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1. Introduction

Recent progress in carriers for drug delivery showed great potentials for being used for targeted therapy with minimized toxicity.1,2 The biocompatible amphiphilic copolymers with well-defined architectures, which can self-assemble into core-shell micelles in aqueous media,3,4 are one of the challenging and rewarding areas in polymer science. The hydrophobic micellar cores can serve as a reservoir for hydrophobic drugs and the hydrophilic shell offers the stability of the carriers in physiological environment.5,6 The micelles can protect the encapsulated drug from contacting with the environment in body and offer the enhanced permeation and retention (EPR) effect in solid tumors.7,8 In addition, the micelles can stay in bloodstream long enough to provide accumulation at the target area, thereby increasing therapeutic efficiency.9

Intelligent drug delivery systems have been developed based on amphiphilic copolymer micelles that can release the encapsulated cargos in physiological conditions have attracted increasing interests.10,11 The regulations of drug release via the specific environmental conditions, external stimuli, or biological triggers in pathologic tissues are attractive to increase the specific drug delivery. Moreover, the stability of the drug carriers during storage and circulation also attracted increasing attentions. Chemical crosslinking of the shell or core of drug carriers to hold the carriers in a thermodynamically frozen state have been tried.12-16 The stabilized carriers that could release the cargos in response to pathological triggers have attracted specific attentions.17-23

The causal relationship between inflammation, innate immunity and cancer has been widely accepted that the tumor microenvironment is largely orchestrated by inflammatory cells,24,25 which are abundance of trypsin, an essential enzyme in body and generally overexpressed in the inflamed and tumor tissues.26,27 Trypsin can cleave the peptide bonds involved in lysine and arginine,28 which can be used as a trigger for targeted release. In recent years, it was found that glutathione (GSH) is the most abundant thiol species in cytoplasm and the major reducing agent in biochemical processes, which forms high reducing environments in cells. Tumor cells and the normal cells are different in reducibility greatly due to that the GSH concentration in cancer tissues is about 7-10 folds as that in normal tissues.29 Moreover, the GSH concentration in blood...
plasma is about 2–10 µM, which is much lower than that in the cytosol (10 mM). The notable difference in redox potential between the mildly oxidizing extracellular space and the cancer tissues provides the opportunities for designing drug carriers for specific intracellular drug delivery for cancer therapy. The exchange reaction of disulfide bonds/thiol can be triggered by GSH and could be used for the fabrication of drug carriers.33

Natural polymers with thiol groups/disulfide bonds have the attracting advantages of the biocompatibility and renewability, and can be used in biomedicine and biomaterials. Keratin is such a kind of cysteine-rich structural protein with excellent biocompatibility.32 Keratin is also rich in lysine and arginine, which enables it to be cleaved in vivo by trypsin33 to produce nontoxic polypeptides and amino acids.34 On the other hand, the abundant disulfide bonds/thiol groups in keratin could be used for the fabrication of functional materials for various applications,35,36 such as drug carriers that respond to GSH stimuli in pathological environment. However, keratin itself is insoluble in aqueous media, and modification is needed for using keratin as the building block of drug carriers. Poly(N-(2-hydroxypropyl)methacrylamide) (PHPMA) is a water-soluble, biocompatible, non-immunogenic, and nontoxic polymer that enables selective delivery into tumor tissue.37 PHPMA and its copolymers have been investigated on anticaner drug delivery, tumor specific antisense oligonucleotides and site-specific delivery to the gastrointestinal tract.38,39

In this work, novel keratin graft poly(N-(2-hydroxypropyl) methacrylamide) (K-g-PHPMA) copolymers were synthesized and characterized. The graft copolymers can self-assemble into micelles in water and the keratin rich core can be cross-linked via disulfide bonds through oxidation of the thiol groups on keratin, by which to stabilize the micelles. The micelles can efficiently load doxorubicin (DOX). The release of DOX from the K-g-PHPMA micelles in the presence of GSH as well as trypsin was investigated. Cellular uptake experiments demonstrate that the DOX-loaded micelles can release DOX in response to GSH in live cells.

