Supplementary information for

Injectable drug-loaded hydrogel based on supramolecular polymeric prodrug

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Experimental Details

Materials

Dichloromethane and triethylamine (TEA) were dried over CaH₂ and distilled just before use. Dichloromethane and triethylamine (TEA) were dried over CaH₂ and distilled just before use. Dextran (Mw 40 KDa) was purchased from Shanghai Ceneral-reagent Titan Chem. Co., Ltd, China. Carboxymethyl chitosan (CMCTS; substitution degree 80%, deacetylation degree 90 %) was obtained from Zhejiang AOXING of China. Doxorubicin hydrochloride (DOX•HCl) was purchased from Shanxi Sciphar Hi-tech Industry Co., Ltd, China. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC; 99%; Aladdin, China), 4-dimethylaminopyridine (DMAP; 99%; Aladdin, China), toluenesulfonyl chloride (TsCl; 99 %; Aladdin, China), 4-formylbenzoic acid (FBA; 98 %, TCI, Japan), 1-adamantanemethanol (AD; 99 %, TCI, Japan) and other reagents were used as received.

Synthesis of polyaldehyde dextran (PAD)

Dextran (3.00 g) was dissolved in water (100 mL) at 50 °C. After cooling to the room temperature, sodium periodate (3.96 g, 18.52 mmol) was added into the solution. The reaction was conducted at 25 °C for 2 h. Then ethylene glycol (1.15 g, 18.52 mmol) was added into the solution proceeded for 2 h. The solution was dialysed against water for 48 h to remove the by-products and then freeze dried (yield: 2.70 g, 90%).

Determination of aldehyde concentration in PAD

The concentration of aldehyde groups in PAD was determined according to the established procedure described elsewhere.1 In brief, accurately weighed PAD (0.15 g) was dissolved in hydroxylamine hydrochloride solution (25 mL, 0.25 M), into which 2 drops of methyl orange reagent (0.05 %) was added. The mixture was allowed to stand for 3 h, titrated with a standardized
solution of 0.1 M sodium hydroxide until the red-to-yellow end point was reached by matching the
colour of the sample solution with that of a blank one. The titration was performed in 5 replicates.
The aldehyde concentration (AC) was calculated from the equation:

\[ AC \text{ (mmol/g)} = \frac{(V_{\text{sample}} - V_{\text{blank}})C}{W} \]

Where \( V \) (mL) is the volume of standardized sodium hydroxide solution, \( C \) (mmol/mL) is the
concentration of standardized sodium hydroxide solution and \( W \) (g) is the weight of sample. The
aldehyde concentration in PAD is 8.67 mmol/g.

**Synthesis of toluenesulfonyl \( \beta \)-cyclodextrin (Tosyl-CD)**

Tosyl-CD was prepared on the basis of Zhang’s method.\(^2\) Briefly, \( \beta \)-CD (20.0 g, 16.3 mmol) was
suspended in water (150 mL), and NaOH (2.16 g, 54.7 mmol) dissolved in water (7 mL) was
added dropwise to the suspension. Toluene sulfonic chloride (3.36 g, 17.6 mmol) dissolved in
acetonitrile (10 mL) was slowly added dropwise. After 2 h of stirring at 25°C, the precipitate was
removed by filtration and the filtrate was neutralized by hydrochloric acid. The resulting white
precipitate was recovered by filtration. Tosyl-CD was obtained from recrystallization of the white
precipitate from hot water and dried in vacuum to a constant weight (yield: 2.16 g, 10 %).

**Synthesis of aminated \( \beta \)-cyclodextrin (Amino-CD)**

Amino-CD was synthesized according to Hidenori’s procedure with some modifications.\(^3\)
Tosyl-CD (3.00 g) dissolved in ethylenediamine (40 mL) was stirred at 50 °C for 36 h.
Approximately half of the unreacted ethylenediamine was removed through rotary evaporation,
and the residue was precipitated into acetone. The precipitate was dissolved in water. amino-CD
was reprecipitated from acetone and dried in vacuum to a constant weight (yield: 2.43 g, 89 %).
Synthesis of 1-adamantanemethyl 4-formylbenzoate (AD-CHO)

4-Formylbenzoic acid (1.00 g, 6.7 mmol) was suspended in anhydrous dichloromethane (60 mL), and anhydrous triethylamine was added dropwise until the mixture became totally transparent. Then 1-adamantanemethanol (1.00 g, 6.0 mmol), DMAP (0.086 g, 0.70 mmol) and EDC (2.3 g, 12.0 mmol) were added sequentially. The mixture was stirred at room temperature for 48 h under an argon atmosphere. Afterwards, the pure product was obtained through the silica gel column chromatography, using a mixture of ethyl acetate and petroleum ether (volume ratio 1:5) as the eluent. Finally, the solvent was removed through rotary evaporation and the product was dried in a vacuum to a constant weight (yield: 1.25 g, 62 %).

