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## ARTICLE

# In situ forming interpenetrating hydrogels of hyaluronic acid hybridized with iron oxide nanoparticles

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Four derivatives of hyaluronic acid (HA) bearing thiol (HA-SH), hydrazide (HA-hy), 2-dithiopyridyl (HA-SSPy), and aldehyde groups (HA-al) respectively were synthesized. Thiol and 2-dithiopyridyl as well as hydrazide and aldehyde make up two chemically orthogonal pairs of chemo-selective functionalities that allow in situ formation of interpenetrating (IPN) disulfide and hydrazone networks simultaneously upon mixing of the above derivatives at once. The formation of IPN was demonstrated by comparing it with the formulations of the same total HA concentration but lacking one of the reactive components. The hydrogel comprised of all four components was characterized by larger elastic modulus than the control single networks (either disulfide or hydrazone) and the three component formulations gave the softest hydrogels. Moreover, a hydrazone cross-linkage was designed to contain a 1,2-diol fragment. This allowed us to partially disassemble one type of network in the IPN leaving another one unaffected. Particularly, treatments of the IPN with either sodium periodate or dithiothreitol resulted in disassembly of the hydrazone and disulfide networks respectively and thus softening of the hydrogel. Contrary, the single network hydrogels completely dissolved under the corresponding conditions. In corroboration with this, enzymatic degradation of the IPN by hyaluronidase was also substantially slower than the degradation of the single networks. In order to further improve the mechanical properties of the elaborated injectable IPN, it has been in situ hybridized with iron oxide nanoparticles (IONPs). The mesh size of the IPN was smaller than the size of the IONPs resulting in the nanoparticles retention in the matrix under equilibrium swelling condition. However, these nanoparticles were released upon enzymatic degradation suggesting their use as MRI tags for non-invasive tracking of the hydrogel material *in vivo*. Additionally, this injectable hybridized hydrogel with encapsulated IONPs can be used in hyperthermia cancer therapy.

## Introduction

Formation of hydrogels in situ using bio-orthogonal reactions allows their application *in vivo* directly after formulation from aqueous solutions at room temperature and physiologic pH. This property of *in vivo* injectability is stipulated by that bio-orthogonal cross-linking reactions do not require toxic initiators, condensing agents, low molecular weight cross-linkers or monomers; do not produce undesirable side products; and proceed with high degree of chemoselectivity. As a result, in situ formed chemical hydrogels attract especial attention in biomedical applications. The most appealing feature of the in situ formed hydrogels in tissue engineering is non-invasive administration via injection avoiding surgical implantation.<sup>1-3</sup> In situ preparation of hydrogels from

suitably functionalized natural biocompatible polymers (polysaccharides and proteins) may be considered as the most safe construction mimicking *in vivo* production of extracellular matrix (ECM). To date, chemoselective reactions have been known as an influential tool in creation of sophisticated biomolecular assemblies to develop soft materials<sup>4</sup> based on polymers, nanoparticles and smart hydrogels networks.<sup>5</sup> Chemical and bio-orthogonality of these reactions allowed simultaneous construction of multicomponent systems with complex hierarchy by selective connection between the pre-defined components.<sup>6-8</sup>

For the purpose of in situ preparation of a bio-compatible hydrogel, we selected hyaluronic acid (HA) as hydrophilic, biodegradable and non-immunogenic polymer that makes it a perfect material for various biomedical applications, especially in tissue engineering.<sup>9-11</sup> HA has been previously considered for this purpose and many chemoselective cross-linking reactions were applied after suitable functionalization of HA which include azide-alkyne cycloaddition click reaction,<sup>12</sup> aqueous Diels-Alder chemistry,<sup>13</sup> peroxidase-catalyzed oxidation of tyramine,<sup>14</sup> oxime coupling,<sup>15</sup> Michael addition of thiols to acrylates,<sup>16</sup> and thiol-disulfide exchange reaction.<sup>17</sup> However, very often these hydrogels have weak mechanical properties which might be related to imperfect cross-linking of polymer chains which starts right after

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mixing of reactive polymer precursors. Mechanical integrity is one of the most important properties of hydrogels in many tissue engineering applications, especially in bone regeneration.<sup>18,19</sup> The formation of interpenetrating networks (IPNs) or double networks<sup>20</sup> is an effective method to improve the mechanical strength of hydrogels.<sup>20-22</sup> From a synthetic viewpoint; IPNs can be classified in two main groups: sequential IPNs and *in situ* IPNs. In the former case, a first network is prepared and swollen in a solution containing precursors for a second network which is then formed within the first network.<sup>21</sup> Most often this procedure was performed via two sequential radical (photo) polymerization reactions.<sup>23</sup> In case if the conditions of the two networks formation can be distinguished, all the soluble components for IPN formation can be then mixed at once and the combined mixture is permitted to undergo two networks formation upon the adjustment of the conditions sequentially. For example, a mixture of solubilized collagen and glycidyl methacrylate-modified HA were converted into IPN by first incubation of the mixture at 37°C to allow the fibrillogenesis of collagen followed by UV exposure to photocross-link the HA chains.<sup>24</sup> In this latter case, both networks can be formed theoretically at once, by allowing two selective and independent cross-linking processes to occur.<sup>21,25</sup> One can envisage that combination of two cross-linking processes may still affect each other due to steric hindrances imposed by the partially formed networks. Most probably, due to this limitation, *in situ* formation of IPNs has been rarely reported by researchers.<sup>3,25-27</sup> Most of the double network hydrogels were prepared sequentially, and they exhibited better mechanical properties than the corresponding single network hydrogels.<sup>3,25</sup>

