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Supporting information *for*

Bioinspired pH and Magnetic Responsive Catechol-Functionalized Chitosan Hydrogels with Tunable Elastic Properties

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Materials: The following chemicals were reagent grade and were used as received. Chitosan (92.4% deacetylated, M_w = 92 KDa, $Đ$ =1.5, Kraeber & Co GMBH), hydrocaffeic acid (HCA, 98%, Alfa Aesar), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 98%, Gl biochemicals), Iron(III) chloride ($FeCl_3$, 98%, Sigma Aldrich), Iron(II) chloride ($FeCl_2$, 98%, Sigma Aldrich), ethanol (Emsure), NaOH (Merck), $NaHCO_3$ (GCE Laboratory Chemicals), HCl (HCl, 37%, Merck), D_2O (99.9%D, Cambridge Isotope Laboratories, Inc), Dextran-FITC (M_w = 20 KDa, Sigma), and DCl (35wt%, 99.9%D, Sigma Aldrich). Deionized water was produced in house with a Milli-Q apparatus (Millipore) and used for all experiments. Regenerated cellulose dialysis membranes (MWCO 6-8 KDa) were supplied by Fisher Scientific.

General Methods: Accurate volumes (10−1000 µL) were measured with calibrated automatic pipettes (Eppendorf Research). Accurate pH values were measured with a pH meter (Jenway 3510) equipped with an electrode from Mettler Toledo (diameter=0.5cm); alternatively, a special pH indicator paper was used. Sonication of particle solutions was performed using an Elmasonic S100H sonicator from Alphasonics in the sweep mode at ~30 °C. Dialysis was performed at room temperature against water (pH 3.5) for a period of three nights using regenerated cellulose dialysis membranes whose MWCO is 6-8 KDa and water was changed twice per day.

Analytical Techniques:

Nuclear Magnetic Resonance (NMR): Spectra were acquired on a Bruker Acance I spectrometer equipped with a variable temperature (VT) module (resonance frequency of 400.13 MHz for ^{1}H) and a QNP probe. Unless otherwise specified, for ^{1}H experiments, 90° pulses and pulse sequence recycle times of 3 s were used. 1D ^{1}H spectra were obtained with 96 scans and 32K data points and were reprocessed using MestReNova software (v6.2). Chemical shifts were referenced with respect to the water peak (δ_{HDO} = 4.52 ppm at 323K). [1]

UV-Vis spectroscopy: Spectra were recorded using a Lambda 35 UV/VIS Spectrometer (PerkinElmer) using quartz cuvettes with a path length of 1cm. Mixtures of HCA-chit with and without $FeCl_3$ (HCA:Fe^{3+} / 3:1) were transferred to a cuvette, diluted, and spectra were measured before and after pH adjustment using NaOH (1 and 6M). Absorbance of hydrogels was conducted by dispersing the gels in their solutions in the cuvette.

Rheology: The rheological properties were recorded on Physica MCR501 from Anton Paar at 20 °C with a protecting cell to minimize evaporation. Solutions were subjected to dynamic (oscillatory) experiments using a cone-plate rheometer (diameter = 25 mm, angle = 1°) with an intercone-plate gap of 52 μm. Likewise, hydrogels were characterized dynamically using a parallel plate geometry (diameter = 10 mm) with an inter-plate gap less than 1mm. All experiments were conducted in the linear viscoelastic regime with a strain of 0.5% and after 2 hours of gelation. All hydrogels were initially prepared in cylindrical vials with a diameter of ~ 20 mm. After formation of the hydrogel discs, the samples were smoothly removed from the vial and placed between the rheometer plates for analysis. Once the upper plate was brought in contact with the hydrogel, the hydrogel edges were carefully trimmed down to the final diameter of 10 mm to ensure that the hydrogel was well in place between the two plates. In the case of gels treated with EDTA or HCl, rheology measurements were first conducted prior to treatment, and then immediately again after treatment for accurate comparison.

