Acid degradable poly(vinylcaprolactam)-based nanogels with ketal linkages for drug delivery†

Yang Wang,ab Jin Zheng,a Yefei Tiana and Wuli Yang*ab

To improve the biocompatibility and biodegradability of nanocarriers, well-defined poly(vinylcaprolactam)-based acid degradable nanogels were fabricated for drug delivery via precipitation polymerization in water, where synthetic ketal-based 2,2-dimethacryloxy-1-ethoxypropane (DMAEP) acted as a cross-linker, and N-(2-hydroxypropyl)methacrylamide (HPMA) served as a co-monomer. Expectedly, we observed that the temperature and pH of the environment play important roles in the performance of the nanogels. The nanogels were reduced in size upon increasing the temperature and showed higher volume phase transition temperature (VPTT) with higher concentration of HPMA. With the incorporation of ketal linkages, the nanogels showed accelerated degradation profiles by lowering the pH and increasing temperature of the incubation medium. When used as nanocarriers of anticancer drug doxorubicin (DOX), compared to non-degradable nanogels with similar components, the acid-degradable nanogels displayed more effective drug controlled release behaviour, low drug leakage of DOX at neutral pH while rapid and sufficient release from the nanogels under acidic conditions. The results of the cytotoxicity and hemolysis assays further highlighted that the acid-degradable nanogel produced no hemolysin but showed excellent viability to normal cells, and the DOX-loaded nanogel exhibited higher proliferation inhibition against tumor cells.

Introduction

Recent advances in stimuli-responsive polymer nanomaterials for cancer therapy have led to the development of smart drug delivery systems that can reduce side effects and enhance the delivery efficiency of anticancer drugs.1–5 Among the polymer nanomaterials studied and applied, nanogels, as a novel family of nanoscale materials, are very promising candidates as drug delivery systems since they exhibit excellent properties such as high colloidal stability for prolonged circulation in the bloodstream, and a large surface area enabling encapsulation of the bioactive drugs.6,7 The stimuli-sensitive nanogels, capable of responding to environmental stimuli such as temperature, pH, light, glucose and redox potential, have been engineered to allow for controlled release and site-specific targeting of the loaded drugs modulated by environmental stimuli.8,9

As certain malignancies are locally characterized by a distinct hyperthermia, thermosensitive nanogels which can respond to temperature changes by changing their network mesh size have been conveniently used to control the drug loading or to tune the drug release from these nanogels.10 As a kind of thermosensitive nanomaterials, poly(N-vinylcaprolactam) (PVCL)-based nanogels as drug carriers have been widely investigated in recent years due to their sharp thermosensitive phase transition and better biocompatibility.11,12 As shown by recent studies, heterogeneous polymerization, including precipitation polymerization (emulsifier-free emulsion polymerization),13 inverse mini/micro emulsion polymerization,14 distillation–precipitation polymerization15 and so on have been widely employed to prepare polymer nanogels. Among these strategies, the method of precipitation polymerization, carried out in an aqueous medium with no or minimal surfactant, is popular for the preparation of thermally sensitive nanogels. In the preparation of nanogels, divinyl cross-linkers are frequently incorporated to achieve a highly stable polymer network.16,17

It will be highly desirable if the nanogels can disintegrate into non-toxic degradation products, and simultaneously lead to a burst release of drugs. Cross-linkers that degrade or hydrolyze in response to environmental stimuli, such as acidic pH,18 redox potential,19 enzyme,20 or light,21 have been introduced into the nanogels to trigger the drug release, as their...
degradation results in swelling or total dissolution of the nanogels which enhances the diffusivity of the embedded drugs.

The existing typical biological features in vivo can favour the drug release. For instance, the pH differences between intracellular lysosomes (pH 4.0–4.5), endosomes (pH 5.0–5.5), and extracellular compartments (pH 7.4) can act as a trigger for intracellular drug release if an acid-cleavable cross-linker is incorporated into the nanogels. The development of acetal or ketal-based polymer networks has led the way due to the non-acidic degradation products, terminated with dioi and carboxyl end groups. Recently, Sui et al. synthesized an acetal-based polymer nanogel by reaction of the hydroxyl pendant groups of the polymer and 1,4-cyclohexanedimethanol divinyl ether after the reversible addition-fragmentation chain-transfer polymerization (RAFT). The release rate of the loaded drug molecules from the nanogels can be modulated by the pH of the environment.

Zhong et al. prepared core-crosslinked pH-sensitive degradable nanogels, where acid-labile acetal and photo-crosslinkable acryloyl groups existed in the hydrophobic polycarbonate block for intracellular paclitaxel (PTX) release. Haag’s group fabricated new pH-cleavable cell-laden microgels with excellent long-term viabilities by strain-promoted azide–alkyne cycloaddition (SPAAC), which exhibited excellent abilities of protecting cell viabilities and controlling the release of cells triggered by benzacetal hydrolysis. Another example is a novel stable thermo-responsive and acid-degradable nanogel with a core containing acid-labile acetal cross-linker via a RAFT polymerization technique, where the protein-loaded acid-degradable nanogel showed a controlled release profile of protein.

