Thermo-sensitive amphiphilic poly(*N*-vinylcaprolactam) copolymers: synthesis and solution properties

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Thermo-sensitive amphiphilic copolymers, PVCL-PTTrpAMT and PVCL-PVP-PTTrpAMT of hydrophilic *N*-vinylcaprolactam (VCL), *N*-vinylpyrrolidone (NVP), and hydrophobic *N*-*t*-Boc-tryptophanamido-*N'*-methacryl thioureas (TrpAMT) monomers, were synthesized and characterized by ¹H NMR, UV-spectroscopy, and GPC-MALLS. The cloud point (CP) measurement showed that hydrophobic PTTrpAMT and hydrophilic PVP segments significantly altered the phase transition temperature of PVCL with comparable molecular weight in aqueous solution. The CP of PVP-PTTrpAMT solution was 38.0°C, lower by 5.0°C than that of unmodified PVCL. In the presence of phosphate buffer saline (PBS), the CP value of the PVCL polymer decreased by ~2.0°C in comparison to that of the aqueous solution. Fluorescent spectroscopy and TEM studies revealed that PVCL-PTTrpAMT and PVCL-PVP-PTTrpAMT self-assembled into the spherical micelles, 30–70 nm in diameter, at concentrations over their CMCs in an aqueous solution. Cytotoxicity tests demonstrated that the PVCL copolymers were not harmful to cell viability, which may favor the use of the copolymers as potential thermo-sensitive polymers in pharmaceutical applications. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: amphiphiles; self-assembly; solution properties; thermo-sensitive; *N*-vinylcaprolactam

INTRODUCTION

Thermo-responsive amphiphilic copolymers have recently attracted extensive attention because of both their intrinsic properties^[1–3] and their wide range of potential applications in medicine,^[4] biotechnology,^[5] ecology,^[6] and pharmacology.^[7] An aqueous solution of this type of polymer is characterized by a phase separation upon heating, and the system exhibits a lower critical solution temperature (LCST).^[8,9] At a low temperature, the solution is homogeneous, but when the temperature exceeds a certain value called the cloud point (CP) of the mixture, a macroscopic phase separation occurs. The LCST corresponds to the minimum of the phase separation curve.^[10,11]

Belonging to the category of thermo-sensitive polymers, poly(*N*-vinylcaprolactam) (PVCL) has recently attracted a great deal of interest.^[12–15] PVCL is a well-known polymer exhibiting the LCST behavior. It shows a dissolution/precipitation transition in water at temperatures close to body temperature.^[16] Also, PVCL is stable against hydrolysis, non-toxic, and biocompatible.^[17] These properties make it an interesting candidate for biomedical applications.^[5,18]

To further modulate the features of PVCL aqueous solutions, PVCL has been modified via copolymerization with *N*-vinylpyrrolidone (NVP),^[19] methacrylic acid,^[20] *N*-vinylimidazole,^[21] and alkyl acrylate derivations labeled with pyrene or naphthalene,^[22] as well as via grafting with PEO as an effective stabilizing agent.^[23] The studies of the properties of hydrophobically modified PVCL polymers suggest that the polymers can associate to form polymeric micelles via hydrophobic interactions in aqueous solution below the LCST.^[22,24] But to the best of our knowledge, the reports on detailed thermo-response and association mechanisms of amphiphilic PVCL copolymers simultaneously

modified by hydrophobic and hydrophilic segments in aqueous solution are very limited.^[23,24]

Herein, amphiphilic PVCL copolymers PVCL-PTTrpAMT and PVCL-PVP-PTTrpAMT of hydrophilic *N*-vinylcaprolactam (VCL), NVP, and hydrophobic *N*-*t*-Boc-tryptophanamido-*N'*-methacryl thioureas (TrpAMT)^[25] monomers were synthesized. The PVP segments chosen here mainly give rise to the hydrophilic and biocompatibility feature of the copolymer, while the TrpAMT moieties with tryptophan residues here not only provide the polymer with its hydrophobic character, but it can strongly bind with DNA^[26,27] This strong interaction with DNA in turn plays an important role in enhancing polymerase chain reaction (PCR) amplification of DNA template,^[25] which would have potential applications in gene delivery system or biology detection. The chemical structures of PVCL-PTTrpAMT and PVCL-PVP-PTTrpAMT are all depicted in Fig. 1.