2. Experiment section

2.1 Materials

Keratin was extracted from wool. The details of the extraction, the amino acids components and the molecular weight of keratin have been provided in previous work.36 2-Mercapto-ethanol (AMRESCO), Pyrene (Alfa Aesar), 2,2’-azobis(2-methylpropionamide) dihydrochloride (V-50, Alfa Aesar), N-(2-hydroxypropyl)methacrylamide (HPMA, Polymerscience), and Ellman’s reagent, 5,5’-dithiobis-(2-nitro-benzoic acid) (DTNB, Alfa Aesar) were used as received. Glutathione (reduced), glutathione monoester (GSH-OEt), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 4’,6’-diamidino-2-phenylindole (DAPI), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich and used as received. Doxorubicin hydrochloride (DOX, Beijing Huafeng United Technology Co.) was used as received. Water with resistivity of 18.2 mΩ·cm from Milli-Q Reference Water Purification System (Millipore) was used for the reaction and purification of the graft copolymers. All other reagents were purchased from local chemical suppliers and used as received.

2.2 Synthesis of K-g-PHPMA copolymers

The synthesis of K-g-PHPMA copolymers is shown in Scheme 1. Typically, keratin (0.200 g) was dispersed in 4 mL Milli-Q water and HPMA (0.020 g) was added. The mixture was purged with N₂ for 20 min and then initiator V-50 (0.004 g) was added. The reaction mixture was stirred continuously for 24 h under 50 °C for the graft copolymerization, during which homopolymer PHPMA, could also be produced in the reaction mixture. After graft copolymerization, the reaction mixture was transferred into a dialysis bag with the molecular weight cut-off (MWCO) of 3.5 kDa and then dialyzed against Milli-Q water for 24 h to remove unreacted monomer HPMA, the initiator V-50, and the homo-polymers PHPMA. The out water for dialysis was replaced every 12 h. After dialysis, the mixture was lyophilized to produce the graft copolymers. 1H NMR (400 MHz, D₂O) δ (ppm) 0.85 (s, 3H, -CH₃), 1.63 (d, -CH), 3.0 (d, -CH₂), 3.77 (s, -CH), 1.05 (s, -CH₃).

![Scheme 1. The synthesis route of K-g-PHPMA.](attachment:image.png)

The PHPMA content in the graft copolymers can be tailored by varying the feeding ratios of monomer HPMA to keratin. The content of PHPMA in the copolymers was evaluated by weighing and element analysis. Due to that there is no suitable solvent for dissolving keratin and K-g-PHPMA copolymers and used as the eluent for gel permeation chromatography (GPC) measurements. Moreover, the peaks of keratin on the 1H NMR spectra could not be assigned to specific groups and used for estimating the degree of polymerization of PHPMA. Therefore, the degree of polymerization of PHPMA was estimated as follows. The moles of –SH groups of keratin were estimated by Ellman’s assay (DTNB). In details, a calibration curve of the absorbance at λ = 412 nm as a function of –SH concentration was first obtained. Then, 50 µL DTNB (10 mM, pH 7.0) was added in the solutions of keratin or K-g-PHPMA in PBS (pH 8.0, 100 mM). The absorbance of the solutions at 412 nm was measured and compared to the calibration curve to result the content of –SH in the solutions, by which the content of –SH in...
the K-g-PHPMA copolymer was calculated. The graft density (DS), defined as the average number of –SH occupied by PHPMA chains grafted onto keratin backbones to the total number of –SH groups of keratin before grafting copolymerization, was calculated by 

\[ DS = 1 - \frac{n_{\text{SH}}}{n_K} \]

where \( n_{\text{SH}} \) and \( n_K \) are the number of moles of –SH groups in K-g-PHPMA and keratin, respectively. The content of PHPMA in the graft copolymers (\( C_{\text{PHPMA}} \)) was estimated by element analysis and used for calculation of the average molecular weight of PHPMA side chains (\( M_w \)). Therefore, \( M_w \) can be estimated by 

\[ M_w = C_{\text{PHPMA}}/(n_K \times DS) \]

The copolymers with different components are distinguished by DS in this work. The details of the experiments and the synthesized copolymers are listed in Table 1.

### 2.3 Preparation of K-g-PHPMA micelles and core crosslinking

K-g-PHPMA copolymers (0.050 g) were dispersed in 25 mL Milli-Q water and stirred for several hours, during which core-shell micelles were formed due to the amphiphilicity of the polymers. The keratin rich core was then cross-linked by the conversion of thiol groups into disulfide bonds in presence of DMSO (10%, v/v). The crosslinking was performed at 45 °C for 24 h. After crosslinking, the solutions were dialyzed against Milli-Q water (MWCO 7 kDa) to remove DMSO. The conversion of thiol groups in K-g-PHPMA solution with concentration of 1 mg mL\(^{-1}\) was monitored by DTNB assay during oxidizing. At a certain time intervals, 2.0 mL mixture was sampled, and then 50 µL DTNB (10 mM, pH 7.0) was added. The absorbance at 412 nm was measured on a Shimadzu UV-1601PC spectrophotometer to estimate the content of thiol groups in the system.