Swelling Measurements: Chemical, physical, were prepared as described below. After 9 hours, the hydrogels were weighed and their swollen mass, m_s, measured. The gels were then dried by freeze drying and their dry mass m_d measured. The mass swelling ratio was measured as:

$$Q\ (g/g) = (m_s - m_d)/m_d.$$

We also verified that the dry mass after freeze drying was the same as that before gel formation, which confirmed minute differences in dry masses attributed to residual water and metal ions.

Vibrating Sample Magnetometer (VSM): The magnetic properties of the materials were determined using a Lakeshore 7404 Vibrating Sample Magnetometer (VSM) in an applied field range of 0–10 kOe. To this end, physical and chemical hydrogels with an initial known concentration of Fe_2O_3 (2.5 and 5%wt.) were prepared and samples of known masses (10-20mg) were analyzed two hours after gelation. Samples were prepared by cutting a small piece of the gel and removing excessive water by pressing against tissue paper. The sample was then weighed and encased in Teflon tape to prevent further evaporation. These Teflon coated sample pellets were then placed in a VSM powder sample holder and the magnetic properties measured at room temperature. The total

mass of the hydrogel (gel+ MNP) was considered when measuring the magnetic properties and the results are obtained per gram of hydrogel.

Transmission Electron Microscopy (TEM): TEM studies were performed on a JEOL 2010 TEM (accelerating voltage 200 kV) at the FACTS lab facility of Nanyang Technological University. 50 mg of the magnetic nanoparticles were dispersed in 1 ml of ethanol and for 5 min sonicated. One drop of solution containing the particles was then placed on a carbon coated, formvar covered, 200 mesh Cu-grid and after 1 day of drying placed inside the TEM using a single tilt holder. Images were taken using a Gatan 794 CCD camera.

Dynamic Light Scattering (DLS): 1 ml of glycerol solution (molecular biology grade) containing 50 mg of the magnetic particles, was added to a plastic cuvette, degassed under vacuum and measured with a Malvern Zetasizer – Nano ZS equipped with a 4 mW He-Ne solid-state laser operating at 633 nm. The sample was measured at room temperature three subsequent times, each measurement consisting out of an average of 30 runs for 10 sec.

Synthesis of hydrocaffeic acid functionalized chitosan (HCA-chit): The product was synthesized based on the work of Lee and coworkers with some modifications. [2, 3] To this end, chitosan (1g, 1eq glucosamine units) was dissolved in 100 mL of 1%HCl (5.5M) overnight. The pH was adjusted to 5.2 using $NaHCO_3$, and the mixture was diluted with 50 mL ethanol. Hydrocaffeic acid HCA (0.74g in 5mL H_2O, 0.7eq) and EDC (2.18g in 6mL EtOH/H_2O: 1/1, 2eq) were successively added to the mixture whose final pH was 4.8. After 18 hours of reaction under nitrogen atmosphere and in dark, the product was purified by dialysis and freeze dried to yield a fluffy white solid. The degree of substitution, *DS*, was measured from ^{1}H NMR and was found to be 15.5% according to the following equation:

$$\%DS = \frac{A_{HCA}}{A_{CH3}} \times DA$$

Where A_{HCA} and A_{CH3} are the area of the protons of HCA at 6.85ppm and that of CH_3 of the acetyl group at 2.1ppm, respectively. *DA* is the degree of acetylation and was found to be 7.6%.

