Electronic Supplementary Information (ESI)

Biodegradable pH-Responsive Hydrogels for Controlled Dual-Drug

Release

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Methods

FTIR analysis of Polymers:

FTIR of PLLA-PEG-PLLA and MA-PLLA-PEG-PLLA-MA (Fig. S1). Specific peaks: 1741 cm⁻¹ (C=O of ester), 1110 cm⁻¹ (ether group, C-O-C), 1635 cm⁻¹ (C=C of methacrylates).

The gel content and yield of hydrogels

The gel content and yield were analyzed gravimetrically (Fig. S3). The dried hydrogel at constant weight (W_c) was immersed in deionized water for 48 hours in order to removal of the unreacted monomers. The hydrogel was then dried in a vacuum oven at 50 °C until constant weight (W_f). The gel content yield of the hydrogels was calculated by using following equations:

$$Gel\% = \frac{W_f}{W_c} \times 100$$

Yield\% = $\frac{W_c}{W_c} \times 100$

Where W_i is the total mass of the monomers of the MA-PLLA-PEG-PLLA-MA, AA, NIPAM and photo-initiator I_{2959.}

Drug loading and encapsulation efficiency of hydrogels

The concentration of the drug solution was obtained by using the standard drug concentration curves (Fig. S5A-D). Therefore the drug loading efficiencies of DOX and TET were calculated. Each polymer was analyzed with three repeats. Drug loading was determined by calculating the difference of the drug weight dissolved in the drug solutions before and after immersing the hydrogels over the weight of dried hydrogels. The experiments were repeated three times and the average values were obtained.

Encapsulation efficiency was calculated according to the following equation:

Encapsulation efficiency (%) =
$$\frac{\text{Drug weight loaded in a hydrogel sample}}{\text{Drug weight in the solution before drug loading}}$$

The equation for the calculation of the swelling ratios of hydrogels:

$$SR = \frac{Q_t}{Q_0} \times 100\%$$

Where Q_t is the weight of the swelling hydrogels at time point t in a buffer, Q_0 is the weight of the dry hydrogel before the swelling experiment.

Hydrogel degradation calculation:

Weight Loss (%) =
$$\frac{W_0 - W_t}{W_0} \times 100\%$$

Where W_t was the weight of a hydrogel at the time t during the degradation and W_0 was the initial weight of the dry hydrogel.

Calculation of in vitro dual drug release:

$Q = C_n V_n + \sum_{n-1}^{i=1} C_i \ V_i$

Where Q was cumulative molar amount of the released drug, C_n was the concentration of TET or DOX in the buffer at time t, V_n was the volume of the buffer in the drug release experiment (8 mL/vial), and V_i was the volume of the collected buffer from each vial (4 mL/time point/vial).

Cell culture

Human adipose-derived stem cells (hADSCs) were isolated and purified in the lab following a literature procedure.[1] In detail, hADSCs were isolated from the armpit fat of an 18 year old female patient after plastic surgery in the Affiliated Second People's Hospital of Changzhou. The fat tissue (about 30 g) was cut into small pieces and washed for three times with PBS (50 mL) to remove blood (1500 rpm, 5 min). The cleaned fat tissue was then suspended in collagenase I solution in PBS (0.1 wt%) and incubated in 37 °C for 60 min. The fat suspension was then filtered through 100 and 200 mesh cloths respectively before centrifugation (1500 rpm, 5 min). The pellet was re-suspended in erythrocyte lysis buffer (20 mL) for 10 min. After centrifugation (1500 rpm, 5 min), the pellet was re-suspended and cultured in a tissue culture flask (25 mL) in Dulbecco's Modified Eagle's medium (DMEM) supplemented with fetal bovine serum (10%), antibiotics (penicillin and streptomycin, 100 units per mL), bFGF (1 ng/100 mL), NaHCO₃ (0.044 M) and tetracycline (0.3 wt%) in an incubator with 5% CO₂ at 37 °C. Three days later the medium was refreshed. The medium was refreshed every 2 to 3 days depending on the growth of cells. The cells were passaged until 90% confluence and then re-seeded into fresh tissue culture plates in a 1 to 3 split, with the cells passaged 3 times before use.

HeLa cells were cultured in low glucose DMEM supplemented with fetal bovine serum (10%), antibiotics (penicillin and streptomycin, 100 units per mL) in an incubator with 5% CO₂ at 37 °C. The medium was refreshed every 2 to 3 days depending on the growth of cells. The cells were harvested for experiments when 90% confluent.

Cytotoxicity of polymers

The cell culture medium was complemented with DMEM containing 0, 10, 20, 50 and 100% of the degradation supernatant of the hydrogels (soaked in DMEM for 24 h). It was found that the media was poorly toxic when used for hADSCs cell culture (Fig. S8).

