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# Preparation, characterization and drug release behavior of polyion complex micelles

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ABSTRACT

Double-hydrophilic block copolymer composed of poly(N-vinylpyrrolidone) (PVP) and poly(styrene-alter-maleic anhydride) (PSMA) has been synthesized by reversible addition-fragmentation chain transfer (RAFT) polymerization. Poly(N-vinylpyrrolidone)-block-poly(styrene-alter-maleic anhydride) (PVP-b-PSMA) thus formed was characterized by gel permeation chromatography (GPC), $^1$H nuclear magnetic resonance ($^1$H NMR) spectroscopy and FTIR spectroscopy. In acid solution, this block copolymer spontaneously formed polyion complex (PIC) micelles with a cationic polyelectrolyte, chitosan. The PSMA/chitosan polyelectrolyte complex formed an inner core while PVP chains surrounded it as a shell. Transmission electron micrographs (TEMs) and dynamic light scattering (DLS) showed the PIC micelles to be spherically shaped, with mean hydrodynamic diameter around 146 nm. The model drug coenzyme A (CoA) was loaded into the micelles and the in vitro drug release behavior was investigated. We found that by manipulating the pH value and salt concentration of the release solution, it was possible to control the releasing rate of CoA.



## 1. Introduction

During the last two decades, polymeric micelles with a core–shell structure have received considerable attention due to their potential applications in the field of drug delivery (Kataoka et al., 2001; Torchilin, 2001; Nishiyama and Kataoka, 2006). Initially, polymeric micelles were formed mainly by the self-assembly of amphiphilic block copolymers (Riess, 2003). Surrounded by a hydrophilic outer shell, the inner core is formed by hydrophobic polymer segments, which can incorporate various hydrophobic drugs. Beside hydrophobic interactions, electrostatic interaction between two oppositely charged polyelectrolytes can also allow the formation of polymeric micelles, which are termed "polyion complex (PIC) micelles" (Harada and Kataoka, 1995; Oh et al., 2006; Adams et al., 2008). This system has been successfully used for the delivery of plasmid DNA (Itaka et al., 2003; Miyata et al., 2004; Wakebayashi et al., 2004), proteins (Jaturanpinyo et al., 2004; Mustafaev, 2004), heparin (Dufresne and Leroux, 2004; Yang et al., 2006) and oligonucleotides (Jeong et al., 2003; Kim et al., 2007). As with conventional micelles, the PIC micelles possess many advantages such as a simple preparation, the good structural stability, high drug loading capacity, prolonged circulation in the blood, targeted delivery, low toxicity, etc. (Nishiyama et al., 2003; Tian et al., 2005; Husseini and Pitt, 2008). In addition, PIC micelles have their unique characteristics. For example, the micelles can encapsulate a variety of therapeutic agents such as hydrophobic compounds, hydrophilic compounds, metal complexes, and charged macromolecules; the preparation of micelles are carried out in aqueous without introducing any organic solutions, which can eliminate the side-effect caused by residual solvents.

Chitosan (CS) is naturally originated and characterized as non-toxic biomaterials with good biocompatibility (Amornchai et al., 2004), which is found in several insects and fungi, particularly in crab shell and lobsters. It contains a large number of hydroxyl and amino groups, and the primary amine groups render special properties that make CS very useful in pharmaceutical applications, such as, cancer treatment, wound dressing, contact lenses or controlled drug release (Agnihotri et al., 2004; Guo et al., 2007; Li et al., 2007). At relatively low pH (<6.5), CS is water-soluble and displays a polycationic character because of the protonation of amino groups ($pK_a$ value equal to 6.5) (Rinaudo et al., 1999).

Poly(N-vinylpyrrolidone) (PVP) is a typical neutral water-soluble polymer. Since its discovery in the 1930s, the unique feature of PVP has been attractive in the chemical, pharmaceutical, and material fields because of the properties including the solubility in water and organic solvents, very low toxicity, good biocompatibility and high complexation ability (Wan et al., 2005; Bartolozzi et al., 2007; Lu et al., 2007).

