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Self-assembled micelles of *N*-phthaloylchitosan-g-polyvinylpyrrolidone for drug delivery

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ABSTRACT

A novel amphiphilic graft copolymer *N*-phthaloylchitosan-g-polyvinylpyrrolidone (PHCS-g-PVP) was synthesized by grafting polyvinylpyrrolidone (PVP) onto a chitosan derivative whose amino groups were protected by phthaloyl groups. Polymeric micelles were prepared by the dialysis method, and showed a low critical micelle concentration (CMC) of 0.83 mg/L detected by fluorescence spectroscopy. Prednisone acetate was incorporated in the polymeric micelles. The loading capacity was found to be around 44.6 wt%. Morphological investigation by transmission electron micrograph (TEM) showed that the micelles were round in shape. The mean particle diameter of the drug-loaded micelles were about 143.3 nm, much bigger than the unloaded micelles which had a unimodal size distribution with an average diameter of 89.8 nm as measured by dynamic light scattering (DLS). In vitro tests showed release of prednisone acetate from the micelles was continuous with no initial burst. All the results suggest that the nano-size core–shell micelles might be used in controlled drug delivery system.

1. Introduction

Delivering water-insoluble drugs, reducing severe systemic toxicities and increasing the utilization of drugs by improving their pharmacokinetics posed many challenges for drug delivery system (DDS) and drug development (Praneet, Tanasait, Amornrut, Theerasak, & Auayporn, 2007). Recently, several types of drug carrier, such as microspheres, liposomes, nanoparticles (Chang, Joseph, & Gardella, 2005) and polymeric carriers, have been investigated as DDS, but non-selective scavenging of these carriers by the reticuloendothelial system (RES) is a serious problem (Praneet et al., 2007). A polymeric micelle drug carrier system based on amphiphilic graft or block copolymers solves these problems by utilizing some preferable characteristics of polymeric micelles where the hydrophobic segments are segregated from the aqueous domain to form an inner core surrounded by a highly hydrated outer shells. The hydrophobic core acts as a reservoir for poorly water-soluble drugs (Sui, Yin, Chen, Zhang, & Kong, 2006), which keep a satisfactory aqueous stability irrespective of high contents of hydrophobic drug incorporated into the inner core of the micelle (Fukashi et al., 1998). Furthermore, small polymeric micelles (<200 nm) can avoid physical clearance by filtration in the lungs and in the spleen or excretion through the kidneys. As its unique core–shell structure and nano-size, it not only protects drugs from inactivation and prevents their sudden release in bloodstream in the physiological environment, but also can reduce the drug toxicity and make them suitable as long-circulating drug carriers. Therefore, much interest has been focused on the polymeric micelles as DDS (Jindrich, 2003; Prabaharan & Gong, 2008; Praneet et al., 2007; Ye et al., 2008).

Polymeric micelles used for DDS in intravenous administration must be of no danger to human body, so the segment used in graft or block copolymer should be non-toxic, biodegradable and biocompatible (Praneet et al., 2007). Chitosan is an abundant, nontoxic and biocompatible natural polymer (Agnihotri & Aminabhavi, 2004; Jayakumar, Prabaharan, Reis, & Mano, 2005; Trong, Chia, & Wen, 2002). So in recent years, the production of chitosan spheres for DDS was developed by some specific processing techniques, such as suspension cross-linking, spray–drying coagulation, emulsification/solvent evaporation. However, it was insoluble in water and cannot form micelles in water. Therefore, to overcome the problems above, some modified chitosan e.g. *N*-octyl-*O*-sulfate chitosan (Zhang, Ping, Zhang, & Shen, 2004) and (2-hydroxyl-3-butoxyl)-propylcarboxymethyl-chitosan (Sui et al., 2006), *N*-succinyl-chitosan (Zhu, Chen, & Yuan, 2006) have been reported for the preparation of polymeric micelles. These polymeric micelles were based on chitosan derivatives modified by small molecules, but the work related to the modification with synthetic polymers was limiting (Praneet et al., 2007). Praneet, Tanasait, Amornrut, Theerasak, and Auayporn (2006) prepared an amphiphilic *N*-phthaloylchitosan-g-poly (ethylene glycol) methylether (PHCS-g-mPEG) in homogeneous phase using a key reaction intermediates (Li, Zhuang, Mu, Wang, & Fang, 2004), *N*-phthaloylchitosan. Introduction of bulky phthaloyl groups into chitosan destroyed inter-

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and intra-hydrogen bonds of chitosan and resulted in its solublility in common organic solvents. The PHCS-g-mPEG self-assembled to form nano-size core–shell micelles (80–170 nm), and the release of camptothecin (CPT) from the micelles was sustained. In drug delivery system, this sustain effect not only can reduce drug toxicity, but also can reduce the side effects, to some extent, it is able to achieve controlled drug release.

