Versatile injectable supramolecular hydrogels containing drug loaded micelles for delivery of various drugs

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In this study, a novel glycol chitosan-Pluronic F127 conjugate (GC-PF127) was produced by adding a carboxyl group to the end of Pluronic F127 followed by an amidation reaction between carboxylated Pluronic F127 and glycol chitosan. Because of the hydrophobic aggregation of PPO segments in Pluronic F127, GC-PF127 self-assembles into micelles in aqueous solution at room temperature. The micelles can effectively load amphiphilic doxorubicin (DOX) and partly load negatively charged protein superoxide dismutase (SOD) at neutral pH with different mechanisms, but cannot load hydrophilic 5-fluorouracil (5-FU). The respective DOX/GC-PF127, SOD/GC-PF127, and 5-FU/GC-PF127 micelle solutions can be converted to injectable supramolecular hydrogels by addition of α-CD. The strength and dissociation rate of the hydrogels can be tuned by the α-CD concentration. The hydrogel structure can recover immediately after extrusion of the hydrogel using a syringe. The hydrogels have prolonged and almost linear release behaviours for various drugs. The hydrogels release free drugs as well as drug loaded micelles via the dissociation of the hydrogels under physiological conditions, which may enhance the targeting ability and therapeutic efficiency. The released micelles sequentially release free drugs that further prolong the release of the drugs. GC-PF127 hydrogels are versatile injectable hydrogels which can deliver various drugs.

Introduction

Injectable hydrogels have attracted much attention for drug delivery due to their tunable properties, controllable degradation, high water content, and administration in a minimally invasive manner.1,2 Drugs, especially delicate bioactive drugs, such as proteins, can be encapsulated directly into hydrogels in situ at room temperature without any contact with organic solvents.3 As a significant property in the application of injectable hydrogels, shear-thinning makes the hydrogels injectable simply under shear stress and recover rapidly after removal of the shear stress.4 Supramolecular hydrogels based on the formation of polypseudorotaxane between cyclodextrins (CDs) and polymers have shown great potential as injectable hydrogels due to their thixotropic nature.5,6 In supramolecular hydrogels, CDs thread onto a polymer chain, forming a supramolecular complex via the CD/polymer hydrophobic interactions; the reversible hydrogen bonds between CDs as well as crystalline domains drive the complex gelation.1,2,3,5 Such hydrogels can be broken by shear force and show gel–sol reversible transformation property and excellent biocompatibility. The supramolecular hydrogels fabricated by the self-assembly of α-CD and the copolymers containing PEO segments have been studied and explored for various biomedical applications.4–8 For example, Ni et al. reported supramolecular hydrogels produced by the self-assembly between Pluronic, commercial PEO–PPO–PEO triblock copolymers and α-CD.10 The hydrogels are thixotropic and reversible, and potentially suitable for use as an injectable drug delivery system. Ma et al. recently reported supramolecular hydrogels produced from heparin-Pluronic F127 micelles and α-CD for co-encapsulation and prolonged release of camptothecin and granulocyte colony-stimulating factor synchronously.11

The drug release kinetics of the supramolecular hydrogels is determined by the erosion of the hydrogels due to de-threading of PEO chains from the cavities of α-CDs.1 A potential advantage of this feature is that release kinetics would be less dependent on the properties of drugs, hence rendering the delivery system more widely applicable to different drugs. On the other side, the controlled release of drugs is a challenge for the supramolecular hydrogels, and their release rate is generally too fast. To solve this problem, Li et al. introduced interpolymeric hydrophobic interactions to prolong the release of loaded model drugs.1 Zhu et al. introduced coordination interaction between platinum(II) atoms and carboxyl groups of PAA blocks to prolong the release of cisplatin from PEG-b-P(AA-cisplatin)/α-CD hydrogels and then from PEG-b-P(AA-cisplatin) micelles sequentially.9

