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Polymeric Hydrogel Nanocapsules: A Thermo and pH Dual-responsive Carrier for Sustained Drug Release

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Abstract: Hydrogel capsules show attractive prospects in drug delivery recently because of high drug loading and sustained release behavior. In this study we reported a simple and convenient route to fabricate poly (acrylic acid)-poly (N-isopropylacrylamide) (PAA-PNIPAm) hydrogel capsules by using hydroxypropylcellulose-poly (acrylic acid) (HPC-PAA) complexes as the templates. The capsules showed a high drug loading (∼280% to the weight of capsules) for Doxorubicin hydrochloride. The release of drug from the capsules was responsive to the temperature and pH of the surroundings, showing a low-rate but sustained release behavior favorable for low-toxic and long-term therapy. Together with the convenient preparation, high drug loading, dual responsivity as well as the sustained release feature, it is implied that this polymeric hydrogel capsule might be a promising candidate for new drug carriers.

Keywords: Hydrogel capsules; Sustained release; High drug loading; Dual responsivity

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Introduction

In the past few decades, polymer-based drug vehicles have attracted significant interests in the development of drug delivery system mainly owing to the tailorability, easy-functionalization and biocompatibility of polymers [1-5]. Polymeric drug vehicles such as nanoparticles, micelles, nanogels, micro/nano capsules are very advantageous in the control of drug distribution in the living organism, prolonging the biological activity of drugs, improving the therapeutic effect and reducing the administration frequency [6-10]. It is revealed that capsule-like drug carriers could easily realize high drug payload and achieve a better sustained release behavior due to their larger inner cavities [11-14], hence many efforts have been devoted to the synthesis of polymeric capsules. Layer-by-Layer (LbL) assembly is a widely used route to the preparation of polymeric capsules with well-defined chemical and structural properties, which potentially afford a large degree of control over functional properties of polyelectrolyte containers, such as their permeability to low molecular weight compounds or macromolecules [15,16]. For example, Möhwald's group has reported a series of pH-sensitive LbL capsules, of which the wall could be triggered by pH or ionic strength to switch on and

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off between 'open' and 'closed' state for guest macromolecules [17-19]. Caruso group has recently found that when disulfide links are used for cross-linking of the hydrogel capsules system, a disulfide exchange reagent can be used to trigger release of a model drug [20-22]. However, there are some intrinsic shortcomings in the LbL approach, e.g., difficult to achieve high yield owing to the multistep preparation, absent of structural robustness of the shell upon template removal, and great challenge to refill the hollow interior with functional substance. As a result, a convenient fabrication procedure to the capsules with satisfactory loading capacity and expected stimuli-responsive behavior is highly desired in terms of practical applications of the capsules in biomedical area.

Recently various approaches have been developed for the massive preparation of polymeric capsules [23-25], and template method is especially attractive by precipitating polymers on the surface of granular templates or removing selectively the core of the templates [26-28]. Kozlovskaya group developed a series of weak polyacid based cross-linked capsules by hydrogen-bonded self-assembly as drug carriers [29-31]. In our previous work, various poly (acrylic acid) (PAA)-based micro/nanogels were prepared by using the precipitation polymerization method [32-34]. The obtained nanogels showed a high drug loading capacity and a sustained release feature for water-soluble drug molecules. In this study, in order to enlarge the inner space of the PAA-based particles and gain further high drug loading capacity, we optimized the synthetic strategy in which HPC-PAA was performed as templates followed by the surface polymerization of N-isopropylacrylamide (NIPAm) and the removal of HPC cores, leading to the PAA-PNIPAm hydrogel capsules finally. These capsules exhibit a new high drug loading of ~280% for anticancer drug Doxorubicin hydrochloride (Dox). The *in vitro* release and anticancer effect on the human intestine cancer cell LoVo show a responsive behavior upon the change in temperature and pH value of the surroundings. In combination of the convenient preparation, high drug loading, dual responsivity as well as the sustained release feature, this polymeric hydrogel capsules show potential application in drug controlled-release system. Importantly, the multiple carboxylic groups on the capsules surface allow functionalization on the capsules with a variety of macromolecules at mild conditions, such as targeting group. This makes the capsules ideal candidates for the target therapy.