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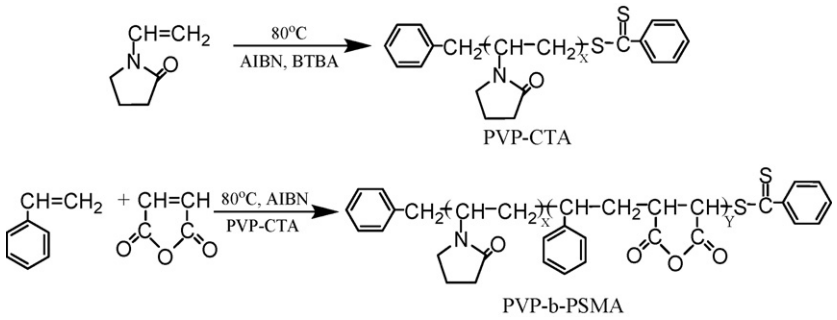



**Scheme 1.** Synthesis scheme of PVP-b-PSMA block copolymer.

In this study, we synthesized the new double-hydrophilic block copolymer poly(N-vinylpyrrolidone)-block-poly(styrene-alter-maleic anhydride) (PVP-b-PSMA) by PVP as hydrophilic nonionic segment. Mixing of PVP-b-PSMA with CS in acetate buffer led to the spontaneous formation of PIC micelles. The micelles have a spherical shape with core–shell architecture, in which the core of polyion complexes formed from negatively charged PSMA segments and positively charged CS is surrounded by the shell of nonionic and hydrophilic PVP segments. In order to access the PIC micelles' applications in the controlled release of water-soluble drugs with charge or potential ion-functional group, CoA was chosen as a model drug and encapsulated into the PIC micelles. The release behavior of CoA from micelles was also investigated at different experimental conditions in this paper. The results suggest that the PIC micelles have great potential in drug delivery systems.

## 2. Materials and methods

### 2.1. Materials

Chitosan (CS) was obtained from Tokyo Kasei Kogyo Co., Ltd. and the degree of deacetylation was 0.85. Styrene (St) was supplied by Tianjin number one Chemical Reagent Factory. Maleic anhydride (MAn) was obtained from China National Pharmaceutical Group Shanghai Chemical Reagents Co. and used as received. N-vinylpyrrolidone (VP) was purchased from Fine Chemical Industry of Nankai University (Tianjin, China) and freshly distilled with reduced pressure before use. S-Benzyl dithiobenzoate (BTBA) was synthesized and purified according to the methods reported in the literature (Chong et al., 2003; Davies et al., 2005). Coenzyme A (CoA) was purchased from Aldrich. All other chemicals used were of analytical grade, without further purification.

### 2.2. Preparation of PVP-b-PSMA

The block copolymer PVP-b-PSMA was synthesized by a reversible addition-fragmentation transfer (RAFT) polymerization using AIBN as an initiator and BTBA as a chain transfer regent (CTA). The synthesis of the block copolymer is shown in Scheme 1. In brief, 1.4 g VP (12.6 mmol), 0.022 mmol of BTBA and 0.0035 g AIBN (0.021 mmol) were dissolved in 1,4-dioxane and deoxygenated by nitrogen gas for 30 min. After reacting at 80 °C for 4 h, the solutions were quenched in ice water to stop the polymerization. The resulting homopolymer (PVP-CTA) were recovered by precipitation in diethyl ether. The second block of PSMA was prepared from 1.1 g macroRAFT agent (PVP-CAT) and 0.033 mol of St/MAn mixture (1:1, mol/mol) in analogy to PVP-CTA. The copolymer was precipitated into methanol and dried in vacuo up to constant weight.

### 2.3. FTIR characterization

Infrared spectrum was recorded using an AVATAR-360FTIR spectrometer by incorporating samples in KBr disks.

