

## Supporting Information

### **Title: “Smart” drug loaded nanoparticle delivery from self-healing hydrogel enabled by dynamic magnesium-biopolymer chemistry†**

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## **MATERIALS AND METHODS**

*Materials.* Tetraethyl orthosilicate (TEOS), magnesium chloride, ammonium chloride, DL-dithiothreitol, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxybenzotriazole (HOBt), doxorubicin hydrochloride, Hoechst 33342, formaldehyde solution, and 10000U/mL penicillin - 10 mg/mL streptomycin (Penn-Strep) solution were purchased from Sigma-Aldrich. HA (150 kDa) was purchased from Lifecore Biomedical (US). Initiator Irgacure 2959 was purchased from BASF. Ammonia aqueous was purchased from Scharlau. Dulbecco's modified eagles medium (DMEM) with high glucose, fetal bovine serum (FBS), were purchased from GE Healthcare HyClone. 3,3'-Dithiobis(propionic hydrazide) **1** was prepared according to the literature procedures.<sup>[1]</sup>

*Preparation of silica nanoparticles.* Monodispersed silica nanoparticles were prepared by Stöber method with slight modification.<sup>[2]</sup> Briefly, 4.5 mL of TEOS, 9 mL of ammonia aqueous, 61.75 mL of ethanol and 24.75 mL of H<sub>2</sub>O were magnetically stirred together under room temperature for 2 hours. Silica nanoparticles were collected by centrifugation (4000 rpm) and washed with ethanol 2 times.

*Preparation and characterizations of MgSiO<sub>3</sub> nanoparticles.* 100 mg of silica nanoparticles were suspended homogeneously in 20 mL of H<sub>2</sub>O. 71.4 mg of magnesium chloride, 534.9 mg

of ammonium chloride and 1 mL of ammonia aqueous were dissolved in 20 mL of H<sub>2</sub>O.<sup>[3]</sup> The dispersion of silica nanoparticles was homogeneously mixed with the solution of magnesium chloride and ammonia and the combined mixture was transferred into an autoclave. The hydrothermal synthesis was performed at 160°C for 12 hours.<sup>[3]</sup> MgSiO<sub>3</sub> nanoparticles were collected by centrifugation and washed with H<sub>2</sub>O and ethanol. The characterization of MgSiO<sub>3</sub> nanoparticles were performed by SEM (Zeiss 1550, Germany), EDXS, and TEM (FEI Tecnai F30, US). N<sub>2</sub> adsorption and desorption (ASAP 2020, Micromeritics) was used to measure pore distribution of MgSiO<sub>3</sub> nanoparticles.

*Synthesis of HA-BP biopolymer.* HA with covalently attached bisphosphonate groups was synthesized in two steps according to our previously reported procedure.<sup>[4,5]</sup> In the first step, native HA was modified with thiol groups to give HA-SH derivative. Briefly, HA (400 mg, 1 mmol of disaccharide repeating units) was dissolved in 25 mL distilled water. Dihydrazide linker **1** (11.9 mg, 0.05 mmol) and HOBt (135 mg, 1 mmol) dissolved in 6 mL of acetonitrile:water mixture (1:1 v/v) were added into the HA solution. The pH of reaction mixture was adjusted to 4.7, after which EDC (57.5 mg, 0.3 mmol) was added. The reaction mixture was subsequently stirred overnight at room temperature. Reaction mixture of pH was then increased to 8.5 with 1 M NaOH solution followed by addition of DTT (54 mg, 0.25 mmol). The mixture was stirred for another day and finally dialyzed against acidified water (pH 3.5) containing 0.1M NaCl using dialysis tube with MW cutoff of 3.5 kDa. After that, the dialysis against acidified water (pH 3.5) was performed two times more and the dialyzed solution was lyophilized. Yield of HA-SH was 390 mg. The structure of HA-SH was analyzed by <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O). In the second step, HA-SH (200 mg) was dissolved in 12.5 mL of degassed distilled water. Acrylated bisphosphonate derivative **2** was added into HA-SH solution at BP:thiol molar ratio of 6:1 followed by addition of photoinitiator Irgacure 2959 (4 mg). The thiol-ene addition reaction between HA and **2** was initiated by exposure to

ultraviolet light (36W UV timer lamp, CNC international BV, Netherlands) for 10 min under stirring. Thereafter, the reaction mixture was dialyzed against acidified water (pH 3.5) containing 0.1M NaCl once and against acidified water (pH 3.5) twice in dialysis tube with MW cutoff of 3.5 kDa. The dialyzed solution was finally lyophilized to obtain 190 mg of HA-BP. The structure of HA-BP was analyzed by  $^1\text{H}$  NMR (400 MHz,  $\text{D}_2\text{O}$ ) and  $^{31}\text{P}$  NMR (400 MHz,  $\text{D}_2\text{O}$ ).

