Hollow Poly(*N*-isopropylacrylamide)-*co*-poly(acrylic acid) Microgels with High Loading Capacity for Drugs

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ABSTRACT: A convenient approach has been developed for the preparation of microsize hydrogels composed of crosslinked poly(acrylic acid) (PAA) and poly(*N*-isopropylacrylamide) (PNIPAm). First, semi-interpenetration polymer networks of hydropropylcellulose (HPC) and PNIPAm-*co*-PAA copolymer are formed through the copolymerization and crosslinking of monomer acrylic acid and *N*-isopropylacrylamide in HPC aqueous solution. After the selective removal of HPC from networks due to ionization of PAA units and disruption of hydrogen bonding with increasing pH, PNIPAm-*co*-PAA microgels are obtained, whose volume is confirmed to be responsive to both temperature and pH. Doxorubicin hydrochloride (Dox) can be encapsulated in PNIPAm-*co*-PAA microgels with high drug loading driven by the electrostatic interaction, and a sustained-release characteristic of Dox from the microgels is observed under physiological pH value and temperature. *In vitro* cell experiments, the drug-loaded microgels can be taken up by LoVo cells and release their payload in cell cytoplasm without loss of drug efficacy. This indicates that PNIPAm-*co*-PAA microgels might be a potential drug delivery carriers especially for water-soluble or polypeptide drugs. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 124: 4678–4685, 2012

Key words: microgels; drug carriers; high drug loading; pH sensitive; thermosensitive

INTRODUCTION

It is of great value to design one target drug carrier that can maintain drugs' efficacy in treating disease, and at the same time minimize their toxicity against normal tissues. In recent years, stimuli-responsive polymeric carriers, including nanoparticles, micelles, microgels, microcapsules,[1–5] and so forth have attracted considerable interests in drug delivery field. As intelligent drug carriers, stimuli-responsive polymers could release drugs selectively in pathogenic position with control by transforming their volumes via swelling/deswelling induced by external stimulus, such as the variation of pH value, temperature, concentration of salt or antigen.[6–10] The utilization of the stimuli-responsive polymers could facilitate the decrease of drug dosage and the toxicity on normal tissues by allowing drugs to be released to desirable site. This is particularly attractive for the loading of anticancer drugs.

The pH and temperature are two of the most important stimulus in the medicine field. The pH-sensitive polymeric drug carriers not only can provide protection for oral polypeptide drug but also distinguish the normal or pathological tissues, organs, and cellular compartments due to the pH differences between them.[11–13] Temperature-sensitive polymeric drug carriers are usually used to control the drug release/loading arising from their reversible deswelling/swelling properties at different temperature.[14] In many cases, certain malignancies could cause increasing of temperature and decreasing of extracellular pH around tumor site at the same time. Thus, if one designed drug carrier can response simultaneously to the two important stimulus of pH and temperature, better efficiency and lower side effects as well as controllable delivery for drugs can be expected.

Many dual-responsible nanoparticles have been fabricated from polymers. Hoffman and coworkers synthesized a crosslinked hydrogel based on poly(*N*-isopropylacrylamide) (PNIPAm) and poly(acrylic acid) (PAA),[15] which can be used to load polypeptide or macromolecular drug at low temperature

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and exhibit the combined pH- and temperature-sensitive properties. However, the drug loading process for the hydrogel requires organic solvents to facilitate drugs solubility. Soppimath et al. fabricated pH- and thermo-responsive nanoparticles of poly(*N*-isopropylacrylamide-*co*-*N*,*N*-dimethylarylamide-*co*-10-undecenoci acid),[16] and it was found that weak acidic environment could induce nanoparticle aggregation and consequently trigger the release of the loaded drug. However, the low drug loading capacity (LC) of about 2.7 wt % is the main limitation for the practice application of these nanoparticles. It is generally accepted that higher LC and milder loading condition are two of the most important criteria for an excellent drug carrier. To date, most of the dual-responsible polymeric carriers are utilized to load hydrophobic drugs.[17] The attempts to load polypeptides or macromolecules on the surfaces of polymers or hydrogels are manipulated; however, little progress has been achieved to acquire polymeric carries with high LC for hydrophilic or peptide drugs.[18]