Experimental Section

Materials

Hydroxypropylcellulose (HPC, M_w=100,000 Da), N, N′-Methylenebisacrylamide (MBAAm) and N-isopropylacrylamide (NIPAm) were purchased from Acros Chemical Company. Sodium dodecyl sulphate (SDS) and Acrylic acid (AA) were purchased from Shanghai Guanghua Chemical Company. Doxorubicin hydrochloride (Dox) was purchased from Beijing Huafeng United Technology Company. All the other reagents were of analytical grade and used without further purification. Distilled water was used for all polymerization and treatment processes. Human intestinal cancer LoVo cells were obtained from Shanghai Institute of Cell Biology (Shanghai, China).

Preparation of the HPC-PAA template particles

The HPC-PAA template particles were synthesized by direct polymerization of AA in HPC aqueous solution. In a typical run, 0.20 g of HPC was dissolved in 35 mL aqueous solution containing 0.20 g of AA. The solution was stirred at room temperature until it became clear. Then 500 μL of ascorbic acid (0.10 mol/L) and hydrogen peroxide (H_2O_2) (0.10 mol/L) solution were separately added into the above solution to initiate the polymerization of AA at 35°C under the protection of nitrogen gas. When the polymerization of AA reached a certain level, opalescent suspension occurred, indicating the formation of HPC-PAA template particles. The reaction was allowed to proceed at 35°C for 1 h.

Preparation of PAA-PNIPAm hydrogel capsules

The PAA-PNIPAm hydrogel capsules were prepared as follows: 15 mL aqueous solution containing 0.20 g of NIPAm, 0.15 g of MBAAm and 0.05 g of SDS was added into the HPC-PAA template particles suspension. Then the polymerization of NIPAm was carried out at 35 °C under a nitrogen stream and magnetic stirring. The reaction kept on for another 2 h before being cooled down to the room temperature, resulting in the HPC-PAA-PNIPAm complex particles. Appropriate amount of HPC-PAA-PNIPAm particles were redispersed in water and the pH of the suspension was adjusted to 8.0 using 0.1 mol/L of NaOH [34]. The PAA-PNIPAm hydrogel capsules were finally obtained after several centrifuging/washing cycles with water.

Preparation of Dox-loaded PAA-PNIPAm hydrogel capsules

The Dox-loaded PAA-PNIPAm hydrogel capsules were prepared by an incubation method at pH = 7.0. A certain content of PAA-PNIPAm hydrogel capsules were mixed with the Dox solution of a predetermined concentration (0.4, 0.8, 1.2, 1.6, 2.0 mg/mL) at room temperature. The mixed solution was incubated at 37°C for 12 h to allow Dox entrapment in the hydrogel capsules to reach an isothermal equilibrium. The drug loading efficiency and loading capacity of PAA-

PNIPAm hydrogel capsules were determined by separating capsules from the aqueous medium containing free Dox via centrifugation (12,000 rpm, 20 min). The amount of free Dox was measured on a UV-vis spectrometer at 495 nm. The loading efficiency (LE) and loading capacity (LC) were calculated with the following equations:

*LE (%) = weight of drug entrapped in hydrogel capsules/ weight of drug fedinitially*100%*

*LC (%) = weight of drug entrapped in hydrogel capsules/ weight of dry hydrogel capsules*100%*

In vitro release

The Dox release from the hydrogel capsules was evaluated by the dialysis method. Dox-loaded PAA-PNIPAm hydrogel capsules were placed in a dialysis bag (MWCO = 14,000 Da) and dialyzed against the PBS solution with predetermined pH value (pH = 4.0, 5.0 and 7.4) at particular temperatures (T = 25, 30 and 37°C), respectively. The released drug outside the dialysis bag was sampled at selected time intervals and measured with a UV-vis spectrometer.

In vitro cytotoxicity

The cell viability for free Dox, empty and Dox-loaded PAA-PNIPAm hydrogel capsules on the LoVo cell lines were evaluated by MTT assay. LoVo cells (5000 cells/well) were cultured in RPMI 1640 containing 10% fetal bovine serum in a 96-well multiplate. Afterwards, the cells were exposed to free Dox, empty and Dox-loaded PAA-PNIPAm hydrogel capsules respectively for 48 h (at 37°C, 5% CO_2), and the medium was replaced with MTT solution (0.50 g/L), and cells were incubated for another 2 h. The resulting blue formazan was solubilized in DMSO, and the absorbance at 560 nm was measured with a plate reader. The MTT reduction of untreated cell was set as 100%, and that of treated cells was expressed as a percentage of untreated cells.

