Electronic Supplementary Material (ESI) for Polymer Chemistry.

SUPPORTING INFORMATION

Influence of ions to modulate hydrazone and oxime reaction kinetics to obtain dynamically cross-linked hyaluronic acid hydrogels

Shujiang Wang,[a,b,c] *Ganesh N. Nawale,*[b,c] *Oommen P. Oommen,*[d] *Jöns Hilborn,*[b] *and Oommen P. Varghese,**[b]

[a]Maisonneuve-Rosemont Hospital Research Centre & Dept. of Ophthalmology, University of Montreal, Montreal, Canada.

[b]Translational Chemical Biology Laboratory, Department of Chemistry, Ångström Laboratory, Uppsala University, 751 21, Uppsala, Sweden.

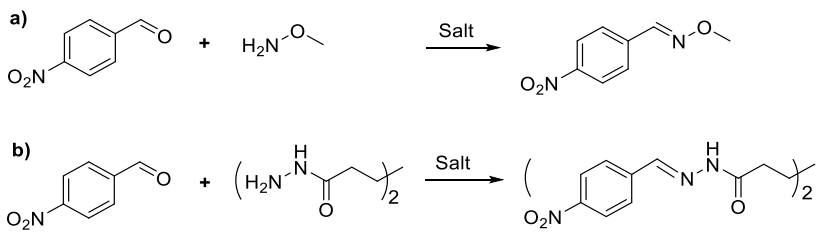
Email-oommen.varghese@kemi.uu.se

[d]Bioengineering and Nanomedicine Lab, Faculty of Medicine and Health Technologies and BioMediTech Institute, Tampere University, Korkeakoulunkatu 3, Tampere-33720, Finland.

Materials and reagents

All reagents, including (aminooxy)methane, 4-nitrobenzaldehyde, sodium chloride, sodium periodate, lithium chloride, lithium perchlorate, potassium periodate, magnesium chloride, calcium chloride, sodium bromide, sodium sulphate, *N*-hydroxyphthalimide, 1,8-diazabicyclo(5.4.0)undec-7-ene (DBU), dibromobutane, glacial acetic acid, 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide (EDC), hydrochloric acid, 2,4,6-trinitrobenzenesulfonic acid solution (TNBS, 5% w/v in H_2O) were purchased from Sigma–Aldrich (Sweden). Phosphate buffer was prepared from sodium phosphate dibasic heptahydrate (mw: 268 g/mol) and sodium phosphate monobasic monohydrate (mw: 138 g/mol). Briefly 20.209 g of sodium phosphate dibasic heptahydrate and 3.394 g of sodium phosphate monobasic monohydrate salts were added to the 800 mL of distilled water to obtain 0.0754 M and 0.0246 M of respective salts and final desired pH was adjusted using HCl or NaOH followed by diluting of solution till 1 L. Hyaluronic acid (HA, 150 kDa) was purchased from Lifecore Biomedical, LLC (Chaska, MN). Lambda 35 UV/Vis spectrophotometer from PerkinElmer instruments was used for spectroscopic analysis. Rheological properties of hydrogels were analyzed using AR2000 Advanced Rheometer (TA Instruments) with a custom-made aluminum parallel plate with a diameter of 8 mm.

Pseudo-first-order acylhydrazone and oxime ligation kinetics analyzed by UV-vis spectroscopy.



Scheme S1. Reaction scheme of oxime and acylhydrazone formation monitored by UV-vis spectroscopy in 10 mM phosphate buffer (pH 7.4) containing 10 % DMF.

The pseudo-first-order reaction kinetics study was performed following our previously reported method.[1] For the acylhydrazone and oxime formation studies, 0.064 mM of nitrobenzaldehyde and 1 mM of adipic dihydrazide (2 mM of hydrazine functional group) or 2 mM of methoxyamine were used as the model substrate. The kinetics for the reaction were studied employing various concentrations (50 mM-1 M) and different type of salt (NaCl, KCl, LiCl, $LiClO_4$, NaBr, $MgCl_2$, and Na_2SO_4) (Scheme **1**). Briefly, samples were mixed by pipetting in a 3 mL standard quartz cuvette with a path length of 1 cm. Phosphate buffer (PB, 10 mM, pH 7.4) containing 10 % (v/v) DMF and the calculated amount of salt was used as a reference. Further, 6 µl of 4-nitrobenzaldehyde (32 mM stock solution in DMF) was added to 2.964 mL of the solvent mentioned above mixture, and UV-Vis absorbance (from 250 nm to 400 nm) were recorded. The reaction was initiated by the addition of 30 µl hydrazide or oxyamine (200 mM stock solution in 10 mM phosphate buffer, pH 7.4) and absorbance was recorded at specific time intervals. All the reactions were performed at 23 °C. Absorbance at the 307 nm was plotted against time, the pseudo-first-order reaction rate was calculated using equations (S1 -S3), and representative examples pseudo-first-order rate kinetics was plotted as in Fig. 1.