### 2.4. $^1$H NMR characterization

$^1$H NMR spectrum was obtained on a Bruker ARX400 nuclear magnetic resonance spectrometer at 400 MHZ using DMSO as the solvent.

### 2.5. GPC measurements

The molecular weights of PVP-CTA and PVP-b-PSMA were determined by gel permeation chromatography (GPC; Wyatt Technology). GPC analyses were performed in THF, using monodispersed polystyrene standards for calibration.

### 2.6. Preparation of polyion complex micelles and drug-loaded micelles

Given amounts of PVP-b-PSMA and chitosan were dissolved in acetate buffer pH 4.65 to polymer concentration of 0.2 mg/ml. These solutions were filtered through a medium pore sintered glass to remove dust. Polyion complex micelles were then prepared by mixing these solutions in a certain volume ratio. Thereafter, the micelles were incubated for 48 h in a water bath set at 37 °C to stabilize the complexes.

The drug-loaded micelles were preformed by using CoA as water-soluble drug in the same method as previously but by replacing PVP-b-PSMA solution with PVP-b-PSMA/CoA mixture, in which the concentration of CoA was 0.36 mg/ml. After incubation in a 37 °C water bath for 48 h, the solution was put into a dialysis bag with MWCO of 8000–14,000 and subjected to dialysis against 30 ml of acetate buffer (pH 4.65) to remove the non-encapsulated drug. The amount of non-capsulated drug in the dialysate was analyzed by UV–visible spectrophotometer (UV-540, US) at 260 nm, using a standard calibration curve experimentally obtained with CoA/water solutions. The drug entrapment efficiency (EE) (Hu et al., 2006) was then calculated based on the following formula:

$$\mathrm{EE}\,(\%) = \left(\frac{\text{the total charged amount of CoA} - \text{the amount of non-encapsulated CoA}}{\text{the total charged amount of CoA}}\right) \times 100 \quad (1)$$

### 2.7. Dynamic light scattering (DLS) measurements

The average size and distribution of PIC micelles were evaluated by DLS measured at 25 °C, using a ZetaSizer Nano-ZS90 spectrogoniometer (Malvern Instrument, Worcs, UK). The measurements

were carried out with a detection angle of 90°. Prior to measurements, the micelles solution (0.2 mg/ml) was passed through a 0.45 μm pore size filter.

### 2.8. Transmission electron microscope (TEM) observation

TEM (JEM-100CX II, Japan) was used to observe the morphology of micelles. A drop of freshly prepared micelles solution was placed on a carbon-coated copper grid and dried at room temperature. The specimen on the copper grid was not stained. Observation was done at 80 kV.

### 2.9. In vitro drug release

The release of CoA from micelles was also followed using UV–vis spectroscopy. After dialysis, the drug-loaded micelle solutions in dialysis bags were directly immersed into 100 ml solutions with different pH value and salt concentration. Aliquots of 5.0 ml were withdrawn from the solution periodically and immediately replaced by an equal volume of corresponding solution after each sampling. The amount of CoA release from micelles was measured using UV absorbance at 260 nm.

## 3. Results and discussion

### 3.1. Synthesis of PVP-b-PSMA

In this study, PVP-b-PSMA was synthesized by RAFT-mediated polymerization, using BTBA as RAFT agent. Shown in Fig. 1 are the FTIR spectra of PVP-CTA and PVP-b-PSMA. According to curve a, Homopolymer PVP is characterized by the stretching vibration band of C=O (imide) at 1674 $cm^{-1}$. It is seen that this band can also be found in curve b, which indicates that the geometry of PVP is preserved inside the copolymer. In addition, FTIR bands at 1858 and 1784 $cm^{-1}$ are the characteristic bands of PSMA, which corresponds to in-phase and out-of-phase stretching of the carbonyl groups in the five-member ring of maleic anhydride (Tenhaeff and Gleason, 2007). The signals at 1600, 1493, 1455 and 707 $cm^{-1}$ are due to the $\nu_{C-C}$ of the benzyl ring. Fig. 2 shows $^1$H NMR spectrum of PVP-b-PSMA in DMSO. The characteristic peaks of St and anhydride were observed at 7.1 ppm for five protons of the phenyl ring and 3.7 ppm, respectively. The characteristic signals of PVP include: the methine proton of the PVP backbone at 3.7 ppm, the methylene protons adjacent to the nitrogen atom at 3.3 ppm. The simultaneous appearance of the resonance characteristic of PVP and PSMA protons indicates that the resulting product combines the