In this work, we, for the first time, pursued *in situ* formation of bio-compatible IPN hydrogel by simultaneous two chemoselective cross-linking reactions. For this purpose, we studied the possibility of hydrazone and thiol-disulfide exchange chemoselective reactions to occur simultaneously upon mixing of four components that can be grouped into two independent pairs of mutually reactive polymer precursors. Particularly, a hydrazone network was envisaged to form upon combination of hyaluronic acid derivatives bearing hydrazide (HA-hy) and aldehyde (HA-al) groups, while the disulfide network was planned to form upon mixing of thiol- and 2-dithiopyridyl derivatives of HA (HA-SH and HA-SSPy respectively). To achieve a single mixing-triggered IPN formation, HA-SH was initially combined with HA-al, whereas HA-SSPy was combined with HA-hy without any sign of cross-reactivity. Thus, the suggested system does not need any low molecular weight cross-linker and is performed in buffered aqueous solution permitting 3D cell encapsulation. For further improvement of mechanical properties of the hydrogel system, we anticipated 3D encapsulation of water soluble iron oxide nanoparticles (IONPs). Such hybridization of HA hydrogel has also the utility of non-invasive imaging of the hydrogel by magnetic resonance imaging (MRI) and in hyperthermia treatment of tumors. Enzymatic degradation of hybrid HA-IONP hydrogel is expected to release IONPs and change the contrast of the hydrogel implant for visualization of the hydrogel degradation *in vivo*. Due to interaction of HA with IONPs, the released nanoparticles are also expected to acquire

an HA coat that may promote selective uptake by cancer cells in case of the hydrogel nanocomposite placement near tumor. Basing on binding of HA to CD44, a receptor up-regulated in many cancer cells, a strategy of tumor targeting was devised in which HA is used as a homing device.<sup>28</sup> Particularly, HA-coated IONPs were synthesized for drug delivery and imaging of macrophages and cancer cells.<sup>29,30</sup> Mechanical strength of the novel prepared HA-IONP IPN hydrogel was characterized by rheology. Enzymatic degradation of the prepared hydrogels was performed by applying hyaluronidase, while breaking of the hydrazone and disulfide cross-linkages in the gels was achieved with sodium periodate and dithiothreitol (DTT) respectively. UV-Vis spectrophotometry was used for evaluation of the release/retention of IONPs from the swollen HA-IONP hybrid hydrogel.

## Materials and methods

### Materials and instruments

Hyaluronic acid (HA) sodium salt (MW 150 kDa) was purchased from Lifecore Biomedical. *N*-Hydroxybenzotriazole (HOBt) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Fluka. Hyaluronidase (Hase, Aldrich), DL-dithiothreitol (DTT), sodium periodate (NaIO<sub>4</sub>), *tert*-butyl carbazate (TBC), and water soluble iron oxide nanoparticles (IONPs, average size = 10 nm, Aldrich) were purchased from Aldrich Chemical Co. Sodium cyanoborohydride was purchased from Acros Organics. Trinitrobenzene sulfonic acid (TNBS) was from Sigma. All solvents were of analytical quality (p.a.) and were dried over 4 Å molecular sieves. Dialysis membranes Spectra/ Por 6 (3500 g/mol cutoff) were purchased from VWR international. <sup>1</sup>H-NMR spectra were recorded in D<sub>2</sub>O with a JEOL JNM-ECP Series FT NMR spectrometer at a magnetic field strength 9.4 T, operating at 400 MHz. UV-vis absorption spectra were recorded using UV-Vis spectrometer (Lambda 35, PerkinElmer Instruments). Rheological characterizations of all hydrogels were performed using an AR2000 Advanced Rheometer (TA Instruments) with aluminium parallel plate geometry of 8 mm diameter. Frequency sweeps from 0.1 to 10 Hz were performed by monitoring storage (G') and loss moduli (G'') at a fixed normal force (0.015 N) and a fixed strain. All experiments were repeated three times. 2-(2-Pyridinyldithio)ethyl hydrazinecarboxylate **1**,<sup>31</sup> 3,3'-dithiobis (propionic hydrazide) **2**,<sup>32</sup> and tartaric acid dihydrazide **3**<sup>33</sup> were synthesized according to the literature procedures.

### Synthesis of 2-dithiopyridyl modified hyaluronic acid (HA-SSPy)

Hyaluronic acid (HA, MW = 150000 Da) (400 mg, 1 mmol of disaccharide repeating units) was dissolved in de-ionized water at the concentration of 8 mg/mL. 2-(2-Pyridinyldithio) ethyl hydrazinecarboxylate **1** (49 mg, 0.2 mmol) was dissolved in 4 mL of methanol and the solution was added to the solution of HA. *N*-hydroxy benzotriazole (153 mg, 1 mmol) in 5 mL of 1:1 (v/v) mixture of acetonitrile-water was also added to the HA solution under stirring. The pH of the resultant solution was adjusted to 4.8, after which EDC (48 mg, 0.25 mmol) was finally added to the mixture. The reaction mixture was stirred

overnight at room temperature. After the amide coupling, the mixture 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, the solution was lyophilized to give 385.1 mg of HA-SSPy (96% yield). The incorporation of pyridyl group was verified by  $^1\text{H-NMR}$ . Specifically, the newly appeared peaks at 7.45, 7.92 – 8.06, and 8.45 ppm corresponding to four aromatic protons of the pyridyl group were integrated. This indicated that 10 % of HA disaccharide units were modified.

#### Synthesis of thiol modified hyaluronic acid (HA-SH)

HA ( $MW = 150000$  Da) (400 mg, 1 mmol of disaccharide repeating units) was dissolved in de-ionized water at concentration 8 mg/mL. Dihydrazide linker **2** (35.7 mg, 0.15 mmol) was added to the HA solution at the reagent/HA disaccharide molar ratios 0.15:1. *N*-hydroxybenzotriazole (153 mg, 1 mmol) in 5 mL of 1:1 (v/v) mixture of acetonitrile-water was also added to the HA solution under stirring. Molar ratio of HOBt to HA disaccharide was 1:1. The pH of the resultant solution was adjusted to 4.8 after which the coupling reaction was initiated by addition of solid EDC (28.8 mg, 0.15 mmol) to the reaction mixture. The mixture was stirred overnight and then basified to 8.5 with 1M NaOH. DTT (208 mg, 1.5 mmol) was added to the solution. 10-fold molar excess of DTT relative to the estimated amount of disulfide linkages in the HA derivative was used to ensure the cleavage of disulfide bond by the reagent. The mixture was stirred overnight, after which the solution was 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. After lyophilization of the dialyzed solution, it was obtained 369 mg of the thiol-modified HA (HA-SH) (92% yield). The degree of incorporation of thiol (10 %) groups in HA-SH was verified by comparison of integration of the  $-\text{CH}_2\text{CH}_2\text{SH}$  side chain peaks at 2.58 and 2.73 ppm with the acetamido moiety of the *N*-acetyl-D-glucosamine residue of HA.

