Supporting Information

Modular Approach to Functional Hyaluronic Acid Hydrogels Synthesis Using Orthogonal Chemoselective Reactions

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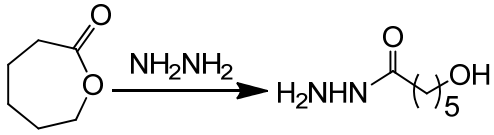
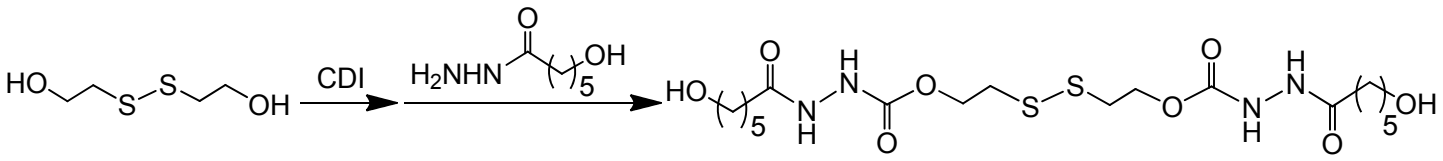
General. 1,1'-Carbonyldiimidazole (CDI), 1-aminomethylpyrene hydrochloride, ε-caprolactone, DL-dithiothreitol, and hydrazine were purchased from Aldrich Chemical Co. *N*-hydroxybenzotriazole (HOBt), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), 2,2'-dithiobisethanol, and hyaluronic acid (HA) sodium salt of the molecular weight 130 kDa was purchased from Lifecore Biomedical. The reagents were used as received. 3,3'-Dithiobis(propionic hydrazide) **2**[1] and 2-(2-pyridinyldisulfanyl)ethanol[2] were synthesized according to the literature procedures. All solvents were of analytical quality (*p.a.*) and were dried over 4Å molecular sieves. Dialysis membranes Spectra/Por® 6 (3500, g/mol cut off) were purchased from VWR International. The NMR experiments (δ scale; *J* values are in Hz) were carried out on Jeol JNM-ECP Series FT NMR System at a magnetic field strength of 9.4 T, operating at 400 MHz for ^{1}H. UV-vis absorption spectra were recorded using UV-Vis spectrometer (Lambda 35, PerkinElmer Instrumdents). Fluorescence spectra were recorded on LS 45 Luminescence Spectrometer (PerkinElmer Instrumdents).

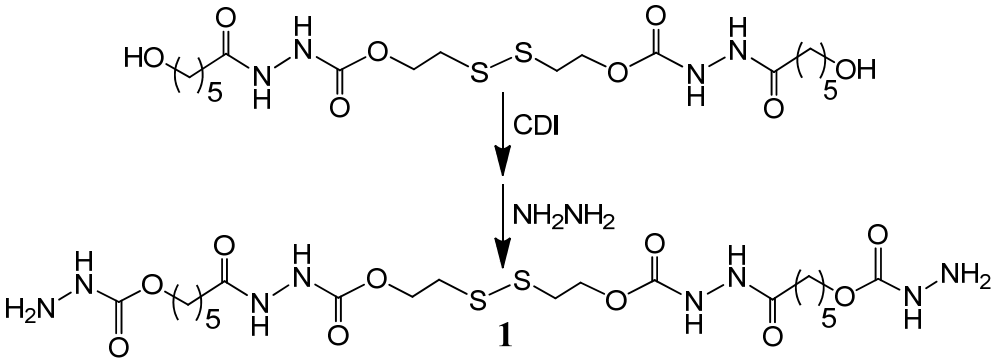


6-Hydroxycaproic acid hydrazide. ε-Caprolactone (4.4 mL, 41.23 mmol) was dissolved in 40 mL of ethanol. Hydrazine (2 mL, 41.23 mmol) was added dropwise to the obtained solution under vigorous stirring. The reaction mixture was heated to reflux for 20 h and then cooled to room temperature. The obtained colorless crystals were filtered off and dried under vacuum. Yield: 3.87 g (26.45 mmol, 64.2%). ^{1}H-NMR (D_2O): 3.47