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Figure 1. Chemical structures of amphiphilic polymers PVCL-PTTrpAMT and PVCL-PVP-PTTrpAMT.

To gain insight into the thermo-sensitive properties and self-aggregation behaviors of the PVCL copolymers, CP measurement and fluorescent spectroscopy were carried out. The aggregate morphologies of the copolymers were also investigated by TEM in some detail. The cytotoxicity of the resulting amphiphilic copolymers was evaluated by MTT test.

EXPERIMENTAL

Materials

N-vinylcaprolactam (VCL, 99%, Acros) and *N*-vinylpyrrolidone (NVP, 99%, Acros) were purified by vacuum distillation. 2,2'-Azobisisobutyronitrile (AIBN, 99%) was recrystallized from methanol. *N*-[(tert-butoxy)carbonyl]tryptophan (*N*-t-Boc-Trp) (98%, Aldrich), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2-tetrazolium bromide (MTT, 99%, Sigma), and gastric carcinoma cell SGC-7901 were used as received. Phosphate buffer saline (PBS) was prepared with KH_2PO_4 and Na_2HPO_4 with pH (≈ 7.4). The hydrophobic monomer, *N*-[(tert-butoxy)carbonyl]tryptophanamido-*N'*-methacryl thiourea (TrpAMT), was prepared as described previously.^[25] Other reagents were used as received.

Polymerizations

All polymerizations were conducted in dimethylformamide (DMF), using the concentrations of monomer(s) and AIBN, listed in Table 1. A typical polymerization procedure for polymerization is as follows: the monomers, AIBN and a stirring bar were added into a Schlenk tube and sealed with rubber septum. The tube was flushed with nitrogen for 30 min and subsequently deoxygenated DMF (8 ml) was introduced into the tube. Then, the tube was immersed into an oil bath pre-heated to 70°C and heated for 20 hr. After that, the reaction mixture was cooled to room temperature, and the polymer was isolated by precipitation from

DMF into diethyl ether. The polymers were purified further by two additional precipitations from THF into hexane, filtered, and finally dried under vacuum at room temperature.

Analysis

^1H NMR spectra were obtained using a Varian Mercury VX-300 MHz spectrometer using CDCl_3 as solvent. Gel-permeation chromatography/multi-angle laser light scattering (GPC/MALLS) analyses were carried out on a DAWN[®]DSP multi-angle laser photometer with a model P100 pump (Thermo Separation Products, San Jose, USA) equipped with two 300 nm Waters Styragel solvent-saving columns and a Waters 2410 differential refractive-index detector at 50°C to determine the molecular weight (M_n) and polydispersity index (PDI) of the polymer, using polystyrene as standards and DMF as a mobile phase, at a flow rate of 1.00 ml/min.

UV spectra were measured using a TU-1901 spectrometer. A series of different concentrations of *N*-t-Boc-Trp as standard solutions were prepared in DMF. The molar absorption coefficient was obtained from the plot of absorption intensity at 282 nm as a function of the *N*-t-Boc-Trp concentration at room temperature. The tryptophan residue content of polymer was established by measuring the absorption of a solution prepared from a carefully weighed amount (m_p) of the PVCL copolymer in a known volume (V_p) of DMF solution. The tryptophan residue concentration, $[\text{Trp}]$, was then estimated from the absorption value at 282 nm in DMF. Then, the tryptophan residue content (χ , mol/g) for the copolymer could be calculated from

$$\chi = \frac{[\text{Trp}]}{m_p/V_p} \quad (1)$$

The CPs of the PVCL copolymers solutions were determined by CP measurements, which were done visually by following the variation of the turbidity of polymeric aqueous solutions with temperature. Polymeric aqueous solution (or PBS buffer) (0.5 mg/ml) in a sample tube was immersed in a MiTRe 4000 thermostated cell with a circulating water bath. The heating rate was regulated to around 0.1°C/min and CP was defined as the temperature at which the solution started to turn cloudy. The reproducibility of the determination was $\pm 0.5^\circ\text{C}$.