Polyvinylpyrrolidone (PVP) has been used in the biomedical field due to its excellent biocompatibility, non-toxicity and physical inertia (Haruhiko et al., 1999; Irene, Roberto, Etienne, & Emo, 2007). Recently, there has been a growing interest in grafting modification of PVP for drug targeting carriers. Park et al, (2003) synthesized galactosylated chitosan graft polyvinylpyrrolidone (GCPVP) and then prepared the GCPVP/DNA complex which showed small sizes and narrow size distribution, and the results showed that the release of DNA from the GCPVP/DNA complex was dependent on both the concentration of chondroitin sulfate added and the molecular weight of chitosan.

Based on the unique properties of PHCS and PVP, we synthesized amphiphilic graft copolymer (PHCS-g-PVP) by a condensation reaction between the hydroxyl group of PHCS and carboxyl terminated group of PVP. Both nano-size blank micelles and prednisone acetate-loaded micelles were prepared by dialysis and in vitro release of prednisone acetate from drug-loaded micelles was also studied.

2. Experimental

2.1. Materials and reagents

Chitosan {CS, degree of deacetylation = 96% was determined by linear potentiometric titration (Jia & Li, 2001), viscosity average molecular weight = 18000, determined in 0.1 M acetate acid/0.2 M NaCl aqueous solution at 25 ± 0.5 °C by means of Ubbelohde Viscometer, according to the Mark–Houwink equation, $K = 1.81 \times 10^{-3}$, $\alpha = 0.93$ (Li et al., 2004; Maghami and Roberts, 1988)}, was purchased from Yuhuan Ocean Biochemical Co. Ltd. (Zhejiang). *N*-vinyl-2-pyrrolidone (NVP) was obtained from J&K Chemical Ltd and used after distillation under reduced pressure. 3-Mercaptopropionic acid (MPA) provied by Chenghui-Shuangda Chemical Co. Ltd. (Jinan) was used after distilled under reduced pressure. *N*,*N*-azobisisobutyronitrile (AIBN) provided by Shanghai Chemical Reagent Co. Ltd. was used after recrystallization with methanol. *N*,*N*-dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) were distilled under reduced pressure from calcium hydride. All other reagents and solvents were used without further purification.

2.2. Synthesis of carboxyl terminated PVP (PVP–COOH)

Carboxyl terminated PVP (PVP–COOH) was prepared by radical polymerization using MPA as a chain transfer agent (Haruhiko et al., 1999; Park et al., 2003). NVP (0.25 mol), MPA (0.013 mol) and AIBN (0.001 mol) were dissolved in 20 ml ethanol. The solution was degassed by bubbling with nitrogen for 30 min. Polymerization was carried out at 70 °C for 24 h. After reaction, the polymer was precipitated into an excess of diethyl ether, then purified by repeated precipitation in diethyl ether. Finally the product was dried in a vacuum. The molecular weight was 7200, measured by potentiometric titration with 0.1 M NaOH.

2.3. Preparation of N-phthaloylchitosan (PHCS)

N-phthaloylchitosan was synthesized as previously reported (Keisuke, Hiroyuki, Yuya, & Shimojoh, 2002). To a solution of 5.6 mmol of phthalic anhydride in definite *N*,*N*-dimethylformamide (DMF) containing 5% (v/v) water was added (1.86 mmol pyranose) of highly deacetylated chitosan, and the mixture was heated in nitrogen at 120 °C with stirring. After 8 h of reaction, the resulting mixture solution was cooled to room temperature and poured into ice water. The precipitate was collected on a filter, washed fully with methanol at room temperature, and dried under vacuum at room temperature overnight. The degree of substitution of phthaloyl groups within PHCS was determined to be about 1.12, calculated by elemental analysis.