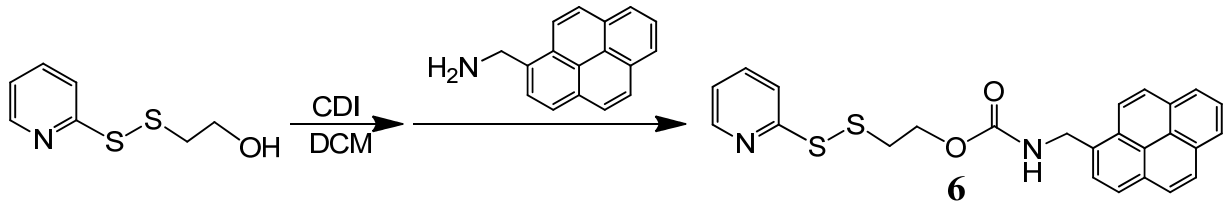
(2H, t, HOC<u>H$_2$</u>–, *J* = 6.6 Hz), 2.10 (2H, t, –C<u>H$_2$</u>CONHNH$_2$, *J* = 7.3 Hz), 1.52 - 1.39 (4H, m, –C<u>H$_2$</u>CH$_2$C<u>H$_2$</u>–), 1.25 - 1.17 (2H, m, –CH$_2$C<u>H$_2$</u>CH$_2$–).



Disulfanediylbis(ethane-2,1-diyl) bis(2-(6-hydroxyhexanoyl)hydrazinecarboxylate). 2,2'-Dithiobisethanol (771 mg, 5 mmol) was placed in a round-bottom flask which was degassed and then filled with argon. 15 mL of dichloromethane (DCM) was added to the flask followed by CDI (1.7 g, 10.5 mmol) under stirring. Stirring was continued for another 2 h under argon at room temperature. 6-Hydroxyhexanoic hydrazide (1.46 g, 10 mmol) was dissolved in hot DMF (15 mL) in a separate flask. After cooling to room temperature, DMAP (61 mg, 0.5 mmol) followed by pyridine (0.81 mL, 10 mmol) were added to the DMF solution. The solution of the CDI-activated 2,2'-dithiobisethanol was added dropwise to the solution of 6-hydroxyhexanoic hydrazide over 1 h at room temperature. The mixture was stirred for 18 h and DMF was evaporated under vacuo. The concentrate was co-evaporated with toluene and the residue was triturated with a mixture of DCM (200 mL) and diethyl ether (50 mL). The insoluble waxy product was collected by decantation, and dried under vacuo overnight. Yield: 2.34 g (4.69 mmol, 94 %). ^{1}H-NMR (CD$_3$OD): 4.35 (4H, t, 2×–OC<u>H$_2$</u>CH$_2$S–, *J* = 6.2 Hz), 3.54 (4H, t, 2×–C<u>H$_2$</u>OH, *J* = 6.4 Hz), 2.98 (4H, m, 2×–OCH$_2$C<u>H$_2$</u>S–), 2.22 (4H, t, 2×–C<u>H$_2$</u>C(O)–, *J* = 7.3 Hz), 1.65 (4H, m, 2×–C<u>H$_2$</u>CH$_2$OH), 1.55 (4H, m, 2×– C<u>H$_2$</u>CH$_2$C(O)–), 1.40 (4H, m, 2×–C<u>H$_2$</u>CH$_2$CH$_2$OH). ^{13}C-NMR: 157.0, 63.2, 61.4, 37.0, 33.4, 32.0, 25.1, 25.0.



Disulfanediylbis(ethane-2,1-diyl) bis(2-(6-((hydrazinecarbonyl)oxy)hexanoyl)hydrazinecarboxylate) 1. The compound **6** (2.34 g, 4.69 mmol) was co-evaporated with dry pyridine two times and finally dissolved in the same solvent (25 mL). CDI (1.83 g, 11.27 mmol) was added to the solution and the reaction mixture was stirred at room temperature for 2 h under argon atmosphere. The reaction mixture was then cooled to -5°C on an ice/NaCl-bath and hydrazine (455 μL, 9.39 mmol) was added to the mixture in three portions over 40 min while maintaining the temperature at -5°C. The mixture was stirred at -5°C for 1.5 h and then allowed to warm up to room temperature for another 17 h. Pyridine was evaporated and the residue was co-evaporated with toluene two times. The residue was re-dissolved in a minimal amount of methanol and the concentrated methanolic solution was poured into 200 mL of DCM, stirred for 15 minutes and then kept for 1 h at -20°C. The insoluble waxy product was collected by decantation, and dried under vacuo overnight. Yield: 2.11 g (3.43 mmol, 73 %). ^{1}H-NMR (CD_3OD): 4.29 (4H, m, 2×–OC$\underline{H_2}$$CH_2$S–), 4.02 (4H, m, 2×–C$\underline{H_2}$OC(O)NHN$H_2$), 2.96 (4H, m, 2×–$OCH_2C\underline{H_2}$S–), 2.21 (4H, m, 2×–C$\underline{H_2}$C(O)–, J = 7.3 Hz), 1.64 (8H, m, 2×–C$\underline{H_2}$$CH_2C\underline{H_2}$–), 1.42 (4H, m, 2×–$CH_2C\underline{H_2}CH_2$–). ^{13}C-NMR: 174.6, 174.3, 156.9, 64.8, 63.2, 37.1, 33.2, 28.5, 25.1, 24.8.



