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Hydrazone crosslinked hyaluronan-based hydrogels for therapeutic delivery of adipose stem cells to treat corneal defects

Laura Koivusalo[a,*,1], Jennika Karvinen[b,1], Eetu Sorsa[b], Ilari Jönkkäri[c], Jari Väliaho[b], Pasi Kallio[b], Tanja Ilmarinen[a], Susanna Miettinen[a], Heli Skottman[a], Minna Kellomäki[a,b]

[a] BioMediTech Institute and Faculty of Medicine and Life Sciences, University of Tampere, Arvo Ylpön katu 34, FI-33520 Tampere, Finland
[b] BioMediTech Institute and Faculty of Biomedical Sciences and Engineering, Tampere University of Technology, Korkeakoulunkatu 3, FI-33101 Tampere, Finland
[c] Faculty of Engineering Sciences, Tampere University of Technology, Korkeakoulunkatu 6, FI-33101 Tampere, Finland

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ABSTRACT

Corneal blindness is a worldwide problem, plagued by insufficient amount of high-quality donor tissue. Cell therapy using human adipose stem cells (hASCs) has risen as an alternative to regenerate damaged corneal stromal tissue, the main structural and refractive layer of the cornea. Herein we propose a method to deliver hASCs into corneal defects in hyaluronan (HA)-based hydrogels, which form rapidly *in situ* by hydrazone crosslinking. We fabricated two different HA-based hydrazone-crosslinked hydrogels (HALD1-HACDH and HALD2-HAADH), and characterized their swelling, degradation, mechanical, rheological and optical properties and their ability to support hASC survival. To promote hASC attachment and survival, we incorporated collagen I (col I) to the more stable HALD1-HACDH hydrogel, since the HALD2-HAADH hydrogel suffered swift degradation in culture conditions. We then used an organ culture model with excised porcine corneas to study the delivery of hASCs in these three hydrogels for stromal defect repair. Although all hydrogels showed good hASC survival directly after encapsulation, only the collagen-containing HALD1-HACDH-col I hydrogel showed cells with elongated morphology, and significantly higher cell metabolic activity than the HALD1-HACDH gel. The addition of col I also increased the stiffness and reduced the swelling ratio of the resulting hydrogel. Most importantly, the corneal organ culture model demonstrated these hydrogels as clinically feasible cell delivery vehicles to corneal defects, allowing efficient hASC integration to the corneal stroma and overgrowth of corneal epithelial cells.

1. Introduction

Corneal blindness due to trauma, burns and various inherited or acquired diseases is a worldwide problem, with estimated 1.5 to 2 million new cases annually [1]. Currently these cases are only treatable by transplantation of a donor cornea; a procedure restricted by immune reactions and graft failure, as well as a continuous shortage of suitable donor tissue [2]. The limitations of corneal transplants have driven the search for alternative treatment options, particularly by means of tissue engineering and stem cell therapy. Mesenchymal stem cells (MSCs) have gained great interest in corneal regeneration due to their immunomodulatory and antiangiogenic properties [3], as well as for their capability to inhibit corneal scarring [4]. Human adipose stem cells (hASCs) are an abundant and accessible source of adult MSCs [5], which have also been shown to differentiate towards corneal stromal keratocytes *in vivo* when delivered to the corneal stroma [6,7]. However, simple stromal injection of hASCs in saline solution results in only low amount of integrated cells and insufficient new collagen production [6,8], whereas hydrogel delivery increases the survival of hASCs in the corneal stroma [7].

Collagen I (col I) and hyaluronan (HA) are natural extracellular matrix (ECM) components, present in varying abundance in different tissues. Col I is the main component of ECM in the corneal stroma, where it exists as highly regular fibrils for combined mechanical strength and high transparency [9]. HA is a high molecular weight polysaccharide, which has a high capacity to retain water and is degraded *in vivo* by hyaluronidase enzymes [10,11]. Rather than use HA in its native form, it can be modified through the carboxyl acid and hydroxyl groups in the D-glucuronic acid and *N*-acetyl-D-glucosamine sugar residues. HA-based hydrogels have also been previously suggested for corneal stromal repair, but to date they have required external crosslinkers and need to be preformed prior to implantation [7].

* Corresponding author.
E-mail address: laura.koivusalo@uta.fi (L. Koivusalo).
[1] Authors contributed equally.