#### Synthesis of hydrazide modified hyaluronic acid (HA-hy)

HA ( $MW = 150000$  Da) was dissolved in de-ionized water at concentration 8 mg/mL. Linker **3** 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 0.2 M and added to the solution of HA. Molar ratio of HOBt to HA disaccharide was 1 : 1. The pH of the resultant solution was adjusted to 4.7 after which the coupling reaction was initiated by addition of solid EDC (1 molar equivalents per HA disaccharide unit) 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 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-hy. The incorporation of hydrazide groups was verified by spectrophotometrically through reaction with trinitrobenzene sulfonic acid (TNBS).<sup>34</sup> It indicated that 10% of the HA disaccharide units were modified with hydrazide-terminated side chains.

#### Synthesis of aldehyde modified hyaluronic acid (HA-al)

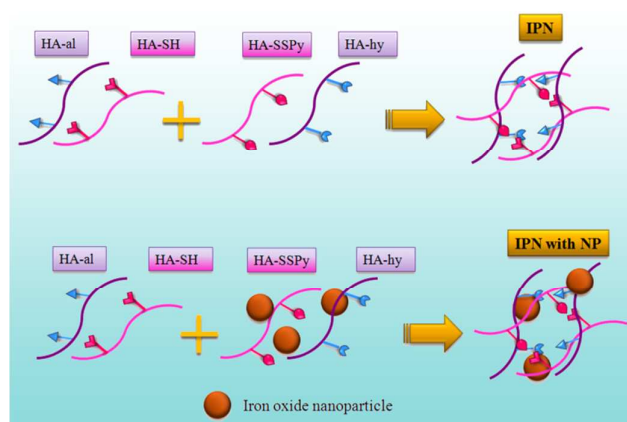
HA-hy was dissolved in de-ionized water at concentration 8 mg/mL. 0.15 M aqueous solution of sodium periodate was added to the above HA solution in 1:1 molar ratio between periodate and HA disaccharide units. The mixture was stirred for 10 min at room temperature, after which ethylene glycol (10 molar equivalents per amount of periodate used) was added to the mixture. The mixture was stirred in the dark overnight. The reaction solution was dialyzed against pure water two times ( $M_w$  cutoff = 3500) and finally lyophilized (60% yield). The amount of aldehyde groups was obtained by reaction with *tert*-butyl carbazate (TBC) followed by reduction with  $\text{NaBH}_3\text{CN}$ . Briefly, HA-aldehyde ( $\approx 20$  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  $\text{NaBH}_3\text{CN}$  (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.  $^1\text{H}$  NMR of the obtained product was examined and the peak corresponding to the *tert*-butyl substituent ( $(\text{CH}_3)_3\text{CONH}-$ ,  $\delta = 1.38$  ppm) was compared with the peak of HA acetamide protons at 1.9 ppm. Degree of aldehyde functionalization in the obtained HA-al derivative ( $\approx 10\%$ ) was calculated from the amount of reacted TBC reagent.

#### Preparation of IPN hydrogels

HA-SH, HA-SSPy, HA-hy, and HA-al were dissolved separately in PBS at 4% concentration. To make hydrogels of 300  $\mu\text{L}$  volume, equal volumes of HA-SH and HA-al solutions (75  $\mu\text{L}$  of each) were combined in a glass vial, while equal volumes of HA-SSPy and HA-hy solutions (75  $\mu\text{L}$  of each) were combined in another glass vial. Finally, the combined HA-SH and HA-al solution was added to the combined HA-SSPy and HA-hy solution, and vortexed for 10 seconds, which resulted in almost instantaneous gel formation. The hydrogel was set for 24 hours and then incubated in 3 mL of PBS for another 24 hours.

#### Preparation of hybrid IPN hydrogels

HA-SH, HA-hy, and HA-al were dissolved separately in PBS at 4% concentration, while HA-SSPy was dissolved at 6.7% concentration. To make hydrogels of 300  $\mu\text{L}$  volume, equal volumes of HA-SH and HA-al solutions (75  $\mu\text{L}$  of each) were combined in a glass vial. In a separately glass vial, 30  $\mu\text{L}$  of



Scheme 1. Representation of the double network and iron oxide hybridized double network.

IONPs dispersion was added to 45  $\mu\text{L}$  of HA-SSPy solution and the obtained combined solution was finally mixed with 75  $\mu\text{L}$  of HA-hy solution. Finally, the combined HA-SH and HA-al solution was added to the combined HA-SSPy, HA-hy and IONPs solution. After vortexing for 10 seconds, a hybrid double network hydrogel was formed. The hydrogel was set for 24 hours and then incubated in 3 mL of PBS for another 24 hours. Scheme 1 illustrates preparations double network hydrogels with and without encapsulated IONPs.