### 2.4 Preparation of DOX-loaded micelles and drug release

DOX-loaded micelles were prepared as follows. DOX (0.014 g) was dissolved in 14 mL Milli-Q water, and K-g-PHPMA (0.028 g) was added. The mixture was stirred for 3 h, and then 1.4 mL DMSO was added to crosslink the micellar core, after which the mixed solutions were dialyzed against (MWCO 7 kDa) Milli-Q water (replaced every 12 h) for 24 h to remove the free DOX and DMSO. The volume of the obtained DOX-loaded micellar solutions were adjusted to 28 mL after dialysis to result solutions with copolymer concentration of 1 mg mL\(^{-1}\). The DOX loading content (\( C_l \)) and encapsulation efficiency (\( E_e \)) of the micelles were calculated by 

\[ C_l (%) = \left( \frac{W_{\text{DOX},L}}{W_M} \right) \times 100 \]

\[ E_e (%) = \left( \frac{W_{\text{DOX},L}}{W_{\text{DOX},f}} \right) \times 100 \]

where \( W_{\text{DOX},L} \), \( W_{\text{DOX},f} \) and \( W_M \) are the weight of the DOX loaded in the micelles, the feeding DOX, and the micelles, respectively. \( W_{\text{DOX},L} \) was estimated as follows. The micelles solutions (200 µL) were mixed with 2.0 mL methanol and the DOX loaded in the micelles was dissolved in the methanol. The absorbance of DOX in the resulted mixed methanol solutions at 495 nm was compared with a working curve of the absorbance of DOX on the UV-vis spectra at 495 nm as a function of DOX concentration in methanol. The K-g-PHPMA copolymers and methanol have no obvious absorbance at 495 nm and the background was subtracted.

Fluorescence quenching experiments were performed on a Perkin Elmer LS 55 fluorescence spectrometer at excitation wavelength of 500 nm. Free DOX solution and DOX-loaded micelles (K\(_{0.15}\)-g-PHPMA) were used. The concentration of DOX was 5.0 µg mL\(^{-1}\) and K\(_{0.15}\)-g-PHPMA was 0.5 mg mL\(^{-1}\). \( \Gamma^2 \) in KI was used as the fluorescence quenching agent. The level of \( \Gamma^2 \) (KI) varied from 0.0 to 0.6 M. The ionic strength of the solutions was kept at constant by adding NaCl.

The DOX release experiments were performed as follows. The DOX-loaded micelles solutions (12 mL) were loaded in a dialysis bag (MWCO 7 kDa) to dialyze against 150 mL PBS (pH 7.4) at 37 °C in a beaker with constant stirring and the micellar concentration was 1 mg mL\(^{-1}\). The concentrations of GSH of the copolymer solutions and the PBS solution outside the dialysis bag were kept at 10 mM or 10 µM during the release experiments to mimic the redox conditions in cells and blood plasma, respectively. At a certain time intervals, 3.0 mL solution was taken from the outer side of the dialysis bag. The DOX released from the micelles in PBS was determined as follows. The absorbance of DOX in the PBS solution outside the dialysis bag, locates at 481 nm, was compared with a working curve of the absorbance of DOX in water (481 nm) as a function of DOX concentration, by which the released DOX from the micelles was calculated. The release profiles of the DOX-loaded micelles in the presence of trypsin (0.04 mmol L\(^{-1}\)) and GSH (10 mM), and control experiments with only trypsin or without GSH were carried out according to the similar procedure.

### 2.5 Characterizations

The \(^1\)H NMR spectra were recorded on a Bruker 400 MHz Advance NMR instrument using D\(_2\)O as solvent at 25 °C and the pH of the mixture for \(^1\)H NMR was adjusted to 8.0 using NaOH. Elemental analysis of the graft copolymer was performed on a Flash EA 1112 Elemental Analyzer. Dynamic light scattering (DLS) experiments were carried out on the ALV/SP-150 spectrometer equipped with an ALV-5000 multi-\(\tau\) digital time correlator and a solid-state laser (ADLS DPY 425II, output power ca. 400 MW at \(\lambda=632.8\) nm) as light source. All the K-g-PHPMA copolymer solutions were filtered through the Millipore Millex-FH nylon filter (0.45 µm). The data were collected at the scattering angle of 90° at 25 °C. The hydrodynamic radius (\(\langle R_g \rangle\)) and its distribution were estimated by using CONTIN program. UV-vis spectra were recorded on a Shimadzu UV-1601PC spectrophotometer.

