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Temperature/pH Dual Responsive Microgels of Crosslinked Poly(*N*-vinylcaprolactam-*co*-undecenoic acid) as Biocompatible Materials for Controlled Release of Doxorubicin

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ABSTRACT: Undecenoic acid functionalized thermo/pH responsive microgels, poly(*N*-vinylcaprolactam-*co*-undecenoic acid) [poly(VCL-*co*-UA)], were synthesized by precipitation emulsion copolymerization. The microgels exhibit reversible thermo/pH responsive phase transition behavior, which can be tuned by varying the monomer feed ratio. The lower critical solution temperatures (LCSTs) of the materials are close to body temperature. As a result, when temperatures rise above ca. 37°C, a rapid thermal gelation process occurs, accompanied by a phase transition, resulting in expulsion of encapsulated compound. *In vitro* experiment evaluated its applicability as a drug carrier for controlled release of an anticancer agent (doxorubicin) and showed that the drug encapsulation efficiency (EE), releasing rate, and kinetics are dependent on the temperature and pH value as expected. Minimal cytotoxicity of the microgels was observed by a cytotoxicity assay using 3T3 fibroblast cells. Our finding suggests that the poly(VCL-*co*-UA) based microgels may be considered a promising candidate for temperature or pH-controlled delivery of anticancer drugs. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 41146.

KEYWORDS: biomaterials; drug delivery systems; microgels

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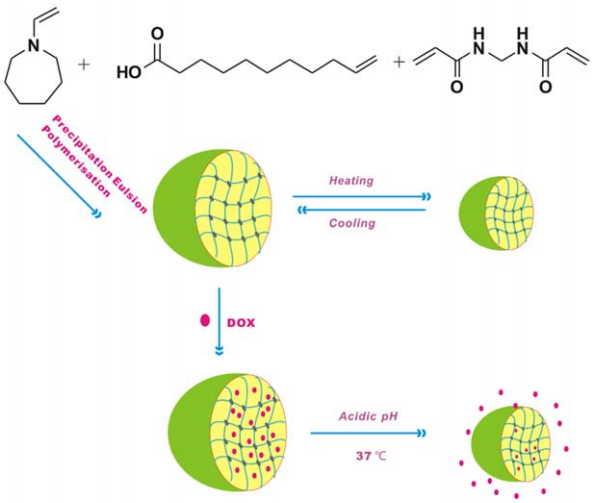
INTRODUCTION

Polymeric micro/nanohydrogels have been proposed and investigated extensively as promising approaches for enabling incorporation and controlled release of anticancer drugs, proteins, and nucleic acids.[1–6] In addition, microgels also could improve the selective accumulation of drugs at tumor sites via the due to their tunable size (from nanometers to several micrometers) and their can be decorated with biospecific targeting groups hydrophilic surface.[7–12] Compared with other conventional drug delivery systems, thermo/pH-responsive microgel delivery systems not only can effectively shield and protect the encapsulated drugs until they reach a tumor site, but also can discharge at switchable release kinetics, that is, slow release while circulating in the blood and rapid release at target tumor sites.[13] For example, thermoresponsive microgels can be used in hyperthermia treatment for localized drug delivery at tumor sites and pH responsive microgels can be designed for drug release in slight acidic tumor microenvironments.[14–19] Consequently, development of thermo/pH-responsive microgels serving as functional drug delivery vehicles has attracted considerable interest due to their enhanced stability, drug loading capacity, and well-modulated drug release in response to physiological and pathological stimuli.

