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Supporting Information

Fabrication of an injectable hydrogel with photothermal effects from tannic acid for local drug delivery and synergistic photothermal-chemotherapy

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Contents

1. Materials and methods

TA and DOX·HCl were obtained from Aladdin Reagents (Shanghai, China). 4-arm-PEG-SH (20 kDa) was purchased from Huateng Pharma (Hunan, China). $FeCl_3 \cdot 6H_2O$ was provided by Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Tyrosinase was obtained from Hefei Bomei Biotechnology Co., Ltd. DMEM was purchased from Gibco (USA). Fetal bovine serum (FBS) was provided by Bioind (ISR). Penicillin sodium and streptomycin were purchased from Dalian Meilun biological Co., Ltd. CCK-8 kit and apoptosis assays kit were obtained from MultiSciences (Lianke) Biotech Co., Ltd. (Hangzhou, China). Live/Dead cell staining kit was provided by Life Technologies Co. (USA). Fourier transform infrared spectroscopy (FTIR) characterizations were performed on the Bruker Vertex 70+Hyperion 2000 (Germany). Rheological tests were carried out on the Thermo Scientific HAAKE RheoStress 6000 rheometer (USA). Scanning electron micrograph (SEM) images characterizations were recorded on the Hitachi SU8010 (Japan). Raman spectroscopy characterizations were conducted on the JOBIN YVON HR800 (France). UV-vis spectroscopy characterizations were taken on the Shimadzu UV1900i (Japan). 808 nm laser (MDL-H-808 nm-5W-BH80521) was purchased from the Changchun Laser Optoelectronics Technology Co. Ltd (China). The temperature changes were recorded by an infrared camera (Fotric 225s, China).

2. Experimental section

2.1 Gelation tests of the TP, TPF and DOX@TPF hydrogels

TA was dissolved in distilled water at the concentration of 2 wt%. The solutions containing different concentrations of 4-arm-PEG-SH were obtained by diluting it's stock solution (10 wt%) with deionized water. After transferring proper volumes of TA to the 4-arm-PEG-SH solution, followed by the addition of tyrosinase, mixed samples containing varied concentrations of 4-arm-PEG-SH (1.9, 2.4, 2.8, 3.3, 3.5, 3.8, 4.7 and 5.9 wt%), TA (0.2, 0.3, 0.4, and 0.5 wt%) and tyrosinase (0, 10, 50, 100, 200, 300, 500 U/mL) were obtained. The sample was then stored at room temperature for gelation, and their gelation time was determined by a tube-inversion method. When the tube was inverted and the sample contained did not fall down within 30s, the hydrogel was considered formed.

The TPF gels were obtained by adding designed volumes of $FeCl_3$ solution (Fe^{3+}/TA feeding ratios = 0, 0.33:1, 0.67:1, 0.8:1, 1:1 and 1.2:1) into the aqueous solution containing TA (0.2 wt%), 4-arm-PEG-SH (2.4 wt%), and tyrosinase (10 U/mL). The time of gelation was determined by tube-inversion method.

The DOX@TPF hydrogels were prepared by adding different amounts of DOX·HCl into the solution of TPF (TA = 0.2wt%, 4-arm-PEG-SH = 2.4 wt%, Fe^{3+}/TA feeding ratio = 0.8:1, tyrosinase = 10 U/mL). The different amounts of DOX·HCl encapsulated in the TPF gel were 0.2, 1, 2, 10, 20 and 400 μg, respectively. The same gelation process was performed as that of the TPF gel.

2.2 FTIR characterization

The samples of FTIR spectra were prepared by using deuterium oxide (D_2O) as a solvent, and loaded into KBr cuvettes. All spectra were scanned in the range of 4000-400 cm^{-1} with an average of 64 scans at a resolution of 2 cm^{-1} on the Bruker Vertex 70+Hyperion 2000 (Germany).