In this study, a convenient approach has been developed for the preparation of microsize hydrogels based on PAA and PNIPAm through the copolymerization and crosslinking of acrylic acid and *N*-isopropylacrylamide in hydropropylcellulose (HPC) aqueous solution. Semi-interpenetration polymer networks of PNIPAm-*co*-PAA/HPC are fabricated driven by the hydrogen bonding interaction between hydroxyl groups of HPC and carboxyl groups of PAA.[19] After selective removal of HPC molecules due to ionization of PAA units and disruption of hydrogen bonding with increasing pH, PNIPAm-*co*-PAA microgels are obtained, which exhibit both temperature- and pH-responsive feature. The loading and releasing behaviors of PNIPAm-*co*-PAA microgels are evaluated in mild condition using anticancer drug Doxorubicin (Dox) as a hydrophilic model drug, which show a superhigh drug LC for Dox, and a sustained-release characteristic under physiological pH and temperature. *In vitro* cell experiment results indicate that drug-loaded PNIPAm-*co*-PAA microgels can be taken up into LoVo cells effectively, implying their potential application in clinical diagnosis.

EXPERIMENTAL

Materials

HPC (Mw, 100 kDa), *N*, *N*′-methylenebisacrylamide (MBAAm) and NIPAm were purchased from Acros Organics Company (Geel, Belgium). Doxorubicin hydrochloride (Dox) was purchased from Shanghai Aladdin Reagent Company (Shanghai, China). All other reagents were of analytical grade and used without further purification. Human intestinal cancer LoVo cells were obtained from Shanghai Institute of Cell Biology (Shanghai, China).

Preparation of PNIPAm-*co*-PAA microgels

In a typical run, 0.2 g of HPC, 0.2 g of NIPAM, and 0.15 g of MBAAm crosslinker were dissolved in aqueous solution containing 0.05 g of AA. When the solution temperature was heated to 35°C, 500 μL of 0.1 mol/L ascorbic acid solution and 500 μL of 0.1 mol/L hydrogen peroxide (H_2O_2) solution were added into the reaction system to initiate the copolymerization of NIPAm and AA under the N_2 protection. It was observed that the solution turned into opalescent suspension after about 10 min, indicating the formation of PNIPAm-*co*-PAA/HPC networks. The reaction was allowed to proceed at 35°C for 2 h. Afterward, the pH of resultant suspension was adjusted to around 8 using 0.1 mol/L NaOH to remove HPC from the polymer networks, and then the suspension was centrifuged at 12,000 rpm for 20 min. The sedimentation was washed with water three times and redispersed with distilled water. The obtained suspension was the PNIPAm-*co*-PAA microgels.

Dox loading in PNIPAm-*co*-PAA microgels

Dox loading in PNIPAm-*co*-PAA microgels was carried out via incubation method. Briefly, appropriate amount of PNIPAm-*co*-PAA microgels suspension was added into Dox solution with a predetermined concentration. The mixed solution was kept at 37°C without stirring overnight, to allow Dox absorption reaching an isothermal equilibrium on the microgels.

Evaluation of drug entrapment

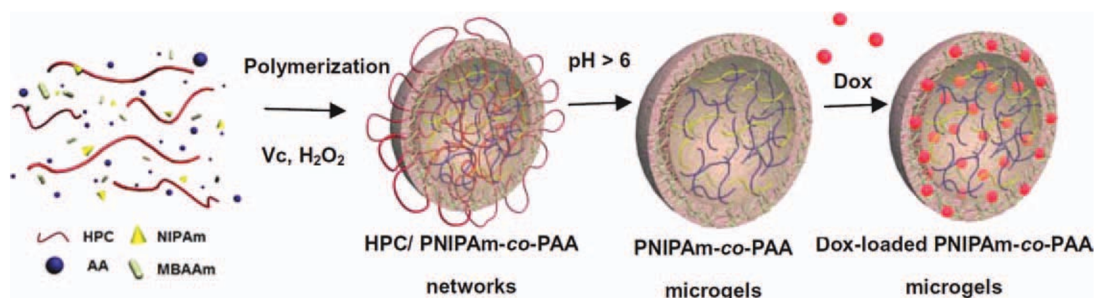
The loading efficiency (LE) and LC of PNIPAm-*co*-PAA microgels were determined by separating microgels from the aqueous medium containing free Dox through centrifugation (12,000 rpm/min, 40 min). The amount of Dox was determined by using an absorbance at 490 nm based on a calibration curve. The drug LE and LC of the microgels were calculated as follows:

$$\mathrm{LE}(\%) = (\text{total amount of drug} - \text{free drug}) / \text{total amount of drug} \times 100\%$$

$$\mathrm{LC}(\%) = (\text{total amount of drug} - \text{free drug}) / \text{dry microgel weight} \times 100\%$$

Dox release in phosphate-buffered saline (PBS)

The release of the drug from PNIPAm-*co*-PAA microgels in PBS was evaluated by dialysis method.