#### Preparation of hybrid disulfide cross-linked hydrogels

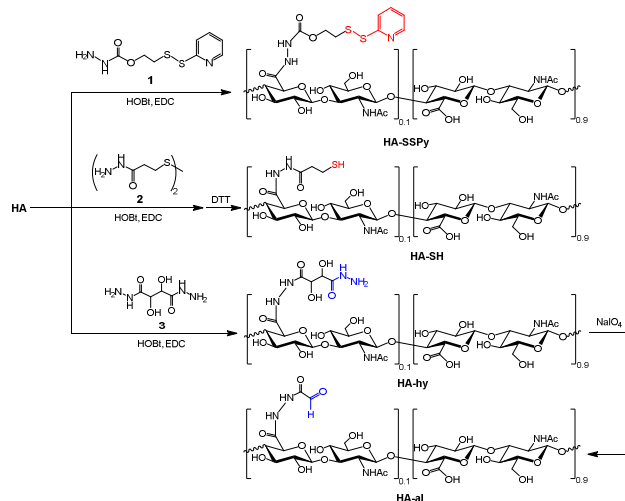
HA-SH was dissolved in PBS at 2% concentration, while HA-SSPy was separately dissolved in PBS at 2.5% concentration. To make hydrogels of 300  $\mu\text{L}$  volume, 30  $\mu\text{L}$  of IONPs dispersion was added to 120  $\mu\text{L}$  of HA-SSPy solution and the obtained combined solution was finally mixed with 150  $\mu\text{L}$  of HA-SH solution. The rest of the procedure was the same as for the formation of hybrid double network hydrogel.

#### Enzymatic degradation of hydrogels

Double network hydrogel with and without IONPs, and hydrazone cross-linked hydrogel with HA-SSPy were placed in 3 mL of PBS containing hyaluronidase at 1000 U/mL (1 mg/gel sample). At predetermined intervals of time (25, 55, 115, 175, 240, 300 min) the hyaluronidase solution was replaced to a fresh one. After 300 min of incubation, the concentration of Hase was increased to 2000 U/mL (2 mg/gel sample). The degradation was continued with the replacement of the enzyme solution at 355, 475, and 535 min. Finally, the enzyme concentration was increased to 8000 U/mL (8 mg/gel sample) and the degradation was continued with the replacement of the enzyme solution at 595, 630, and 660 min. At each time point, the mass of hydrogel samples was measured.

#### Chemical disassembly of hydrazone and disulfide networks

Double network hydrogel and hydrazone cross-linked hydrogel with HA-SSPy were placed in 3 mL of PBS containing 6 mg of  $\text{NaIO}_4$  (9.35 mM). After 45 minutes the hydrazone cross-linked



Scheme 2. Synthesis of HA derivatized with 2-dithiopyridyl (HA-SSPy), thiol (HA-SH), hydrazide (HA-hy), and aldehyde groups (HA-al).

hydrogel was completely dissolved, while double network hydrogel did not. Therefore, the double network hydrogel was taken from the solution and characterized by rheology. Similarly, double network hydrogel and disulfide cross-linked hydrogel were placed in 3 mL of PBS containing 11.6 mg of DTT (25 mM). The disulfide cross-linked hydrogel was dissolved after 30 minutes, whereas double network hydrogel did not. The double network hydrogel after DTT treatment was also characterized by rheology.

#### Assay of retention/release of IONPs from hybrid hydrogel

Three hydrogels, disulfide cross-linked hydrogel and its analogue hybridized with IONPs as well as IPN-IONPs hydrogel were prepared, set for 24 hours, and finally placed into 3 mL PBS each. After 24 hours of incubation, the solutions were examined by UV-Vis spectrophotometer. After the measurements, the hydrogels were washed two times more for 1 and 24 hours respectively. The absorbance of these washings was also measured.

#### Cytotoxicity study

Double network hydrogels of 100  $\mu\text{L}$  volume were prepared as previously described. After setting for 45 minutes, the hydrogels were repeatedly washed with 500  $\mu\text{L}$  of full cell culture medium (5  $\times$  5min) in order to adjust pH to neutral. 500  $\mu\text{L}$  of the cell culture medium was finally added into each hydrogel for 24 hours to acquire a leaching medium. C2C12 cells were seeded onto 96-well plate (5000 cell per well) and cultured in 200  $\mu\text{L}$  of cell culture medium containing 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin at 37°C and 5%  $\text{CO}_2$  humidified atmosphere for 12 hours. The cell culture medium was replaced with the leaching medium and culturing in the leaching medium was continued for 24 hours. Cell viability was assessed with CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit (Promega, USA) according to the manufacturer's protocol. Normal cell culture medium was used as a control group and each group had 5 repeats. Cell

experiments were performed following a protocol approved by the research and ethics advisory board of Uppsala University.

## Results and discussion

### Synthesis of HA derivatives

The incorporation of 2-dithiopyridyl and hydrazide groups to HA have been accomplished in one step by using the corresponding modifying reagents **1** and **3** (Scheme 2). Carbodiimide-mediated amide coupling of **1** occurred at carbazate terminal of the reagent thus providing the attachment of 2-dithiopyridyl functionality to HA backbone. On the other hand, we previously noticed that coupling of dihydrazide linker **3** occurred exclusively at one terminal most probably due to the rigidity of the linker.<sup>5</sup> This permitted hydrazide functionalization of HA in which the hydrazide terminal group is linked to the HA backbone through a 1,2-diol spacer. The obtained HA-hy derivative was partly used as-synthesized for hydrogels formation as well as it served as a starting material for the synthesis of aldehyde-derivatized HA (HA-al, Scheme 2). Particularly, treatment of HA-hy with sodium periodate led to the oxidative cleavage of the 1,2-diol group and generation of the aldehyde group. The last, thiol-modified HA component (HA-SH) for the formation of hydrogels was obtained via coupling of symmetrical dihydrazide linker **2** to HA carboxylate groups followed by reductive cleavage of the disulfide bond of the linker with dithiothreitol (DTT) as reported in the literature.<sup>32</sup>