2.3 SEM characterization

For SEM characterization, the lyophilized gels were cut into thin slices and loaded onto a silicon wafer, following by being coated with a thin gold layer via vacuum-drying before being visualized on the Hitachi SU8010 (Japan).

2.4 Rheological tests

The rheological tests of TP, TPF and DOX@TPF hydrogels were carried out on the Thermo Scientific HAAKE RheoStress 6000 at 25 °C by placing 300 μL of hydrogels on a parallel plate of 20 mm dimeter. (1) Dynamic strain sweep tests were implemented from 0.01 to 2000% strain at a fixed angular frequency of 1 Hz. (2) The dynamic frequency sweep measurements were conducted from 0.1 to 10 Hz with a fixed strain at 1%. (3) Alternate-step sweep tests were performed at a fixed angular frequency (1 Hz) from a small strain (γ = 1%) to a large strain (γ = 2000%) with 100 s for every strain interval.

2.5 Raman spectrum characterization

Raman spectra were measured by using a Raman microscope (JOBIN YVON HR800, France) with a 785 nm laser light as excitation source, and the laser power was set at 25 mW. All spectra of lyophilized samples were scanned in the range of 2000-300 cm^{-1} with a spectral resolution of 1 cm^{-1}.

2.6 Photothermal properties of the TPF hydrogel

To assess the photothermal performance of the prepared TPF gel, 200 μL of TPF gels containing different Fe^{3+} concentrations (Fe^{3+}/TA feeding ratios = 0, 0.33:1, 0.67:1, 0.8:1, 1:1 and 1.2:1) were firstly placed in a 48-well plate. The samples were then irradiated using an 808 nm NIR laser at the power density of 2 W/cm^2 for 5 min and the temperatures changes were monitored by an infrared camera (Fotric 225s, China).

The photothermal conversion efficiency (η) of the TPF hydrogel was measured by following previously reported methods. The sample (TA = 0.2 wt%, 4-arm-PEG-SH = 2.4 wt%, Fe^{3+}/TA feeding ratio = 0.8:1) was irradiated to a steady maximum temperature with 808 nm laser at 2 W/cm^2, and then was allowed to cool down to room temperature. Where η was calculated according to the following equations:

$$\eta = \frac{hS\left(T_{max} - T_{max,water}\right)}{I\left(1 - 10^{-A_\lambda}\right)} \quad (1)$$

where h means heat transfer coefficient, S represents the surface area of the container, T_{max} refers to the maximum steady-state temperature, I represents the power density of laser, and A_λ means the absorbance of the sample at 808 nm.

The value of hS was determined according to the rate of temperature drop when NIR was switched off. Thus hS can be obtained as following:

$$hS = \frac{mC_p}{\tau_s} \quad (2)$$

where m and C_p are the mass and heat capacity of solvent, τ_s is the time constant of heat transfer and determined by applying the linear time data from the cooling period versus -lnθ:

$$\tau_s = \frac{t}{-\ln\theta} \quad (3)$$

where t is the time of cooling process and θ is defined as the ratio of $\triangle T$ to $\triangle T_{max}$:

$$\theta = \frac{\triangle T}{\triangle T_{max}} \quad (4)$$

where $\triangle T$ is defined as $T - T_{surr}$ (T and T_{surr} are the sample temperature and ambient temperature, respectively). $\triangle T_{max}$ is calculated by $T_{max} - T_{surr}$.

2.7 Photothermal stability of the TPF hydrogel

The photothermal stability of the TPF hydrogel was characterized by on–off cycles of NIR laser irradiation. The TPF hydrogel (TA = 0.2 wt%, 4-arm-PEG-SH = 2.4 wt%, Fe^{3+}/TA feeding ratio = 0.8:1) was exposed to 808 nm laser irradiation (2 W/cm^2) for 5 min, followed by naturally cooling down to room temperature without irradiation for 7 min. The temperature changes were recorded for three on–off cycles.