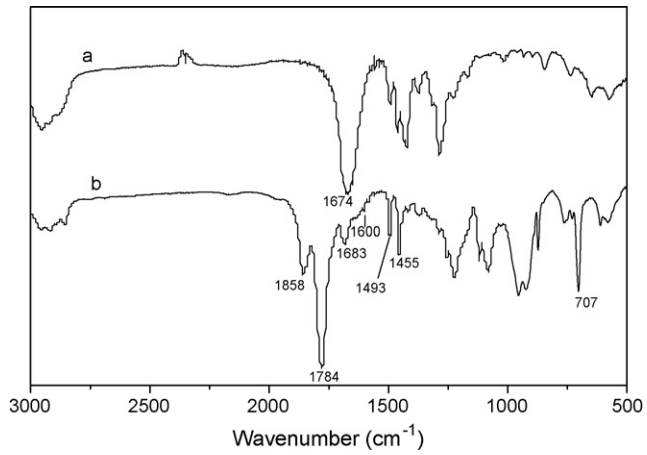



**Fig. 1.** The FTIR spectra of the PVP-CTA (a) and PVP-b-PSMA (b).

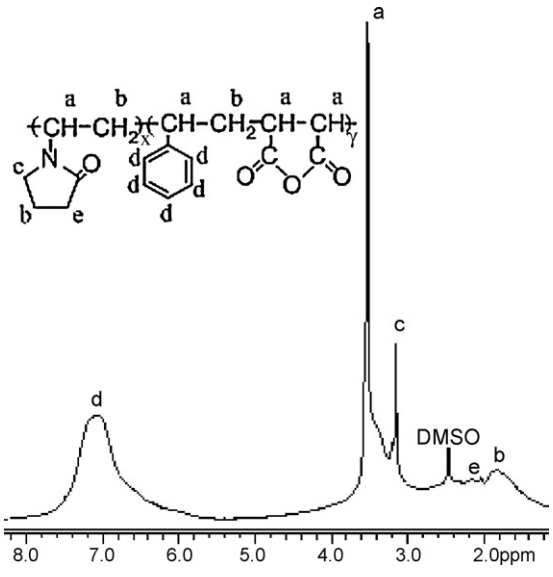



**Fig. 2.** $^1$H NMR spectra of PVP-b-PSMA in DMSO (temperature: 25 °C).

structural features of PVP and PSMA, i.e., the PVP-b-PSMA block copolymer was successfully obtained. Table 1 shows the GPC data of the obtained PVP-b-PSMA. It has a narrow molecular weight distribution, and the Mn was determined to be $1.53 \times 10^5$ g/mol with Mw/Mn = 1.32. From the molecular weight of PVP block (52,800) and PSMA block (153,000–52,800), we can calculate the degree of polymerization (DP) values of PVP and PAMPS blocks, which were 475 and 491, respectively. Therefore, the obtained block copolymer was denoted as $PVP_{475}$-b-$PSMA_{491}$.