Cell uptake

4T1 mouse breast cancer cells were incubated with PAA-PNIPAm hydrogel capsules in a humidified atmosphere with 5% CO_2 at 37°C. After incubation for 2 h to allow the 4T1 cells to internalize the capsules, the noninternalized capsules were removed through washing three times with PBS solution. Cell nucleolus was stained by 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI). Cells were observed using a laser confocal scanning microscope (LCSM, Zeiss LSM 710, Germany) at an excitation wavelength of 543 nm.

Characterizations

The morphology of particles and capsules was observed by transmission electron microscopy (TEM; JEOL TEM-1005) and scanning electron microscopy (SEM, HITACHI S-4800). The samples were dripped onto nitrocellulose-covered copper grid at room temperature without staining. The hydrodynamic diameter (D_h) and size distribution of particles and capsules were measured by dynamic light scattering (DLS) using a Nano ZS system (Malvern Instruments Corporation, UK). Each sample was diluted to the appropriate concentration with water and repeatedly conducted for 3 times at 25°C, obtaining the mean diameter. Zeta potential of the PAA-PNIPAm hydrogel capsules was determined by Zetaplus (Malvern Instruments Corporation, UK). All analyses were performed on samples adjusted with 0.01 M NaCl solution in order to maintain a constant ionic strength. Each measurement was triplicated and the result was the average of three runs.

Results and discussion

The strategy to prepare the PAA-PNIPAm hydrogel capsules is proposed in Fig. 1. The HPC-PAA template particles are prepared by the direct polymerization of AA monomers in HPC aqueous solution according to the method reported by Hu et al. [35]. Hydrogen bonding interaction between hydroxyl groups of HPC and carboxyl groups of PAA induces the formation of HPC-PAA particles in the aqueous solution. The size of the templates can be readily tuned from 100-1000 nm by varying the feed ratio of HPC to AA from 10:1 to 1:2.5 [33]. In this study, the [HPC]:[AA] was fixed at 1:1. After polymerization and cross-linking of monomer NIPAm on the surfaces of the HPC-PAA templates, cross-linked HPC-PAA-PNIPAm complex particles with core-shell structure were formed. It should be noted that increasing the pH of system could cause ionization of PAA as well as dissociation of hydrogen bonds and consequently the HPC component of the templates would re-dissolve in the solution and thus the templates would be broken. Hence, cross-linked PAA-PNIPAm hydrogel capsules could be obtained easily just after adjusting the pH of the solution to 8.0.

Figure 2 illustrates the TEM and SEM images of particles at each step. As shown in Fig. 2(a), HPC-PAA template particles possessed morphology of round shape with an average diameter of about 135 nm. After the polymerization and crosslinking of PNIPAm on the template surface at the second step, the size of the particles increased to about 230 nm. Close observation of the particles were indicative of core-shell feature with a dark core and a dusky shell (Fig. 2(b)). After the removal of the template, the particles still kept the sphere morphology as seen in Fig. 2(d). By further TEM

