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RESEARCH PAPER

Stable and biocompatible genipin-inducing interlayer-crosslinked micelles for sustained drug release

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Abstract To develop the sustained drug release system, here we describe genipin-inducing interlayer-crosslinked micelles crosslinked via Schiff bases between the amines of amphiphilic linear-hyperbranched polymer poly(ethylene glycol)-branched polyethylenimine-poly(ε-caprolactone) (PEG-PEI-PCL) and genipin. The generation of Schiff bases was confirmed by the color changes and UV-Vis absorption spectra of polymeric micelles after adding genipin. The particle size, morphology, stability, in vitro cytotoxicity, drug loading capacity, and in vitro drug release behavior of crosslinked micelles as well as non-crosslinked micelles were characterized. The results indicated that genipin-inducing interlayer-crosslinked micelles had better stability and biocompatibility than non-crosslinked micelles and glutaraldehyde-inducing interlayer-crosslinked micelles. In addition, genipin-inducing interlayer-crosslinked micelles were able to improve drug loading capacity, reduce the initial burst release, and achieve sustained drug release.

Keywords Crosslinking · Drug controlled release · Genipin · Polymeric micelles · Schiff base · Nanomedicine

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Introduction

Polymeric micelles are typically formed by self-assembly of amphiphilic copolymers in aqueous medium or organic solvents by non-covalent driving forces (hydrogen bonding, van der Waals force, coordination bonding, electrostatic interaction, hydrophobic interaction, solvent effect, space stacking effect, etc.) (Lefevre et al. 2009). Polymeric micelles exhibit various formations such as spherical, worm-like, rod, vesicle, and so on (Blanazs et al. 2009; Holder and Sommerdijk 2011; Letchford and Burt 2007). So far, polymeric micelles have been widely applied in the fields of biology, medicine, catalysis, separation, and molecular optoelectronic devices (Amjad et al. 2017; Deng et al. 2012; Gong et al. 2012; Li et al. 2015). Polymeric micelles as drug carriers have the following advantages (Ahmad et al. 2014): (1) low critical micelle concentration (CMC) and high thermodynamic-kinetic stability; (2) long-term circulation in plasma due to the hydrophilic shell and high water-insoluble drug loading capacity owing to the hydrophobic core; (3) small particle size with a narrow distribution and passive targeting in tumor through the enhanced permeability and retention (EPR) effect; (4) sustained drug release to control the drug concentration in body fluids and reduce the drug side effect; and (5) active targeting through modifying the surface of polymeric micelles (such as conjugating a ligand or antibody) or using stimuli-responsive amphiphilic copolymers.

However, polymeric micelles injected into the body are susceptible to dilution, temperature, ionic strength,

pH, biological molecules in the blood, and other factors so that the micelle structure is damaged and the encapsulated drug is prematurely released (Owen et al. 2012). Therefore, improving the stability of polymeric micelles in the blood circulation is one of the important research fields in the drug delivery systems (Attia et al. 2013; Honda et al. 2013; Ke et al. 2014; Logie et al. 2014). The crosslinking is able to improve the stability of polymeric micelles (Deepagan et al. 2016; Li et al. 2014; Wu et al. 2014; Zhang et al. 2016; Zhang et al. 2013). The crosslinking is mainly divided into three kinds in accordance with the reaction regional of polymeric micelles (O'Reilly et al. 2006): core-crosslinking, shell-crosslinking, and interlayer-crosslinking. For the core-crosslinking, the crosslinker is difficult to enter the core so that the crosslinking reaction is not exhaustive. Furthermore, the crosslinking reaction in the core may affect drug loading performance and drug activity (Read and Armes 2007). For the shell crosslinking, the crosslinking reaction usually requires high diluted solution to avoid the cross-reaction of the crosslinker between the micelles (Blencowe et al. 2009). As a more advanced method than the core crosslinking and the shell crosslinking, the interlayer crosslinking is generally formed in the interfacial region between the core and the shell through the reactive groups such as carboxyl groups and the crosslinker (van Nostrum 2011).

