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Comparison of two polymeric carrier formulations for controlled release of hydrophilic and hydrophobic drugs

Haiyan Chen · Yueqing Gu · Yuzhu Hu

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Abstract Two temperature sensitive drug carriers, poly (*N*-isopropylacrylamide-co-acrylic acid) (PNIPA-co-AA) and poly (*N*-isopropylacrylamide-vinyl pyrrolidone-acrylic acid) (PNIPA-VP-AA), were successfully synthesized through free radical mechanism. The diameters of PNIPA-co-AA and PNIPA-VP-AA particles can be regulated to be less than 100 nm, which were related to surfactant sodiumdodecyl sulfate and initiator ferrous ammonium sulfate, respectively. The lower critical solution temperature (LCST) of them can be manipulated to be higher than 40 °C, which was correlated to amount of acrylic acid (AA) that was copolymerized with NIPA. Hydrophilic anti-tumor drugs, 5-fluorouracil (5-Fu) and hydrophobic drug thalidomide were entrapped into PNIPA-co-AA and PNIPA-VP-AA, respectively. For different interaction mechanism between drug and carrier, 5-Fu was prone to be entrapped in PNIPA-co-AA with loading efficiency larger than 10% (w/w), while thalidomide was entrapped in PNIPA-VP-AA up to 80% (w/w). Fluorescein, an angiography agent, was used to evaluate the drug loading mechanism between PNIPA-VP-AA and poor water-soluble drug. In vitro drug release behavior from these two drug carriers were significantly different and showed temperature dependent, which demonstrated that PNIPA-co-AA and PNIPA-VP-AA are promising candidates for different controlled drug delivery system.

H. Chen · Y. Gu (✉)
Department of Biomedical Engineering, School of Life Science and Technology, China Pharmaceutical University, Nanjing, P. R. China
e-mail: guyueqing@hotmail.com; njhuyuzu@jlonline.com

H. Chen · Y. Hu
Department of Analytical Chemistry, School of Basic Science, China Pharmaceutical University, Nanjing, P. R. China

Introduction

Drug distribution in biological tissues depends on the drug's physical-chemical property, especially on its affinity to specific tissue [1–3]. Lipo-water distribution coefficient of drugs is the main factor to influence its affinity to tissue. Thalidomide is a poorly water-soluble and novel anti-tumor drug in the treatment of refractory multiple myeloma [4, 5]. 5-fluorouracil (5-Fu) is a water soluble anti-cancer drug, targeting cancer of the digestive system and breast, among others [6]. Enhancement of anticancer activity and intra-tumor distribution of these two representative hydrophilic and hydrophobic anticancer drugs is essential for chemotherapy [7–9]. Targeted and controlled drug carriers may offer a promising way to achieve this goal. With the new drug carrier, the retention time of drug in specific tissue site will increase and the toxic effect on normal tissue will decrease. Thus, a better therapeutical outcome can be achieved.

Polymeric hydrogel poly *N*-isopropylacrylamide (PNIPA), which is sensitive to environmental temperature stimulus and undergoes a volume phase transition at lower critical solution temperature (LCST) of about 34 °C [10, 11], is one of the most promising novel formulations in the development of smart drug delivery system [12–14]. When *N*-isopropylacrylamide (NIPA) copolymerized with other hydrophilic monomers with specific functional group such as acrylamide [15], *N*-(hydroxymethyl) acrylamide [16], the obtained co-polymeric hydrogel may have better hydrophilicity and site-specific function compared to PNIPA itself. And thus, water-soluble drug can be entrapped into its network. In contrast, if hydrophobic monomers were copolymerized with NIPA, the co-polymeric particles may form stable micelle aggregating in aqueous solution with hydrophobic cores mainly composed

of hydrophobic moiety. This sort of co-polymeric micelle may load more hydrophobic drug [17, 18].

Taking advantage of its environmental sensitivity, PNIPA particle can be used as a targeted drug carrier for sustained drug release. The particle size is a key factor in targeted drug delivery. As reported, the particles with different size will accumulate in different organs. Superparamagnetic iron oxide (SPIO) agent AMI-121 with 300 nm particle size, which is approved by FDA, targets to GI lumen, while AMI-277 with 20–40 nm size targets to lymph nodes and blood pool [19]. To avoid being scavenged by the reticuloendothelial system (RES) [20, 21] and improve tumor accumulation potential, particles size need to be controlled less than 100 nm, which is also our purpose for this work.