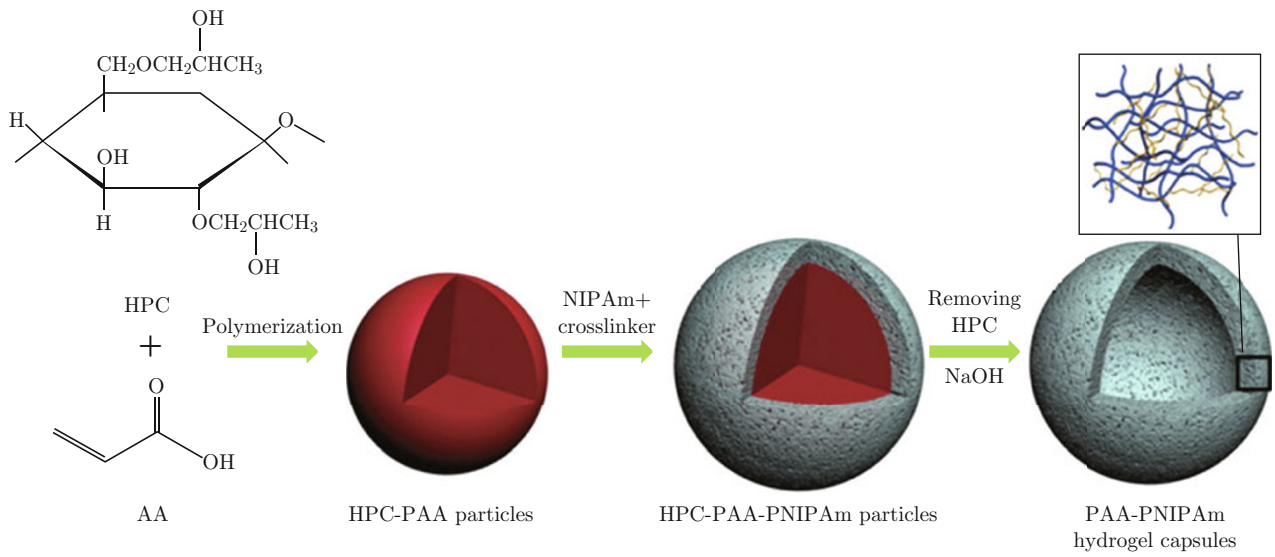

Fig. 1 Schematic representation of the preparation of PAA-PNIPAm hydrogel capsules.

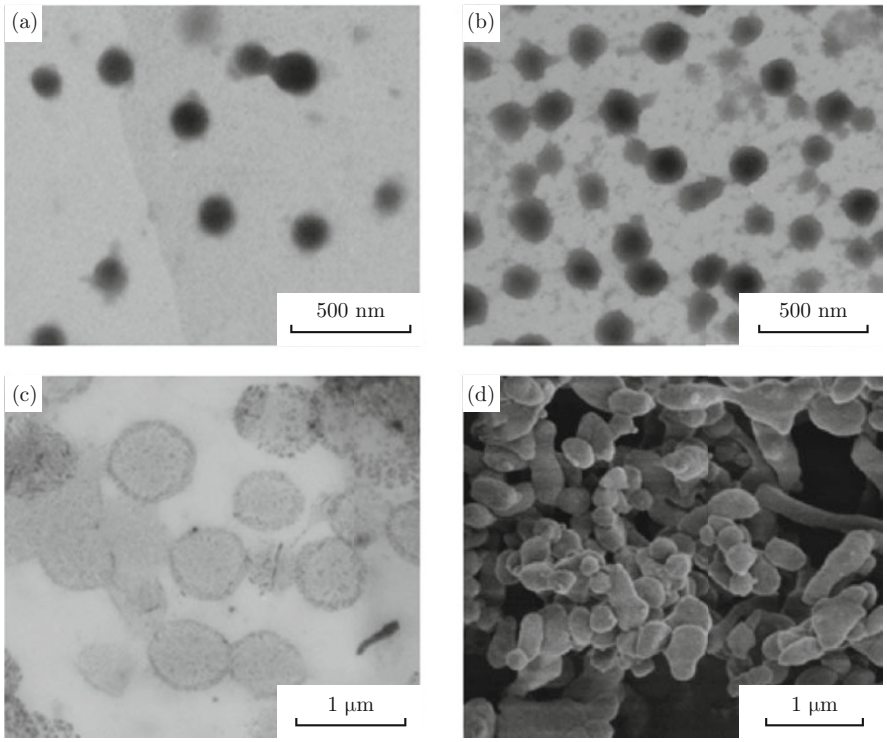

Fig. 2 TEM images of (a) HPC-PAA particles (pH = 2.4); (b) HPC-PAA-PNIPAm composite particles (pH = 2.4); and (c) PAA-PNIPAm hydrogel capsules (pH = 8.0). (d) SEM image of PAA-PNIPAm hydrogel capsules after frozen drying treatment.

measurement, they were characterized by a thin shell and a fairly large inner cavity (Fig. 2(c)). The wall thickness is about 50 ± 12.5 nm according to the TEM image. More interestingly, some mesopores penetrating from shell to the hollow interior of PAA-PNIPAm hydrogel capsules can be seen in the Fig. 2(c), which were probably the molecular imprint left by HPC after stripping from the HPC-PAA-PNIPAm particles. Compared to the HPC-PAA-PNIPAm particles, the size of PAA-PNIPAm hydrogel capsules increased significantly to about 560 nm, which maybe result from the intense swelling and the electrostatic repulsion generated by the complete ionization of PAA at pH = 8.0. Additionally, in virtue of removing the core, the restriction imposed by the hydrogen bonding between HPC and PAA was gradually released. This induced the particles to swell as well, accounting partially for their size increase. The size distributions of the particles in three steps