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Glycol chitosan (GC) is a biocompatible, biodegradable, highly water-soluble and low toxic material which contains a number of primary amines. It has been reported that hydrophobic modified GC conjugates form self-aggregates in aqueous solution and can be used as antitumor drug carriers, which display prolonged blood circulation time, decreased time-dependent excretion from the body, and elevated tumor accumulation due to the enhanced permeability and retention (EPR) effect. For example, Park et al. reported hydrophobically modified GC by chemical conjugation of fluorescein iso-thiocyanate or doxorubicin to the backbone of GC. The concentration of the self-aggregates in blood was as high as 14% of dose at 1 day after intravenous injection and was still higher than 8% even at 3 days. When the doxorubicin loaded self-aggregates were administered into the tumor-bearing mice via the tail vein, they exhibited lower toxicity than but comparable antitumor activity to free doxorubicin. Lee et al. conjugated a hydrophobic photosensitizer, protoporphyrin IX, onto GC to demonstrate that the hydrogels release free drugs as well as dually responsive injectable hydrogel, prepared by cross-linking of GC with benzaldehyde-capped PEO–CD into the micelle solutions. The micelles and hydrogels were characterized by a combination of techniques. The results demonstrated that the hydrogels release free drugs as well as drug loaded micelles via the dissociation of the hydrogels under physiological conditions. The released micelles sequentially release free drugs that further prolong the release of the drugs.

**Experimental section**

**Materials**

Glycol chitosan (GC, n = 400, Mw 82 kDa) was purchased from Wako Pure Chemical Industries, Ltd. Pluronic F127 (PF127, EO100–PO95–EO100, Mw 12.6 kDa), 1-ethyl-3-(3-dimethylaminoproyl)carbodiimide hydrochloride (EDC, 99%), and N-hydroxysuccinimide (NHS, 99%) were obtained from Sigma-Aldrich. α-CD (98%) was purchased from Junsei Chemical Co. Ltd. Doxorubicin hydrochloride (DOX, 99%) was from Zhejiang Hisun Pharmaceutical Co. Ltd. 5-Fluorouracil (5-FU) was from Bangcheng Chemical Co. Ltd. Bovine Cu,Zn-superoxide dismutase (SOD, 6000 U mg⁻¹) was from Yili BioChem Company. Pyrogallol acid, 4-dimethylaminopyridine (DMAP), succinim anhydride (SA), 1,4-dioxane, anhydrous diethyl ether, triethyl amine (TEA), methylene chloride, hydrochloric acid, and sodium hydroxide were of analytical grade and obtained from Sinopharm Chemical Reagent Co. Ltd. KB cell line was from American Type Culture Collection. DMEM cell culture medium and fetal bovine serum were from Gibco BRL Life Technologies Inc. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxymethyl)-2-(4-sulfophenyl)-2H-tetrazolium) was from Promega Co.

**Synthesis of terminal carboxylated PF127 (PF127-SA)**

PF127-SA was prepared according to the literature with some modification. Briefly, 6 g PF127 (0.48 mmol) was dissolved in 50 mL 1,4-dioxane under stirring for 24 h, and then 130 mg DMAP (1.06 mmol) and 150 μL TEA (1.09 mmol) were added to activate the terminal hydroxyls at room temperature for 2 h. Subsequently, 100 mg SA (1.0 mmol) dissolved in 5 mL 1,4-dioxane was dropwise added into the activated PF127 solution and the mixture reacted at room temperature for 48 h to produce PF127-SA. PF127-SA was precipitated in cold diethyl ether and then further purified by dissolution in methylene chloride and precipitation in cold diethyl ether repeatedly for 3 times. Finally, PF127-SA was dried at 30 °C under vacuum overnight. The yield is 70–75%.

**Synthesis of GC-PF127**

GC-PF127 was prepared using EDC/NHS chemistry. Briefly, 400 mg GC (0.005 mmol) and 2.4 g PF127-SA (0.19 mmol) were respectively dissolved in 40 mL of 0.01 mol L⁻¹ pH 5.0 phosphate buffer for 24 h; 300 mg EDC (1.31 mmol) and 300 mg NHS (2.61 mmol) were added into PF127-SA solution to activate the carboxyl groups at room temperature for 30 min. Subsequently, the GC solution was slowly added into the activated PF127-SA solution and their mixture reacted at room temperature for 24 h. After reaction, the solution was dialyzed (cut off Mw 14 kDa) against water. Purified GC-PF127 was obtained after filtration and lyophilization of the solution. The yield is about 91%.

**Characterization of PF127-SA and GC-PF127**

FTIR spectra were recorded on a Thermo Nicolet spectrometer (NEXUS-470). FTIR samples were prepared by pressing lyophilized PF127-SA and GC-PF127 samples with KBr.
spectra were obtained on a spectrometer (Bruker DMX500) at 25 °C. NMR samples were prepared by dissolving PF127-SA and GC-PF127 samples in D_2O.

