Supplementary Information (SI).

Cartilage acellularized matrix derived highly porous and injectable hydrogels with reduction and NIR light dual-responsive drug release properties for application in anti-tumor therapy

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Supplementary Information

1. Materials

Poly(ethylene glycol) (OH-PEG-OH, M_n-1000 g mol^{-1}, Tokyo Chemical Industry (TCI)), *p*-toluenesulfonyl chloride(99%, Sigma-Aldrich), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC.HCl, 99 %, TCI), N-hydroxysuccinimide (NHS, 98 %, Sigma Aldrich), 5-norbornene-2-carboxylic acid (98 %, TCI), triethylamine (TEA, 99 %, Sigma Aldrich), glutathione (GSH, reduced form, TCI) glutaric anhydride (GA, 98 %, TCI), hydroxylamine hydrochloride ($HONH_2 \cdot HCl$, 99 %, Sigma Aldrich), doxorubicin hydrochloride (DOX, Boryung Pharm. Co.), 4-(Aminomethyl)benzonitrile hydrochloride (98%,Matrix Scientific), and zinc(II) trifluoromethanesulfonate (98%, TCI) were used as received. Anhydrous hydrazine was obtained from the distillation of hydrazine monohydrate (98%, TCI). Acetone, dichloromethane (DCM), diethyl ether, n-hexane, methanol, isopropyl alcohol (IPA) and ethyl acetate were purchased from Duksan Pure Chemicals. Cells from a human fibroblast cell line and human Caucasian colon adenocarcinoma cells (HT29 cell line) were obtained from American Type Cell Culture Collection (ATCC). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum, L-glutamine, trypsin-EDTA solution (10×), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT, 97.5%) and was purchased from Sigma-Aldrich.

2. Measurements and instruments

2.1. Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were obtained by using a JEOL (JNM ECZ-400) Fourier transform nuclear magnetic resonance spectrometer (FT-NMR 400 MHz). Chemical shifts were measured in ppm using deuterated solvents. NMR spectra were processed using MestRENova 6.0.2 software (Mestre lab Research S.L).

2.2. Fourier transform infrared (FTIR) spectroscopy

FTIR spectra were recorded on an Agilent Technologies Cary 630 FTIR spectrophotometer fitted with a diamond single reflection ATR unit. All spectra were acquired by directly placing a small amount of a dried sample on a clean crystal of the instrument and were referenced against background spectra obtained by scanning the clean crystal before addition of the sample.

Transmittances were recorded in the range 4000-650 cm^{-1} and data were processed using the Agilent MicroLab software suite.

2.3. Size exclusion chromatography (SEC)

SEC was recorded on an Agilent 1200 GPC-SEC system furnished with a differential refractive index detector. The mobile phase was HPLC grade THF at 25 °C at a flow rate of 1 mL/min. Separations were performed on a PLgel Mixed-D columns (300 × 7.8 mm, 5 µm bead size, Polymer Labs UK) fitted with a matching guard column (50 × 7.8 mm). The number average molecular weight (M_n), was calculated based on a standard calibration method using poly(styrene) narrow molecular weight standards in the range of 100 Da–500 kDa. Chromatographs were analyzed using Polymer Labs Cirrus 3.0 software.

2.4. Ultraviolet-visible spectroscopy (UV-Vis)

All UV-Vis spectra were recorded on a UV-vis spectrophotometer Optizen POP using quartz cuvettes. A sample volume of 2 mL was used and all spectra were referenced against a background of an appropriate blank.

2.5. Confocal laser scanning microscopy

Confocal laser scanning microscopy was performed using a Zeiss 510 Meta Confocal microscope. A 488 nm wavelength laser was used to excite the Calcein AM (excitation/emission maxima = 485/530 nm) and ethidium bromide stain (excitation/emission maxima = 301/603) was excited with a laser of 633 nm wavelength. The control untreated cells (without micelles) were used to subtract the background or auto fluorescence.

2.6. Thin-layer chromatography (TLC) and column chromatography

Thin-layer chromatography (TLC) was performed using a Merck TLC silica gel 60 F_{254}, aluminum-back TLC plates with an F254 fluorescent indicator, and 175–225-µm thick silica gel layer. Column chromatography was performed using silica gel with 60 Å porosity, 230–400 mesh.