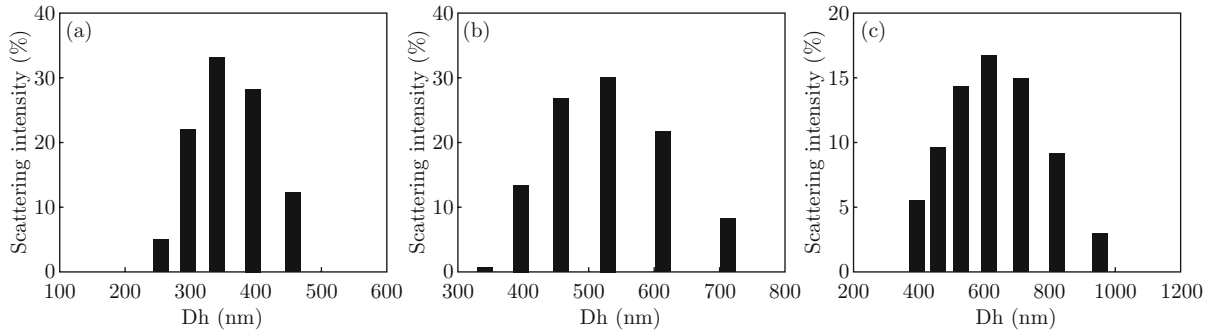

Fig. 3 The hydrodynamic diameter distribution of (a) HPC-PAA particles (pH = 2.4); (b) HPC-PAA-PNIPAm composite particles (pH = 2.4); and (c) PAA-PNIPAm hydrogel capsules (pH = 8).

measured by DLS are shown in Fig. 3, which illustrates the agreeable trend of particles size with the TEM observations. The diameters from DLS measurements obviously greater than those from TEM measurements could be attributed to the stretching PNIPAm chains in the aqueous solution of 25°C and the shrinking PNIPAm chains under ultrahigh vacuum condition [36].

Considering the large space inside the capsules, the PAA-PNIPAm hydrogel capsules are expected to be drug carriers with superior loading capacity which is regarded as an important factor for the performance of drug carriers [37]. Here, a water-soluble antitumor agent, Doxorubicin hydrochloride (Dox), is selected as a model drug to evaluate its drug loading ability. The drug loading process proceeds easily by incubating the PAA-PNIPAm hydrogel capsules in Dox water solution at 37°C for 12 h. The excessive Dox was removed by centrifugation, followed by repetitive washing with distilled water, leading to the drug-loaded hydrogel capsules. The UV-vis spectrum with the Dox characteristic absorption peak 495 nm (Fig. 4(a)) and LCSM section image of the Dox loaded PAA-PNIPAm hydrogel capsules (Fig. 4(a) inset) confirmed a successful entrapment of Dox into the capsules. Figure 4(b) depicted the loading capacity (LC) and loading efficiency (LE) of the PAA-PNIPAm hydrogel capsules corresponding to different feeding Dox concentrations at pH = 7.0. Along with the rise of the feeding drug concentration, increasing in the LC was observed. The maximum LC of ~280% was exhibited at the feeding Dox concentration of 1.6 mg/mL. Compared to analogous PAA-PNIPAm polymer particles [34,38], the PAA-PNIPAm hydrogel capsules show higher loading capacity for Dox. The high loading of Dox in the PAA-PNIPAm hydrogel capsules may arise from the charge controlled permeability mechanism, i.e., negatively charged PAA in the capsules shell at neutral medium induces positively charged Dox to penetrate through the capsule shell and then deposit spontaneously into the interior of the capsules [39,40]. As previously reports, the incubation process usually exhibited lower loading capacity compared to the encapsulation method. However, the PAA-PNIPAm hydrogel capsules herein still show high loading capacity using the incubation method, which suggests their convenient and low-cost loading procedure for drugs in future application.