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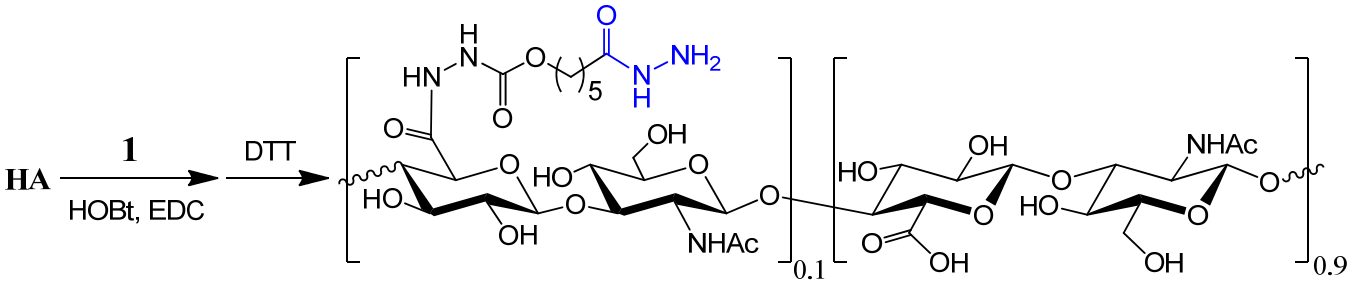
2-(Pyridin-2-yldisulfanyl)ethyl (pyren-1-ylmethyl)carbamate 6. 2-(2-Pyridinyldisulfanyl)ethanol[2] (300 mg, 1.6 mmol) was dissolved in 9 mL of DCM. CDI (312 mg, 1.93 mmol) was added to the solution and stirred at room temperature for 1.5 h, after which 1-aminomethylpyrene hydrochloride (515 mg, 1.93 mmol) and triethylamine (0.4 mL, 2.89 mmol) were added to the reaction mixture. Since 1-aminomethylpyrene hydrochloride was not dissolving in DCM, 3 mL of dry DMF was added and the mixture was stirred overnight at room temperature. The mixture was then dissolved in 200 mL of ethyl acetate and washed with water (3×15 mL). The organic phase was dried over $MgSO_4$ and evaporated. The crude material was purified by silica gel flush column chromatography. The product was eluted from the column with 20 % pentane/DCM. Yield: 625 mg (1.4 mmol, 88%). ^{1}H-NMR ($CDCl_3$): 8.36 – 7. 96 (10H, m, pyrene protons + pyridinyl proton), 7.68 (1H, d, pyridinyl, J = 8.1 Hz), 7.52 (1H, t, pyridinyl, J = 7.68 Hz), 6.88 (1H, t, pyridinyl, J = 5.9 Hz), 5.35 (1H, broad triplet, NH), 5.06 (2H, d, –C<u>H$_2$</u>NH–, J = 5.5 Hz), 4.37 (2H, t, –SCH$_2$C<u>H$_2$</u>O–, J = 6.0 Hz), 3.07 (2H, t, –C<u>H$_2$</u>S–, J = 6.0 Hz).



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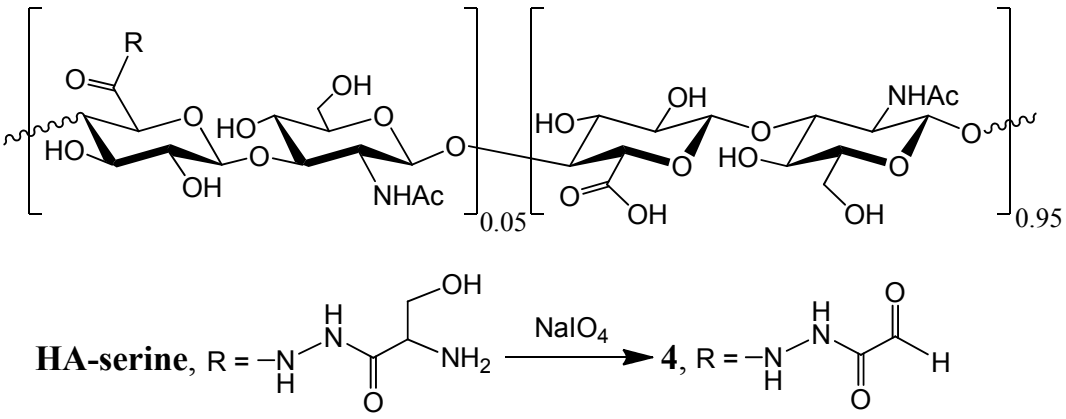
Synthesis of hydrazide-modified HA 5. HA was dissolved in de-ionized water at concentration 8 mg/mL. Linker **1** was added to the HA solution at the reagent/HA disaccharide molar ratio specified in the Table 1 and