### 3.2. Formation and characterization of PIC micelles

PIC micelles were prepared by simple and direct mixing of PVP-b-PSMA and chitosan solutions at a certain volume ratio in acetate buffer pH 4.65. This pH was elected to realize a condition where both the carboxylic groups in MAn and the amino groups in chitosan have an ionic form (Khanal et al., 2005). The micelle solution remained transparent for long storage times and no precipitation was observed, suggesting the high storing stability of PIC micelles. Fig. 3a shows the particle size distribution of PIC micelles based on number average by dynamic light scattering. It was clear that the prepared PIC micelle had a narrow size distribution with an average diameter of around 146 nm. The morphology of micelles was observed by transmission electron microscopy. As shown in Fig. 4a, the chitosan/PVP-b-PSMA micelles are well dispersed as individual nanoscaled micelles with regularly spherical shape, with the size of 90–120 nm. The micelle size determined by TEM is lower that measured by DLS, which is due to the shrinkage of the micelles during the drying process of TEM sample (Pan et al., 2007).

By the method described in the Section 2.6, the CoA loaded micelles were also prepared with a drug entrapment efficiency of 72.0%. The drug-loaded micelles showed a spherical morphology with a size of 120–160 nm as observed by TEM (Fig. 4b). As expected, the hydrodynamic diameter of the drug-loaded micelles (204 nm, Fig. 3b) determined by DLS was slightly larger than that measured by TEM. By comparison with the size of blank and drug-

**Table 1**
GPC data of PVP-CTA and block copolymer PVP-b-PSMA.

| Polymer | Mn ($\times 10^{-4}$) | Mw ($\times 10^{-4}$) | Mw/Mn |
|---|---|---|---|
| PVP-CTA | 5.28 | 6.60 | 1.25 |
| PVP-b-PSMA | 15.3 | 20.2 | 1.32 |

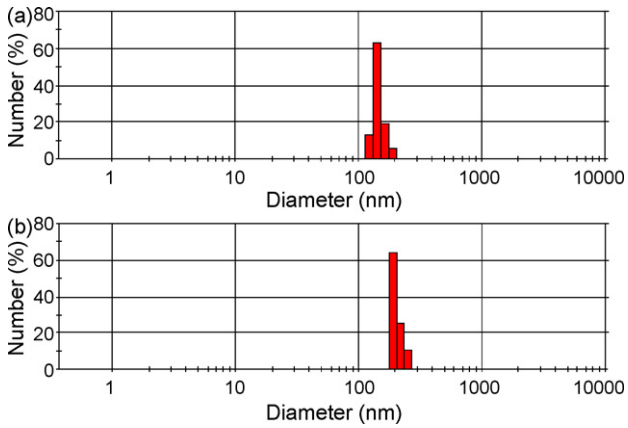



**Fig. 3.** The DLS results of chitosan/PVP-b-PSMA micelles (a) and CoA-loaded chitosan/PVP-b-PSMA micelles (b).

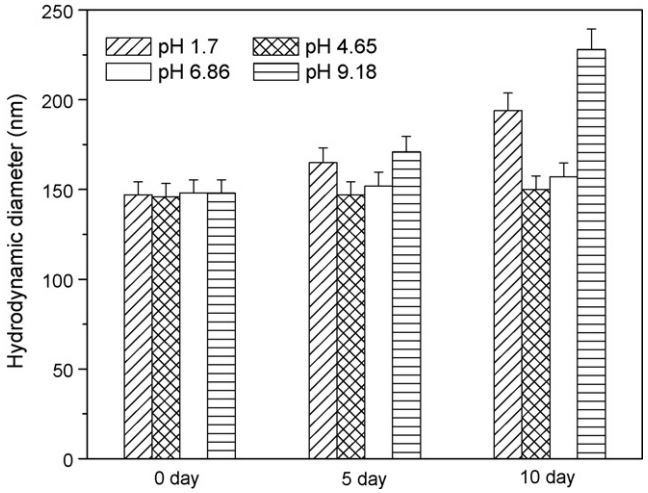



**Fig. 5.** Effect of pH and storage time on the particle size of PIC micelles prepared in acetate buffer pH 4.65.

loaded micelles, it appears that the average diameter of drug-loaded micelles is a little larger than that of blank micelles. This is because CoA was entrapped into the hydrophobic micelle cores by electrostatic and hydrogen bonding interactions, which will result in the small increase of micelle size.