Herein we developed novel thermosensitive and acid-degradable P(VCL-ketal-HPMA) nanogels by incorporating an acid-cleavable ketal cross-linker. As shown in Scheme 1, the nanogels were prepared via precipitation polymerization and hydrophilic N-(2-hydroxypropyl)methacrylamide (HPMA) was used as a co-monomer. As HPMA has been carefully investigated in biomedicine due to its non-immunogenicity and non-toxicity, the introduction of HPMA can modulate the volume phase transition temperature (VPTT) of the nanogels and improve the nanogel biocompatibility. Such well-fabricated acid-degradable nanogels are applicable to effectively load the anticancer drug DOX, and exhibit an acid-triggered drug release profile as the nanogels can be degraded into individual short linear polymer chains under acidic conditions. Subsequently, hemolysis assays and cytotoxicity assays of blank nanogels against normal cells and DOX-loaded nanogels to cancer cells were also conducted. Non-degradable nanogels were prepared by introducing permanent cross-linker N,N'-methylenebisacrylamide for comparison.

**Experiment**

**Materials and methods**

N-Vinylcaprolactam (VCL, 99%) was obtained from Sigma-Aldrich. N-(2-Hydroxypropyl)methacrylamide (HPMA) was synthesized as described in a recent report. Ketal cross-linker 2,2-dimethacryloxy-1-ethoxypropane (DMAEP) was synthesized by condensation methods described in a recent report. N,N'-Methylenebisacrylamide (MBA) were purchased from Shanghai Aladdin Chemistry Co. Ltd. Potassium persulfate (KPS), sodium dodecyl sulfate (SDS), and sodium bicarbonate (NaHCO3) were obtained from Shanghai Chemical Reagents Company. KPS was recrystallized from water. Doxorubicin (DOX), in the form of the hydrochloride salt, was obtained from Beijing Huafeng United Technology Company. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and other biological reagents were purchased from Sigma Corp. The other chemicals were of analytical grade and were used as received.

1H NMR was performed on a Bruker 500 MHz spectrometer. Fourier transform infrared (FTIR) analysis was conducted on a Nicolet Nexus-440 FT-IR spectrometer. UV-vis spectra were obtained using a Perkin-Elmer Lambda 35 spectrophotometer. The PVCL-based nanogels were negatively stained with 1% phosphotungstic acid for their morphology characterization using a Hitachi H-600 transmission electron microscope (TEM). The hydrodynamic diameter and light scattering intensity of the nanogels were measured using a dynamic light scattering (DLS) particle size analyzer (Malvern Nano ZS90) at a scattering angle of 90°. The average molecular weight and molecular weight distribution (Mw/Mn) of linear polymer chains were estimated by Gel Permeation Chromatography (GPC) measurements. GPC was performed on a HP Agilent series 1100 Chromatograph equipped with a G1310A pump, a G1362A refractive index detector, and a G1315A diode-array detector. Poly(ethylene oxide) (PEO) standard samples were used for calibration and 0.1 M NaNO3 aqueous solution at an elution rate of 1 mL min⁻¹ was used as the mobile phase. The cellular images were acquired using a confocal laser scanning microscope (CLSM, Leica TCS SP5).

**Preparation of PVCL-based nanogels**

The P(VCL-ketal-HPMA) nanogels cross-linked with ketal cross-linker DMAEP were prepared by precipitation polymerization and 1 wt% of the sum (VCL + HPMA) but different mass ratios of VCL to HPMA (VCL/HPMA: 100/0, 97/3, 95/5, 92/8, 90/10) were used (Table 1). A typical procedure for the preparation of
nanogels is as follows: 950 mg VCL, 50 mg HPMA, 40 mg SDS, 30 mg NaHCO$_3$, and 40 mg DMAEP were dissolved in 100 mL pure water in a three-neck flask equipped with a reflux condenser, then the reactor contents were heated to 70 °C and kept under a nitrogen atmosphere. After mechanical stirring of 200 rpm for 0.5 h, a shot of 25 mg KPS dissolved in 2 mL water was added and the polymerization was initiated. The polymerization reaction was allowed to continue under a nitrogen atmosphere. After mechanical stirring of 200 rpm for 0.5 h, a shot of 25 mg KPS dissolved in 2 mL water was added and the polymerization was initiated. The polymerization reaction was allowed to continue under a nitrogen atmosphere with stirring for 6 h. The reaction mixture was subsequently cooled to room temperature, while maintaining the stirring and nitrogen flow to prevent possible aggregation. Finally, the obtained nanogels were dialyzed at pH 8.0 for a week via a dialysis bag (molecular weight cut off 14 000) to remove the unreacted reagents and impurities. By contrast, the polymer P(VCL-co-HPMA) without cross-linker and P(VCL-cross-HPMA) nanogels cross-linked with stable cross-linker MBA were synthesized as described in the preparation of P(VCL-ketal-HPMA) nanogels.