the obtained mixture was stirred till complete dissolution of the reagent. *N*-hydroxybenzotriazole (HOBt) was separately dissolved in a 1:1 (v/v) mixture of acetonitrile-water at concentration 0.2M and added to the solution of HA. Molar ratio of HOBt to HA disaccharide was 1. The pH of the resultant solution was adjusted to 4.7 after which the coupling reaction was initiated by addition of solid EDC (see Table 1 for EDC/HA disaccharide feeding ratio) to the reaction mixture. The mixture was stirred overnight. The pH of the reaction solution was slightly increasing during this time which evidenced the coupling of the reagents to HA carboxylates. The reaction solution was basified to 8.5 with 1M NaOH and DTT was added to the solution. 5-Fold molar excess of DTT relative to the applied reagent **1** was used to ensure the cleavage of disulfide bond of the reagent. The mixture was again stirred overnight, after which the solution was acidified to 3.5 with 1M HCl and transferred to a dialysis tube (M_w cutoff = 3500). After exhaustive dialysis against dilute HCl (pH 3.5) containing 0.1 M NaCl, followed by dialysis against dilute HCl, pH 3.5 two times, the solution was lyophilized to give the hydrazide-modified HA **5**. The incorporation of hydrazide groups was verified by ^{1}H-NMR. Specifically, the peaks corresponding to the native HA protons, such as acetamide protons at 1.9 ppm, 2', 3', 4', 5', and 6'-protons of HA disaccharide unit at 3.2 – 4.0 ppm, as well as anomeric 1'-protons at 4.4 ppm, were compared with newly appeared peaks corresponding to the α, β, γ, δ, and ε methylene protons of the of the grafted side chains (see Figure 1 for designation). This indicated that 10% of the HA disaccharide units were modified with hydrazide-terminated side chains. Additionally, the presence of free hydrazide groups was confirmed spectrophotometrically by reaction with trinitrobenzene sulfonic acid (TNBS).[3]

Synthesis of hydrazide-thiol dually modified HA 3. The procedure was analogous to the synthesis of HA derivatives **5** except that two reagents, **1** and **2**, were used in the coupling step for preparation of dually modified HA derivative **3**. The degree of incorporation of thiol (10%) and hydrazide (10%) groups in **3** was verified by comparison of integration of the –C*H*$_2$C*H*$_2$SH side chain peaks at 2.58 and 2.73 ppm and the –

$OCH_2CH_2CH_2CH_2CH_2CONHNH_2$ side chain peaks at 4.90, 2.23, 1.57 and 1.30 ppm with the acetamido moiety of the *N*-acetyl-D-glucosamine residue of HA.



Synthesis of aldehyde-modified HA 4. 370 mg (0.96 mmol of disaccharide units) of the HA-serine derivative,[4] was dissolved in 45 mL of de-ionized water. 0.15 M aqueous solution of sodium periodate (5.8 ml, 0.96 mmol) was added to the HA-serine solution which caused formation of a gel. The gel was diluted with 60 mL of water which led to partial dissolution of the gel. After 5 min, ethylene glycol (0.5 mL, 9.6 mmol) was added to the mixture. The mixture was stirred in the dark overnight during which the gel was completely dissolved. The reaction solution was dialyzed against pure water two times (M_w cutoff = 3500) and finally lyophilized. Yield – 347 mg (94.4%). The amount of aldehyde groups was obtained by reaction with *tert*-butyl carbazate (TBC) followed by reduction with $NaBH_3CN$. Briefly, HA-aldehyde (~20 mg) was dissolved in 2 ml of water and to this solution was added the 0.5 M aqueous solution of TBC (10-fold excess per molar amount of sodium periodate that was used in the preparation of HA-aldehyde derivative). The mixture was stirred for 1 h at room temperature after which 0.5 M aqueous solution of $NaBH_3CN$ (equimolar amount to that of TBC) was added to the mixture. The mixture was allowed to react for 24 h at room temperature. The TBC-modified HA was recovered by dialysis in 3500 MW cut off tubing against water twice. ^{1}H NMR of the obtained product was examined and the peak corresponding to the *tert*-butyl substituent ($(C\underline{H}_3)_3COCONHNH$–, δ = 1.38 ppm) was

compared with the peak of HA acetamide protons at 1.9 ppm. Degree of aldehyde functionalization in **4** (~ 5%) was calculated from the amount of reacted TBC reagent.