This paper describes preparation of temperature sensitive copolymeric nanoparticle formulations, poly (*N*-isopropylacrylamide-co-acrylic acid) (PNIPA-co-AA) and poly (*N*-isopropylacrylamide-vinyl pyrrolidone-acrylic acid) (PNIPA-VP-AA). Similarity and difference between PNIPA-co-AA and PNIPA-VP-AA were compared. Two types of anti-cancer drug with different water-solubility, 5-Fu and Thalidomide, were entrapped into different carriers and in vitro release profiles of them were compared. The mechanism of drug loading between PNIPA-VP-AA and poor water-soluble drug was also investigated.

Methods

Materials

N-Isopropylacrylamide (NIPA) was obtained from Aldrich-Chemie (Steinheim, Germany). N, N′-methylene-bis-acrylamide (BIS) with analytical grade was obtained from Tianjing Chemical Institute (Tianjing, China). Ferrous ammonium sulfate (FAS), ammonium persulfate (APS), *N*-vinyl pyrrolidone (VP) and acrylic acid (AA) were obtained from Huakang Technology Company (Jiangsu, China). Fluorescein was purchased from Shengyang Regent Company (Shengyang, China). Thalidomide was kindly given by Changzhou Medicals (Jiangsu, China) as a gift. All other chemicals were analytical grade. Water for all reactions, solution preparation and polymer purification was double distilled.

Preparation of PNIPA-co-AA, PNIPA-VP-AA and PNIPA nanoparticles

Two PNIPA-co-AA copolymers with NIPA: AA ratios of 80:20 mol/mol (PNIPA-co-AA1) and 65:35 mol/mol (PNIPA-co-AA2) were synthesized by precipitation polymerization method [11]. In details, 3.779 g *N*-isopropylacrylamide (NIPA), 0.070 g N, N′-methylene-bis-acrylamide (BIS), 0.395 g sodiumdodecyl sulfate (SDS), and different amount of acrylic acid (AA) were added to 100 mL double distilled water and stirred under nitrogen purging for 20 min at room temperature. The polymerization was initiated by adding 0.166 g Potassium persulfate (KPS) and the process of polymerization reaction lasted for 4 h under nitrogen atmosphere at a temperature of 70 ± 2 °C. The nanohydrogels were cooled down to room temperature and then dialyzed (10 kDa molecular weight cutoff) for 2 weeks in double distilled water with fresh water being changed several times each day. The dialyzed solution of nanohydrogel was lyophilized immediately to obtain dry powder for subsequent use. Lyophilized powder is easily re-dispersed in aqueous solution.

PNIPA-VP-AA nanoparticles with different NIPA: AA ratios of 80:20 mol/mol (PNIPA-VP-AA1) and 65:35 mol/mol (PNIPA-co-AA2) were prepared [22]. The detail procedure is as follows: 0.302 g NIPA, 33 μL VP and different amount of AA were added to 90 mL distilled water. Cross-linked agent BIS (56 mg) was also added to cross-link the polymer chain. Nitrogen gas was purged at least for 30 min to remove dissolved oxygen. 50 μL of saturated APS solutions and 100 μL of FAS (0.045 g/mL) were added to initiate the polymerization reaction. The polymerization was carried out at 29 ± 1 °C for 20 h in nitrogen atmosphere. The aqueous solution was cooled down to room temperature and then dialyzed (10 kDa cutoff) for 3 days in double distilled water with fresh water being changed five times each day. The dialyzed aqueous solution of nanoparticles was lyophilized to obtain dry powder.

To compare PNIPA-co-AA with well-known hydrogel, PNIPA nanohydrogel with constant LCST was synthesized according to the procedure of PNIPA-co-AA, except that AA monomer was not included.

Characterization of PNIPA-co-AA and PNIPA-VP-AA nanoparticles

Measurement of nanoparticle size by Laser Scattering Analyzer (LSA)

The mean particle diameter and poly-dispersed index (P.I.) of PNIPA-co-AA1 and PNIPA-VP-AA1 were determined by Mastersizer 2000 Laser Particle Size Analyzer (Malvern, British) at 25 °C. A helium-neon laser (10 mW max, Wand wavelength of 633 nm) was used as the light source. P.I. was utilized to evaluate the distribution of nanoparticle population.