Scheme 1 The scheme of preparation of PNIPAm-*co*-PAA microgels. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

A purified Dox-loaded PNIPAm-*co*-PAA microgels solution with known Dox concentration was placed inside a dialysis bag (MWCO, 14 kDa) and dialyzed against PBS solution at 37°C. The released drug outside of the dialysis bag was sampled at defined time periods and measured by UV spectrometer.

In vitro cytotoxicity

Cell viability of empty PNIPAm-*co*-PAA microgels, free Dox, and Dox-loaded PNIPAm-*co*-PAA microgels on the LoVo cell lines was evaluated by MTT assay. LoVo cells (5000 cells/well) were cultured in RPMI 1640 containing 10% fetal bovine serum in a 96-well multiplate. Then, the cells were exposed to empty PNIPAm-*co*-PAA microgels, free Dox, and Dox-loaded PNIPAm-*co*-PAA microgels respectively, for 48 h, and MTT solution was added. Cell viability was measured by the formed formazan absorbance at 570 nm.

Cell uptake

LoVo cells were incubated with Dox-loaded PNIPAm-*co*-PAA microgels in a humidified atmosphere with 5% CO_2 at 37°C. After incubation for 2 and 4 h to allow the LoVo cells to internalize the microgels, the noninternalized microgels were removed through washing three times with PBS solution. Cell nucleolus was stained by 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DPAI). Cells were observed using a laser confocal scanning microscope (LCSM, Zeiss LSM 710, Germany) at an excitation wavelength of 543 nm.

Characterization methods

Fourier transformation infrared (FT-IR) spectra were measured on a Bruker IFS 66V vacuum-type spectrometer. The PNIPAm-*co*-PAA microgels suspension was lyophilized to dry powder, and the obtained dried powder was mixed with KBr and pressed to a plate for measurement. The hydrodynamic diameter and zeta potential of PNIPAm-*co*-PAA microgels were recorded by dynamic light scattering (DLS) using a Nano ZS with 4 mW He-Ne laser (Malvern Instruments Corp., UK) and analysis in CONTIN mode. The concentration of each sample was adjusted to be about 0.01% (w/v) with distilled water in the case of zeta potential examination. All DLS measurements were done with a wavelength of 633 nm and an incident angle of 90° at 25°C. The morphology of PNIPAm-*co*-PAA microgels was observed by transmission electron microscopy (TEM; JEOL TEM-1005). The sample was dropped onto nitrocellulose-covered copper grid at room temperature without staining.

RESULTS AND DISCUSSION

Morphology and structure of PNIPAm-*co*-PAA microgels

Through the copolymerization and crosslinking of monomers NIPAm and AA in HPC solution, semi-interpenetration polymer networks of PNIPAm-*co*-PAA/HPC are fabricated driven by the hydrogen bonding interaction between hydroxyl groups of HPC and carboxyl groups of PAA.[20] Increasing the pH value of system can cause ionization of PAA and destroy hydrogen bonds between them,[21] thus HPC can be selectively removed from the polymer network, resulting in the formation of PNIPAm-*co*-PAA microgels (Scheme 1). The synthetic strategy does not require adscititious surfactants usually used in the preparation of PNIPAm-based microgels,[22–24] avoiding the hazard of toxic surfactants due to incomplete removal. Actually, the HPC herein plays the role of surfactant in the microgels preparation to stabilize the microgels and avoid polymer aggregation in the solution. As we know, the hydroxyl group of HPC could interact with the carboxyl group of PAA by hydrogen bonds in this system; on the other hand, the residual uncomplexed hydroxyl groups of HPC remain their hydrophilicity. So the HPC in this study becomes "amphiphilic" and works as stabilizer agent to prevent polymer particles from clustering. In control