Hydrogel formation and characterization. HA hydrogels were prepared in cylindrical glass vials (10 mm in diameter) from 2% w/v precursor solutions (**3** or **5** and **4**). In a typical gelation experiment, HA-aldehyde **4** and HA-nucleophile **3** or **5** were dissolved separately in water and then mixed by addition of the HA-nucleophile solution (0.5 mL) to the HA-aldehyde solution (0.5 mL) followed by brief vortexing for a few seconds. Gelation time was determined by a flow test as the point at which the mixture would no longer flow under the force of gravity. The gels were left in the capped vials for 4 hours to complete cross-linking reactions and then swelled in 10 mL of PBS buffer for 8 days. The swollen hydrogels were applied to the bottom plate of AR2000 rheometer (TA Instruments Inc., UK). The mechanical properties were measured at a frequency of 0.1 to 10 Hz at 25 ºC using 8 mm aluminum plate geometry. The gap was adjusted starting from the original sample height and compressing the sample to reach a normal force of ca. 50 mN (gap sizes were between 7 and 8 mm). To confirm that (**3** + **4**) hydrogel forms doubly cross-linked network after swelling for 8 days in PBS buffer, it was also treated with DTT in PBS buffer (50 mM) for 24 h. The elastic modulus of the gel after exposure to DTT was then measured and was found to be decreased from 333±2 Pa to 174.8±2.6 Pa.

Hydrogel swelling. Equilibrium swelling measurements were carried out by weighing the swollen gels at predetermined time intervals after removing the gels from swelling media and blotting off the surface water (W_t). The PBS buffer was renewed after each measurement at predetermined time point. The gel swelling ratio was calculated as W_t / W_{solid}, where $W_{solid} = 0.02$ g – is a mass of HA components in the freshly prepared gels.

Modular synthesis of the pyrene-immobilized HA hydrogel. The initial hydrazone cross-linked hydrogel was prepared as described above from 2% w/v PBS solutions of HA derivatives **3** and **4**. Two minutes after mixing of the solutions, the formed hydrogel (0.5 mL) was transferred into a vial containing 10 mM solution of the pyridyl disulfide derivative of pyrene **6** in 2 mL DMSO (the molar ratio of the reagent:thiol groups available in the gel was 16:1). The gel was thus exposed to the reagent for 30 h after which it was removed from the reagent solution and placed into 2 mL of pure DMSO for 10 h to wash out the unbound **6**. This procedure was repeated 5 times over a period of two days. 50 µL from each washing solution were diluted till 3 mL with pure DMSO and the resultant samples were examined by UV-vis (Figure S4). As it can be seen from Figure S4 no unbound pyrene derivative **6** has been left in the gel after 5 successive DMSO washings. The weight of the gel after all DMSO washing cycles was 1.644 g. The DMSO-swollen gel was then placed into 4 mL of PBS buffer to