Determination of LCST of PNIPA-co-AA and PNIPA-VP-AA nanoparticles

Optical transmittances at 500 nm of PNIPA-co-AA and PNIPA-VP-AA aqueous solutions (3%, w/w) were monitored by Lambda 35 UV-visible spectrophotometer (Perkin Elmer, America). Sample and reference cells were thermostat adjusted from 25 °C to 50 °C. And at least 3 min was allowed for the sample to reach equilibrium temperature. The transmittance of nanoparticles solution was measured as a function of temperature. LCST was defined as the temperature at which the transmittance of the nanoparticle solution is 10% deviation from the initial value.

Measurement of drug loading efficiency

Measurement of the 5-Fu loading efficiency in PNIPA-co-AA

PNIPA-co-AA nanohydrogel solutions were incubated with 5-Fu solution at 20 °C for 24 h. The 5-Fu was entrapped into the nanohydrogel and the drug loaded nanohydrogel solutions were dialyzed (10 kDa molecular weight cutoff) regularly with fresh double distilled water until there was no 5-Fu to be measured outside the dialyzed bag. The solution in dialysis bag was then lyophilized to obtain dry powder for subsequent use. Each of the dialyzed solutions were collected for the measurement of free 5-Fu by a UV spectrophotometer at 265 nm. The amount of entrapped 5-Fu was calculated as the 5-Fu feeded in the solution subtracted the 5-Fu measured in dialyzed solutions. Drug loading efficiency was defined as $E\% = ([5\text{-Fu}]_{entrapped})/([5\text{-Fu}]_{total}) \times 100$.

Measurement of thalidomide loading efficiency in PNIPA-VP-AA

PNIPA-VP-AA is water-soluble and has temperature-sensitivity in water, but its physical property may change greatly in water-organic solvent systems. To investigate the influence of organic solvents on LCST of PNIPA-VP-AA, transmittances (500 nm) of PNIPA-VP-AA2 in different water-organic solvent systems were monitored by UV-visible spectrometer when the environmental temperature (45 °C) was kept higher than LCST of PNIPA-VP-AA2 aqueous solution. The drug loading process included organic solution dropping into aqueous solution and this process may change the physical property (especially LCST) of PNIPA-VP-AA. This study may provide a reference for water-insoluble drug loading.

Physical entrapment of thalidomide in PNIPA-VP-AA nanoparticle was carried out as follows: Thalidomide was dissolved in N, N′-dimethylformamide (10 mg/mL), which was added into 10 mL PNIPA-VP-AA solution (4 mg/mL) slowly with vigorously stirring until turbidity appeared. The solution was then filtered through micro-pore membrane (0.2 μm) and thalidomide blocked by filter membrane was quantified by using UV spectrometer. The filtrate was then lyophilized to get dry powder for subsequent use. Drug loading efficiency was defined as $E\% = ([\text{thalidomide}]_{total} - [\text{thalidomide}]_{blocked})/([\text{thalidomide}]_{total}) \times 100$.

In vitro release of drug loaded particles in aqueous buffer

In vitro drug release behaviors of 5-Fu and thalidomide loaded particles at different temperatures (below and above the LCST) were investigated. A certain amount of lyophilized powder of PNIPA-co-AA2 loaded with 5-Fu and PNIPA-VP-AA2 loaded with thalidomide were dispersed in 10 mL phosphate buffer of pH 7.4 and placed into a dialysis bag with molecular weight cut-off of 12,000. The dialysis bags were then submerged in phosphate buffer at either 25 °C or 45 °C. At fixed time intervals, the external aqueous phase was replaced by the fresh buffer and drug concentration in the sampled solution was measured.