$$\%Conversion = 100\times\frac{A_t}{A_{max}} \quad \text{(S1)}$$

$$C_{-CHO} = \%Conversion\times 6.4\times 10^{-7} \quad \text{(S2)}$$

$$lnC_{-CHO} = -k_{obs}t \quad \text{(S3)}$$

where A_t is an absorbance at time t, A_{max} is maximum absorbance when t=∞, C_{-CHO} is a concentration of aldehyde (M) at time t (h), k_{obs} is the observed pseudo-first-order rate constant (h^{-1}).

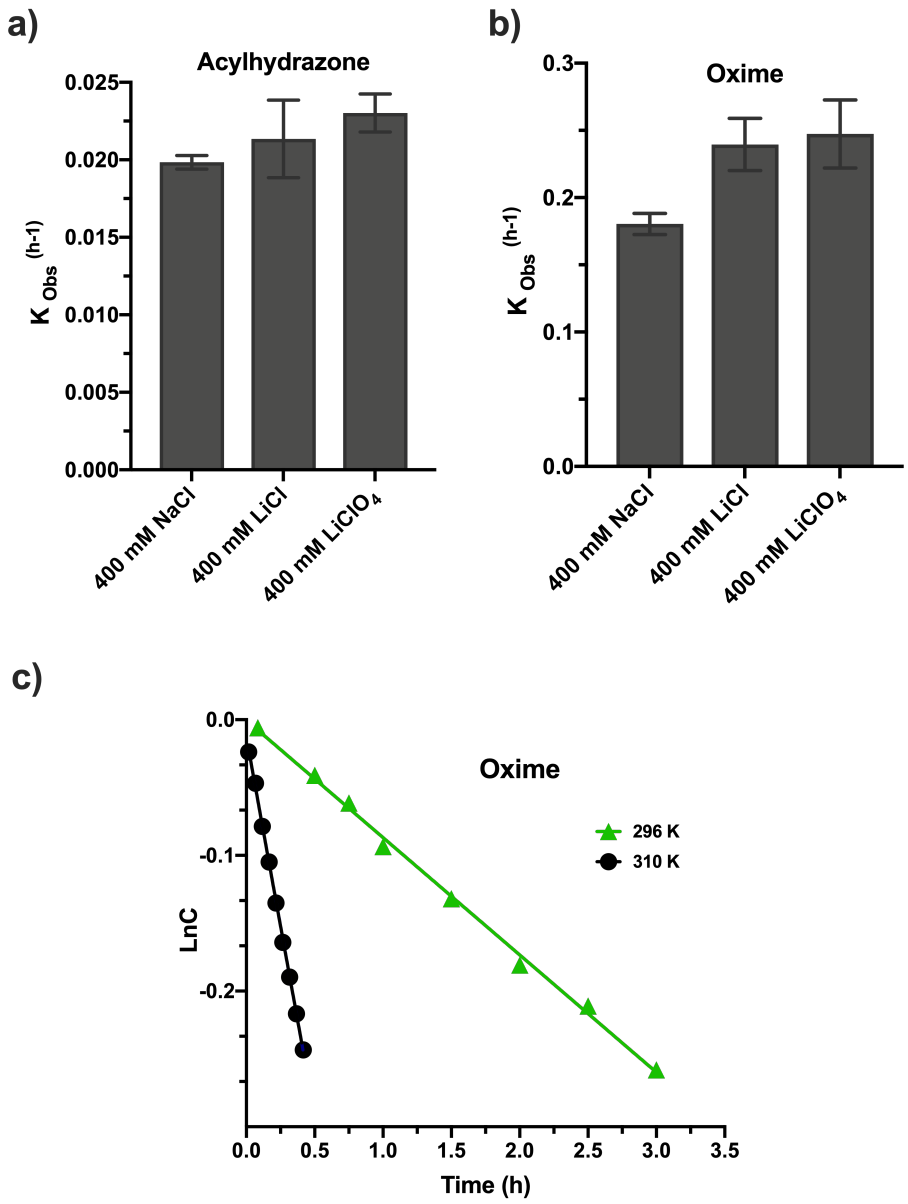
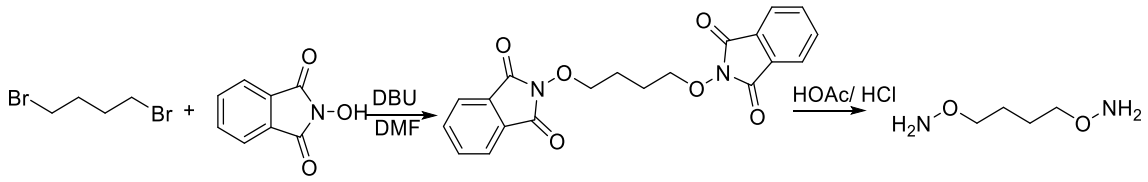