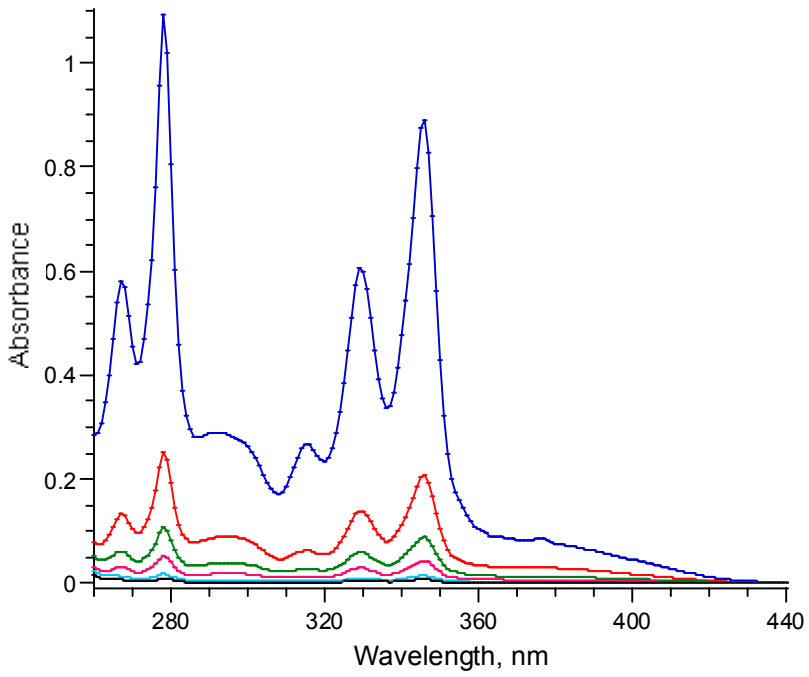


Figure S4. Absorption spectra of the DMSO washing solutions obtained after incubation of fresh DMSO solvent with the pyrene-conjugated gel. Blue, red, green, pink, light blue, and black curves correspond to the first, second, third, forth, fifth, and sixth washing solutions respectively.

exchange the solvent to water. Overall, swelling in fresh PBS buffer was repeated three times over another two days. 100 μL of the last PBS washing solution was diluted till 3 mL with PBS buffer and UV-vis absorption was measured (Figure S5, red curve). The mass of the hydrogel was reduced to 457 mg during exchange of DMSO to water, which indicated decreased swelling capacity of the hydrophobically modified hydrogel in water.

Enzymatic degradation of the pyrene-immobilized HA hydrogel. After removing of the unbound reagent **6** from the pyrene-immobilized HA hydrogel and the solvent exchange to PBS buffer, it was treated for 96 h with 4 mL of hyaluronidase dissolved in PBS buffer at concentration 250 U/mL. The enzymatic digest was then filtered through 0.8 μm filter which afforded approximately 4 mL of the final solution (Figure S5, insert). 100 μL of this solution was diluted till 3 mL with PBS buffer and UV-vis absorption was measured (Figure S5, blue curve). Figure S5 shows that no pyrene derivatives were present in the swelling buffer where the pyrene-immobilized HA hydrogel was immersed prior its degradation with hyaluronidase. However, incubation of the gel in the presence of hyaluronidase resulted in the release of pyrene in water soluble form, i.e. pyrene-HA conjugates.

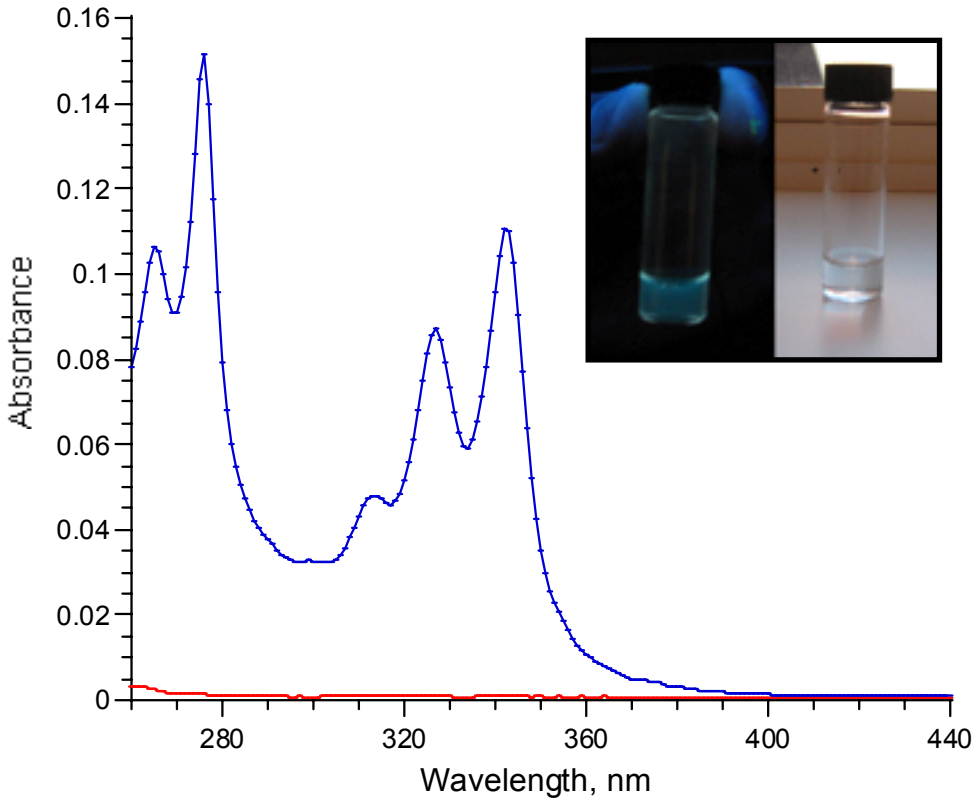


Figure S5. Comparison of the UV-vis spectra of the final PBS washing solution for the pyrene-conjugated HA gel (red curve) and the solution obtained from degradation of the gel by hyaluronidase (blue curve). Insert: photos of the solution obtained after hyaluronidase degradation of the pyrene-conjugated HA gel as seen in UV light and in day light.