In vitro HeLa cell killing and antibacterial activity

DOX and TET were used as drug models for testing the controlled drug release from the hydrogels. Dual-drug loaded hydrogels were sterilized under UV illumination for 30 min. 100 mg of hydrogel was socked in 4 mL of Dulbecco's Eagle Medium (DMEM, ThermoFisher Scientific) supplemented with 10 % FBS (NQBB) and 1 % penicillin/streptavidin (Sigma-Aldrich) in a 48-well plate before incubation at 37 °C. The supernatant from each well was collected at 1 h and 12 h and mixed with freshly complemented DMEM containing 0, 10, 20 and 50% v/v of the collected supernatant and subsequent culturing of cells in a 48-well plate for 24 h (2×10⁴ cells per well). Then the medium were removed from the plate and replaced with 200 µL DMEM containing 20 µL CCK-8 (TJBiolite). The well plates were placed into an incubator for 2 h at 37 °C before analysis with a microplate reader (Epoch) at 450 nm. Cell viability was calculated using the following equation:

Cell Viability % =
$$\frac{A_{\text{sample}} - A_{\text{blank}}}{A_0 - A_{\text{blank}}} \times 100\%$$

Where the A_{sample} was the UV absorption of the wells with cells cultured in DMEM containing the supernatant of the degraded hydrogel, A_{blank} was the absorption of the corresponding DMEM without cells, A₀ was the absorption of the wells with cells cultured in pure DMEM.

Doxorubicin activity was evaluated by measuring the viability of HeLa cells incubated in DMEM containing the released drugs from the hydrogels after incubation with the dual-drug loaded hydrogels for 1 or 12 hours respectively at 37 °C. HeLa cell viability was lowered to 80% when the media was supplemented with 10% of the

solution from the drug-released supernatant (after 1 h of release). Cell viability decreased to 56% if the culture medium was supplemented with 50% of the drug released supernatant (after 12 h release) (Fig. S9).

Antimicrobial activity of the loaded hydrogels

To assess the antibacterial capability of the hydrogels loaded with TET antibacterial activity against *E. coli* was tested using the inhibition zone method.[2] Five dual-drug loaded hydrogel samples were examined with the corresponding blank hydrogels without drugs were used as the negative controls. The diameters of the inhibition zones were measured and calculated (Fig. S10).

Physical properties of the hydrogels

Compression test: The compressive modulus of the hydrogels was measured using a WDT-10 electronic universal testing machine with a constant preload force of 0.01 N and a force ramp of 0.5 N/min to 18 N.

Rheological test: Oscillatory rheological characterization of the hydrogels were performed on an MCR301 rheometer equipped with a Peltier plate. Strain sweeps were performed on samples from 0.1% to a maximum strain of 1000% to determine the linear viscoelastic region. Dynamic oscillatory frequency sweeps were conducted from 0.1 to 100 rad/s with a constant strain amplitude of 1%.

The compression modulus of hydrogels was measured at room temperature. In this study, all the cylindrical hydrogels (10 mm in diameter and 10 mm in height) were soaked in PBS (pH 7.4) for 48 hours before testing. During the measurement, the preloaded force was set at 0.01 N with an increasing rate of 0.5 N/min until reaching 50 N. Analysis showed that the hydrogels S1, S2 and S3 all recovered to their original sizes after removing the pressure due to higher crosslinking density (Fig. S11A), but S4 and S5 were destroyed during the measurements with bigger strain changes (67% and 57%) than others (~20%). The slopes of the curves showed that the compression modulus of the hydrogels were ascending in S1>S2>S3>>S4>S5, with S3 some 7 times stronger than S4 or S5 (Fig. S11B). It shows that the larger the molecular weight of the cross-linker, the softer the hydrogels (in pH 7.4 buffer), and also presented greater shrinkage when the pH of the buffers was reduced from 7.4 to 1.2.

The storage moduli (G') and loss moduli (G'') of hydrogels were measured as the function of frequency (Fig. S11C). The results show that the storage moduli were almost consistent during the increasing of the frequency from 0.1 to 10 rad/s. All of the storage moduli was larger than corresponding loss moduli indicating the formation of the stable cross-linked networks of hydrogels.[3]

Data analysis

The data are expressed as mean and standard error of the population. Two-group comparisons were performed using a student's T-test and two-tailed p values < 0.05 were considered statistically significant.



Fig. S1 FTIR spectra of PEG, PLLA-b-PEG-b-PLLA (PLEA) and MA-PLLA-b-PEG-b-PLLA-MA (PLEA-MA) macro cross-linkers.



Fig.S2 GPC spectrum of PLLA-b-PEG-b-PLLA (PLEA) and MA-PLLA-PEG-PLLA-MA (PLEA-MA) (1 mg/mL) eluting with THF with a flow rate of 1 mL/min.



Fig. S3 Gel content and yield of the hydrogels (S1-5). Errors are STDEV and n=3.



Fig. S4 LCST analysis of hydrogels. (A) Transmittance of hydrogels at pH1.2 at various temperatures ranging from 25 to 40 °C; (B) Transmittance of hydrogels at pH7.4 at various temperatures ranging from 25 to 40 °C. The hydrogels were immersed in buffers until equilibrium was reached and cut to size and put into the quartz cuvette with buffer for measurement. The quartz cuvettes with hydrogel samples were warmed up before scanning on a UV/Vis spectrometer.



