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Electronic Supplementary Information

Robust, highly elastic and bioactive heparin-mimetic hydrogels

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1. Preparation and characterization of graphene oxide (GO)

Graphene oxide (GO) was prepared from natural graphite flakes by a modified Hummers method.[1] Briefly, 5 g graphite and 3.75 g $NaNO_3$ were placed in a flask. Then, 150 mL H_2SO_4 was added with stirring in an ice-water bath, and then 20 g $KMnO_4$ was slowly added for over 1 h. The mixture was stirred in an ice water bath for 2 h, followed by a vigorously stirring for 3 days at room temperature. Then, the mixture was diluted with DI water (500 mL) slowly, and the excessive $KMnO_4$ was decomposed by H_2O_2 (30 wt.%, 15 mL). The insoluble precipitations were removed by centrifugation. The resulted GO solution was filtered and washed with HCl (10 wt.%, 1 L) and DI water for several times to remove the metal ions. The pristine brown GO solution was dialyzed with DI water for 1 week before use to remove any residual salts and acids.

The prepared GO was further characterized with several methods. Morphological observation was carried out with a transmission electron microscope (TEM) (JEM-1200EX, JEOL, Japan). Two to three drops of the solution (1 mg/mL) were spread on a 3 mm copper grid and dried at ambient temperature to prepare the TEM sample. A Multimode Nanoscope V scanning probe microscopy (SPM) system (Bruker, USA) was used to obtain atomic force microscopy (AFM) images. The samples were prior prepared by dropping and drying on silicon wafers for the AFM analysis. FTIR spectrum was acquired on a FTIR spectrometer (Nicolet 560, USA) between 500 and 4000 cm^{-1}, using the KBr disk method. A Q500 Thermogravimetric analyzer (TA instruments, USA) was used to get the thermogravimetric analysis (TGA) curves of the hydrogels under a dry nitrogen atmosphere, the temperature was ranged from 50 ºC to 700 ºC at a heating rate of 10 ºC/min.

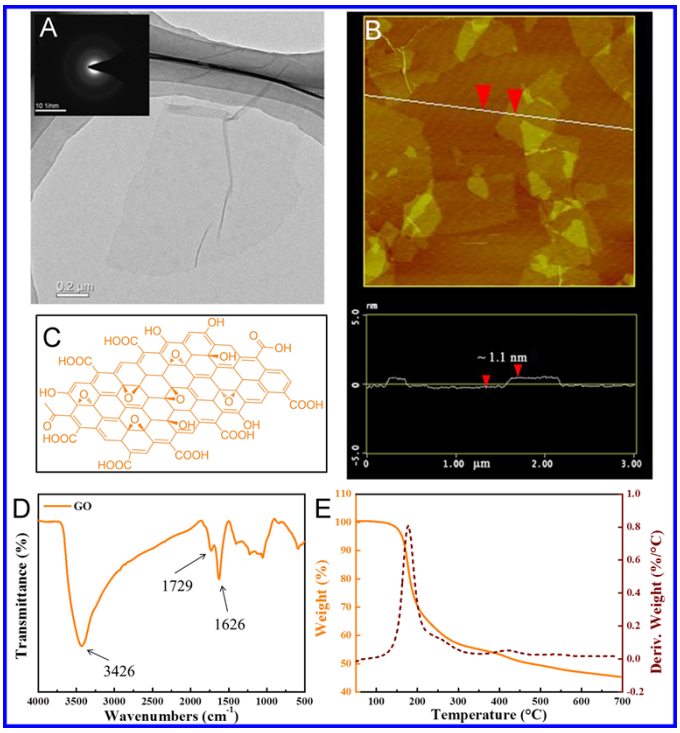

Figure S1. (A) TEM images and corresponding SAED patterns of GO. (B) Typical AFM images and cross-section analyses of GO. (C) Chemical structure of GO. (D) FTIR spectrum for the prepared GO. (E) The TGA image for the prepared GO.