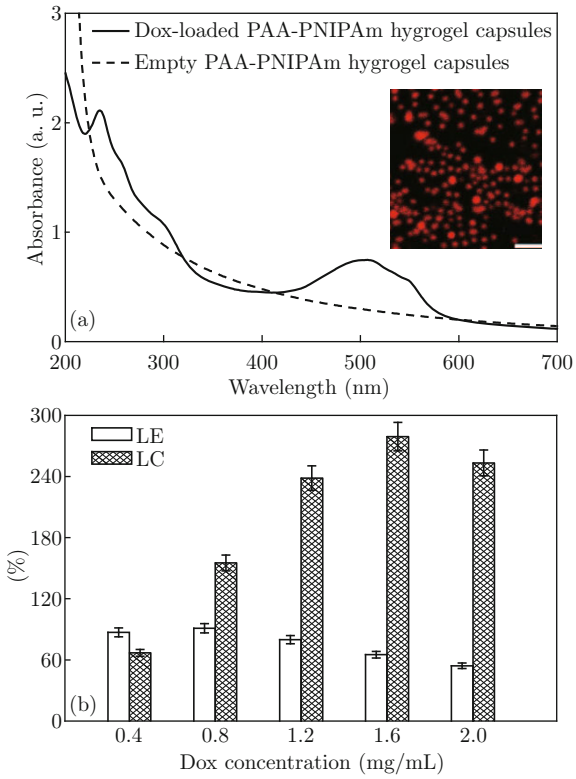

Fig. 4 (a) UV-vis spectra of Dox-loaded PAA-PNIPAm hydrogel capsules and empty PAA-PNIPAm hydrogel capsules; (inset) the LCSM image of the Dox-loaded PAA-PNIPAm hydrogel capsules, the scale bar is 2 μm; (b) LC and LE of PAA-PNIPAm hydrogel capsules corresponding to different feeding Dox concentrations at pH = 7.0.

Upon changing the environmental conditions, the loaded drugs were released from the capsules in a controlled manner. Figure 5 described the release profiles of Dox from PAA-PNIPAm hydrogel capsules in different pH and temperature, showing a low-rate but sustained release characteristic compared with free Dox.

For instance, at pH = 7.4 and the temperature of 37°C, about 27% of the loaded Dox was released in the early 10 h and 33% of the total Dox was liberated continuously in the next 100 h. This implied that the utilization of the PAA-PNIPAm hydrogel capsules as the Dox carrier was favorable for low-toxic and long-term therapy for cancer.

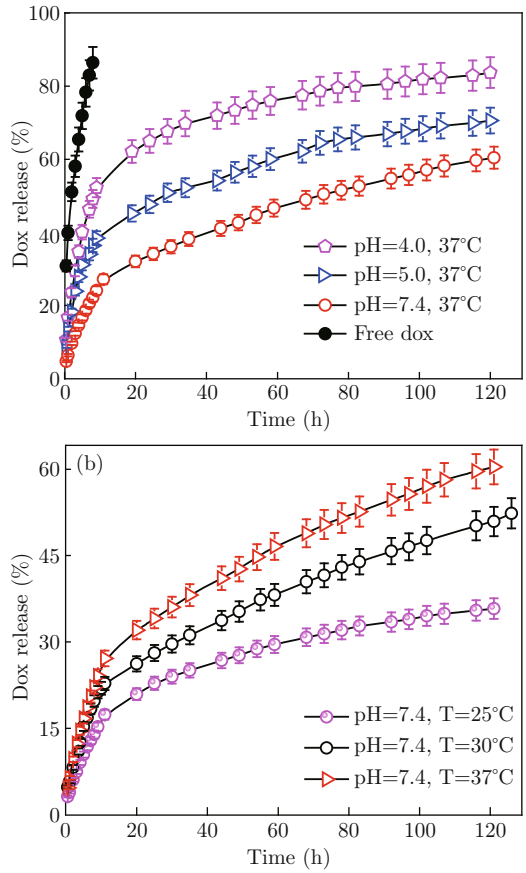

Fig. 5 Release profiles of Dox from the hydrogel capsules in PBS with different (a) pH and (b) temperatures.

Figure 5(a) revealed that the release behavior significantly correlates with the pH of release medium. The release rate of Dox from PAA-PNIPAm hydrogel capsules accelerates with the pH decreasing. This could be explained by the following two aspects. On one hand, the electrostatic interaction between PAA and Dox was actively responsive to the pH variation. At pH = 7.4, PAA chains and Dox molecules were fully ionized with opposite charges and the electrostatic interaction between them was too strong to make the entrapped Dox escape out rashly. As the pH drops down to 4.0, PAA chains were mainly protonated and the bonding force would be weakened greatly, boosting Dox to get off the cavity with ease. On the other hand, deswelling/swelling of the hydrogel capsules also had a certain effect on Dox release. In order to confirm the influence of pH on the size of hydrogel capsules, PAA-PNIPAm hydrogel capsules were incubated in a set of buffer solution with different pH values (pH = 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0) for 24 h. The size and zeta potential of samples at each pH were shown in Fig. 6. It could be found that as the pH increasing from 2.0 to 6.0, a remarkable increase in the size of PAA-PNIPAm hydrogel capsules was observed from 365 nm to 578.4 nm. This could be explained that the ionization degree of PAA chains gradually enlarges and the electrostatic repulsion between COO^- groups of PAA causes the whole sphere to be swelling increasingly. Particularly, in the pH range from 4.0 to 6.0, the size showed a dramatic increase possibly being associated with the pK_a of 4.75 for PAA. When the pH was beyond 6.0, there was little change in the size due to the complete deprotonation of PAA. Meanwhile, the zeta potential also revealed a similar variety trend. The gradual shrinking of the capsules could undoubtedly narrow their inner volume and force more entrapped Dox to release out of the cavity. This pH-dependent releasing behavior was favorable for the antitumor drug delivery system, since tumor cells were often at a lower pH level of 5.7-7.8 [41], which would induce a faster drug release in the diseased cells than in the normal cells.