To corroborate micelle formation of the amphiphilic copolymers, steady-state fluorescence spectra were obtained on a Shimadzu RF-5301PC spectrometer (Japan) at 25°C using pyrene as a fluorescent probe. Aliquots of solution of pyrene (6×10^{-7} M) in acetone (0.3 ml) were added to the cells, the acetone was fully evaporated, and an aliquot solution of the polymer (3 ml) was added at different concentrations to afford a final pyrene concentration of 6×10^{-8} M. The probes were equilibrated overnight at room temperature, before emission spectra were recorded at 360–450 nm, with an excitation wavelength of

Table 1. Summary of the polymerization conditions

Polymers	[NVCL] (mol/L)	[NVP] (mmol/L)	[TrpAMT] (mmol/L)	[AIBN] (mmol/L)
PVCL	0.8	—	—	8.08
PVCL-PTTrpAMT	0.8	—	16.6	8.08
PVCL-PVP-PTTrpAMT	0.8	64	16.6	8.08

342 nm. The emission and excitation slit-widths were 1.5 and 3.0 nm, respectively.

A solution of the copolymer (0.6 mg/ml) in distilled water was sonicated, applied onto a Formvar-membrane-coated Cu grid, dried at 20°C, and then analyzed by JEM-100CXII TEM (Hitachi; Japan) to determine the morphology and size of the aggregation of the copolymer.

The cytotoxicity of polymers was evaluated by means of MTT assay as described previously,^[28] all steps were performed in Hank's balanced salt solution (HBSS). First, the cells were attached to the wells of a 96-well plate, and incubated for 12 hr at 37°C. The polymer in HBSS (200 µl) was added at different concentrations, and the wells were incubated for 48 hr at 37°C. Then, MTT (2.0 mg/ml, 10 µl) was added to the wells, and the plate was incubated for an additional 4 hr at 37°C before the MTT solution was removed. Negative controls (HBSS only) were treated similarly as above, but without polymer. The UV absorbance values from the wells were measured at 492 nm with a Multiskan MK3 Sunrise reader. Cell viability (in % relative to the negative control) was then calculated from the absorbance values (eqn 2).

$$\text{Cell viability (\%)} = \text{OD}_{492(\text{polymer})} / \text{OD}_{492(\text{control})} \times 100 \quad (2)$$

Where $\text{OD}_{492(\text{polymer})}$ is the absorbance value for the cells treated with polymer, $\text{OD}_{492(\text{control})}$ is the absorbance value for the cells of the negative control.

RESULTS AND DISCUSSION

Synthesis and characterization of the polymers

Amphiphilic copolymers, PVCL-PTTrpAMT and PVCL-PVP-PTTrpAMT, were prepared by the free radical solution copolymerization of VCL, NVP, and TrpAMT in DMF. PVCL was also synthesized to serve as a reference material. The copolymers obtained were characterized by ¹H NMR spectra to establish the copolymerization of the monomers. Figure 2 shows the ¹H NMR spectra of PVCL-PTTrpAMT and PVCL-PVP-PTTrpAMT. The characteristic peaks corresponding to the vinyl groups of monomers between $\delta(\text{H})$ 4.8

and 6.5 ppm disappeared completely in CDCl_3 , and the indole resonances occurred at $\delta(\text{H})$ 6.9–7.6 ppm, suggesting the successful copolymerization of the monomers.

The hydrophobic segment contents, i.e., the tryptophan residue contents of the copolymers were determined by UV absorption, assuming that the extinction coefficient of the chromophore, *N*-*t*-Boc-Trp, linked to the copolymers is identical to that of the corresponding model compound, *N*-*t*-Boc-Trp. The hydrophobic contents level of PVCL-PVP-PTTrpAMT was slightly higher than that of PVCL-PTTrpAMT, suggesting that introducing PVP here may favor the copolymerization of the hydrophobic monomer (Table 2). The molecular weights and polydispersities of the resulting polymers were measured by GPC-MALLS and summarized in Table 2.