Since pH will influence the ionization of chitosan and PVP-b-PSMA, it was therefore necessary to determine its influence on the micelles. After the PIC micelles were prepared in acetate buffer pH 4.65, the pH of the micellar solution was adjusted to desired values by addition of 0.1 M HCl or 0.1 M NaOH, which can avoid significant deviations in the micellar concentration and the ionic strength of medium. The chitosan/PVP-b-PSMA micelles at various pH values were characterized immediately after formation, and also after 5 and 10 days storage at room temperature. As shown in Fig. 5, for the freshly prepared micelles, the micellar size has no significant difference at all pHs tested. Ten days later, the particle size for micelles at pH 4.65 remains almost the same and there is a slight increase in the size of the micelles at pH 6.86; while the micelles at pH 1.7 and 9.18 increase their size sharply from 165 and 171 nm (5 days) to 194 and 228 nm (10 days), respectively. This could be explained by the different ionization state of chitosan and PVP-b-PSMA in different pH solutions. When the solution pH increases from 4.65 to 6.86 and 9.18, the $OH^-$ in solutions diffuse gradually into the micellar inner cores; the deprotonation degree of the carboxylic acid groups in MAn and the amino groups of chitosan both increase. This will weaken the electrostatic attraction between chitosan and PVP-b-PSMA and strengthen the repulsion between the de-protonated carboxylic acid. As a result, the micellar cores expand gradually and in a loose state, and so the micellar size increases. Similarly, when the solution pH decreases from 4.65 to 1.7, $H^+$ in solutions diffuses gradually into the micellar inner cores and the protonation degree of CS and MAn increases. The weakening of the electrostatic attraction between chitosan and PVP-b-PSMA and strengthening of the repulsion between the protonated CS will also make the micellar cores expand. So the micellar size increases at pH 1.7 accordingly. In addition, the counterion diffusion into the cores is increasing as time goes on and the effect of pH on the micellar size is dramatically. Based on the above results, the present pH-sensitive PIC micelles may be considered as candidates for controlled drug delivery systems.

### 3.3. *In vitro release test of CoA*

As we know, PIC micelles can incorporate ionic drugs into the core due to its ionic nature. CoA (the molecular weight is 767.54) is a water-soluble ionic drug and widely used in the medical area. The chemical structure of CoA is shown in Scheme 2. CoA can modulate the metabolism of sugar, fat and protein in the body. It

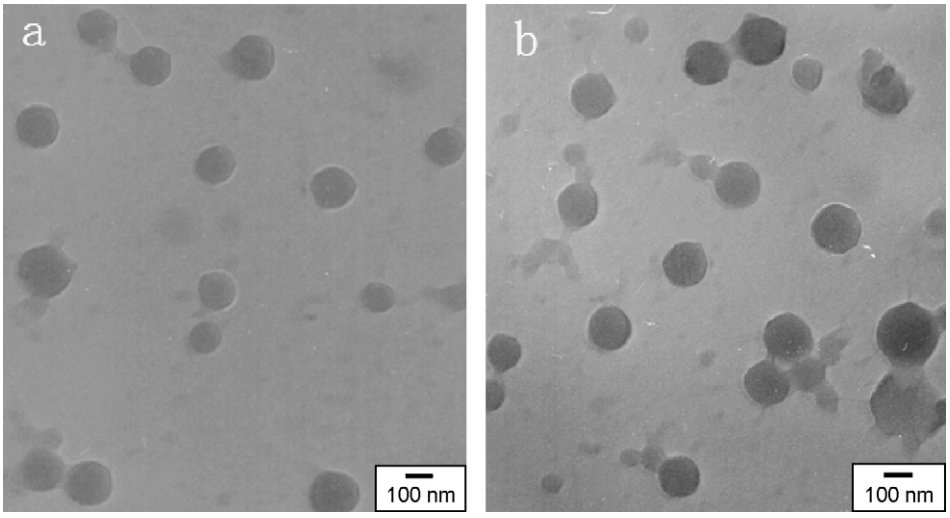
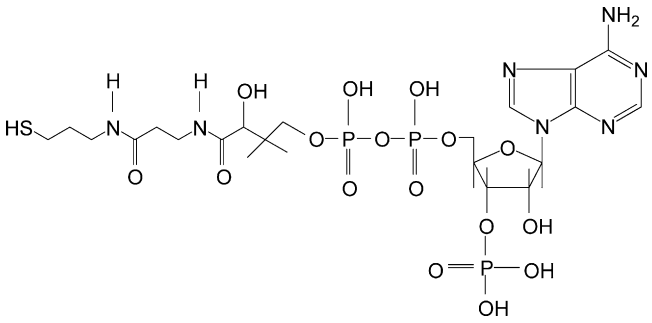