### Interpenetrating hydrogels

First, interpenetrating 2% disulfide and 2% hydrazone networks were prepared by mixing of two solutions containing orthogonal HA derivatives, i.e. HA-SH and HA-al as well as HA-hy and HA-SSPy. Each of the derivatives was prepared as a 4% solution in PBS affording an IPN with 4% total concentration of HA. For comparison, two other samples were prepared with the same total HA concentration (i.e. 4%) but lacking one of the reactive components. Particularly, disulfide gel with encapsulated HA-al was formed when HA-hy component of the four-component

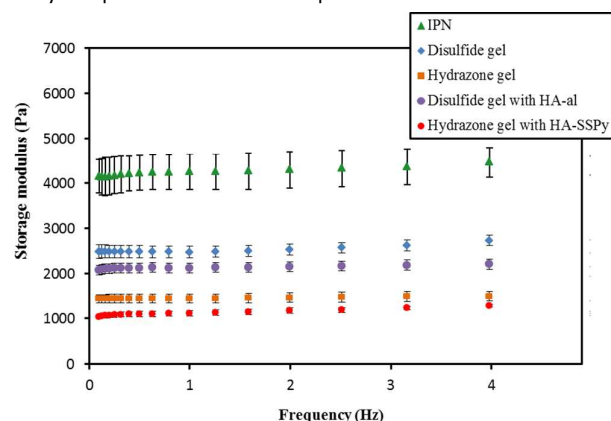


Fig. 1 Frequency sweeps of the single network hydrogels (disulfide and hydrazone), three-component formulations (i.e. [HA-SH + HA-SSPy + HA-al] and [HA-hy + HA-al + HA-SSPy]) and double network hydrogel.

formulation (i.e. HA-SH, HA-al, HA-SSPy, and HA-hy) was substituted to the equal mass amount of HA-al. Analogously, HA-SH was substituted with equal mass amount of HA-SSPy to form a hydrazone gel with physically encapsulated HA-SSPy.

Rheological analysis of the prepared hydrogels (i.e. single networks including disulfide and hydrazone hydrogels, double network and three components hydrogels) confirmed our assumption of increasing mechanical strength for HA IPN as compared with its single network counterparts. As shown in Figure 1, the storage modulus ( $G'$ ) of IPN was sufficiently higher than that of the other types of hydrogels. Particularly, the storage modulus of IPN was two times more than that of the disulfide gel and three times more than that of the hydrazone gel. On the other hand, mechanical strength of the three component hydrogels decreased relative to four and two component hydrogels. The third component, i.e. HA-SSPy in hydrazone gel and HA-al in disulfide gel, might have an interfering steric effect during the cross-linking reaction. As a result, the cross-linking density of the formed three component hydrogel matrix could be lower, thus reducing its mechanical strength. We observed that the hydrogel comprised of all four components (affording two networks) had the largest mechanical strength (green triangles in Figure 1) whereas the three component formulation containing HA-hy + HA-al + HA-SSPy gave the softest hydrogel (red circles in Figure 1).

The formation of IPN was also verified by characterizing morphology of the prepared hydrogels (Figure 2). Scanning electron microscopy (SEM) images showed that the porosity of the double network was lower than that of the single disulfide cross-linked hydrogel. Porosity of the hydrogel containing three components (i.e. HA-SH + HA-SSPy + HA-al, Figure 2c) was somewhat in between for the double (Figure 2a) and the single (Figure 2b) network hydrogels. This observation clearly indicated higher cross-linking density that was achieved after mixing of four HA components leading to the formation of two independent and interpenetrating disulfide and hydrazone networks.

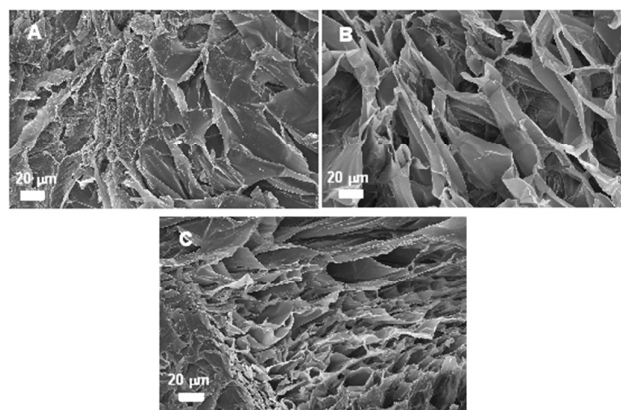


Fig. 2 SEM images of (a) double network hydrogel, (b) single disulfide network hydrogel, and (c) three component formulation [HA-SH + HA-SSPy + HA-al].

### Hybrid IPN hydrogels

To further enhance mechanical properties of the *in situ* forming hydrogels, provide their MRI imaging *in vivo*, and impart additional magnetic field-induced heat mediator properties, the double network hydrogel was *in situ* hybridized with water soluble iron oxide nanoparticles. Our mixing-triggered formation of hydrogels allows for *in situ* 3D encapsulation of any water soluble molecules, nanoparticles or even cells. In this study, dispersion of iron oxide nanoparticles (10 nm) in water was used for the preparation of the nanocomposite hydrogels.

To this point, the dispersion of IONPs was combined with HA-SSPy solution of a desired concentration and either double network or a single disulfide hydrogels hybridized with IONPs were subsequently prepared. In Figure 3, mechanical strength of the hybridized hydrogels and their non-hybridized analogs was compared before and after swelling in PBS. From Figure 3, it can be seen that the hybridized IPN hydrogel had the highest storage modulus ( $G' \approx 6000$  Pa). Generally, hybridization with IONPs resulted in increase of the strength of both single network and double network hydrogels. The enhancement of the gel strength was first of all due to the increase of the solids concentration in the hydrogels in which IONPs acted as a nano-filler agent. We have incorporated 0.15 mg of IONPs in each hydrogel of 300  $\mu$ L volume. Taking into account that average diameter of IONPs was 20 nm, it corresponded to  $\approx 7 \times 10^{12}$  particles of overall volume 0.03  $\mu$ L. In case of IPN, a hydrogel sample contained in total 12 mg of HA which was equivalent to  $\approx 0.18 \times 10^{23}$  disaccharide repeating units. Assuming homogeneous distribution of IONPs, each single particle was surrounded by  $\approx 2.57 \times 10^9$  of disaccharide repeating units. This indicates that IONPs did not contribute much as a filler material but rather interacted with HA polymer chains thus functioning as additional cross-linkers. Iron oxide can be bond to sulfur<sup>35</sup> of the modified side groups of HA as well as to oxygen<sup>36,37</sup> which is abundant in even non-modified HA. Equilibration of the as-prepared hybrid IPN hydrogel in PBS led to further increase of its

storage modulus. Contrary,  $G'$  of the hybrid disulfide hydrogel slightly decreased after swelling.