*Preparation and characterizations of  $\text{MgSiO}_3\cdot\text{HA-BP}$  composite hydrogel.* The hydrogel was formed upon mixing equal volumes of 4% (w/v) of HA-BP solution and 12% (w/v) of dispersion of  $\text{MgSiO}_3$  nanoparticles in water. Rheological characterizations of composite hydrogel were performed using AR2000 Advanced Rheometer (TA Instruments) with 8 mm diameter of aluminum parallel plate geometry at room temperature. In order to investigate the kinetics of hydrogel formation, 25 minutes after the mixing, the still liquid composition was placed on a rheometer plate and mechanical properties were measured as a function of time sweep to monitor changes of storage modulus ( $G'$ ) and loss modulus ( $G''$ ). Moreover, frequency sweep experiment (from 0.1 to 1 Hz) at a fixed 10% of strain was also performed on a hydrogel after complete setting. In order to investigate the self-healing properties of HA-BP• $\text{MgSiO}_3$  composite hydrogel, storage modulus ( $G'$ ) and loss modulus ( $G''$ ) of hydrogel were monitored with sequential shear strains of low (10%) -high (500%)-low (10%) sweep at fixed frequency (1 Hz). Strain sweep of hydrogel was performed from 1% to 500% strain at fixed 1 Hz to study shear-thinning properties of hydrogel. Additionally, SEM was used to observe microstructure of lyophilized  $\text{MgSiO}_3\cdot\text{HA-BP}$  composite hydrogel. Herein, HA-BP with 22.5% BP modification and molecular weight 150 kDa was used.

*Stability of  $\text{MgSiO}_3\cdot\text{HA-BP}$  composite hydrogel in PBS at different pH values.* Composite hydrogel was formed by mixing 200  $\mu\text{L}$  of 4% (w/v) of HA-BP solution and 200  $\mu\text{L}$  12%

(w/v) of dispersion of MgSiO<sub>3</sub> nanoparticles. Hydrogel after complete setting was placed into 3 mL of PBS (pH of the buffer was adjusted either to 5.0 or 7.4). MgSiO<sub>3</sub> nanoparticles were collected with centrifugation at each time point of 1, 2, 4, 8, and 24 hours, followed by washing with distilled water and methanol. The incubation PBS (3 mL) was refreshed at each time points. The collected nanoparticles were dried under vacuum. The mass of the released MgSiO<sub>3</sub> nanoparticles was measured.

*Dox loading into MgSiO<sub>3</sub> nanoparticles.* 1.4 mg of Dox was dissolved in 32 mL of H<sub>2</sub>O, and 80 mg of MgSiO<sub>3</sub> nanoparticles was added into the above solution. Mixture was magnetically stirred under room temperature overnight. MgSiO<sub>3</sub>@Dox nanoparticles were collected by centrifugation at 4000 rpm. The free drug was removed together with the supernatant. Loading of Dox into the nanoparticles was determined by measuring the unbound drug in the supernatant by fluorescence spectroscopy (excitation wavelength = 490 nm). Loading efficiency was calculated using the following equation:

$$\text{Loading efficiency} = \frac{M(\text{initial Dox}) - M(\text{Dox in supernatant})}{M(\text{initial Dox})}$$

*Release of free Dox in PBS at different pH values.* Dox loaded hydrogel was prepared by mixing 100 µL of 4% (w/v) HA-BP solution and 100 µL of 12% (w/v) MgSiO<sub>3</sub>@Dox nanoparticles dispersion. The hydrogel was incubated with 3 mL of PBS at different pH values (5.0 and 7.4). At each time point (1, 2, 4, 8, and 24 hours), PBS was collected and refreshed with the new 3 mL of PBS. The supernatant was collected after centrifugation, and fluorescence emission spectrum of the supernatant was recorded (excitation wavelength = 490 nm). The MgSiO<sub>3</sub>@Dox nanoparticles released from the hydrogel during incubation and separated by centrifugation were subsequently placed in 3 mL of PBS under different pH

values (5.0 and 7.4) for another 10 days. The nanoparticles were again centrifuged out and the supernatant was analyzed by fluorescence spectroscopy to detect the released free Dox.

*Toxicity study with hydrogel released media.* Human breast cancer cell line (MCF-7) was used to study *in vitro* cytotoxicity of MgSiO<sub>3</sub>@Dox nanoparticles derived from acidic (pH 5.0) decomposition of HA-BP•MgSiO<sub>3</sub>@Dox hydrogel. Composite hydrogel was formed under sterile conditions by mixing 100 µL of 4% (w/v) HA-BP solution with 100 µL of 12% (w/v) MgSiO<sub>3</sub>@Dox nanoparticles dispersion. It corresponded to 200 µg of Dox loading per hydrogel sample. The hydrogels were put into 3 mL PBS at pH 5.0 similarly to the release study. The released media were collected at different time points (1, 2, 4, 8, and 24 hours) after which a fresh PBS was added to the hydrogel samples. MCF-7 cells were cultured in DMEM medium containing 10% (v/v) FBS and 1% (v/v) Penn-Strep at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. 7000 cells in 190 µL cell culture medium were added into each well of a 96-well plate. The plate was placed in incubator for 2 h to ensure the cells attachment. 10 µL of the medium derived from the incubation of HA-BP•MgSiO<sub>3</sub>@Dox hydrogel in PBS at pH 5.0 was added to each well with the cells. This medium contained the released MgSiO<sub>3</sub>@Dox nanoparticles and polymeric HA-BP resulted after the hydrogel decomposition. The medium collected at different time points was examined for the toxicity. After incubation for 24 hours, the cells were washed several times using fresh PBS and the cells viability was analyzed using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent kit (Promega, USA) according to the manufacturer's protocol. Briefly, 20 µL of MTS reagent together with 180 µL of medium was added into each well and the plate was incubated at 37°C for 2 hours. The optical density (OD) of each sample was determined at 490 nm using plate reader (Infinite® 200 PRO, Tecan, Switzerland). The control group was performed using the composite hydrogel without

