Electronic Supporting Information (ESI)

Co-assembled supramolecular hydrogels of doxorubicin and indomethacin-derived peptide conjugates for synergistic inhibition of cancer cell growth

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Experimental section

Materials

All the Fmoc-amino acid (Fmoc-Gly-OH, Fmoc-Phe-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Arg(Mtr)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-His(Trt)-OH), 2-chlorotrityl chloride resin, 1-Hydroxybenzotriazole (HOBt), O-benzotriazole-N,N,N,N-tetramethyluronium tetrafluoroborate (TBTU) were obtained from GL Biochem (Shanghai, China). Doxorubicin hydrochloride was obtained commercially from Dalian Meilun Biotech Company, Ltd. (Dalian, China). Indometacin (IDM), Trifluoroacetic acid(TFA), and N,N-diisopropylethylamine (DIEA) were purchased from Aladdin Reagent Corporation (Shanghai, China). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was obtained from Biosharp Company (Hefei, China). All reagents used are of at least analytical reagent grade. Fetal bovine serum (FBS) was purchased from Hyclone Corporation. RPMI-1640 cell culture medium was purchased from Giboco Corporation. A549 and NIH/3T3 were obtained from American Type Culture Collection (ATCC), The deionized (DI) water (18.25 MΩ cm) was produced by UlUPure water system (UPR-II-10 T, Sichuan ULUpure Tech.).

Synthesis and purification of IDM-1

The IDM-1 was prepared by standard Fmoc-solid phase peptide synthesis techniques (SPPS). The 2-chlorotrityl chloride resin (1.0~1.2 mmol/g) was swelled in a shaker vessel with dry dichloromethane (DCM). Then the first amino acid Fmoc-His(Trt)-OH (2 equiv.) was loaded onto resin with DIPEA (2 equiv.) for 2 h. After washed with DCM for three times, the blocking solution (80:15:5 of DCM/MeOH/DIPEA) was added for 20 min, 20% piperidine in DMF was used to remove the Fmoc protecting group on the resin. Then the next Fmoc-protected amino acid/HOBt/TBTU/DIEA (3:3:3:6 relative to resin) in DMF was coupled to the free amino group for at least 3 h . In the last coupling step, IDM was used to produce IDM-1. The IDM-1 was cleaved from the resin using 99% TFA .The TFA was removed under *vacuo* and the crude product was poured into ice-cold ether. The crude IDM-1 was purified on a Shimadzu preparative HPLC with a Shim-Pack PRC-ODS column. The product was eluted with a linear gradient of 10% Acetonitrile to 90% over 30 minutes. Characterizations of products, with purity > 90%, were performed with HPLC (Shimadzu, Japan), ^{1}HNMR (500 MHz, Agilent, USA) and TOF-MS (Q-TOF. micro. Waters), respectively.

Characterization of IDM-1

^{1}HNMR spectroscopy (500 MHz, Agilent, USA) was performed using d6-DMSO as a solvent. Mass spectrometry was performed on a waters Q-TOF (Q-TOF. micro. Waters) equipped with electrospray ionization (ESI) source operating in positive mode. Transmission electron microscopy (TEM) was performed using a HT7700 (HITACHI, Japan) operated at an accelerating voltage of 120 kV. The Samples for TEM were prepared by diluting preformed IDM-1 hydrogels (1.5 wt.%) and IDM-1 with 0.1eq DOX hydrogel (1.5 wt.%), a small volume of solution was applied to a copper grid. Circular dichroism (CD, Jasco J-810, JASCO, Japan) of IDM-1 (0.05 wt.%, at pH 7.4) and IDM-1 with 0.1 equiv. DOX solutions (0.05 wt.%, at pH 7.4) was performed in a 0.5 mm quartz cell and recorded over a wavelength range of 190-260 nm. Dynamic light scanning (DLS; Malvern instruments, Nano-ZS90, Britain) measurements were conducted to measure the zeta potential distribution of IDM-1/DOX. 1 mL IDM-1 stock solution (0.05 wt.%) with different ratios of DOX (0.05 to 0.3 equiv.).

Doxorubicin-triggered hydrogel formation and drug loading

The sol-gel phase transition of the IDM-1 was determined by the tube inversion method in PBS buffers. Compound of IDM-1 was firstly dissolved in PBS buffer with 0.1 M HCl/NaOH adjusting the pH to 7.4. Doxorubicin was dissolved in PBS buffer at the concentration of 1 mg/mL as a stock solution. Subsequently, different equiv. of doxorubicin was added to the peptide solution. A homogeneous mixed solution was obtained by mixing and IDM-1/DOX hydrogel was formed within 5 minutes (the final peptide concentration = 1.5 wt.%).

