Acta Biomaterialia 38 (2016) 23–32

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Full length article

Hyaluronic acid-fibrin interpenetrating double network hydrogel prepared *in situ* by orthogonal disulfide cross-linking reaction for biomedical applications

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ARTICLE INFO

Article history:
Received 6 November 2015
Received in revised form 22 April 2016
Accepted 27 April 2016
Available online 28 April 2016

Keywords:
Interpenetrating hydrogel
Fibrin
Hyaluronic acid
Biodegradation
3D cell culture

ABSTRACT

To strengthen the mechanical properties of a fibrin gel and improve its applicability as a scaffold for tissue engineering (TE) applications, a strategy for the *in situ* preparation of the interpenetrating network (IPN) of fibrin and hyaluronic acid (HA) was developed on the basis of simultaneous and orthogonal fibrinogenesis and disulfide cross-linking. The synthetic pathway included the preparation of mutually reactive HA derivatives bearing thiol and 2-dithiopyridyl groups. Combining thiol-derivatized HA with thrombin and 2-dithiopyridyl-modified HA with fibrinogen and then mixing the obtained liquid formulations afforded IPNs with fibrin-resembling fibrillar architectures at different ratios between fibrin and HA networks. The formation of two networks was confirmed by conducting reference experiments with the compositions lacking one of the four components. The composition of 2% (w/v) fibrin and 1% (w/v) HA showed the highest storage modulus (G′), as compared with the single network counterparts. The degradation of fibrin in IPN hydrogels was slower than that in pure fibrin gels both during incubation of the hydrogels in a fibrin-cleaving nattokinase solution and during the culturing of cells after their encapsulation in the hydrogels. Together with the persistence of HA network, it permitted longer cell culturing time in the IPN. Moreover, the proliferation and spreading of MG63 cells that express the hyaluronan receptor CD44 in IPN hydrogel was increased, as compared with its single network analogues. These results are promising for tunable ECM-based materials for TE and regenerative medicine.

Statement of Significance

The present work is devoted to *in situ* fabrication of injectable extracellular matrix hydrogels through simultaneous generation of networks of fibrin and hyaluronic acid (HA) that interpenetrate each other. This is accomplished by combination of enzymatic fibrin cross-linking with orthogonal disulphide cross-linking of HA. High hydrophilicity of HA prevents compaction of the fibrin network, while fibrin provides an adhesive environment for *in situ* encapsulated cells. The interpenetrating network hydrogel shows an increased stiffness along with a lower degradation rate of fibrin in comparison to the single fibrin network. As a result, the cells have sufficient time for the remodelling of the scaffold. This new approach can be applied for modular construction of *in vitro* tissue models and tissue engineering scaffolds *in vivo*.

1. Introduction

The general idea of tissue engineering (TE) is based on the three-dimensional (3D) culturing of cells in suitable resorbable scaffolds followed by the implantation of the cell-seeded scaffolds *in vivo*. Hydrogels are 3D networks composed of cross-linked hydrophilic polymer chains. Hydrogels have been used as TE

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http://dx.doi.org/10.1016/j.actbio.2016.04.041
1742-7061/

scaffolds and in cell therapy because they provide the cells with highly hydrated tissue-like environment [1]. 3D encapsulation of cells in hydrogels is conducted *in situ*, i.e. during the polymer chains cross-linking; this affords a network to which the cells become entrapped and possibly attached. Ideally, hydrogels should mimic extracellular matrix (ECM) by providing a complex natural cocktail of tissue-specific signals. This is often achieved by supplying the cells with growth factors that have a very well defined role in tissue development. Particularly, the stimulation of cellular growth as well as the proliferation and differentiation in hydrogel matrices often rely on the simultaneous *in situ* encapsulation of the cells and growth factors. Among many factors that have to be considered in hydrogel design for TE, mechanical properties and the rate of scaffold degradation are important for providing adequate mechanical support for the cells during culture and space for scaffold remodelling. Moreover, in the case of load-bearing tissue, the scaffold must provide sufficient temporary support after implantation *in vivo* to withstand *in vivo* stress and loading [2]. Natural macromolecules such as collagen, fibrin and hyaluronic acid (HA) have widely been used to generate hydrogels with 3-dimensionally encapsulated cells as they possess many favourable properties for biomedical applications [3].

Fibrin is formed during the physiological coagulation cascade after thrombin-mediated cleavage of fibrinogen in the presence of Ca^{2+}. Fibrinogen is an approximately 45-nm-long plasma protein composed of two sets of three polypeptide chains, Aα, Bβ and γ, which are joined together by six disulfide bridges. Upon activation by the serine protease thrombin, fibrinogen monomers have a great tendency to self-associate in a half-staggered manner to form insoluble fibrin, a 3D porous network structure. Furthermore, the activated blood coagulation factor XIIIa belonging to a family of transglutaminases rapidly cross-links γ chains in the nascent fibrin polymer by introducing intermolecular (γ-glutamyl)-lysine chemical cross-links between the lysine of one γ-chain and the glutamine of another γ-chain. Fibrin sealants have been clinically used as a haemostatic agent in cardiac, liver and spleen surgery [4]. Due to their ability to support cell proliferation and differentiation, fibrin hydrogels have also been used in a variety of TE applications, including engineering of cardiovascular, ocular, muscle and bone tissues in the last decade [5]. However, fibrin has three major disadvantages: (1) compaction upon gel formation, (2) low mechanical strength, and (3) rapid degradation precluding proper tissue regeneration [5–9]. To improve the poor mechanical properties of fibrin, the combination of fibrin with other materials was suggested to obtain constructs with improved mechanical strength [10–13].

