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# Electronic Supplementary Information

## Sustained delivery of gemcitabine via in situ injectable mussel-inspired hydrogel for local therapy of pancreatic cancer

Li Xu[a], Shengnan Tang[b], Huiquan Yang[c], Min Liang[a], Pengfei Ren[a], Dandan Wei[a], Jian He[b], Weiwei Kong[e], Peidang Liu[c,d], Tianzhu Zhang[a*]

[a] State Key Laboratory of Bioelectronics, National Demonstration Center for Experimental Biomedical Engineering Education, School of Biological Science and Medical Engineering, Southeast University, Nanjing 210096, China

[b] Department of Nuclear Medicine, Nanjing Drum Tower Hospital, The Affiliated Hospital of Nanjing University Medical School, Nanjing 210008, China

[c] School of Medicine, Southeast University, Nanjing 210009, China

[d] Jiangsu Key Laboratory for Biomaterials and Devices, Southeast University, Nanjing 210096, China

[e] The Comprehensive Cancer Centre of Drum Tower Hospital, Medical School of Nanjing University & Clinical Cancer Institute of Nanjing University, Nanjing 210008, China

*Corresponding author. E-mail address: zhangtianzhu@seu.edu.cn

**Materials and characterization instruments**

Carboxymethylcellulose (CMC, 250 kDa) and dopamine hydrochloride (DA) were purchased from Aladdin Industrial Inc.(China). Carboxymethylchitosan (CMCS, DS ≥ 80%) was purchased from Shanghai Macklin Biochemical Co., Ltd (China). Other reagents were obtained from Sinopharm Chemical Reagent Co., Ltd (China). RPMI1640 culture medium was obtained from Gibico (USA). A CCK-8 cell proliferation assay kit, and LIVE/DEAD strain assay were purchased from Kaiji Biotechnology Co., Ltd (Jiangsu, China). Other reagents were obtained from Sinopharm Chemical Reagent Co., Ltd. (China) and used as received without any further purification.

**Fabrication of precusors and preparation of hydrogels.**

In this work, CMCS is a commercially available material and used without any further purification. OCMC-$DA_4$ was synthesized according to our previous report with slight modifications (Scheme 1). Copolymers were prepared by a facile synthetic approach developed in our laboratory previously. As schematically illustrated in Scheme 1, 4 g of CMC was added in 200 mL of distilled water with vigorous stirring overnight. Sodium periodate (3.5 g) was dissolved in 40 mL of distilled water and added into CMC solution in five equal portions. Then, covered with tinfoil, the solution was stirred for 12 h at room temperature under light-free condition, followed by adding 2.5 mL of ethylene glycol and stirring for another 2 h to stop the reaction. The

reaction kettle was wrapped with several layers of tinfoil shielded from light, i.e. to avoid auto oxidation. The solution was dialyzed against distilled water for 3 days. The product (oxidized-carboxymethylcellulose, OCMC) was lyophilized.

To synthesize the OCMC-$DA_4$, 1.0 g of OCMC was completely dissolved in 50 mL of water. Later, ～82.6 mg dopamine in 5 mL of water was added to the OCMC solution. The mixture was stirred for 1 h with light-free. The product was dialyzed against water. Finally, the dry OCMC-DA was obtained through lyophilizing.

The CMCS/Gem and OCMC-DA were dissolved at the indicated weight proportions in normal saline (NS) directly to form solutions (20 and 40 mg/mL). Then, a predetermined amount of gemcitabine was directly dissolved in the CMCS micelles solution to form a homogeneous solution. Finally, the two precursor solutions were mixed together by a double syringe with equal volume ratios to form hydrogels, namely, OCMC-$DA_4$/$CMCS_2$/Gem hydrogels (Gem@Gel). The synthesis of the OCMC-$DA_4$/$CMCS_2$ and $OCMC_4$/$CMCS_2$ hydrogel were consistent with previous method.

**Physicochemical characterization**

As mentioned, simulations and characterizations can be performed under different conditions, and here that other advantages would be put into good use. Methods for the detailed SEM, TEM, TGA and H-NMR have been described below

**FTIR-ATR spectroscopy**

The FTIR-ATR spectra of CMC, OCMC, OCMC-DA, and lyophilized OCMC-$DA_4$/$CMCS_2$ gel were recorded with a PerkinElmer Spectrum Two FTIR-ATR spectrophotometer (PerkinElmer, USA) in the range 4000-500 $cm^{-1}$. All samples were completely dried and crushed.