Among various temperature sensitive materials, poly(*N*-isopropylacryamide) (PNIPAAm) has a lower critical solution temperature (LCST) of ca. 32°C in aqueous solution.[20–24] Such thermal phase transition around physiological temperature of PNIPAAm is the reason for it being the most extensively investigated thermosensitive polymer for potential clinical applications. However, the clinical applicability of PNIPAAm-based microgels is limited due to neurotoxicity of its monomer.[24] Instead, a biologically compatible polymer, poly(*N*-vinylcaprolactam) (PVCL), has been developed with similar thermoresponsive characteristics to PNIPAAm, that is, PVCL can gradually and reversibly change from hydrophilic to hydrophobic in water when temperature is increased in the range of 0–35°C.[25,26] In contrast to PNIPAAm and *N*-isopropylacrylamide, both PVCL and *N*-vinylcaprolactam have been reported



Scheme 1. Preparation strategy of dual responsive microgels of poly(VCL-*co*-UA) and thermo/pH triggered release of DOX. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

to be much less cytotoxic, and hydrolysis of the amide group of PVCL does not produce small amide compounds that are potentially unsafe.[25,27–29] The LCST of PVCL can be easily tuned by controlling the polymer molecular weight and using a hydrophilic/hydrophobic comonomer.[30] Unlike the extensive literature on PNIPAAm-based copolymers, only a few reports have evaluated the potential application of PVCL-based responsive microgels with various functional derivatives as drug delivery systems.[27]

Since Gao et al. reported the synthesis of the PVCL-based nanogels by radical precipitation polymerization in 1999,[26] Forcada et al. performed systemic investigation on the PVCL-based nanogels and studied the kinetics of synthesis, competitive reactions between the propagation of PVCL chain and its hydrolysis during copolymerisation with ionizable comonomers such as acrylic acid (AA). They reported that higher polymerization yield and better colloidal characteristics of the nanogels could be achieved by adding the desired amount of acrylic acid 30 min after the polymerization reaction instead of mixing all of the reagents at once.[26,29–31] Recently, PVCL-based microgels comprised of ionic monomer such as AA and methyl methacrylate (MMA),[33,34] *N,N*-dimethylaminoethylmethacrylate (DMAEMA),[35] acrylamidoglycolic acid (AGA),[36] sodium acrylate (NaA),[26] and chitosan[37] have been reported, which typically show pH-dependent phase separation behavior in aqueous solution. The loading and release of various drugs by these functionalized PVCL microgels have also been studied.[33–40] For example, Subha et al. described synthesis of poly (VCL-*co*-AGA) via free radical emulsion polymerization and reported temperature/pH dual responsive release of 5-flurouracil (5-FU).[36] Demirel et al. prepared P(VCL-*co*-DMAEMA) nanogels by surfactant-free emulsion polymerization and Rhodamine B (RhB) was chosen as a model drug to demonstrate temperature/pH and ultrasound-enhanced pulsatile release for long-term and one-shot applications.[35] Poly(VCL-*co*-MMA) nanogels with size less than 50 nm were loaded with etoposide and targeted to B16F10 melanoma cells. A decrease in the number of cancerous cells was observed as a result of etoposide released from the microgels, indicating that these microgels may hold promise as drug delivery systems for cancer treatment.[38] Yang et al. prepared a series of multi-responsive disulfide crosslinked poly(VCL-*s*-*s*-MAA-3)-PEG microgels via precipitation polymerization in sodium bicarbonate buffer, which had excellent stability and distinct thermo/pH sensitivity.[39]

The fatty acid, 10-undecenoic acid (UA), has been used in drug delivery due to its biocompatibility, pH sensitivity, and easy copolymerization with other vinyl monomers.[1,41,42] In this work, we synthesized a new poly(*N*-vinylcaprolactam-*co*−10-undecenoic acid) [poly(VCL-*co*-UA)], in which 10-undecenoic acid was employed as the hydrophobic and pH sensitive segment. The thermal response mechanism and the effect of UA on thermal response were extensively discussed from the perspective of hydrogen bonding interaction. Furthermore, to assess the potential of poly(VCL-*co*-UA) microgels for temperature and pH-controlled drug delivery, *in vitro* release experiments using an encapsulated anticancer drug, doxorubicin (DOX), were performed under different physiological conditions. Finally, the biocompatibility of the microgels was also investigated using a cytotoxicity assay.

EXPERIMENTAL

Materials

N-vinylcaprolactam (VCL, 98% purity), 10-undecenoic acid (UA), and ammonium persulfate (APS, 98%) were purchased from Sigma Aldrich Chemicals. Sodium dodecyl sulfate (SDS, 98.5%) and *N,N′*-methylenebis(acrylamide) (BIS, 98%) were purchased from J&K Chemical Ltd. and used as received. VCL was recrystallized from n-hexane/toluene mixture. APS was recrystallized from deionized water and dried under vacuum.