2.7. Determination of free-amine content of CAM and degree of substitution of CAM-Nb

The free amine content of CAM and degree of substitution of CAM-Nb was analyzed by using ninhydrin assay according to a modified procedure reported previously.[1] Briefly, 4 mg of dried

CAM or CAM-Nb were mixed in 2 mL of DI water and 1 mL of freshly prepared 2% ninhydrin solution (in absolute ethanol). The reaction mixture was heated at 100 °C for 30 min and cooled with ice water to end the reaction. The optical densities of blue/violet color mixture were measured by using a UV-visible spectrophotometer at 570 nm wavelength. The free amine content of CAM was calculated by using glycine as a standard amino acid. For this purpose, a standard curve of glycine was made with various concentration of glycine after treating with ninhydrin reagent. The degree of substitution was determined using the following equation:

$$\text{Degree of substitution } (\%) = 1 - \left(\frac{A_f}{A_u}\right) \text{ x } 100$$

$A_{f \text{ and }} A_u$ represent the absorption of norbornene functionalized CAM and un-functionalized CAM, respectively. The determined degree of substitution was found to be 49% of original amine contents.

2.8. Rheology

Rheological properties of hydrogels were characterized using a Discovery HR-2 hybrid rheometer (TA instruments) equipped with a flat steel plate geometry (20 mm, gap size 500 µm) at a temperature of 25 °C. The oscillatory time sweep measurements (600 s) were recorded at a continuous strain of 1% and an angular frequency of 10 rad/s. The frequency sweep measurements were performed at varying angular frequency ranging from 0-100 rad/s. The *in situ* gelation time of the hydrogel was determined by the intersection of elastic storage modulus, G′, and the viscous loss modulus, G″,

2.9. Swelling studies

The swelling properties of hydrogels were characterized by a gravimetric method. The freeze dried hydrogel samples were weighed on an analytical balance and immersed in PBS at 25°C. The hydrogel samples were removed at predetermined time intervals, quickly blotted free of surface water using a filter paper, weighed again on an analytical balance, and returned to the swelling medium. The equilibrium swelling ratio was considered when the mass of swollen hydrogels did not change over time. The swelling ratio was calculated from the following equation and expressed as a percentage.

$\%SR = (W_S - W_D) / W_D \text{ x } 100$

Where, W_S and W_D are the weights of swollen and dried hydrogels, respectively.

3. Supplementary Methods

3.1. Preparation and characterization of cartilage acellularized matrix (CAM) powder

Cartilage acellular matrix (CAM) was extracted using established protocol with modifications.[2] Briefly, cartilage was dissected from hind and fore legs of 6-month-old porcine's knee joints (Obtained from local slaughter house) and was immediately washed with phosphate-buffered saline and lyophilized. Subsequently, lyophilized tissues were pulverized by using a cryogenic milling system to obtain cartilage powder, which was then decellularized by first treating it with a 10 mmol hypotonic buffer (Tris–HCl, pH 8.0) for 12 h at room temperature and then with 1% sodium dodecyl sulfate (SDS) solution in Tris-buffered saline (10 mmol NaCl, pH 7.6) for 2 h. The SDS residues were removed by washing with DI water (5 times) and centrifugation at 10000 rpm for 10 minutes. Next, the pellets were treated with DNase (100 U/mL) for 12 h at 4°C to remove any residual genetic material. Afterwards, enzymatic digestion of decellularized cartilage powder was accomplished by treating it with 0.1% pepsin solution in 0.5 N HCl for 24 h at room temperature. The digested cartilage powder was then neutralized with sodium hydroxide solution to pH 7.4, and dialyzed (MWCO ~ 2 kDa) against DI water for 24 h to remove remaining salts. Finally, the CAM suspension was lyophilized and then pulverized by using a cryogenic milling system to obtain fine CAM powder.

The free amine contents of CAM powder were determined by ninhydrin assay. Briefly, 4 mg of CAM powder was dissolved in 2 mL DI H_2O in a test tube and mixed with 1 mL of 2% ninhydrin solution in absolute ethanol. The test tube was heated to 100 °C for 30 minutes and then was cooled down by using an ice bath. The optical absorbance of resulting purple solution was measured at 570 nm using a UV-Vis spectrophotometer. The percentage of free amino groups in the CAM powder was calculated from a standard curve of glycine following the same procedure. The percentage of free amine contents of CAM was determined to be 4 % (Wt. %).