### 2.6 Cytotoxicity and cellular uptake of K-g-PHPMA

The cytotoxicity of the graft copolymers was estimated by MTT assay. MCF-7 cell (ECACC, European Collection of Cell Cultures, UK) were cultivated in Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen, US) containing 10% fetal bovine serum (FBS, Invitrogen, US) and 1% antibiotics at 37°C in 5% CO\(_2\) atmosphere. The cells were seeded into 96-well plates at
5×10⁴ cells per well in 200 µL culture medium and incubated for 24 h. The culture medium was then removed and 200 µL medium containing K-g-PHPMA copolymers was added into the cells. The cells were then incubated for another 24 h. The culture medium was removed and the wells were washed with PBS for three times. After which 100 µL culture medium and 10 µL of 5 mg mL⁻¹ MTT assay solution in PBS were added into each well and the cells were incubated for another 4 h. The unreacted MTT was removed carefully and 150 µL DMSO was added to dissolve the blue formazan crystals. The absorbance was then measured by the microplate reader (Multiskan MK3, Thermo Electron Co.) at a wavelength of 570 nm for each cell. The relative cell viability (%) was calculated by comparing the optical density at 570 nm with untreated cells.

MCF-7 cells for cellular uptake experiments were grown in DMEM supplemented with 10% FBS at 37 °C with 5% CO₂ in a 95% humidified atmosphere in an incubator. The cells were subcultured every 48 h. Before the experiments, the cells were harvested by trypsinization using a 0.05% (w/v) trypsin/0.03% (w/v) EDTA solution. The DOX-loaded K-g-PHPMA micelles were the same as that used for in vitro release experiments. MCF-7 cells were plated on microscope slides in a culture dish (5×10⁴ cells per well) using 1 mL of DMEM media supplemented with 10% FBS in a humidified atmosphere with 5% CO₂ at 37 °C for 24 h, and then treated with prescribed amounts of GSH-OEt for 2 h. Cells were washed with PBS for three times and incubated for another 0.5 h or 1.5 h in DMEM containing 250 µg mL⁻¹ DOX-loaded K-g-PHPMA mixture with a final DOX concentration of 10 µg mL⁻¹. Cells without GSH-OEt pretreatment were used as control. Then the culture medium was removed and the cells were washed with PBS for three times. The cells were fixed using 4% paraformaldehyde for 30 min at 37 °C and rinsed in PBS for three times. The cell nuclei were stained with DAPI for 15 min and washed with PBS for three times. Fluorescence images of the cells were obtained using a confocal laser scanning microscopy (FV 1000-IX81, Olympus, Japan).

3. Results and discussion

3.1 Synthesis of K-g-PHPMA

The synthesis of K-g-PHPMA copolymers was actualized through graft copolymerization of monomer HPMA using V-50 as initiator and –SH as chain transfer agent. The synthetic route has not involved any additional macrorinitiator or chain transfer agent that are needed in other synthetic methods of graft copolymers. The synthetic route can be used for graft copolymerization water-soluble monomers onto backbones with –SH groups. The resultant K-g-PHPMA copolymers were characterized by ¹H NMR (Fig. 1). For comparison, the ¹H NMR spectra of keratin and PHPMA are also shown in Fig. 1. The results show that all the signals of PHPMA protons appear on the ¹H NMR spectrum of K-g-PHPMA, confirmed the successful synthesis of K-g-PHPMA copolymer. Graft copolymers with different grafting ratios can be obtained by varying the feeding ratios of HPMA to keratin. The content of thiol groups in keratin is 1.04 mmol g⁻¹, which was estimated by DTNB assay. The experimental details and the resultant graft copolymers are listed in Table 1. The DS, the content of PHPMA and the average molecular weight of PHPMA side chains in the graft copolymers increase with the increase in the feeding ratio of monomers to keratin.