Synthesis of the Thermo/pH Sensitive Poly(VCL-*co*-UA) Microgels

A series of thermo/pH sensitive poly(VCL-*co*-UA) crosslinked microgels were prepared via free radical precipitation polymerization with some modification to the method reported previously.[39] Polymerization was performed in a three-neck flask with condenser attached. The VCL monomer (1.13 g), $NaHCO_3$ (17 mg), UA (50 mg), and crosslinker BIS (30.8 mg) were dissolved in 95mL of deionized water containing 27 mg of SDS. The solution was maintained at 70°C under nitrogen, and stirred at 200 rpm. After all the reagents were dissolved and mixed well, 45 mg of APS was dissolved in 1 mL deionized water and injected into the mixture to initiate the polymerization reaction. The reaction was maintained under nitrogen atmosphere at 70°C for 5 h. The resulting dispersions were dialyzed (cut-off: 8000–14,000 Da) against water for 2 weeks to ensure complete removal of unreacted monomers and other small molecules. The synthesis protocol is depicted in Scheme 1.

The feed molar ratio of crosslinker and monomers (VCL and UA combined) was maintained at 50 : 1, and the molar ratio of VCL : UA was varied as shown in Table I.

Characterization of the Microgels

Chemical structure of poly(VCL-*co*-UA) microgels was analyzed by ^{1}H-NMR (400 MHz, $CDCl_3$, Bruker ARX 400).

Table I. Synthesis and Characterization of Poly(VCL-*co*-UA) Microgels

Sample ID	Feed ratio UA (molar%)	Yield (%)[a]	D_h[b] (nm)	LCST[c] (°C)	Zeta potential[d] (mV)	EE (%)[e]
PVCL	0	61.2	241	31.8 ± 1.58	−3.7	40.66 ± 1.01
PVU5	5	55.1	260	35.1 ± 2.03	−10.6	43.2 ± 1.63
PVU10	10	51.6	283	37.2 ± 2.27	−15.8	45.85 ± 1.73

[a] Determined by gravimetric analysis.
[b] Determined by dynamic light scattering at 25°C.
[c] Determined by UV-vis spectroscopy.
[d] The zeta potential of the copolymer solutions was measured at pH 7.4 by a Zetasizer Nano ZS instrument at room temperature.
[e] Encapsulation efficiencies (EE) was determined by ultrafiltration steps. Dox unloaded was isolated from the microgels buffer solution by ultrafiltration (ultrafiltration membrane MWCO 50000, Millipore). The isolated solution was measured using a UV-Vis spectrometer at 485 nm.

The average hydrodynamic diameter of the poly(VCL-*co*-UA) microgels in water were measured by dynamic light scattering (DLS) equipment using a (Brookhaven BI-200SM goniometer) equipped with a solid state laser source emitting at 532 nm and fitted with an external water-bath and thermostat. Samples were allowed to equilibrate thermally for 15 min before measurements were taken at each temperature. Measurements Readings were recorded in triplicate with a 20 s integration time for each measurement.

The thermoresponsive properties of poly(VCL-*co*-UA) microgels were investigated by UV-vis spectroscopy (Perkin-Elmer Lambda 35 instrument). The transmittance of microgel suspension was recorded at $\lambda = 500$ nm with increasing temperature from 20 to 40°C at a heating rate of 1.0°C/min. Before measurements were taken, the solution was equilibrated for 10 min at the desired temperature. The LCST is defined as the temperature corresponding to a 50% reduction in the initial value of transmittance.[20,41]

For morphological characterization, microgels suspended in water were dropped and dried on carbon tape. After drying, the samples were attached to an aluminum SEM stub and sputter-coated with gold, which were visualized at 10 kV with a scanning electron microscope (SEM; JEOL, JSM-7500F).