Pyrene fluorescence emission spectra were recorded on a fluorescence spectrophotometer (FLS-920, Edinburgh) to investigate the hydrophobic aggregation of the polymers. Pyrene acetone stock solution (1 × 10^{-3} mg mL^{-1}) was added into polymer aqueous solutions with desired pH, temperature, and polymer concentration; the final pyrene concentration was 1.5 × 10^{-6} mg mL^{-1}. The mixtures were stirred for 2 days and then the fluorescence emission spectra were recorded at an excitation wavelength of 335 nm.

Both dynamic light scattering (DLS) and ζ-potential measurements were conducted on a ZetaSizer Nano ZS90 (Malvern Instruments) at 25 °C and 90° scattering angle to obtain z-average hydrodynamic diameter (D_h) and ζ-potential values. The samples were prepared by diluting GC-PF127 solutions to 5 mg mL^{-1} concentration with the same pH aqueous solutions.

**In vitro cytotoxicity of GC-PF127**

Cell viability against GC-PF127 was measured by MTS assay. KB cells were seeded in 24-well plates and incubated with DMEM medium containing 10% fetal bovine serum and 100 IU mL^{-1} penicillin G sodium and 100 μg mL^{-1} streptomycin sulfate. After 24 h of incubation at 37 °C, the cells were treated with 0.5 mL per well of fresh media containing GC-PF127 with 0.1, 0.5, 1, 5, and 10 mg mL^{-1} concentrations in triplicate. After 72 h of incubation, the cell media were replaced by 120 μL fresh medium containing 16.7% MTS, and the cells were incubated for another 2 h, then, the optical density was read on a BioTek microplate reader.

**Preparation of DOX, 5-FU or SOD loaded GC-PF127 micelles**

The preparation conditions of DOX, 5-FU, or SOD loaded GC-PF127 micelles are shown in Table 1. DOX aqueous solution of 12 mg mL^{-1}, 5-FU aqueous solution of 10 mg mL^{-1}, and SOD aqueous solution of 12 mg mL^{-1} were separately added into 10 wt% GC-PF127 aqueous solutions at 4 °C. The pH was adjusted to 7.4 for the DOX/GC-PF127 mixture and 6.8 for the 5-FU/GC-PF127 mixture as well as the SOD/GC-PF127 mixture. The final concentrations of DOX, 5-FU, and SOD were 6, 5, and 1.2 mg mL^{-1}, respectively; the final concentration of GC-PF127 was 5 wt%.

The mixed solutions were slowly stirred at 37 °C for 24 h to produce DOX, 5-FU, or SOD loaded GC-PF127 micelles. The resultant micelle solutions were stored at 25 °C. Free DOX, 5-FU, or SOD in the micelle solution was isolated by an ultrafiltration membrane (Millipore), whose cut off M_w was 50 kDa for DOX and 5-FU isolated solutions and 100 kDa for SOD isolation. The loading efficiency was calculated using the following equation:

\[
\text{Loading efficiency} (%) = \frac{\text{drug in feed} - \text{free drug}}{\text{drug in feed}} \times 100\%
\]

**Preparation of supramolecular hydrogels**

Supramolecular hydrogels were prepared by adding α-CD into the DOX, 5-FU or SOD loaded GC-PF127 micelle solution to reach a α-CD concentration of 7, 8, or 9 wt%. The mixture was stirred at 25 °C to dissolve the α-CD and then to fabricate supramolecular hydrogels by inclusion complexation of the micelles with α-CD in an aqueous system. The gelation time was visually observed by a vial inversion method when the sample solution did not flow.

**Characterization of DOX, 5-FU or SOD loaded GC-PF127 micelles and hydrogels**

Transmission electron microscopy (TEM) observations were carried out on a Philips CM120 electron microscope. The TEM sample was prepared by depositing a drop of diluted micelle solution onto a carbon-coated copper grid. Excess solution was removed using filter paper. The copper grid was dried naturally. Scanning electron microscopy (SEM) images were observed on a TS 5136 MM scanning electron microscope. The sample was prepared by coating the freeze-dried hydrogel with gold for 30 s. XRD patterns of the freeze-dried hydrogels were acquired on an X’pert PRO X-ray powder diffractometer at a rate of 2° min^{-1} in a 2θ range of 5–50°. Storage modulus (G’), loss modulus (G”), and shear viscosity were measured on a rotational rheometer (ARES 2000, TA Instruments). The hydrogel samples after 24 h of consolidation were measured using a cone-plate geometry of 25 mm diameter, a cone angle of 0.1°, and a gap of 0.0508 mm at 25 °C. Dynamic frequency sweep from 0.1 to 100 rad s^{-1} with a strain of 0.1% was performed within the linear viscoelastic region to record G’ and G”. Steady rate sweep from 0.01 to 10 s^{-1} was conducted to record the viscosity.