3.2. Synthesis of α-tosyl-ω-hydroxy polyethylene glycol

α-tosyl-ω-hydroxy polyethylene glycol was synthesized by follwing a previous protocol with modifications.[3] Briefly, PEG (1000 g/mol, 10 g, 10 mmol, 3 mol. eq.) was dried by azeotropic distillation in dry toluene under reduced pressure and then was dissolved in 100 mL of dry dichloromethane in a 3-neck round bottom flask. Triethylamine (0.438 g, 0.603 mL, 4.332 mmol, 1.3 mol. eq.) was added in one portion by using a glass syringe with vigorous stirring at 0 °C. Afterwards, *p*-toluenesulfonyl chloride (0.635 g, 3.33 mmol, 1 mol. eq., pre-dissolved in 10 mL dry DCM) was added into the reaction mixture over 15 minutes by using a dropping funnel (1-2 drops per second). The reaction mixture was stirred at 0 °C for 3 hours and then at room temperature for 12 hours. Afterwards, the mixture was neutralized with 1M HCl solution, and washed with 3 x 100 mL DI water and then with 2 x 100 mL brine, by using a separating funnel. The organic layer was dried over anhydrous magnesium sulphate and solvent was evaporated under reduced pressure. Finally, the product was purified by flash column chromatography (50% methanol in ethyl acetate, R_f = 0.71, yield = 53%). ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.83-7.81 (meta protons of benzene ring, d, *J* = 8.3 Hz, 2H), 7.37-7.35 (ortho protons of benzene ring, d, *J* = 8.0 Hz, 2H), 4.18 (CH2-O-S-, m, 2H), 3.86–3.47 (O–CH2–CH2–O–, m, 471H), 2.47 (CH3 s, 3H). ^{1}H NMR (400 MHz, DMSO-d_6, δ): 7.79 ((meta protons of *p*-toluene sulfonyl, d, *J* = 8.2 Hz, 2H), 7.49 (ortho protons of *p*-toluene sulfonyl, d, *J* = 8.1 Hz, 2H), 4.57 (CH2-OH, t, *J* = 5.4 Hz, 1H), 4.13 – 4.11 (CH2-O-S-, m, 2H), 3.72–3.26 (O–CH2–CH2–O–, m, 470H), 2.43 (CH3, s, 3H).

3.3. Synthesis of (4-(cyano) benzylamino)-5-oxopentanoic acid

4-(aminomethyl) benzonitrile hydrochloride (2 g, 11.86 mmol, 1 mol. equi.) was dissolved in 100 mL of extra pure acetonitrile in a dry 2-neck round bottom flask and was purged with nitrogen gas 30 minutes. Afterwards, triethylamine (5 mL, 35.58 mmol, 3 mol equi.) was introduced into the system by using a glass syringe. After stirring for 30 minutes, glutaric anhydride (1.48 g, 13.04 mmol, 1.1 mol. equi., pre-dissolved in acetonitrile) was added into the flask with the help of a glass syringe and the reaction was refluxed at 85°C for 24 h. After cooling the contents and removing the solvent by a rotary evaporator, the solid crystals were dissolved in 100 mL DI water and acidified to pH 3 by using 1N HCl, and then extracted with 3 x 100 mL ethyl acetate. The combined organic layers were washed with 3 x 100 mL DI water, and then with 100 mL of brine, and then dried over anhydrous magnesium sulfate. Finally, the organic solvent was removed under reduced

pressure to obtain white solid. (91% yield). ^{1}H NMR (400 MHz, DMSO-d_6, δ): 1H NMR (400 MHz, DMSO) δ 12.05 (s, 1H), 8.44 (t, J = 5.9 Hz, 1H), 7.78 (d, J = 8.5 Hz, 2H), 7.42 (d, J = 8.6 Hz, 2H), 4.33 (d, J = 6.0 Hz, 2H), 2.26 – 2.15 (m, 4H), 1.79 – 1.69 (m, 2H).