2.4. Synthesis of PHCS-g-PVP copolymer

The whole grafting procedure of PHCS-g-PVP was showed in Scheme 1. PHCS (0.40 mol) was stirred with PVP–COOH (1 mol) in 20 ml DMF solution. 1-Hydroxybenzotrizole (HOBt) (3 mol) was added as catalyst and stirred at room temperature until fully dissolved. Then DCC (3 mol) was added. After 48 h reaction at room temperature, the copolymer was dialyzed against distilled water, and then the precipitate was collected, washed fully with ethanol, and dried under vacuum at room temperature overnight to obtain white particles. The graft content (G%) was 292.3%, calculated as follows: $G\% = (W_g - W_0)/W_0 \times 100$, where W_g and W_0 are the weight of graft copolymers and PHCS, respectively.

2.5. Preparation of polymeric micelles

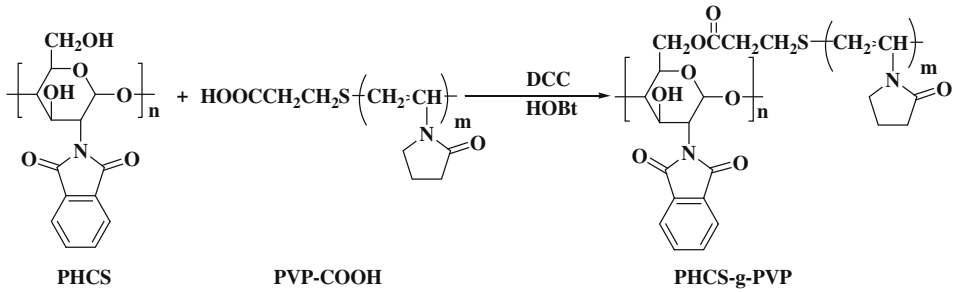
Polymeric micelles of PHCS-g-PVP were prepared by the dialysis method (Praneet et al., 2006; Yokoyama et al., 1999). Distilled water was added slowly (1 drop min^{-1}) into DMSO solution of graft copolymer (1 mg/ml) under vigorous stirring until slightly turbid. Then the solution was put into a dialysis bag (MWCO = 14,000) and dialyzed against distilled water at −4 °C for 48 h, with the distilled water being changed every 12 h. The micelles solution was purified by ultrafiltration using a filtration membrane of 0.45 μm.

2.6. Measurement of critical micelle concentration

Critical micelle concentration (CMC) of PHCS-g-PVP was determined by fluorescence spectroscopy (Praneet et al., 2006; Zhang et al., 2004). Aliquots of pyrene solutions in diethyl ether (5 μl) was placed into each of a series of tubes and the diethyl ether was evaporated, 4 ml aqueous polymer solutions at different concentrations ($1–0.42 \times 10^{-6}$ mg/ml) were added to each tube containing the pyrene residue ($[py] = 6 \times 10^{-7}$ M), then the mixture was sonicated for 30 min and stored overnight at room temperature to reach the dissolution equilibrium of pyrene in the aqueous phase. An excitation spectrum was measured at 336 nm, and emission spectra were recorded ranging from 350 to 550 nm. From the pyrene emission spectra, the intensity ratios (I_1/I_3) of the first band (374 nm) to the third band (385 nm) were analyzed as a function of polymer concentration. The critical micelle concentration (CMC) value was determined at the onset of a decrease in the plot of the polymer concentration versus ratio of I_1/I_3.

2.7. Drug loading

The incorporation of prednisone acetate into polymeric micelles was carried out by a dialysis method (Praneet et al., 2006). 5 mg of copolymer and prednisone acetate (5 mg) were dissolved in 2 ml DMSO in a glass tube. The mixture was stirred at room temperature until completely dissolved, then distilled water was added slowly (1 drop min^{-1}) into the mixture under vigorous stirring until slightly turbid, the mixture was placed in a dialysis bag (MWCO = 14000), dialyzed against distilled water over night.



Scheme 1. Synthesis of PHCS-g-PVP.

Drug-loaded micelles were purified by filtration with a 0.45 μm pore-sized microfiltration membrane.

Prednisone acetate concentration was measured at 242 nm with a UV–Vis spectrometer and was calculated based on the standard curve: c (mg/L) = A/0.0231, where A is the UV absorbance at 242 nm (Wei, Zheng, Zhou, Cheng, & Zhuo, 2006).