We observed that swelling of the hybrid hydrogels in aqueous solution did not result in a visual release of IONPs from the prepared materials. It suggests that IONPs are indeed strongly bonded to the HA polymer chains of the matrices. To prove this assumption, the release/retention of IONPs was assayed by UV-Vis absorption. The absorption peak of IONP is observed at 207 nm wavelength. Calibration curve for the maximum absorption of IONPs versus their concentration in PBS was first obtained (Figure S1). The hybridized and non-hybridized single disulfide cross-linked networks were chosen for the characterization of IONPs release and retention in the hydrogels. These hydrogels were separately placed into 3 mL PBS. After 24 hours of incubation, the solutions were examined by UV-Vis spectrophotometry. The hydrogels were then additionally washed two times more for 1 and 24 hours respectively and the absorbance of the obtained solutions after washings was also examined.

UV-vis spectra of the washing solutions are presented in Figure 4. During the initial hydrogels swelling for 1 hour, both hydrogels released some compounds or particles absorbing in UV-vis region. It should be noted that disulfide cross-linking by action of a thiol on 2-thiopyridyl residue results in elimination of 2-thiopyridine which absorbs light at 270 and 345 nm. This side product should be trapped in the formed matrix and subsequently released during the first washing round. Moreover, the amount of 2-thiopyridine released from the pure disulfide cross-linked hydrogel and its IONPs hybridized analogue should be equal because of the identical amounts of HA-SH and HA-SSPy used in the preparation of these hydrogels. Indeed, absorption intensities at 270 and 345 nm were found relatively similar for the first washings of both hydrogels. However, much higher intensity of absorption at 207 nm was detected in the first washing of the hybrid hydrogel comparatively with its non-hybridized counterpart. This absorption band corresponds to IONPs and it was expected to observe some amount of the released nanoparticles in the hybrid hydrogel. The slightly higher absorption intensities at 270 and 345 nm in the UV-vis spectrum of the hybrid hydrogel washing can also be explained by the released IONPs that have non-zero absorptions at wavelengths longer than 207 nm. Alternatively, these slightly enhanced absorptions at 270 and 345 nm might be due to binding of 2-thiopyridine to IONPs which are released from the hydrogel with the 2-thiopyridine surface coating layer. No compounds or particles absorbing in UV-vis region could be detected in the third washing solution of both hydrogels. Nevertheless, the hybrid hydrogels stayed yellow-colored after three washings indicating that only

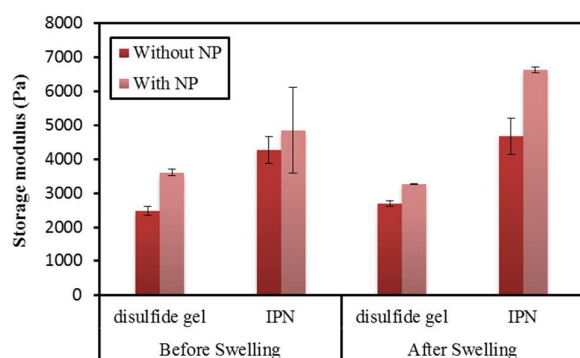


Fig. 3 Effect of iron oxide nanoparticles on hydrogel strength. Disulfide cross-linked hydrogel and IPN were examined at frequency of 1 Hz.

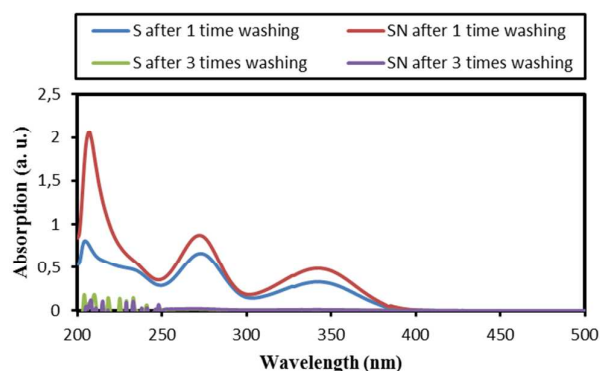


Fig. 4 UV-Vis absorption of washings of disulfide cross-linked hydrogels non-hybridized (S) and hybridized with iron oxide nanoparticles (SN) after one and three washings with PBS.

IONPs loosely bound on the surface of the hydrogel could be released during first washings while the rest of the nanoparticles were permanently trapped in the bulk of the hydrogel. Table 1 reveals the maximum absorption intensities of the solutions obtained after one and three times washing of the studied hydrogels. Because 2-thiopyridine does not absorb at 207 nm, we could calculate the amount of the released IONPs from the hybrid hydrogel (Table S1). Around 15  $\mu\text{g}$  of IONPs was released in the first fraction which corresponds to only 10% of the amount loaded during the preparation of the hybrid hydrogel ( $\approx 150 \mu\text{g}$  in 30  $\mu\text{L}$  solution).

The obtained results unequivocally showed feasibility of *in situ* entrapment of IONPs. The disulfide *in situ* cross-linked hydrogel does not leak the entrapped nanoparticles which become permanently associated with the hydrogel matrix. Thus, the iron oxide hybridized HA hydrogel can potentially be utilized in biological application such as hyperthermia of cancers.