ABC triblock copolymers have significant advantages in the preparation of the interlayer-crosslinked micelles (Bastakoti et al. 2013; Wang et al. 2010; Wu et al. 2013; Zhao and Liu 2015). ABC triblock copolymers, which are made up of hydrophilic block A as the shell for stabilizing polymeric micelles, hydrophilic/hydrophobic block B as the interlayer for the crosslinking, and hydrophobic block C as the core for loading the drugs. The crosslinker is easy to enter the interlayer to crosslink polymeric micelles and drug loading capacity should be not affected by the crosslinking reaction. More importantly, the crosslinking reaction probably carries out in high concentration. At present, three-layer polymeric micelles are mainly crosslinked via click chemistry of alkyne-azide in the presence of copper catalyst (Jiang et al. 2009), UV irradiation of photo-crosslinkable moiety (Kim et al. 2009), amidation chemistry of carboxyl groups with the addition of activator (Fujii et al. 2005), the quaternization of 2-(dimethylamino)ethyl methacrylate (DMA) by adding the bifunctional alkyl iodide, 1,2-bis(2-iodoethoxy)ethane (BIEE) (Jiang et al. 2007), and the reaction of N-hydroxysuccinimide (NHS)-activated acrylic acid, and diamine (Samarajeewa et al. 2013). However, to our knowledge, there are no relevant reports on the employment of interlayer-crosslinked micelles via Schiff bases.

In our previous report, amphiphilic linear-hyperbranched polymer PEG-PEI-PCL was synthesized through the condensation reaction of poly(ethylene glycol)-branched polyethylenimine (PEG-PEI) and monocarboxy-capped poly(ε-caprolactone) (PCL-COOH). PEG-PEI-PCL formed stable three-layer polymeric micelles by self-assembly in aqueous solution, which were used as the stabilizer to prepare highly dispersed platinum nanoparticles (PtNPs) (Dai et al. 2016). Here, we describe interlayer-crosslinked micelles via Schiff bases between the amines of PEG-PEI-PCL and the crosslinker (genipin or glutaraldehyde) for the first time (Scheme 1). Compared to the reported methods of the crosslinking reaction, the preparation of genipin-inducing interlayer-crosslinked micelles via Schiff bases is very simple and operable without using any chemicals as the catalyst or the condensing agent. The formation of Schiff bases was explored by the solution color changes in vision and UV-Vis absorption spectra of polymeric micelles. The micelle properties (morphology, particle size, stability, in vitro cytotoxicity, drug loading capacity, and in vitro drug release behavior) of genipin-inducing interlayer-crosslinked micelles were investigated by comparing non-crosslinked micelles and glutaraldehyde-inducing interlayer-crosslinked micelles.