loaded Dox. Blank control group was used to normalize the cells viability, for which 10  $\mu$ L of cell culture medium was used in place of the medium derived from the hydrogels incubation.

*Cellular uptake of Dox@MgSiO<sub>3</sub> nanoparticles decomposing from hydrogel.* MCF-7 cells in cell culture medium were seed onto 96-well plate (5000 cells in 190  $\mu$ L/well) and grown to near 70-80% confluence. 10  $\mu$ L of the medium derived from the incubation of the Dox@MgSiO<sub>3</sub>•HA-BP hydrogel under pH 5.0 and collected at time point of 8 hours was added into each well. The cells were incubated at 37°C for 3 hours, and subsequently washed with PBS for several times to remove MgSiO<sub>3</sub>@Dox nanoparticles that were not internalized by the cells. 200  $\mu$ L of formaldehyde (4%) in PBS was added into each well for 10 min at room temperature to fix the cells. The cells were subsequently washed with PBS for 3 times, incubated with 200  $\mu$ L of Hoechst 33342 (10  $\mu$ g/mL) for 15 min at room temperature to stain the cells nuclei, and finally washed with PBS for 3 times. Cellular uptake of MgSiO<sub>3</sub>@Dox nanoparticles was observed by fluorescence microscopy (ECLIPSE-Ti, Nikon, Japan) using red channel for Dox and UV channel for Hoechst 33342.

*Statistical analysis:* All quantitative experiments were performed with three samples and the results were shown as means  $\pm$  SD. Statistical analyses were carried out using ANOVA with a Student's T-Test (\*\*p<0.01).

## RESULTS

*Preparation of MgSiO<sub>3</sub> nanoparticle and HA-BP biopolymer.* Silica nanoparticles were prepared by Stöber method as previously reported.<sup>[2]</sup> The size of silica nanoparticles was determined by scanning electron microscopy (SEM). According to SEM analysis, the particles were found with the size around 350 nm (Fig. S1a). Further, hydrothermal treatment of SiO<sub>2</sub> nanoparticles with ammonia in the presence of MgCl<sub>2</sub> allowed us to prepare MgSiO<sub>3</sub>

nanoparticles. Detailed morphology of  $\text{MgSiO}_3$  nanoparticles was observed by SEM and transmission electron microscopy (TEM) (Fig. S1b and Fig. S1c). TEM images show that the size of  $\text{MgSiO}_3$  nanoparticles was around 350 nm. TEM images revealed also the hollow structure of  $\text{MgSiO}_3$  nanoparticles with a dark edge and grey center. This type of structure was expected basing on the assumption that dissolution of  $\text{SiO}_2$  and its conversion into  $\text{MgSiO}_3$  takes place from the surface of silica nanoparticles. This process should lead first to the formation of silica core-magnesium silicate shell type of particles.<sup>[3]</sup> Formation of  $\text{MgSiO}_3$  shell may subsequently slower the formation of magnesium silicate phase in the core of the particles after silica dissolution. The surface of  $\text{MgSiO}_3$  nanoparticles was extremely rough with many needle-like nano-spikes. Energy-dispersive X-ray spectroscopy (EDXS) analysis of the nanoparticles showed that they contained Mg, Si, and O elements (Figure S1d). Moreover, porosity of hollow  $\text{MgSiO}_3$  nanoparticles was investigated by  $\text{N}_2$  adsorption and desorption analysis (Fig. S1e,f). The  $\text{N}_2$  isotherm of  $\text{MgSiO}_3$  nanoparticles was of type IV indicating mesoporous structure of  $\text{MgSiO}_3$  hollow nanoparticles. The average pore size determined from the absorption curve was 1.35 nm. Brunauer-emmett-teller (BET) surface area of  $\text{MgSiO}_3$  nanoparticles calculated from  $\text{N}_2$  isotherm was 268  $\text{m}^2/\text{g}$ . Basing on the obtained data, we could conclude that  $\text{MgSiO}_3$  nanoparticle prepared through hydrothermal method was mesoporous with high surface area making these nanoparticle promising as drug carriers.