Study of drug loading mechanism of PNIPA-VP-AA

To study the drug loading mechanism of PNIPA-VP-AA, the influences of different amount of PNIPA-VP-AA2 on fluorescent intensity of fluorescein was monitored. An appropriate concentration of fluorescein aqueous solution was selected by establishing linear range of it to exclude self-quenching possibility. The fluorescent intensity of fluorescein with different concentration from 6×10^{-6} mg/mL to 9×10^{-5} mg/mL was studied. Fluorescence spectra of fluorescein were measured by using a Shimadzu RF5300-PC Fluorescence Spectrophotometer with a 150 W xenon lamp. The xenon lamp in our own laboratory acts as the excited light sources whose light spectrum range is of 200–600 nm. The wavelength of 320 nm was selected to excite the fluorescence. The emission spectrum with peak intensity at 520 nm was obtained. Temperature for measurement was kept at 25 °C.

Various amounts of PNIPA-VP-AA2 were added into 10 mL fluorescein solutions with selected concentration (3×10^{-5} mg/mL) and mixed vigorously for at least 5 min. Fluorescent spectra of these solutions were measured at 25 °C.

Results and discussions

Size distribution of PNIPA-co-AA and PNIPA-VP-AA

The size distribution of a typical PNIPA-co-AA nanoparticle is shown in Fig. 1. The particles have an average size of 42 nm at 25 °C with narrow size distribution. Ram et al reported that the SDS can regulate the PNIPA particles size within the range of 100–2,500 nm [23], which is too large for particle to accumulate in tumor sites. In this basis, we adjusted the feed amount of SDS to obtain the PNIPA-co-AA nanohydrogel with size distribution less than 50 nm. As expected, electrostatic repulsion force of the monomers in the process of reaction is strengthened accordingly with the increase of the surfactant sodiumdodecyl sulfate (SDS), which results in the shorter chain of the polymer, and thus, the smaller size of polymeric nanoparticles. However, irregular size distribution of PNIPA-co-AA nanohydrogel was caused by excess SDS feeding, which was observed in our experiments.

Figure 2 shows the size distribution of PNIPA-VP-AA1 nanoparticles. The particles have minimal size around 10 nm and also show narrow size distribution. In the reaction process, we found that FAS is the main factor to determine the particle size and morphology. The control of feed amount of FAS is very important for inadequacy or excess of FAS will probably result in failure in the synthesis of particles. Also, we found that it's necessary to use freshly prepared FAS solution because FAS is easily oxidated in air.

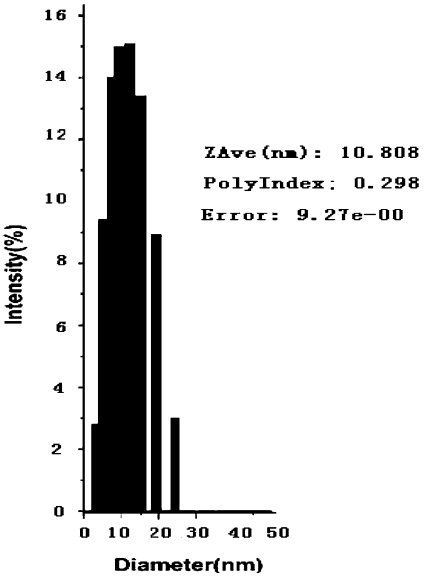

Fig. 2 Size distributions of PNIPA-VP-AA1 particles measured by Laser Scattering Analyzer measurement at 25 °C

LCST of PNIPA-co-AA and PNIPA-VP-AA nanoparticles

Figure 3 shows the transmittance transitions of both types of nanoparticle solutions in different environmental temperature. As defined in Section Methods, LCST was the temperature at which the transmittance of the nanoparticles solution is 10% deviation from the initial value. PNIPA acted as a reference for its LCST (33 °C) was constant and well known. LCST of PNIPA-co-AA and PNIPA-VP-AA were raised to higher value compared to PNIPA. Although

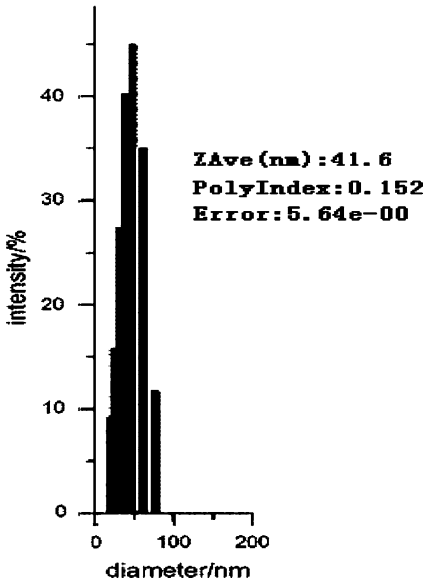

Fig. 1 Size distribution of PNIPA-co-AA1 copolymeric nanohydrogel measured by Laser Scattering Analyzer measurement at 25 °C