2. Preparation of the heparin-mimetic hydrogels

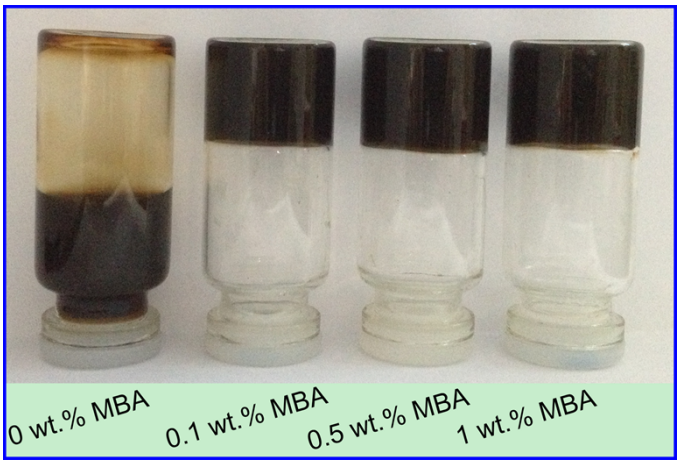

Figure S2. Photographs of the synthetic hydrogels in inverted glass bottles with various amount of cross-linker MBA.

3. Characterization of the heparin-mimetic hydrogels

Atomic force microscope (AFM) is found to be the most powerful tool for the visualization of GO sheets, and is used to investigate the interaction between GO and the polymers. In order to prepare the test samples, the concentration of the monomers in the GO suspension was decreased to avoid gelation; meanwhile, no crosslinker is

added to avoid the formation of crosslinked structure. The synthesis condition was described as follows: 50 mL GO suspension (contained 18.75 mg of GO) was firstly added to a 100 mL flask, then the monomers of AA (450 mg) and SSNa (300 mg) were introduced to synthesize heparin-mimetic polymers. Under N_2 flow protection and vigorous stirring, 7.5 mg of initiator APS was added. The flask was then immersed in a 70 °C oil bath for 24 h to insure the sufficient reaction. The obtained suspension was dialyzed with DI water for 1 week and then centrifuged at 14 800 g more than 3 times to make sure that the unreacted monomers, soluble polymers and the initiator were removed completely. Then the atomic force microscopy (AFM) images of the samples were acquired using a Multimode Nanoscope V scanning probemicroscopy (SPM) system (Bruker, USA).

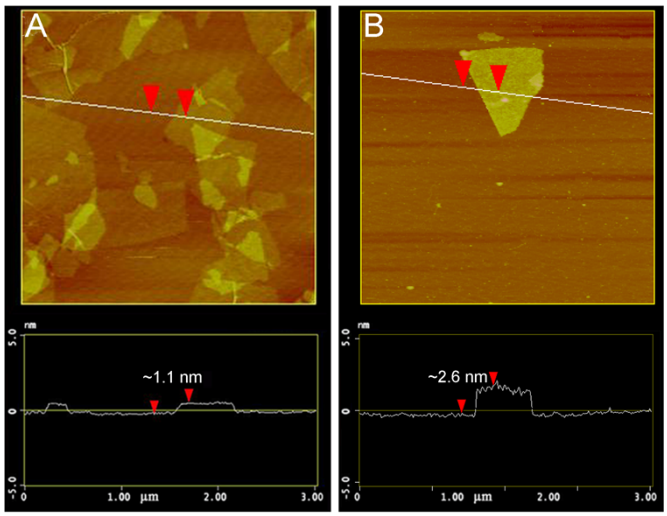

Figure S3. AFM images and cross-section analyses of GO (A) and heparin-mimetic polymer grafted GO (B).

Fig. S3 shows the AFM images and the corresponding height profiles of the GO and polymer grafted GO, respectively. As shown in Fig. S3A, the representative AFM image and cross-section analysis of the GO nanosheet clearly exhibits some wrinkles with an average thickness of about 1.1 nm, indicating the single-layered GO nanosheets. For the heparin-mimetic polymer grafted GO, some protuberances are observed on the sheet back-bones with the increased height of about 2.6 nm. Meanwhile, when incubating poly(styrene sulfonate) with GO at the same condition as that of B in Fig. S3, there is no obviously thickness increase, which indicate that the increased thickness in the figure is not caused by the adsorption of heparin-mimetic polymers on GO. Notably, even though it is recognized that polymer chains can be directly grafted onto the graphitic surface by using such radical addition/coupling reaction between polymer radicals and the double bonds of surfaces, and it is still an open challenge to provide more direct evidence for the detailed reaction mechanism. The results demonstrated that the monomers could be successful grafted onto the GO nanosheets; therefore, we believed that the GO covalently doped hydrogels could be constructed via utilizing GO as bridges or covalent cross-linkers to connect the heparin-mimetic polymer chains by free radical polymerization methods.