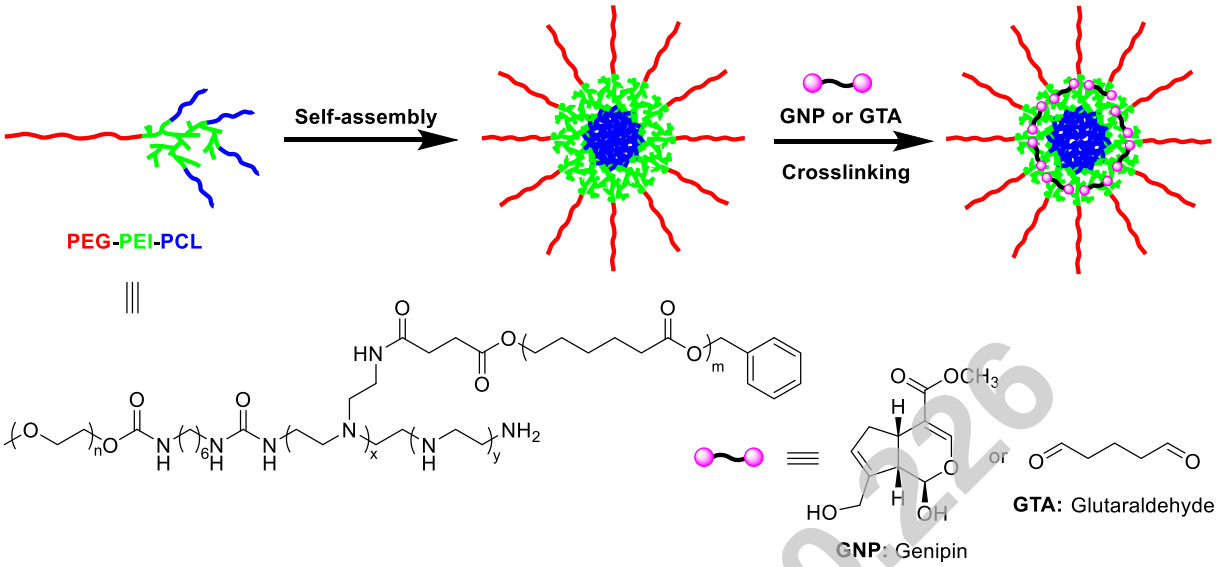
Materials and methods

Synthesis of PEG-PEI-PCL

PEG-PEI-PCL was synthesized as described in our previous report (Dai et al. 2016). The molecular weight of PEG-PEI-PCL is 5000–1800–5200 Da with total molecular weight of 12,000 Da. The weight ratios of PEG, PEI, and PCL are 41.7, 15, and 43.3%, respectively.

Micelle formation and crosslinking

PEG-PEI-PCL (5 mg) was dissolved in 2 mL of *N*,*N*-dimethylformamide (DMF) and 5 mL of deionized water was added dropwise. The solution was dialyzed in a dialysis bag (MWCO 3500 Da) against deionized water



Scheme 1 Formation of interlayer-crosslinked micelles via conjugated Schiff bases

for 2 days and lyophilized. The obtained micelles were re-dissolved in 5 mL of deionized water and passed through a membrane filter with a pore size of 0.22 μm.

Genipin/glutaraldehyde-inducing interlayer-crosslinked micelles were prepared by adding a certain amount of the crosslinker (the molar ratios of the crosslinker and the amines of PEG-PEI-PCL are 0.25, 0.5, 1, 2, 4, and 8) to the micelle solution, followed by stirring at room temperature for 24 h. The solution was passed through a membrane filter with a pore size of 0.22 μm. UV-Vis absorption spectra were recorded by a Perkin-Elmer Lambda Bio 40 UV-Vis spectrophotometer in a scanning range of 200–700 nm at room temperature.

Methotrexate encapsulation and determination

PEG-PEI-PCL (10 mg) and methotrexate (2.0 mg) were dissolved in 4 mL of DMF. Methotrexate-encapsulated interlayer-crosslinked micelles in deionized water were produced using the same procedure described above. One milliliter of aliquot of methotrexate-encapsulated interlayer-crosslinked micelles were freeze-dried and then dissolved in DMF (chromatographic grade). UV absorbance at 303 nm was measured to determine the amount of loaded methotrexate with a Perkin-Elmer Lambda Bio 40 UV-Vis spectrophotometer. The drug loading content (DLC) and entrapment efficiency (EE) were calculated as follows:

$$\text{DLC}\ (\%) = \text{weight of loaded drug/weight of polymer and loaded drug} \times 100$$

$$\text{EE}\ (\%) = \text{weight of loaded drug/weight of drug in feed} \times 100.$$

Micelle characterization

The size and size distribution were determined by dynamic light scattering (DLS). Measurements were carried out and repeated three times at 25 °C with a scattering angle (θ) of 90° in optically homogeneous quartz cylinder cuvette by using a Beckman Coulter N4 Plus submicron particle sizer. A JEOL-3011 transmission electron microscope (TEM) was used to characterize the morphology of micelles. The samples for TEM analysis were prepared as follows: One drop of the solution was added onto a carbon-coated copper grid. After 3–5 min, most of the solution was removed by touching the edge of the filter paper until the grid surface is nearly dry. A drop of 1% phosphotungstic acid solution (pH 7.2 adjusted with NaOH) was added onto the copper grid for negative staining. One to 2 min later, the staining solution was removed by touching on a

piece of the filter paper. The grid was allowed to dry under ambient conditions.