3.4. Synthesis of Tetrazine(benzylamino)-5-oxopentanoic acid

Tetrazine(benzylamino)-5-oxopentanoic acid was synthesized according to a previously published protocol with modifications.[4] Briefly, (4-(cyano)benzylamino)-5-oxopentanoic acid (1 g, 4.08 mmol, 1 mol. equi.) was mixed with formamidine acetate salt (2.12 g, 20.39 mmol, 5 mol. equi.) and zinc triflate (0.074 g, 0.20 mmol, 0.05 mol. equi.) in a dried round bottom flask. Afterwards, freshly distilled anhydrous hydrazine (3.26 mL, 102 mmol, 25 mol equi.) was carefully introduced into the flask dropwise. After vigorous gas release, the resulting mixture was stirred at room temperature for 24 h. Subsequently, sodium nitrite (2.82 g, 40.80 mmol, 10 mol equi., dissolved in 5 mL of DI water) was added and followed by the dropwise addition of 1N HCl at 0 °C. After solution turned bright pink color and gas evolution stopped (consuming approximately 150 mL of 1N HCl, pH ~ 3), the mixture was extracted with 3 x 100 mL dichloromethane, washed with 3 x 100 mL brine, and dried over anhydrous magnesium sulfate. After filtration, dichloromethane was removed by using a rotary evaporator. Finally, the product was purified by recrystallization in cold isopropyl alcohol. (bright pink solid, 53% yield). ^{1}H NMR (400 MHz, DMSO) δ 12.06 (s, 1H), 10.58 (s, 1H), 8.52 – 8.40 (m, 3H), 7.53 (d, J = 8.6 Hz, 2H), 4.40 (d, J = 5.9 Hz, 2H), 2.28 – 2.17 (m, 4H), 1.77 (p, J = 7.3 Hz, 2H).

3.5. Statistical analysis

All experiments were carried out in triplicates and data are expressed in mean ± standard deviation. The significance of tests was calculated by Student's *t*-test. The difference between two samples was perceived statistically significant for $p < 0.05$, and very significant for $p > 0.01$.

4. Supplementary Figures

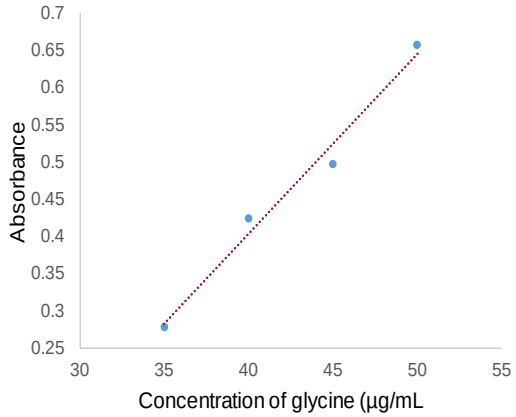

Fig. S1. Standard curve of glycine to determine free-amine contents of CAM and degree of substitution of CAM-Nb by using a ninhydrin assay. Absorbance was measured by using a UV-visible spectrophotometer at 570 nm.