2.8 Degradation in vitro

The degradation properties of the DOX@TP and DOX@TPF hydrogels were evaluated by monitoring the change in mass with time. Hydrogels were immersed in 5 mL PBS buffers (pH = 7.4 and 6.5) and incubated at 37 °C. At predetermined time intervals, they were removed from PBS buffers, washed with distilled water, freeze-dried and weighed. The degree of degradation rate (DR) was calculated based on the following formulas:

$$DR = \frac{W_0 - W_t}{W_0} \times 100\%$$

where W_0 is the weight of the hydrogel samples at 0 h, and W_t is the weight of the hydrogel samples at time t. For each test, three parallel samples were measured and an average result was displayed.

2.9 Drug release in vitro

200 μL of DOX@TP (TA = 0.2 wt%, 4-arm-PEG-SH = 2.4 wt%, DOX·HCl = 2 mg/mL) and DOX@TPF (TA = 0.2 wt%, 4-arm-PEG-SH = 2.4 wt%, Fe^{3+}/TA feeding ratio = 0.8:1, DOX·HCl = 2 mg/mL) hydrogels were immersed in 2 mL PBS of varying pH values (7.4 and 6.5) to perform controlled drug release in an orbital shaker (100 rpm, 37 °C). At predetermined time intervals, 300 μL of solution was taken out for quantification of DOX·HCl by using the UV-vis spectrophotometer (Shimadzu UV1900i, Japan) at a wavelength of 480 nm, and an equal volume of fresh dissolution medium PBS was added to maintain a constant volume.

2.10 In vitro cytotoxicity.

The DOX@TP (TA = 0.2wt%, 4-arm-PEG-SH = 2.4 wt%) and DOX@TPF (TA = 0.2 wt%, 4-arm-PEG-SH = 2.4 wt%, Fe^{3+}/TA feeding ratio = 0.8:1) hydrogels were placed in a 96-well plate and were washed with PBS for three times prior to cell seeding. The doses of DOX within gels in the 96-well plate were set as 0, 0.01, 0.05, 0.1, 0.5, and 1 μg. A549 cells were placed at a density of 3×10^3 cells per well onto the hydrogels and were co-incubated for 48 h. After that, the

supernatant was taken out and 10% CCK-8 solution was added for another 1 h at 37 ° C before the optical absorbance was recorded using a microplate reader (MD, SpectraMax Plus 384, USA) at 450 nm and 650 nm in order to quantify the viability of cells.

$$\text{cell viability}\ (\%) = \frac{OD_{450}\text{sample} - OD_{650}\text{sample}}{OD_{450}\text{control} - OD_{650}\text{control}} \times 100\%$$

2.11 In vitro photothermal-chemotherapy

The sample from different groups (PBS, TPF, DOX@TPF, TPF+NIR, DOX@TPF+NIR) was placed in a 96-well cell culture plate and the A549 cells were seeded onto the hydrogels at a density of 3×10^3 cells per well. After 4 h of incubation, the groups of TPF+NIR and DOX@TPF+NIR were exposed to 808 nm laser irradiation at a density of 2 W/cm^2 for 5 min. The cell viability was tested by CCK-8 assay after the cells were cultured for 48 h.

2.12 Live/dead assay

A549 cells at a density of 5×10^4 cells per well were subjected to treatment by the sample from different groups (PBS, TPF, DOX@TPF, TPF+NIR, DOX@TPF+NIR). After 48 h incubation, cell culture medium was removed and cells were rinsed with PBS for three times. Then 100 μL of staining solution (containing 2 μM Calcein AM and 4 μM PI) was added to each well, and the mixture was incubated at 37 ^{o}C for 15 min before the photos were taken under a fluorescence microscope (Olympus IX71, USA).

