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Novel Amphiphilic Poly(*N*-vinylpyrrolidone) Block Copolymer: Aggregative Behavior and Interaction with DNA

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Summary: Novel block copolymers poly(N-vinylpyrrolidone)-*block*-poly[(*tert*-butoxy) carbonyl] tryptophanamido-*N*′-methacryl thiourea (PVP-*b*-PTAM-I, II and III) were synthesized by atom transfer radical polymerization (ATRP) in DMF using PVP-Cl as macroinitiator. The structures of the copolymers were characterized by UV-vis and GPC-MALLS. The results revealed that the copolymers with controlled molecular weight and relatively low polydispersity (PDI < 1.34) were obtained through ATRP. By means of dynamic light scattering (DLS) and transmission electron microscopy (TEM), we demonstrated that copolymer PVP-*b*-PTAM self-aggregated to form spherical micelles in aqueous solution and the size of the micelles increased with increasing hydrophobic contents. The interaction of PVP-*b*-PTAM with DNA was explored using ethidium bromide (EB) quenching experiments. The interaction between PVP-*b*-PTAM and DNA markedly depended on both the copolymer concentration and composition. The PVP-*b*-PTAM-II and III with higher hydrophobic contents exhibited highly complexed DNA ability at low copolymer concentration, such as 0.017 mg/mL, relative to PVP-*b*-PTAM-I. As the copolymer concentration further increased for PVP-*b*-PTAM-II and III, they first exhibited a sharply decreased affinity for DNA and then kept steady. The interaction mechanism between the amphiphilic copolymers and the EB-DNA complex was discussed in detail.

Keywords: ATRP; DNA; interaction; PVP block copolymers; self-assembly

Introduction

In recent years, the field of non-viral gene therapy has gained increasing attention.[1–4] It is widely believed that non-viral gene therapy can overcome some problems inherent to current viral-based therapies.[5] Currently, an alternative approach for gene delivery evaluating non-ionic amphiphilic polymers are found to promote gene delivery in vivo, in tissues such as skeletal and cardiac muscle.[3,4] It has been discovered that muscular cells can be transfected by naked DNA,[6] but only at a very low efficiency. However, keeping in mind that conditioning DNA with neutral amphiphilic block copolymers such as the well known pluronics, block copolymers of poly(ethylene oxide) and poly(propylene oxide) were revealed to be much more efficient in transfection assays than naked DNA.[3,4,7]

Poly(*N*-vinylpyrrolidone) (PVP) is a very interesting polymer because it is well water-soluble, biocompatible, and has been extensively used in the pharmaceutical industry.[8] PVP and its copolymers have been applied in various drug delivery systems, including microspheres, nanoparticles, liposomes, and polymer conjugates.[9–13] The research of non-ionic amphiphilic PVP copolymers as a promising fluorescent probe for measurements of biomacromolecules[14] and a promoter for enhancement of DNA amplification by polymerase chain reaction[15] have been

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reported. PVP alone as a potential booster of the gene expression using naked DNA was also explored, and the results demonstrated that PVP could enhance gene expression of the naked DNA in tissues, such as skeletal muscle.[16,17]

In our case, we preferred non-ionic amphiphilic PVP block copolymers with a good biocompatibility and well-defined structure. From this point of view we prepared a well-defined PVP macroinitiator by atom transfer radical polymerization (ATRP).[18] In this contribution, a hydrophobic functional monomer *N-t-Boc*-tryptophanamido-*N′*-methacryl thiourea (TAM) has been synthesized,[15] and novel amphiphilic PVP block copolymers (PVP-*b*-PTAM) are synthesized via ATRP using the PVP-Cl as macroinitiator. The appended tryptophanamido moieties in the copolymer chain have ability to interact with DNA by intercalating, π-stacking, and hydrophobic interactions, as well as by hydrogen bonding.[19–21] The aggregation behaviors of the copolymers were investigated by dynamic light scattering (DLS) and transmission electron microscopy (TEM). The interaction between PVP-*b*-PTAM and DNA was explored in detail, and the interaction mechanism is discussed.