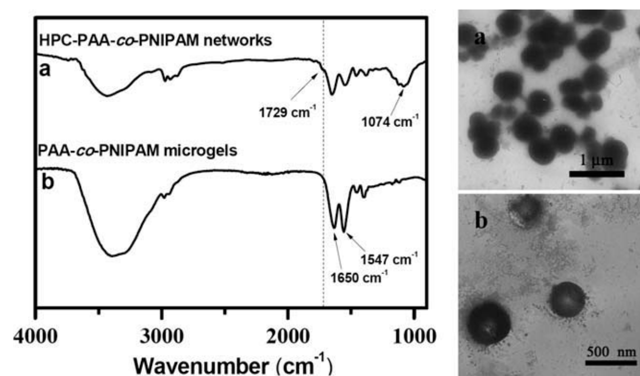


Figure 1 FTIR spectra and TEM images of microgels (a) before and (b) after NaOH solution treatment.

experiment without HPC, macroscopical precipitation of PNIPAm-*co*-PAA will be produced after the addition of initiators.

FT-IR spectra and TEM images of microgels before and after HPC removal are shown in Figure 1. For PNIPAm-*co*-PAA/HPC networks, FT-IR spectrum exhibits the characteristic absorption bands of HPC (1074 cm^{-1}), PNIPAm (1650 cm^{-1}, 1547 cm^{-1}), and PAA (1729 cm^{-1}). The networks show the morphology of spherical particles with the mean diameter of 390 nm. In the FT-IR spectrum of PNIPAm-*co*-PAA microgels obtained after the pH adjustment, the intensity of absorption band at 1074 cm^{-1} that is corresponding to the ether linkages of HPC is decreased greatly, revealing that the HPC is mostly removed from the networks. In addition, the characteristic band corresponding to the carbonyl group in PAA (1729 cm^{-1}) is also absent, resulting from ionization of PAA after alkalization treatment. TEM image of the PNIPAm-*co*-PAA microgels indicates that the spherical particles are interior-hollow, arising from the removal of HPC. Both FT-IR and TEM results are in consistence with the formation mechanism of the microgels.

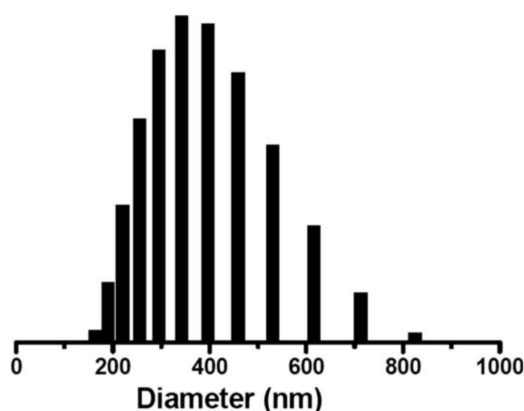


Figure 2 The size distribution of PNIPAm-*co*-PAA microgels (pH = 7.0).

TABLE I
Dependence of the Amount of Monomers on the Size of PNIPAm-*co*-PAA Microgels

HPC (g)	AA (g)	NIPAm (g)	Mean diameter (nm)	PDI
0.2	0.2	0.2	1244	0.126
0.2	0.1	0.2	516	0.087
0.2	0.05	0.2	382	0.075
0.2	0.02	0.2	269	0.063
0.2	0.1	0.3	674	0.116
0.2	0.1	0.4	1081	0.325

The size distribution of PNIPAm-*co*-PAA microgels in Figure 2 is measured by DLS, which shows the mean diameter of 382 nm, in agreement with the TEM observations. To investigate the effect of monomers on the size of microgels, we varied the added amount of monomer AA and NIPAm. It should be noted that the concentration of HPC in all the cases was fixed at 4×10^{-3} g/cm^3 for maintaining the same viscosity of polymerization system. And the amount of crosslinker should be controlled to be larger than 0.15 g, otherwise the microgels will dissociate when pH > 6 without close crosslinking. The amount of crosslinker and initiator however has little effect on size of the microgels. From Table I, it can be seen that a lower HPC/AA ratio leads to large sizes and wider size distribution of microgels owing to the decrease of uncomplexed HPC chain segments acting as stabilizer and increase of hydrophobic complexed HPC/PAA chain segments. The size of microgels as well as its distribution is also found to be increasing with the amount of NIPAm. This indicates that the size of microgels increases with the AA amount and could be controlled by changing the amount of AA in the preparation.