In Vitro Drug Loading and Release

DOX-loaded microgels were prepared by a simple protocol. Briefly, 2 mg DOX·HCl neutralized with three-mole excess of triethylamine in 3 mL DMSO and the solution was stirred to dissolve the DOX. Totally, 10 mg of microgels was then suspended in the drug solution. The mixture was dialyzed against 500 mL deionized water for 48 h to remove the free drug. The DOX-loaded microgels were filtered and freeze-dried. The UV absorbance of the supernatant was measured at 490 nm to determine the amount of supernatant DOX. The DOX content of the microgels was calculated by subtracting the DOX in the supernatant from that of the total DOX added to the loading solution. The encapsulation efficiency (EE) of DOX was calculated according to eq. (1)

$$\mathrm{EE}(\%) = \frac{D_a}{D_t} \times 100 \quad (1)$$

where D_a is the actual amount of the drug within the microgel network and D_t is the amount of the drug in loading solution.

In vitro drug release experiments were performed using previously reported methods.[43,44] 10 mg of a DOX-loaded poly(VCL-*co*-UA) microgels was suspended in 10 mL of a pH 7.4 buffer solution. The entire system was kept at 25°C or 37°C with continuous magnetic stirring. After a predetermined period, 3 mL was drawn out from release system for analysis, and 3 mL of fresh medium was added into the release buffer. The DOX concentration of each aliquot was determined by UV analysis following construction of a calibration curve. The release percentage of DOX was calculated using eq. (2):

$$\text{Drug release } (\%) = \frac{M_t}{M_\infty} \times 100 \quad (2)$$

Where M_t is the amount of DOX released from the microgels at time t and M_∞ is the amount of the DOX in the microgels.

Cytotoxicity Test

The cytotoxicity of the microgels was evaluated by the CCK-8 method. The tests were performed with three PVCL polymers with varying UA percentage (Table I). Eight concentrations of the polymers between 0.1 and 10 mg/mL were tested at 37°C. The incubation time in the CCK-8 test was 48 h. Briefly, NIH/3T3 cell cultured in complete DMEM containing 10% fetal bovine serum, 50 mg/mL gentamycin. Cells (104 cells/100 μL/well) were seeded into 96-well flat bottom tissue-culture plates in complete culture medium. Drug solutions were added after overnight and incubated for 24 h in a humidified atmosphere at 37°C and 5% CO_2. After incubation, 10 μL of CCK-8 (Dojindo, Japan) solution predissolved in phosphate-buffered saline was added to each well. After incubation for 1 h, the absorbance of each sample wells was measured by a microplate reader (Thermo Scientific Varioskan Flash) at the wavelength of 450 nm. The percentage of viable cells in each well was presented as the ratio of absorbance of treated and untreated control cells.

RESULT AND DISCUSSION

Chemical Analysis of the Poly(VCL-*co*-UA) Microgels

Poly(VCL-*co*-UA) microgels were prepared by emulsion free radical polymerization using APS as an initiator and BIS as a crosslinker. To confirm the polymerization of poly(VCL-*co*-UA), ^{1}H-NMR spectroscopy measurements were carried out. The chemical structure of the synthesized copolymers and the NMR spectrum are shown in Figure 1. The polyVCL peak positions

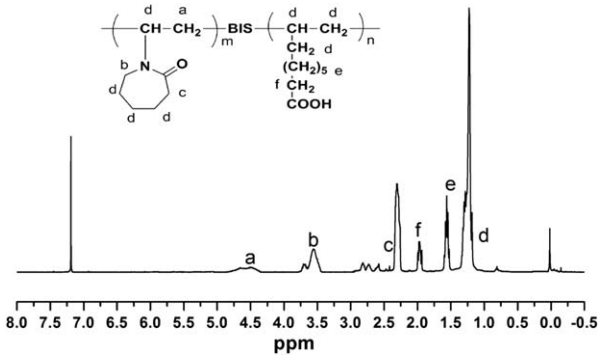


Figure 1. ^{1}H-NMR (CDCl3) spectrum of poly(VCL-*co*-UA).

were reported and accordingly poly(VCL-*co*-UA) were in good agreement with previously reported data.[33,37,42] The success of polymerization was evidenced by the absence of vinylic proton signals. In the spectrum of the synthesized copolymers, the disappearance of the peaks at δ4.8-5.0 (CH_2=$CHCH_2$) and δ5.7-5.9 (CH_2=$CHCH_2$) reveals that the polymerization was complete, because the monomers with carbon double bonds have been converted to polymer with C—C single bonds.