Thermo-responsive properties of the polymers

The phase transitions of the PVCL polymers were studied by CP measurement. The CP values for the polymers in aqueous solution were obtained and summarized in Table 2. The value of CP was slightly lower by 0.8°C for the PVCL-PTTrpAMT than that of the unmodified PVCL. Generally, introduction of the hydrophobic segments along a thermo-sensitive water-soluble polymer chain leads to a decrease of CP.^[30] For the thermo-sensitive PVCL, the effect of molecular weight on the phase transition temperature of PVCL aqueous solution is a distinctive feature of this polymer, and the phase transition temperature dramatically decreased with decreasing molecular weight (M_n), especially for PVCL with molecular weight ranging from 5×10^4 to 6×10^3 .^[29] In this study, the M_n of PVCL-PTTrpAMT are lower than that of the unmodified PVCL. Thus, under this condition, the slight change in CP does not reflect the real effect of the hydrophobic or hydrophilic segments on the phase transition of PVCL.

The referenced CP value of PVCL with M_n of 21,000 was 38.4°C, which is in agreement with that of the PVCL with comparable molecular weight in this study (Table 2). Therefore, some real significant modification effect could be evaluated by comparing the phase transition of PVCL copolymers with those of PVCL cited here with comparable M_n . The CP is significantly affected by

Figure 2. ¹H NMR spectra of PVCL-PTTrpAMT and PVCL-PVP-PTTrpAMT in CDCl_3 .

Table 2. The compositions and solution properties of the PVCL polymers

Polymers	M_n^a (g/mol)	PDI ^a	$[\chi]^b$ (mol/g)	CP ^c (°C)		Yield (%)
				Water	PBS	
PVCL	21,000	1.30	—	38.8	37.2	74.3
PVCL ^d	21,000	—	—	38.4	—	—
PVCL ^d	13,000	—	—	43.0	—	—
PVCL-PTTrpAMT	13,000	1.26	6.7×10^{-5}	38.0	36.5	70.3
PVCL-PVP-PTTrpAMT	12,000	1.47	7.5×10^{-5}	43.2	41.0	78.2

^a From GPC-MALLS analysis.
^b The tryptophan residue content $[\chi]$ calculated from UV absorption.
^c From the cloud point (CP) for the polymers solution (0.5 mg/ml).
^d Cited from the literature.^[29]

introduction of the hydrophobic segments, PTTrpAMT, with a decrease in value by 5.0°C compared to that of unmodified PVCL cited with M_n of 13,000. The large decrease in CP is attributed to the lower degree of solubility of the copolymer as the result of strong hydrophobicity of the hydrophobic segments. Furthermore, introduction of more water-soluble hydrophilic PVP caused a great increase by 5.2°C in CP, compared to that of PVCL-PTTrpAMT. This comparison indicates that hydrophobic PTTrpAMT and hydrophilic PVP segments indeed significantly altered the phase transition temperature of PVCL in aqueous solution.

Influence of PBS on LCST of the polymers

Salts are known to affect the phase behavior of PVCL aqueous solution on polymer–water interaction.^[31] To investigate the effect of salt on the CP of the PVCL polymers, PBS, possessing the water-association properties, as salt here was chosen in order to mimic physiological condition. The decrease by ~2°C in the CP value for the polymers aqueous solution was observed after the addition of PBS (Table 2). This result can be explained by the following way: in the presence of PBS, the strong association of water near anions and cations of PBS decreased the mobility of the molecules of water. Most of the water molecules were involved in this strong association, allowing the dehydration of PVCL aqueous solution, resulting in a reduction in phase transition temperature of the polymer.^[32]

Self-assembly behavior

The association behaviors of the PVCL copolymers in aqueous solution were assessed by extrinsic fluorescent probe methodology, which gives the critical micelle concentrations (CMCs) of the copolymers. Pyrene here was chosen as the fluorescent probe because of its special photophysical properties with strong hydrophobicity in water but being preferentially solubilized in the hydrophobic core of micellar assemblies in amphiphilic copolymer solution.^[33] The change in the microenvironment of pyrene is related to the change of its photophysical properties, and can be detected by monitoring the fluorescence intensity ratio at 393 versus 373 nm (I_{393}/I_{373}). This ratio is plotted as a function of the copolymer concentration in Fig. 3.

