Electronic Supplementary Information

Selenium-containing thermogel for controlled drug delivery by coordination competition

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1. EXPERIMENTAL SECTION

1.1. Materials

Methoxyl poly(ethylene glycol) (mPEG) with molecular weight (MW) ~550 or 750, stannous octoate ($Sn(Oct)_2$, 92.5~100%), *N,N'*-Dicyclohexylcarbodiimide (DCC, 99%), 4-dimethylaminopyridine (DMAP, 99%), hexamethylene diisocyanate (HMDI, 99%), 3- chloropropionic acid (98%), selenium powder (~100 mesh, 99.5%) were products of Sigma-Aldrich. Monomers glycolide (GA) and D,L-lactide (LA) were purchased from Jinan Daigang Biomaterial Co., Ltd (China). Cisplatin (99.6%) was obtained from Shandong Boyuan Pharmaceutical Co., Ltd (China). Other reagents were used as received without further purification.

1.2. Synthetic procedures

Synthesis of mPEG-PLGA diblock copolymer

mPEG-PLGA diblock copolymer was synthesized by ring-opening copolymerization of LA and GA using mPEG as the macro-initiator. For Polymer-1, mPEG (27.5 g, MW 550) was added into a three-neck flask and dried under vacuum at 120 ˚C for 3 h. After cooling the system to 90 ˚C, LA (55.6 g) and GA (14.9 g) were added under an argon atmosphere and then heated under reduced pressure at 90 ˚C for 30 min. Next, a certain amount of $Sn(Oct)_2$ (90 mg) as the catalyst was injected to the reaction system. After reacting at 150 ˚C with continuous stirring for 12 h under the protection of argon, a vacuum was applied to remove the unreacted monomers for 3 h. The obtained crude products were dissolved in dichloromethane and precipitated in diethyl ether at -20 ˚C for 24 h. After decanting the supernatant, the residual solvent was removed under vacuum for several days to obtain the final product (yield: 87%). Polymer-2 was synthesized by a similar procedure.

Synthesis of 3,3'-selenodipropionic acid (SePA)

SePA was synthesized according to the previous publications.[1, 2] All the reactions were performed under an argon atmosphere to prevent the oxidization of selenium products. Milli-Q water was used in this study and the dissolved oxygen in water was removed

by blowing argon for 30 min. Sodium hydrogen selenide (NaHSe) was firstly synthesized by slowly adding sodium borohydride solution (2.27 g, 60 mmol) into the selenium powder (2.37 g, 30 mmol) suspension in water with magnetic stirring in an ice bath. After finishing the reaction, a colorless NaHSe solution was obtained. Meanwhile, 3-chloropropanoic acid (6.50 g, 60 mmol) was dissolved in water and its pH was adjusted to 8.0 by adding of sodium carbonate with the color of solution changing from light pink to pale yellow. Then, the 3-chloropropanoic acid solution was injected into the colorless NaHSe solution, followed by stirring the reaction mixture overnight under argon. After filtration, a clear yellow liquid was obtained and its pH was adjusted to pH 3~4 using 1 mol/L HCl, producing a turbid suspension with an amount of crimson substances. The filtrate was then extracted three times by ethyl acetate. The collected organic layers were washed with water twice to remove water-soluble by-products. After drying using anhydrous magnesium sulfate for 3-4 h, the solvent was removed *via* rotary evaporation and the crude products was recrystallized from ethyl acetate to obtain the final white solid, SePA (yield: 50%).

Synthesis of mPEG-PLGA-mPEG

A conventional triblock copolymer mPEG-PLGA-mPEG (Control-1) was synthesized *via* coupling reaction between HMDI and the end-hydroxyl group of mPEG-PLGA. In brief, Polymer-1 (6.70 g) was dissolved in 80 mL toluene. The residual moisture in polymers was removed by azeotropic distillation of toluene to a final volume of 25 mL. HMDI (0.2994 g) and $Sn(Oct)_2$ (30 mg) were then introduced into the polymer solution and the mixture was stirred at 60 ˚C for 6 h under an argon atmosphere. After the completion of reaction, diethyl ether was used to precipitate the product for 24 h. Finally, the resultant precipitate was dried under vacuum at room temperature until future use (yield: 63%).

