried out under a nitrogen atmosphere at 20 °C. To After 80 min, deoxygenated DPA (4.569 g, 21.40 mmol), and methanol (6 mL) mixture were injected into the flask and reacted for 42 h. Then the deoxygenated DMA (0.8589 g, 5.35 mmol) and methanol (1 mL) mixture was injected into the flask and reacted for 25 h. Finally, the reaction solution was diluted by addition of 2-propanol (about 200 mL) and then passed through a silica column to remove the catalyst, the solvent removed by rotatory evaporator, and the

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copolymer washed by acetonitrile for 6 times within 3 days and dried in a vacuum oven at 50 °C for 2 days.

3.6. Preparation of Vesicles. The following protocol was adopted. $PMPC_{25}$-*b*-$PDMA_{23}$-*b*-$PDPA_{136}$ triblock copolymers (40.00 mg) was dissolved into dilute aqueous HCl (pH 2; 50.00 g; the initial copolymer concentration was 0.08%) and stirred for at least 1 h. Then the pH was slowly adjusted over a 20 min period from pH 2 to the trigger points (pH 6.2) using 0.10 M aqueous NaOH and then from pH 6.4 to pH 7.0 using 0.01 M aqueous NaOH over the same time scale. The final copolymer concentration after pH adjustment was calculated by the final weight of solution. The process should be gentle to avoid the polymer precipitation, especially for copolymers with longer PDPA block. For larger scale of vesicle preparation (e.g., 300 mg copolymer), only 0.10 M aqueous NaOH was used to adjust the pH. When the initial copolymer concentration was below 0.05 wt %, a faster dropping rate of NaOH water was used because no precipitate problem was observed. Different copolymers have different triggering points, which can be easily recorded according to the plateau of solution pH against added the volume of added NaOH water.

The procedure for preparing $PMPC_{25}$-*b*-$PDMA_{17}$-*b*-$PDPA_{37}$ micelles is similar to the preparation of polymer vesicles.

3.7. Cross-Linking of Vesicles by BIEE. At first, BIEE (40.0 μL) was dissolved in methanol (2.00 mL) to get 2.00 vol % stock solution. Then, BIEE stock solution (8.00 μL, 9.18×10^{-7} mol) was added into $PMPC_{25}$-*b*-$PDMA_{21}$-*b*-$PDPA_{136}$ copolymer vesicle solution (0.067 wt % copolymer concentration, 2.00 g, 7.65×10^{-7} mol DMA unit, pH 7.0). The molar ratio of BIEE to DMA unit was 1.2:1. When the ratio is 0.5:1, 8.00 g of vesicle solution and 13.4 μL of BIEE stock solution were used. When the ratio is 0.25:1, 12.0 g of vesicle solution and 10.0 μL of BIEE stock solution was used. The solutions were reacted for 4 days at room temperature. For the ^{1}H NMR studies, either D_2O (at pH 7) or D_2O/DCl (at pH 2) was directly mixed with the above H_2O vesicle solution. The water suppression software option was selected during ^{1}H NMR measurement.

3.8. Drug Loading and Release of $PMPC_{25}$-*b*-$PDPA_{107}$-*b*-$PDMA_{14}$ Vesicles. The controlled release of drug was achieved according to the following protocol. 15.0 mg $PMPC_{25}$-*b*-$PDPA_{107}$-*b*-$PDMA_{14}$ copolymer was dissolved in 10.0 mL fresh DOX·HCl solution (ca. 0.3 mg/mL; pH 1.75). Then this polymer/DOX mixture solution was adjusted to pH 7.4 by using 0.05 M, 0.005 M and 0.0005 M aqueous NaOH solutions to form DOX-loaded polymer vesicles by stirring for 12 h. The final volume of the solution was 35.2 mL. The unloaded free drug was removed by dialysis using a dialysis tube (cutoff M_n = 8000−14000) according to a reported procedure.[40] The dialysis tube was immersed in 1000 mL tris buffer (0.01 M; pH 7.4) and dialyzed at 20 °C with 300 r/min of stirring (in a 1000 mL beaker). Fresh tris buffer (0.01 M; pH 7.4) was renewed for 5 times in 2.5 h (0.5 h each). Fluorescence spectroscopy before and after dialysis were measured to calculate drug loading efficiency. The vesicle/DOX mixture after removing free drug was divided into six parts, and immediately transferred into a new dialysis tube (cutoff M_n = 8000−14000). The final drug release process was carried out by dialyzing 5.0 mL of DOX-loaded vesicles in the dialysis tube against 100 mL tris buffer (0.01 M; pH 7.4 or pH 5.0) in a beaker (250 mL), at 37 °C and 190 r/min of stirring rate. The volume of liquid in the beaker (outside of the dialysis tube) was ensured around 100 mL during the measurement. At different time intervals, the liquid in the beaker were measured with fluorescence spectroscopy (excitation at 461 nm and emission at 591 nm) and the cumulative release curve of DOX was obtained. The calibration curve was shown in Figure S6, Supporting Information. The drug loading content (DLC) and drug loading efficiency (DLS) were calculated according to the following equation.[40,42]

$$\begin{aligned}\text{DLC (\%)} &= (\text{weight of drug encapsulated in vesicles}\\ &\quad /\text{weight of polymer}) \times 100\%\\ &= 1.0084\ \text{mg}/15.0\ \text{mg} \times 100\%\\ &= 6.70\%\end{aligned}$$

$$\begin{aligned}\text{DLE (\%)} &= (\text{weight of drug encapsulated in vesicles}\\ &\quad /\text{weight of drug in feed}) \times 100\%\\ &= 1.0084\ \text{mg}/3.0\ \text{mg} \times 100\%\\ &= 33.6\%\end{aligned}$$

4. CONCLUSION

In summary, a new class of ABC, ACB triblock copolymers and A(B+C) diblock-statistical copolymer with various compositions and sequences were synthesized by ATRP in methanol at room temperature. DLS, NMR and TEM studies revealed that those block copolymers with relatively long PDPA block can form polymer vesicles whereas the copolymer with relatively short PDPA block can form polymer micelles in water. The vesicles formed by PMPC-*b*-PDMA-*b*-PDPA, PMPC-*b*-PDPA-*b*-PDMA and PMPC-*b*-P(DMA-*stat*-DPA) block copolymers have various IEPs and pH trigger points by changing the block copolymer sequence and composition. Furthermore, the reactive PDMA chains can be incorporated into the vesicle membrane, or serve as vesicle corona, or the middle shell, which provides an effective strategy regarding the immobilization of vesicles by selective quaternization of PDMA with BIEE. The degree of cross-linking can be tuned by varying the molar ratio of PDMA to BIEE. ^{1}H NMR and DLS studies revealed the PDPA membrane of cross-linked vesicles can be opened at low pH and closed at high pH, suggesting the possibility for tuning of permeability of cross-linked vesicle membrane. Moreover, these vesicles are authentic polymeric analogues of conventional surfactant-based liposomes and have biomedical applications as nanosized drug delivery vehicles, showing obvious retarded drug delivery property.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figures S1−S6, showing ^{1}H NMR spectra, correlation functions of DLS study, TEM images, and a calibration curve. 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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