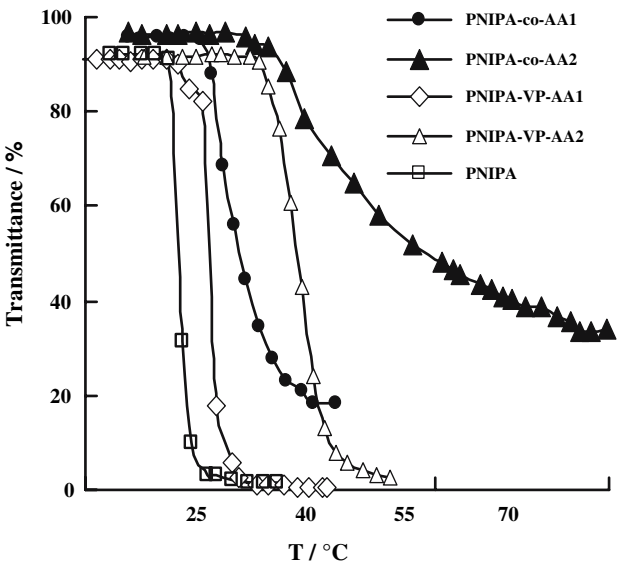

Fig. 3 Transmittance (at 500 nm) of PNIPA, PNIA-co-AA (1, 2) and PNIPA-VP-AA (1, 2) nanoparticles plotted against temperature

Table 1 LCST for different prescription of PNIPA-co-AA and PNIPA-VP-AA

Nanoparticle	NIPA/AA (mol/mol)	VP/NIPA (mol/mol)	LCST (°C)
PNIPA-co-AA1	80:20	/	36 ± 1
PNIPA-co-AA2	65:35	/	42 ± 1
PNIPA-VP-AA1	80:20	0.015	37 ± 1
PNIPA-VP-AA2	65:35	0.015	43 ± 1

monomer VP was copolymerized to form PNIPA-VP-AA, PNIPA-co-AA and PNIPA-VP-AA with the same NIPA: AA ratios have similar LCST, which was shown in Table 1. However, the transmittance profile of PNIPA-VP-AA is much steeper than that of PNIPA-co-AA, which indicated PNIPA-VP-AA is more sensitive to temperature, as shown in Fig. 3.

LCST of PNIPA-co-AA and PNIPA-VP-AA is strongly influenced by hydrophilic monomer AA. The more hydrophilic moiety causes stronger hydrogen bond interaction in the aqueous solution, requiring more energy to destroy the hydrogen bond. And thus, the collapsing of polymer nanoparticles will occurred in higher temperature, and the particles will have higher LCST.

Different therapeutic cases require different phase transition temperature. LCST higher than physiological temperature but lower than clinical hyperthermia (between 38 °C and 42 °C), which is easily and safely achieved without inducing deleterious side effects on surrounding healthy tissue [24, 25], is especially benefic for the targeted and controlled drug release in specific-sites (such as tumor sites) by regulating the tissue temperature.

Comparison of 5-Fu and Thalidomide loaded into different nanoparticle carriers

PNIPA-co-AA nanohydrogel is hydrophilic and contained a lot of water content in its interspaces when the environmental temperature is below its LCST. Accordingly, 5-Fu with good solubility can be dissolved in aqueous solution and then be entrapped in PNIPA-co-AA nanohydrogel with good loading efficiency up to 10% (w/w).

Using similar method, the loading efficiency of thalidomide entrapped into PNIPA-co-AA nanohydrogel was only 0.8% (w/w). Results demonstrated that PNIPA-co-AA is a promising carrier for water-soluble drug in the controlled and targeted drug delivery system.