Synthesis of Bi(mPEG-PLGA)-Se conjugate

Bi(mPEG-PLGA)-Se conjugate was synthesized by coupling SePA with mPEG-PLGA in the presence of DCC and DMAP. In brief for Conjugate-1, Polymer-1 (9.35 g) was dissolved in anhydrous tetrahydrofuran (THF). Then, SePA (0.57 g) and DMAP (0.17 g) were added into the polymer solution under an argon atmosphere. DCC (1.14 g) was

dissolved in THF and injected into the ice-cooled mixture system. The reaction was stirred in ice bath for 4 h and then additional 48 h at room temperature under an argon atmosphere. After the completion of reaction, DCC was converted into N,N'-dicyclohexylurea (DCU) by addition of several drops of water. After filtration, a transparent yellow solution was obtained and then the solvent was removed by rotary evaporation. The synthesized conjugates were purified by dissolving crude products in dichloromethane and then precipitating in diethyl ether. The final product was obtained after drying *in vacuo* (yield: 79.6%). Conjugate-2 was synthesized by a similar procedure.

1.3. Characterization

NMR Characterization

^{1}H NMR spectra were recorded on a 400 MHz proton FT-NMR spectrometer (AVANCE III HD, Bruker) using DMSO-d_6 or $CDCl_3$ as the solvent and tetramethylsilane (TMS) as the internal standard. ^{77}Se NMR spectra were recorded on a 500 MHz spectrometer (AVANCE III HD, Bruker) with dimethyl selenide as the external reference, 95.382MHz, using DMSO-d_6 as the solvent.

Gel Permeation Chromatography (GPC)

A gel permeation chromatography system (Agilent 1260) equipped with a refractive index detector was used to determine the MWs and distributions of synthesized polymers. THF was selected as the eluting solvent at a flow-rate of 1 mL/min at 35 ˚C and MWs were calibrated based on the monodispersed polystyrene standards.

Sol-Gel Transition

The sol-gel transition temperature was determined *via* the test tube inverting method.[3, 4] Polymer solutions (25 wt%) were prepared in distilled water and equilibrated at 4 ˚C overnight. Then, 2-mL vials containing 0.5 mL polymer solutions were immersed in a water bath at predetermined temperature for 15 min to reach the equilibrium. Temperature increment was set as 1 ˚C per step. If no visual flow was observed within 30 s after inverting the vials, the sample was regarded as a gel.

Dynamic Rheology Analysis

Dynamic rheology analysis was carried out to investigate the sol-gel transition using a dynamic stress-controlled rheometer (Kinexus, Malvern). After loading the polymer solutions (4 ˚C, 1.5 mL) on the cone plate (diameter: 60 mm, cone angle: 1˚), a thin layer of low-viscosity silicone oil was overlaid onto the fringe to minimize the evaporation of solvent. Storage modulus G' and viscosity (η) were recorded with an oscillatory frequency ω of 10 rad/s and a heating rate of 0.5 ˚C/min.

X-ray Photoelectron Spectroscopy (XPS)

XPS measurements were performed on a photoelectron spectrometer (Axis Ultra Dld, Shimadzu/Kratos) using Si wafer as substrates.

Dynamic Light Scattering (DLS)

The size of micelles was recorded on a nanoparticle analyzer Zetasizer (Zetasizer Nano, ZS 90, Malvern) at 25 ˚C. Before measurement, solutions were filtrated by a 0.45 μm filter and the light scattering angle was set as 90˚. Hydrodynamic diameter of particles was calculated by the Stokes-Einstein equation. CONTIN method was employed to analyze the intensity-intensity time correction function and average hydrodynamic diameter was denoted in Z-average.