Experimental Part

Materials

Macroinitiator poly(*N*-vinylpyrrolidone)-Cl (PVP-Cl) (M_n 18 600 g/mol, PDI 1.26) was synthesized according to the procedure for the polymerization of *N*-vinylpyrrolidone (NVP).[18] The cyclic ligand, 5,5,7,12,12,14-hexamethyl-1,4,8,11-tetra-azacyclo-tetradecane (Me_6Cyclam), was synthesized according to the method described by Hay and Lawrance.[22] CuCl (Aldrich, 98%) were purified by stirring in acetic acid, washed with methanol, and then dried under vacuum. The hydrophobic monomer *N*-[(*tert*-butoxy)carbonyl]tryptophanamido-*N′*-methacryl thiourea (TAM) was prepared as described previously.[15] *N*,*N*-Dimethylformamide (DMF) (Shanghai Chemical Reagents Co.) was purified by distillation before use. Ethidium bromide (EB) and Herring Sperm DNA (Sigma Co.) were used as received. All the other reagents (Shanghai Chemical Reagents Co.) were used as received.

Characterization

Gel permeation chromatography-multi-angle laser light scatting (GPC-MALLS) is convenient for determination of the true molecular weight and molecular weight distribution of polymer without a standard sample. Number molecular weights M_n, and polydispersity index (PDI) M_w/M_n of the samples were determined by a DAWN DSP multi-angle laser photometer in DMF at a flow rate of 1.00 mL/min at 25 °C. UV-vis spectra were taken on a TU-1901 spectrometer (China) using DMF as solvent. Dynamic light scattering (DLS) (Malvern Nano ZS90 Instruments, Ltd., U. K.) was carried out with an aqueous solution of the block copolymers (0.6 mg/mL) for the determination of the particle size. The size and morphology of copolymer aggregation were observed by a JEM-100CXII transmission electron microscope (Japan). Steady- state fluorescence spectra were obtained on a Shimadzu RF-5301PC spectrometer (Japan).

Preparation of Amphiphilic Block Copolymer PVP-*b*-PTAM

In a typical run, a 25 mL round-bottom flask was charged with DMF (5 mL), TAM, macroinitiator PVP-Cl, and catalyst CuCl/Me_6Cyclam with the following relative molar ratio: TAM/PVP-Cl/CuCl/Me_6Cyclam = 5:1:1:1. The flask was sealed with a rubber septum and evacuated, and back filled with nitrogen three times. The flask was placed in a preheated oil bath and maintained at 60 °C for 24 h. The reaction mixture was diluted with $CHCl_3$ and passed through a neutral alumina column to remove the copper catalyst. The resulting solution was then concentrated and the copolymer was precipitated into excess diethyl ether to remove the unreacted hydrophobic momo-

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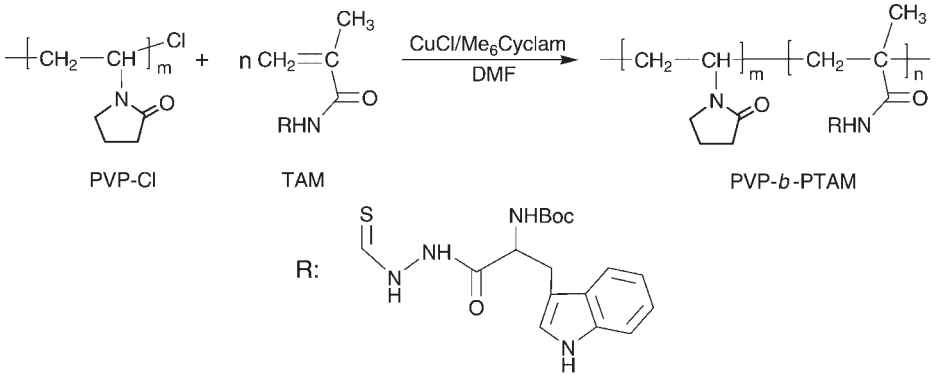

Figure 1.
Synthesis route of block copolymer PVP-*b*-PTAM via ATRP.

mer. The obtained copolymer was dried under vacuum to provide PVP-*b*-PTAM-I. This protocol was repeated varying the molar ratio of TAM to PVP-Cl to obtain amphiphilic block copolymers PVP-*b*-PTAM-II and III.

Results and Discussion

Synthesis and Characterization of the Block Copolymers

The synthesis route of amphiphlic block copolymer PVP-*b*-PTAM is given in Figure 1. UV-vis absorption and GPC-MALLS were used to characterize the composition of amphiphlic block copolymers (Table 1). The exact PTAM content of the copolymer was determined by UV-vis absorption at 270 nm, using a standard calibration curve experimentally obtained with a TAM/DMF solution, and M_n was calculated. The results showed that the contents of PTAM in copolymers increased with the molar ratio of TAM to PVP-Cl. The molecular weights and polydispersity index (PDI) M_w/M_n were determined by GPC-MALLS in DMF. Although the molecular weights determined by GPC-MALLS were close to theoretical values ($M_{n,th}$), the results determined and calculated by UV-vis were considered more exact.[23] The molecular weight distributions of copolymers determined by GPC-MALLS were narrow between 1.27 and 1.34. It demonstrated that well-defined PVP block copolymers were successfully obtained using PVP-Cl as macroinitiator via ATRP. These results confirmed that the molecular weight and the unit composition of the block copoly-

Table 1.
The compositions of the three block copolymers PVP-*b*-PTAM.