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Hydrogel components, which gel upon mixing without external crosslinking agents, have the added advantage that they can be injected directly to the defect site where they are able to fill even irregularly shaped defects.

Hydrazone crosslinking has been widely used to prepare hydrogels for tissue engineering applications. Hydrazone crosslinking is a reaction between aldehyde- and hydrazide-groups and belongs to the group of pseudo click reactions (pseudo refers to moderate orthogonality). These reactions have many favorable properties, i.a. high reactivity, simple reaction conditions, no toxic reagents or side products, and high yields [12]. HA can be modified with complementary reactive aldehyde and hydrazide groups to enable this crosslinking. Aldehyde groups can be generated from vicinal diol groups of HA using periodate oxidation [13], or by incorporating an amino-glycerol side chain *via* an amidation reaction and selective oxidation of the pendent group of HA [14]. The latter method provides a less invasive way to modify the polymer. Hydrazide groups can be produced *via* reaction with either adipic acid dihydrazide [15,16] or carbodihydrazide [17]. The polyanionic behavior of HA at physiological pH hinders the adhesion of proteins and cells, which can be overcome by addition of other ECM binding sites, such as collagen [18]. Collagen can be incorporated to the previously described hydrogels, for example through imine formation, although it should be noted, that neutralized collagen can also form a gel on its own at 37 °C.

The aim of the study was to create transparent HA-based hydrogels for the delivery of hASCs for regeneration of the corneal stroma. In this study, we fabricated two HA-based hydrazone crosslinked hydrogels, and characterized their swelling, degradation, mechanical, rheological and optical properties and their ability to support hASC survival. We then further incorporated human col I into the more stable hydrogel, with the aim to promote hASC attachment and survival. In order to demonstrate proof-of-concept, we used an organ culture model with excised porcine corneas to evaluate the clinical relevance of the HA-based hydrogels for hASC delivery to stromal defects.

2. Materials and methods

2.1. Materials and general methods

Hyaluronic acid sodium salt (M_w = 1.5 × 10^5 g/mol) was purchased from Lifecore (Chaska, MN, USA). Adipic acid dihydrazide (ADH), hyaluronidase from bovine testes (Type I–S, 400–1000 units/mg solid), hydroxylamine hydrochloride, acetic acid, sucrose, 1-hydroxybentzotriazole (HOBt), carbodihydrazide (CDH), 3-amino-1,2-propanediol, t-butyl carbazate (TBC), picrylsulfonic acid solution (5% (*w/v*) in H_2O, TNBS), sodium cyanoborohydride, sodium periodate, sodium acetate, ethylene glycol, 1-Ethyl-3-[3-(dimethylamino)propyl] carbodiimide (EDC), dimethyl sulphoxide (DMSO), and deuterium oxide (99.9 atom% D, contains 0.05 wt% 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid, sodium salt) and collagen type I from human placenta were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium chloride was purchased from J.T. Baker (Holland). All solvents used were of analytical quality. Milli-Q water was used in synthesis and determinations. Dialysis membranes (Spectra/Por®cut-off 3500, 12–14,000 and 25,000 g/mol) were purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA, USA).

NMR-spectra were measured with Varian Mercury 300 MHz NMR Spectrometer (Palo Alto, USA). Samples (5 mg) were dissolved in deuterium oxide (600 μL) containing internal standard (0.05 wt% 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid, sodium salt). FTIR-spectra from hyaluronan components and formed hydrogels were measured on a Perkin Elmer Spectrum One ATR-FTIR Spectrometer (Waltham, MA, USA) in the spectral range of 400 to 4000 cm^{-1}.