For those hydrogel groups, a further centrifugation was performed at 10,000 rpm for 10 min to collect supernatants for measurement of DOX content by UV−vis spectrophotometer. The drug loading content (DLC) and drug loading efficiency (DLE) were calculated with the following formula:

$$\text{DLC \%} = \frac{W_{DOX(gel)}}{W_{DOX(gel)} + W_{hydrogel}} \times 100\%$$

$$\text{DLE \%} = \frac{W_{DOX(gross)} - W_{DOX(supernatant)}}{W_{DOX(gross)}} \times 100\%$$

Rheology

Rheological experiments of hydrogels were conducted on a Discovery HR-2 instrument (TA Instruments, USA) using a set of parallel plates with a diameter of 60 mm and a thickness of 0.3 mm. All experiments were performed at 37 °C. Dynamic time sweep measurements of the IDM-1 hydrogel (2 wt.%, pH 7.4) and IDM-1/DOX hydrogel (2 wt.%, 0.1equiv. DOX, pH 7.4) were performed with a strain of 0.2% and a frequency of 6.280 rad/s at 37 °C for 3600 seconds. Dynamic frequency sweep measurements were performed from 0.1 to 100 rad/s, with a fixed strain at 0.2% (all the gels were in the linear viscoelastic (LVE) region at this strain). Dynamic strain sweep were performed in the region of 0.1% to 100% strain with a frequency of 6.280 rad/s.

Quantum yields

A UV−vis spectrophotometer (UV-1800, Shimadzu Corp, Japan) was used for record of the UV-vis spectra, and a fluorescence spectrophotometer (RF-5301, Shimadzu Corp. Japan) for record of fluorescence spectra. All samples were prepared by mixed the DOX (20μg/mL) with different concentrations of IDM-1 solution (40-4000 μg/ mL). The relative quantum yield φ_x was then calculated using DOX as standard:

$$\varphi_x = \frac{Em_x}{Em_{std}} \times \frac{Abs_{std}}{Abs_x}$$

where $Em_x$and Em_{std} are the integrated areas of DOX in IDM-1/DOX or DOX solution in emission spectra, and Abs_x and Abs_{std} are the absorbance of IDM-1/ DOX and DOX in UV-vis spectra.

Calibration curve and in vitro drug release

UV-vis spectra of DOX was used to setup calibration curves (obtained by linear fitting) of DOX (absorption wavelength: 490nm, 1 to 60 μg/mL) with a UV−vis spectrophotometer (UV-1800, Shimadzu Corp, Japan). The HPLC elution curves was used to setup calibration curves of IDM-1 (0.05 to 1.0 mg/mL) with an analytic HPLC machine (LC-20AD, Shimadzu Corp, Japan, detection wavelength: 265 nm).

IDM-1 Hydrogels (2 mL, 1.5 wt.%) containing 0.1 equiv. doxorubicin were formed in an Eppendorf tube at 37 °C. After 12 h, 2 mL of release buffer of varying pH or ionic strength (i.e. NaCl) was added on the surface of hydrogels. At pre-determined time intervals, 1 mL of incubated solution was taken out, and replaced with fresh release medium. The quantity of released drugs was determined with UV-vis or HPLC using pre-established calibration curves.

To study the release mechanism, Ritger-Peppas equation was employed for data fitting[1]:

$M_t / M_\infty = k \bullet t^n$

Where M_t / M_∞ is fractional drug release, M_t is the amount of drug released at time t, M_∞ is the maximum amount of drug released at time ∞, t is the release time, k is a rate constant of kinetic release, and n is the diffusion exponent, characteristic of the drug release mechanism. For n<0.45, it indicates that the release behavior follows the Fickian diffusion, whereas the non-Fickian release behavior has a value of n between 0.45 and 1.00 since hydrogels belong to non-swelling cylindrical devices.

Cytotoxicity Study

The A549 cells were cultivated in RPMI 1640 with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic at 37 °C in 5% CO_2. Stock solution of IDM was prepared in dimethyl sulfoxide (DMSO) and diluted for cell treatment, with final concentration of DMSO in the medium ≤ 0.2% for not affecting cell growth. A total volume of 100 μL A549 cells were seeded in a 96-well plate containing 3,000 cells per well. Subsequently, they were treated with various concentrations of DOX (54 – 864 nM), IDM (25 – 400 μM), IDM-1 (25 –400 μM) and combination of DOX

(54 – 864 nM) with IDM or IDM-1(fixed at 10 μM). Cells without drug treatment served as the control. After 48 h incubation, the MTT assays were performed. Briefly, 20 μL MTT reagents (5 mg/mL) were added to each well, and then incubated for 4 h in the darkness at 37 °C. Afterwards, solutions were aspirated and formazon crystals at the bottom of each well were dissolved in 200 μL DMSO. Absorbance was then measured at wavelength of 492 nm using a microplate reader (Tecan Sunrise, Austria). All experiments were repeated for at least three times. Cell viabilities of drug treated groups were normalized with control and plotted with DOX concentration in Origin software (USA), with nonlinear curve fitting utilized calculate IC_{50} values (i.e. DOX concentrations required for achieving 50% cell viability).

The quantitative analysis of the synergistic effects of drugs was performed using the combination index (CI) theorem of Chou-Talalay, which offers quantitative definition of the additive effect (CI = 1), synergism (CI < 1), and antagonism (CI > 1) in drug combinations[2]. The general equation for the combination index $^{n}(CI)_{x}$ for *n* drugs at *x*% inhibition is described as,

$$^{n}(CI)_{x=}\sum_{j=0}^{n}\frac{(D)_{j}}{(D_{x})_{j}}$$

where $(D)_j$ is the concentrations of *n* drugs used in combination to achieve *x*% drug effect, and $(Dx)_j$ is the concentrations of each drug alone to achieve the same effect.