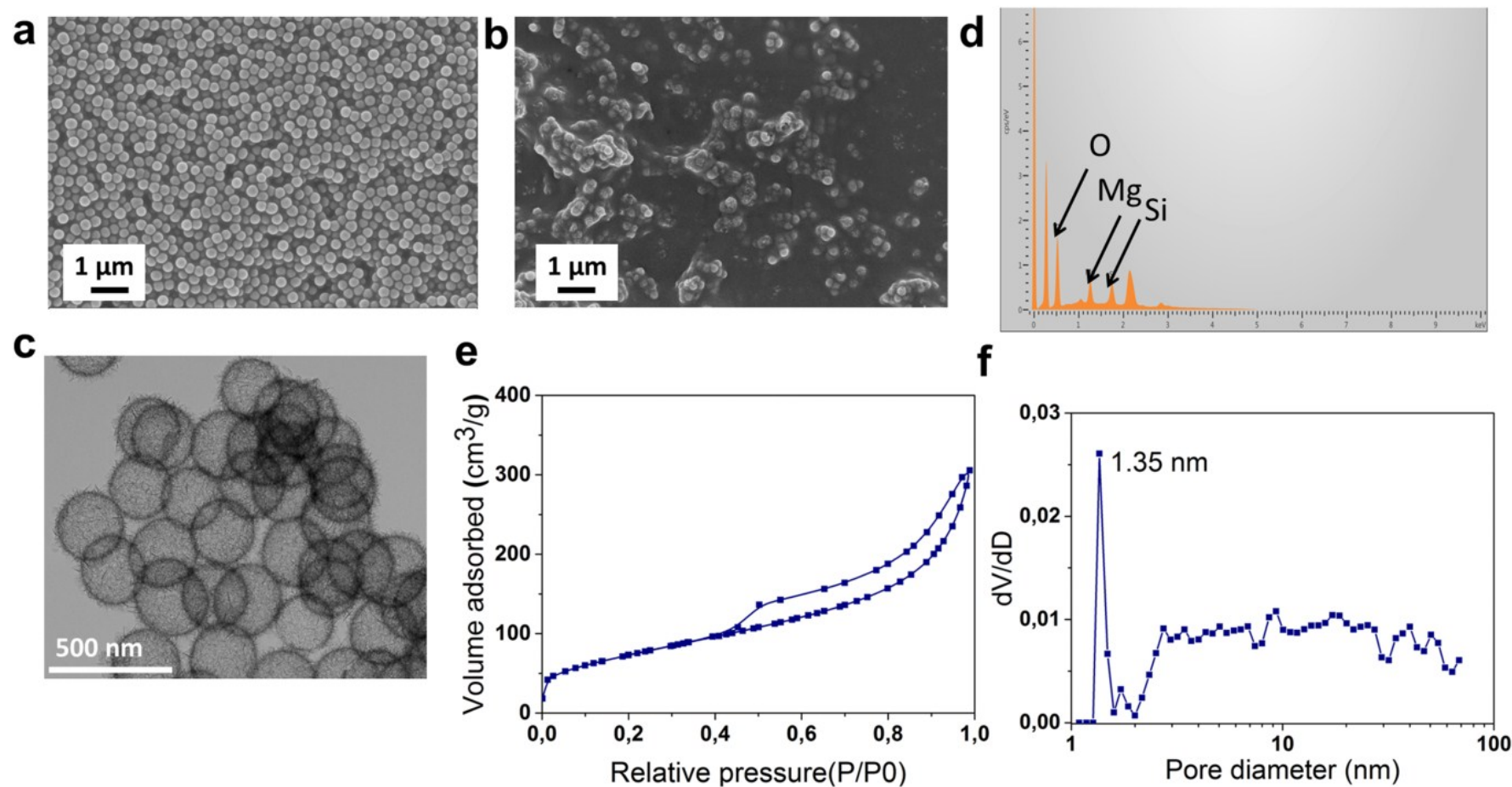
*Preparation of HA-BP biopolymer.* The synthesis of HA-BP was performed according to our established procedure,<sup>[4,5]</sup> i.e. in two steps (Fig. S2). In the first step, thiolated HA (HA-SH) was prepared by carbodiimide (EDC) mediated coupling of carboxylate groups of native HA with hydrazide groups of disulfide linker **1** followed by in situ reduction of a disulfide bond with dithiothreitol (DTT). The degree of modification with thiol groups (9 %) was verified by  $^1\text{H}$ -NMR by comparison of integration of the  $-\text{CH}_2\text{CH}_2\text{SH}$  side chain peaks at 2.81 and 2.68 ppm with the acetamide group of the *N*-acetyl-D-glucosamine residue of HA backbone (Fig.

S3c). BP groups were subsequently attached to HA backbone using photo-initiated thiol-ene addition reaction between thiol groups of HA-SH and acrylated BP reagent **2** (Fig. S2). The structure of HA-BP was confirmed by  $^1\text{H}$ -NMR analysis which showed appearance of peak at 2.18 ppm that is characteristic for methylene protons adjacent to the bridging carbon of BP ( $-\text{CH}_2\text{C}(\text{OH})(\text{PO}_3\text{H}_2)_2$ ). Degree of BP modification calculated by comparing integral of this peak with the integral for the acetamide moiety at 1.9 ppm was found to be 22.5% which pointed that in average 2.5 groups were linked per one sulfhydryl group (Fig. S3a). Additionally, phosphorus peak at 18.27 ppm was observed by  $^{31}\text{P}$ -NMR (Fig. S3b) which further confirmed successful conjugation of BP groups to HA backbone.