Transmission Electron Microscopy (TEM)

Micelles were observed by transmission electron microscopy (Tecnai G2 20 TWIN, FEI) with an accelerating voltage of 200 kV. Samples were prepared by placing one drop of solution on a copper grid coated with a superthin carbon film, dried under an infrared lamp and observed without staining.

Coordination of cisplatin with Bi(mPEG-PLGA)-Se conjugate

Excess cisplatin was added into the Bi(mPEG-PLGA)-Se aqueous solution with Pt/Se ratio of 10/1 to study the coordination process. The solution was stirred in the dark at room temperature with a constant speed of 450 rpm. Then, 0.1 mL mixture was taken out at scheduled time and diluted with 15 mL water, followed by dialysis (MWCO of 3500) against deionized water for 24 h to remove the excess uncoordinated cisplatin molecules in the conjugate solution. The Pt/Se coordination ratio was determined by analyzing the platinum and selenium contents in the dialysates using inductively coupled plasma optical emission spectrometer (ICP-OES, PE-8000, PerkinElmer). The

selenium and platinum contents in the samples were measured at 196.026 and 265.945 nm, respectively, and calculated with the standard curve of each atom.

In *vitro* release study

The cisplatin-loaded (8 mg/mL) Bi(mPEG-PLGA)-Se conjugate aqueous solutions (25 wt%) with different coordinating ratios were obtained by taking out the solutions at different mix time, namely 1, 2 and 4 d. 0.5 g of the cisplatin-loaded conjugate solution was transferred into a 10-mL test tube and then incubated in a shaking bath (37 ˚C, 50 rpm) for 15 min to form an *in situ* hydrogel. Afterwards, 9 mL phosphate buffer saline (PBS, pH 7.4) containing 0.025 wt% sodium azide with or without 10 mM glutathione was introduced as the release media. At designed intervals, 5 mL release media was taken out and replaced with the same amount of fresh PBS to maintain the sink condition. The cisplatin-loaded mPEG-PLGA-mPEG gel formulation with the same drug loading amount was set as the control. The Pt released amount in the release medium was analyzed using ICP-OES and the cumulative release curve was calculated correspondingly.