### Acid-triggered degradation of nanogels

The turbidity change of the nanogels in response to different pH values was monitored by dynamic light scattering (DLS) measurement to estimate the acid degradation of nanogels. Briefly, samples at an identical particle concentration of 1.0 mg mL$^{-1}$ were prepared in 0.01 M phosphate buffers of pH 5.0, 6.5, and 7.4, respectively. Subsequently, the prepared samples were incubated in a shaking bath at 37 °C with a rotation speed of 200 rpm, and were withdrawn at predetermined intervals to measure the scattering light intensities at 90° by DLS. In addition, samples prepared in the buffer of pH 5.0 were incubated at 45 °C to investigate the temperature influence on the profile of the acid degradation. The ratio of the scattering intensity of nanogels at a predetermined interval to that of the initial non-degradable nanogels was calculated to determine the relative turbidity. The degraded polymer solutions were collected and the molecular weight of the degraded polymers was measured in 0.1 M NaNO$_3$ aqueous solution by GPC after the polymer solutions were filtered through a 0.45 μm filter without strong press.

### Drug loading and release

DOX, chosen as a model anticancer drug, was loaded into nanogels using an incubation method. Typically, DOX aqueous solution (1 mg mL$^{-1}$) was adjusted to pH 7.4 using 0.1 M NaOH. Then 10 mg of nanogels was ultrasonically dispersed into 2 mL DOX solution and incubated under magnetic stirring for 24 h at room temperature. The DOX-loaded nanogels were separated from the dispersion by centrifugation and washed with phosphate buffer of pH 7.4 twice to remove the surface adsorption of DOX. The DOX amount loaded into the nanogels was calculated by subtracting the mass of DOX in the total supernatant from the total drug mass in the initial solution by UV-vis spectrometry at 480 nm against the pre-established calibration curve.

To investigate the pH-dependence of the drug release profiles of nanogels, various buffer solutions of different pH values containing 0.15 M NaCl were chosen: sodium acetate buffer for pH 5.0, phosphate buffers for pH 6.5 and 7.4. Typically, 1 mg of DOX-loaded nanogels was dispersed in 1 mL corresponding buffer and then placed into a dialysis bag, which was subsequently immersed into a glass reservoir containing 100 mL of the above buffer solution and gently shaken at 37 °C. In order to investigate the temperature influence on the drug release behaviour, the drug release experiments were respectively conducted in the buffer solutions of pH 5.0 and 7.4 at different temperatures. At predetermined time intervals, 2 mL of the external buffer solution was withdrawn from the reservoir and analyzed by UV-vis at 480 nm. To retain a constant volume, 2 mL of fresh buffer medium was added back to the reservoir after each sampling. All drug-release experiments were performed in triplicate and the results were expressed as the average data with standard deviations.

**In vitro cell and hemolysis assays**

The cytotoxicity assay of DOX-loaded nanogels against HeLa cells (human cervical carcinoma cells, cancer cells) and blank nanogels against HK-2 cells (human tubular epithelial cells, normal cells) were assessed by standard MTT assay. HeLa cells and HK-2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) FBS (fetal bovine serum), penicillin (100 U mL$^{-1}$) and streptomycin (100 mg mg$^{-1}$) at 37 °C and 5% CO$_2$. Typically, HeLa cells were seeded at a density of 5 × 10$^3$ cells per well and incubated in 96-well plates for 24 h to allow cell attachment. Then the cells were treated with blank nanogels, DOX-loaded nanogels, and free DOX at various concentrations and incubated for 48 h at 37 °C, respectively. Next, 20 μL of MTT solution (5 mg mL$^{-1}$ in PBS) was replaced with fresh DMEM containing MTT (5 mg mL$^{-1}$), and the cells were incubated for another 4 h. Then, the supernatant was removed, and 150 μL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan. The relative cell viability was calculated by comparing the absorbance of treated cells at 570 nm to that of control cells. The results were expressed as mean values of three measurements. The same process of cytotoxicity to blank nanogels against HK-2 cells was followed as mentioned above.

The cellular uptake and intracellular release behaviours of DOX-loaded nanogels and free DOX were performed using confocal laser scanning microscopy (CLSM). HeLa cells were...
incubated in a six-well plate at 37 °C with the DOX-loaded nanogels and free DOX, respectively. At predetermined intervals, the culture media were removed, and the cells were washed two times with PBS prior to the CLSM observation.