Using pyrene as fluorophore, steady-state fluorescence spectra were measured with a FluoroMax-2 fluorimeter with a slit width of 5 nm for both excitation and emission. All spectra were run on air-equilibrated solutions. For fluorescence emission spectra, the excitation wavelength was set at 339 nm, and for excitation spectra, the emission wavelength was 390 nm. The scanning rate was set at 125 nm min^{-1}. All tests were carried out at 25 °C. To prepare sample solutions, a known amount of pyrene in acetone was added to 5.0 mL of tube and the acetone was evaporated. To each tube was then added a stock sample solution, which was first mixed ultrasonically for 5 s and left to stand overnight at room temperature. The pyrene concentration was kept at 6.0×10^{-7} M.

In vitro cytotoxicity assay

The extracts of genipin, glutaraldehyde, non-crosslinked micelles, genipin-inducing interlayer-crosslinked micelles, and glutaraldehyde-inducing interlayer-crosslinked micelles were prepared by dissolving a certain amount of samples into 2 mL of cell culture medium (DMEM) containing 2×10^{-3} M glutamine, 10% FBS, and 50 units P/S. The extracts were filtered through a 0.22-μm membrane filter and diluted in DMEM to different concentrations. COS7 cells were seeded into a 96-well plate, 5000 cells/well, in 100 μL complete DMEM. After the cells have grown at 37 °C for 24 h in an atmosphere containing 5% CO_2 and 95% air, 100 μL of extract solutions at different concentrations was added to the wells containing cells and complete DMEM (100 μL) such that the final concentration of extract solution for cell culture was half of the original concentration. Cells were cultured with extracts for 24 h at 37 °C, and then 20 μL of MTT solution (5 mg mL^{-1}) in PBS was added to each well and cultured at 37 °C for a further 4 h. One hundred fifty microliters of DMSO was added to each well to dissolve the formed purple crystals derived from MTT. The absorbance of the solution was measured using a microplate reader (Bio-Rad 550, USA) at 570 nm. The percent relative viability related to the control well containing complete DMEM without extract was calculated by the following equation:

$$\text{Cell viability}\ (\%) = \left(\mathrm{OD_{sample}} - \mathrm{OD_{blank}}\right) / \left(\mathrm{OD_{control}} - \mathrm{OD_{blank}}\right) \times 100$$

where OD_{sample} is the absorbance of the solution containing cells cultured with samples; OD_{blank} is the absorbance of the medium; and $OD_{control}$ is the absorbance of the cells cultured with the medium only.

In vitro drug release behavior

The methotrexate-loaded micelle solution was placed in a dialysis bag (MWCO 3500 Da). The dialysis bag was sealed and immersed in 40 mL of phosphate-buffered saline (PBS) (pH 7.4, 0.1 M). An in vitro drug release study of methotrexate-loaded micelles was carried out in a shaking water bath at 37 °C. Three milliliters of solution was taken out, and the same volume of PBS solution was added after each sampling at predetermined time intervals. The drug concentration was determined by measuring the absorbance of methotrexate at 303 nm. The rate of drug release was measured by the released concentration of methotrexate at predetermined time intervals according to the calibration curve of methotrexate.

Results and discussion

Amphiphilic linear-hyperbranched polymer PEG-PEI-PCL is able to form three-layer polymeric micelles by self-assembly in aqueous solution (Dai et al. 2016). The interlayer should be able to be crosslinked via Schiff bases between the amines of PEG-PEI-PCL and the crosslinker (genipin or glutaraldehyde) (Yang et al. 2007). The DLS measurements were carried out to monitor the variations in size of three-layer polymeric micelles that reacted with genipin (Fig. 1). The molar ratios of the crosslinker and the amines of PEG-PEI-PCL are 0.25, 0.5, 1, 2, 4, and 8, respectively. An initial steep decrease and then stable micelle diameters are observed when the molar ratio of the crosslinker and