Volume Phase Transition

Incorporating functional co-monomers into VCL-based polymers is expected to shift the lower critical solution temperatures (LCSTs) and change the temperature range over which the phase transition occurs. This was firstly assessed by dynamic light scattering (DLS) experiments, and the results are shown in Figure 2.

As illustrated in Figure 2, the particle size of poly(VCL-*co*-UA) microgels is larger than that of pure PVCL microgels at pH 7.4.This increase in size may be attributed to the addition of hydrophilic -COO^- group of UA, which makes the microgels absorb more water and swell more at room temperature. The particle size remains roughly constant until ca.25°C, before diminishing rapidly between 30 and 35°C, after which the size is largely invariant on further heating.

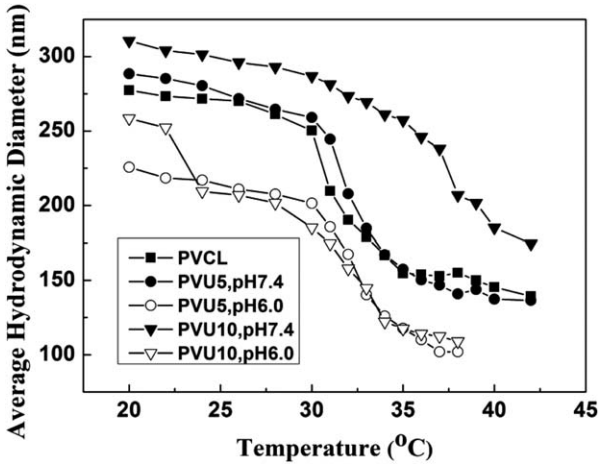


Figure 2. Temperature dependence of the hydrodynamic diameter for dilute (0.5 wt %) p(VCL-*co*-UA) microgel suspension at pH 6.0 and 7.4, respectively.

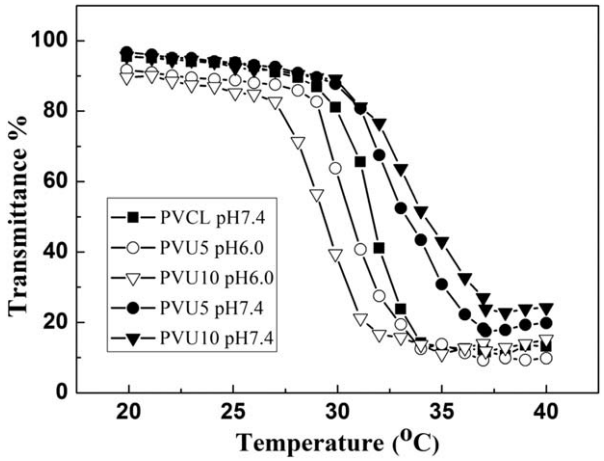


Figure 3. The optical transmittance at 500 nm of poly(VCL-*co*-UA) microgel suspension with different compositions and pH as a function of temperature (microgel concentration: 0.3 mg/mL).

The hydration of PVCL results from the formation of water network structures surrounding the hydrophilic groups of the polymer to produce stable water-network structures at room temperature.[25,26] As the temperature increases, the hydrogen bonds between the PVCL chains and water molecules are broken, hydrophobic interactions between the polymer chains become dominant, and the network structure of the microgels progressively collapse.

The size of the PVU-5 and PVU-10 exhibits apparent increase between pH 6.0 and 7.4 (225 nm vs. 270 nm and 256 nm vs. 310 nm, respectively), which can be attributed to the deprotonation of the -COOH groups of UA to become -COO^- at a higher pH. The results also showed that hydrodynamic diameter at pH 7.4 is bigger than at acidic conditions pH 6.0 in the copolymers. In addition, the hydrodynamic diameter increase with more incorporation of UA in recipe, consistent with literature reports.[42,44,45] These could be attributed to negative charge density on the microgel surface due to the ionization of the carboxyl groups from UA, resulting in an increase of the electrostatic repulsion of copolymer chains with the increase of UA content.