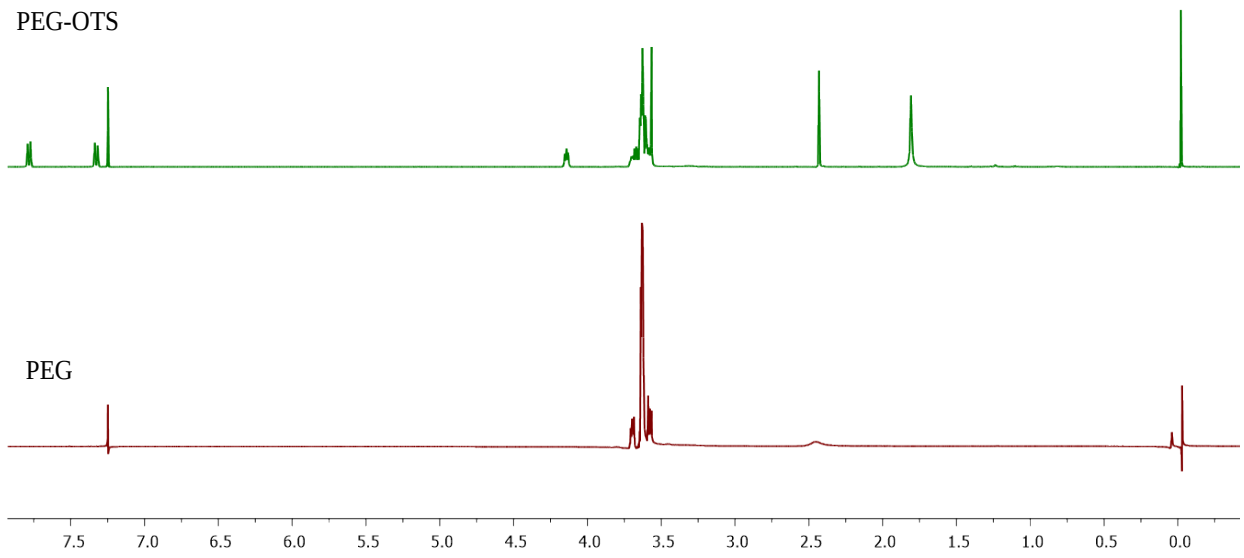

Fig. S2. ^{1}H NMR spectrum of PEG and monotosylated-PEG (HO-PEG-OTS) in $CDCl_3$.

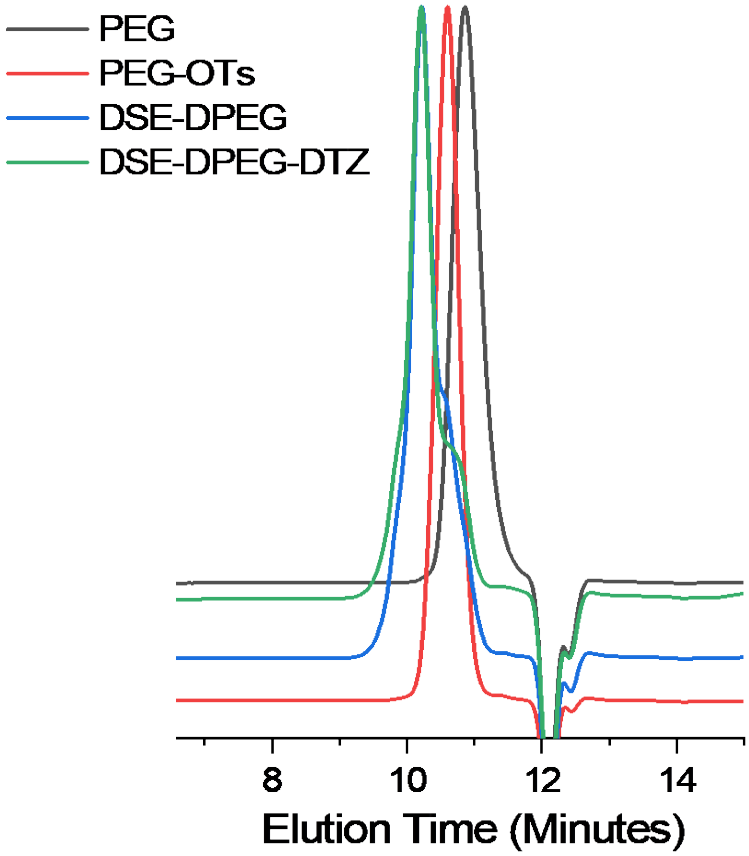

Fig. S3. GPC/SEC traces of starting materials and highly water soluble diselenide based IEDDA click-able crosslinker (DSe-DPEG-TZ) in THF at room temperature.