**Disassociation of the hydrogels**

The disassociation of the hydrogels was investigated by immersing 0.5 mL of the hydrogel in 1 mL PBS (0.01 mol L^{-1} pH 7.4 phosphate buffer containing 0.15 mol L^{-1} NaCl) at 37 °C. At

<p>| Table 1 Preparation conditions, loading efficiency, size, and ζ-potential of DOX, 5-FU, or SOD loaded GC-PF127 micelles |</p>
<table>
<thead>
<tr>
<th>Samples</th>
<th>GC-PF127 (mg mL^{-1})</th>
<th>Drug (mg mL^{-1})</th>
<th>pH</th>
<th>Loading efficiency (%)</th>
<th>D_h (nm)</th>
<th>ζ-Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC-PF127</td>
<td>50</td>
<td>0</td>
<td>6.8</td>
<td>0</td>
<td>10 ± 1</td>
<td>22.7 ± 0.3</td>
</tr>
<tr>
<td>DOX/GC-PF127</td>
<td>50</td>
<td>6</td>
<td>7.4</td>
<td>83.7 ± 2.6</td>
<td>323 ± 13</td>
<td>10.1 ± 1.3</td>
</tr>
<tr>
<td>5-FU/GC-PF127</td>
<td>50</td>
<td>5</td>
<td>6.8</td>
<td>2.9 ± 2.0</td>
<td>9 ± 1</td>
<td>23.0 ± 0.1</td>
</tr>
<tr>
<td>SOD/GC-PF127</td>
<td>50</td>
<td>1.2</td>
<td>6.8</td>
<td>37.9 ± 3.2</td>
<td>6 ± 1, 111 ± 15</td>
<td>14.2 ± 0.2</td>
</tr>
</tbody>
</table>
a predetermined time interval, the PBS was taken out, the hydrogel was weighed, and fresh PBS was added in succession.

**In vitro release of DOX, 5-FU, and SOD from the micelles and hydrogels**

**In vitro** release of the loaded drug from the hydrogels was investigated by immersing 1 mL of the drug loaded hydrogel in 10 mL PBS at 37 °C. At a predetermined time interval, 2 mL of the PBS was taken out and then 2 mL of fresh PBS was added. The release of the loaded DOX and SOD from the hydrogels was also investigated by dialysis of 1 mL hydrogel against 10 mL PBS at 37 °C. Similarly, at a predetermined time interval, 2 mL of the PBS was taken out and 2 mL of fresh PBS was added. All the release processes were carried out in triplicate. The absorption at 480 nm was measured on a spectrophotometer (Shimadzu UV-2550) to obtain the free DOX concentration in the release buffer.

The absorption at 268 nm was measured to obtain free 5-FU concentration in the release buffer.

The SOD activity in the release buffer was measured by the assay of pyrogallol autoxidation according to our previous report. The control experiments verified that GC-PF127 do not influence the DOX, 5-FU, and SOD assays.

**Results and discussion**

**Synthesis and characterization of PF127-SA and GC-PF127**

Scheme 1 shows the synthesis approach of GC-PF127. Firstly, PF127 was activated by adding a carboxyl group to the end of PF127 via an esterification reaction between PF127 and SA. Secondly, PF127-SA was conjugated to GC via an amidation reaction between the amino group of GC and the carboxyl group of PF127-SA with EDC and NHS as activator and coupled agents.