Critical Micelle Concentration (CMC)

The CMC was determined using a Malvern Zetasizer, NANO ZS90 (Malvern Instruments Limited, U.K.) equipped with a laser light scattering spectrometer at 632.8 nm under room temperature (25 °C). Solutions containing different concentration of peptides were tested, and the light scattering intensity was recorded for each concentration analyzed.

Confocal laser scanning microscopy (CLSM)

Cellular uptakes of IDM-1/DOX hydrogel or free DOX in A549 cells were visualized with a confocal laser scanning microscope (CLSM, LSM800, Zeiss, Germany) and processed using a ZEN imaging software. Briefly, A549 cells were seeded in a petri-dish and grown for 24 h at 37°C followed by an incubation with IDM-1/DOX or free DOX for 2 h at 37°C or 4°C. Subsequently, the medium was discarded and the cells were washed with PBS, fixed with 4% paraformaldehyde and stained with DAPI for final observations. For inhibition of integrin receptor, cells were pre-incubated with free RGD (0.2 mg/mL) for 2 h prior to addition of IDM-1/DOX.

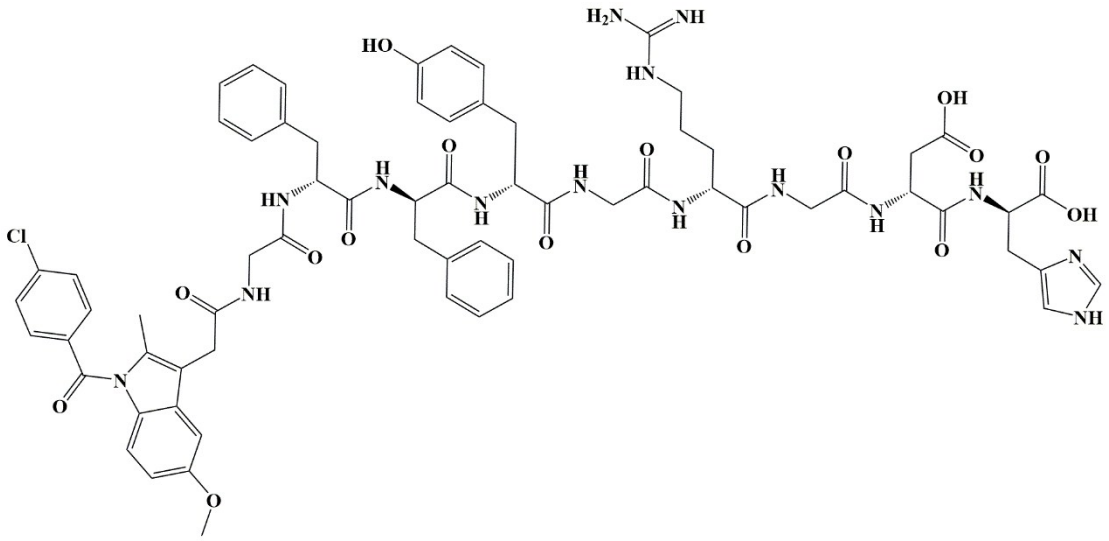

Figure S1. The chemical structure of IDM-1.

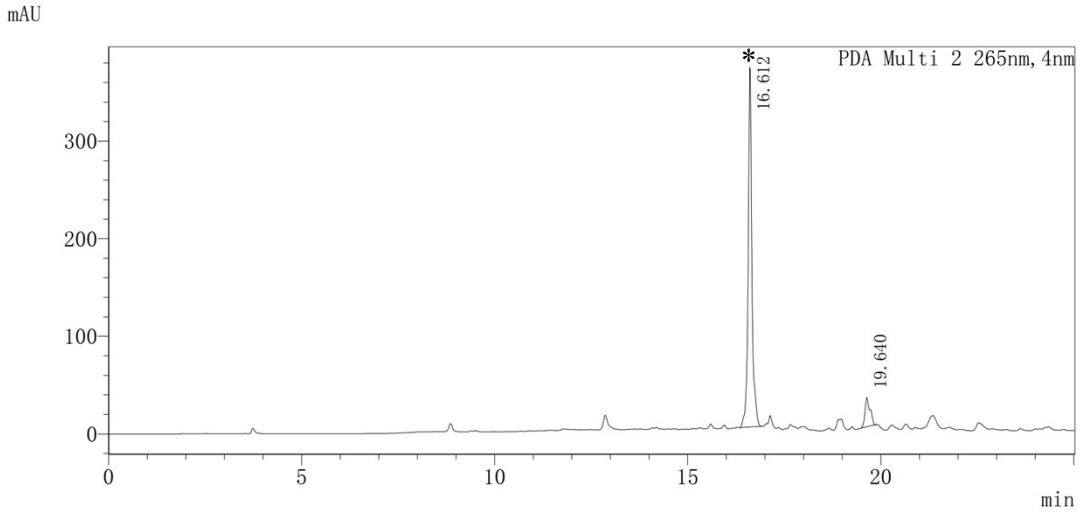

Figure S2. HPLC elution curve of IDM-1. The HPLC conditions were as follows: a linear gradient of 10% to 90% of acetonitrile over 25 min at a flow rate of 1.0 mL/ min, detected at 265 nm. *: The peak of IDM-1. Purity: > 90%.