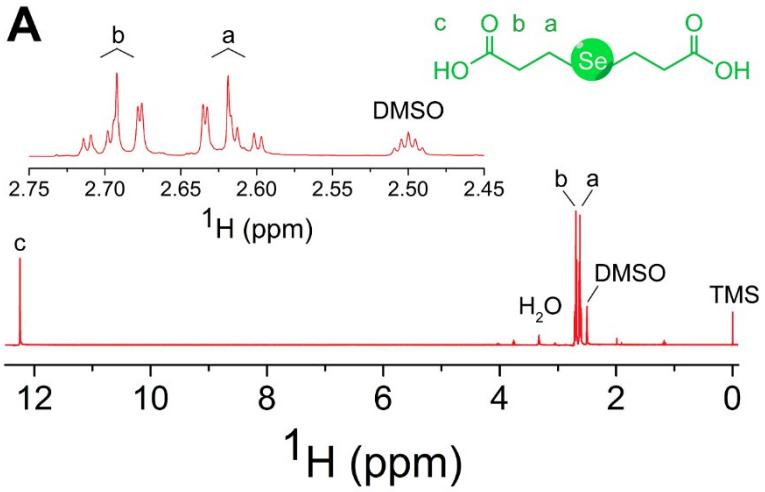
In *vitro* cytotoxicity test

Cytotoxicity of mPEG-PLGA-mPEG polymer and Bi(mPEG-PLGA)-Se conjugate was evaluated using a cell counting kit-8 (CCK-8) assay. NIH 3T3 or SKOV-3 cells were cultured in DMEM or McCoy 5A containing 10% fetal bovine serum (FBS) with 100 U/mL penicillin and 100 mg/mL streptomycin and incubated under a humidified environment at 37 ˚C with 5% CO_2. Cells were seeded in a 96-well plate at a cell density of 5×10^3 per well and incubated for 6 h. Subsequently, the upper medium was replaced with 200 μL of fresh culture medium containing various concentrations of mPEG-PLGA-mPEG polymer or Bi(mPEG-PLGA)-Se conjugate. After 24 h incubation, the medium was replaced with 200 μL fresh medium containing 20 μL CCK-8 solution and then cells were incubated for another 3 h. Finally, optical density of each well was measured at 450 nm using a microplate reader (ELx808, Biotech). The cell viability of culture medium without polymer treatment as the blank control was defined as 100%.

2. RESULTS

The synthetic procedure of Bi(mPEG-PLGA)-Se conjugate was illustrated in Scheme 1. Firstly, SePA, a coupling agent bearing two carboxylic groups, was prepared according to the previous publications.[1, 2] Meanwhile, diblock copolymer methoxyl poly(ethylene glycol)-b-poly(D,L-lactide) (mPEG-PLGA) with appropriate mPEG/PLGA ratio was synthesized *via* ring-opening polymerization of D,L-lactide (LA) and glycolide (GA) in the presence of mPEG using stannous octoate as the catalyst. Finally, SePA was covalently linked to the hydrophobic end of two mPEG-PLGA polymer chains *via* esterification reaction, resulting in the formation of BAB-type Bi(mPEG-PLGA)-Se conjugate.