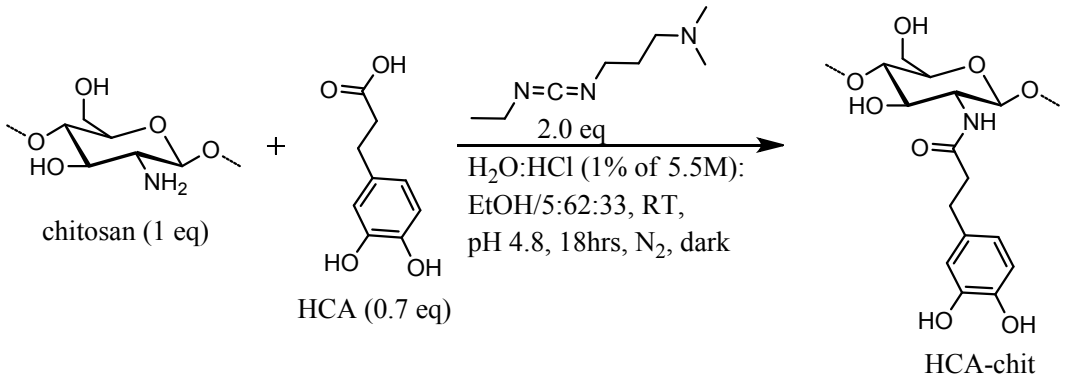
Synthesis of magnetic nanoparticles Fe_2O_3: $FeCl_3 \cdot 6H_2O$ (0.2 M) and $FeCl_2 \cdot 4H_2O$ (0.1M) were dissolved in ultrapure Milli-Q water (200 mL) and heated to 80 °C under nitrogen. 0.14 M NaOH was added dropwise over 20 min and the solution was maintained at 80 °C for 30 min. The solution was then cooled and magnetically separated. The precipitate was repeatedly washed with water till neutral pH was obtained. The particles were then dried and stored under an inert atmosphere. Magnetite nanoparticles (Fe_3O_4) were converted to maghemite (γ-Fe_2O_3) by oxidation in air at 150 °C for 1.5 hrs. The structure and composition of the maghemite was studied by a Shimadzu 6000 X-ray Diffractometer (XRD) with CuKα radiation of wavelength 1.54056 Å, in the 2θ range of 20°–70° at a scan rate of 2° min^{-1}. Phase identification was performed by matching peak positions and relative intensities to reference JCPDS files. The crystallite size was calculated using the Scherrer formula.

Synthesis of chemical and physical hydrogels from hydrocaffeic acid functionalized chitosan (HCA-chit): In a typical experiment, HCA-chit (50 mg) was dissolved in 5mL 1%HCl (5.5M) overnight. To induce chemical gelation, the pH of 1mL mixture was raised to ≅13 using NaOH (6M). For physical gel synthesis, a 1mL solution of HCA-chit was mixed with $FeCL_3$ (20μL, 0.16M) in a ratio of 1:3/Fe^{3+}:HCA. The pH was either raised to ~6 using NaOH (1M) or to 12 using NaOH (6M) to afford bis- and tris-complex physical gels, respectively. The chemical hydrogel looked yellowish-orange in color whereas the bis- and tris-complex physical hydrogels were blue-purple and dark red, respectively.

Synthesis of magnetic chemical and physical hydrogels from hydrocaffeic acid functionalized chitosan (HCA-chit): HCA-chit (50 mg) was dissolved in 5 mL 1%HCl (5.5M) overnight. Fe_2O_3 (16mg) was dissolved in water (2mL) and sonicated for one hour. To a 1mL HCA-chit solution, a respective amount of Fe_2O_3 (32 or 64 μL) was added to afford the target composition (2.5 or 5 wt.%) and the mixture was further sonicated. For chemical gelation, the pH of mixture was raised to ~13 using NaOH (6M). For the physical gel, 20 μL of $FeCl_3$ (0.16M) solution was added to the HCA-chit mixture (1mL) containing the particles in a ratio of 1:3/Fe^{3+}:HCA and the pH was raised to 12 using NaOH (6M) to afford two types of tris-complex physical hydrogels with 2.5 and 5 wt.% magnetic nanoparticles, respectively.