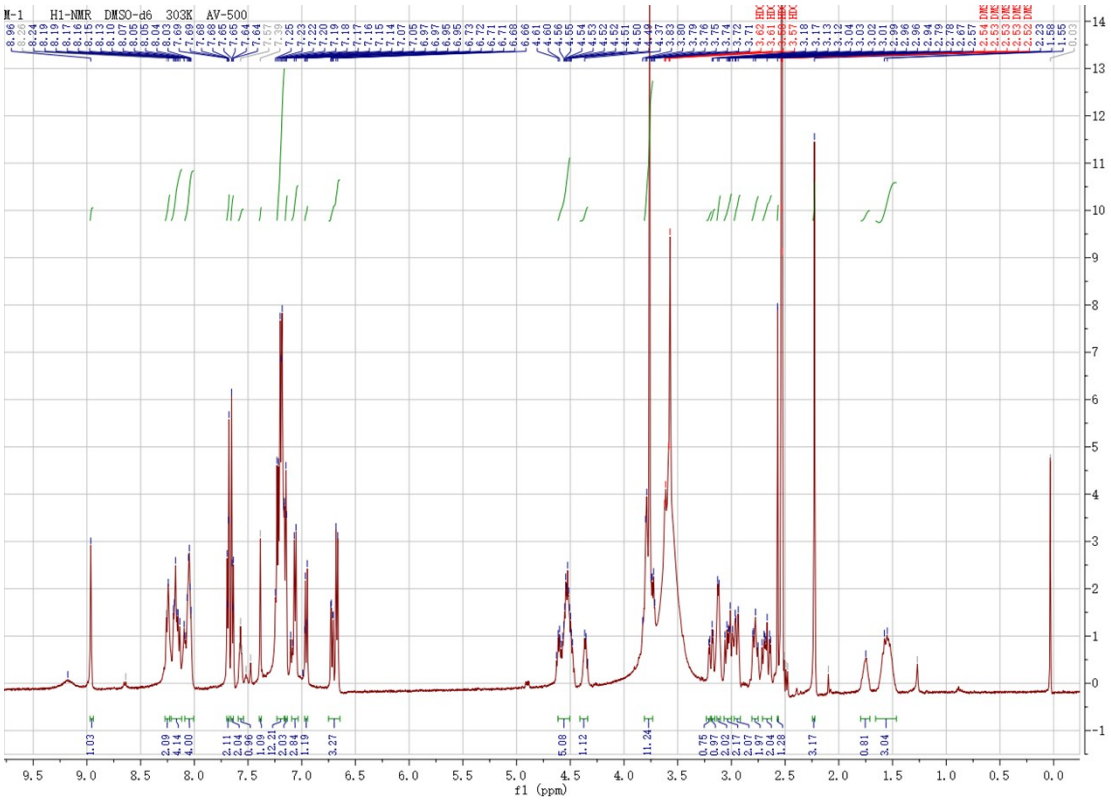

Figure S3. [1]HNMR spectrum of IDM-1 (500 MHz, d6-DMSO).

[1]HNMR (500 MHz, DMSO) δ 8.96 (s, 1H), 8.26-8.24 (d, J = 10 Hz, 2H), 8.19-8.13 (m, 4H), 8.10-8.04 (m, 4H), 7.69-7.68 (d, J = 5 Hz, 2H), 7.65-7.64 (d, J = 5 Hz, 2H), 7.57 (s, 1H), 7.39 (s, 1H), 7.23-7.16 (m, 12H), 7.16-7.15 (d, J = 5 Hz, 2H), 7.07-7.05 (d, J = 10 Hz, 2H), 6.97-6.95 (d, J = 10 Hz, 1H), 6.73-6.71 (d, J = 10 Hz, 1H), 6.68-6.66 (d, J = 10 Hz, 1H), 4.63-4.53 (m, 5H), 4.37-4.34 (m, 1H), 3.80-3.71 (m, 11H), 3.21-3.20 (d, J = 5Hz, 1H), 3.18-3.17 (d, J = 5Hz, 1H), 3.13-3.12 (d, J = 5Hz, 2H), 3.06-3.01 (m, 2H), 2.96-2.94 (d, J = 10Hz, 2H), 2.80-2.75 (m, 2H), 2.71 – 2.64 (m, 2H), 2.57 (s, 1H), 2.23 (s, 3H), 1.75(s, 1H), 1.58 –1.55 (m, 3H).

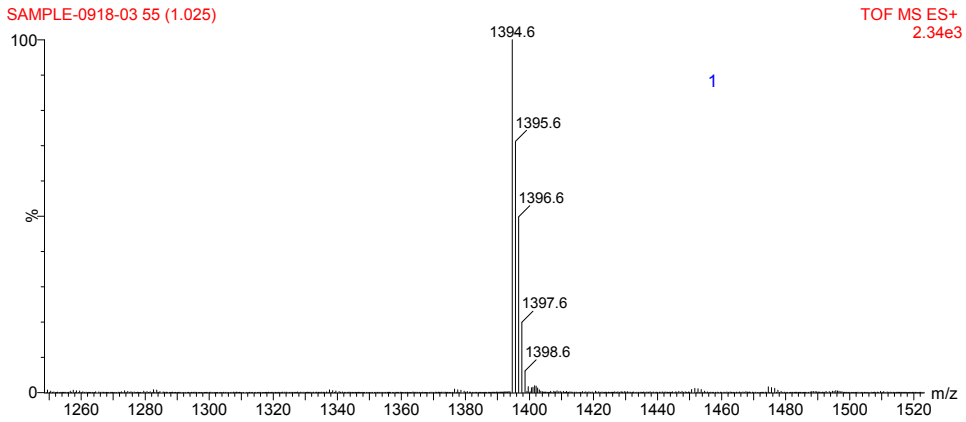

Figure S4. TOF-MS spectrum of IDM-1. calc.: 1393, obsvd.: [M+H] = 1394.6.