2.13 Flow cytometry analysis

Apoptosis of A549 cells were evaluated using a dead cell apoptosis kit containing Annexin V-FITC and PI. The sample from different groups (PBS, TPF, DOX@TPF, TPF+NIR, DOX@TPF+NIR) was placed in a 24-well cell culture plate. A549 cells were seeded onto the surface of the hydrogels at a density of 5×10^4 cells per well. The groups of TPF+NIR and DOX@TPF+NIR were exposed to 808 nm laser irradiation at a density of 2.0 W/cm^2 for 5 min after 4 h of incubation. After 48 hours, cells were trypsinized and resuspended in PBS twice. Finally, cells were co-stained with Annexin V-FITC and PI for 15 min following the protocol from the supplier before flow cytometry analysis (Becton, Dickinson and Company ACCURI C6, USA).

2.14 In vivo inhibition of tumor growth

BALB/c mice (4 weeks, male) were purchased from Shanghai SLAC Laboratory Animal Co.

LTD. (China). All animal experiments were performed according to the protocol approved by the Institutional Animal Care and Use Committee (IACUC) Tianjin Hospital. To establish the in vivo tumor model, 5×10^6 A549 cells suspended in 100 μL 1×PBS were subcutaneously inoculated into the lateral axilla of each BALB/c mouse after anesthetization with isoflurane. When the tumors had grown to a mean volume of around 100 mm^3, the mice were randomly divided into five groups (n = 5 in each group) and intratumorally injected with 20 μL of sample (PBS, TPF, DOX@TPF, TPF+NIR and DOX@TPF+NIR). The dose of DOX·HCl was 3 mg/kg, and the concentrations of TA and 4-arm-PEG-SH were 0.2 wt% and 2.4 wt%, respectively, Fe^{3+}/TA feeding ratio = 0.8:1. After 2 h post intratumoral injection, the mice in the TPF+NIR and DOX@TPF+NIR groups were further irradiated by NIR light (808 nm, 2 W/cm^2, 5 min) every 2 days during the treatment process of 14 days. The temperature change of the tumor site during irradiation was recorded using a thermal imaging camera. The body weight and tumor size of each mouse were recorded every other day.

2.15 Histological analysis

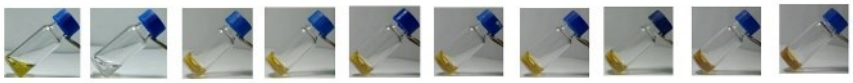
After 14-day treatment, major organs including heart, liver, spleen, kidney and lung were excised from the sacrificed mice, fixed in 4% paraformaldehyde aqueous solution overnight, embedded in paraffin and sectioned into slices with a thickness of 4 μm. The as-obtained tissue sections were analyzed by an optical microscope after treatment with hematoxylin and eosin (H&E) staining.

2.16 Statistical analysis

All the tests were performed in triplicate at least. The data were presented as the mean ± standard deviation. Statistical analyses were performed on Student's t-test (*P<0.05, ** P<0.01 and *** P<0.001).

3.Gelation tests of the TP and TPF gels

Table S1 Summary of the conditions and properties of TP hydrogels with varied amounts of TA and 4-arm-PEG-SH

Sample	Sol 1	Sol 2	Gel 1	Gel 2	Gel 3	Gel 4	Gel 5	Gel 6	Gel 7	Gel 8
TA (wt%)	1	0	0.2	0.2	0.2	0.2	0.2	0.3	0.4	0.5
PEG (wt%)	0	1	1.9	2.4	2.8	3.3	3.8	3.5	4.7	5.9
TA : PEG molar ratio	-	-	1:0.8	1:1	1:1.2	1:1.4	1:1.6	1:1	1:1	1:1
-OH : -SH molar ratio	-	-	1:0.13	1:0.16	1:0.19	1:0.22	1:0.26	1:0.16	1:0.16	1:0.16
pH	7.4	7.4	7.4	7.4	7.4	7.4	7.4	7.4	7.4	7.4
Gel images										
Gelation time (min)	-	- [a]	10.5	8	5.6	3.5	1.2	5.5	3.4	1.1