Hemolysis assays of the P(VCL-ketal-HPMA-8) nanogel were conducted against rat RBCs which were separated from fresh rat blood by centrifugation at 3000 rpm for 20 min to remove plasma and other cell debris. After washing three times with normal saline, purified RBCs were resuspended in normal saline to obtain 2% (v/v) RBC suspension. Then, different volumes of stored nanogel (15 mg mL⁻¹) diluted in saline were added to 2.5 ml of 2% rat RBC suspension to make the final particle concentration 0.1, 0.2, 0.5 and 1 mg mL⁻¹, respectively. After incubation at 37 °C for 4 h, the samples were centrifuged at 2500 rpm for 10 min and the supernatants were observed for indication of haemolysis. Distilled water served as positive control and normal saline as negative control.

Results and discussion
Preparation of PVCL-based nanogels
In this work, by using a precipitation polymerization method, a series of PVCL-based nanogels were prepared at different mass ratios of HPMA and VCL by incorporating acid-cleavable cross-linker DMAEP. The chemical structure and acid-hydrolysis of ketal cross-linker DMAEP was further confirmed by 1H NMR (Fig. S1 and S2, ESI†). In these experiments, KPS was used as an initiator, and NaHCO₃ acted as a buffer to keep the reaction basic to avoid the hydrolysis of the cross-linker and VCL.³² The typical recipes and tested results are shown in Table 1. With an increase in the feeding amount of HPMA from 0 to 10 wt%, the hydrodynamic diameter (Dh) of the P(VCL-ketal-HPMA) nanogels increased from 90 to 230 nm with narrow size distribution (PI < 0.1). This result was owing to the higher hydrophilicity of comonomer HPMA. In precipitation polymerization, when the polymer chains reach a critical chain length, they become insoluble and precipitate, forming colloidal unstable particle nuclei. As the feeding amount of HPMA increased, the growing polymer chains became more hydrophilic and were unable to undergo precipitation until they reached a longer chain length. Therefore, the particle nuclei number decreased, resulting in larger particles.³³

Fig. 1 illustrates the representative TEM images of P(VCL-ketal-HPMA) nanogels with different HPMA contents (Fig. S3, ESI†). All the nanogels exhibited uniform and regular spherical morphology, consistent with the narrow size distribution. The size of the nanogels observed in TEM increased from 68 ± 10 nm (3 wt% HPMA) to 108 ± 20 nm (10 wt% HPMA). In comparison with the sizes measured by DLS and TEM, the size measured by DLS was larger than that presented in the corresponding TEM images. The explanation for the result maybe that the nanogels for DLS measurement are swollen in the aqueous solution while those for TEM observation are collapsed and air-dried. The difference in sizes measured by DLS and TEM means that the nanogels have obvious swelling behaviour. For instance, the size of the P(VCL-ketal-HPMA-8) nanogel measured in the swollen state is 206 nm but 98 ± 10 nm in the shrunk state (TEM).

The introduction of the monomer VCL, HPMA and cross-linker DMAEP into PVCL-based nanogels was characterized by FTIR spectroscopy (Fig. 2). As exhibited in the FTIR spectrum of PVCL-based nanogels, the absorptions at 1624 and 1482 cm⁻¹ could be attributed to amide I and II bands of PVCL.³⁵ In the spectra of DMAEP and DMAEP-crosslinking nanogel, the typical ester C=O band of cross-linker DMAEP could be clearly observed at 1726 cm⁻¹,³⁶ while no absorption appeared at 1726 cm⁻¹ in the spectrum of MBA-crosslinking nanogel. This observation further confirmed the successful incorporation of the acid-degradable cross-linker into the polymer network. The bands appearing at 1533 cm⁻¹ were attributed to C–N stretching and N–H bending vibration of amide from PHPMA.³⁷ As expected, the characteristic peaks were observed clearly in the FTIR spectra of the HPMA-introduced nanogels. The 1H NMR spectroscopy measurement was carried out. Only the signals of the peripheral linear polymer chains were observed from the 1H NMR spectrum (Fig. S4, ESI†), as the cross-linker located inside. The PVCL and PHPMA characteristic positions were in good agreement with previous reports.³⁸–⁴⁰

Temperature sensitivity of P(VCL-ketal-HPMA) nanogels
The temperature-sensitivity of the nanogels was analyzed by monitoring the change in hydrodynamic diameter as a function
of HPMA content with increasing temperature from 20 to 65 °C. Fig. 3 shows the deswelling behavior of the P(VCL-ketal-HPMA) nanogels with different feeding amounts of HPMA. As largely observed in the case of PVCL-based particles, all the nanogels exhibited a decrease in hydrodynamic diameter with an increase in temperature, corresponding to a volume phase transition.\(^{31}\) For example, the P(VCL-ketal-HPMA-8) nanogels presented a size of about 210 nm at 20 °C while decreased to about 100 nm at 60 °C. When the temperature rises, the outstretched polymer chains become more hydrophobic and collapsed, causing a decreased size.