Figure S1. Field effect comparison of rate constant and temperature dependent salt catalyzed oxime formation. Reactions were performed using 64 µM 4-nitrobenzaldehyde and a) 1 mM adipic dihydrazide or b) 2 mM (aminooxy)methane in 10 mM phosphate buffer (PB, 7.5 mM Na_2HPO_4 and 2.5 mM of NaH_2PO_4) PB containing 10 % DMF. c) The reaction was performed in 2 mM (aminooxy)methane in 10 mM phosphate buffer (PB, 7.5 mM Na_2HPO_4 and 2.5 mM of NaH_2PO_4) PB containing 10 % DMF and 100 mM NaCl.

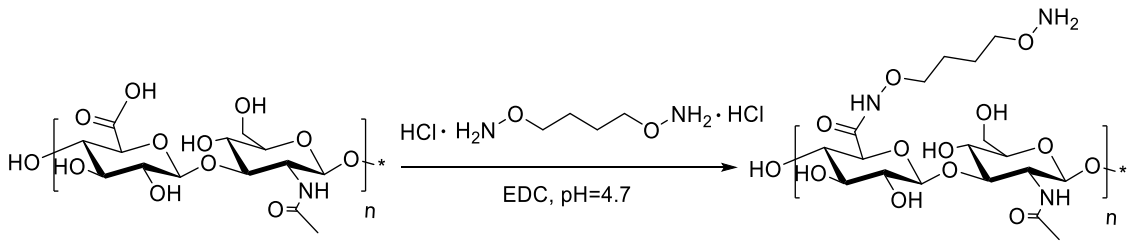
HA-aldehyde and HA-hydrazide derivative were prepared following our previously reported methods.[2-3]

Synthesis of bis(oxyamine) linker and oxyamine modified HA



Scheme S2. A synthetic strategy for O,O'-(butane-1,4-diol)bis(hydroxylamine).

The bis(oxyamine) linker was prepared following reported procedure.[4] Briefly, N-hydroxyphthalimide (4.75 g, 29.20 mmol) was dissolved in 30 mL of DMF, and DBU (4.36 mL, 29.20 mmol) was added dropwise. Thereafter, dibromobutane (3 g, 13.90 mmol) was added, and the mixture was heated at 85 °C for 1 h. The resultant solution was poured into ice and the precipitate was filtered and washed with 13 mL of cold H_2O and 8 mL of cold CH_3CN. The crude 1,2-diphthalimidooxybutane was recrystallized from butanol. Further, a suspension of 1,2-diphthalimidooxybutane in glacial acetic acid/HCl (12 mL, 30/20 v/v) was heated at 115 °C for 3 h to afford a clear solution. Further, the reaction mixture was concentrated under vacuum, and 1.5 mL of H_2O was added to the residue. The suspension was filtered, and the residue was washed with HCl (6 M). The filtrate was collected and dried under vacuum. The product was finally recrystallized from EtOH: H_2O (5:1, v/v) to obtain the pure product (Scheme **2**). The integrity of the sample was evaluated using the ^1H NMR (Figure S2a).



Scheme S3. A synthetic strategy for oxyamine-modified HA derivative.

The HA-oxyamine derivative was prepared following the modified EDC coupling strategy.[5] Briefly, 200 mg of HA (150 kDa, 0.50 mmol of disaccharide units) was dissolved in H_2O (50 mL). Further, the bis(oxyamine) linker (116 mg, 0.60 mmol) was added to the HA solution and stirred until the reaction mixture was homogeneous. After adjusting the pH to 4.7, EDC (11.5 mg, 0.06 mmol) was added, and the reaction mixture was stirred overnight. The reaction mixture was subsequently dialyzed against HCl (pH 3) containing 0.1 M NaCl for two days followed by HCl (pH 3) for one day, thereafter the pH of the obtained reaction mixture was adjusted to 7.4 using 0.1 M NaOH and subsequently dialyzed against distilled water for one day. Reaction mixture after dialysis was lyophilized to furnish the HA- oxyamine derivative.