Entry	PVP-Cl[a)]	TAM[a)]	Yield[b)]	TAM[c)]	M_n[c)]	$M_{n,th}$[d)]	M_n[e)]	M_w/M_n[e)]
	m	*n*	%	mmol/g	g/mol	g/mol	g/mol	
PVP-Cl							18 600	1.26
PVP-*b*-PTAM-I	1	5	89	0.07	19196	20825	19940	1.27
PVP-*b*-PTAM-II	1	16	80	0.20	20415	25720	26820	1.30
PVP-*b*-PTAM-III	1	32	75	0.55	24629	32840	32750	1.34

a) *m* and *n* are the feed molar ratio in the polymerization;
b) Yield of copolymers determined by gravimetry;
c) Calculated from UV-vis absorption;
d) Based on the initial monomer to macroinitiator ratio assuming quantitative initiation;
e) Obtained from GPC-MALLS in DMF.

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mers could be controlled by varying the molar ratio of functional monomer to macroinitiator via ATRP.

Aggregation Behaviors of PVP Block Copolymers

The self-aggregation behaviors of the copolymer PVP-*b*-PTAM were explored by a fluorescent-probe method, dynamic light scattering (DLS), and transmission electron microscopy (TEM) observation.

Micelle Properties

The association behavior of PVP-*b*-PTAM in aqueous media was assessed by using a steady-state fluorescent-probe methodology, which allowed determining the critical micelle concentration (CMC) of PVP-*b*-PTAM. Pyrene was chosen as the extrinsic fluorescent probe because of its special photophysical properties.[24] The change in the microenvironment of pyrene was related to the change of its photophysical properties,[25] and could be detected by monitoring the fluorescence intensity ratio at 393 *vs* 373 nm (I_{393}/I_{373}). This ratio value was plotted as a function of the copolymer concentration in Figure 2.

As demonstrated in Figure 2, an abrupt increase in the I_{393}/I_{373} ratio of the fluorescent probe was observed upon increasing the copolymer concentration, indicating that micelle formation was taking place in solution. The concentration at the abrupt increase in the I_{393}/I_{373} ratio represents the CMC value,[26] which was determined as 25.1 mg/L for PVP-*b*-PTAM-II. The CMC values of PVP-*b*-PTAM-I and III obtained by same operation were 35.2 and 20.2 mg/L, respectively (Table 2). The results show that the CMC of the copolymer decreases with an increase of the hydrophobic segment content in the copolymer with the same hydrophilic segment.

Table 2.
Characteristic solution properties of the copolymers.

Sample	CMC[a)]	*D*[b)]	Mean diameter[c)]
	mg/L	nm	nm
PVP-*b*-PTAM-I	35.2	–	64 ± 8
PVP-*b*-PTAM-II	25.1	30 ± 5	121 ± 12
PVP-*b*-PTAM-III	20.2	210 ± 20	260 ± 19

[a)] Obtained from fluorescence probe measurements;
[b)] Diameter (*D*) was obtained by TEM;
[c)] Measured average hydrodynamic diameter from DLS.

Aggregation Behaviors

To further explore the association behaviors of PVP-*b*-PTAM, the DLS measurements were also carried out with an aqueous solution of the block copolymers (0.6 mg/mL). The size distributions of the solutions revealed the existence of copolymer aggregates with diameters that ranged

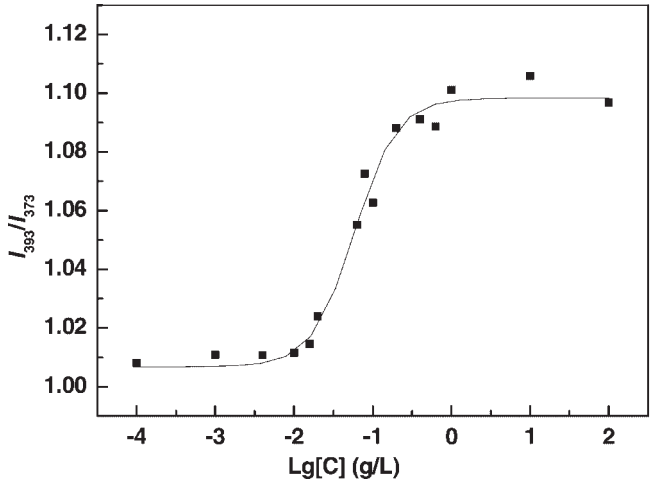

Figure 2.
Plot of I_{393}/I_{373} in the excitation spectra *vs* the concentration of the PVP-*b*-PTAM-II in aqueous solution, pH = 5.8.