Fig. 1 presents FTIR spectra of PF127, PF127-SA, GC, and GC-PF127. The peaks at 1658 cm⁻¹ (amide I, C═O stretching) and 1603 cm⁻¹ (N–H bending of the primary amine group). In the spectrum of GC-PF127, the absorption peak at 1603 cm⁻¹ almost disappears and the peak at 1556 cm⁻¹ (amide II band, N–H bending) appears, indicating that some of the primary amine groups in the GC have reacted with the carboxyl groups in PF127-SA and amide bonds formed. Moreover, the peak of C═O stretching (1737 cm⁻¹) of the carboxyl group is significantly weakened in the GC-PF127 spectrum compared with the peak in the PF127-SA spectrum due to the formation of the amide bonds. On the other hand, the presence of the peak at 1737 cm⁻¹ in the GC-PF127 spectrum indicates that some of the carboxyl groups in PF127-SA do not form amide bonds with GC.

Fig. 2 shows ¹H-NMR spectra of PF127, PF127-SA, GC and GC-PF127. The peak at 2.70 ppm in the PF127-SA spectrum corresponds to the methylene protons (CH₂CH₂COOH) of the succinic groups, further confirming that the carboxyl group was conjugated to the end of PF127. According to the peak areas of the methyl protons (δ = 1.18 ppm, OCH₂CH(CH₃)O) and the methylene protons (δ = 2.70 ppm, CH₂CH₂COOH) in the PF127-SA spectrum, about 65% of the hydroxyl groups in PF127 were converted to carboxyl groups in PF127-SA. That is, each PF127 molecule has 2 hydroxyl groups in which about 1.3 hydroxyl groups were conjugated with carboxyl groups on average. The
peaks at 2.08 and 2.74 ppm in the GC spectrum correspond to the methyl protons in N-acetyl groups CH₂C(O) and C₂ protons in N-unsubstituted glucosamine units of GC, respectively. From the peak areas of 2.08 ppm and 2.74 ppm as well as the polymerization degree of 400 in GC, we can estimate that the number of N-acetylated glucosamine units in GC is 43 per 400 sugar units, that is, the deacetylation degree of GC is about 89%. The peaks of 2.08 ppm and 2.74 ppm also appear in the GC-PF127 spectrum. The reaction between GC and PF127-SA through an amide bond is further confirmed by the splitting of the resonance peak of the methylene protons from one at 2.70 ppm into two at 2.63 ppm and 2.50 ppm. From the peak areas of 2.08 ppm (N-acetyl protons of GC) and 1.18 ppm (OCH₂CH(CH₃)₂O of PF127) in the GC-PF127 spectrum, we can calculate about 34 PF127 molecules being grafted onto GC sugar units in each GC molecule on average, thus the molecular weight of GC-PF127 is about 510 kDa. In the reaction, the molar ratio of GC to PF127-SA was 1 : 38 in feed. The 1H-NMR result demonstrates that most of the PF127-SA molecules were conjugated onto GC.

The micellization of amphiphilic GC-PF127 in aqueous solution was investigated using pyrene as a fluorescence probe. It was reported that once the micelles form in aqueous solution, the hydrophobic pyrene can be encapsulated into hydrophobic microdomains that causes a decrease of the intensity ratio of the first to third band (I₁/I₃) in the pyrene fluorescence emission spectrum. Fig. 3 shows that the I₁/I₃ ratio decreases with the increase of GC-PF127 concentration at 37 °C, and the critical micellization concentration (CMC) of GC-PF127 is 0.37 mg mL⁻¹. This CMC value is about an order of magnitude larger than the CMC value of PF127, which is 0.035 mg mL⁻¹. This result is also larger than the CMC value of 0.124 mg mL⁻¹ of heparin-conjugated PF127. The reason may be that the GC chain is positively charged and the electrostatic repulsion results in the micellization of GC-PF127 at higher concentration.

The influence of temperature and pH on the I₁/I₃ ratio of pyrene fluorescence in GC, PF127, and GC-PF127 aqueous solutions was investigated, and the results are shown in Table 2. Because GC is soluble in aqueous solution, the I₁/I₃ ratios in GC solutions do not have pH and temperature sensitive changes, which are close to the ratio of pyrene in water. PF127 is an amphiphilic and thermosensitive polymer containing two hydrophilic PEO blocks and one hydrophobic PPO block. The I₁/I₃ value of PF127 solution is about 1.8 at 4 °C and changes to 1.44 at 25 °C because the hydrophobicity of PPO blocks increases at higher temperature. Further increasing the temperature to 37 °C, the I₁/I₃ values do not change significantly. The I₁/I₃ values of GC-PF127 solutions at different pH and temperature are the same as those of PF127 solutions, that is, GC-PF127 forms micelles at 25 and 37 °C at the concentration studied. The DLS result (Table 1) and TEM image (Fig. 4A) confirm that GC-PF127 forms micelles at room temperature with a Dₙ of 10 nm.