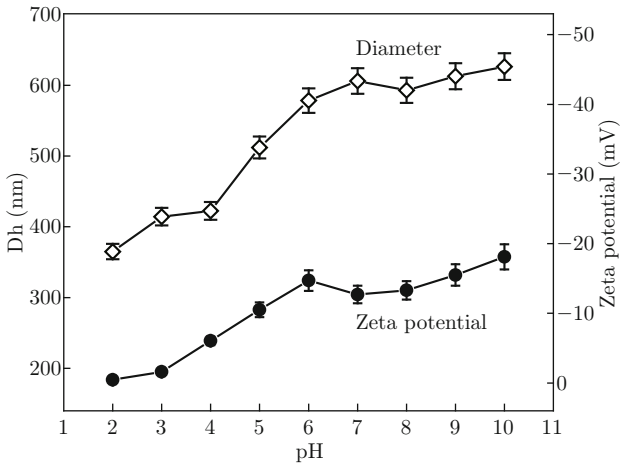

Fig. 6 Size and zeta potential of PAA-PNIPAm hydrogel capsules at different pH.

It is noteworthy that PNIPAm belongs to thermo-responsive polymers, so the PAA-PNIPAm hydrogel capsules serving as drug carriers are expected to exhibit a thermo controlled-released character. In order to verify this inference, the Dox-loaded hydrogel capsules were dialyzed in PBS (pH = 7.4) at different temperatures. The release profiles at different temperatures depicted in Fig. 5(b). Based on the previous analysis, it was concluded that the obtained PAA-PNIPAm hydrogel capsules, of which the drug release was dual-responsive to both temperature and pH, and could be able to intelligently distinguish between normal and pathological tissues, achieving better targeting efficiency

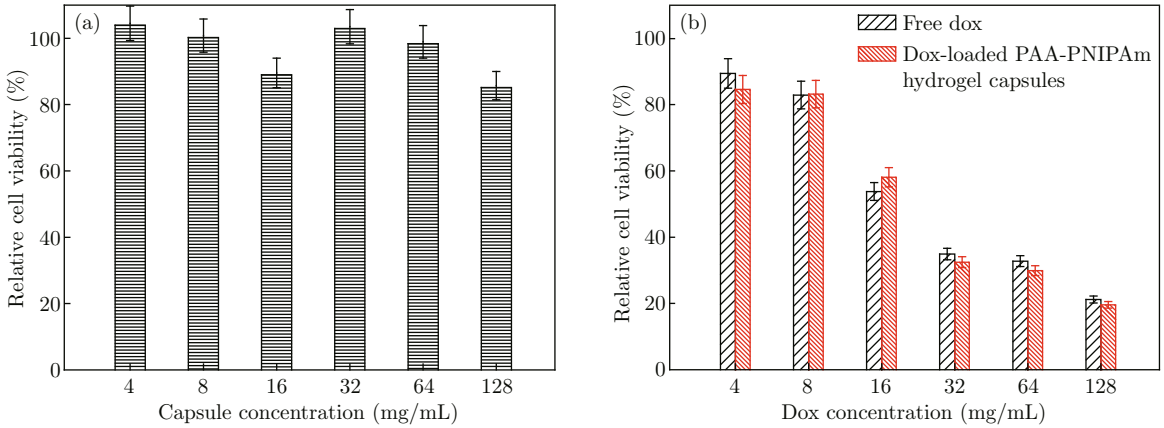

Fig. 7 In vitro cytotoxicity of (a) empty PAA-PNIPAm hydrogel capsules; and (b) Dox-loaded PAA-PNIPAm hydrogel capsules, free Dox against LoVo cell line at normal concentration.