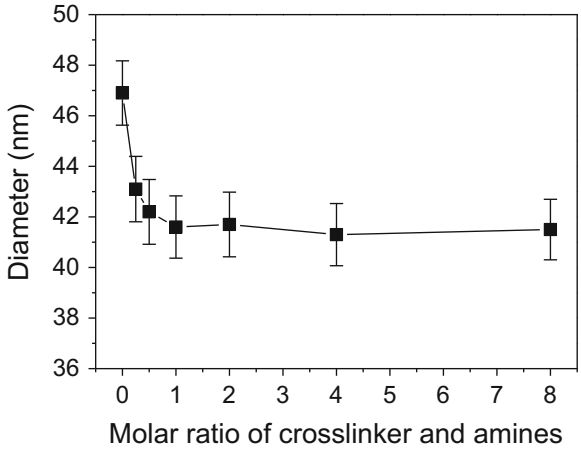


Fig. 1 Variations in size with increasing the molar ratio of the crosslinker (genipin) and the amines of PEG-PEI-PCL determined by DLS. The concentration of polymeric micelles is 1.0 mg/mL

the amines is beyond 1. Thus, the molar ratio (2) of the crosslinker and the amines of PEG-PEI-PCL is chosen in the following experiments.

To confirm the crosslinking reaction, photo images (Fig. 2) of crosslinked micelles and non-crosslinked micelles in aqueous solution were taken. Genipin aqueous solution, glutaraldehyde aqueous solution, and non-crosslinked micelle solution are clear and transparent. However, the color of the micelle solution turns gradually from transparent to yellow, to brown, and finally to dark blue after adding genipin. The blue coloration is due to the double bonds of the genipin-crosslinking molecules, thus confirming the generation of Schiff bases between the amines of PEG-PEI-PCL and genipin (Elzoghby et al. 2013b; Imsombut et al. 2010; Song et al. 2009). Glutaraldehyde-inducing interlayer-crosslinked micelle solution does not have significant change in color. Generally, glutaraldehyde has stronger reaction ability with the amines to form Schiff bases

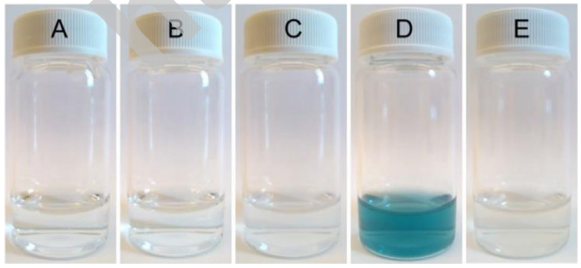


Fig. 2 Pictures of **a** 0.4 mg/mL of genipin, **b** 0.25 mg/mL of glutaraldehyde, **c** non-crosslinked micelles at the concentration of 1.0 mg/mL, **d** genipin-inducing interlayer-crosslinked micelles, and **e** glutaraldehyde-inducing interlayer-crosslinked micelles in aqueous solution

than genipin (Lai et al. 2010). Therefore, it is reasonable to believe that the interlayer in three-layer polymeric micelles of PEG-PEI-PCL is crosslinked via Schiff bases between the amines of PEG-PEI-PCL and glutaraldehyde.

UV-Vis spectra (Fig. 3) of crosslinked micelles and non-crosslinked micelles in aqueous solution were detected to further confirm the crosslinking reaction. Genipin aqueous solution has the characteristic peak at 240 nm of genipin. Non-crosslinked micelle solution does not have a significant characteristic peak. After adding genipin to the micelle solution, the absorption peak of genipin at 240 nm significantly decreases and the new absorption peaks (290 and 600 nm) are attributed to the double bonds of the genipin-crosslinking molecules, which indicate the generation of Schiff bases between the amines of PEG-PEI-PCL and genipin (Elzoghby et al. 2013a).

The size and size distribution of crosslinked micelles and non-crosslinked micelles in aqueous solution were determined by dynamic light scattering (DLS) with the distribution profile shown in Fig. 4. The non-crosslinked micelles have an average diameter of 46.9 nm based on the intensity-averaged values by DLS. The mean diameters of 41.9 and 42.4 nm are observed after genipin-inducing crosslinking and glutaraldehyde-inducing crosslinking, respectively. The diameters of interlayer-crosslinked micelles are slightly lower than those of non-crosslinked micelles. The morphologies of crosslinked micelles and non-crosslinked micelles were characterized by transmission electron