**In vitro cytotoxicity of GC-PF127**

Cell viability against GC-PF127 was assayed to evaluate the biocompatibility and cytotoxicity. The data in Fig. 5 reveal that GC-PF127 has no inhibition effect on KB cell proliferation up to 10 mg mL⁻¹ concentration after 72 h incubation. This result is reasonable because GC is a biocompatible, biodegradable, and low toxic material and PF127 has been approved by FDA. Fig. 5 indicates that GC-PF127 has good biocompatibility, which is an important property for its biomedical applications.

**Preparation and characterization of DOX, 5-FU or SOD loaded GC-PF127 micelles**

It was reported that hydrophobic drugs can be loaded in the hydrophobic PPO microdomains. In this study, we used GC-PF127 micelles to load amphiphilic antitumor drug DOX. DOX has a pKₐ of 8.2, its hydrochloride salt is water-soluble, but the solubility decreases gradually with the increase of the solution pH because of the increase of the hydrophobicity. DOX loaded GC-PF127 micelles were prepared by adjusting the mixed solution from acidic to pH 7.4 and then from 4 °C to 37 °C to allow deprotonation of the DOX molecules partly and formation of hydrophobic microdomains in the PPO segments. Table 1 shows that about 83.7% of the DOX was loaded into GC-PF127 micelles. The Dₙ of DOX/GC-PF127 micelles is 323 nm and the ζ-potential is 10.1 mV. The TEM image (Fig. 4B) shows that DOX/GC-PF127 micelles are spherical in shape approximately.

We also investigated the loading of hydrophilic antitumor drug 5-FU in GC-PF127 micelles. The ultrafiltration separation result (Table 1) reveals that 5-FU cannot be loaded into GC-PF127 micelles. Table 1 shows that the size and ζ-potential of the mixture of 5-FU and GC-PF127 are about the same as those of GC-PF127 micelle solution. The TEM image of the mixture (Fig. 4C) presents GC-PF127 micelles as well as 5-FU particles which formed during the drying process. 5-FU particles have larger size and higher electron density compared with GC-PF127 micelles.

We used positively charged GC-PF127 micelles to load negatively charged protein SOD at pH 6.8. Bovine Cu,Zn SOD is a dimer with a molecular weight of 31 kDa and an isoelectric point of pH 5.4. SOD carries negative charges when the
solution pH is higher than 5.4. Table 1 shows that the loading efficiency is about 37.9% at pH 6.8. The DLS result shows two kinds of micelles in the mixture of SOD and GC-PF127: one is 6 nm and the other is 111 nm. TEM images reveal that the larger micelles are clusters composed of smaller micelles (Fig. 4D) and then the clusters become relatively uniform (Fig. 4E) when the mixture was incubated for 6 and 24 h, respectively. The TEM and DLS results indicate that the negative SOD molecules cause the positive GC-PF127 micelles to be close to each other at the beginning, and then the clusters change to uniform SOD/GC-PF127 complex micelles. The \( \zeta \)-potential changes from 22.7 to 14.2 mV after addition of SOD (Table 1). The 37.9% of the SOD loading efficiency suggests that the binding between SOD and GC-PF127 is not strong. The data in Table 1 and Fig. 4 demonstrate that GC-PF127 micelles can effectively load DOX, partly load SOD, but hardly load 5-FU.

The release of DOX, 5-FU, or SOD from the micelles under physiological condition was investigated and the results are shown in Fig. 6. About 18% of the DOX was released and/or diffused from DOX/GC-PF127 micelles in the first 6 h, then the release rate decreases. Because 5-FU cannot bind with GC-PF127, about 81% of the 5-FU was diffused from the GC-PF127 micelle solution in the first 2 h. Table 1 shows that only 37.9% of the SOD was loaded in the micelles, which means that the free SOD can diffuse rapidly from the micelle solution. Fig. 6 shows that about 43% and 90% of the SOD was diffused and released from the micelle solution in the first 6 and 48 h, respectively. The released SOD molecules have full activity.