Fig. S5 The standard drug concentration curves in PBS (pH 7.4) and acidified PBS (pH1.2). A: TET at pH1.2; B: TET at pH7.4; C: DOX at pH1.2 and D: DOX at pH7.4.



Fig. S6 The UV absorption curves of the drug tetracycline hydrochloride (black line), doxorubicin (red line), and the hydrogel loaded with both drugs (blue line).



Fig. S7 Optical images of hydrogel S4 under different conditions: (1) Dried hydrogel; (2) The hydrogel after drug release at pH1.2 (most of the Dox is retained in the hydrogel); (3) The hydrogel after drug release at pH7.4; (4) The dried hydrogel with the loaded drug (Dox). The insert numbers are the diameter of the hydrogel showing swelling at different pH's.



Fig. S8 Cytotoxicity analyses of hADSCs cultured in DMEM containing 0, 10, 20, 50 and 100% hydrogel (S4) of supernatant (this stock supernatant solution was prepared by incubating for 24 h 100 mg of the hydrogel in 4 mL of DMEM). hADSCs were cultured in DMEM media (the desired volume of hydrogel stock DMEM) on PS tissue culture well plates for 24 hours before the CCK8 test (Student's T-test, all concentrations were compared to 10%, p>0.2).

(A)



Drug concentration in the supernatant of DMEM incubated with drug-loaded hydrogels (mg/mL (μ M)). The concentrations of Doxorubicin used in the cell cultures (above) were 10%, 20% and 50% of these values

Incubation time	DOX		TET	
(h)	1	12	1	12
S 1	0.0064 (12µM)	0.032 (58µM)	0.026(58µM)	0.051 (115µM)
S2	0.0087 (15µM)	0.037 (67µM)	0.039(88µM)	0.068(153µM)
S 3	0.013 (23µM)	0.038 (69µM)	0.041(92µM)	0.056 (126µM)
S4	0.016 (29µM)	0.051 (93µM)	0.057(128µM)	0.081 (182µM)
S5	0.023 (42µM)	0.062 (113µM)	0.05 (113µM)	0.081 (182µM)

(C)



Fig. S9 The released drug supernatants were collected following incubation of the drug-loaded hydrogels (100 mg) in DMEM (4 mL) for 1 and 12 h. In cell culture this supernatant was used at 10%, 20% and 50% v/v.

(A) HeLa cell viability when cultured in DMEM with the hydrogel-drug-supernatants.

(B) The levels of released drug (Dox) in the table were calculated by analysis of the drug release profiles. If 100% of the drug were released this would equate to 0.4 mg of drug in 4 mL (0.1 mg/mL or 184 μ M Dox). This is then diluted into the cell culture media (1 in 10; 1 in 5 and 1 in 1).

(C) HeLa cell viability when cultured in DMEM with varying concentrations of DOX. The control (0%) is cells cultured in DMEM without any drug. The culture time was 24 hours after the addition of DMEM+Drug. The error is STDEV and n=3.

(B)



(B)

Sample No.	Diameters of inhibition zones		
	(mm)		
S1	23±0.4		
S2	22±0.3		
S 3	25±0.5		
S 4	33±0.8		
S5	35±0.7		

(C)



Fig. S10

(A) Images of inhibition zones of various hydrogels loaded with DOX and TET placed on agar gels with *E. coli* cultured in an incubator at 37°C at 75% humidity for 24 h. S1-5 are hydrogel samples with loaded drugs and S1'-5' the equivalent hydrogel without drug loading.

(B) Table of the diameters of the *E. coli* inhibition zones on agar plates with dual-drug loaded hydrogels. Note: The diameter of the inhibition zones of the controls were all zero.

(C) HeLa cell viability when cultured in DMEM with various percentages of Tetracycline. The controls (0%) were cells cultured in DMEM without any drug. The culture time was 24 hours after the addition of DMEM+Drug (n=3). As expected HeLa cells were viable in the presence of tetracycline.



Fig. S11 Mechanical properties of the hydrogels S1-S5. (A) Compression modulus analysis by sweeping the applied forces on hydrogels from 0.01 to 50 N and then removing the force at the same sweeping rate; (B) The compression modulus of hydrogels at a fixed strain of 20%; (C) Rheological analysis of hydrogels using an oscillatory frequency sweep of the hydrogels from 0.01 to 100 rad/s in 1% strain amplitude.

References

[1] P. A. Zuk, M. Zhu, P. Ashjian, D. A. DeUgarte, J. I. Huang, H. Mizuno, Z. C. Alfonso, J. K. Fraser, P. Benhaim and M. H. Hedrick, *Mol. Biol. Cell*, 2002, **13**, 4279-4295.

[2] X. P. Feng, G. R. Wang, K. Neumann, W. Yao, L. Ding, S. Y. Li, Y. Sheng, Y. Jiang, M. Bradley, R. Zhang, *Mater. Sci. Eng. C, Mater. Biolog. Appl.*, 2017, **74**, 270-278.

[3] J. Raeburn, G. Pont and L. Chen, Soft Matter, 2012, 8, 1168-1174.