pH-response of the PNIPAm-*co*-PAA microgels

It is well-known that PAA is a pH-responsive polymer.[25] The response of the PNIPAm-*co*-PAA microgels to the pH variation is evaluated by the changes of their surface charge density and hydrodynamic diameter. PNIPAm-*co*-PAA microgels were suspended in water and the pH was adjusted with HCl or NaOH solution. The zeta potentials of microgels as a function of pH are shown in Figure 3, which indicate that the zeta potential is close to zero when pH < 3, increases almost linearly with pH in the range of 3–6, and reaches a maximal and constant value of about -14.5 mV at pH = 6–10. It implies that the carboxyl groups of PAA are completely deprotonated above pH = 6. Correspondingly, the diameter of PNIPAm-*co*-PAA microgels also increases with pH and keeps at about 365 nm above pH = 6. That is to say, the diameter of the microgels increases with the increase of surface charge,

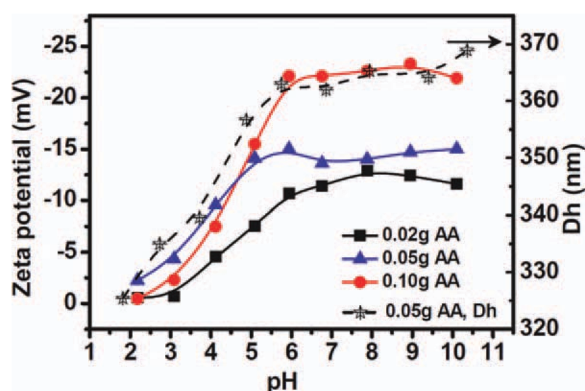


Figure 3 Zeta potentials and diameters of PNIPAm-*co*-PAA microgels at different pH. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

suggesting that electrostatic repulsion generated by the ionization of PAA in the microgels could induce the stronger swelling of microgels. Additionally, the PNIPAm-*co*-PAA microgels obtained with other AA amount exhibit similar change tendency with pH, except that the maximal negative zeta potential is valued as −12 mV for the sample from 0.01 g AA and −23 mV for that from 0.1 g AA. It is indicated that the PNIPAm-*co*-PAA microgels indeed have promising pH-responsive property as expected.

Thermoresponse of the PNIPAm-*co*-PAA microgels

Since PNIPAm is a thermoresponsive polymer, the PNIPAm-*co*-PAA microgels are expected to show thermoresponsible property as well. To confirm it, the microgels suspension (pH = 7.0) was kept at the temperature from 25 to 60°C and the mean diameters were measured by DLS. A parameter is introduced to scale the effects of temperature on the volume of microgels. α is so-called deswelling or swelling ratio calculated by the ratio of the hydrodynamic diameters in shrunken state to that at 25°C. As shown in Figure 4, a significant deswelling of microgels starts near 32°C, which corresponds to the lowest critical solution temperature (LCST) of 32°C for PNIPAm. It is worthy to note that the sizes of PNIPAm-*co*-PAA microgels will decrease all along even when the temperature is up to 60°C, whose temperature range of deswelling is much wider than pure PNIPAm microgels reported in other cases.[26] The special difference might be caused by the loose structure of microgels[27–29]; it is reasonable that the shrinkage of the microgels absent of HPC with hollow structure is much easier than that of solid particles. The existence of HPC will restrict the shrinkage of the spheres. The PNIPAm-*co*-PAA microgels show fast kinetic response in a wide temperature range of at least from 32 to 60°C. Furthermore, the size of microgel would reswell up to original size after cooling of this solution down to 25°C (data not shown), indicating that the swelling–deswelling response to temperature was reversible. Same thermoresponse of microgels was also observed when the medium pH = 3.0 and 10.0. In other words, PNIPAm-*co*-PAA microgels have great potential as intelligent drug delivery carriers because of the large variation of the mean diameter and the wide deswelling temperature range of 32–60°C that covers the most temperature range for the pharmaceutical application of around body temperature 37°C.