Release kinetics of dextran-FITC from hydrogels: HCA-chit (50 mg) was dissolved in 2.5 mL of 55 mM HCl overnight. On the next day, 2 μL of dextran-FITC solution (10 mg/mL) was added into 200 μL freshly prepared HCA-chit solution before formation of gels as described above. The gel was then transferred to the tube ended with dialysis membranes (MWCO: 100 KDa) in both sides, and the gel-containing tube was further exposed to either pH 12 buffer solution (phosphoric acid/di-Sodium hydrogen phosphate solution, Sigma), or pH 6 phosphate buffered saline solution with constant shaking. At predetermined point, the buffer solutions were periodically collected and replenished with fresh ones. The fluorescence intensity of the collected solutions was measured on an Infinite M200 microplate reader (Tecan, Switzerland) at an excitation wavelength of 495 nm and an emission wavelength of 518 nm. The dextran-FITC concentration in the solution was calculated based on the standard curve calibrated with dextran-FITC solutions of known concentrations. For rhodamine B loading, the details of the preparation of MNP-incorporated physical gels were given below as a typical example. 2 μL of rhodamine B solution (10 mg/mL) and 8 μL of Fe_2O_3 (25 mg/mL) were first added into 200 μL freshly prepared HCA-chit solution in a 2 mL Eppendorf tube. Then, 5 μL of $FeCl_3$ solution (26 mg/mL) was mixed with above solution mixture and pH was raised to ~13 by adding 15 μL of NaOH (6M). After 30 min, the hydrogel was transferred into dark room for fluorescence imaging with UV lamp. The preparation method of chemical and physical hydrogels is the same as above without adding $FeCl_3$ solution (for chemical gels) or MNP solution (for physical gels).

Figures and Schemes



Scheme S1. Synthetic scheme for the synthesis of hydrocaffeic acid functionalized chitosan.

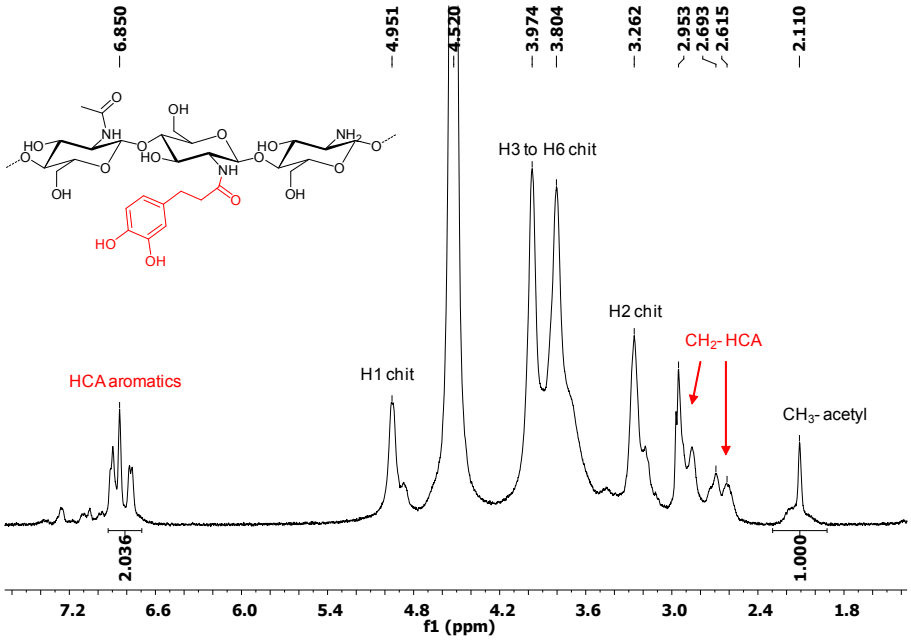


Figure S1. [1]H NMR spectrum of hydrocaffeic acid functionalized chitosan (HCA-chit). Conditions: 400.13 MHz, 9mg/mL, DCl (50 mM), 323K. Note that the spectrum is referenced with respect to the HDO peak at 4.52 ppm. [1]