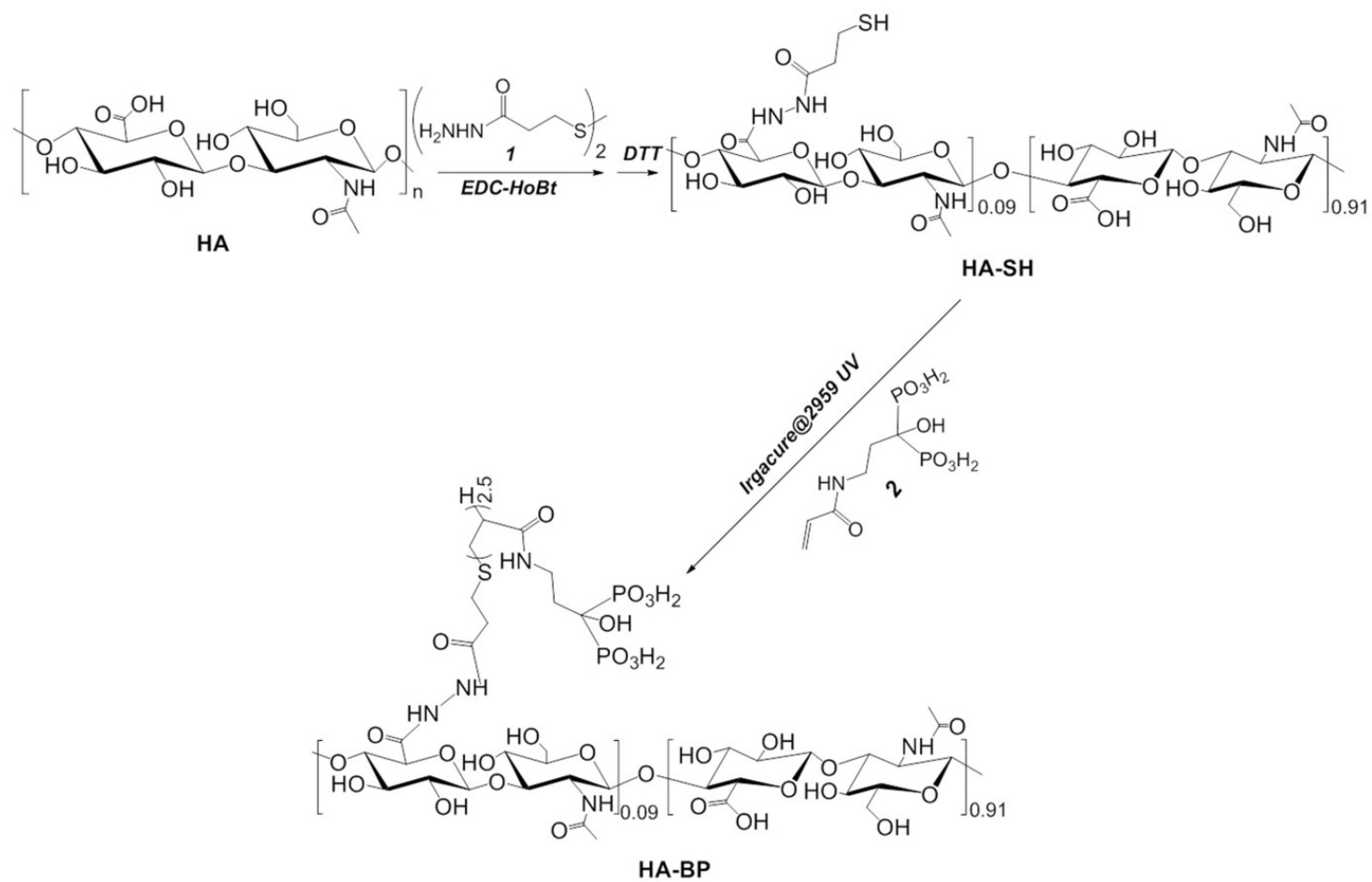
**Table S1.** Hydrogel formation via coordinate bonds depends on concentration of chelator groups and metal ions and hence on concentration of HA-BP and  $\text{MgSiO}_3$  components.

<i>HA-BP concentration (w/v %)</i>	<i>MgSiO<sub>3</sub> NPs concentration (w/v %)</i>	<i>State of the mixture</i>
0.5	6	Liquid
1	6	Liquid
1.5	6	Liquid
2	6	Gel
2	4	Weak gel
2	2	Liquid