### Degradation of hydrogels

Strong association of IONPs in the disulfide cross-linked HA matrix opens the possibility of using the nanoparticles as MRI contrast agents for tracing the degradation of the IONPs hybridized hydrogel *in vivo*. It is expected that the hydrogel will be resorbed *in vivo* due

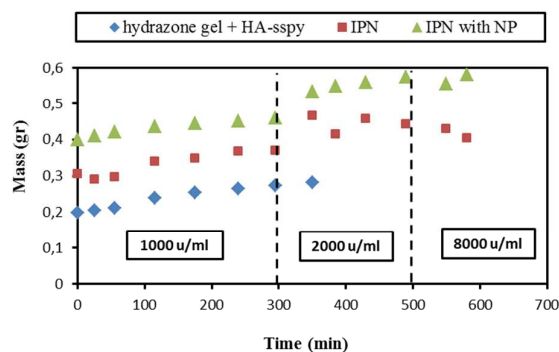


Fig. 5 Change of mass of hydrogels during enzymatic degradation by hyaluronidase over 600 minutes.

to enzymatic degradation. This will lead to gradual liberation of the IONPs from the matrix and change of the contrast of the hydrogel implant. To test this hypothesis, the degradation of IPNs and single network hydrogel (hydrazone hydrogel with entrapped HA-SSPy derivative) were subjected to degradation with hyaluronidase (Hase).

The enzymatic degradation of hydrogels was studied by measuring the hydrogel mass during their incubation in buffer solution containing hyaluronidase (Hase). The enzyme concentration was gradually increased from 2000 to 4000 and then to 8000 unit/mL in order to ensure the hydrogel degradation (Figure 5). It was detected that the mass of hydrogels increased slightly with time when the hydrogels were exposed to Hase. Hase catalyzes cleavage of HA backbone along the glycosidic bonds.<sup>38</sup> Thus, cross-linking density of the hydrogels should be gradually decreased leading to the increase of swelling of the hydrogels and the increase of their mass during the time of degradation.

It was observed that the hydrazone gel was disintegrated after about six hours while the IPN hydrogels were still intact after five days of incubation even at 8000 unit/mL of hyaluronidase concentration. This observation indicates that double network hydrogels are more resistant to enzymatic degradation and corroborates with their strong mechanical properties. It is also an indirect evidence of that these hydrogels will last longer *in vivo*. The controlled degradation of the HA hydrogels *in vivo* is crucial in tissue engineering<sup>11,39</sup> and drug delivery.<sup>11,40,41</sup> *In situ* formation of hybrid double network HA hydrogel can be thus a promising strategy to reduce fast enzymatic degradation *in vivo* and more effectively utilize release of drugs through an enzymatic pathway of hydrogel implant degradation.

*In situ* forming IPN hydrogels contain disulfide linkages in the disulfide network and 1,2-diol fragments in the hydrazone network. It gives a tool to study the formation of the double network through its selective chemical degradation. Particularly, a disulfide linkage can be cleaved with a reducing agent such as dithiothreitol (DTT), while 1,2-diol is known to be oxidatively cleaved with sodium periodate. Single hydrazone and disulfide networks can be used as references, respectively. It is expected that one type of network in the double network hydrogel partially disassembles by incubation in the solution of one of the cleaving agents (9.35 mM  $\text{NaIO}_4$  or 25 mM DTT) and leave another network unaffected. This is because  $\text{NaIO}_4$  does not break the disulfide linkage, while DTT does not affect 1,2-diol or hydrazone linkages. Treatment of IPN with either  $\text{NaIO}_4$  (Figure S2) or DTT (Figure S3) did not lead to the gel dissolution while hydrazone and disulfide cross-linked single network hydrogels were completely dissolved upon treatment with sodium periodate (after 43 minutes) and DTT (after 30 minutes) respectively. However, treatment of the double network hydrogel with either  $\text{NaIO}_4$  or DTT led to only softening of the hydrogel (cylindrical geometry of IPN maintained for 24 hours after treatment). Particularly, storage modulus ( $G'$ ) of hybrid IPN dropped from around 5500 Pa to 1300 Pa and 700 Pa (in viscoelastic region of frequencies from 0.1 to 1 Hz) for DTT and  $\text{NaIO}_4$  treated gels respectively (Figure 6). It proves that one of the networks (either hydrazone or disulfide) was disrupted during such treatments. It should lead to a decrease of overall cross-linking density of the hydrogel and some leakage of iron oxide

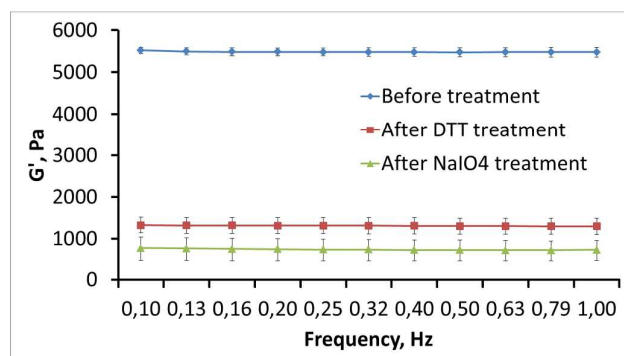


Fig. 6 Frequency sweeps of IPN hybridized with IONPs before and after treatment with either DTT or  $\text{NaIO}_4$ .

nanoparticles. Indeed, UV-vis spectroscopic examination showed that  $28.2 \pm 1.2$  and  $26.4 \pm 0.9$   $\mu\text{g}$  of IONPs was released from the hybrid IPN hydrogel during DTT and  $\text{NaIO}_4$  treatment respectively. It corresponded to  $18.8 \pm 0.8$  % and  $17.6 \pm 0.6$  % of the initially loaded IONPs. The obtained results thus further proved that two independent networks were indeed formed simultaneously upon mixing of four orthogonally reactive HA derivatives. On the other hand, enzymatic degradation or chemical disassembly of hybrid IPN hydrogel provided a mean of release of IONPs from the hydrogel.