![H NMR spectra of K-g-PHPMA, keratin, and PHPMA in alkaline D₂O (pH about 8.0) at 25°C.](image)

**Fig. 1** ¹H NMR spectra of K-g-PHPMA, keratin, and PHPMA in alkaline D₂O (pH about 8.0) at 25°C.

<table>
<thead>
<tr>
<th>Sample</th>
<th>mₖ/mₘₕₐₜₐ</th>
<th>G¹ (%)</th>
<th>G² (%)</th>
<th>DS²</th>
<th>Keratin cont. (wt%)³</th>
<th>Mₙ (Da)</th>
<th>C₁ (wt%)³</th>
</tr>
</thead>
<tbody>
<tr>
<td>K₀.ₐ₉ₙ₉-PHPMA</td>
<td>1:0.48</td>
<td>6.0</td>
<td>9.3</td>
<td>0.09</td>
<td>94.0</td>
<td>715</td>
<td>22.5</td>
</tr>
</tbody>
</table>
| K₁.ₐ₅ₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕ₅ | 1000-IS-11, Olympus, Japan).

3.2 Micellization behaviours of K-g-PHPMA in aqueous solution

Amphiphilic copolymers composed of hydrophobic and hydrophilic components can self-assemble into nanoparticles in selective solvents. For K-g-PHPMA copolymers, the keratin backbone is hydrophobic, and the PHPMA side chain is hydrophilic, which offer K-g-PHPMA copolymers the self-assembly properties in selective solvents. The micellization of amphiphilic copolymers depends on polymer concentration. The critical micellization concentration (CMC) of Kₐ₃₉₉-PHPMA copolymers was determined by pyrene assay (Fig. S1, ESI†). The CMC of K₀.ₐ₉₉₉-PHPMA, K₁.ₐ₅ₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕ₅
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Progressive increase in the conversion of the –SH groups and polymeric micelles are capable of retaining drugs, proteins and by oxidizing the thiol groups into disulfide bonds. Fig. 3 shows bonds as a function of time during the crosslinking procedure.

Further treatment of the samples with DMSO leads to side reactions, oxidized into disulfide bonds in the presence of DMSO without effects on the hydrodynamic radius of the micelles.

The concentration of the solutions was 0.5 mg mL−1, which is above the CMC of the graft copolymers. The variation of PHPMA component of the graft copolymers has no obvious effects on the hydrodynamic radius of the micelles.

3.3 DOX loading and triggerable release

Stimuli-responsive drug delivery systems can adapt to surrounding environments and regulate transportation of cargos to targeted sites. An excellent polymer drug carrier should have the desired encapsulating ability of the guest molecules and stability in blood stream. Moreover, the drug carriers could release the loaded cargos in response to physiological relevant triggers, specifically after reaching the target tissues. The encapsulation ability of K-g-PHPMA micelles for DOX has been estimated and the drug content of the keratin-g-PHPMA micelles are listed in Table 1. The highest DOX content in the K-g-PHPMA micelles is about 22.5% in K0.09g-PHPMA micelles, which is quite good compared with those polymeric nanoparticulate delivery vehicles. Meanwhile, it was found that the drug content of the DOX-loaded micelles increases with keratin content in K-g-PHPMA copolymers (Table 1), suggesting that keratin plays a vital role in the loading of DOX. Keratin is a kind of natural protein. The amide groups, as well as the carbonyl and amine groups, can interact with the carbonyl, hydroxyl, and amino groups in the DOX molecules via hydrogen bonds, which stabilizes the DOX in the inner core of the micelles prepared from K-g-PHPMA copolymers (Scheme 2). The multiple interactions between the active groups of keratin and DOX molecules contribute to the high drug loading capacity of K-g-PHPMA micelles.

Upon systemic administration, part of the micellar drug carriers prepared from copolymers could be dissociated since the concentration of copolymers in body fluids is much lower than CMC. Crosslinking of the core or shell of the micelles could avoid the dissociation of the carriers. Cross-linked polymeric micelles are capable of retaining drugs, proteins and nucleotides, and releasing them under certain stimuli environments. Thiol groups of cysteine in keratin can be oxidized into disulfide bonds in the presence of DMSO without side reactions, so the micelles that formed through the self-assembly of the K-g-PHPMA copolymers can be cross-linked by oxidizing the thiol groups into disulfide bonds. Fig. 3 shows the conversion of –SH groups of K-g-PHPMA into disulfide bonds as a function of time during the cross-linking procedure. The concentration of the copolymer was kept 1 mg mL−1. The content of the –SH groups in the system was estimated by Ellman’s method. The results indicate that about 60% of the –SH groups was converted into disulfide bonds after 24 h. Further treatment of the samples with DMSO leads to progressive increase in the conversion of the –SH groups and the conversion will achieve 95% after 110 h.