FTIR spectra of the hydrogels were acquired on a FTIR spectrometer (Nicolet 560, USA) between 500 and 4000 cm^{-1}, using the KBr disk method. The hydrogels were completely dried in a vacuum oven at 40 °C for 24 h before the respective FTIR experiments were carried out.

A Q500 Thermogravimetric analyzer (TA instruments, USA) was used to get the thermogravimetric analysis (TGA) curves of the hydrogels under a dry nitrogen atmosphere, and the temperature was ranged from 50 ºC to 700 ºC at a heating rate of 10 ºC/min.

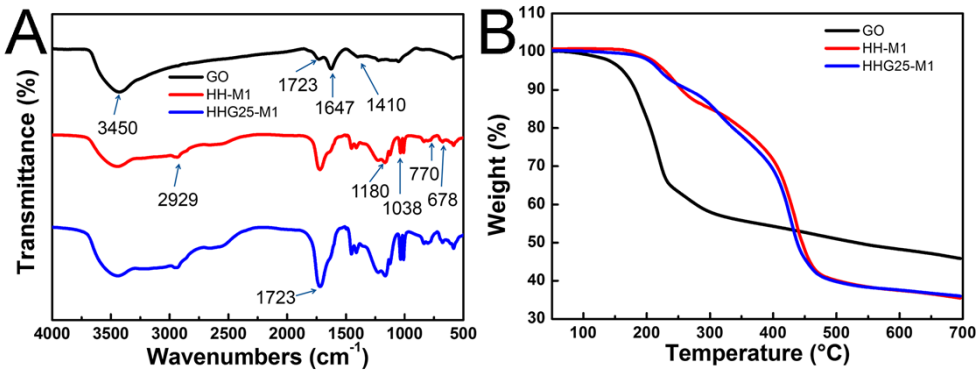

Figure S4. (A) FTIR spectra for GO, HH-M1 and HHG25-M1 hydrogels. (B) The TGA curves for GO, HH-M1 and HHG25-M1 hydrogels.

To calculate the composition of the hydrogel, elemental analyses were performed on a CARLO ERBA 1106 elemental analyzer (Italy) for carbon (C), hydrogen (H), sulfur (S) and nitrogen (N) with a carrier gas (He, at a flow rate of 100 ml/min) at a combustion temperature of 1000 °C using the samples.[2] The composition of HH-M1 (constructed with AA, SSNa and MBA) could be conveniently calculated from the content of S (only SSNa contained) and N (only MBA contained) as shown in Table S1.

Table S1. The weight compositions of the hydrogel HH-M1.

Sample	Composition		
	AA (wt.%)	SSNa (wt.%)	MBA (wt.%)
Monomers before reaction	59.41	39.60	0.99
The synthetic hydrogel HH-M1[a]	53.84	45.13	1.03

[a] Elemental analysis data from CARLO ERBA 1106 elemental analyzer, the test was repeated three times.

For the GO hybrid hydrogels HHG5-M1 and HHG25-M1, the compositions could not be calculated directly from the elemental analyses, since both the AA and GO contain the same elements of carbon, hydrogen and oxygen. Herein, the content of GO is supposed to be constant, since GO owns relatively large size compared to the monomers and can be rarely lost during the reaction process. After the polymerization, the hydrogels are dialyzed to remove the small molecules, then dry to constant weight

to calculate the yielded weight of the heparin-mimetic hydrogels. Thus, the approximate composition of GO could also be calculated after weighting the obtained dry hydrogel. Then the compositions of the GO hybrid hydrogels could be further calculated as shown in Table S2.

Table S2. The weight compositions of the GO hybrid hydrogels.

Sample		Composition			
		GO (wt.%)	AA (wt.%)	SSNa (wt.%)	MBA (wt.%)
HHG5-M1	Monomers before reaction	0.49	59.11	39.41	0.99
	The synthetic hydrogel	0.53	53.41	45.04	1.02
HHG25-M1	Monomers before reaction	2.42	57.97	38.65	0.97
	The synthetic hydrogel	2.67	52.41	43.92	1.00