Synthesis of adamantane-modified doxorubicin (AD-DOX)

AD-CHO (0.20 g, 0.66 mmol) and DOX•HCl (0.30 g, 0.55 mmol) were dissolved in dimethyl sulfoxide (20 mL), and triethylamine (0.45 mL, 3.24 mmol) was added. The reaction continued in the dark at room temperature for 48 h. Most of solvent was removed through rotary evaporation and the crude product was washed with water and precipitated from ethyl acetate. The pure product was collected by centrifugation at 2000 rpm for 30 min and dried in a vacuum to a constant weight (yield: 0.38 g, 81 %).

Synthesis of β-cyclodextrin-modified polyaldehyde dextran (PAD-CD)

PAD (0.50 g, 4.34 mmol aldehyde group) and amino-CD (0.14 g, 0.12 mmol) were dissolved in 0.2 M pH 7.4 PBS. The mixture was stirred in the dark at room temperature for 48 h to obtain the PAD-CD solution. The solution was dialysed against water for 48 h and then freeze dried (yield: 0.55 g, 86 %).
**Synthesis of supramolecular polymeric prodrug (PAD-CD/AD-DOX)**

PAD (0.50 g, 4.34 mmol of aldehyde group) and amino-CD (0.14 g, 0.12 mmol) were dissolved in 0.2 M pH 7.4 PBS. AD-DOX (0.098 g, 0.12 mmol) was added under ultrasonic treatment and then the mixed solution was stirred for 48 h. The free AD-DOX was removed by centrifugation at 2000 rpm. The supernatant was filtered through 0.45 μm membrane filter to obtain the PAD-CD/AD-DOX solution. The solution was dialysed for 48 h against water and freeze dried (yield: 0.57 g, 88%).

**Preparation of blank hydrogels**

CMCTS (2.00 g, 8.21 mmol) and PAD-CD (2.00 g, 13.2 mmol) were separately dissolved in 0.01 M pH 7.4 PBS at a concentration of 4 wt%. The blank hydrogels were formed by mixing of CMCTS and PAD-CD solutions at a volume ratio of 8:5 at 37 °C.

**Preparation of DOX-loaded hydrogels**

CMCTS (2.00 g, 8.21 mmol of amino group) and PAD-CD/AD-DOX (2.00 g, 13.2 mmol of aldehyde group) were separately dissolved in 0.01 M pH 7.4 PBS at a concentration of 4 wt%. The DOX-loaded hydrogels were formed by mixing of CMCTS and PAD-CD/AD-DOX solutions at a volume ratio of 8:5 at 37 °C.

**Characterization**

Proton nuclear magnetic resonance (¹H NMR) spectroscopy was carried out using a Bruker Avance DMX500 NMR spectrometer (500 MHz) at room temperature with CDCl₃, D₂O or DMSO-d₆ as the solvent. Fourier transform infrared spectroscopy (FT-IR) was performed using a VECTOR 22 spectrometer. The amount of DOX was determined by using a Shimadzu UV2550
UV-vis spectrophotometer at the absorbance wavelength of 480 nm.

**Scanning electron microscopy (SEM)**

The morphologies of the hydrogels were examined using field emission scanning electron microscopy (FESEM, HITACHI, SU8010) operating at an acceleration voltage of 30 kV. The samples were frozen in liquid nitrogen, quickly cut and then lyophilized. The freeze-dried samples were mounted on aluminium stubs, using double-sided adhesive tape. Cryosection was employed to observe the hydrogel morphology after sputter-coating with an ultrathin layer of gold.

**Rheological experiments**

Mechanical behaviors of the hydrogels were characterized using a TA AR-G2 stress-controlled rheometer (TA Instruments, US), equipped with a parallel plate of 20 mm in diameter. Storage modulus (G’) and loss modulus (G’’) of hydrogels were monitored over frequency ranging from 0.5 to 50 rad/s. Frequency sweeping tests were performed at 37 °C and a given strain amplitude of 2 %.