**TGA measurements**

Thermogravimetric analysis (TGA) was measured on a Netzsch Model TG 209 F3 instrument from 300 K to 870 K at a rate of 10 K/min with nitrogen protection, and the results as shown in the Figure S2.

**Morphology of the hydrogels**

The hydrogels were prepared and freeze-dried using a SCIENTZ-12N Freeze Dryer. Microscopic morphology studies of the cross section of the freeze-dried hydrogel were carried out on an Ultra Plus field emission scanning electron microscope (Ultra Plus, Carl Zeiss, Germany). In order to observe the morphology of micelles in aqueous polymer solution at a preset temperature, the obtained polymer was fully dissolved in phosphate-buffered saline (PBS, pH 7.4) at room temperature under mild agitation to form the micelle solution. The morphology of micelles was observed under a transmission electron microscope (TEM, JEM-2100, JEOL, Japan). The aqueous polymer solutions were dropwise added to coppers on a piece of filter paper, dried at room temperature overnight, and then observed using TEM with the acceleration voltage at 200 kV. Additionally, the distribution of micelle size (with a

copolymer concentration of 1 wt.%) at different time periods was detected using dynamic light scattering (DLS, Nano-ZS 90, Malvern, Worcestershire, UK).

**$^1$H NMR and GPC Characterization**

The OCMC-DA was analyzed using a 600 MHz 1H NMR spectrometer (AVANCE III HD, BRUKER, Switzerland) for investigating the chemical structure of OCMC-DA. $D_2O$ was used as a solvent in the presence of tetramethylsilane (TMS) as the internal standard. The MWs and molar mass dispersity ($Đ_M$) values of the synthesized copolymers were confirmed on a gel permeation chromatography (GPC, Agilent 1260) system. Pure water was chosen as the eluent with the flow rate at 1.0 mL/min and the operating temperature at 35 °C.

**Rheological Properties of the Hydrogels**

The dynamic rheological properties of the hydrogels with or without drugs were characterized using a rheometer (MCR 302, Anton Paar GmbH, Austria). Briefly, 300μL of each hydrogel precursors were mixed and placed in the disk mold to form a column (D=20 mm) with a gap of 5～10 mm. The mixture was kept at room temperature with a consistently humid environment for 1 h to ensure the hydrogel formed completely. The hydrogel was loaded onto the table of a rheometer, and the equipment was run in the frequency sweep mode in a range of 0.1－10 Hz. To evaluate the hydrogels' gelation time, a time sweep test was conducted. The precursors of OCMC-$DA_4$/$CMCS_2$-Gem are injected at some time point during the

solidification, achieving the flow of injectable formulations under modest pressure and rapid setting after the injection, which is unambiguous to define ($T_{gel}$). Using a double-tube syringe, 500 μL of each hydrogel precursor solution were injected onto the rheometer plate; changes of their storage moduli (G′) and loss moduli (G″) were monitored at a constant frequency (1 Hz), temperature (25°C), and strain (1.0%) during 5 min. The gelation time was recorded when G' became higher than G".

**Equilibrium Swelling and Degradation of Hydrogels**

The hydrogel was prepared as in the previous approach using a disc mold. The initial weight of the hydrogel was determined after it was formed completely. Then, the hydrogel was immersed in distilled water for 24 h at room temperature. The equilibrium swelling behavior (ER) of the hydrogel was determined using the following equation

$$ER(\%) = (W_s\text{-}W_d)/W_d \times 100\% \quad (1)$$

where $W_s$ and $W_d$ represent the weights of the swollen and dry samples, respectively.