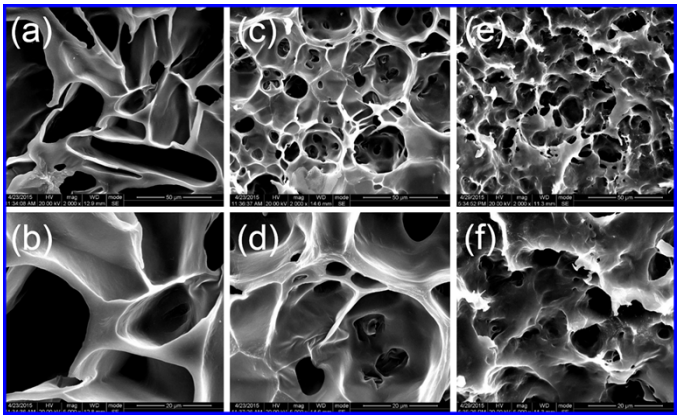

Figure S5. SEM images of the cross-section views of HHG25-M0.1 (a, b), HHG25-M0.5 (c, d) and HHG25-M1 (e, f). Voltage: 20.0 kV; magnification: 2000 × with the scale bar of 50 μm for a, c, e; 5000 × with the scale bar of 20 μm for (b, d, f).

4. Mechanical properties of the hydrogels

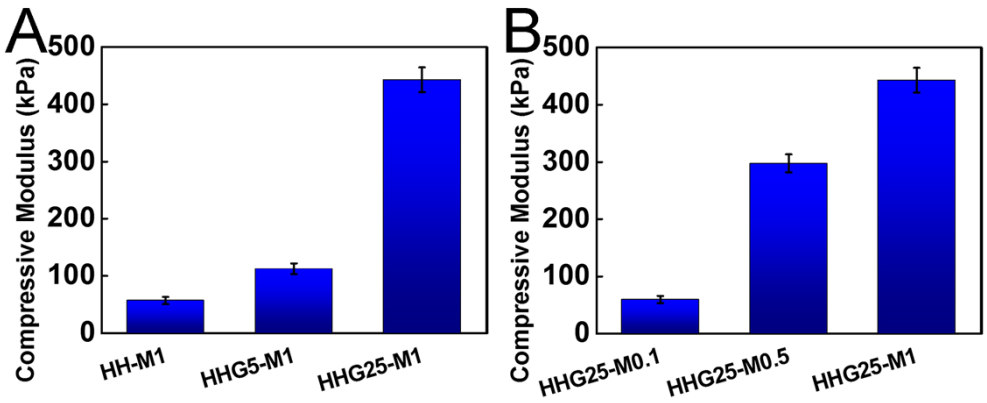

Figure S6. (A) Compressive modulus of the hydrogels with various amounts of GO. (B) Compressive modulus of the hydrogels with various amounts of cross-linker MBA.

5. Clotting time test

To evaluate the anticoagulant ability of the heparin-mimetic hydrogels, an automated blood coagulation analyzer CA-50 (Sysmex Corporation, Kobe, Japan) was applied to measure activated partial thromboplastin time (APTT) and thrombin time (TT).[3] The platelet-poor plasma (PPP) was collected after centrifuging at 4000 rpm for 15 minutes from healthy human fresh blood, which was collected with vacuum tubes containing sodium citrate as an anticoagulant (anticoagulant to blood ratio, 1:9 v/v).

For the anticoagulant tests, the freeze dried powder specimens were dispersed in PBS at the concentration of 5 mg/mL. the APTT test method was described as follows: 100 μL PPP and 5 μL suspension were incubated at 37 °C for 30 min, then 50 μL of the incubated PPP was taken out and added into a test cup, and then the APTT was measured by adding 50 μL of APTT agent (Dade Actin Activated Cephaloplastin Reagent, Siemens) and 50 μL of $CaCl_2$ solution (0.025 M). The TT test was carried out with a similar process to the APTT test, except that the added amount of Test Thrombin Reagent (Siemens; incubated 10min before use) to the test cup was 100 μL.

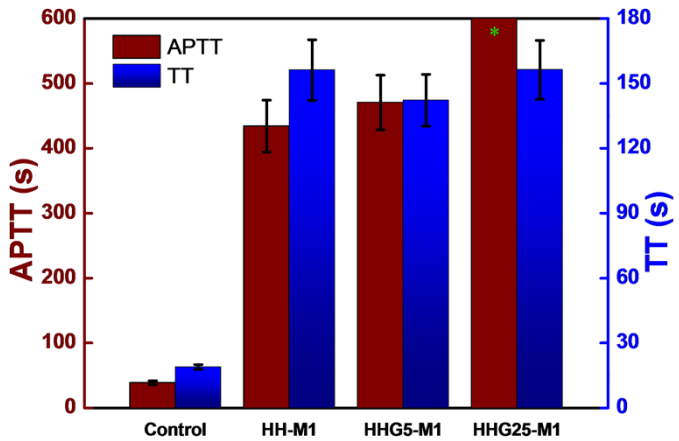

Figure S7. APTT and TT values for the hydrogels. For the control group, 5 μL PBS was added instead. Values are expressed as means ± SD (n = 3). The marks (*) meant

incoagulable.