[a] The gelation process did not occur in 48h

Table S2 Summary of the conditions and properties of TP hydrogels triggered by tyrosinase

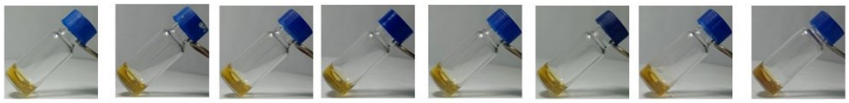
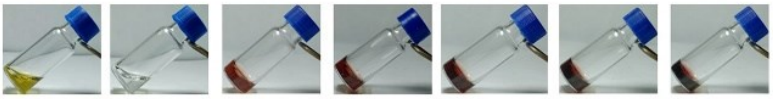
Sample	Gel 2	Gel 9	Gel 10	Gel 11	Gel 12	Gel 13	Gel 14	Gel 15
TA (wt%)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
PEG (wt%)	2.4	2.4	2.4	2.4	2.4	2.4	2.4	2.4
Tyrosinase (U/mL)	0	5	10	50	100	200	300	500
pH	7.4	7.4	7.4	7.4	7.4	7.4	7.4	7.4
Gel images								
Gelation time (min)	8	7.2	6.7	5.3	3.4	2.5	1.6	0.8

Table S3 Summary of the conditions and properties of TPF hydrogels

Sample	Sol 1	Sol 2	Gel 16	Gel 17	Gel 18	Gel 19	Gel 20
TA (wt%)	1	0	0.2	0.2	0.2	0.2	0.2
PEG (wt%)	0	1	2.4	2.4	2.4	2.4	2.4
TA : PEG molar ratio	-	-	1:1	1:1	1:1	1:1	1:1
Tyrosinase (U/mL)	10	10	10	10	10	10	10
TA : Fe^{3+} molar ratio	-	-	1:0.33	1:0.67	1:0.8	1:1	1:1.2
pH	7.4	7.4	7.4	7.4	7.4	7.4	7.4
Gel images							
Gelation time (min)	-	- [a]	5.3	4	3.5	2.5	1.8

[a] The gelation process did not occur in 48h

4. Rheological analysis of TP gels

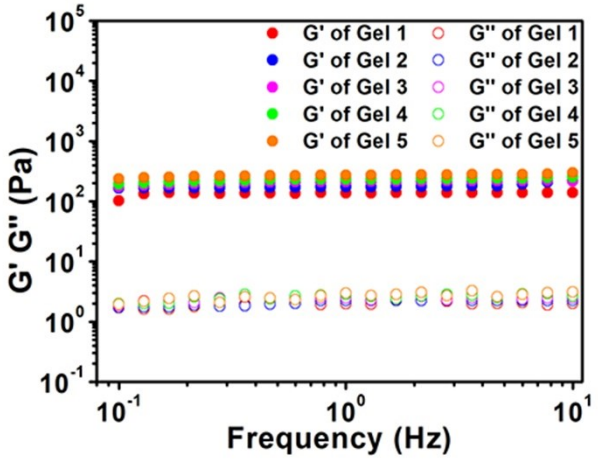


Fig. S1 Frequency dependence of the dynamic storage moduli (G') and the loss moduli (G'') of the TP gel containing different concentrations of 4-arm-PEG-SH (1.9, 2.4, 2.8, 3.3, and 3.8 wt%).