During the equilibrium swelling period, the hydrogel was measured as an initial mass. Then, in vitro degradation tests were conducted by monitoring the weight changes of oven-dried samples in the degradation process. Briefly, each hydrogel was placed in the test tube containing 5 ml of PBS (pH 7.4) at 37°C with mild agitation (50 rpm). At predetermined time intervals, hydrogels were moved from the PBS solution and then dried under vacuum at room temperature to constant weight, which was recorded

every 24 h over the degradation period. All experiments were done in triplicate. The extent of degradation (weight loss ratio) was expressed using the following equation

$$\triangle W(\%) = (W_i - W_r)/W_i \times 100\% \quad (2)$$

where $W_i$ and $W_r$ are the initial and remaining weights, respectively, of each sample.

**Lap Shear Adhesion Testing.**

The design of mussel-inspired synthetic adhesives is also under fast growth, accompanied with the mechanistic study on the structure-property relationship. Lap shear adhesive bonding of the hydrogel with Gem was carried out with a modified version 13 of the ASTM F2255 standard method. Another substrate was placed on top of the first to form a lap shear joint of 25 × 25 $mm^2$. All glass substrates were pretreated by rinsing with ethanol and deionized water and then dried before test. For dry environment groups, the as-prepared joint with a stainless steel clip was placed at room temperature for 3 h after being pressed by a 500 g weight for 5 min. For adhesion tests under underwater conditions, due to the low viscosity of the copolymer, the exact underwater condition was not applicable. Instead, the adherends were pre-wetted first for 2 h, and the adhesive solution was applied to the surface while it was still wet. The two adherends, pressed by a 500 g weight for 5 min, were then fixed using a stainless steel clip, and immediately immersed into water and completely soaked for 12 h before test (37℃, 50% RH). Finally, the samples of the underwater group were taken out and dried in air for another 3 h. For constancy, the amount of the adhesive was fixed at 100 μL in all cases. The maximum force at joint failure

divided by the overlap area provided the adhesion strength. Each sample was tested a minimum of 4 times and averaged.

**In vitro drug release**

The mechanism of release kinetics of Gem from the drug-loaded hydrogel were assessed. Generally, 1.0 mL OCMC-$DA_4$ solution was injected to the bottom of a 15-mL centrifuge tube (φ = 78 mm) to form a certain of homogeneous agent. Soon afterwards, 1.0 mL $CMCS_2$ precursor solution loaded with 0.5 mg/mL Gem was carefully added into the above solution at ambient temperature to completely allow in situ gelation and then incubated under gentle shaking (37 °C, 50 rpm) for 30 min. Then, 10 mL of phosphate-buffer saline (PBS, pH 7.4) as the release medium was carefully added into each tube along the wall. At predetermined intervals (within 2 weeks), 2 mL of supernatant was collected from each vial, followed by adding 2 mL of fresh PBS during the in vitro release process. A high-performance liquid chromatography (HPLC, Primaide 1749L) system provided with a $C_{18}$ reverse-phase column (Agilent, 4.6 × 150 mm, 5 μm) and a UV detector was employed to determine the amount of drug released at each time point. For the detection of Gem, the column was eluted with water/acetonitrile (90/10, v/v) at a flow rate of 1.0 mL/min for 2 min, followed by changing the component of acetonitrile in the mobile phase from 10% to 50% within 3.5 min. The UV absorption wavelength was set at 268 nm. All data measured at pH 7.2 are as the mean of three individual experiments to assess the drug release mechanism.

## Cellular experiments

### Cell culture

BxPC-3 cells were purchased from the American Type Culture Collection (ATCC, Rockville, USA), which were cultured in RPMI-1640 growth medium with 10% FBS, 50 U/mL penicillin and 50 U/mL streptomycin. The cells were maintained at 37 °C with humidified 5% $CO_2$ and were cultured according to ATCC recommendations.

### In vitro cytotoxicity assays and antitumor activity

The relative cytotoxicity of Gem against BxPc-3 human pancreatic adenocarcinoma cell was assessed by the Cell Counting Kit-8 (CCK-8) assay. Cells were seeded into 96-well plates at a density of 5000 cells/well in the presence of 100 μL culture medium containing 10% heat-inactivated FBS and incubated for 24 h. Then, the medium was replaced with 200 μL of fresh medium containing Gem solution at indicated concentrations (n = 5 for each group). After incubation for 48 h at 37 °C, the cells were subjected to CCK-8 assay, and the absorbance at 450 nm was measured using a microplate reader (Bio-Rad). The relative viability of treated cells was calculated as the percentages of the untreated control group. The results of cytotoxicity were recorded by a microplate reader at 450 nm. The cell viability was calculated using the following equation

$$\text{Cell viability}(\%) = (A_t - A_0)/(A_c - A_0) \times 100\% \quad (3)$$

where $A_t$, $A_0$, and $A_c$ represent the absorbance of the sample groups, blank groups and

control groups, respectively.