The anticoagulant ability of the heparin-mimetic hydrogels is investigated by using standard APTT and TT tests. APTT can give the inhibited efficacy of both the intrinsic (or referred to as the contact activation pathway) and the common plasma coagulation pathways including factors II, V, X, XII or fibrinogen.[3] While, TT shows the clot formation time for the thrombin converted fibrinogen into fibrin in platelet-poor plasma (PPP).[4] As shown in Fig. S7, both the APTT and TT values for the HH-M1 increase obviously compared to the control group, indicating that the sulfonic acid group and carboxylic acid group contained hydrogels can partially mimic the anticoagulant activity of heparin. After the doping of GO, the APTT value increases gradually with increasing the GO ratio, indicating that GO may also contribute to the heparin-mimetic functionality.

6. Protein adsorption

The protein adsorption tests were carried out by using bovine serum albumin (BSA) and bovine serum fibrinogen (BFG) as model proteins to assess the binding affinity of the hydrogels to proteins. The hydrogels were immersed in 10 mL of PBS containing BSA or BFG with a concentration of 3 mg/ml, and incubated at 37 °C for 12 h to reach adsorption equilibrium. The concentration of the residual protein in the PBS was determined by an UV–VIS spectrophotometer (UV-1750, Shimadzu Co., Ltd, Japan) at the wavelength of 278 nm, and then the adsorbed protein amount was calculated.

Earlier report has indicated that a considerable number of proteins can interact with the polysaccharide heparin, together making up the class of heparin-binding proteins, such as proteases, growth factors, chemokines, lipid-binding proteins, pathogens and adhesion proteins.[5] Many latest reports have presented that heparin-mimetic structures can also show high protein-binding ability as that of heparin. Nguyen et al. showed that an important heparin-binding protein, basic fibroblast growth factor (bFGF), could be stabilized by conjugation of a synthetic heparin-mimetic polymer, poly(sodium 4-styrenesulfonate-co-poly(ethylene glycol) methyl ether methacrylate) (p(SS-co-PEGMA)).[6] And many other heparin-mimetic polymers could also conjugate with growth factors, which have been listed in Table S3.

The calculated protein adsorption amounts of HH-M1, HHG5-M1 and HHG25-M1 for BSA were 207.4, 236.8 and 254.2 mg/g, respectively; and those for BFG were 181.5, 206.1 and 223.2 mg/g, respectively. Thus, we may conclude that the heparin-mimetic hydrogels show high binding affinity to various proteins, which may include growth factors.

Table S3. Growth factors and proteins conjugated by heparin-mimetic polymers.

Heparin-mimetic polymers	Growth factors and proteins	References
Poly(sodium 4-	basic fibroblast growth factor	Nguyen et al.[6]

styrenesulfonate-co-poly(ethylene glycol) methyl ether methacrylate) (p(SS-co-PEGMA))	(bFGF)	
Poly(sodium 4-styrenesulfonate-co-poly(ethylene glycol) methacrylate) (pSS-co-pPEGMA)	basic fibroblast growth factor (bFGF) vascular endothelial growth factor (VEGF)	Christman et al.[7]
sulfonate groups contained peptide nanofibers	vascular endothelial growth factor (VEGF) hepatocyte growth factor (HGF) fibroblast growth factor-2 (FGF-2)	Mammadov et al.[8]
A heparinoid isolated from marine shrimp	vascular endothelial growth factor (VEGF) epidermal growth factor (EGF) fibroblast growth factor-2 (FGF-2)	Dreyfuss et al.[9]
sodium styrene sulfonate and acrylic acid based hydrogels	bovine serum albumin (BSA) bovine serum fibrinogen (BFG)	In this study

7. Cell culture

Human umbilical vein endothelial cells (HUVECs) were grown in R1640 medium supplemented with 10% fetal bovine serum (Hyclone, USA), 2 mmol L-glutamine and 1 vol.% antibiotics mixture (10,000 U penicillin and 10 mg streptomycin). Cultures were maintained in a humidified atmosphere of 5% CO_2 at 37 °C (Queue Incubator, France). Confluent cells were detached from the culture flask with sterilized PBS and 0.05% trypsin/EDTA solution. The culture medium was changed every day.[10]