The loading capacity was calculated from the formula:

$$\text{Loading capacity}(\%) = \frac{M_0}{M_0 + M_p} \times 100$$

where M_o is the amount of drug-loaded in the polymeric micelles, and M_P is the amount of copolymer. M_o was calculated by subtracting the amount of unloaded drug from the initial feed drug amount. The amount of unloaded drug was analyzed by measuring the absorbance at 242 nm of the dialysis fluid. The amount of prednisone acetate incorporated into polymeric micelles was about 4.03 mg, and the loading capacity was 44.6 wt%.

2.8. In vitro drug release

Drug release from prednisone acetate-loaded micelles was measured using a dialysis bag (MWCO = 14000) as described previously (Praneet et al., 2006). 3 ml prednisone acetate-loaded micelles were placed in a dialysis bag and immersed in the medium (PBS, 0.1 M, pH = 7.4), which kept at 37 °C. At certain time intervals, 3 ml aliquots of the medium were withdrawn and the same volume of fresh medium was added. The amount of prednisone acetate released from micelles was measured using UV–Vis spectrometer at 242 nm. All experiments were performed in triplicate.

The cumulative drug release was calculated from the formula cumulative drug release (%) = $(M_t/M_o) \times 100$. Where M_t is the amount drug release from micelles at time t, and M_o is the amount of drug-loaded in PHCS-g-PVP polymeric micelles.

2.9. Characterization

Fourier-transform infrared (FT-IR) transmission spectra were obtained from samples in KBr pellets using a Bruker IFS66v/S FT-IR spectrophotometer. ^{1}H NMR (nuclear magnetic resonance) spectra were recorded on an AV-300 M NMR spectrometer. The morphology of the copolymers aggregates was studied by H-600, Japan transmission electron micrograph (TEM). Dynamic light scattering (DLS) measurements were carried out with a BI-200SM, Brookhaven instrument. UV–Vis measurements were carried out at 25 °C with a Perkin–Elmer LS55 (America) spectrophotometer.

3. Results and discussion

3.1. Preparation and characterization of graft copolymers

Carboxyl terminated PVP was prepared by radical polymerization using 3-Mercaptopropionic acid as chain transfer agents. Fig. 1a shows the FT-IR of PVP–COOH. The strong signals at 1673.9 cm^{-1} and 1288.3 cm^{-1} attributed to the C=O stretching vibration (Amide I) and C–N stretching vibration in the PVP ring (Irene et al., 2007). The stretching vibration of O–H appears at 3464.3 cm^{-1}. As indicated in ^{1}H NMR of PVP–COOH (Fig. 2a), the $SCH_2C\mathbf{H}_2$ peaks appear at 1.4–1.7 ppm and the peaks of CH_2C=O appear at 2.2–2.4 ppm. The strong signals at 3.2 and 3.5–3.6 ppm were due to the protons of $CHC\mathbf{H}_2$ and $C\mathbf{H}CH_2$ in PVP ring (Irene et al., 2007). The above results proved the synthesis of PVP–COOH.

N-phthaloylchitosan was synthesized by chitosan reacting with excess phthalic anhydride. The FT-IR spectrum of *N*-phthaloylchitosan was shown in Fig. 1c, compared with that of original chitosan (Fig. 1b), PHCS showed the characteristic absorptions peaks at 1776.1 cm^{-1} and 1712.5 cm^{-1} referring to the carbonyl anhydride, and the absorptions peak at 721.3 cm^{-1} belonging to the aromatic ring (Keisuke et al., 2002; Li, Zhuang, Mu, Wang, & Fang, 2008). The ^{1}H NMR (Fig. 2b) of *N*-phthaloylchitosan, showed mainly two parts of broad peaks: 2.8–5.0 ppm belonged to the chitosan backbone hydrogens and 7.8–8.0 ppm assigned to the phthaloyl group (Praneet et al., 2007). These results verified that the PHCS was synthesized.