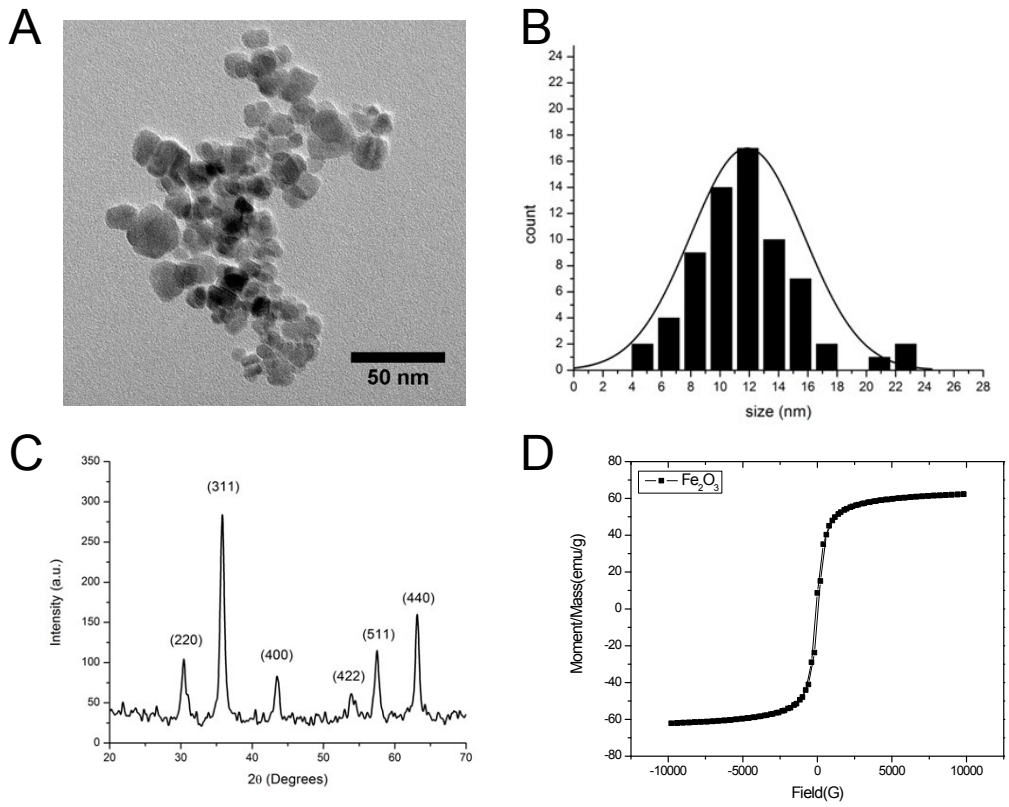


Figure S2. (A) Bright field TEM micrographs of γ-Fe_2O_3 nanoparticles. **(B)** Size distribution of MNPs, with an average size of ~11.8 nm and a standard deviation of 3.8. **(C)** XRD patterns of MNPs with peaks matching those of γ Fe_2O_3. **(D)** Room temperature VSM measurement of synthesized Fe_2O_3 shows a magnetization value of 62 emu/g with a weak hysteresis behaviour.

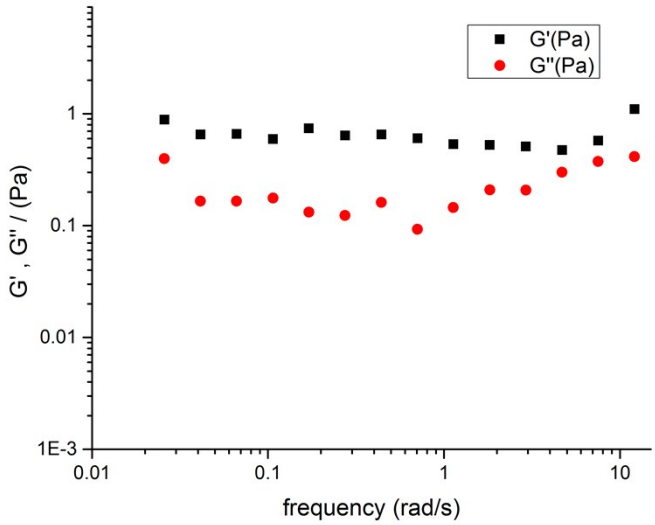


Figure S3. Rheology properties of HCA-Chit/Fe^{3+} at pH 3. The storage modulus is in the range 0.8 - 1 Pa, which is orders of magnitude lower than at pH 6 and pH 13 (compare with Fig. 2A of the main text).