The volume phase transition temperature (VPTT) calculated from the \(D_h\) versus \(T\) curves for each nanogel is shown in Table 2. The VPTT change of shifting from 31.3 (3 wt% HPMA) to 44.0 °C (10 wt% HPMA) indicated the nanogels underwent volume phase transition at higher temperatures with increasing feeding amount of HPMA. It could be noted in Fig. 3 that the P(VCL-ketal-HPMA-10) nanogels had the highest VPTT and the broadest phase transition range.\(^{42,43}\) As more HPMA fed in the polymerization improved the hydrophilicity of the polymer chains, the balance of hydrophilic and hydrophobic forces would be broken at higher incubation temperature, which resulted in a higher VPTT and a broader phase transition range.\(^{42,43}\)

In principle, one of the criteria for the temperature-sensitive nanoparticles as the potential drug delivery systems is that the vehicle can remain swollen in the bloodstream but quickly collapsed in tumor tissues. In this sense, premature drug leakage can be avoided in the bloodstream while rapid drug release occurs when reaching the tumor sites. In view of this, the P(VCL-ketal-HPMA-8) nanogel, exhibiting an applicable VPTT of 39.5 °C which is higher than the physiological temperature (37 °C) and close to the physiological temperature, was chosen as a drug carrier for further study.

**Acid-triggered degradation of P(VCL-ketal-HPMA) nanogels**

Successful incorporation of the ketal-bonded cross-linker into the nanogel network would result in the degradation of the nanogels via acid-catalytic scission of the cross-linking points. The degraded polymer could be easily eliminated through the excretion pathway in vivo, resulting in reduced cytotoxicity.\(^{44}\)

The turbidity measurement by DLS was used to trace the degradation degree of the P(VCL-ketal-HPMA) nanogel with 8 wt% HPMA at pH 5.0, 6.5 and 7.4, respectively. As displayed in Fig. 4a, it could be obviously observed that the degradation rate of the nanogels was pH-dependent. At pH 7.4, the relative turbidity of the nanogel was almost 100% in 24 h, indicating no degradation. At pH 6.5, the relative turbidity decreased to 65.4% in the same time. And at pH 5.0, by contrast, the relative turbidity rapidly decreased to 45% in 0.5 h and to 16% in 1 h. The above results are related to the acid-triggered hydrolysis profiles of the cross-linker DMAEP (Fig. S5, ESI†). In an acidic medium, one oxygen atom of the ketal group was protonated, which facilitated water molecules to attack the carbonyl carbon and subsequently resulted in the degradation of the ketal compounds.\(^{45}\) Thus, under acidic conditions, the ketal linkages in the polymer network were cleaved, which made the skeleton network of nanogels to disassemble into free linear P(VCL-co-HPMA) chains and resulted in complete dissolution of the nanogels. It could be found that the hydrolysis rate of P(VCL-ketal-HPMA) nanogels was slower than that of the ketal cross-linker DMAEP (Fig. S4, ESI†) at the same pH value. The half-life of the P(VCL-ketal-HPMA) nanogels at pH 5.0 was 0.45 h, and that of the ketal cross-linker DMAEP was 0.23 h. This may relate to the more time required for the diffusion of the buffer solution into the polymer network and inadequate contact between ketal linkages and buffer solutions.\(^{46}\) It could be seen from Fig. 4b that in the buffer solution of pH 5.0, the acid-degradation rate of the nanogels at 45 °C is faster than that at 37 °C expectedly. The half-life of nanogels at pH 5.0 decreased from 0.45 h to 0.15 h when the temperature was increased from 37 °C to 45 °C. This result coincided with that of the polyether polyketals reported by Kizhakkedathu and co-workers.\(^{47}\) The higher temperature could promote the acid-hydrolysis of the inside ketal bonds, resulting in a faster acid-degradation rate.

The sizes of the P(VCL-ketal-HPMA) nanogel with 8 wt% HPMA before and after degradation were determined by DLS analysis for further investigation (Fig. 5). The size of the nanogels decreased from about 200 nm to about 10 nm after

---

**Table 2.** VPTT of P(VCL-ketal-HPMA) nanogels fed with different amounts of HPMA

<table>
<thead>
<tr>
<th>Sample code</th>
<th>VPTT (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P(VCL-ketal-HPMA-3)</td>
<td>31.3</td>
</tr>
<tr>
<td>P(VCL-ketal-HPMA-5)</td>
<td>36.0</td>
</tr>
<tr>
<td>P(VCL-ketal-HPMA-8)</td>
<td>39.5</td>
</tr>
<tr>
<td>P(VCL-ketal-HPMA-10)</td>
<td>44.0</td>
</tr>
</tbody>
</table>
2 h of incubation in an acidic environment. Correspondingly, it could be observed from that there was no obvious spherical morphology in the TEM image of the degraded nanogels (Fig. S6, ESI†). These results all indicated that the nanogels could break down into linear polymers under acidic conditions. As a control, the linear polymer P(VCL-co-HPMA) containing 8 wt% HPMA was synthesized. The size of the linear P(VCL-co-HPMA) was about 5 nm, approaching the size of the degraded nanogels. Besides, the nanogel dispersion appeared as a milky emulsion before degradation (Fig. 5, inset A), but as a clear solution after degradation (Fig. 5, inset B) which was similar to the appearance of the synthetic linear copolymer (Fig. 5, inset C). The above results suggested that the scission of the ketal cross-linking units under acidic conditions resulted in the complete dissolution of the nanogels.