Graft copolymer (PHCS-g-PVP) was synthesized in homogeneous phase by a condensation reaction between the hydroxyl group of PHCS and carboxyl terminated group of PVP. Compared to the FT-IR spectrum of PHCS, the obtained PHCS-g-PVP (Fig. 1d) presented new absorption peaks at 1186.2 cm^{-1} and 1089.5 cm^{-1}, which referring to the characteristic dissymmetrical and symmetry stretching vibration peaks of C–O–C of the ester group,

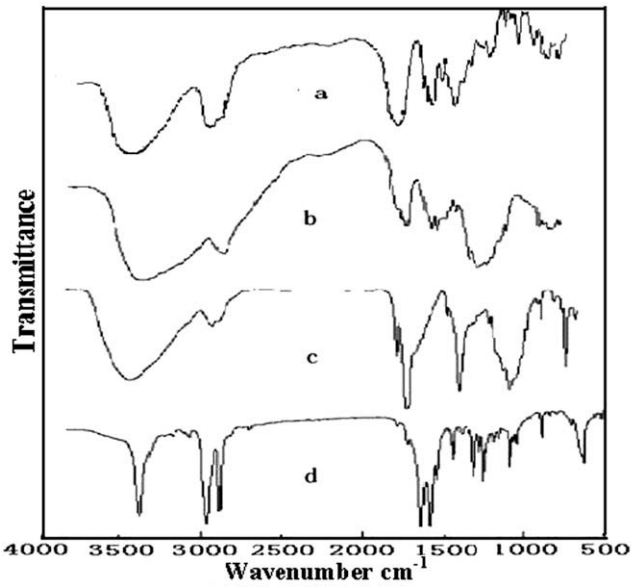


Fig. 1. FT-IR spectra of (a) PVP–COOH, (b) CS, (c) PHCS, and (d) PHCS-g-PVP.

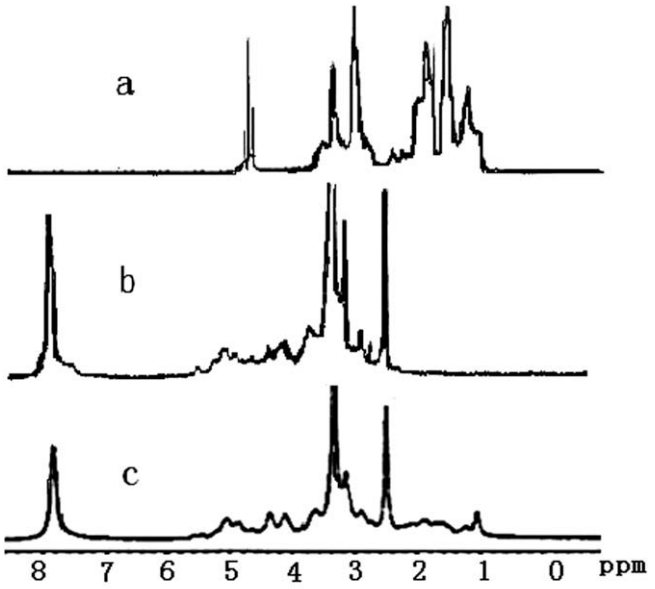


Fig. 2. ^{1}H NMR spectra of (a) PVP–COOH in D_2O, (b) PHCS in DMSO-d_6, and (c) PHCS-g-PVP in DMSO-d_6.

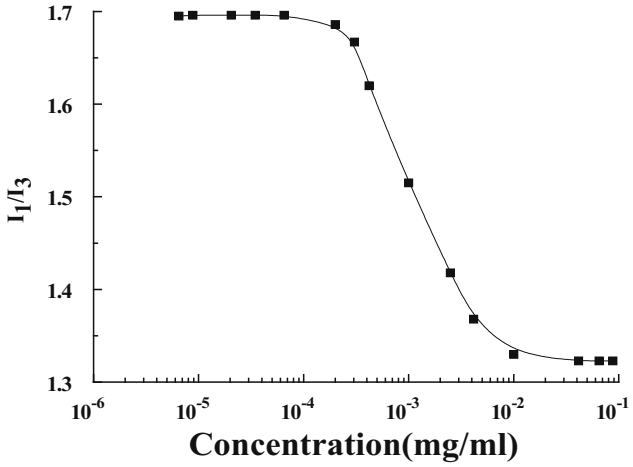


Fig. 3. Plot of the ratio of intensities (I_1/I_3) of selected emission bands in the pyrene fluorescence spectrum as a function of PHCS-g-PVP concentration. λ_{ex} = 336 nm. [py] = 6.0×10^{-7} M.

the peaks at 1643.5 cm^{-1} and 2929.9 cm^{-1} belonging to the carbonyl group and C–H (–CH_2CH_2), respectively. The evidence implied the successful introduction of the PVP chains on PHCS. Compared with PHCS, the ^{1}H NMR spectrum (Fig. 2c) of graft copolymer showed new proton peaks at 1.4–1.7 ppm and 2.2–2.4 ppm assigned to C–H ($SCH_2C\mathbf{H}_2$) and $CH_2C{=}O$ of PVP graft chain, respectively. This further confirmed the PVP were successfully grafted onto chitosan chains.