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Fig. 2 The hydrodynamic radius of K-g-PHPMA copolymers measured by DLS. The initial concentration of the solutions was 0.5 mg mL−1.

Fig. 3 The conversion of thiol groups into disulfide bonds as a function of time during the oxidization using DMSO at 45 °C. The concentration of the solution was 1 mg mL−1.

Fig. 3 The conversion of thiol groups into disulfide bonds as a function of time during the oxidization using DMSO at 45 °C. The concentration of the solution was 1 mg mL−1.

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The successful loading of DOX in the core of the micelles was proved by the fluorescence quenching study of DOX.\textsuperscript{20} Collisional quenching constants, $K_Q$, were estimated from Stern-Volmer plots of the fluorescence quenching data of free DOX and DOX loaded in the micelles by

$$\frac{F_0}{F} = 1 + K_Q [I^{-1}]$$  \hspace{1cm} (3)

where $F_0$ and $F$ are the maximal fluorescence intensity of DOX at initial state and that with I$^{-1}$. $K_Q$ is the collisional quenching constant that relates to the polarity around accessible fluorophor.\textsuperscript{21} $K_Q$ for free DOX and the encapsulated DOX in micelles obtained by linear fitting using Eq. 3 (Fig. S2, ESI$^\dagger$), is 23.5 and 6.8 M$^{-1}$, respectively. The results indicate that the free DOX in aqueous media are ready to be quenched by I$^{-1}$. However, the encapsulated DOX in the micelles are somewhat difficult to be quenched by I$^{-1}$. The results confirm that DOX molecules mainly locate in the micellar cores.\textsuperscript{20}

Disulfide bonds can be cleaved into thiol groups by reduction, and this reversible reaction plays an important role in the conformation and activity of proteins.\textsuperscript{23} The release of DOX encapsulated in K-g-PHPMA micelles was investigated at GSH concentration of 10 mM and 10 µM, corresponding to the level of GSH in cells and blood plasma, respectively. Fig. 4 shows the accumulated release profiles of DOX from the cross-linked K-g-PHPMA micelles. At GSH concentration of 10 mM, the plateau was achieved at 12, 24 and 36 h, for micelles prepared from K$_{0.09}$g-PHPMA, K$_{0.15}$g-PHPMA and K$_{0.34}$g-PHPMA (Fig. 4a and Fig. S3, ESI$^\dagger$), respectively. It can be seen from Fig. 4a that about 45% of the loaded DOX can be finally released from the micelles of K$_{0.09}$g-PHPMA. Unlike that of 10 mM, less DOX has been found to be released at the GSH concentration of 10 µM for all the micelles of the copolymers. The results indicate that GSH may play a significant role on the release of DOX. For the micelles prepared from K$_{0.09}$g-PHPMA copolymer, a control experiment of the release of DOX in PBS buffer without GSH was performed. It can be seen that only 10% of the loaded DOX can be released after 24 h, which is even lower than that at 10 µM GSH (Fig. 4a). The drug release of the K-g-PHPMA copolymers with different content of keratin shows the similar behavior (Fig. S3, ESI$^\dagger$). Above results confirm that the release of DOX from the micelles is triggered by GSH and accelerated at the GSH concentration of the cytoplasm level (10 mM). The GSH triggerable release of the loaded DOX could attribute to that the disruption of the crosslinks weakened the tight structure of keratin core of the micelles and accelerated the diffusion of DOX from the micelles, especially at a relatively higher GSH concentration of intercellular level.