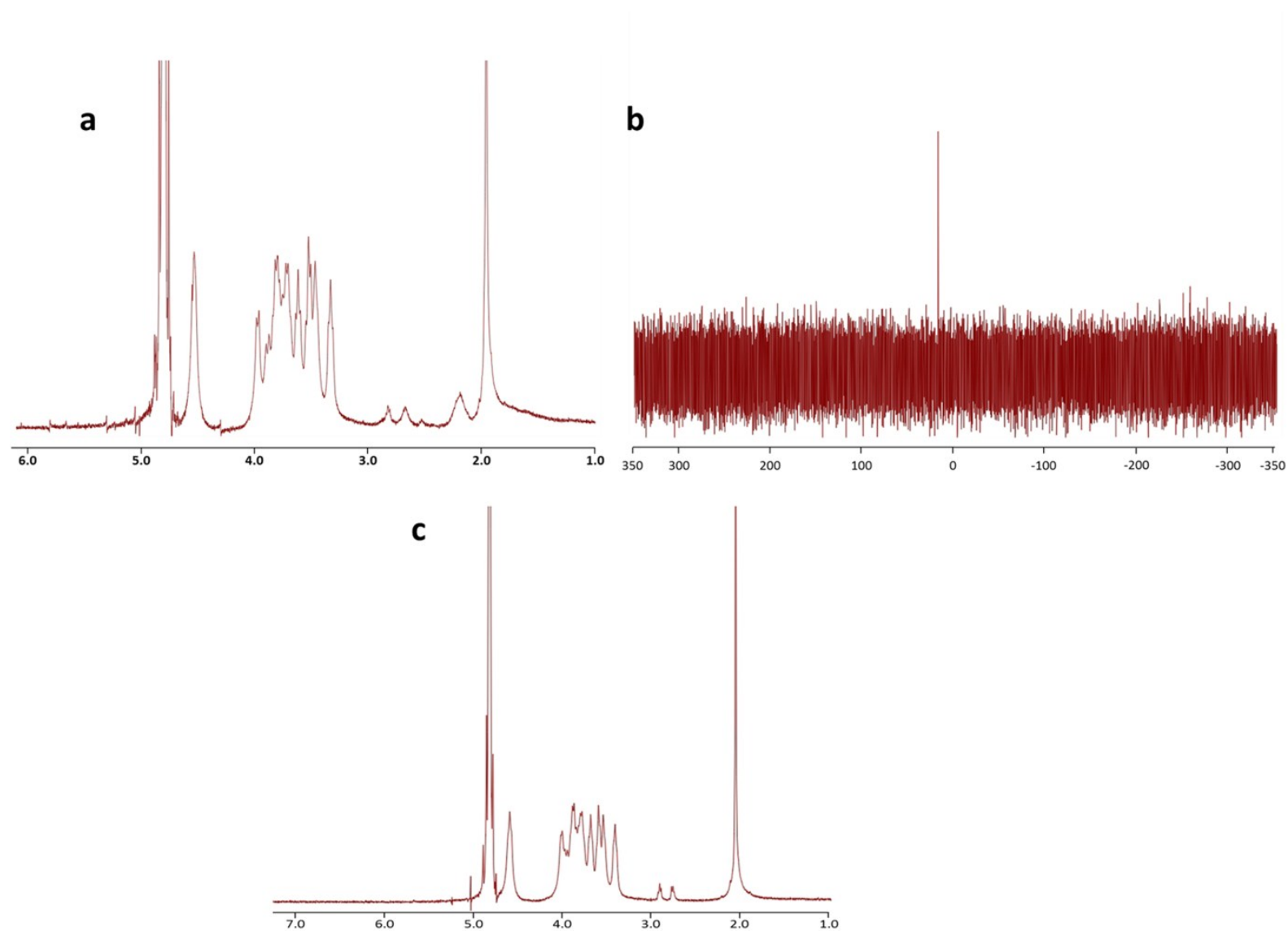




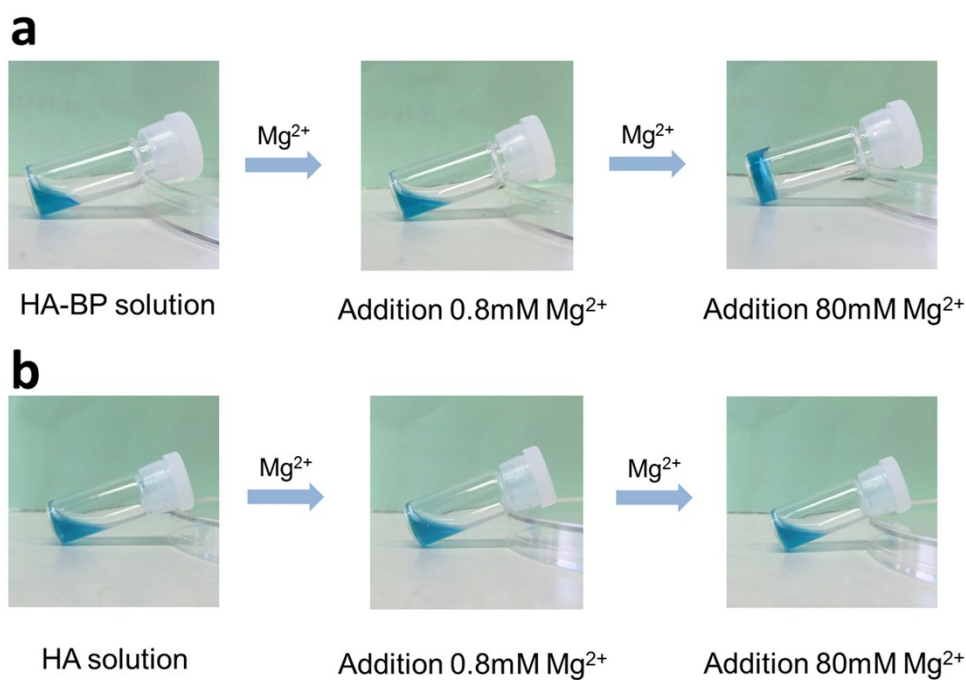
**Fig. S1.** (a) SEM image of silica nanoparticles. (b) SEM image of MgSiO<sub>3</sub> nanoparticles. (c) TEM image of MgSiO<sub>3</sub> nanoparticles. (d) EDX spectrum of MgSiO<sub>3</sub> nanoparticles showing the presence of magnesium, silicon, and oxygen. (e) N<sub>2</sub> adsorption-desorption isotherm of MgSiO<sub>3</sub> nanoparticles. (f) Pore-size distribution curve.