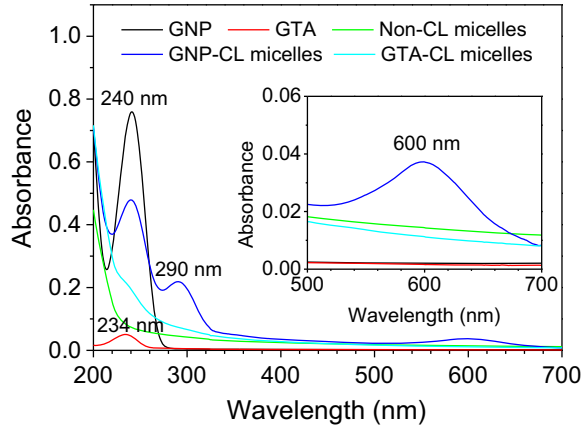


Fig. 3 UV-Vis spectra of genipin (GNP), glutaraldehyde (GTA), non-crosslinked micelles (non-CL micelles), genipin-inducing interlayer-crosslinked micelles (GNP-CL micelles), and glutaraldehyde-inducing interlayer-crosslinked micelles (GTA-CL micelles) in a scanning range of 200–700 nm

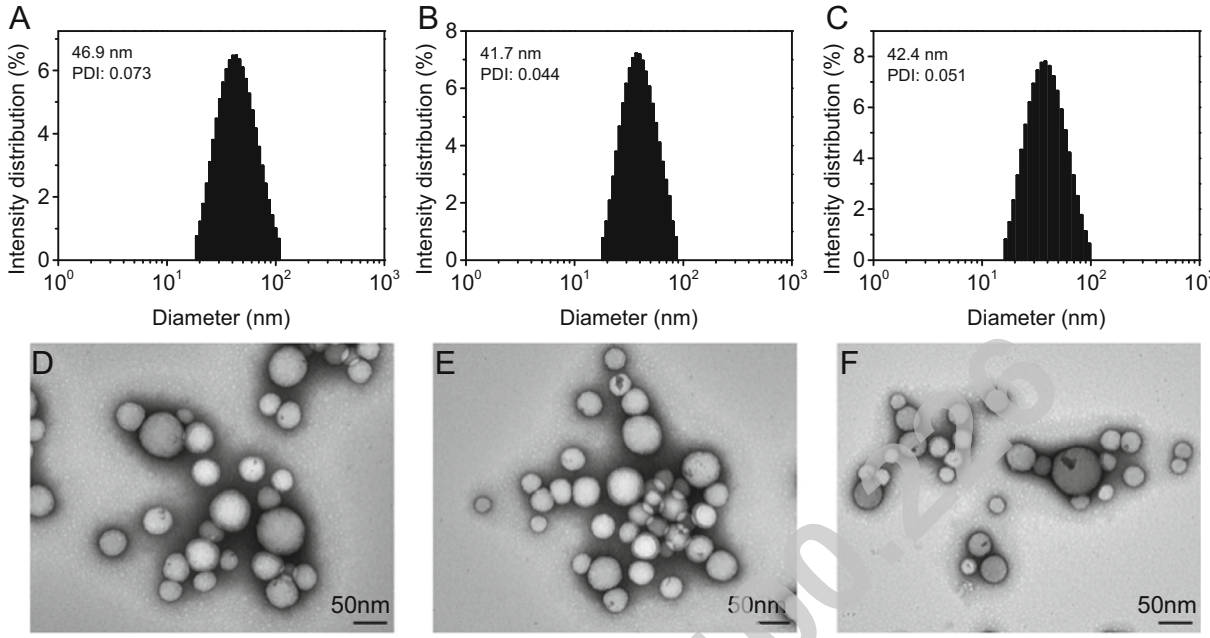


Fig. 4 Size distribution profile determined by DLS of **a** non-crosslinked micelles, **b** genipin-inducing interlayer-crosslinked micelles, and **c** glutaraldehyde-inducing interlayer-crosslinked micelles. Morphology images characterized by TEM of **d** non-crosslinked micelles, **e** genipin-inducing interlayer-crosslinked micelles, and **f** glutaraldehyde-inducing interlayer-crosslinked micelles. Scale bar 50 nm

microscopy (TEM) as shown in Fig. 4. The particles for non-crosslinked micelles and interlayer-crosslinked micelles are both nano-sized spheres. The average diameters of non-crosslinked micelles, genipin-inducing interlayer-crosslinked micelles, and glutaraldehyde-inducing interlayer-crosslinked micelles are 44, 35, and 31 nm estimated from the TEM images, respectively.