2.2. Synthesis of aldehyde-modified hyaluronans

Aldehyde groups were introduced to HA according to previously reported method [14]. Reaction scheme is shown in Fig. S1 (a). Briefly, HA (400 mg) was dissolved in deionized water (60 mL). 3-amino-1,2-propanediol (182 mg), and HOBt (153 mg) pre-dissolved in 1:1 (*v/v*) mixture of acetonitrile-water (2 mL) were added, and pH of the solution was adjusted to 6 (1 M HCl). EDC (58 mg) was added to the mixture under nitrogen and stirred overnight. Derivatized polymer was dialyzed with MW cutoff 3500 membrane against dilute HCl (pH 3) containing 0.1 M NaCl for 48 h and against dilute HCl (pH 3) for 24 h. Purified polymer was lyophilized to obtain a white cotton-like product (2,3-dihydroxypropyl amide derivative of HA). This product (200 mg) was dissolved in deionized water (25 mL). Sodium periodate (107 mg) pre-dissolved in deionized water (0.5 mL) was added to the solution in the dark at room temperature (RT) and stirred for 5 min. Ethylene glycol (0.06 mL) was added to inactivate unreacted periodate and solution was stirred for 2 h. Derivatized polymer was dialyzed with MW cutoff 3500 membrane against deionized water for 24 h. Purified polymer was lyophilized to obtain a white cotton-like product, HALD1. ^{1}H NMR (D_2O, 300 MHz) 2,3-dihydroxypropyl amide derivative of HA: δ 4.53 (br s, 1H), 3.83–3.34 (m, 10H), 2.00 (s, 3H). HALD1 (Fig. S3 (a), D_2O, 300 MHz): δ 9.57 (s, 1H), 4.53 (br s, 1H), 3.65 (sharp s, 1H), 3.83–3.34 (m, 10H), 2.01 (s, 3H). FTIR (Fig. 1 (a), cm^{-1}): 1732 (ν(C═O) of −C(O)H), 1643 (ν(C═O) of *sec*. amide), 1617 (d(N−H) of −NHC(O)−), 1558 (d(N−H) of sec. amide).

Alternatively, a periodate oxidation was used to generate aldehyde groups from vicinal diol groups of HA according to previously reported method [13] with small modifications. Reaction scheme is shown in Fig. S1 (b). Briefly, sodium hyaluronate (0.500 g) was dissolved in deionized water (100 mL). Sodium periodate (0.30 g) was dissolved in deionized water (2.7 mL), added dropwise and stirred for 4 h in the dark at RT under nitrogen. Ethylene glycol (4 equivalents) was added to inactivate any unreacted periodate and the solution was then stirred for 1 h. Derivatized polymer was dialyzed with MW cutoff 25,000 membrane against deionized water for three days. Purified polymers were lyophilized to obtain white cotton-like product, HALD2. ^{1}H NMR (Fig. S3 (b), D_2O, 300 MHz): HALD2: *δ* 4.47 (m, 1H, H1 of glucose unit), 3.84–3.34 (m, 5H, H2–5 of glucose unit), 2.02 (s, 3H, −NHC(O)CH3). FTIR (Fig. 1 (b), cm^{-1}): HALD2: 1721 (ν(C═O) of −C(O)H), 1633 (ν(C═O) of −NHC(O)− and −C(O)OH), 1618 (d(N−H) of −NHC(O)−).

Degree of substitution (DS%) of HALD components were determined with TNBS method similarly to [19,20]. Briefly, HALD (20 mg) was dissolved in acetate buffer (2 mL, 0.1 M, pH 5.2) and added to TBC solution in acetate buffer (1 mL, 0.0348 g, 10-fold excess per molar amount of sodium periodate used). The mixture was allowed to react for 1 h at RT. Sodium cyanoborohydride (1 mL, 0.0166 g, equimolar amount to TBC) in acetate buffer was added and allowed to react for 24 h at room temperature under nitrogen. The polymer was dialyzed with MW cutoff 25,000 membrane against 0.1 M NaCl for 24 h and for a further 24 h in deionized water. Purified polymer was lyophilized to obtain a white cotton-like product. The ^{1}H NMR spectrum was measured and the DS% was determined from the integration of ^{1}H NMR peaks. ^{1}H NMR (D_2O, 300 MHz): δ 2.0 (3H, $NHCOCH_3$) and 1.4 (9H, t-Boc).

2.3. Synthesis of hydrazide-modified hyaluronans

Hydrazide groups were introduced to HA according to previously reported method [17]. Reaction scheme is shown in Fig. S1 (c). Briefly, HA (408 mg) was dissolved in deionized water (100 mL). Carbodihydrazide (90 mg) and HOBt (153 mg) were added to the solution and pH was adjusted to 4.7 (0.1 M NaOH). EDC (19.17 mg) was added under nitrogen and stirred for overnight. Derivatized polymer was dialyzed with MW cutoff 3500 membrane against dilute HCl (pH 3.5) containing 0.1 M NaCl for 48 h and against deionized water for 24 h. Purified

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