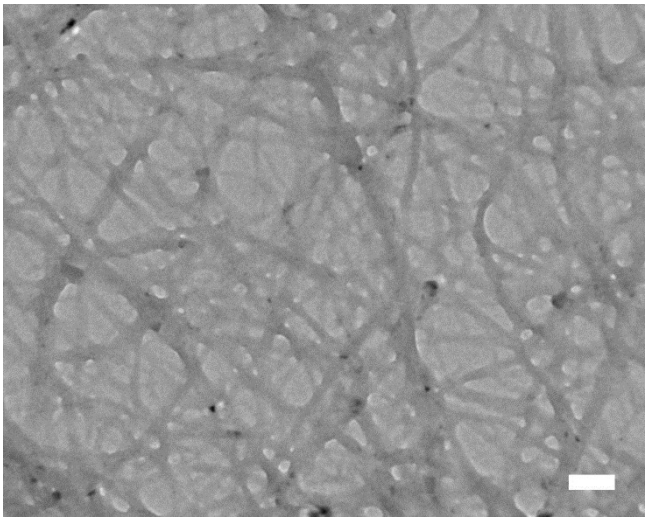

Figure S5. TEM images of **Nap-1** hydrogel with 0.1 equiv. of DOX. Bar, 100 nm.

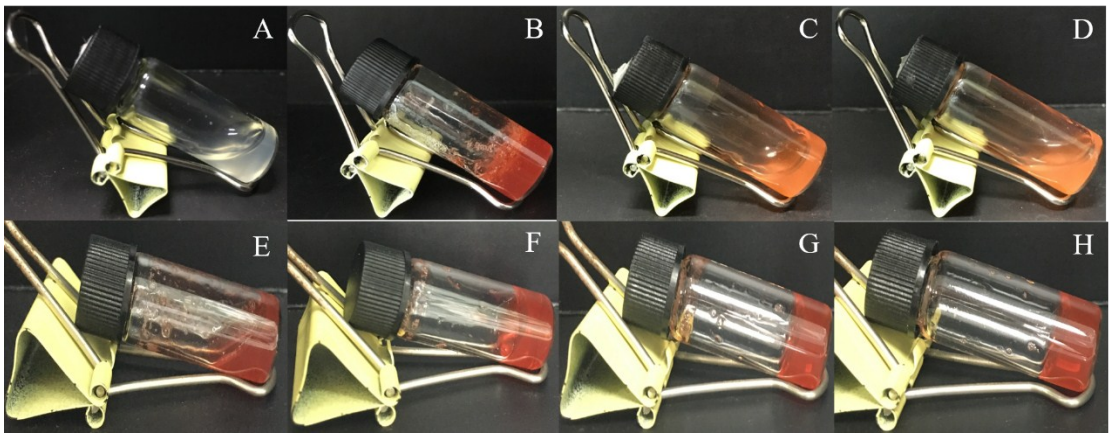

Figure S6. Optical images of 1.5 wt.% IDM-1 (A) and IDM-1/DOX (B) at pH 7.4, and 1.5 wt.% IDM-1/DOX at pH 6.5 (C) & pH 5.5 (D). Sol/gel images of 1.5 wt.% IDM-1/DOX after addition of 2 M NaCl (E), 0.4 M imidazole (F), 0.4 M histidine (H) or 0.4 M EDTA at pH 7.4.

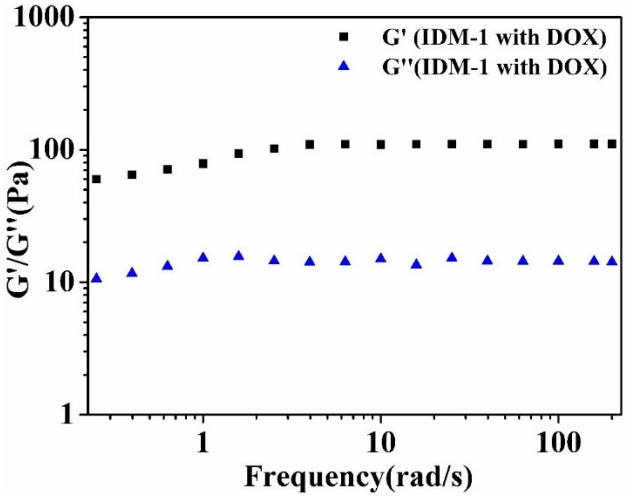

Figure S7. Dynamic frequency sweep of IDM-1/DOX hydrogels (2 wt.% IDM-1; 0.1 equiv. DOX).