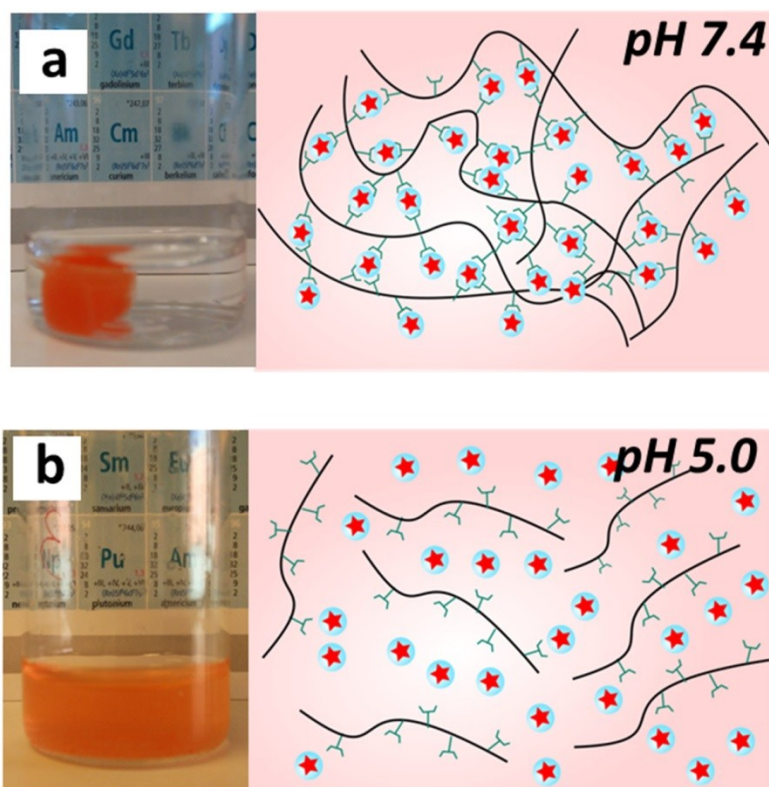
**Fig. S2.** Synthesis of HA-BP.



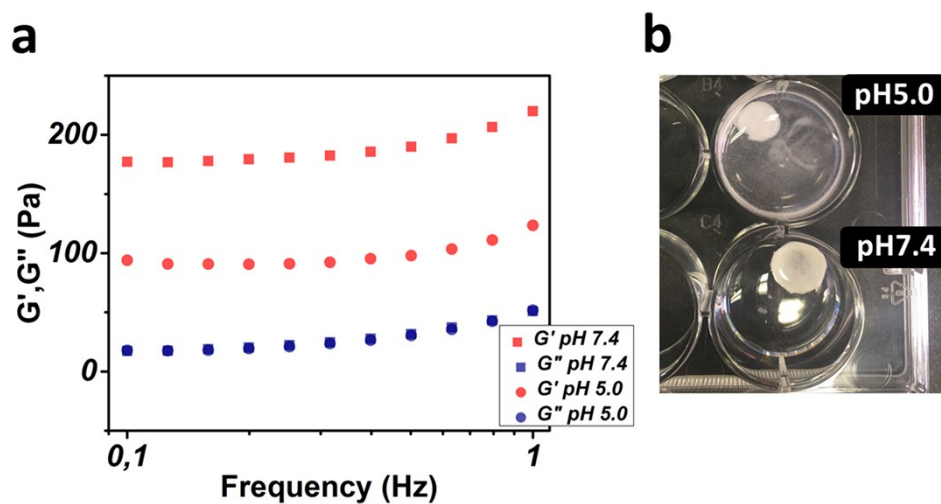
**Fig. S3.** (a)  $^1\text{H}$ -NMR spectrum and (b)  $^{31}\text{P}$ -NMR spectrum of HA-BP. (c)  $^1\text{H}$ -NMR spectrum of precursor HA-SH derivative.