GPC measurement was further used to analyze the molecular weight of the degraded nanogels. As shown in Fig. 6, the GPC results showed that the weight-average molecular weight of the degraded polymers is 1500 g mol\(^{-1}\) with narrow molecular weight distribution (\(M_w/M_n\) 1.07). We further measured the molecular weight of the synthetic copolymer poly (VCL-co-HPMA) by GPC (Fig. S7, ESI†) and found that the copolymer had a close molecular weight (\(M_w\), 2100 g mol\(^{-1}\)) and narrow molecular weight distribution (\(M_w/M_n\), 1.05). These results indicated that the nanogels had a uniform polymer network inside and through acid catalysis could be disassembled into short linear polymers which corresponded to the oligomers composing the nanogels. As mentioned above, the VPTT of the P(VCL-ketal-HPMA-8) nanogels was 39.5 °C, higher than and close to the physiological temperature (37 °C), which meant the degraded linear polymers of the nanogel also had a lower critical solution temperature (LCST) higher than 37 °C so as to be easily metabolized as solution. Therefore, when the nanogels were degraded by disconnecting the cross-linking units, the short linear polymers from disassembled nanogels could be easily eliminated through the renal excretion pathway in vivo.\(^48\)

**Drug loading and release**

As mentioned above, in view of the applicable VPTT of nanogels, the P(VCL-ketal-HPMA) nanogel with 8 wt% HPMA was chosen to investigate the drug loading and release of nanogels. Besides, the drug loading and release behaviour of the non-degradable P(VCL-cross-HPMA) nanogel with the same HPMA feeding amount were also investigated as a control. DOX, a DNA interacting drug widely used in chemotherapy,\(^49\) was used as a model anti-cancer drug to test the loading and releasing properties. The nanogels were loaded with DOX (1: 0.2 w/w) using an incubation method in a highly concentrated DOX solution at pH 8.0 for 24 h, and magnetic stirring could favour the diffusion of drug molecules into the pores of the nanogels. The incubation drug loading method takes advantage of the sponge-like nature of the nanogels, thereby allowing solute molecules to partition into the porous network.\(^50\) This drug loading method has been widely used to prepare drug delivery systems based on thermosensitive nanogels.\(^51\) The zeta potentials before and after drug loading were −19.5 mv and −18.4 mv, respectively. The lower negative potential indicated that there were enough negative charges on the surface to stabilize the particles. The drug loading results indicated that, for the P(VCL-ketal-HPMA) nanogel, the DOX loading content and encapsulation efficiency were 13 wt% and 74 wt%, respectively. The contrast of the non-degradable P(VCL-cross-HPMA) nanogel also presented a similar drug loading capacity, giving a DOX loading content of 11 wt% and an encapsulation efficiency of 62 wt%. The higher drug loading content and encapsulation efficiency may result from the hydrophobic interaction between the drug and polymer and hydrogen bonding to the amide groups on the polymer side chains.\(^52\)

Based on the aforementioned discussions, the acid-degradable property of the nanogels would result in the acid-triggered drug release profiles due to the different pH domains throughout the body. The effect of pH on the drug release from the DOX-loaded P(VCL-ketal-HPMA-8) nanogel was studied directly through investigating the in vitro drug release profiles at different pH values (5.0, 6.5 and 7.4). For comparison, the drug release profiles of the non-degradable P(VCL-cross-HPMA-8) nanogel were also studied under the same conditions. The release curves of DOX in 48 h are shown in Fig. 7. It was found that the two nanogels both presented pH-dependent drug release profiles that the total amount of drug release increased upon a decrease of the pH value. At pH 7.4, the two nanogels released similar amounts of drug in 48 h, i.e. 13 wt% for P(VCL-ketal-HPMA) nanogel and 12 wt% for the P(VCL-cross-HPMA) nanogel, which confirmed the stability of the nanogels and a low premature drug release under physiological pH conditions. The low drug leakage was ascribed to the hydrophobic and hydrogen-bond interaction between DOX and the polymer, and the swollen state of the nanogels due to the higher VPTT at pH 7.4 (39.5 °C). In the acidic environment of
pH 5.0 and 6.5, the drug release at the same time scale from the two DOX-loaded nanogels was obviously accelerated. At pH 5.0, the DOX-loaded nanogels showed 96 wt% and 46 wt% of drug released from the DOX-loaded P(VCL-ketal-HPMA) and P(VCL-cross-HPMA) nanogels, respectively. As for P(VCL-ketal-HPMA) nanogels, the acid-triggered drug release profiles were related to the acid-degradation of the nanogels. The acid-triggered cleavage of the cross-linking linkages inside made the nanogels to swell and even dissolve, and hence facilitated the release of DOX from the nanogels. As for P(VCL-cross-HPMA) nanogels, the pH-dependent drug release profiles might be ascribed to the pH-sensitivity of DOX itself. By comparing the drug release profiles of the two nanogels under the same acidic conditions (pH 5.0), it can be explicitly observed that DOX released from the P(VCL-ketal-HPMA) nanogels much faster than from P(VCL-cross-HPMA) nanogels. The comparably accelerated drug release from the acid-degradable nanogels, to some extent, demonstrates that the cleavage of the ketal linkages inside can trigger the drug release.