The stability of crosslinked micelles and non-crosslinked micelles in aqueous solution was investigated by measuring critical micelle concentration (CMC) with pyrene as a hydrophobic molecule. When the polymer concentration is higher than CMC, pyrene accesses to the hydrophobic core of the micelles, leading to the optical changes. Therefore, the intensity of the fluorescence emission spectra of pyrene has a mutation with the increasing of the polymer concentration. The polymer concentration at the mutation is defined as CMC. Figure 5 shows plots of I_3/I_1 versus log of the polymer concentration. CMC of PEG-PEI-PCL is determined to be 0.059 mg/mL. Compared to the non-crosslinked micelles, genipin/glutaraldehyde-inducing interlayer-crosslinked micelles have decreased CMC and show improved stability. The crosslinking enhances the stability of polymeric micelles. In addition, glutaraldehyde-inducing interlayer-crosslinked micelles have lower CMC than genipin-inducing interlayer-crosslinked micelles, which is perhaps because glutaraldehyde has stronger crosslinking ability with the amines to form Schiff bases than genipin (Lai et al. 2010).

Cytotoxicity is an important factor to reflect biocompatibility of polymeric micelles. Polymeric micelles crosslinked by less cytotoxic crosslinkers have better biocompatibility and lower inflammatory response triggered by the host body. As far as we know,

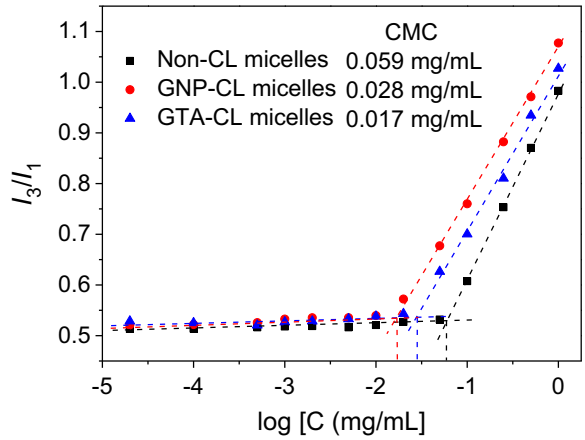


Fig. 5 Plots of I_3/I_1 versus log of the polymer concentration. [Pyrene] = 6.0×10^{-7} M

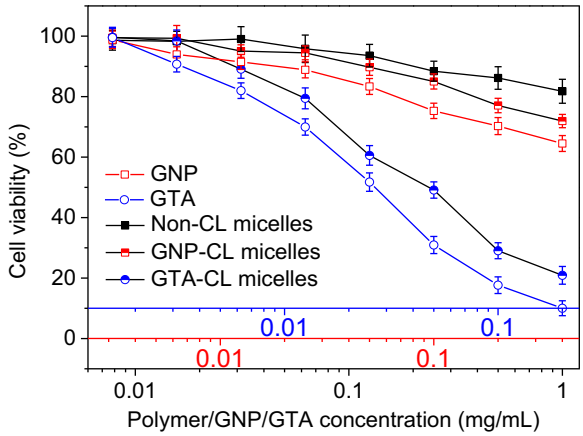


Fig. 6 Relative cell viability at 24 h in COS7 cells in the presence of genipin (GNP), glutaraldehyde (GTA), non-crosslinked micelles (non-CL micelles), genipin-inducing interlayer-crosslinked micelles (GNP-CL micelles), and glutaraldehyde-inducing interlayer-crosslinked micelles (GTA-CL micelles) as demonstrated by MTT assay (average of four times)