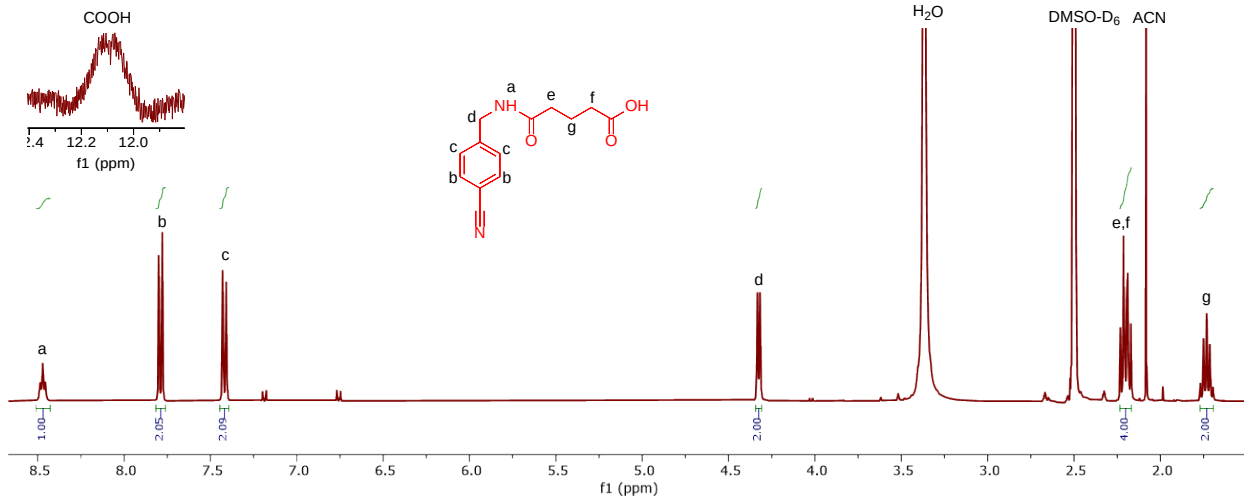

Fig. S4. ^{1}H NMR characterization of 5-((4-cyanobenzyl)amino)-5-oxopentanoic acid intermediate.

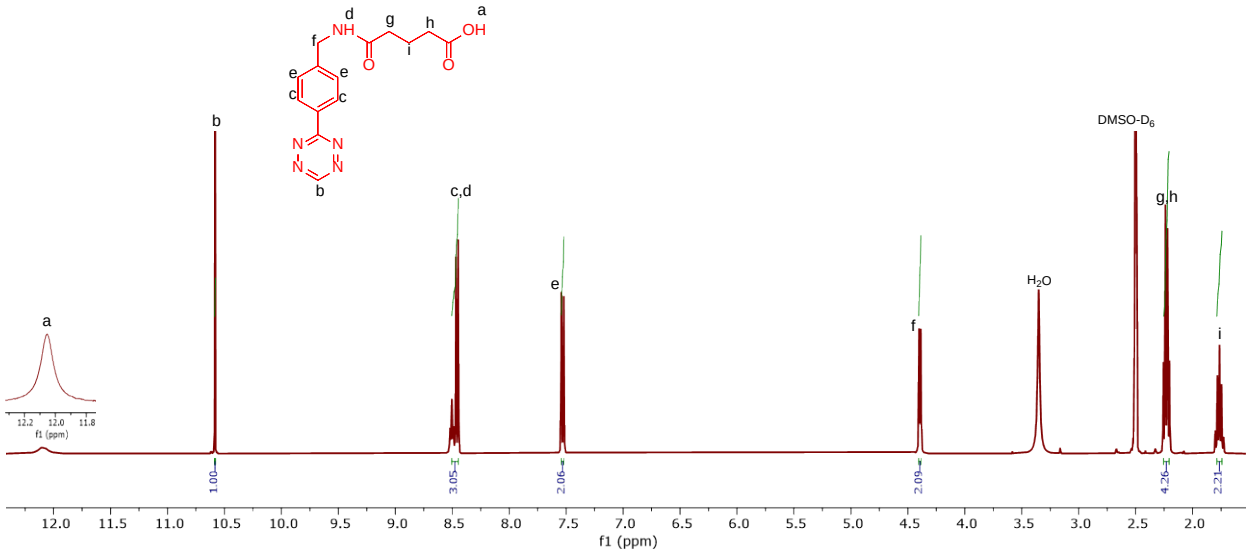

Fig. S5. ^{1}H NMR characterization of Tetrazine-COOH.