PNPIA-VP-AA is composed of hydrophilic and hydrophobic moiety. In aqueous solution, its hydrophilic moiety exposes to water phase while hydrophobic moiety hides inside and then forms micelle. Therefore, PNPIA-VP-AA is suitable for loading water-insoluble drug into its inner cores. When thalidomide was loaded into PNIPA-VP-AA carrier, the loading efficiency can be higher than 80% (w/w). In the same way, we also tested the 5-Fu drug loading to the PNIPA-VP-AA and the results showed that its loading efficiency was only 0.4% (w/w).

During thalidomide loading process, organic solvent DMF was added into PNIPA-VP-AA aqueous solution and this may influence the temperature-sensitivity of PNPA-VP-AA. Therefore, influence of typical organic solvent/water systems on transmittance (at 500 nm) of PNIPA-VP-AA2 (LCST = 43 °C) aqueous solution (3 mg/mL) at a temperature of 45 °C was studied, which is shown in Fig. 4. When the value of V_{DMF}/V_{water} is around 0.2, the transmittance of PNIPA-VP-AA solution has a sudden change, indicating the physical property of PNIPA-VP-AA changes at this value. And the transition value for $V_{ethanol}/V_{water}$ and $V_{methanol}/V_{water}$ are 0.3 and 0.4, respectively. If the transmittance (at 500 nm) of PNIPA-VP-AA in organic solvent/water solution is approximated to 0% at a temperature higher than its LCST, the temperature-sensitive property of PNIPA-VP-AA dose not change and this system is suitable for drug loading study. And if the transmittance is close to 100%, the situation goes to the reverse phase. From the figure, we may conclude that temperature-sensitivity of PNPA-VP-AA disappears when the ratio of organic solvent to water exceeds critical value. Therefore, in Thalidomide loading process, we should control the ratio of DMF/water below 0.2. This provided a reference for

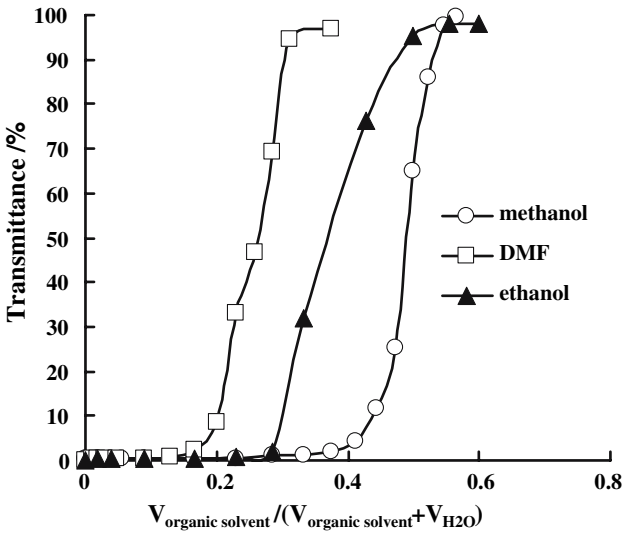

Fig. 4 Influence of typical organic solvent-water systems on transmittance (at 500 nm) of PNIPA-VP-AA2 aqueous solution (3 mg/mL). The environmental temperature (45 °C) was kept higher than LCST of PNIPA-VP-AA2 aqueous solution

hydrophobic drug loading into PNPA-VP-AA aqueous solution.

In vitro release kinetic studies

Figure 5 shows the release profile of 5-Fu from PNIPA-co-AA2 and Thalidomide from PNIPA-VP-AA2 when the environmental temperature higher or lower than their LCST. 5-Fu released from PNIPA-co-AA2 was so slow that it was approximated to 0% (w/w) at the first 20 h and only 1% (w/w) released in 48 h at 25 °C. When environmental temperature for 5-Fu release was kept at 45 °C, nearly 25% of the drug was released in 48 h. However, profile for thalidomide released from PNIPA-VP-AA2 was different. At 25 °C (<LCST), the release ratio of thalidomide was nearly 65% (w/w) in 10 h, while 95% (w/w) of the drug released from PNIPA-VP-AA2 in 0.5 h at 45 °C (>LCST). Because micelle is not so stable, so drug may release from the inner cores even the temperature is below LCST of PNIPA-VP-AA.