glutaraldehyde as biomaterials is usually used to crosslink proteins due to its low cost and high crosslinking efficiency. However, glutaraldehyde has a high cytotoxicity deriving from the formation of toxic products by reactions with the tissue during in vivo biodegradation (Elzoghby et al. 2012; Lai et al. 2010). Therefore, the non-toxic crosslinker has been explored to replace glutaraldehyde. As a natural crosslinker, genipin is less cytotoxic than glutaraldehyde (Imsombut et al. 2010). In vitro cytotoxicity of crosslinked micelles and non-crosslinked micelles in COS7 cells was investigated by MTT assay (Fig. 6). PEG-PEI-PCL is considered to be non-toxic, and the cell viability is beyond 80% even at very high polymer concentration (1.0 mg/mL). Genipin is low cytotoxicity and glutaraldehyde has high cytotoxicity. Interestingly, genipin-inducing interlayer-crosslinked micelles and glutaraldehyde-inducing interlayer-crosslinked micelles are less cytotoxic than the corresponding crosslinker (genipin or glutaraldehyde). The crosslinking reaction between the amines of PEG-PEI-PCL and the crosslinker consumes most of the crosslinker, and the cytotoxicity of interlayer-crosslinked micelles should be from the residual unreacted crosslinker. However, the cell viability in the presence of interlayer-crosslinked micelles is relatively high (>70%), which indicates that interlayer-crosslinked micelles are a type of good biocompatible materials.

Methotrexate, a hydrophobic anticancer drug, was encapsulated by addition to the polymer solution before the self-assembly formation and the crosslinking. The drug loading contents into non-crosslinked micelles, genipin-inducing interlayer-crosslinked micelles, and glutaraldehyde-inducing interlayer-crosslinked micelles are 2.85, 4.21, and 4.54%, respectively. The result indicates that interlayer-crosslinked micelles have a higher drug loading capacity than non-crosslinked micelles. The crosslinking enhances the drug loading capacity of polymeric micelles. In vitro drug release behavior was investigated in PBS (pH 7.4) by monitoring the drug amounts released from the drug-loaded micelle solution that was placed in a dialysis bag. As shown in Fig. 7, 68, 39, and 31% of methotrexate is released from non-crosslinked micelles, genipin-inducing interlayer-crosslinked micelles, and glutaraldehyde-inducing interlayer-crosslinked micelles within 1 day, respectively. Interlayer-crosslinked micelles continuously release the drugs over a prolonged period up to 2 weeks. The crosslinking enhances the sustained release of the drugs.

Conclusions

In summary, we report genipin-inducing interlayer-crosslinked micelles crosslinked via Schiff bases between the amines of amphiphilic linear-hyperbranched polymer PEG-PEI-PCL and genipin. The blue coloration of polymeric micelles after adding genipin and the new absorption peaks in UV-Vis spectra derived from the double bonds of the genipin-crosslinking molecules confirm the

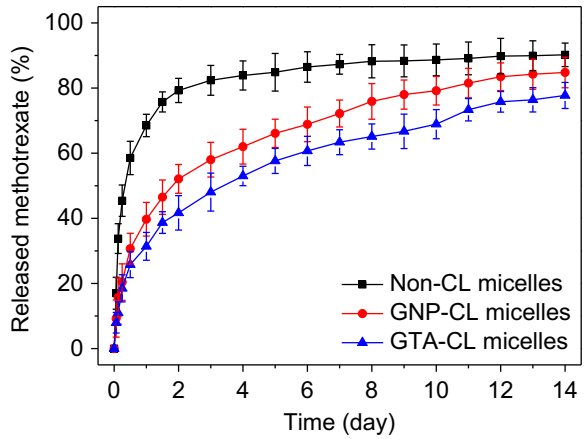


Fig. 7 In vitro methotrexate release profile from non-crosslinked micelles (non-CL micelles), genipin-inducing interlayer-crosslinked micelles (GNP-CL micelles), and glutaraldehyde-inducing interlayer-crosslinked micelles (GTA-CL micelles) at 37 °C

generation of Schiff bases. Genipin-inducing interlayer-crosslinked micelles are nano-sized (~40 nm) spherical nanoparticles. The crosslinking enhances the stability, the drug loading capacity, and the sustained release of polymeric micelles. Of note, genipin-inducing interlayer-crosslinked micelles are a type of good biocompatible materials. In a word, genipin-inducing interlayer-crosslinked micelles having good stability and biocompatibility will be a kind of hopeful biomaterial to be used for sustained drug release.

Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

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