Lower Critical Solution Temperature (LCST)

The LCST is defined as the temperature corresponding to a 50% reduction in the initial value of transmittance. It can be seen in Figure 3 that both the percentage of UA and the pH are important to LCST shift. The protonated PVU microgels at pH 6.0 and 7.4 displayed sharp phase transition and the LCST of 28.7 and 30.1°C, while those ionized at 7.4 showed broad volume phase transition and higher LCST of 34.5 and 35.3°C. These results were related to the increased negative charges originating from carboxyl groups of UA in higher pH condition. As the negatively charged carboxyl groups of UA were completely ionized at higher pH, the noticeably increased negative charges on the particle surface increased the hydrophilicity and stability of microgels and weakened the swelling/collapse behavior. As the UA composition increases, more hydrophilic carboxyl groups of UA are incorporated into the polymer, and the

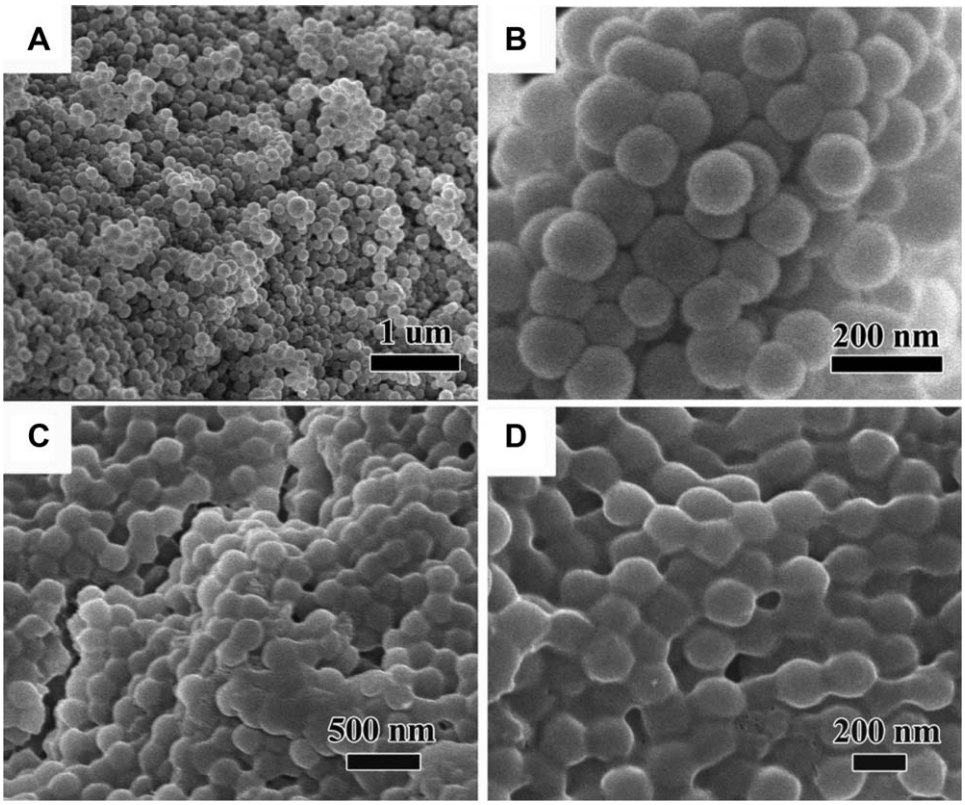


Figure 4. SEM images showing the morphology of poly(VCL-*co*-UA)10% (A, B) and drug-loaded poly(VCL-*co*-UA) (C, D).

amount of hydrogen bonding between the copolymer chains and water molecules increases significantly. This makes it more difficult for water to be excluded from the polymer chains, resulting in a more gradual chain-to-globule transition.[46,47] At low pH (i.e., 6.0), UA becomes hydrophobic due to protonation of its carboxyl group, decreasing the LCST of the polymer and causing microgels to collapse over 31.3°C.