Quantification of pyrene chemical loading into HA gel. The amount of covalently attached pyrene to the HA hydrogel was determined by analyzing the mixture obtained after gel degradation by Hase. 2 mL of the Hase degraded and filtered mixture was dialyzed in 3000 Da cut off dialyzing membrane against water two times (2 × 12h) and freeze-dried which yielded 4 mg of the dry material. Taking into account that the whole volume of the gel digest was 4 mL, this implies that 80% of the HA mass of the pyrene-immobilized hydrogel was degraded (10 mg of HA derivatives **3** and **4** were taken initially for the modular synthesis of the gel). 0.5 mg of the dialyzed and dried Hase digest was dissolved in 1 mL of DMSO under heating. 0.4 mL of the above solution was diluted till 3 mL with pure DMSO and UV-vis absorption was measured. Solutions of 1-

aminomethylpyrene hydrochloride in DMSO (1 – 20 μM) were used as standards to obtain a calibration curve for determining the amount of pyrene groups in the sample. The amount of pyrene in the digest sample was thus determined to be 0.072 μmol/mg, which correspond to 5.8% degree of substitution in the HA-hydrazide-thiol derivative **3**. Therefore, under pyridyl disulfide exchange conditions, 60% of the sulfhydryl groups were consumed for the reaction of pyrene conjugation to the **3** + **4** gel.