The degree of oxyamine modification was quantified using the TNBS assay. Briefly, 1 mg of HA-oxyamine was dissolved in 3 mL borate buffer (pH 9.2). To this solution, 25 µl of 5 % (w/v) TNBS reagent was added, and UV absorbance was recorded at 480 nm after 30 min using 3 mL borate buffer containing 25 µl of TNBS reagent as a reference. The concentration of oxyamine was determined using methoxyamine as the standard. The degree of oxyamine modification was quantified as 7 %. The integrity of the sample was further evaluated using a ^{1}H NMR (Figure S2b).

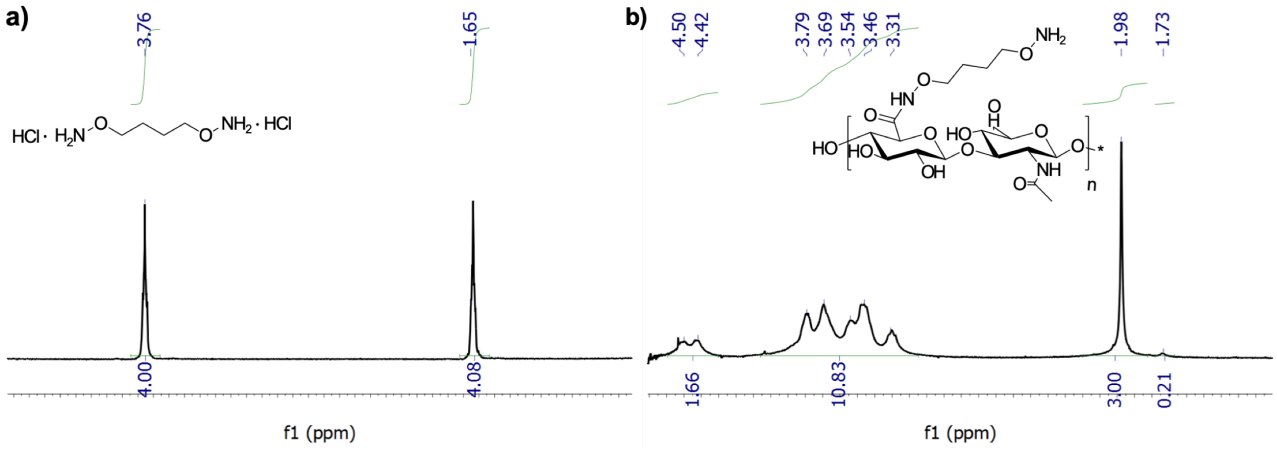
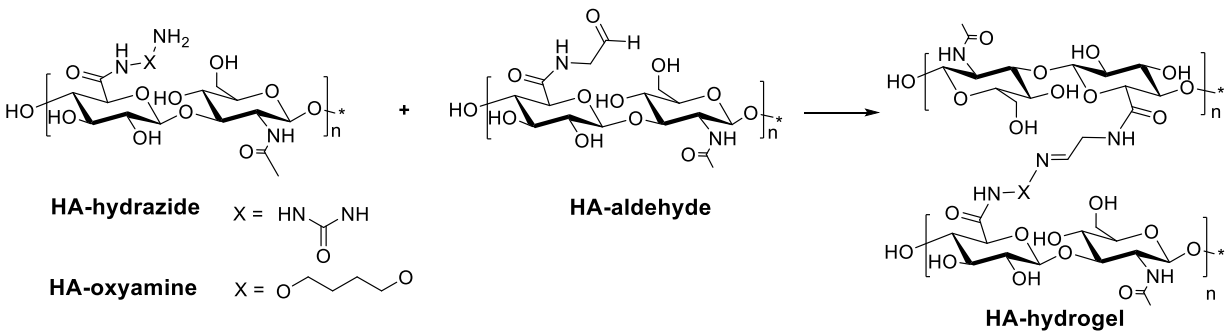


Figure S2. ^{1}H NMR of a) bis(oxyamine) and b) HA-oxyamine derivative.