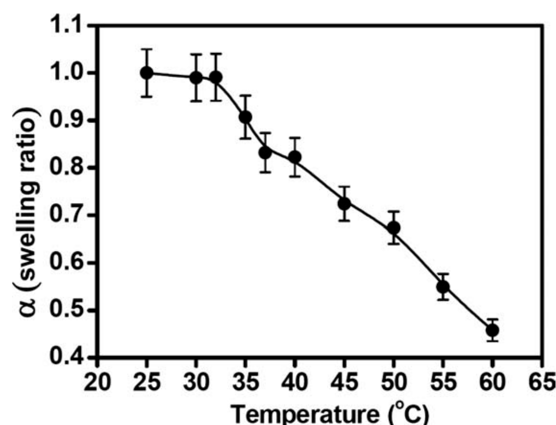


Figure 4 α-temperature curve of PNIPAm-*co*-PAA microgels in the medium pH = 7.0.

Dox loading of PNIPAm-*co*-PAA microgels

As discussed above, PNIPAm-*co*-PAA microgels have a loose structure and hollow interior, which are very attractive for drug loading. A water-soluble anticancer drug, Doxorubicin hydrochloride (Dox), is used as the model drug to measure the LC of PNIPAm-*co*-PAA microgels. Figure 5 demonstrates the LE and LC of PNIPAm-*co*-PAA microgels at different initial Dox concentrations. The LC is about 120% as the initial Dox concentration is larger than 1 mg/mL,

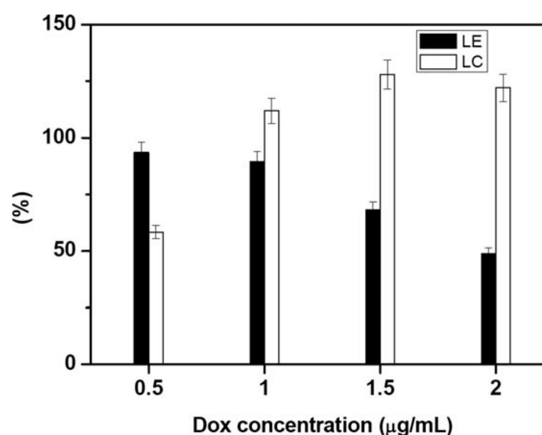


Figure 5 LC and LE of PNIPAm-*co*-PAA microgels at different initial Dox concentrations at pH = 7.0.

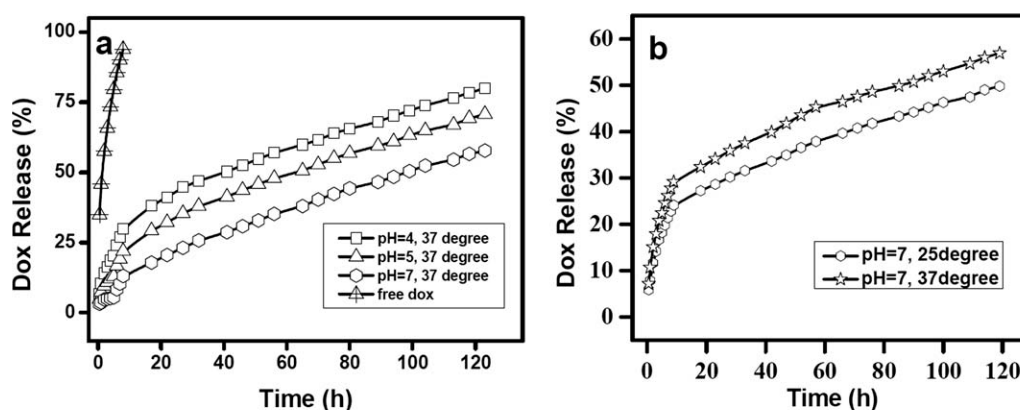


Figure 6 The release profiles of Dox from PNIPAm-*co*-PAA microgels in the medium with different (a) pH and (b) temperature.

which is much higher than that for other analogous Dox-loaded systems.[30–32] Electrostatic interaction between microgels and Dox molecules is the driving force for the drug loading. At neutral medium, the carboxyl groups in the microgels are ionized completely (Fig. 3) and negatively charged, while Dox molecules (pKa = 8.6) are positively charged at this condition, thus Dox can be loaded in the microgels with high LC due to the electrostatic interaction between the COO^- within the microgels and the protonated amino nitrogen in Dox. When the electrostatic interaction between Dox and PAA reach equilibrium, the LC would keep the constant. This is in agreement with the result that the LC shows no obvious increase as the Dox concentration is higher than 1.5 mg/mL. In comparison to covalent attachment or hydrophobic interaction functioned in other drug carriers, electrostatic interaction driven drug-carrier complex are easy to control both in loading and release.[33] In addition, because the molecular weight of HPC is 100,000, the pores formed after HPC removal are much larger than Dox, making it feasible to the transporting of Dox into and out of the microgels.