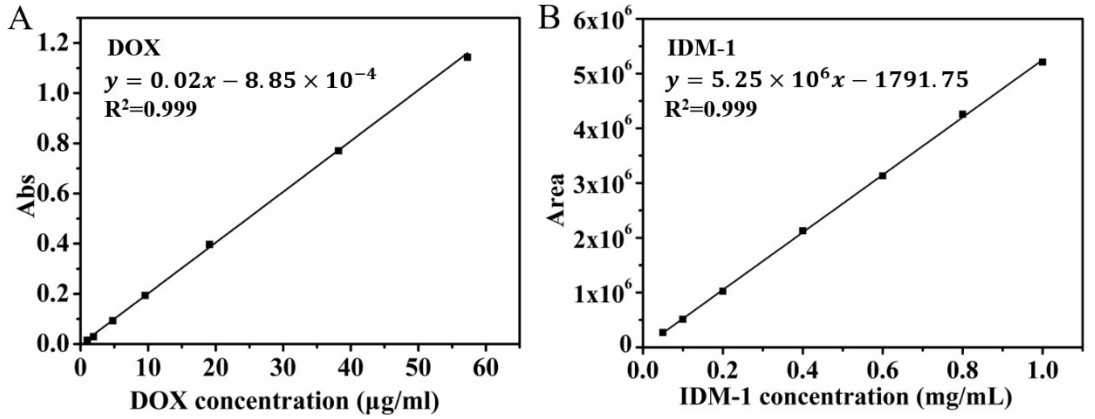

Figure S8. (A) Calibration curve of DOX with UV-vis spectrum; (B) calibration curve of IDM-1 with HPLC elution curves.

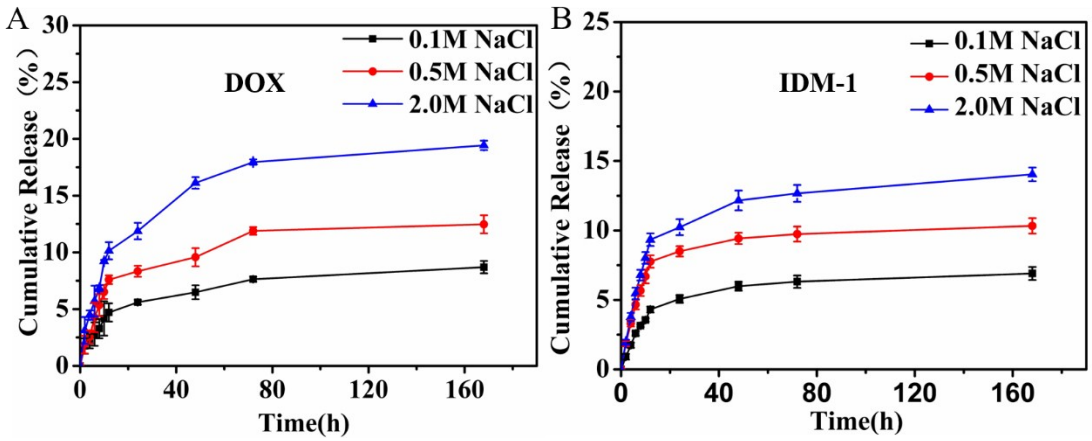

Figure S9. Cumulative release of DOX (A) and IDM-1 (B) from IDM-1/DOX hydrogels (1.5 wt.% IDM-1; 0.1 equiv. DOX) in pH7.4 release buffers of varying concentrations of NaCl.

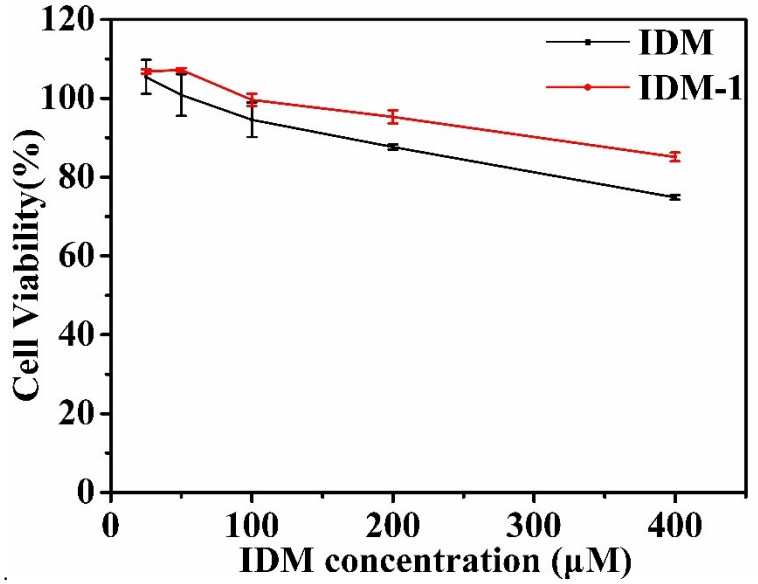

Figure S10. Cell viabilities of NIH/3T3 after treatment of IDM or IDM-1 for 48 h.