**Cytotoxicity of lixivium**

Complete Roswell Park Memorial Institute (RPMI) 1640 culture medium, composed of 90% RPMI 1640 (containing 100 U/mL penicillin and 100 μg/mL streptomycin) and 10% fetal bovine serum, was used to culture the BxPc-3 cells in a $CO_2$ incubator at 37°C. 0.4 mL Gel and Gem@Gel were immersed in 2 mL complete culture medium, and after incubation for 24 and 48 h at 37 °C, the lixivium was collected to evaluate the cytotoxicity of the lixivium from Gel (Gem@Gel). 100 μL cell suspension ($2\times10^4$ cells/mL) was introduced into a 96-well plate and cultured for 24 h, followed by the treatment with lixivium at different proportions in complete culture media for another 24 h. Cell viabilities of these cells treated with different concentrations of Gem for 24 h were utilized as a cytotoxicity refer for the released gemcitabine from Gem@Gel. After that, 10 μL CCK-8 solution was added to each well and incubated with the cells for 3 h. After shaking the plate for 5 min, the CCK-8-containing complete culture medium in each well was then measured at the wavelength of 450 nm by a microplate photometer (Multiskan FC, Thermo Scientific, USA).

**Effects of Gem-loaded hydrogel**

The in vitro anti-tumor activity of the drug-loaded hydrogel composite was evaluated through a 24-well Transwell (Corning) co-culture system. 500 μL BxPc-3 cell

suspension was added to the lower chambers of the 24-well Transwell (40,000 cells/well) and cultured for 24 h. 50 μL PBS, free Gem (10 μM), or a combination of free Gem and hydrogel (10 μM Gem) were directly added to the upper inserts of the Transwell. To create a drug depot, 50 μL Gem@Gel hydrogel solution (10 μM Gem) was added to the upper insert of the transwell and maintained at 37 °C for 30 min to form a micelle crosslinked hydrogel. Subsequently, these hydrogel-containing inserts were placed into the cells contained well plate for further co-culture. For all formulations, the initial medium was replaced by fresh medium after 24 h. The cells were subjected to CCK-8 assay after incubation for 24, 48, and 72 h, and the relative viability was expressed as a percentage compared to the PBS treated cells. All experiments were performed four times and the results are presented as the mean ± standard deviation (S.D.) (n = 3).

Meanwhile, to obtain the optical and fluorescent morphology images of BxPC-3 cell, cells cultured in 24-well Transwell plates were treated with 50 μL PLEL hydrogel, GEM/hydrogel, as described in the previous section. After incubation for 24, 48, and 72 h, BxPC-3 cells were washed with PBS (pH 7.4) three times and fixed with paraformaldehyde solution (4% in PBS at pH 7.4) at room temperature for 30 min. Then, the fixed cells were treated with DAPI (0.1w t.% in PBS) for 5 min to stain the cell nuclei. After that, optical and fluorescent images were observed under a fluorescence microscope (Olympus, Tokyo, Japan) and images were analyzed with ImageJ 7.0 software.

**Cell apoptosis analysis by flow cytometry**

Flow cytometry was performed to evaluate the apoptosis of BxPC-3 cells after exposure to either single or dual drug loaded hydrogel composites. Cells cultivated in 24-well Transwell plates were treated with 50μL PBS, free Gem solution, or GEM@hydrogel as described in the previous section. After 4, 12 and 24 h of cultivation, cells were harvested and stained with the Annexin V-FITC/PI Apoptosis Detection Kit, according to the manufacturer's instruction. The fluorescence of FITC and propidium iodide (PI) were detected by flow cytometry (BD FACSCalibur) and the data were analyzed with FlowJo 7.6.1 software. The results are presented as the percentage of early apoptosis (Annexin V-FITC positive and PI negative), late apoptosis (Annexin V-FITC positive and PI positive), and viable (Annexin V-FITC negative and PI negative) cells. All experiments were performed with three parallel samples and the percentages of apoptotic cells are presented as mean ± S.D. (n = 3).