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in size from about 64 to 260 nm, depending on the copolymer composition (Table 2). It suggested that the size of the copolymer aggregates increased as the amount of hydrophobic segments incorporated increased. TEM photograph of PVP-*b*-PTAM-II also indicated that the copolymer self-assembled mostly into spherical micelles of around 30 nm in diameter (Figure 3). When the hydrophobic PTAM block contents increased, larger polymer micelles with spherical aggregates of about 210 nm were formed for PVP-*b*-PTAM-III (not shown). Compared with the mean sizes of the copolymer micelles determined by DLS, the sizes observed by TEM in the dried model were smaller. The difference in sizes determined by DLS and TEM could be attributed to the dissimilar state (wet or dry) of copolymer micelles, especially for PVP-*b*-PTAM-I and II with lower hydrophobic contents. It shows that the aggregates of PVP-*b*-PTAM-I and II tend to shrink markedly during the drying process. A similar result also was reported for amphiphilic PVP block polymers.[27]

Interaction of the PVP-*b*-PTAM Copolymer with DNA

EB Quenching Experiments

Intercalation of EB into the DNA (to form EB-DNA complexes) could cause a marked increase in the fluorescence of EB.[28] It has been demonstrated that cationic copolymers can reduce the fluorescence of EB-DNA complexes due to binding of cationic copolymers to the DNA with subsequent displacement of EB.[29,30] To further explore the interaction of the PVP-*b*-PTAM copolymer with DNA, the fluorescence spectra were also monitored by recording the fluorescence of EB-DNA complexes in the presence of the copolymer. The addition of increasing amounts of the PVP-*b*-PTAM copolymer to an EB-DNA complex system resulted in a reduction of the relative fluorescence intensity (I/I_0), where I_0 was the fluorescence intensity of the EB-DNA with no copolymer added (Figure 4). Figure 4 shows that the maximal decrease of I/I_0 was about 15, 21, and 25% for PVP-*b*-PTAMM-I, II, and III, respectively, with around 0.017 mg/mL of copolymer. It indicated that the copolymer with a higher hydrophobic moiety content exhibited a stronger ability to interact with the EB-DNA complexes at low copolymer concentration, such as under ~0.017 mg/mL. Upon further increasing the copolymer concentration, for PVP-*b*-PTAM-I, the fluorescence quenching tended to remain steady; while for PVP-*b*-PTAM-II and III, the relative fluorescence I/I_0 first jumped rapidly and then also leveled off. This implies that

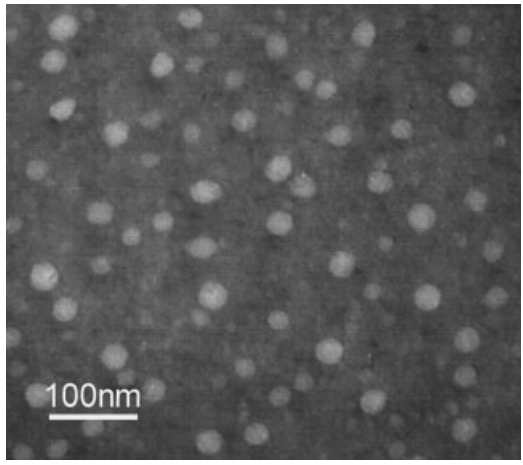

Figure 3.
TEM photograph of PVP-*b*-PTAM-II aqueous solution (0.6 mg/mL).