To investigate the temperature influence on the drug release of the P(VCL-ketal-HPMA-8) nanogel, we conducted the drug release experiments in the medium of pH 7.4 at 37 °C and 41 °C, respectively. As shown in Fig. 8, within 48 h, about 40 wt% of DOX was released from the nanogel at 41 °C, four times as the drug release amount at 37 °C (about 10 wt%). The faster drug release at higher temperature may be related to the collapse of the nanogels at the temperature which is higher than the VPTT of the nanogel. When heated up to the temperature above its VPTT, the nanogels would shrink and squeeze out the encapsulated drug. In acidic environments, the temperature influence on the drug release within 8 h was not significant (Fig. S8, ESI†). Because the nanogels were almost completely degraded in 1 h at pH 5.0, the drug release profiles would not be affected by the temperature, so the difference of the drug release profiles at different temperatures became less significant in an acidic medium.

**In vitro cell and hemolysis assays**

The intracellular drug release against HeLa cells respectively incubated with DOX-loaded P(VCL-ketal-HPMA-8) and P(VCL-cross-HPMA-8) nanogel was further studied by CLSM measurements. After 6 h incubation with DOX-loaded P(VCL-ketal-HPMA) nanogels, red fluorescence from DOX was obviously distributed in the cytoplasm surrounding the cell nucleus (Fig. 9a), and, after 12 h, the strong red fluorescence was observed in the cell nucleus besides the significant fluorescence in the cytoplasm (Fig. 9b). The strong red fluorescence of DOX in the nucleus indicated the rapid DOX release from the nanogels and the localization in the nucleus. As DOX kills tumor cells through interacting with DNA in the nucleus, localization of DOX in the nucleus illustrated that the DOX-loaded P(VCL-ketal-HPMA) nanogels could efficiently kill the cancer cells. By contrast, when HeLa cells were incubated with DOX-loaded P(VCL-cross-HPMA) nanogels for 6 h, almost no red fluorescence was observed in the cells (Fig. 9c), and even for 12 h, the red fluorescence from DOX only appeared in the cytoplasm (Fig. 9d). The faster intracellular drug release of DOX-loaded P(VCL-ketal-HPMA) nanogels suggested that the scission of ketal bonds inside accelerated the intracellular DOX release from the nanogels. The intracellular drug release of free DOX was also studied by CLSM measurements. Within 6 h, strong red fluorescence was observed in the cell cytoplasm and the nucleus (Fig. S9, ESI†). The result of quicker location to the nucleus of the free DOX might correlate two aspects: free DOX could enter the cells quickly by diffusion and it took a period of time for DOX-loaded nanogels to enter the cells by endocytosis and to degrade to release drug in the cell. After 12 h incubation, the red fluorescence in the cell nucleus from the acid-degradable nanogels and free DOX was comparative, which suggested that acid-degradable

![Fig. 7](image_url) **Fig. 7** pH-dependent DOX release profiles of acid-degradable P(VCL-ketal-HPMA-8) nanogel (a) and non-degradable P(VCL-cross-HPMA-8) nanogel (b) at 37 °C at different pH values.

![Fig. 8](image_url) **Fig. 8** DOX release profiles of the acid-degradable P(VCL-ketal-HPMA-8) nanogel in the medium of pH 7.4 at 37 °C and 41 °C.

![Fig. 9](image_url) **Fig. 9** CLSM images of the intracellular DOX release from P(VCL-ketal-HPMA-8) and P(VCL-cross-HPMA-8) nanogels using HeLa cells. (a) 6 h and (b) 12 h incubation of DOX-loaded P(VCL-ketal-HPMA-8) nanogels; (c) 6 h and (d) 12 h incubation of DOX-loaded P(VCL-cross-HPMA-8) nanogels. In each column, images from top to bottom: differential interference contrast microscopy, fluorescence microscopy, and overlays of both images.
DOX-loaded nanogels could sufficiently exert the effect of killing cancer cells after 12 h incubation.\textsuperscript{36}