In vitro Dox release

The Dox release from PNIPAm-*co*-PAA microgels is evaluated by dialysis in PBS at different release conditions. The release profiles at different pH values are depicted in Figure 6(a), which show a sustained-release characteristic compared with free Dox. Dox-loaded PNIPAm-*co*-PAA microgels exhibit a two-sectional release behavior in all cases. A relatively rapid release occurs in initial 10 h, followed by a sustained and slow release over an extended time up to 5 days. The rapidly released Dox may come from the shallow surface of microgels, which can access the aqueous medium in a short diffusion time. After this initial stage, ~ 50% of entrapped Dox would be released in next 110 h. The release amount of Dox is responsive to the environmental pH changes. Decreasing the pH value of PBS can accelerate the release of Dox from the microgels, because the surface charge density of PNIPAm-*co*-PAA microgels would gradually decrease with pH (Fig. 3) and the electrostatic interaction would be weakened likewise, thus Dox could easily go through the channels within the microgels. This pH-dependent releasing behavior is of particular interest in achieving the tumor-targeted Dox delivery. At normal physiological condition (pH = 7.4), most Dox molecules are entrapped in the microgels, and a faster release will occur once they are internalized by tumor cells due to the lower pH value there (pH = 5.7–7.8).[34] In the endosome/lysosome of tumor cells, the release rate can be further accelerated due to the pH values of 4.0–5.0.[35,36] On the other hand, a higher temperature was also found to be helpful for drug release on account of the coil-to-globule transition of PNIPAm chains above the LCST, which makes drug movement within the polymer matrix. *In vitro* release shows clearly that the PNIPAm-*co*-PAA microgels are thermo- and pH-sensitive, and therefore very promising as drug carriers for water-soluble antitumor drugs.

Cytotoxicity and cellular uptake of Dox-loaded PNIPAm-*co*-PAA microgels

To examine the pharmacological activity of Dox released from PNIPAm-*co*-PAA microgels and the potential toxicity of the PNIPAm-*co*-PAA microgels, *in vitro* cytotoxicity against LoVo cell of Dox-loaded and empty microgels was investigated together with a positive control of free Dox (Fig. 7). The inhibitory ratio was determined after 48 h of incubation with a series of concentration of Dox, Dox-loaded microgels, and empty microgels by MTT assay. As shown in Figure 7(a), Dox-loaded microgels exhibit

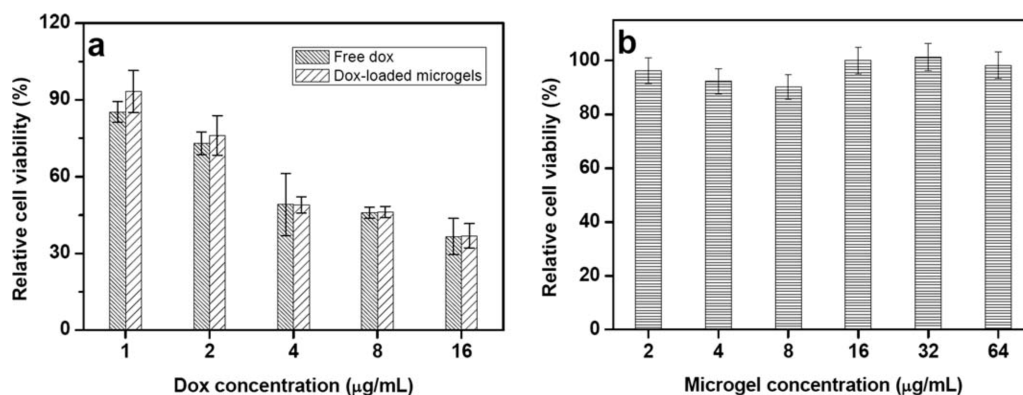


Figure 7 *In vitro* cytotoxicity of (a) Dox-loaded PNIPAm-*co*-PAA microgels, free Dox, and (b) empty PNIPAm-*co*-PAA microgels against LoVo cell line at normal concentration.

comparable cytotoxicity with free Dox at equal Dox concentrations, suggesting that the process of drug loading hardly affects the drug efficacy released from the microgels. Additionally, no significant cytotoxicity is observed with the empty microgels at all used concentrations [Fig. 7(b)].