3.2. Preparation and characterizations of polymeric micelles

In general, the formation of self-assembled micelles occurs as a result of two forces (Sui et al., 2006). One is an attractive force that result in the self-assembly of molecules, while the other repulsive force prevents unlimited growth of the micelles to a distinct macroscopic phase. Amphiphilic copolymer can self-assembly when its solution was dialysized in a poor solvent for either hydrophobic or hydrophilic segment. In our experiment, the self-assembled micelles were prepared by dialysis of a DMSO solution of the PHCS-g-PVP copolymers against distilled water.

The CMC of polymeric micelles were characterized by fluorescence spectroscopy using pyrene as a fluorescent probe. The plot of the intensity ratio I_1/I_3 of the pyrene excitation spectra against the polymer concentration is shown in Fig. 3. As seen from the Fig. 3, below the CMC, the fluorescence intensity in I_1/I_3 was nearly a constant, it indicated that the copolymers exist as single chains, but when the copolymer concentration reached a value which above the CMC, a dramatic decrease in I_1/I_3 was observed, indicating the formation of micelles and dissolution of pyrene into the hydrophobic core of micelles. Compared to low molecular weight surfactants micelles (e.g. 2.3 mg/ml for sodium dodecyl sulfate in water) (Nagasaki, Okada, & Scholz, 1998), the CMC of PHCS-g-PVP with 0.83 mg/L is lower. The polymeric micelles with lower CMC will be suitable as drug targeting devices since they are stable in an aqueous environment and cannot easily dissociate on extremely diluted by blood in intravenous administration, and can prolong circulation in the bloodstream.

The morphology of polymeric micelles was measured by TEM (Fig. 4a). Fig. 4a showed that self-assembled micelles are well dispersed as individual nano-size micelle with regularly spherical shape, and the size of the nano-size micelles were around 40–100 nm in diameter. Fig. 5a showed that PHCS-g-PVP micelles exhibit a unimodal size distribution with an average diameter of 89.8 nm measured by DLS. These are consistent with the TEM results.

3.3. Prednisone acetate incorporation into PHCS-g-PVP micelles

As the inner core of the polymeric micelles is hydrophobic, hydrophobic drug molecules can be physically incorporated and stabilized by hydrophobic interactions. Prednisone acetate is a good anti-inflammation and anti-allergic drug, but it is water-insoluble, here it was used as a model drug to be entrapped into the hydrophobic core of amphiphilic graft copolymer micelles. The prednisone acetate was successfully loaded into the inner core of polymeric micelles with a loading capacity of 44.6 wt%.

The TEM photograph (Fig. 4b) showed that the size of drug-loaded polymeric micelles were approximately 118–200 nm, and it were much bigger than the blank micelles (Fig. 4a), indicating that the dissolution of the prednisone acetate into the micelles made the micelles much bigger. These are consistent with the DLS results (Fig. 5b). The drug-load micelles showed an average size of 143.3 nm. Therefore, if the polymeric micelles were used as DDS, its nano-size can reduce non-selective system scavenging by the reticuloendothelial.

3.4. Drug release measurement

In vitro release of prednisone acetate from drug-loaded micelles was evaluated in PBS (pH = 7.4, 0.1 M) at 310 K. The cumulative drug release pattern of the polymeric micelles is showed in Fig. 6. As seen from the curve, the drug release rate was very stable, no initial burst of release appeared. After 69 h incubation at 37 °C, only 26% drug was gradually released. The stability and low release rate probably attributed to two factors. On the one hand, the interaction between the drug and inner core of micelles is stronger than that between drug and solvent (Zhao, Wang, Winnik, Riess, & Croucher, 1990), on the other hand, since the hydroxyl in PHCS and the carbonyl group of PVP can form intermolecular H-bond with the carbonyl groups and hydroxyl of prednisone acetate, which is prone to stabilize the structure of micelles.