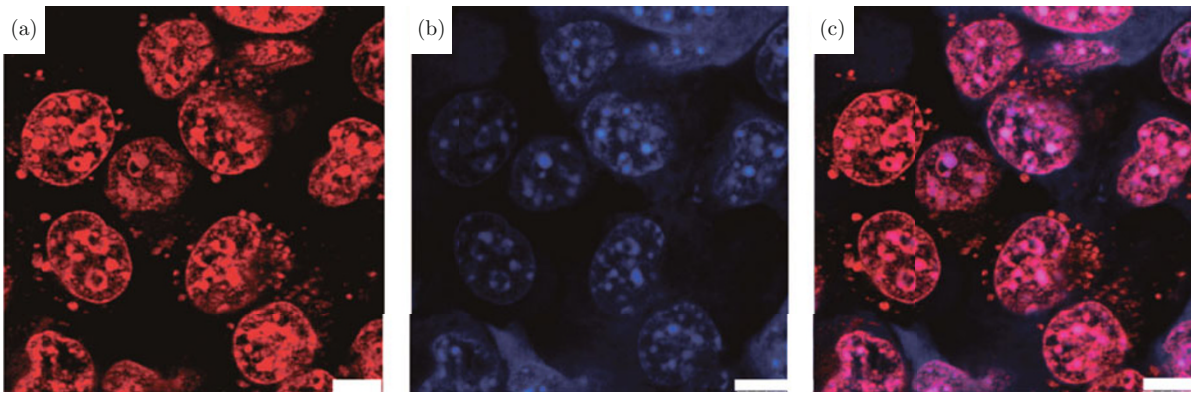

Fig. 8 LCSM images of 4T1 cells incubated with Dox-loaded PAA-PNIPAm hydrogel capsules for 2 h: red from Dox (a); blue from nuclei (b); and overlaid (c). The scale bar is 10 μm.

and treatment efficacy, and foreseeing an application to the drug controlled-released system.

To investigate the potential toxicity of the PAA-PNIPAm hydrogel capsules and the pharmacological activity of Dox released from the hydrogel capsules, empty and Dox-loaded PAA-PNIPAm hydrogel capsules solution were incubated with human intestine cancer cell line LoVo cells to examine their effect on cell viability with a positive control of free Dox. The MTT assay (Fig. 7(a)) showed that, even at high concentrations of 128 μg/mL, PAA-PNIPAm hydrogel capsules still have no significant cytotoxicity. *In vitro* cytotoxicity of Dox-loaded hydrogel capsules and free Dox against LoVo cell were exhibited in Fig. 7(b). It could be observed that the cytotoxicity of Dox-loaded hydrogel capsules was almost equivalent to that of free Dox, implying that the drug efficacy released from the hydrogel capsules was scarcely influenced by the process of drug loading, which was in agreement with our previous work [32,34]. LCSM characterization was used to further examine cellular uptakes of PAA-PNIPAm hydrogel capsules. Figure 8 showed the section images of 4T1 mouse breast cancer cells after incubation with the Dox-loaded hydrogel capsules at 37°C for 2 h. As shown in Fig. 8 (a)-(c), the Dox-loaded hydrogel capsules with red fluorescence arising from Dox were observed to mainly distribute in the cytoplasm of cells, indicating that the hydrogel capsules could overcome cellular barriers to enter the intracellular region, although it carried negative surface charges, which was consistent with the results reported by Savic et al. [42]. Moreover, the red fluorescence in the nuclei came from the Dox migrating and accumulating in the nuclei after releasing from the hydrogel capsules localized in the cytoplasm. These results firmly confirmed that the PAA-PNIPAm hydrogel capsules as a drug carrier had an excellent biosecurity and a high pharmacological activity.

Conclusions

We have demonstrated the convenient preparation of PAA-PNIPAm hydrogel capsules in this work, which were fabricated by a templated strategy in aqueous solution. Hydrophilic antitumor drug Dox was successfully entrapped in these hydrogel capsules based on charge controlled permeability mechanism, leading to a new high loading capacity of ~280% to the weight of the

carriers. More encouragingly, the release of drug from the PAA-PNIPAm hydrogel capsules exhibited a slow-rate but sustainable feature, and behaved the thermo and pH dual-responsivity. *In vitro* cytotoxicity assay indicated that the Dox-loaded PAA-PNIPAm hydrogel capsules have high antitumor activity. Considering the simple and mild preparation procedure, high drug loading capacity as well as the desired dual-responsive controlled release property, the PAA-PNIPAm hydrogel capsules may serve as a promising candidate for intelligent drug delivery system.

Acknowledgements

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