Trypsin in the tumor cells can cleave peptides at the carboxyl side of lysine and arginine. Keratin is rich in lysine and arginine, which means it can be cleaved by trypsin in vivo.\textsuperscript{28} The release of the loaded DOX in the micelles was checked in the presence of trypsin. It shows that the accumulated release of DOX from the micelles reached 80% in just 3 h with trypsin concentration of 0.04 mM (1 mg mL$^{-1}$) and 10 mM GSH contained in the media, and more than 90% of DOX was released after 24 h (Fig. 4a). The results suggest that the sufficient release of DOX can be achieved in the presence of trypsin. To check the effects of solo trypsin on the release behavior of DOX-loaded micelles, the release experiments just in the presence of trypsin were conducted and the results were shown in Fig. 4a. The release of DOX from the micelles is slower than that under 10 mM GSH and 0.04 mM trypsin, but the amount of the released DOX exceeds 80% in 12 h, yet. It indicates the release can be exploded by trypsin and the K-g-PHPMA micelles were dissociated by degradation of keratin in the presence of trypsin. In order to simulate the physiological environment of different consecutive procedures during drug administration, the accumulated drug release of K-g-PHPMA micelles in subsequent media of pure PBS, 10 µM GSH, 10 mM GSH, and 10 mM GSH + 0.04 mM trypsin, corresponding to the conditions of the preparation and storage of drug loaded micelles, blood circulation, and intracellular environment of cancer cells, respectively, was investigated and the results are shown in Fig. 4b. The results show that only about 10% of the

![Fig. 4](image_url)
loaded DOX released under the conditions of the preparation and storage of the micelles and blood circulation. In the intracellular environment of cancer cells, about 60% of the loaded DOX stimuli released by 10 mM GSH and 10 mM + 0.04 mM trypsin. The results confirm the dual biological stimuli responsive properties of K-g-PHPMA micelles, which is of essential clinical interest as this approach offers the opportunity to link drug delivery to a specific pathologic location.50

Above results confirmed the enhancement of trypsin on the DOX release from the K-g-PHPMA micelles. For the purpose to elucidate the mechanism of trypsin on the structure of the micelles, the hydrodynamic radius of the micelles was monitored by DLS when incubating with trypsin. Fig. 5 shows the $<R_h>$ of the micelles prepared from K$_{0.15}$g-PHPMA in the presence of 0.04 mM trypsin at 37 °C as a function of time. The $<R_h>$ of the original micelles is 93 nm and dropped to 55 nm after being incubated with trypsin for 1 h. The size of the micelles kept decreasing and the $<R_h>$ decreased to 24 nm after being incubated with trypsin for 24 h. The decrease in the $<R_h>$ of the micelles in the presence of trypsin is due to that the keratin backbones of K-g-PHPMA was degraded by trypsin, which deconstructs the original micelles to create smaller nanoparticles. The results demonstrate that the collapse of the structure of the micelles leads to the sufficient release of DOX from the micelles.

The loading and physiological stimuli responsive release of DOX in K-g-PHPMA micelles can be depicted schematically in Fig. 6. The DOX molecules were first encapsulated in the core of K-g-PHPMA micelles via co-selfassembly. The core of the micelles was then crosslinked by oxidation of thiol groups into disulfide bonds (Fig. 6b), which are stable during the storage and the blood circulation circumstance. It is known that the tumor or inflammatory cells have the relatively high GSH concentration.26,27,29 When the micelles are taken up by cells, especially by tumor or inflammatory cells, the higher GSH concentration in them will lead to the swelling of the micelles and release part of the loaded DOX (Fig. 6c), which is due to the reducing of the disulfide bonds into the thiol groups. Moreover, the presence of trypsin will cause the degradation of the keratin backbones and lead to the disassembly of the micelles, which results a much higher release of DOX.

Fig. 6 Schematics of preparation of DOX-loaded K-g-PHPMA micelles crosslinked by disulfide bonds and the release of loaded DOX under different physiological stimuli.

3.4 Cytotoxicity and cellular uptake of K-g-PHPMA micelles

Fig. 7 shows the cell viability of the K-g-PHPMA copolymers using MTT assay on MCF7 cells after 24 h incubation. Copolymer solutions with different concentrations were used to treat the cells. The relative cell viability after 24 h incubation exceeds 80% under different concentrations of K-g-PHPMA. The results confirm that K-g-PHPMA copolymers have no cytotoxicity at the copolymer concentration up to 1 mg mL$^{-1}$.