Temperature-responsive nanoparticles undergo structural change when environmental temperature goes over their LCST. Accordingly, drug release from PNIPA-co-AA and PNIPA-VP-AA all demonstrated different kinetic profile when environmental temperature was kept at 25 and 45 °C. In another way, thalidomide showed different release behavior from 5-Fu. Thalidomide released from PNIPA-VP-AA was much faster than 5-Fu from PNIPA-co-AA at 25 °C or 45 °C, which is corresponded to different transmittance transition mechanism when temperature is lower or higher than their LCST. For the micelle structure of PNIPA-VP-AA, water can invade into the inner cores of micelles. At the same time, thalidomide entrapped in the inner cores will partly dissolve in the water and be brought out from the drug carrier. As a result, thalidomide released from PNIPA-VP-AA even at a temperature lower than LCST of drug carrier and the release rate was much faster than that of 5-Fu from PNIPA-co-AA.

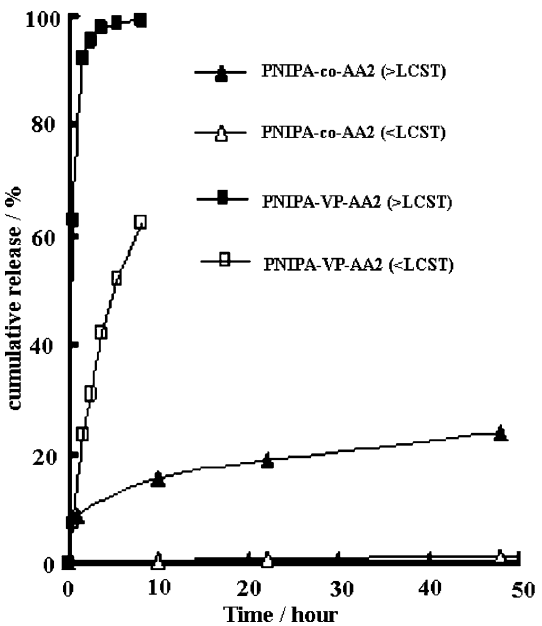

Fig. 5 The release profile of 5-Fu from PNIPA-co-AA2 and thalidomide from PNIPA-VP-AA2 when the environmental temperature lower or higher than their LCST. 25 and 45 °C were selected as the environmental temperature

Interaction between PNIPA-VP-AA and fluorescein

To study the interaction between hydrophobic drug and PNIPA-VP-AA, fluorescein was used as a water-insoluble drug model. Fluorescein is easily influenced by environmental factors. To exclude the influence of self-quenching, appropriate concentration of fluorescein was selected according to the linear range between fluorescent intensity and fluorescein concentration. As showed in Fig. 6, the linear range of fluorescein is from 6×10^{-6} mg/mL to 9×10^{-5} mg/mL, and 5×10^{-5} mg/mL was selected as the concentration to study interaction between PNIPA-VP-AA and fluorescein. Figure 7 shows the emission spectra of fluorescein solutions (5×10^{-6} mg/mL) with various amount of PNIPA-VP-AA added in the solution. Concentration of PNIPA-VP-AA was also plotted against F_x/F_0. F_0 is the fluorescent intensity of 5×10^{-6} mg/mL pure fluorescein solution, while F_x is fluorescent intensity of fluorescein solution with different amount of PNIPA-VP-AA. The multinomial formula was introduced to fit the relationship between PNIPA-VP-AA quantity and F_x/F_0, and R^2 of this formula is 0.998. Figure 7 displayed that more PNIPA-VP-AA2 added, more fluorescent intensity of fluorescein in aqueous solution would be.

Fluorescein is poor water-soluble, so fluorescent intensity of its aqueous solution is low. When PNIPA-VP-AA added into the aqueous solution, micelle structure formed. As long as fluorescein entrapped to the inner cores of PNIPA-VP-AA micelle, its fluorescent intensity strengthened. As a result, more PNIPA-VP-AA added, more fluorescein entrapped inside the hydrophobic core, and thus, stronger fluorescence intensity appeared.

Further, we observed that the fluorescence reach the maximal value 5 min after the addition of PNIP-VP-AA2, which indicated an equilibrium process existed when hydrophobic drug entrapped into PNIPA-VP-AA. To increase drug loading efficiency, more hydrophobic drug needs to be added before the equilibrium arrived.