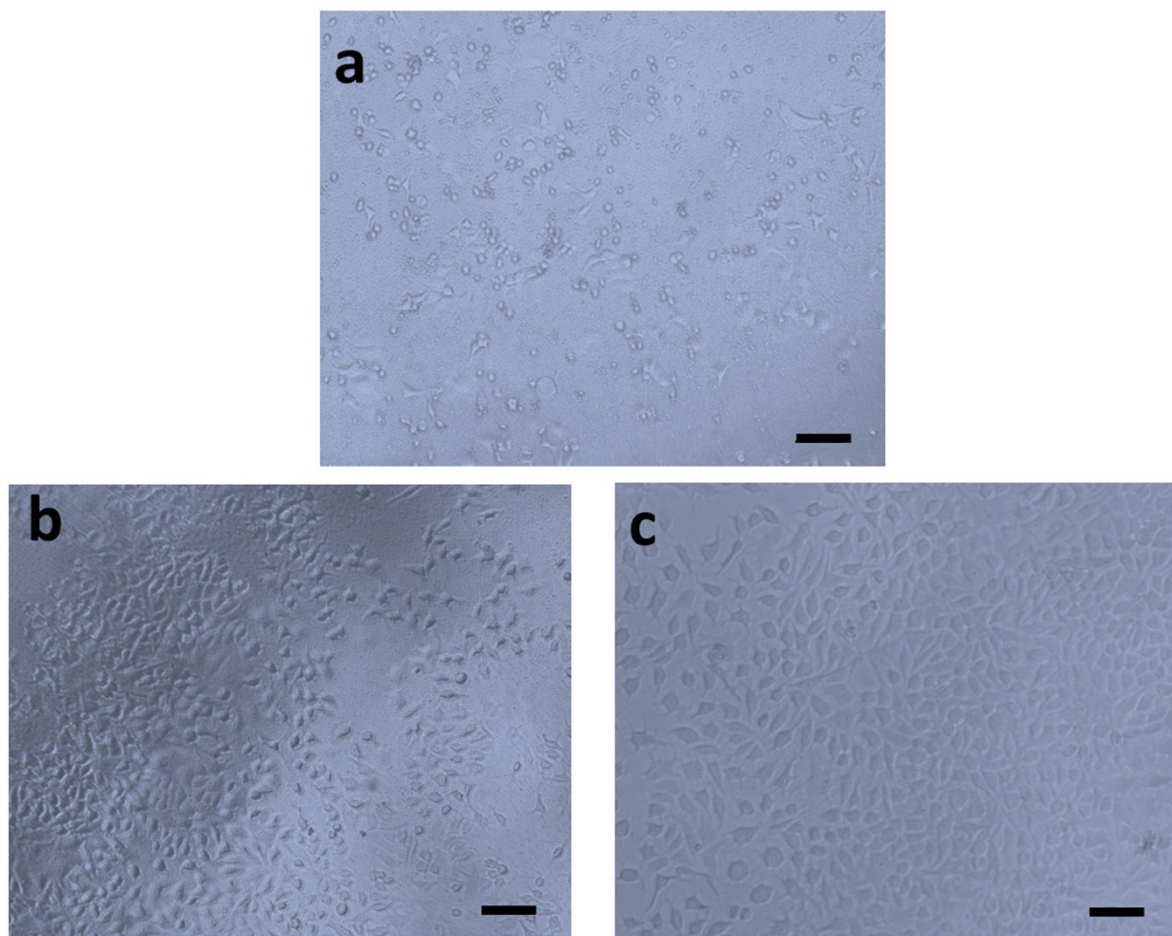
**Fig. S4.** (a) Hydrogel formation upon stepwise addition of low (0.8 mM) and highly (80 mM) concentrated  $MgCl_2$  solution to HA-BP solution. (b) Analogous addition of  $MgCl_2$  to the unmodified HA yields no gel. The concentration of hyaluronic acid in both cases is 4%. The hydrogel is formed only in case of (HA-BP +  $Mg^{2+}$ ) system with soluble  $Mg^{2+}$  ions concentration of 80 mM. Alcian blue dye was present in hyaluronic acid solutions as a stain for contrast visualization.



**Fig. S5.** pH dependent stability of HA-BP•MgSiO<sub>3</sub> composite hydrogel. (a) Hydrogel was stable after incubation in PBS for 5 days at pH 7.4. (b) Hydrogel decomposed completely after incubation in PBS for only 24 hours at pH 5.0.



**Fig. S6.** After preparation of HA-BP•MgSiO<sub>3</sub> composite hydrogels, they were incubated in either neutral (pH 7.4) or acidified (pH 5.0) PBS for 1 hour and evaluated by rheology. (a) Frequency sweep experiment performed for HA-BP•MgSiO<sub>3</sub> hydrogel after incubation. (b) Images of HA-BP•MgSiO<sub>3</sub> hydrogels after swelling at two different pH. It was evident that acidic incubation medium became turbid indicating the release of nanoparticles.



**Fig. S7.** MCF-7 cells morphology after incubation with the released medium derived from HA-BP•MgSiO<sub>3</sub>@Dox hydrogel (a) and from the drug-free HA-BP•MgSiO<sub>3</sub> hydrogel (b) at pH 5.0 and 24 hours of incubation. The cells morphology without any treatment is shown in (c). Scale bar is 100 μm.

## REFERENCES

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