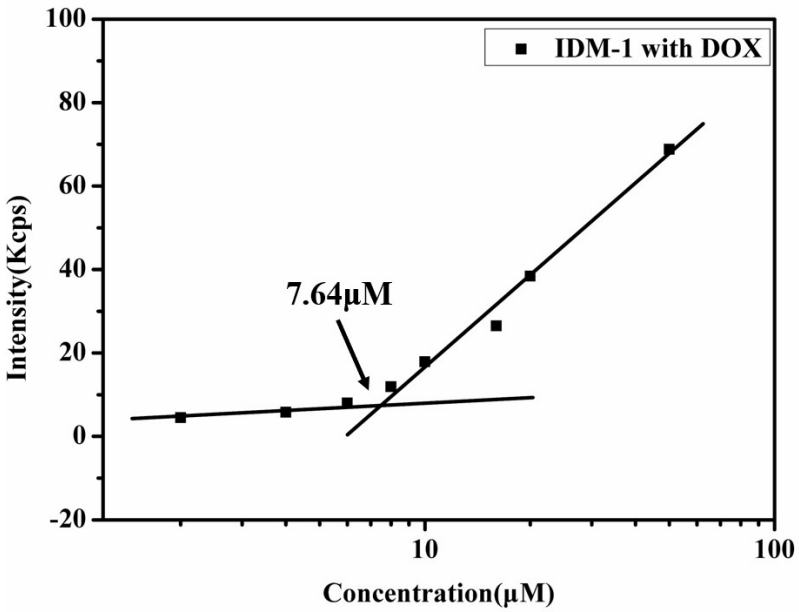

Figure S11. The curves used to determine the CMC value of **IDM-1** hydrogel with 0.1 equiv. of DOX.

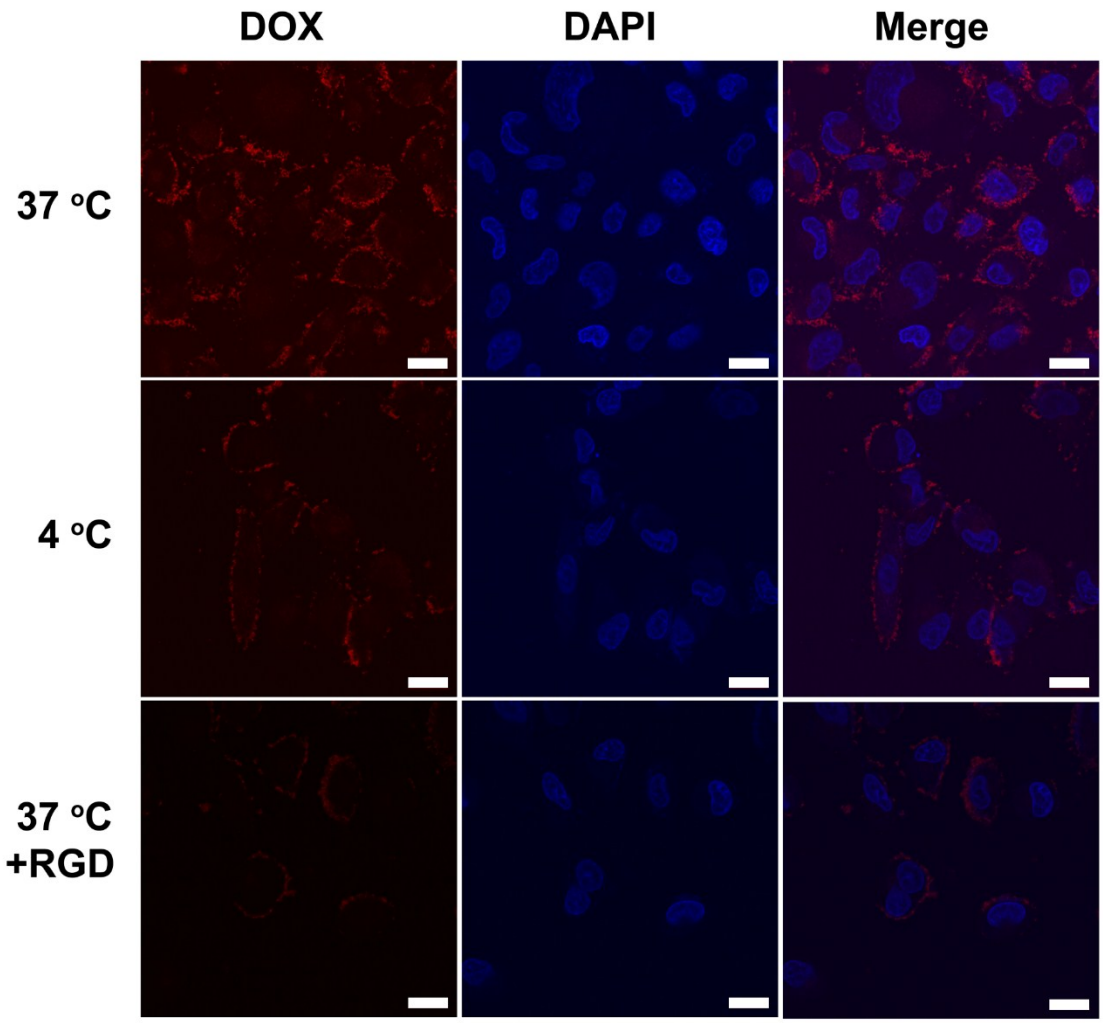

Figure S12. Confocal laser scanning microscopy (CLSM) images of A549 cells with incubation of IDM-1/DOX hybrid hydrogel at 37 °C, 4 °C, or 37 °C with pre-incubation of RGD (200 μM). Red, DOX stain; blue, DAPI stain for cell nuclei. Bar, 20 μm.