^{1}H and ^{77}Se NMR measurements were used to monitor the entire reaction process and all the characteristic proton or Se signal peaks of resultant polymers were well assigned on their NMR spectra, as presented in Fig. S1. In the ^{1}H NMR spectrum of Bi(mPEG-PLGA)-Se conjugate (Fig. S1C), not only a new peak belonging to the proton signals of -$\boldsymbol{CH_2CH_2}$Se- appeared at 2.76 ppm, but also the molar ratio between the peaks a (-$\boldsymbol{CH_2CH_2}$Se-) and c (-OC$\boldsymbol{H_3}$) was approximately 4:3, which provides a direct evidence of the successful introduction of SePA into Bi(mPEG-PLGA)-Se conjugate with high conjugation efficiency. On the other hand, the signal peak of Se downshifted from 194.1 ppm to 200.4 ppm due to the change of chemical environment after coupling reaction (Fig. S1D). GPC chromatograms in Fig. S2 offer a further proof of the successful synthesis of Bi(mPEG-PLGA)-Se conjugate. Both the GPC traces of mPEG-PLGA copolymer and Bi(mPEG-PLGA)-Se conjugate displayed a unimodal distribution, and compared with the mPEG-PLGA copolymer, the MW of Bi(mPEG-PLGA)-Se conjugate increased significantly while the molar-mass dispersity ($Đ_M$) reduced correspondingly.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DMSO
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TMS
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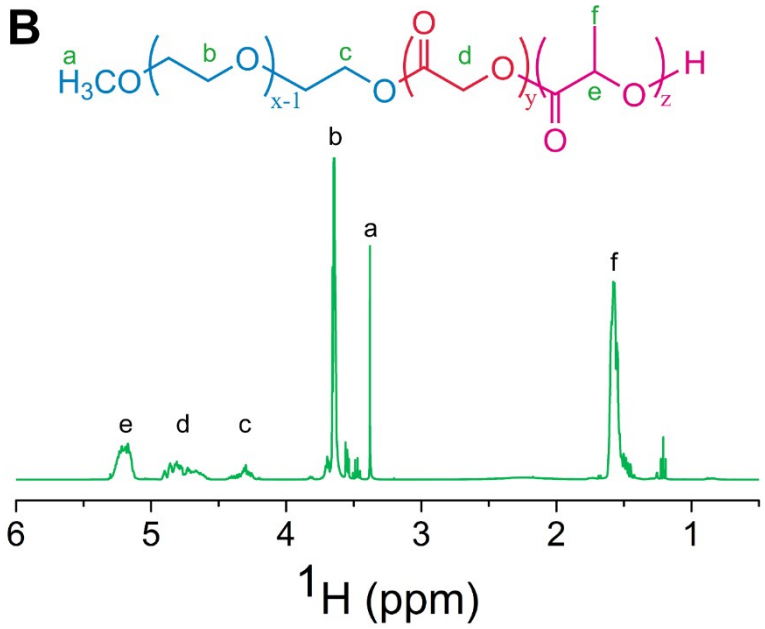
1H (ppm)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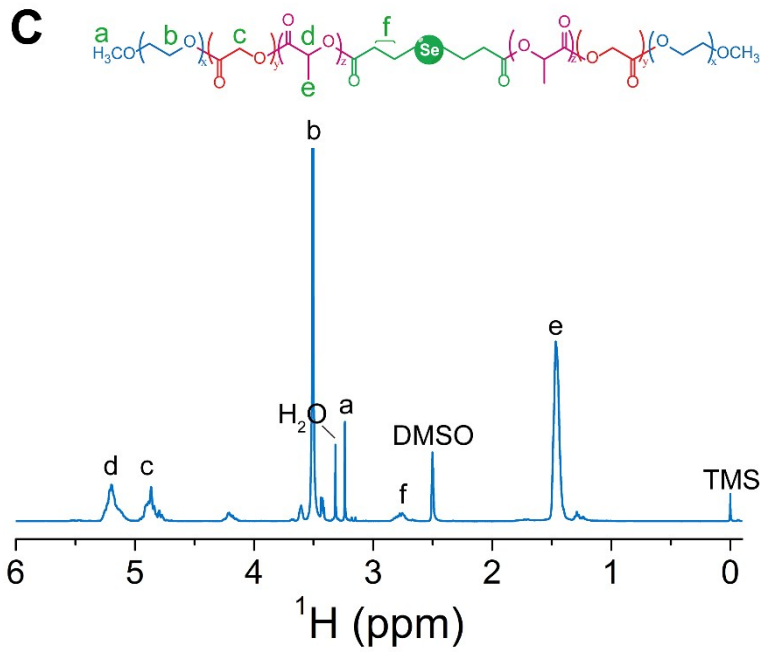
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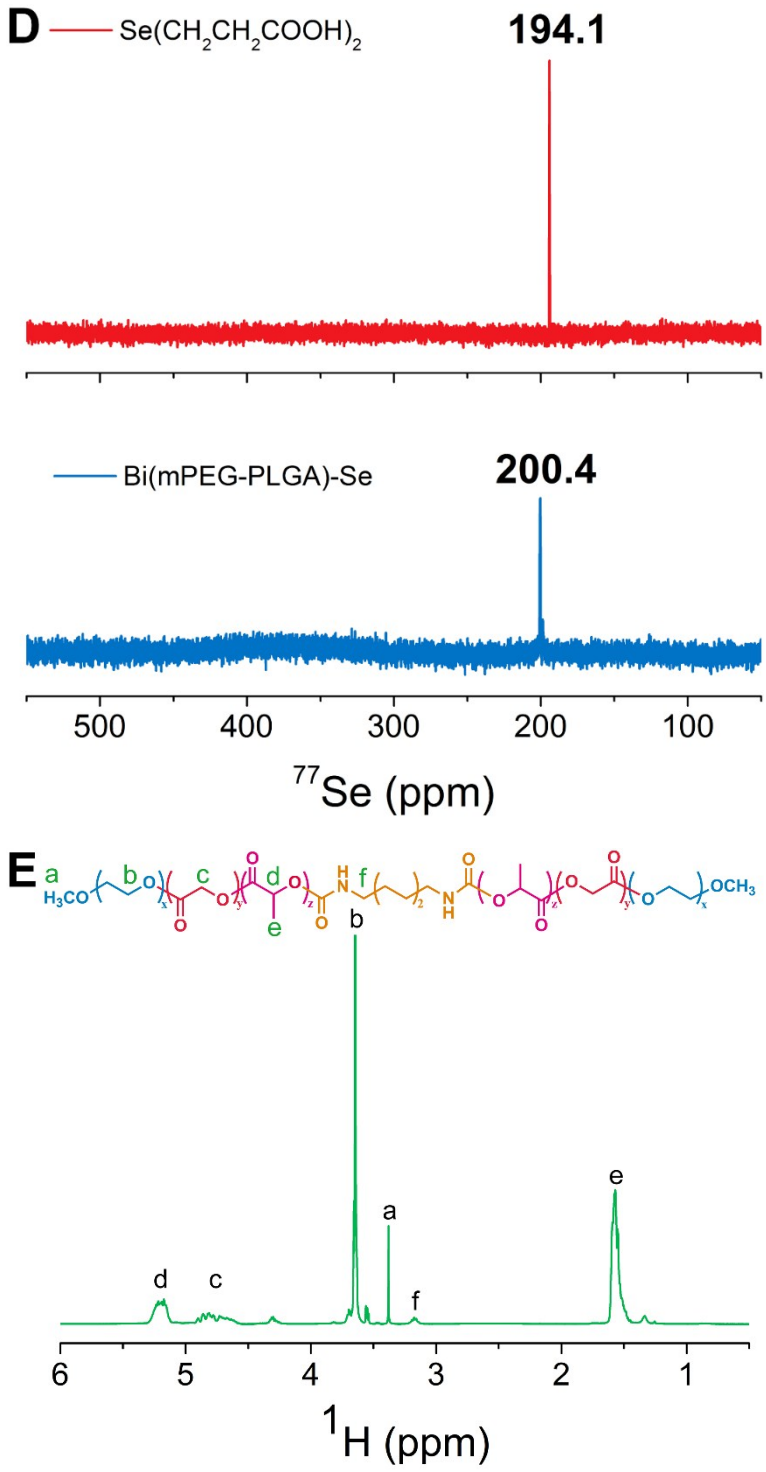