5. The injectability of the TP gel

Fig. S2 Gel patterns formed by the injectable TP hydrogel containing rhodamine B

6. Photothermal conversion efficiency of the TPF gel

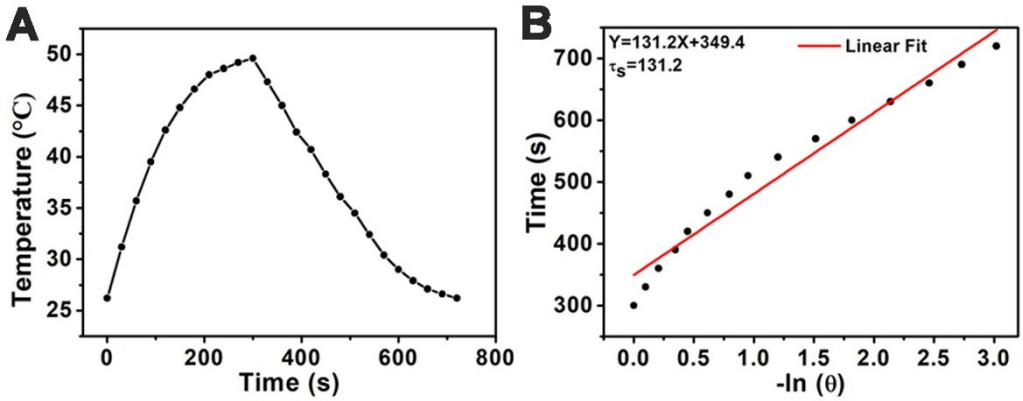


Fig. S3 (A) Photothermal heating curve of the TPF gel (TA = 0.2 wt%, 4-arm-PEG-SH = 2.4 wt%, Fe^{3+}/TA feeding ratio = 0.8:1) under the irradiation with 808 nm laser (2 W/cm^2) for 5 min and the cooling curve after the laser was switched off for 7 min. (B) The linear relationship between the cooling period and -lnθ.

7. A standard curve of DOX·HCl solutions

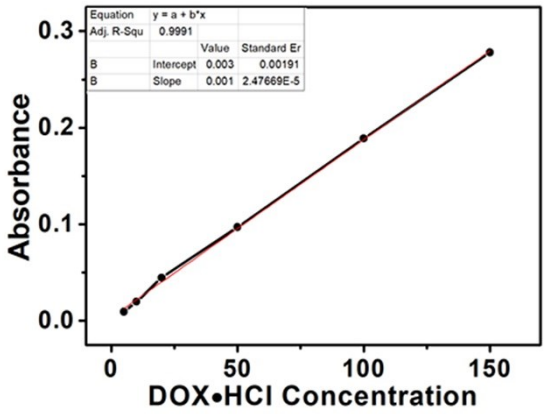


Fig. S4 A standard curve of the solutions containing different concentrations of DOX·HCl (5, 10, 20, 50, 100, and 150 μg/mL).

8. SEM images of the TPF and DOX@TPF gels

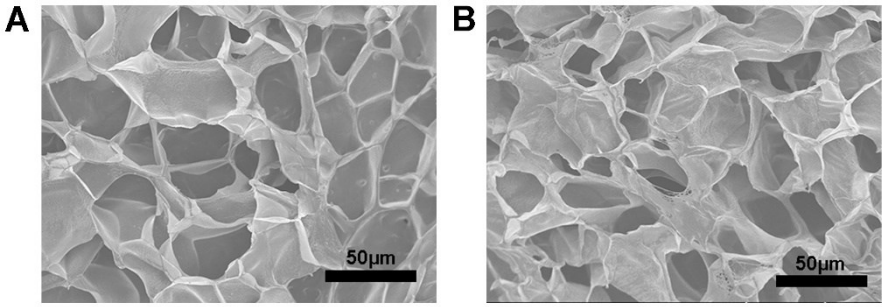


Fig. S5 SEM images of (A) the TPF hydrogel (TA = 0.2 wt%, 4-arm-PEG-SH = 2.4 wt%, molar ratio TA/Fe^{3+} = 1:0.8) and (B) the DOX@TPF hydrogel (TA = 0.2 wt%, 4-arm-PEG-SH = 2.4 wt%, molar ratio TA/Fe^{3+} = 1:0.8, DOX·HCl = 2 mg/mL). Scale bars: 50 μm.