**Preparation and characterization of supramolecular hydrogels**

Supramolecular hydrogels were prepared via inclusion complexation between the PEO chains in GC-PF127 micelles and \( \alpha \)-CDs. Fig. 7 presents that the gelation time decreases when the \( \alpha \)-CD concentration increases from 7 to 9 wt%. This result confirms that the micelle solutions, which contain DOX, 5-FU, or SOD inside and outside the micelles, can be converted to hydrogels after addition of \( \alpha \)-CDs. When the \( \alpha \)-CD concentration is 7 wt%,

**Table 2** Influence of temperature and pH on the \( I_2/I_3 \) ratio of pyrene fluorescence in GC, PF127, and GC-PF127 solutions

<table>
<thead>
<tr>
<th>Polymer</th>
<th>pH 4.0</th>
<th>pH 6.8</th>
<th>pH 4.0</th>
<th>pH 6.8</th>
<th>pH 4.0</th>
<th>pH 6.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC</td>
<td>1.88 ± 0.04</td>
<td>1.87 ± 0.02</td>
<td>1.85 ± 0.03</td>
<td>1.82 ± 0.01</td>
<td>1.83 ± 0.02</td>
<td>1.81 ± 0.01</td>
</tr>
<tr>
<td>PF127</td>
<td>1.79 ± 0.05</td>
<td>1.80 ± 0.01</td>
<td>1.43 ± 0.02</td>
<td>1.44 ± 0.03</td>
<td>1.36 ± 0.05</td>
<td>1.40 ± 0.02</td>
</tr>
<tr>
<td>GC-PF127</td>
<td>1.78 ± 0.02</td>
<td>1.74 ± 0.01</td>
<td>1.40 ± 0.02</td>
<td>1.45 ± 0.03</td>
<td>1.37 ± 0.04</td>
<td>1.38 ± 0.02</td>
</tr>
</tbody>
</table>

\( ^a \) The concentration of PF127 and GC-PF127 was 5 mg mL\(^{-1} \) and the concentration of GC was 1 mg mL\(^{-1} \) for pyrene fluorescence measurements.

![Fig. 4](image1.png) TEM images of GC-PF127 micelles (A), DOX/GC-PF127 micelles (B), 5-FU/GC-PF127 micelles (C), and SOD/GC-PF127 micelles produced after 6 h (D) and 24 h (E) of mixing SOD solution with GC-PF127 micelle solution.

![Fig. 5](image2.png) KB cell viability after 72 h incubation with different concentrations of GC-PF127.

![Fig. 6](image3.png) Accumulative release of DOX, 5-FU, and SOD from the drug loaded micelles in PBS at 37 °C.

![Fig. 7](image4.png) Cell viability (%) as a function of GC-PF127 concentration (mg/mL).

![Fig. 8](image5.png) Influence of temperature and pH on the \( I_2/I_3 \) ratio of pyrene fluorescence in GC, PF127, and GC-PF127 solutions.
the gelation time of GC-PF127 micelle solution is significantly longer than the time of drug loaded micelle solutions. By increasing the α-CD concentration to 8 and 9 wt%, the gelation time of GC-PF127 micelle solution is similar to the time of drug loaded micelle solutions. Fig. 8 shows XRD patterns of free α-CD, free GC-PF127, and freeze-dried supramolecular hydrogel samples. The characteristic diffraction peak of the column crystalline structure of PEO/α-CD inclusion at 2θ 19.8 degree is presented in all the hydrogel samples. The XRD results indicate that the column crystalline structure of the supramolecular inclusion complex acts as a cross-link in the hydrogels. The SEM image of freeze-dried 5-FU/GC-PF127 supramolecular hydrogels (Fig. 9) shows a porous structure due to the high water content. The other freeze-dried supramolecular hydrogels display the same morphology (data not shown).

The strength of supramolecular hydrogels can be tuned by the α-CD concentration. Fig. 10A shows that the higher α-CD concentration results in the hydrogels having a greater storage modulus. At the same 8 wt% α-CD concentration, DOX loaded hydrogels have a smaller storage modulus compared with the other hydrogels (Fig. 10B). Fig. 11 presents that all the hydrogel samples with and without drug have a shear-thinning behaviour, indicating that the shear stress applied on the supramolecular hydrogels can break the column crystalline structure of the PEO/α-CD inclusion complex. On the other hand, the hydrogel structure can recover immediately after extrusion of GC-PF127 and DOX/GC-PF127 hydrogels using a syringe (Fig. 11). Similarly, the other drug loaded hydrogels have the same injectable property and immediate recovery of the hydrogel structure. These merits enable the hydrogels to be administrated by injection and fixed at the site after administration.