Fig. S1 ^{1}H NMR spectra of the indicated samples (A) coupling agent SePA in DMSO-d_6, (B) diblock copolymer mPEG-PLGA (Polymer-1) and (C) Bi(mPEG-PLGA)-Se conjugate (Conjugate-1) in DMSO-d_6. (D) ^{77}Se NMR spectra of SePA and Bi(mPEG-PLGA)-Se with dimethyl selenide as the external reference, 95.382MHz, and DMSO-d_6 as the solvent. (E) ^{1}H NMR spectra of mPEG-PLGA-mPEG (Control-1) in $CDCl_3$.

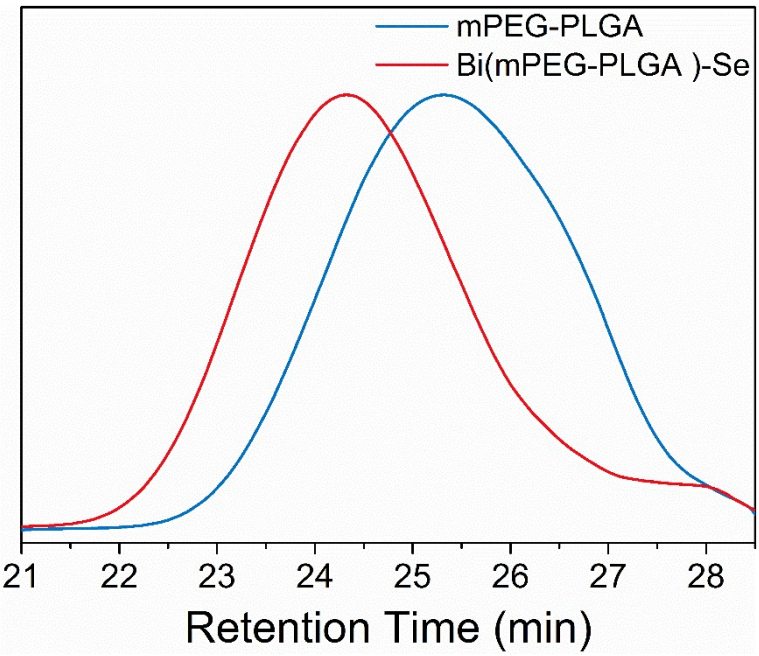

Fig. S2 GPC traces of mPEG-PLGA (Polymer-1) and Bi(mPEG-PLGA)-Se (Conjugate-1).