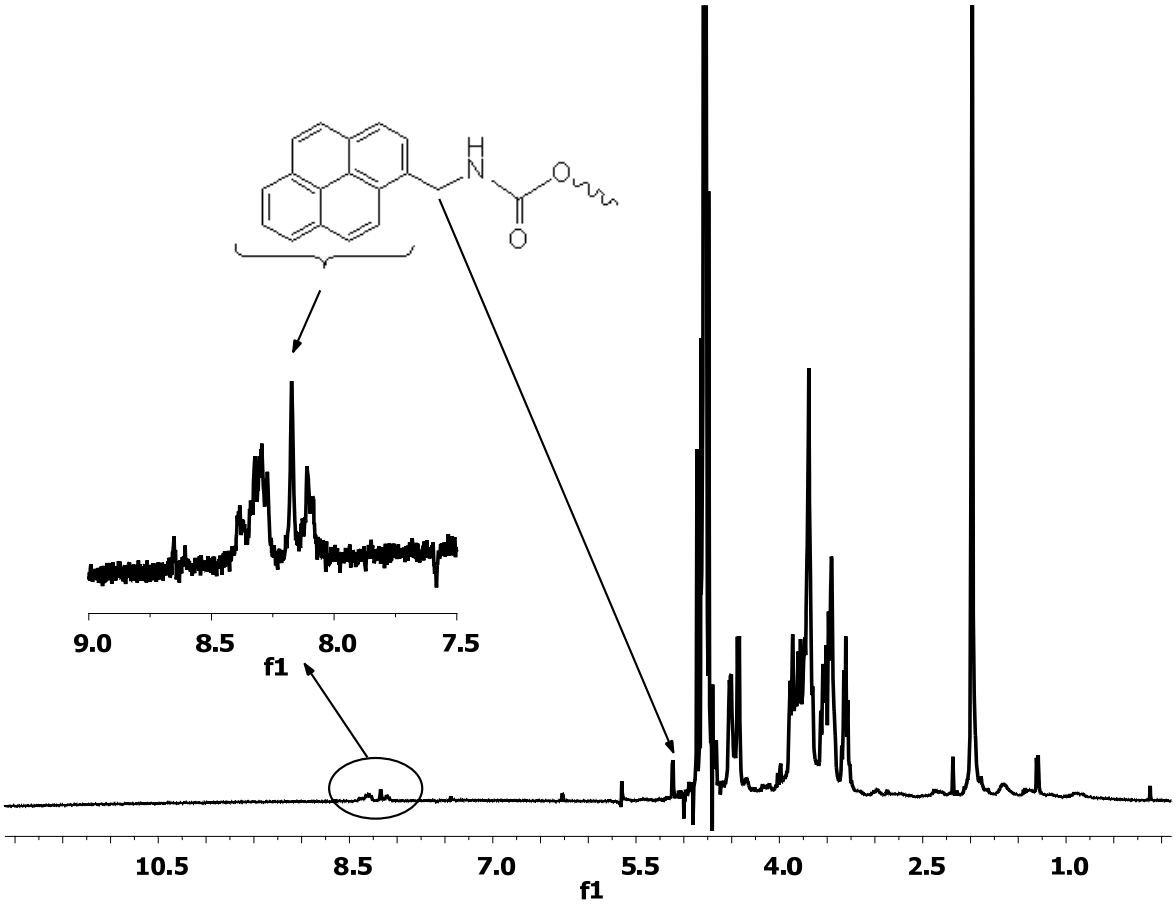


Figure S6. ^{1}H NMR spectra of the pyrene-conjugated HA gel degradation mixture after its dialysis against water two times and subsequent freeze-drying. The spectra were recorded in D_2O.

The gel degraded by Hase and subsequently dialyzed and freeze-dried was also analyzed by proton NMR which confirmed that the gel degrades down to water soluble HA-pyrene conjugates (Figure S6). Signals of aromatic protons were observed in 8.35 – 7.95 ppm region, exactly where the signals of pyrene protons were located for

the reagent **6**. Comparison of integration of these peaks with the acetamido moiety of HA indicated 5.4% of substitution per disaccharide unit of the dually modified HA **3** which is fairly close to the amount determined from the UV measurements.

Atomic force microscopy (AFM). The mixture obtained directly after Hase degradation was filtered through a 0.8 μm filter and one drop of the filtered solution (at concentration 2 mg/mL) was deposited onto a glass slide. The AFM imaging was performed using a Nanosurf Mobile S system. All images were recorded in non-contact mode, using silicon cantilevers oscillating at frequencies of 190 kHz (force constant 48 N/m). For higher sensitivity the cantilevers are aluminum coated on the back side. The typical tip radius was 10 nm. For comparison, the same analysis has been performed with HA derivative **3** at concentration 2 mg/mL (Figure S7).

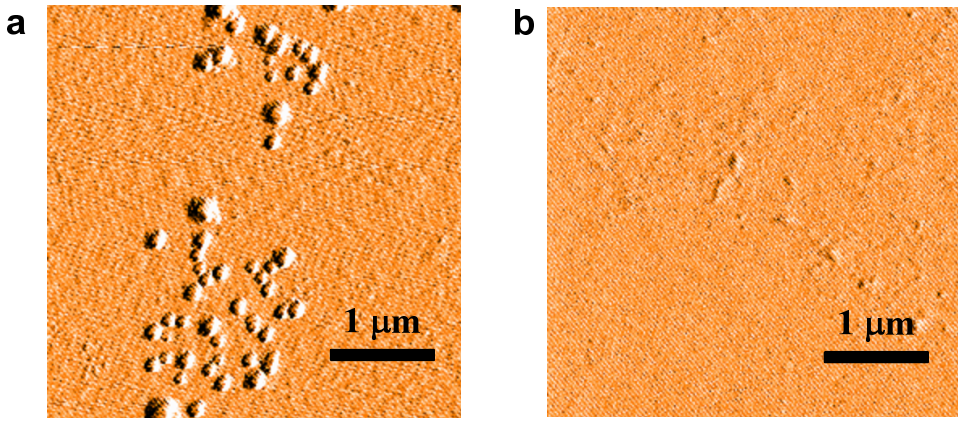


Figure S7. Comparison of the AFM images of the particles formed upon degradation of the pyrene-conjugated HA gel (**a**) and the parent doubly-modified HA **3** (**b**).

Dynamic light scattering (DLS). The size distribution of nanogel particles in PBS buffer was carried out on particle size/zeta potential-meter (Malvern, Zetasizer Nano ZS). The mixture obtained directly after Hase degradation was filtered through 0.8 μm filter and 1 mL of the filtered solution (at concentration 2 mg/mL) was applied for DLS measurement.

Uptake study by inverted fluorescent microscopy. The particles dispersion was sterilized by passing through a 0.8 μm filter. NIH 3T3 cells were cultured on collagen treated glass surfaces in DMEM with 10% FBS. The cells grown to near 75% confluence were seeded at 5000 cells / mL of medium (1.5 mL total volume of media per well). An aliquot (50 μL) of the nanogels (2 mg/mL) was added to 1.5 mL of cell culture media containing cells. The cells were then incubated for 24 hours. After incubation, the medium was removed and the cells were washed with PBS (pH 7.4). The particle uptake was observed with a Leica epi-fluorescence microscope at 20× magnification with monochromatic filter of 494 nm.

References:

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