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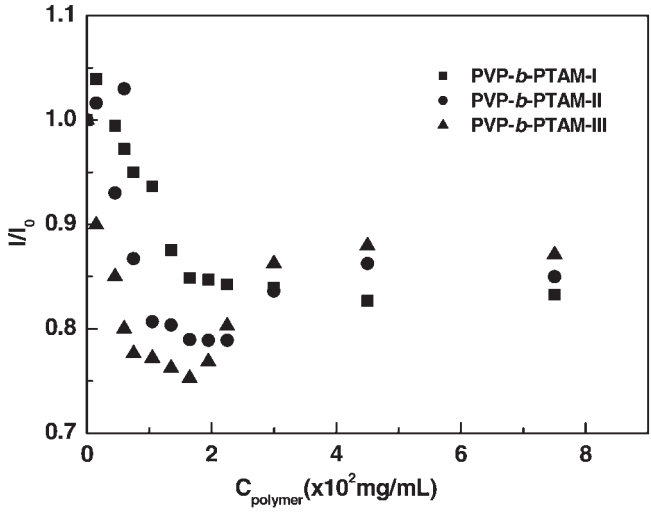

Figure 4.
Variation of EB-DNA fluorescence at 596 nm versus the different concentration of the copolymers in Tris-HCl buffers (pH 7.4), at 25 °C ([EB] = 1 μg/mL, [DNA] = 5 μg/mL).

copolymer self-aggregation and the interaction between the copolymer and the DNA complexes are co-existent in the system in the range of higher copolymer concentration, such as above ~0.017 mg/mL. The results from EB fluorescence quenching studies suggested that the interaction between the block copolymer and the DNA is highly dependent on the copolymer concentration and composition, such as hydrophobic moiety content.

Interaction Mechanism

Cationic copolymers formed strong charge-compensated complexes with anionic phosphate groups on the DNA backbone via electronic interactions.[29,30] While for non-ionic copolymers, they could complex with DNA through multiple hydrogen bonding and hydrophobic interactions.[31] Non-covalent interactions between tryptophan residues and DNA, which include hydrogen bonding, π-stacking and intercalating interactions, have been reported.[19–21] Herein, we propose that the possible binding modes between PVP-*b*-PTAM copolymers and DNA would be π-stacking, intercalating, and hydrophobic interactions, as well as multiple hydrogen bonding. The experimental results showed that the interaction mechanism between PVP-*b*-PTAM and DNA was related to the copolymer composition and copolymer concentration.

It is most likely that the quenching of EB-DNA fluorescence occurred when the copolymer concentration increased and interacted with DNA via non-covalent bonding. At lower copolymer concentrations, the linear reduction of relative fluorescence with increasing copolymer concentration may be attributed to a partially intercalating interaction of the tryptophan residues into DNA. This phenomenon was especially dramatic for PVP-*b*-PTAM-III with higher contents of PTAM segments. As the copolymer concentration further increased, PVP-*b*-PTAM-II and III, with a higher content of PTAM segments, was not able to complex with DNA and exhibited a marked decrease in binding to DNA compared with the PVP-*b*-PTAM-I copolymer. The intra- and inter-molecular hydrophobic interaction of PVP-*b*-PTAM would become dominant with increasing copolymer concentration. For PVP-*b*-PTAM-III, the stronger hydrophobic interaction facilitated self-assembly of the

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copolymer, which resulted in some reduction of the interaction ability between the copolymer and DNA. The transfer of the double-modes between self-assembling and the interacting with DNA in the system is very sensitive to copolymer concentration and composition and finally achieves equilibrium (Figure 4).

The results demonstrated that the interaction mechanism between PVP-*b*-PTAM and DNA was a complicated course. Possible bonding modes such as π-stacking, intercalating, hydrophobic interactions, and multiple hydrogen bonding could facilitate interaction between the copolymer and DNA. The interacting ability of the copolymer was not always proportional to its concentration, and also depended on its composition. The balance between the intra- and inter-molecular hydrophobic interaction and the interaction of the copolymer with DNA played a primary role in influencing the complexing ability of the copolymer with DNA.

Conclusion

In summary, novel amphiphilic block copolymers PVP-*b*-PTAM have been synthesized by ATRP of the hydrophobic monomer TAM initiated with PVP-Cl as macroinitiator. The copolymers had a relatively narrow PDI between 1.27 and 1.34, and the content of PTAM in the copolymers increased with the molar ratio of TAM to PVP-Cl. DLS and TEM observation indicated that the block polymers in water self-assembled into spherical micelles. For the PVP-*b*-PTAM copolymers, fluorescence quenching studies of EB-DNA complexes revealed that the copolymers could interact with DNA efficiently. The ability of the copolymer to interact with DNA was highly dependent on both the concentration and composition of the block copolymers. The strong intra- and inter-molecular hydrophobic interaction facilitated self-assembly of the copolymers and reduced the complexing ability of the copolymer with DNA.

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