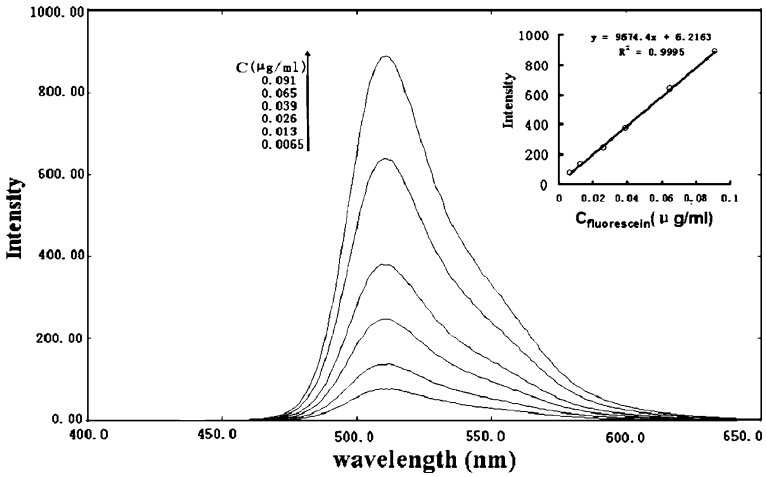

Fig. 6 Emission spectra of fluorescein with different concentration at 25 °C and standard curve of different fluorescein concentration plotted against fluorescent intensity. The concentration range of fluorescein is from 6×10^{-6} mg/mL to 9×10^{-5} mg/mL. The wavelength of 320 nm was selected to excite the fluorescence. The emission spectrum with peak intensity at 520 nm was obtained

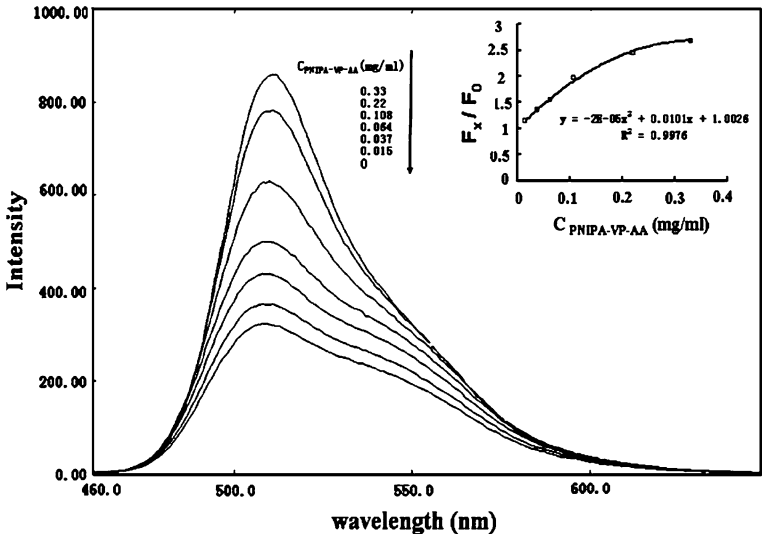

Fig. 7 Emission spectra of fluorescein solutions (3×10^{-5} mg/mL) with various amount of PNIPA-VP-AA added in the solutions. Concentration of PNIPA-VP-AA2 also plotted against F_0/F_x. F_0 is the fluorescent intensity of 3×10^{-5} mg/mL fluorescein solution while F_x is fluorescent intensity of fluorescein solution with different amount of PNIPA-VP-AA2. The wavelength of 320 nm was selected to excite the fluorescence. The emission spectrum with peak intensity at 520 nm was obtained

Conclusion

Two temperature-sensitive drug carrier, PNIPA-co-AA and PNIPA-VP-AA were successfully obtained. Their size can be controlled with in range of 100 nm and LCST was improved to be larger than 37 °C. Hydrophilic and hydrophobic drugs, 5-Fu and thalidomide were entrapped into PNIPA-co-AA and PNIPA-VP-AA, respectively. In vitro drug release behavior of these two drug carriers showed significantly temperature dependent, which all demonstrated that PNIPA-co-AA and PNIPA-VP-AA are promising candidates for targeted and controlled drug delivery system.

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