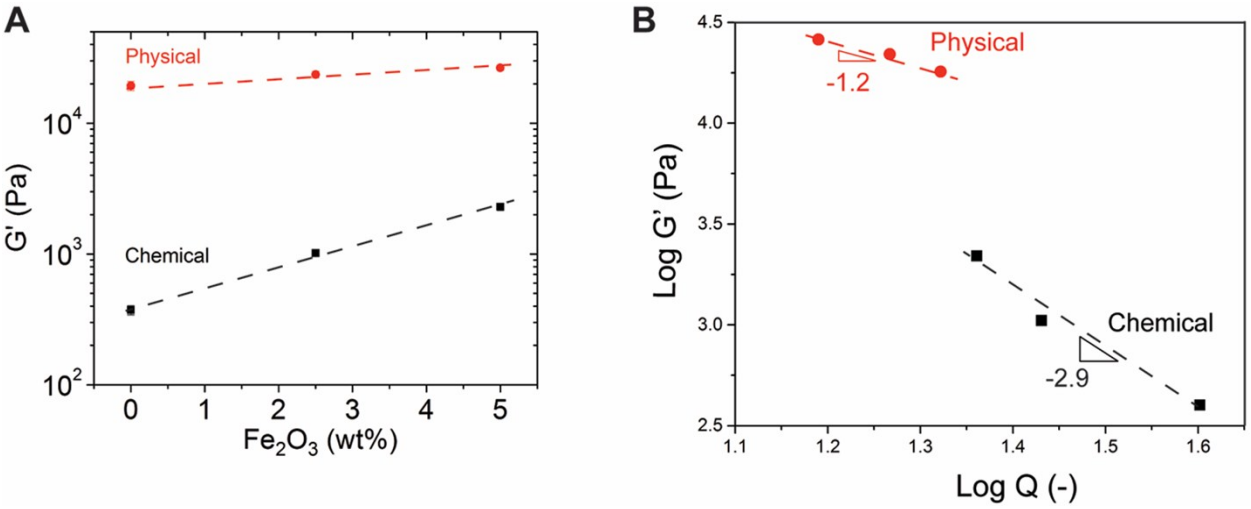


Figure S4. (A) Variation of *G′ vs.* Fe_2O_3 MNPs for the chemical and the physical hydrogels. *G′* values from Fig. 3A of the main text are plotted at 1Hz. **(B)** Storage modulus *G*' versus swelling ratio *Q* (log-log scale) for both type of gels.

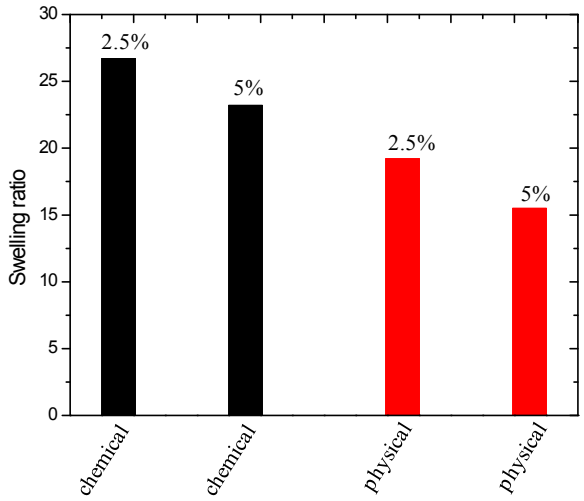


Figure S5. Swelling ratio of chemical and physical hydrogels at two concentrations of Fe_2O_3 (2.5 and 5 wt%.).