Moreover, the selenium content in Conjugate-1 was also determined using ICP-OES. The result showed that selenium content reached about 1.82 wt%, which was very close to the theoretical value of 1.84 wt%. All of these findings indicate that the desired product was successfully synthesized.

Dynamic rheology analysis was applied to monitor the sol-gel transition of the polymer aqueous solutions as presented in Fig. S3. At low or room temperatures, all the polymer aqueous solutions were free-flowing sols with low storage moduli G' (less than 0.01 Pa). As the temperature increased, the moduli increased abruptly for several orders of magnitude for the polymer/water systems, indicating the formation of semi-solid thermogel (Fig. S3A). The viscosity changed in a similar way of modulus (Fig. S3B).

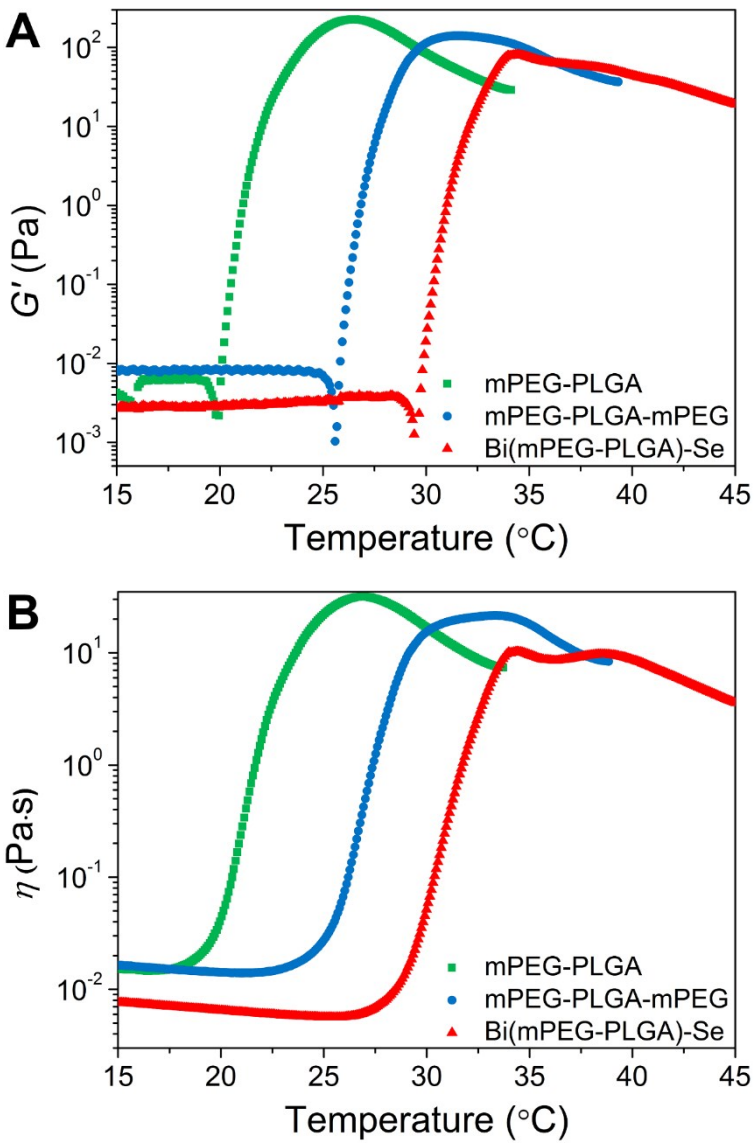

Fig. S3 Storage modulus G' (A) and viscosity η (B) of mPEG-PLGA (Polymer-1), mPEG-PLGA-mPEG (Control-1) and Bi(mPEG-PLGA)-Se (Conjugate-1) aqueous solution (25 wt%) as a function of temperature. Heating rate of 0.5 ˚C/min and oscillatory frequency of 10 rad/s.