In many reported papers on DDS (Sui et al., 2006; Zhang et al., 2004), showed an initial rapid burst of release and then were slowly released. This phenomenon results in large amounts of

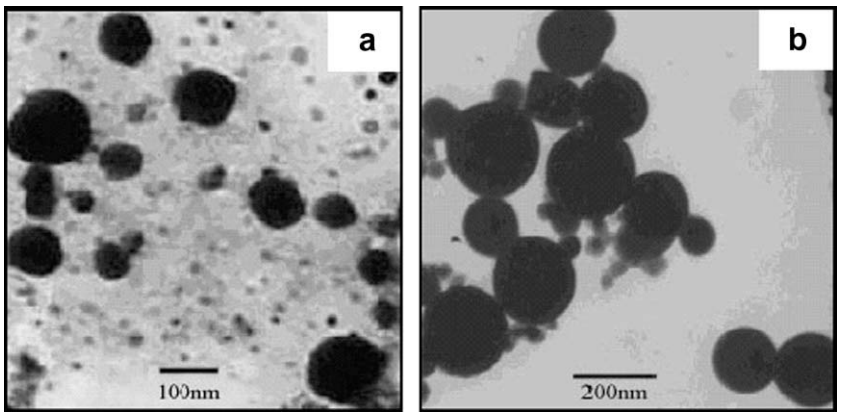


Fig. 4. Transmission electron micrograph (a) blank micelles (×50,000), and (b) Prednisone acetate-loaded micelles (×50,000).

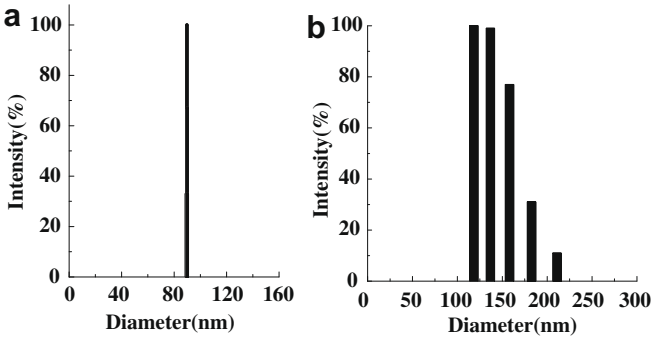


Fig. 5. Particle size distribution of (a) PHCS-g-PVP micelles, (b) prednisone acetate-loaded micelles.

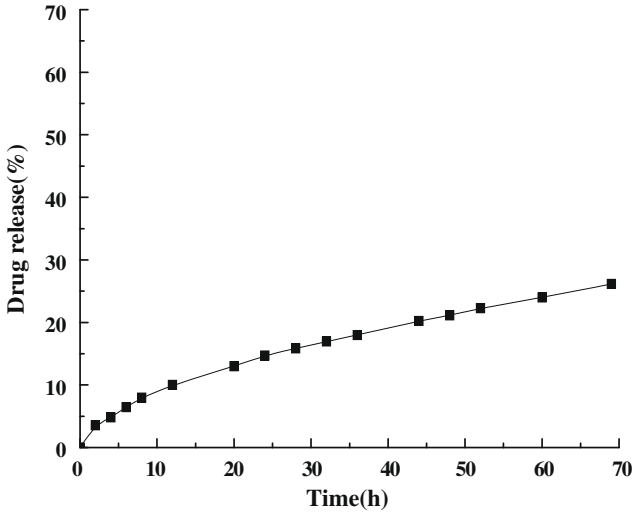


Fig. 6. In vitro release of prednisone acetate in PBS (pH = 7.4) at 310 K.

drugs being wasted before getting to the targeted sites. In addition, the micellar solution used in intravenous administration were often extensively diluted by blood, so they are easily deformable and disassemble which result in the faster leakage of loaded drugs (Ye et al., 2008). Different from other studies, the drug release rate is stable in our drug release experiment and no sudden release appeared, these not only can reduce the number of injections a patient must endure, but also can improve the utilization of drugs.

4. Conclusion

A novel non-toxic, biocompatible and biodegradable graft copolymer (PHCS-g-PVP) was successfully synthesized in homogeneous system. In aqueous solution, the spherical polymeric micelles with nano-size and narrow distribution were formed based on amphiphilic graft copolymer. An anti-inflammation drug, prednisone acetate was incorporated into the micelles with loading capacity around 44.6 wt%. In vitro tests, release of prednisone acetate from the micelles was sustained. Taking the advantage of the nano-size and stable drug release rate, the nanoscope polymeric micelles might be useful as drug carriers to achieve controlled drug release.

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