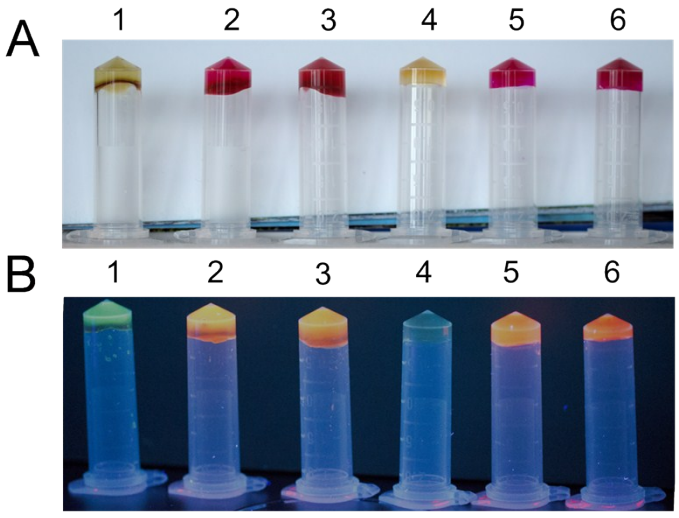


Figure S6. White light images **(A)** and fluorescent images **(B)** of 1, chemical gel; 2, chemical gel loaded with rhodamine B; 3, chemical gel loaded with rhodamine B and MNPs; 4, physical gel; 5, physical gel loaded with rhodamine B; 6, physical gel loaded with rhodamine B and MNPs.

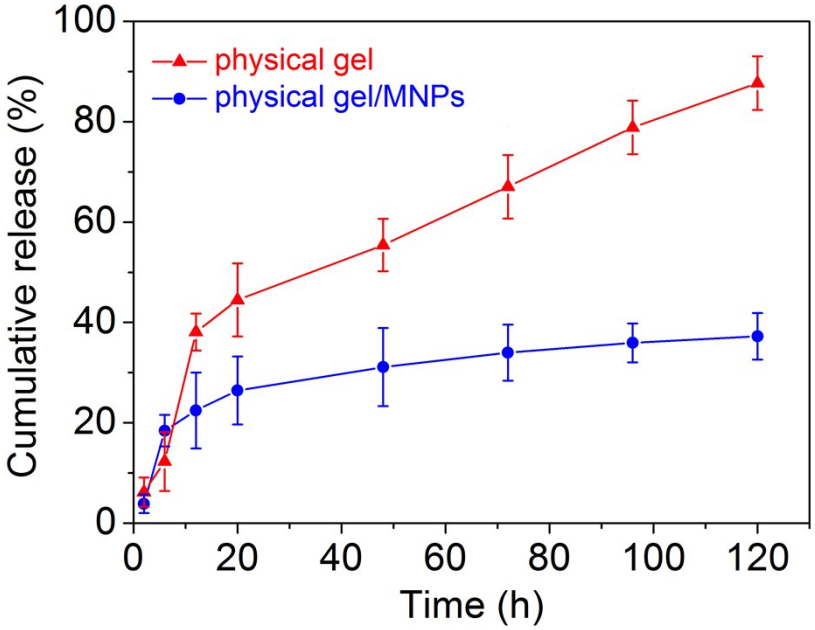


Figure S7. Time-dependent release of dextran-FITC (MW: 20,000) from physical hydrogels at pH 6. The data represent the mean ± SD (n = 3).

References

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2. K. Kim, J. H. Ryu, D. Y. Lee and H. Lee, *Biomater. Sci.* 2013, **1**, 783-790.
3. J. H. Ryu, Y. Lee, W. H. Kong, T. G. Kim, T. G. Park and H. Lee, *Biomacromolecules*, 2011, **12**, 2653-2659.