**Fig. 4.** The TEM images of chitosan/PVP-b-PSMA micelles (a) and CoA-loaded chitosan/PVP-b-PSMA micelles (b).



**Scheme 2.** The chemical structure of CoA.

contains the following three parts: adenosine triphosphate, pantothenic acid, cysteamine. In order to access the PIC micelles' use in biomedical application, we use the micelles to control the release of CoA at different pH and salt concentration. Noteworthy, CoA was incorporated into the hydrophobic micellar cores only by physical interaction (hydrogen bonding and ionic interaction), so the biological activity of CoA is not destroyed in the experiment.

The studies on the release of CoA from chitosan/PVP-b-PSMA micelles were carried out in a buffer solution at different pH values and 37 °C. As shown in Fig. 6, an initial burst was observed with all the buffers within the first 5 h. This might be due to the localization of a little portion of CoA in the outer shell or interfaces between the inner core and outer shell of the PIC micelles. In addition, the release rate of CoA varied with the different pH in released solution. That is to say that the effect of pH of the release medium on the controlled release of CoA is considerable.

According to Fig. 6, the amount and percentage of CoA release is fastest at pH 9.18, then pH 1.7. The least release rate was at pH 6.86. This could be explained by the different structural feature of the PIC micelles and the effect between the both block copolymers and CoA at different experimental conditions. The p$K_a$ of the $-NH_3^+$, $-H_2PO_4$ and –SH in CoA have been reported to be 3.7, 6.6 and 10.4, respectively (Pitman and Morris, 1980). Under pH 6.86 solution, about a half of the phosphoric groups in CoA carry charges. Maleic anhydride in the PVP-b-PSMA is partly negatively charged (the p$K_a$1 and p$K_a$2 of PSMA are 3.0 and 6.3, respectively) (Soer et al., 2008), whereas some chitosan segments remain positively charged (Montilla et al., 2007). On one hand, the electrostatic attraction between PVP-b-PSMA and CS make the micellar cores