**Dissociation and drug release of supramolecular hydrogels**

Dissociation of the hydrogels was investigated in PBS at 37 °C. For GC-PF127 hydrogels, Fig. 12 shows that the hydrogel weights...
decrease almost linearly with the increase of immersion time. Increasing the α-CD concentration from 7 to 9 wt% can reduce the dissociation rate. After 7–9 days of the immersion, the hydrogels disappear completely. For DOX loaded hydrogels, the dissociation time is about 12 days at 8 wt% α-CD concentration, but 5-FU and SOD loaded hydrogels have the same dissociation rates as the hydrogels without drug (data not shown).

The drug loaded hydrogels were immersed in PBS or dialyzed against PBS to investigate the release behaviours of the hydrogels. In immersion condition, the drug loaded micelles and the free drugs can be released from the hydrogels as a result of the dissociation of the hydrogels. The α-CD concentration was 8 wt% in all supramolecular hydrogel samples.

The DOX release rate decreases with the increase of the α-CD concentration which is consistent with the dissociation rate of the hydrogels. Fig. 13A shows that the DOX release in dialysis condition is much slower than the release in immersion condition. The reason is that both free DOX and DOX/GC-PF127 micelles can be released in the immersion condition, but only free DOX in the release buffer was isolated in the dialysis condition. The results in Fig. 13A demonstrate that the hydrogels release DOX/GC-PF127 micelles in a sustained manner via the dissociation of the hydrogels and those released DOX/GC-PF127 micelles release free DOX in PBS in succession. DOX is a broad-spectrum antitumor drug. However, the clinical application of DOX has been hampered by its dose-dependent side effects. Moreover, DOX is rapidly eliminated by enzymes and hydrolytic degradation in plasma after intravenous administration. In this study, the merits of DOX/GC-PF127 hydrogels enable the hydrogels to be injected close to tumor and then release DOX/GC-PF127 micelles in a sustained manner. The released DOX/GC-PF127 micelles can have reduced side effects, enhanced tumor cellular uptake,
enhanced tumor accumulation, and prolonged blood circulation time as those of hydrophobic modified GC nanoparticles. The released DOX/GC-PF127 micelles sequentially release free DOX that prolongs the release of the DOX and increases the antitumor activity of DOX.

5-FU/GC-PF127 hydrogels release free 5-FU molecules by the dissociation of the hydrogels because 5-FU cannot be loaded in GC-PF127 micelles; the hydrogels have almost sustained release behaviours after the first 20% burst release (Fig. 13B). For SOD/GC-PF127 micelles, both free SOD and SOD/GC-PF127 micelles were released in PBS in the immersion condition because only 37.9% of the SOD was inside the micelles. The free SOD released from the micelles/hydrogels in the dialysis condition is almost linear, which is slower than the SOD release in the immersion condition.

In this study, various drug containing GC-PF127 micelle solutions can be converted to injectable supramolecular hydrogels by addition of α-CD. The release kinetics shown in Fig. 6 and 13 demonstrates that the hydrogels release the free drugs as well as the drug loaded micelles via the dissociation of the hydrogels and the released micelles sequentially release free drugs under physiological condition. Scheme 2 is the illustration of the release mechanism. The GC-PF127 supramolecular hydrogels are versatile injectable hydrogels which can deliver various drugs.

**Conclusions**

A novel GC-PF127 conjugate was produced by the addition of a carboxyl group to the end of PF127 followed by an amidation reaction between carboxylated PF127 and GC. GC-PF127 forms micelles at 25 °C. At neutral pH, GC-PF127 micelles effectively load DOX, partly load SOD, but hardly load 5-FU. The GC-PF127 and drug loaded GC-PF127 micelle solutions form injectable supramolecular hydrogels by adding α-CD in the micelle solutions. The strength and dissociation rate of the hydrogels can be tuned by the α-CD concentration. The hydrogel structure can recover immediately after extrusion of the hydrogels using a syringe. Under physiological condition, the hydrogels release free drugs as well as drug loaded micelles via the dissociation of the hydrogels, and the released micelles sequentially release free drugs that further prolong the release.

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**Notes and references**