Figure 3 shows an abrupt increase in the I_{393}/I_{373} ratio of the fluorescent probe upon increasing the copolymer concentration, suggesting that the micelle formation occurs in solution. The concentration at the abrupt increase in the ratio of I_{393}/I_{373} represents CMC value. The CMCs of PVCL-PTTrpAMT and PVCL-PVP-PTTrpAMT were determined as 0.016 and 0.025 mg/ml, respectively. These results suggest that, below the LCST, the amphiphilic PVCL copolymer undergoes some interchain association via hydrophobic interactions between the chromophores and self-assemble into micelles in aqueous solution. The increase in CMC for PVCL-PVP-PTTrpAMT with comparable hydrophobic contents can be explained by the fact that PVCL-PVP-PTTrpAMT is more water-soluble than PVCL-PTTrpAMT caused higher copolymer concentrations to be needed to form stable aggregates.

Morphologies of PVCL copolymer aggregation

Based on the CMC studies of the copolymer, the PVCL copolymers would be mainly present in the form of the micelles at concentrations above the corresponding CMCs in aqueous

Figure 3. Change of the fluorescence intensity ratio (I_{393}/I_{373}) of pyrene as a function of the concentration of the PVCL copolymers, 25°C.

solution. To further determine the micelles morphology of the copolymers, the self-aggregation of the copolymers was investigated by TEM. Figure 4 shows the TEM images of the self-aggregation of PVCL copolymers aqueous solution at concentration of 0.6 mg/ml. PVCL-PTTrpAMT and PVCL-PVP-PTTrpAMT self-aggregated into mostly spherical micelles with diameters of around 30–40 and 50–70 nm, respectively. PVCL-PVP-PTTrpAMT formed slightly larger aggregates than PVCL-PTTrpAMT in aqueous solution. This can be attributed to the following reason: the more water-soluble PVP segments tend to bind more water molecules and swell the aggregated copolymer chains, resulting in the formation of the larger aggregates.

Cytotoxicity of the PVCL polymers

The MTT test was used to evaluate the cytotoxicity of the PVCL copolymers in order to obtain information regarding the cellular viability. Figure 5 shows that the cell viability of the polymer at the concentration ranged from 0.1 to 100 $\mu\text{g/ml}$ was almost above 100% at 37°C, compared to negative controls. Clearly, the polymers with PVCL hydrophilic surfaces did not interfere with cell membrane, at least during the tested polymer concentrations. For PVCL-PTTrpAMT with hydrophobic segments

Figure 4. TEM photographs of the copolymers from dried aqueous solutions, (A) PVCL-PTTrpAMT and (B) PVCL-PVP-PTTrpAMT, 0.6 mg/ml.

Figure 5. Viability values for the thermo-sensitive polymers at concentrations ranging from 0.1 to 100 $\mu\text{g/ml}$, as measured by MTT test at 37°C.

and less hydrophilic segments, the cell viability was actually decreased compared to PVCL at the identical conditions. For PVCL-PVP-PTTrpAMT, the further introduction of the hydrophilic PVP segments had an interesting influence on cytotoxicity of the copolymers: the viability of the cells was mostly higher. This suggests that the PVP segments with more biocompatibility and water-solubility than PVCL favored the viability of the cells and enabled the alteration of the cytotoxicity properties of the PVCL copolymer.^[34,35] These results indicate that at concentrations ranging from 0.1 to 100 $\mu\text{g/ml}$, the amphiphilic PVCL copolymers are benign in cellular contact, which gives us significant support for considering the copolymers as potential thermo-sensitive materials in pharmaceutical application.

CONCLUSIONS

Thermo-sensitive copolymers, PVCL-PTTrpAMT and PVCL-PVP-PTTrpAMT, were synthesized successfully. The hydrophobic PTTrpAMT and hydrophilic PVP segments significantly altered the phase transition temperature of PVCL polymers in aqueous solution. The CP value of PVCL-PTTrpAMT was significantly higher by 5°C than that of unmodified PVCL with comparable molecular weight, and the introduction of hydrophilic PVP segments in PVCL-PTTrpAMT caused its CP to increase due to the higher water solubility of the resulting polymer. In the presence of PBS, there was a decrease by $\sim 2^\circ\text{C}$ in CP for the copolymers as compared with those aqueous solutions. Studies of the association behavior of the PVCL copolymers in aqueous solution revealed that the formation of spherical micelles of the copolymers with around 30–70 nm in diameter took place over their CMCs. MTT test showed that at the concentrations ranging from 0.1 to 100 $\mu\text{g/ml}$, the amphiphilic PVCL copolymers were well tolerated in cell cultures.

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