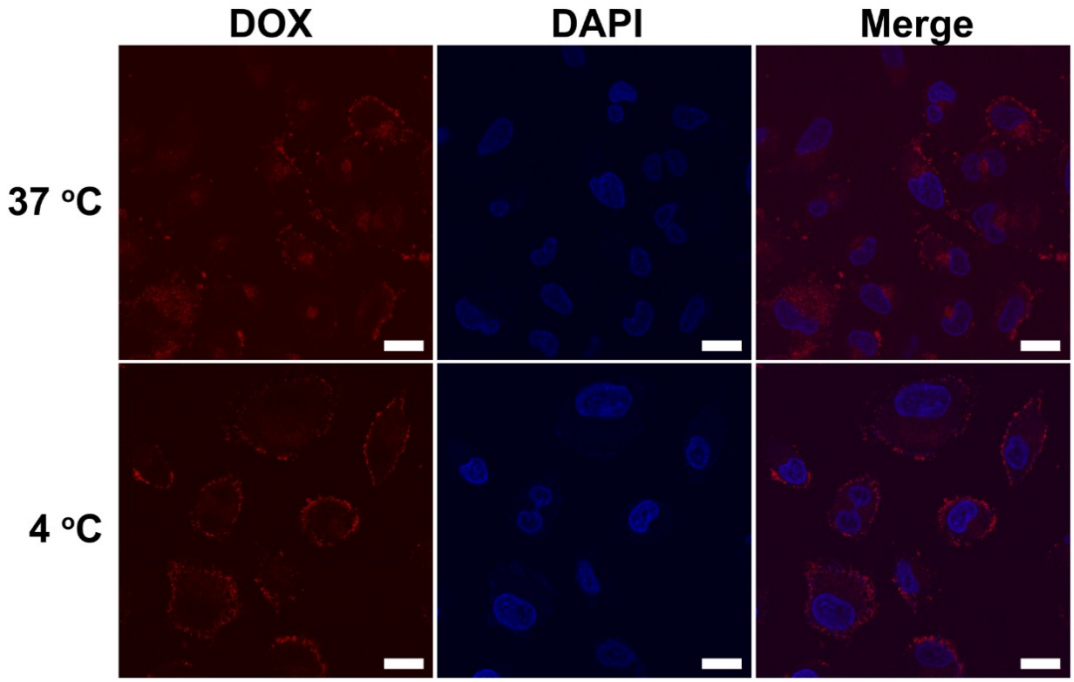

Figure S13. Confocal laser scanning microscopy (CLSM) images of A549 cells with incubation of aqueous solution of DOX at 37 °C or 4 °C. Red, DOX stain; blue, DAPI stain for cell nuclei. Bar, 20 μm.

Table S1. Gelation and DOX-loading capabilities of **IDM-1** and Nap-1.

Sample	MGC (wt.%) w/o DOX at pH 7.4	MGC (wt.%) w/ 0.1 equiv. DOX at pH 7.4	DLE	DLC
Nap-1	1.0	0.8	92.4 ± 1.1%	11.9 ± 0.3%
IDM-1	2.0	1.5	92.7 ± 1.1%	16.6 ± 1.8%[a]

Notes: MGC, minimal gelation concentration; DLE, drug loading efficiency; DLC, drug loading capacity.

[a] DLC of IDM-1 was significantly higher than that of Nap-1. $P < 0.01$.

Table S2. Table of parameters of Ritger-Peppas models for drug release behaviors of **IDM-1**/DOX hydrogel at varying pH conditions in 24 h.

Drugs		**pH conditions**		
		5.5	**6.5**	**7.4**
DOX	**K**	0.0219	0.0068	0.0046
	R^2	0.9462	0.9262	0.9447
	n	0.6282	0.7975	0.7857
IDM-1	**K**	0.0142	0.0135	0.0046
	R^2	0.9292	0.9362	0.9485
	n	0.7042	0.6510	0.7200

Table S3. IC_{50} values and combination index (CI) of DOX and IDM-based drugs in A549 cells.

Drugs	IC_{50} of DOX (nM)	CI[a] value
DOX	784.51 ± 3.35	N/A
Nap-1/DOX	443.68 ± 5.16 [a]	N/A
IDM/DOX	388.05 ± 8.46[a]	0.519 ± 0.012
IDM-1/DOX	274.12 ± 6.70[a,b]	0.376 ± 0.018[c]

Notes: [a] IC_{50} values of Nap-1/DOX, IDM/DOX or **IDM-1**/DOX were significantly lower than that of DOX. [b] IC_{50} value of **IDM-1**/DOX was significantly lower than those of IDM/DOX or Nap-1/DOX. [c] CI values of **IDM-1**/DOX were significantly lower than that of IDM/DOX. $P < 0.01$.

Reference

1. N. A. Peppas, *Journal of controlled release* .2014**, 190**, 31.
2. T. C. Chou; Talalay, P., *Adv. Enzyme Regul.* 1984**, 22**, 27.