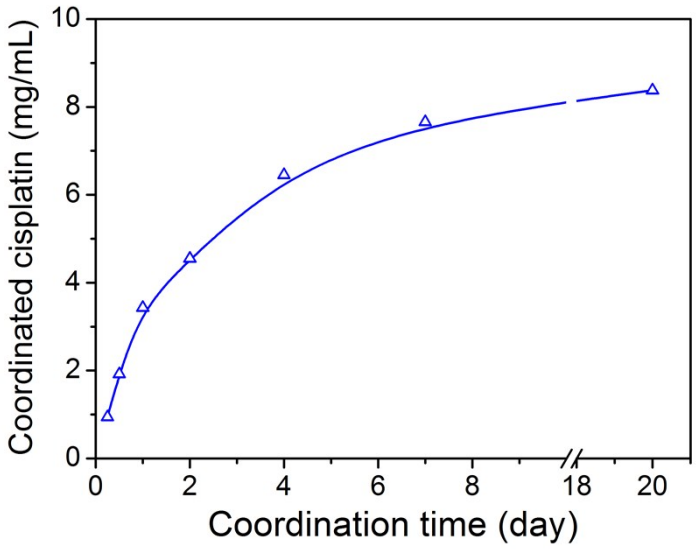

Fig. S4 Coordinated cisplatin amount in the Conjugate-1 aqueous solution (25 wt%) as a function of coordinating time. Excess drugs (> 100 mg/mL) were added. 0.1 mL sample was taken out at the predetermined time points and diluted with 15 mL water, followed by dialyzing against deionized water for 24 h to remove uncoordinated cisplatin in the conjugate solution before measurement via ICP-OES.

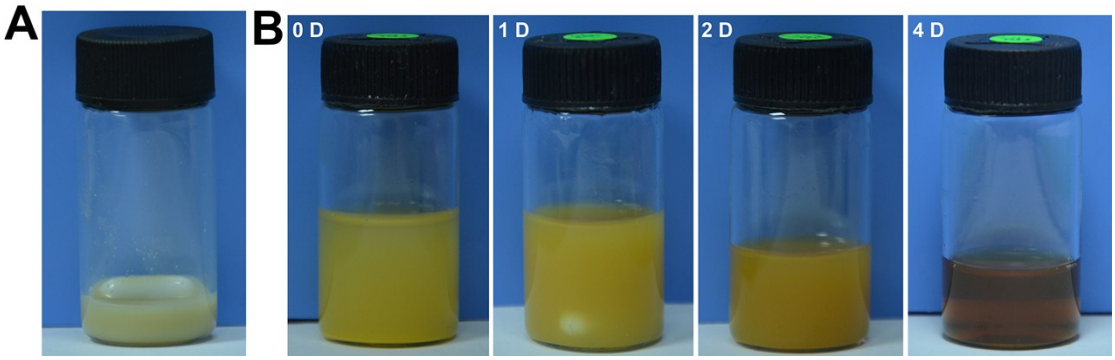

Fig. S5 (A) Photograph of Control-1/cisplatin aqueous system (Control-1: 25 wt%, cisplatin: 8 mg/mL) after 4 d stirring. (B) Photographs of Conjugate-1/cisplatin aqueous system (Conjugate-1: 25 wt%, cisplatin: 8 mg/mL) at different coordinating time points. 1.5 mL of solution was taken out for the drug release experiments at each time point.

References

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