The cellular uptake experiments of DOX-loaded K-g-PHPMA micelles were performed to confirm that the DOX released from the micelles can be internalized into live cells and the release is triggered by GSH. As a neutral molecule, GSH-OEt can be internalized into cells efficiently and hydrolyzed to generate GSH. It was used to manipulate the intracellular GSH concentration in MCF-7 cells and cells without any pretreatment were used as the control.52,53 MCF-7 cells were incubated with 10 µM or 10 mM GSH-OEt in 1 mL of DMEM media supplemented with 10% FBS for 2 h to ensure the concentration of GSH. As shown in Fig. 8a, weak red fluorescence of DOX was observed mainly in the cytoplasm of the control cells after incubated for 0.5 h. In contrast, the fluorescence intensity of the cells pretreated with 10 mM GSH-OEt is dramatically got improved with a much stronger fluorescence in the nucleus (Fig. 8c). However, for MCF-7 cells incubated with 10 µM GSH-OEt, the fluorescence intensity is
much weaker than those cells pretreated with 10 mM GSH-OEt (Fig. 8b). These results confirm that GSH is responsible for releasing DOX from the drug carrier and the release amount of DOX is strongly dependent on the intracellular GSH content. After incubated for 1.5 h, the red fluorescence displayed in all cells showed a noticeable increase in both cytoplasm and nucleus. This phenomenon can be attributed to the diffusion of the released DOX from the micelles into the nucleus of the cells. The results of the cellular uptake experiments indicate that the DOX released from the DOX-loaded micelles can be internalized into cells efficiently and the DOX-loaded K-g-PHPMA micelles can response to GSH in living cells.

![Graph showing relative cell viability](image)

**Fig. 7** Relative cell viability of MCF-7 cells against micellar solution after cultured for 24 h with different concentrations. MCF-7 cells incubated without micelles were used as the control and the cell viability was determined by MTT assay. Each point is the mean of four independent measurements.

The cellular viability of DOX-loaded K-g-PHPMA micelles after uptake of DOX is shown in Fig. S4 (ESI†). The relative viability of cells pretreated with GSH-OEt or buthionine sulfoximine (BSO) was investigated, and cells without any pretreatment before incubating with DOX-loaded K-g-PHPMA micelles were used as control. BSO was used to inhibit the GSH synthesis in living cells. The results confirm that the DOX-loaded micelles can effectively kill the cells and the cell viability decreases with the increase in DOX concentration in the culture medium. Pretreatment of cells with 10 mM GSH-OEt, providing the source of GSH in the living cells, can enhance the cell toxicity of the DOX-laded micelles. The results indicate that the GSH concentration in living cells can stimulate the release of DOX from the micelles. Surprisingly, the cells pretreated with BSO (1.0 mM) before incubated with the DOX-loaded micellar solutions show lower viability (Fig. S5, ESI†). Fig. S6 (ESI†) shows the cellular viability result of MCF-7 cells treated with BSO and the relative cell viability after 48 h incubation exceeds 70% under 1.0 mM BSO, which means BSO didn’t cause obvious cytotoxicity to cells. It can be concluded that the low cell viability of the cells pretreated with BSO (1.0 mM) before incubated with the DOX-loaded micellar solutions is mainly due to that the cytotoxicity of DOX to cells is enhanced, which is similar to those results in literatures.⁵⁴,⁵⁵

![Representative CLSM images](image)

**Fig 8** Representative CLSM images of MCF-7 cells incubated with DOX-loaded K-g-PHPMA micelles: (a) 0.5 h, cells without pretreatment; (b) 0.5 h, cells pretreated with 10 µM GSH-OEt; (c) 0.5 h, cells pretreated with 10 mM GSH-OEt; (d) 1.5 h, cells without pretreatment; (e) 1.5 h, cells pretreated with 10 µM GSH-OEt; (f) 1.5 h, cells pretreated with 10 mM GSH-OEt. Cell nuclei were stained with DAPI.
4. Conclusion

Novel K-g-PHPMA copolymers were successfully synthesized and characterized. K-g-PHPMA can self-assemble into micelles with a keratin rich core stabilized with PHPMA chains. The CMC of the copolymers increases with the PHPMA content in the graft copolymers. The K-g-PHPMA micelles can efficiently load DOX, in which keratin plays a critical role for high drug content. The release behaviour of the DOX-loaded micelles is sensitive to GSH and trypsin. The presence of trypsin helps to achieve the complete release of the loaded DOX. K7 copolymers are found to have no cytotoxicity to cells. In vitro cellular uptake experiments indicate that the DOX released from the DOX-loaded k-g-PHPMA micelles can be internalized into cells efficiently, and the loaded DOX showed an accelerating release into the nucleus of the cells under higher GSH condition. The graft copolymers based on keratin have been proved to have promising applications as excellent drug carriers for cancer therapy.

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Notes and references

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Dual-stimuli sensitive keratin graft PHPMA as physiological trigger responsive drug carriers

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Keratin graft PHPMA copolymers were successfully synthesized and can be used as drug carriers with physiological stimuli responsive properties.