In addition, at high UA contents, a slight increase in pH can cause dramatic changes in the LCST. The temperature-responsive poly(VCL-*co*-UA) copolymer is triggered under a small pH change window as sufficient UA units switch from a hydrophobic state to an ionized state at defined regions between pH 6.0–7.4. Thus, the LCST can be adjusted to desired range at a particular pH value (such as endosomal pH) by changing the UA content of the copolymer.

Microgel Morphology

SEM images in Figure 4 confirm that poly(VCL-*co*-UA) and DOX-loaded poly(VCL-*co*-UA) microgels were almost monodispersed spheres. The average size of the DOX-loaded poly(VCL-*co*-UA) microgels was approximately 150–200 nm with a narrow size distribution. The size data was different between the DLS and SEM analysis methods, because that SEM images were taken on crosslinked dried microgels, while DLS analysis was performed on microgels in the aqueous and swollen state. The hydrodynamic particle size of the DOX-loaded microgelsis ~50% larger than the blank ones (ca., 100–150 nm), which is likely due to the incorporation of large and bulky drug molecules (Mw of Doxorubicin hydrochloride: 579.89 g/mol) within the networks.

In Vitro Release

The DOX was encapsulated into the microgels by the hydrophobic interactions of PVCL moieties as well as the formation of hydrogen bonds. The encapsulation efficiencies of DOX were 43.2%, and 45.8% for the 5% and 10% UA microgels, respectively.

As discussed above, since these PVCL-based microgels are both temperature and pH-sensitive, controlled release of encapsulated compounds could be achieved by varying the temperature and pH conditions. Figure 5(A) show the release profile of DOX from PVU-10 at 37°C and varying pHs (6.0, 6.8, and 7.4) was a function of incubation time. Due to the diffusion-controlled drug release mechanism, the system showed slow release at pH 7.4 (ca., 40% in 10 h), while relatively faster release was detected at pH 6.0 (ca., 80% in 10 h), nearly two folds of the release rate at pH 7.4. In acidic environment, UA became hydrophobic due to its protonation, which lowers the LCST and the DOX molecules are positively charged, both factors caused the drug-loaded nanoparticles to deform and precipitate. Based on these results, we expect that the poly(VCL-*co*-UA) microgels are sensitive to mildly acidic environments and suitable for tumor drug-delivery.

As shown in Figure 5(B), we summarized the release profiles at 25°C, 30°C, and 37°C. The poly(VCL-*co*-UA) microgels exhibited a temperature-dependent release profile of DOX. There was

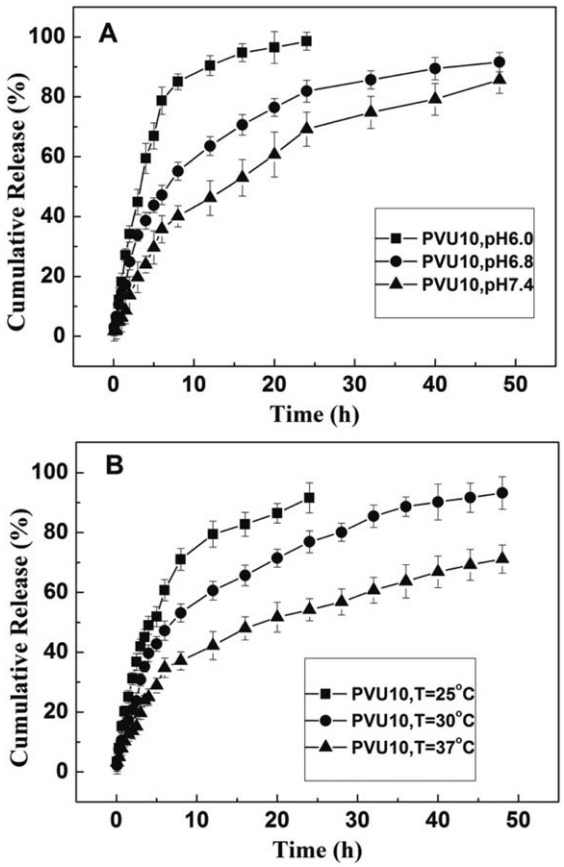


Figure 5. A: Cumulative DOX release from poly(VCL-*co*-UA) microgels at 37°C in buffers with pH values of 6.0, 6.8, and 7.4. B: Cumulative DOX release from poly(VCL-*co*-UA) microgels in PBS (pH 6.8) solution at different temperature. Data were presented as mean ± standard deviation ($n = 3$).