Laser scanning confocal microscopy was used to trace the cellular uptakes of microgels. Figure 8 showed the section images of LoVo cell after the incubation with Dox-loaded PNIPAm-*co*-PAA microgels at 37°C for 2 and 4 h. As shown in Figure 8(a), red fluorescent signals arising from the self-fluorescence of Dox are distributed in the cell cytoplasm, but only little in the cell nuclei. Free Dox was ever reported that can enter and accumulate in the nuclei by diffusion process only after 1-h incubation,[37] while polymer particles are difficult to enter nuclei for the size limitation of nuclear pore. We deduced that the localized dot-shaped fluorescence came from Dox-loaded microgels, suggesting that the microgels could be trapped into cell and remained in the cytoplasm by endocytosis process. On the other hand, a considerable fluorescence change was observed in the cells after 4-h incubation [Fig. 8(b)], which clearly demonstrates that red signals migrate and accumulate in the nuclei. Moreover, LoVo cell was dead under the influence of pharmacological efficacy of Dox judged from the rupture of cell membrane and the outflow of cytoplasm. It could be explained that with the long time uptake, Dox was accumulated in cell nuclei after releasing from the micelles localized in the cytoplasm, giving strong red fluorescence signals. This result could also be understood as the pH-dependent release property of microgels. The pH of cell cytoplasm is about 5.0, much lower than that of the normal physiological condition of 7.4.[38] After the Dox-loaded microgels are taken up to the cell interior, a large amount of Dox is released responsive to acidic stimuli.

CONCLUSIONS

A convenient approach has been developed for the preparation of PNIPAm-*co*-PAA microgels. Semi-interpenetration polymer networks of PNIPAm-*co*-PAA/HPC are first fabricated. After the selective

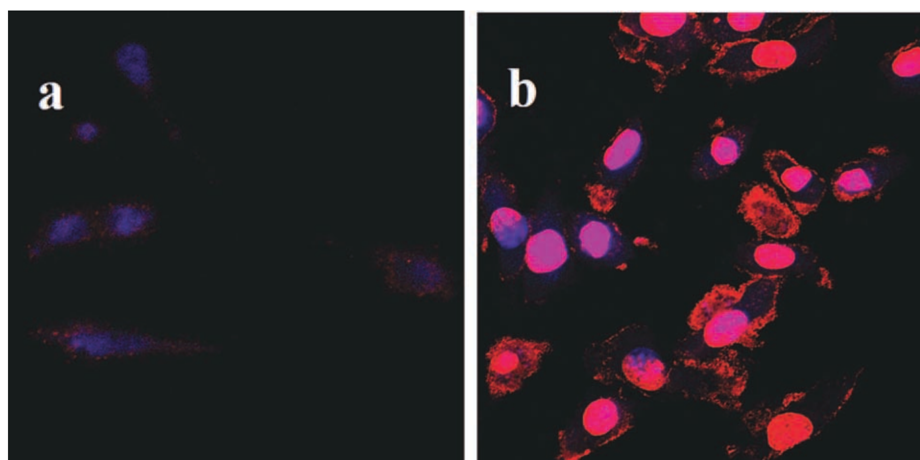


Figure 8 LCSM images of LoVo cells after incubation with Dox-loaded PNIPAm-*co*-PAA microgels for (a) 2 h, (b) 4 h. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

removal of HPC by adjusting pH of the solution, PNIPAm-*co*-PAA microgels with loose structure and hollow interior are obtained, which exhibit the pH- and thermo-responsive properties as expected. Water-soluble Dox can be encapsulated in the PNIPAm-*co*-PAA microgels with high drug loading driven by the electrostatic interactions. A sustained-release characteristic of drug is observed under physiological pH and temperature. The Dox-loaded PNIPAm-*co*-PAA microgels could be taken up by the cell and release their payload in the cell cytoplasm without loss of drug efficacy. This indicates that PNIPAm-*co*-PAA microgels might be a potential drug delivery carriers especially for water-soluble or polypeptide drugs.

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