Hydrogel gelation kinetics

HA-hydrazide (7% modification) and HA-aldehyde (7% modification) were separately dissolved in PB (0.01 M, pH 7.4, containing various salt) to reach a concentration of 16 mg/mL. The hydrogel was prepared (100 μL) by mixing equal volumes of HA-hydrazide and HA-aldehyde solution (Scheme **4**). Immediately after mixing, the material was transferred to a rheometer plate for oscillatory time sweep rheological analysis with a constant frequency at 1 Hz and a controlled gap distance of 1.0 mm. Values of storage modulus (G') and loss modulus (G'') were plotted against time, and gel point (G'=G'') was recorded. For the slow gelation hydrogels, with gel time longer than 15 min, hydrogel precursor mixture was stored in a sealed Eppendorf tube for several min and transferred to rheometer plate right before measurement.



Scheme S4. Reaction scheme of hydrazone/oxime cross-linked HA hydrogel.

Oxime cross-linked HA-hydrogels were prepared as mentioned above using HA-hydrazide (6% modification) and HA-aldehyde (12% modification). Since the gelation of HA-oxime gel (16 mg/mL) is fast, measurement of gelation kinetics in the presence of high concentration or divalent salt is challenging. Therefore, we investigated the salt effect on oxime-cross-linked gelation kinetics by utilizing the lower concentration (12 mg/mL) of HA-oxyamine and HA-aldehyde derivatives.

Hydrogel preparation for swelling analysis and rheological characterization

HA-hydrazide, HA-oxyamine and HA-aldehyde derivatives were dissolved in phosphate buffer (0.01 M, pH 7.4, containing different salts) separately to reach a concentration of 16 mg/mL. Hydrogel for rheological and swelling experiments was prepared by mixing 100 µL HA-oxyamine /HA-hydrazide solution with 100 µL HA-aldehyde solution. Immediately after mixing, the material was transferred to a custom-made cylinder mold, sealed with Parafilm and stored for 24 h before analysis.

Hydrogel Swelling

Completely cross-linked hydrogels (200 µL) were suspended in 2 mL of PBS (10 mM, pH 7.4). Weights of the gel before and after swelling were recorded, and the percentage of swelling was measured from equation S4.

$$sw\% = \frac{w_t - w_0}{w_0} \times 100\% \quad \text{(S4)}$$

where w_t is the weight of gel after swelling time t, w_0 is the weight of gel at the time of gel preparation.

Rheological analysis

Completely cross-linked hydrogels (200 µL) were suspended in 2 mL of PBS (10 mM, pH 7.4) for 24 h to achieve equilibrium of swelling. Thereafter gels were transferred to the rheometer, and the rigidity of the hydrogels was investigated by performing oscillatory frequency sweeps with frequency varies from 0.1-10 Hz at a constant % strain of 1 % and a normal force of 0.03 N.[6]

References

(1) Wang, S.; Nawale, G. N.; Kadekar, S.; Oommen, O. P.; Jena, N. K.; Chakraborty, S.; Hilborn, J.; Varghese, O. P. Saline Accelerates Oxime Reaction with Aldehyde and Keto Substrates at Physiological pH. *Sci. Rep.* **2018,** *8*, 2193.

(2) Oommen, O. P.; Wang, S.; Kisiel, M.; Sloff, M.; Hilborn, J.; Varghese, O. P. Smart Design of Stable Extracellular Matrix Mimetic Hydrogel: Synthesis, Characterization, and In Vitro and In Vivo Evaluation for Tissue Engineering. *Adv. Funct. Mater.* **2013,** *23*, 1273-1280.

(3) Oommen, O. P.; Garousi, J.; Sloff, M.; Varghese, O. P. Tailored Doxorubicin-Hyaluronan Conjugate as a Potent Anticancer Glyco-Drug: An Alternative to Prodrug Approach. *Macromol. Biosci.* **2014,** *14*, 327-333.

(4) Wendeler, M.; Grinberg, L.; Wang, X.; Dawson, P. E.; Baca, M. Enhanced Catalysis of Oxime-Based Bioconjugations by Substituted Anilines. *Bioconj. Chem.* **2014,** *25*, 93-101.

(5) Varghese, O. P.; Kisiel, M.; Martínez-Sanz, E.; Ossipov, D. A.; Hilborn, J. Synthesis of Guanidinium-Modified Hyaluronic Acid Hydrogel. *Macromo. Rapid Commun.* **2010,** *31*, 1175-1180.

(6) Wang, S.; Oommen, O. P.; Yan, H.; Varghese, O. P. Mild and efficient strategy for site-selective aldehyde modification of glycosaminoglycans: tailoring hydrogels with tunable release of growth factor. *Biomacromolecules* **2013,** *14*, 2427-32.