an unfavorable burst release of over 70% of drug in the initial 10 h when incubated at 25°C (swollen state) while the release rate and the total amount released were notably greater than at 37°C (collapsed state). The rapid release occurred at 25°C because at this temperature the polymer is below its LCST. The entrapped drug molecules diffuse out of the poly(VCL-*co*-UA) networks the quickly because microgels are highly porosity and hydrated in the swollen state. In contrast, at 37°C, the network is collapsed and more hydrophobic than at 25°C, so that drug diffusion is significantly hindered.[48,49] The rapid release of ca., 20–25% of DOX in the first few hours at 37°C is probably due to dissolution of loosely bound drug molecules from the surface of microgels.[50]

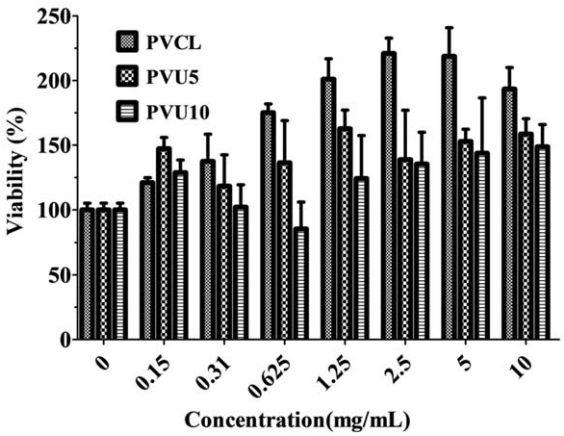


Figure 6. Cytotoxicity of PVCL and poly(VCL-*co*-UA) microgelsin culture of 3T3 cells for 48 h. Data are presented as the average ± standard deviation ($n = 4$).

In Vitro Cytotoxicity Evaluation

Cytotoxicity study of PVCL, poly(VCL-*co*-UA) 5% and poly(VCL-*co*-UA) 10% microgels were performed by the CCK-8 assay using 3T3 murine fibroblast cells. Cells were exposed to the microgels at concentration from 0.15 to 10 mg/mL. The materials showed no obvious toxicity to the cells (cell viabilities ≥90%) up to a concentration as high as 10.0 mg/mL (Figure 6), which not only revealed that PVCL and poly(VCL-*co*-UA) microgels were nontoxic even when applied at a rather high concentration. Moreover, it was also evident that the cells were viable and proliferated in the presence of the microgels, suggesting that the pVCL materials may provide a suitable environment for cells and potentially be used as scaffolds for tissue engineering. Indeed, we propose that these thermosensitive polymers may have the potential as substrate materials for cell adhesion control and cell sheet engineering, or as switchable cell culture scaffold for cell transplantation.[51]

CONCLUSIONS

In this study, we prepared new thermo/pH dual-response microgels via precipitation emulsion polymerization based on crosslinked copolymers of VCL and UA. The obtained microgels showed uniform particle size, and ^{1}H-NMR confirmed that the comonomers (VCL and UA) were well incorporated into the chemical structure. The materials showed thermal and pH responsiveness, which can be adjusted by changing the composition of the co-monomers in the synthesis process. *In vitro* drug release was sensitive to moderately acidic pH environment, suggesting that these poly(VCL-*co*-UA) microgels may be a promising anticancer drug-delivery system to enhance effective killing of cancer cells while reducing side-effects on normal tissues. The pH/temperature responsive properties of the material may provide a promising platform for controlled intracellular release of potent chemotherapeutic drugs as cancer treatment or injectable scaffolds for tissue engineering.

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