HA is a non-sulfated glycosaminoglycan that is widely distributed in the ECM of connective tissues. It plays an important role in the regulation of cell adhesion, tissue morphogenesis and modulation of inflammation. Because of its high hydrophilicity, biocompatibility and unique viscoelastic properties, HA has been used in a number of clinical applications, including eye surgery to facilitate healing and regeneration of the surgical wounds (a surgical aid) [14] and ear and sinus surgery [3]. HA hydrogels are also promising materials in cell culture and drug delivery [15–18]. HA was chemically converted into mutually reactive derivatives that could form hydrogels upon simple mixing in an aqueous medium [16]. Moreover, two orthogonal chemoselective cross-linking reactions were envisioned to form an interpenetrating network (IPN) of HA and fibrin to reinforce fibrin for TE applications [13,19]. Despite of these few examples of combination of HA and fibrin, little is known about which chemoselective chemistries can be tolerated without compromising the formation of the fibrillar structure of fibrin and afford ECM resembling IPNs with improved mechanical properties which last for sufficient time to induce cell proliferation and differentiation.

In this study, we investigated the use of disulfide cross-linked HA hydrogel in preparation of fibrin-HA IPN gels as an injectable and biodegradable scaffold for cell proliferation and differentiation. We first investigated if the obtained hydrogel was composed of two chemical networks of HA and fibrin. Mechanical properties of these new IPN gels were compared with those of single network counterparts as well as with the formulations wherein the conditions for one of the cross-linking reactions were compromised. Fluorescence spectroscopy was utilized for quantification of enzymatic degradation of hydrogels prepared from fluorescently labelled fibrinogen. Finally, viability, spreading and proliferation of cells encapsulated in IPN gels were studied in comparison with the single network analogues. Hydrogel degradation mediated by the cells laden in IPN hydrogels was also performed.

2. Materials and methods

2.1. Materials

HA sodium salt (MW 150 kDa) was purchased from Lifecore Biomedical, USA. 1-Ethyl-3- (3-dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxybenzotriazole (HOBt), 2,2′-dithiodi ethanol, DL-dithiothreitol (DTT) and pyridyl disulfide were purchased from Sigma-Aldrich Chemical Co., Sweden. Disulfanediyl bis(ethane-2,1-diyl) bis(2-(6-((hydrazinecarbonyl)oxy)hexanoyl)h ydrazinecarboxylate) **1** and 3,3′-dithiobis(propionic hydrazide) **2** were synthesized according to the literature procedures [20]. Hydrogels for rheological measurements were prepared with Tissucol (Baxter, Austria) 1.0 mL kit (fibrinogen, aprotinin, thrombin and $CaCl_2$ solution). For degradation studies, hydrogels were prepared from Tissucol Duo 500 (Baxter, Austria) 5.0 mL kit (fibrinogen and thrombin). The fibrinogen component in both fibrin sealant kits contain 10–50 U/mL of the transglutaminase Factor XIII (FXIII), where 1 U corresponds to the amount of FXIII contained in 1 mL of fresh normal plasma. Alexa Fluor© 488-labelled fibrinogen was purchased from Life Technologies, Sweden. Nattokinase was purchased from Japan bioscience Labs, USA. All solvents were of analytical quality. ^{1}H-NMR spectra of HA derivatives were recorded in D_2O with a JEOL JNM-ECP Series FT NMR spectrometer (400 MHz).

2.2. Synthesis of HA-hy-SSPy

HA was dually functionalized with hydrazide and thiol groups in one pot, as previously reported [20]. In detail, 400 mg of HA sodium salt (MW 150 kDa, 1 mmol of disaccharide repeat units) was dissolved in 50 mL of distilled water. Two homodifunctional linkers, dis ulfanediylbis(ethane-2,1-diyl) bis(2-(6-((hydrazinecarbonyl)oxy) hexanoyl)hydrazine carboxylate) (**1** in Scheme 1, 0.15 M equivalent of HA repeat units) and 3,3′-disulfanediyldi(propanehydrazide) (**2** in Scheme 1, 0.15 M equivalent of HA repeat units) were added into HA solution followed by the addition of HOBt (1 M equivalent per HA repeat unit), and the pH was adjusted to 4.7. EDC was added into the mixture at 0.3 M equivalent per HA repeat unit. After the overnight reaction, the pH of the mixture was increased to 8 and DTT was added with 10:1 M ratio to the sum amount of the linkers. After the next overnight treatment, the pH of the mixture was lowered to 3.5 and the mixture was thrice dialyzed against acidified water (pH = 3.5) and finally lyophilized. The obtained HA-hy-SH was analysed by ^{1}H-NMR to find the degree of modification with hydrazide (-hy: 10%) and thiol (-SH: 10%) groups. The degree of incorporation of thiol and hydrazide groups in HA-hy-SH was verified by comparison of integration of the –CH_2CH_2SH side chain peaks at 2.58 and 2.73 ppm and the –O$CH_2CH_2CH_2CH_2CH_2$CONHNH_2 side chain peaks at 4.90, 2.23, 1.57 and 1.30 ppm with the acetamido