The proliferation inhibition of drug-loaded nanoparticles against tumor cells is a very important parameter for effective drug carriers. Thus, the careful investigation on the cytotoxicity of DOX-loaded nanogels with 8 wt% HPMA had been conducted by MTT assay against HeLa cells. As shown in Fig. 10a, the blank nanogels showed no cytotoxicity to HeLa cells even up to a high concentration of 100 μg mL\(^{-1}\). In contrast, a significant proliferation inhibition of HeLa cells was observed when the cells were incubated with either DOX-loaded nanogels or pure DOX for 48 h at a higher DOX dose (Fig. 10b). It was obviously noted that the IC\(_{50}\) values (the concentration of drugs required to reduce cell growth by 50%) for pure DOX, DOX-loaded P(VCL-ketal-HPMA) nanogels and DOX-loaded P(VCL-cross-HPMA) nanogels were determined to be 0.4, 0.55 and 2.87 μg mL\(^{-1}\) individually. The above results of cell viability indicated that the cytotoxicity of DOX-loaded P(VCL-ketal-HPMA) nanogels was higher than that of DOX-loaded P(VCL-cross-HPMA) nanogels, and similar to that of pure DOX. As DOX release was accelerated due to the degradation of P(VCL-ketal-HPMA) nanogels induced by intracellular acidic stimulus, the cell proliferation inhibition was enhanced. The HeLa cells treated with DOX-loaded P(VCL-cross-HPMA) nanogels exhibited a lower cytotoxicity perhaps because the inside network lowered the drug release. In all, the DOX-loaded P(VCL-ketal-HPMA) nanogels displayed higher cytotoxicity with enough efficiency to kill the cancer cells.

To evaluate the biocompatibility of the P(VCL-ketal-HPMA) nanogel as a drug carrier, the in vitro cytotoxicity assay against the normal HK-2 cell line was also conducted by treating the cells with the blank nanogels for 48 h. As shown in Fig. 11, no obvious cytotoxicity could be detected after 48 h incubation of the nanogels at the concentration range of 1–500 μg mL\(^{-1}\), and over 90% of cell viability was observed even up to the concentration of 500 μg mL\(^{-1}\). The low cytotoxicity to normal cells, which might be ascribed to the non-immunogenicity and nontoxicity of PHPMA and PVCCL, shows nanogels have good biocompatibility and are suitable as drug carriers. The hemocompatibility indicated that the P(VCL-ketal-HPMA) nanogel would not produce hemolysin as negative control (Fig. S10, ESI\(^{7}\)). Overall, it can be foreseen that this kind of acid-degradable P(VCL-ketal-HPMA) nanogel is an intriguing candidate for effective drug carriers in tumor therapy.

**Conclusions**

In summary, a new type of thermosensitive and acid-degradable P(VCL-ketal-HPMA) nanogel was successfully prepared via precipitation polymerization using synthetic ketal-bonded DMAEP as a cross-linker, and hydrophilic HPMA as a comonomer. These nanogels, with uniform size and narrow size distribution, underwent adjustable VPTT affected by the feeding amount of HPMA. The ketal linkages provided the nanogels with pH-responsive property of degradation into short linear polymer chains under acidic conditions. The degradation rate was accelerated in the medium with lower pH and higher temperature. In contrast, permanent cross-linker MBA was introduced to form non-degradable P(VCL-cross-HPMA) nanogels. DOX was used as a model anticancer drug and was effectively loaded into both the nanogels. The drug release results indicated that the DOX-loaded P(VCL-ketal-HPMA) nanogels presented more effective drug-controlled release profiles at 37 °C that after 48 h, 13% of DOX was released in blood circulation (pH 7.4), while a rapid and complete drug release (96%) was triggered in the intracellular lysosome environment (pH 5.0). Besides, the drug release can be accelerated by increasing temperature due to strongly collapsed behaviour of the nanogels. The results of in vitro cell assays showed that the nanogels were non-toxic to normal HK-2 cells, and the DOX-loaded P(VCL-ketal-HPMA) nanogels possessed higher cytotoxicity and enough efficiency to kill the HeLa cells in comparison with non-degradable DOX-loaded P(VCL-cross-HPMA) nanogels. The results of the hemolysis assays further confirmed that the acid-degradable nanogel produced no hemolysin and is suitable to be used as a drug delivery vehicle. Though more in vivo testing is necessary to determine the potential of the P(VCL-ketal-HPMA) nanogel as a drug delivery vehicle, the initial results are very promising and demonstrate obvious advantages of using the nanogels as smart drug carriers for drug controlled release in tumor therapy.

**Acknowledgements**

We are grateful for the support from the National Science Foundation of China (Grant No. 51273047 and 51473037) and the “Shu Guang” project (12SG07) supported by Shanghai...
Municipal Education Commission and Shanghai Education Development Foundation.

Notes and references

46 V. Bulmus, Y. Chan, Q. Nguyen and H. L. Tran, Macromol. Biosci., 2007, 7, 446–455.