**Swelling tests**

Swelling tests were performed by immersing the weighed freeze-dried hydrogels in 20 mL 0.01 M PBS at pH 5.8, 6.8 and 7.4 at 37°C in a water bath with horizontal shaking. All tests were conducted in triplicate and the corresponding PBS was refreshed everyday. At a predetermined time interval, the swollen hydrogels were weighed after removing the buffer on the surface. The swelling ratio (SR) was determined by the following equation:

\[ \text{SR} \ (%) = \left( \frac{W_s - W_d}{W_d} \right) \times 100\% \]

Where \( W_s \) is the weight of the swollen hydrogel and \( W_d \) is the weight of the freeze-dried hydrogel.

**In vitro DOX release**
Cylinder-shaped DOX-loaded hydrogels (0.65 mL, 4 wt-%) were immersed in 20 mL 0.01 M PBS at pH 5.8, 6.8 and 7.4 and 37 °C in a water bath with horizontal shaking. At a predetermined time interval, 4 mL incubated solution was removed and replenished with 4 mL corresponding fresh PBS. The amount of DOX released in the PBS was determined by measuring the UV absorbance of the solutions at 480 nm. All release measurements were conducted in triplicate.

**In vitro degradation**

Degradation experiments were implemented by placing blank hydrogels (0.65 mL, 4 wt-%) in 20 mL 0.01 M PBS at pH 5.8, 6.8 and 7.4 at 37°C in a water bath with horizontal shaking. All experiments were performed in triplicate and the corresponding PBS was refreshed everyday. At regular time intervals, the samples were taken out, rinsed with distilled water and then lyophilized. Weight loss was calculated according to the following formula:

\[
\text{Weight loss(%) = } \frac{(W_i - W_r)}{W_i} \times 100\%
\]

Where \(W_i\) is the weight of the initial freeze-dried hydrogel and \(W_r\) is the weight of the residual freeze-dried hydrogel.

**In vitro cytotoxicity**

The in vitro cytotoxicity of blank and DOX-loaded hydrogels were evaluated against HeLa cells (human breast cancer, obtained from Meihua Sui Group) by MTT (Nybio, Japan) assay. All the tests were conducted five times. Free DOX•HCl (300 µg/mL) was dissolved in 0.01 M pH 7.4 PBS and sterilized through 0.22 µm membrane. The cells were pre-incubated in a 24-well plate (6 \(\times\) 10^4 cells per well) with a culture medium of 10 % FBS/α-MEM (Invitrogen Co., Carlsbad, CA) in a humidified 5 % CO₂-containing atmosphere at 37 °C for 24 h. The culture medium was then replaced with fresh medium. Then the cells were further incubated with 100 µL free DOX•HCl,
blank and DOX-loaded hydrogels for 24 h, 48h and 72h, respectively. Non-treated cells were used as negative controls, wells without cells but culture medium were used as the blank. Subsequently, 100 μL MTT reagents were added into each well, and the cells were incubated in the dark for another 4 h. The absorbance of the medium was measured at 570 nm using a microplate reader (Sunrise™ Basic; TECAN, Zurich, Switzerland). Meanwhile, the morphologies of the cells incubated with free DOX, blank and DOX-loaded hydrogels for 24 h, 48 h and 72 h were observed by phase-contrast microscopy (OLYMPUS, CKX41).

**Fig. S1.** $^1$H NMR spectra of (A) AD-CHO in CDCl$_3$ and (B) AD-DOX in DMSO-d$_6$. 
**Fig. S2.** $^1$H NMR spectra of (A) Tysol-CD in DMSO-$d_6$ and (B) Amino-CD in D$_2$O.

**Fig. S3.** $^1$H NMR spectra of (A) dextran in D$_2$O, (B) PAD in DMSO-$d_6$ and (C) PAD-CD in D$_2$O.
Fig. S4. FT-IR spectra of (A) dextran, (B) PAD, (C) PAD-CD.

Fig. S5. $^1$H NMR spectrum of PAD-CD/AD-DOX in D$_2$O.
Fig. S6. FT-IR spectra of (A) CMCTS, (B) PAD-CD/AD-DOX, (C) DOX-loaded hydrogel.

Fig. S7. Swelling curves of the lyophilized hydrogels at pH 5.8, 6.8 and 7.4.
**Fig. S8.** Phase-contrast microscopy images of Hela cells incubated with free DOX, blank and DOX-loaded hydrogels for 24 h, 48 h and 72 h, scale bars are 100 μm for all images.

**Fig. S9.** Standard curves of DOX in 0.01 M pH 3.0 citrate buffer solution (A), 0.01 M pH 5.8 PBS (B), 0.01 M pH 6.8 PBS (C) and 0.01 M pH 7.4 PBS (D).

**References**