MTT assay was used for examining the cell proliferation. After culturing for 48 h, 50 μL of MTT solution (1 mg/mL in PBS) was added to each well and incubated at 37 °C for 4 h, and then the MTT solution was replaced with 400 μL/well ethanol, followed by 30 min shaking. The optical density (OD) of each well was determined with a Microplate reader (model 550, Bio-Rad) at 492 nm. The MTT value for the control group (TCPS) was set as 100%, and the cell growth of the hydrogel was expressed as the percentage value by comparing with that of the control sample. The difference among individual groups was evaluated using the Student's t-test, and the level of significance was chosen as $P < 0.05$.

To further investigate the cell compatibility on the large-size hydrogels, the hydrogels were cut into thin films, then the HUVECs were seeded onto the hydrogel films (1 × 1 cm^2) at a density of approx. 2.5×10^4 cells/cm^2.[11] After culturing for 48 h, MTT assay was performed to quantify the extent of the cell growth on the hydrogels, and fluorescence staining (fluorescein diacetate/propidium iodide (FDA/PI)) and confocal

laser scanning microscopy (CLSM) were also used for the endothelia cells adhesion and morphology.

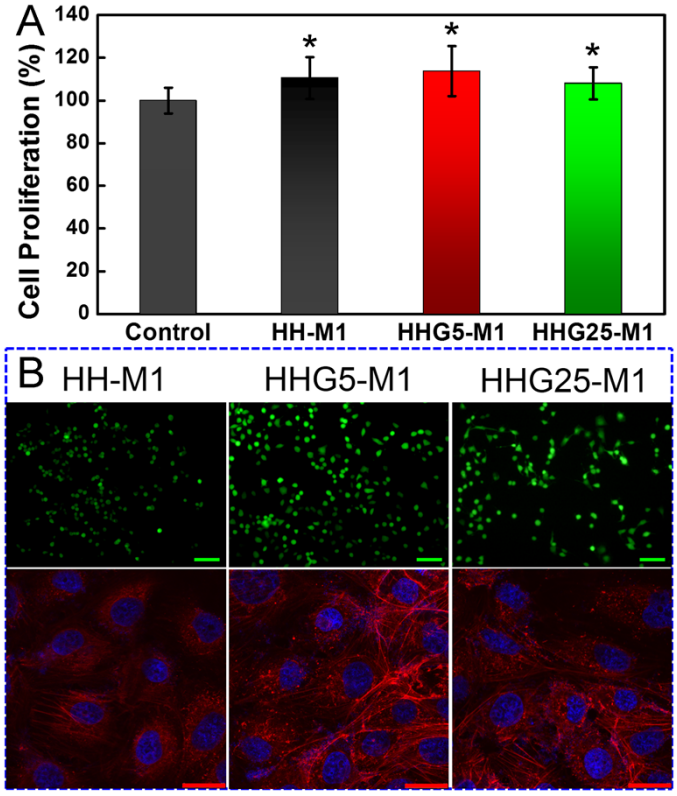

Figure S8. (A) MTT assay was performed to quantify the cell proliferation. Data were normalized to the control group (TCPS). *P < 0.05 compared to the values of the control sample. (B) FDA/PI staining for the cells cultured with the hydrogels (upper, scale bars: 50 μm) and the CLSM images (lower, scale bars: 25 μm).

8. Drug loading properties of the hydrogels

Doxorubicin hydrochloride (DOX) was chosen as the model drug to test the loading abilities of the hydrogels. The experiments were carried out by immersing the hydrogels into 0.5 mg/mL DOX aqueous solution to allow the impregnation of the hydrogels with the model drug. After mildly stirring for 48 h at room temperature to equilibrium, the loading ratio, X (%), could be estimated by using Eq. (1) as shown below:[12]

$$X\ (\%) = \frac{W_2}{W_1} \times 100 \qquad (1)$$

where, W_1 (mg) is the weight of the dried sample without loading drug; W_2 (mg) is the weight of the loaded drug, which is determined by measuring the UV-vis absorbance at 480 nm with an UV-vis spectrometer (UV-1750, Shimadzu Co., Ltd, Japan). The calculated drug loading ratios for the hydrogels of HH-M1 and HHG25-M1 were 49% (equal to 0.49 mg/mg) and 64% (equal to 0.64 mg/mg), respectively.

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