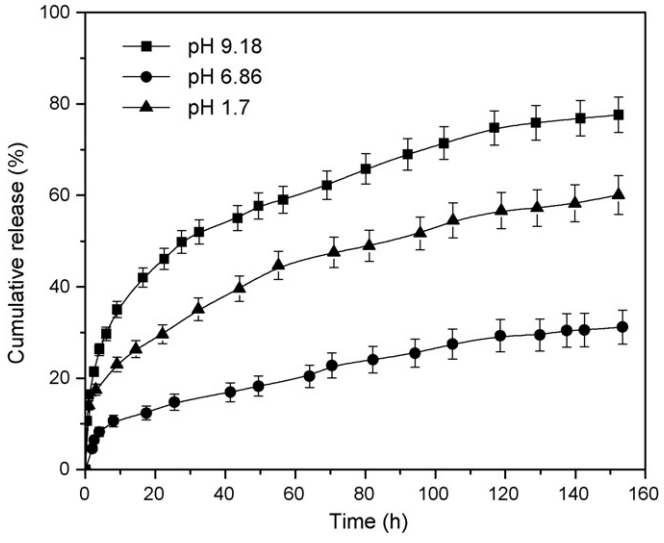



**Fig. 6.** Released profiles for CoA from PIC micelles as a function of the pH of solution.

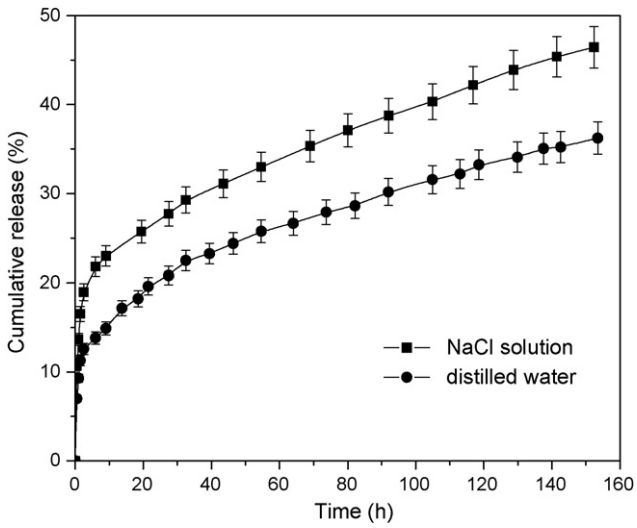



**Fig. 7.** Drug release profiles of PIC micelles in distilled water and 0.3 M NaCl solution.

compact. On the other hand, there are a large number of H-bonds between the polar groups in CoA such as –OH, $-NH_2$, $-H_2PO_4$, –SH, and –NHCO and the groups in the both block copolymers. They all restrain the release of CoA. So in neutral solution, the amount of the drug released was the least. Under acid conditions (pH 1.7), the maleic anhydride was hydrolyzed and protonized to carboxyl group, while the pyridyl group in CoA and residual amide $-NH_2$ on the CS chains are mainly positively charged. Although the H-bonds still existed, the electrostatic repulsion between the positive charge of CoA and $-NH_3^+$ groups on the CS accelerates the release of CoA from the PIC micelles. At pH 9.18 alkaline solution, there are two factors contribute to the release of CoA. Firstly, the complex is destabilized at strongly basic conditions due to the neutralization of the charge on CS, which lead to complex degradation gradually resulting in CoA exposure on the micelle surface. Secondly, the phosphoric groups in CoA and $-COO^-$ on the side chains of MAn are all negatively charged. The strongly electrostatic repulsion between them facilitates the release of CoA. In accordance with this, the release of CoA from the PIC micelles was significantly higher at pH 9.18 compared to pH 1.7. So we can use the PIC micelles to control the release of CoA at different pH values solution.

Drug release profile was also determined in NaCl solution to investigate the ion strength on drug release. Comparing the drug release profile in distilled water with that in NaCl solution (Fig. 7), it can be found that the amount of drug released increases with added salt. At the initial 6 h, the accumulative releases of CoA were 22% in NaCl solution and 14% in distilled water. However, it takes about 69 h to reach the 35% release rate of CoA in NaCl solution, while 138 h to the same release rate in distilled water. It was suggested that a high concentration of salt cause swelling of the micelle cores, which make the drug easily release to the environment (Yang et al., 2005). So adjusting the ion strength can also control the releasing of CoA to a certainty.

## 4. Conclusions

By RAFT, the double-hydrophilic block copolymer PVP-b-PSMA was synthesized. Polyion complex micelles have also been prepared from mixtures of the polyanionic PVP-b-PSMA with the polycations CS. From the TEM and DLS observations, the PIC micelles have a spherical shape with a narrow distribution. In order to assess its application in biomedical area, the model drug CoA was loaded in the PIC micelles and the in vitro drug release behavior was inves-

tigated. The results indicated that by manipulating the pH and salt concentration of the release solution, it was possible to control the rate of the release of CoA. So the PIC micelles had potential applications in drug delivery systems.

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