9. Rheological measurements of the TPF and DOX@TPF gels

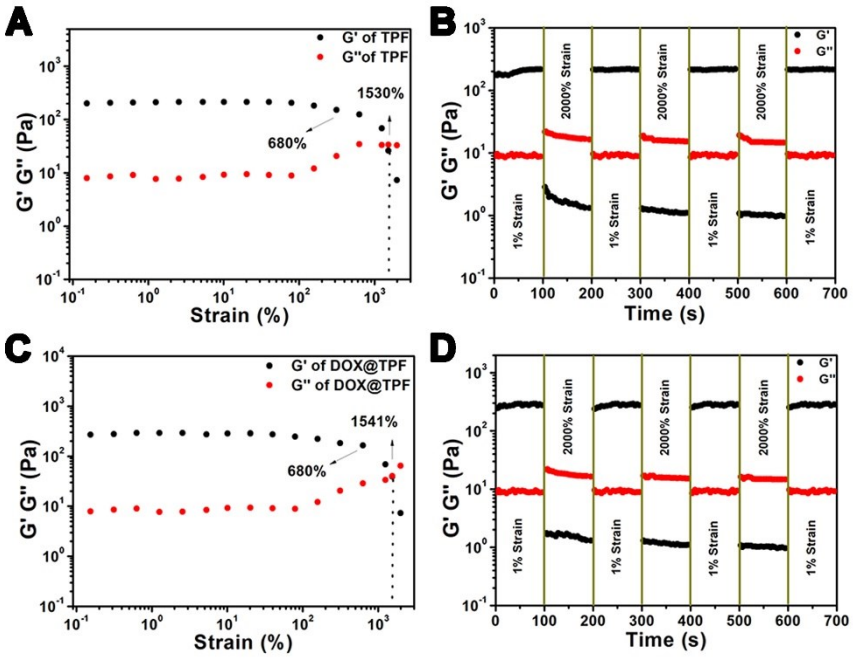


Fig. S6 (A) Strain-dependence of the dynamic storage modulus (G') and loss modulus (G'') of the TPF gel (TA = 0.2 wt%, 4-arm-PEG-SH = 2.4 wt%, molar ratio TA/Fe^{3+} = 1:0.8). (B) Storage moduli (G') and the loss moduli (G'') of the TPF gel under alternate-step strains of 1% and 2000%. (C) Strain-dependence of the dynamic storage modulus (G') and loss modulus (G'') of the DOX@TPF gel (TA = 0.2 wt%, 4-arm-PEG-SH = 2.4 wt%, molar ratio TA/Fe^{3+} = 1:0.8, DOX·HCl = 2 mg/mL). (D) Storage moduli (G') and the loss moduli (G'') of the DOX@TPF gel under alternate-step strains of 1% and 2000%.

10. In vitro degradation of DOX@TP and DOX@TPF gels

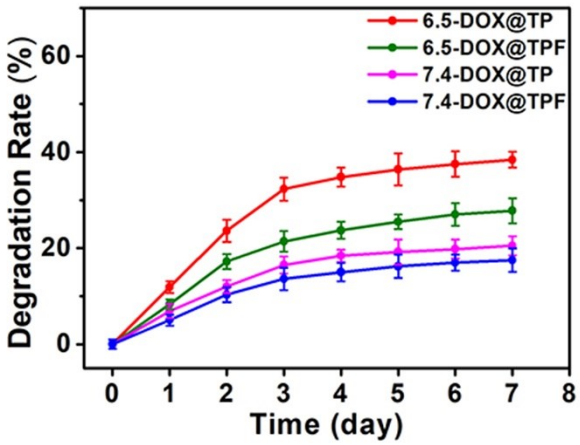


Fig. S7 In vitro degradation of DOX@TP and DOX@TPF hydrogels in PBS buffers under different pH conditions (pH= 7.4 and 6.5) at 37 °C.