**In vivo anti-tumor experiments**

**In vivo anti-tumor efficiency of the Gem-drug loaded hydrogel system**

Balb/c nude mice (female, 18 ± 2 g) were purchased from Yangzhou University Medical Center (Yangzhou, China). The mice were housed in a SPF environment with free access to food and water. All animal experiments were conducted under the approval of the Animal Care Committee of Southeast University and in compliance with the Regulations for the Administration of Affairs Concerning Experimental

Animals of China. Pancreatic cancer models were built through a one-step tissue block graft model. First, BxPc-3 cells ($5\times10^6$ cells) in 100 μL of the culture medium were subcutaneously injected in the right flank regions of nude mice. Subsequently, tumor growth inhibition evaluation was conducted on BxPc-3 subcutaneous mouse models. When the tumor volumes reached around 200–300 $mm^3$, the tumor-bearing mice were randomly distributed into 5 groups (n = 3 each) and treated with 200 μL different formulations via either intratumoral (i.t.) or intravenous (i.v.) injection on day one. The formulations included NS (i.t.), hydrogel (i.t.), free Gem (i.v.), free Gem (i.t.), Gem@Gel (i.t.). Herein, it is worth pointing out that the intratumoral injection of drug loaded hydrogel composite was carried out in this work due to the relatively low shear adhesive strength of the samples. For all drug-containing formulations, the dosages of Gem were retained at 10 mg/kg, respectively. The tumor sizes were measured every two days using a caliper and calculated using the following equation

$$V = L \times W^2/2 \quad (4)$$

where L and W represent the length and width of tumor. The body weight of each mouse was recorded every other day as well. On day 14 post treatment, the animals were sacrificed, tumors and major organs were harvested, weighted and fixed in 4% PBS buffered paraformaldehyde for further examination.

**Detection and retention of intratumoral distribution for drug loaded hydrogel composites**

To evaluate the intratumoral distribution and maintenance of drug-loaded hydrogel composites, water-soluble near-infrared fluorescence dye Cy5.5 was used as substitute for Gem. Pancreatic cancer xenograft models were established as described in the previous section. The tumor-bearing mice were randomly distributed into two groups (n = 3 each) and intratumorally injected with 100μL Cy5.5solution in normal saline (NS; 5μg/mL Cy5.5) or Cy5.5/hydrogel (5μg/mL Cy5.5). Particularly, for these mice in each group, fluorescence images were obtained by an IVIS Lumina III imaging system (Perkin Elmer, Caliper Life Sciences, MA, USA; excitation = 673 nm, emission = 692 nm). The mice were anesthetized, and fluorescence images were acquired at the time interval of 1, 3, 6, 12 and 24 h. The images were analyzed with onboard software.

**Histological and immunohistochemical analysis**

The 4% paraformaldehyde fixed tumors and major organs (heart, liver, spleen, lung, and kidney) were dehydrated through a graded series of ethanol and embedded in paraffin wax, followed by cutting into 5 μm sections. Then, according to the protocols provided by manufacturers, the tissue sections were stained with hematoxylin and eosin (H&E) of all tissues for histology assessment. Meanwhile, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assays of tumors were performed so as to evaluate the apoptotic response in tumor tissues. The H&E slides were analyzed using an inverted microscope (Eclipse LV100ND, Nikon), and the TUNEL slides were analyzed using another inverted

fluorescence microscope (Eclipse Ti-SR, Nikon)

**Statistical analysis**

Statistical analysis was performed using SPSS 23.0 software and all of the results were expressed as means ± S.D (n = 3 or 5). One-way analysis of variance (ANOVA) was adopted to analyze the differences between the control group and the treated groups using Tukey's test. A P value < 0.05 (*) was considered statistically significant. ** and *** indicate statistically significant differences at P < 0.01 and P < 0.001, respectively.