#### Cytotoxicity of IPN hydrogels

Because double network hydrogels were designed for tissue engineering applications, we have evaluated cytotoxicity of the hydrogels. Due to fast gelling kinetics, it is unlikely that endogenous cells surrounding a hydrogel implant will be exposed to the free of the cells cultured in these media was similar to viability of the cells cultured with the control medium not exposed to the gels. Cell functional HA derivatives. Therefore, we evaluated double network hydrogels both with and without encapsulated IONPs after short setting for 45 minutes. The hydrogels after preparation were subsequently incubated in cell culture medium for 24 hours. This medium was added to C2C12 myogenic progenitor cells and the cells were cultured for 24 hours. Figure 7 clearly shows that viability

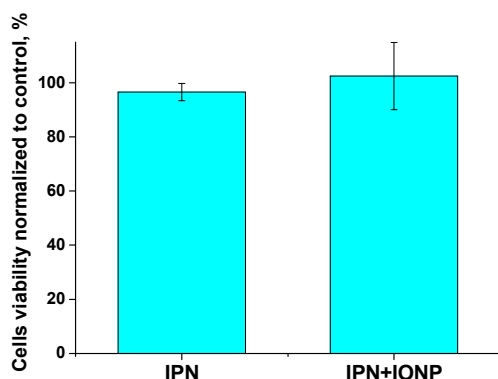


Fig. 7 Cytotoxicity assay of IPNs without and with IONPs. (a) C2C12 cells viability after culture for 24 hours in media derived from incubation with the gels.

morphologies were also similar after culturing under these three conditions (Figure S4). It indicates that the gels did not release any toxic products into culture medium that was later used for the cell culture.

#### Conclusions

We have developed an injectable double network hydrogels based on chemo-selective assembly of hyaluronic acid without any toxic solvents and low molecular weight cross-linkers. These double networks were successfully prepared through in situ mixing of aqueous solutions of suitably derivatized HA components. To further improve the mechanical strength of the double network hydrogel and possibly utilize it in MRI imaging as well as in hyperthermia treatment of tumors, the hydrogel was *in situ* hybridized with iron oxide nanoparticles. The highest mechanical gel strength was observed for the nanocomposite double network. The formation of IPN structure was confirmed by both rheology measurements and degradation studies. From the release/retention studies, it was found that 10 nm iron oxide nanoparticles were not released after three times washing. Strong binding of IONPs to the HA network thus provides useful physic-chemical properties to the resultant hydrogel. Consequently, this novel nanocomposite double network hydrogels can be a good candidate for biomedical application such as cancer therapy, especially in hyperthermia and in MR imaging of the implanted hydrogel materials.

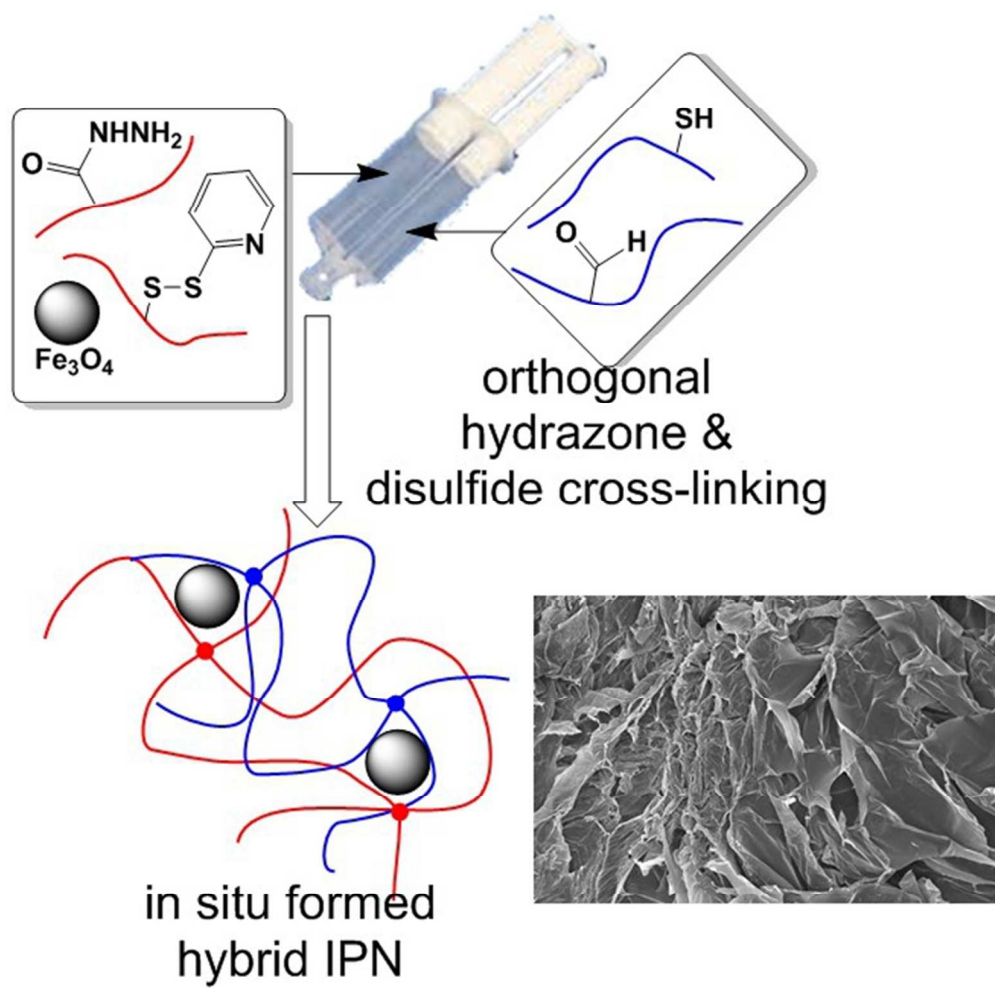
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