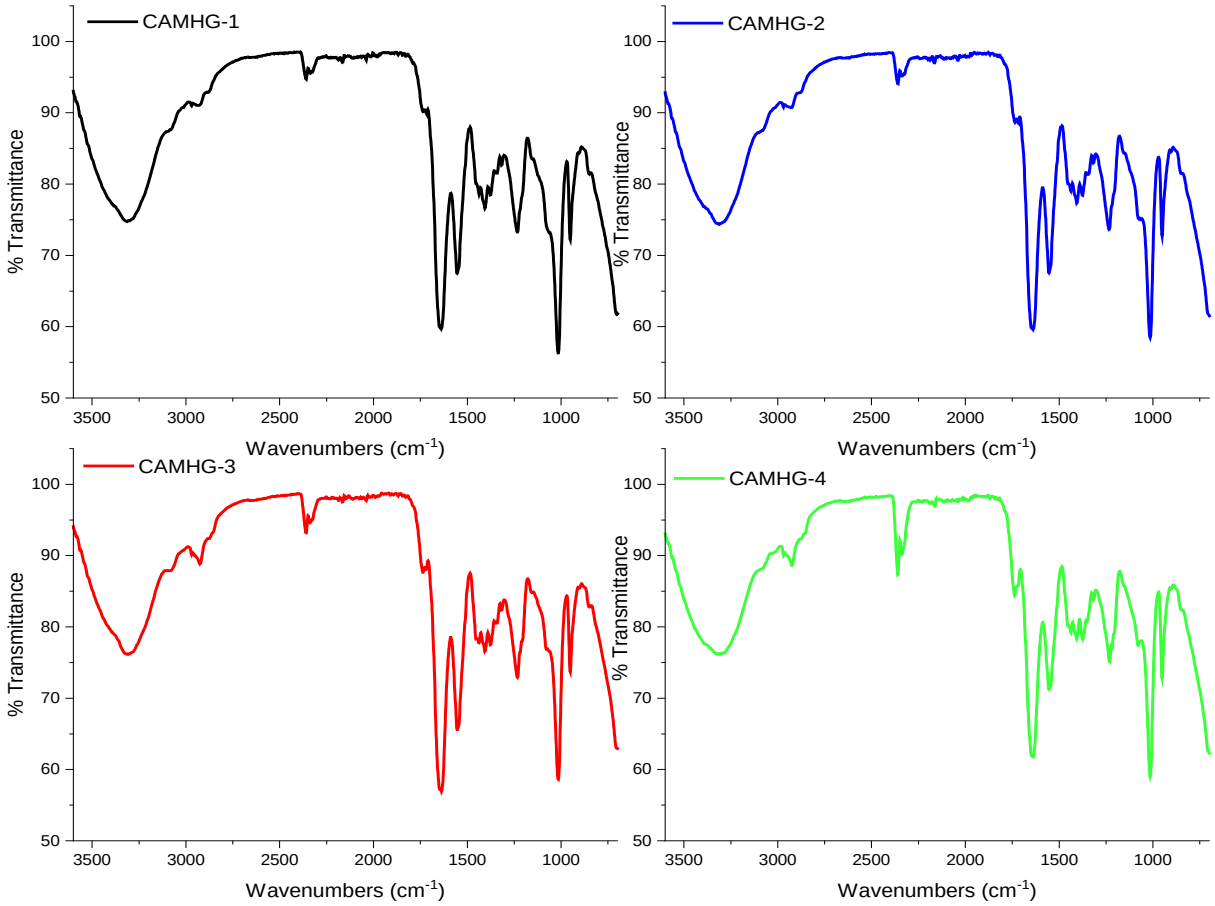

Fig. S6. ATR-FTIR characterization of CAM hydrogels.

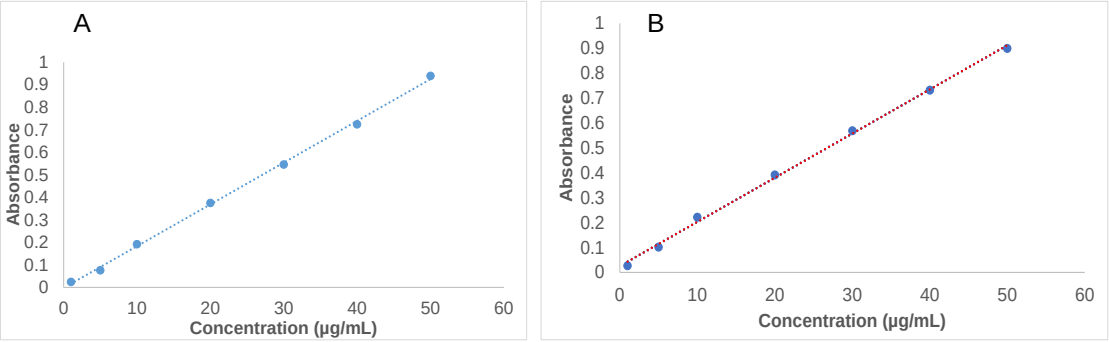

Fig. S7. Standard curves of DOX.HCl to determine drug loading efficiency (%) and drug release assessment. (A) Standard curves of DOX.HCl in PBS (pH = 7.4), and (B) Standard curves of DOX.HCl in 10 mmol GSH. Optical densities were measured by using a UV-visible spectrophotometer at 485 nm wavelength.

5. References

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