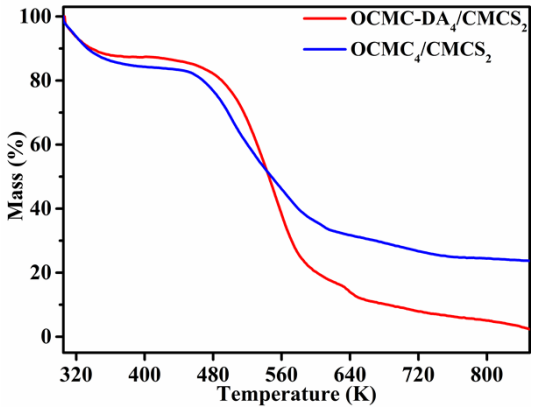


**Figure S1.** The result of thermogravimetric (TG) curves for the dry gels under nitrogen flow.

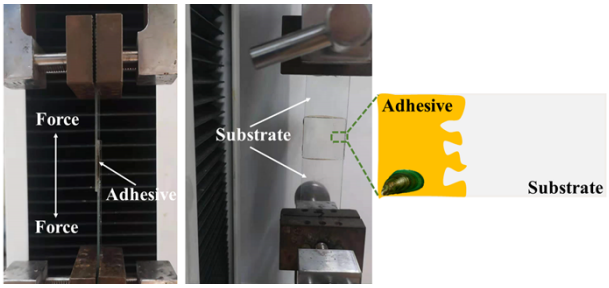


**Figure S2.** Schematic illustration of the lap shear measurement.

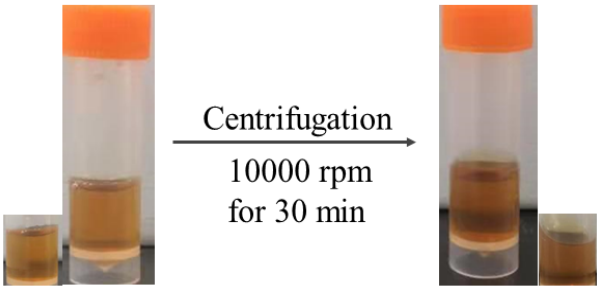


**Figure S3.** The optical photographs of Gem-loaded polymer solution before and after centrifugation.

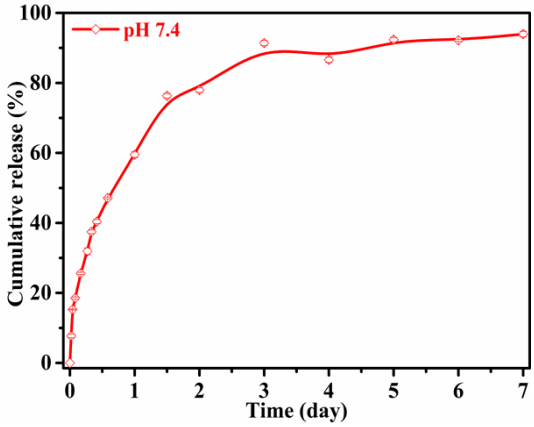


**Figure S4.** In vitro release kinetics of GEM from the hydrogel system in PBS at pH 7.4. Results are shown as average values ± SD (n = 3).

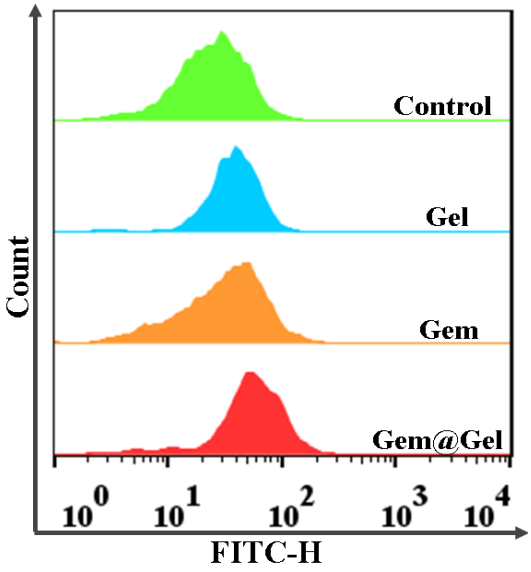


**Figure S5.** Evaluation of ROS generation in BxPC-3 cells for 24 h after various treatments as indicated by flow cytometry.