Carbohydrate Polymers 161 (2017) 277–285

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

A novel photopolymerizable derivative of hyaluronan for designed hydrogel formation

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ARTICLE INFO

Article history:
Received 6 October 2016
Received in revised form 2 January 2017
Accepted 4 January 2017
Available online 10 January 2017

Keywords:
Hyaluronan
Polymerization
Crosslinking
Hydrogel
Photolithography

ABSTRACT

A new photopolymerizable derivative of hyaluronan (methacrylhydrazide-HA, MAHA) was prepared by carbodiimide chemistry. The reaction conditions were optimized for molecular weight (M_w), reaction time and amount of reagents with a degree of methacrylation (DM) ranging from 2% to 58%. Methacrylhydrazide-HA was hydrolytically stable (PBS, 7 days, 37 °C) in contrast to commonly used methacrylester analoque (23% hydrolyzed). MAHA readily photopolymerized into densely crosslinked hydrogels under physiological conditions. The varied DM, M_w, irradiation time (t_{exp}) and macromer concentration in photocrosslinking afforded hydrogels with different physical (swelling ratio, degradation rate) and mechanical properties (stiffness, toughness). Three-dimensional fabrication and surface patterning of MAHA hydrogels were demonstrated by photolithography and light mediated micromolding. A live-dead assay with skin fibroblasts showed convenient biocompatibility of MAHA (16%, 116 kDa) for potential scaffolding applications in tissue engineering and regenerative medicine.

1. Introduction

Photopolymerizable hydrogels derived from naturally occurring polymers such as hyaluronan (HA) have attracted significant interest in tissue engineering due to physical and mechanical properties, biodegradability and ability to be cured *in situ* through a minimally invasive procedure. Photopolymerizable hydrogels are hydrophilic gels covalently crosslinked by exposing a precursor polymer solution to light in the presence of a photoinitiator. The crosslinking reaction is called light-mediated free radical polymerization (photopolymerization) and it can proceed relatively rapidly under physiological conditions (Levett et al., 2014). Moreover photopolymerization is advantageous due to a temporal and spatial control that is achieved by light as the initiation trigger (Ifkovits & Burdick, 2007). Direct cellular encapsulation via photopolymerization is also possible as long as the initiation conditions are mild enough, so that radical concentrations or light intensities are not detrimental to the viability of the cells (Burdick, & Anseth, 2002).

The photopolymerization of polymer solutions has also been utilized in stereolithography (SLP) for the production of three-dimensional (3D) solid objects in a multi-layer fashion (Zorlutuna et al., 2012). This additive fabrication produces 3D scaffolds with designed pore size, geometry, orientation and bulk mechanical properties (Bártolo, Chua, Almeida, Chou, & Lim, 2009).

Another robust and reproducible approach that enables patterning of planar substrates is micromolding. This technology is used for generation of micropatterns in 3D tissue engineering constructs (Zorlutuna et al., 2012). Photopolymerized micromolding was used to generate cell-laden micro-scale hydrogels (microgels) from a variety of biopolymers including HA (Khademhosseini et al., 2006),

Abbreviations: 3D, three dimensional; BTH, bovine testicular hyaluronidase; CAD, computer aided design; CLSM, confocal laser scanning microscopy; COSY, correlation spectroscopy; DMEM, Dulbecco's modified eagle medium; DOSY, diffusion ordered spectroscopy; DM, degree of methacrylation; ε, molar attenuation coefficient; FBS, fetal bovine serum; HA, hyaluronan; GlcA, glucuronic acid; GlcNAc, *N*-acetylglucosamine; HPLC, high-performance liquid chromatography; GMHA, glycidyl methacrylate hyaluronan; HSQC, heteronuclear single quantum coherence; LC–MS, liquid chromatography–mass spectrometry; MAHA, methacrylhydrazide hyaluronan; M_w, molecular weight; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; Q_e, equilibrium swelling ratio; r.t., room temperature; RM, reaction mixture; SEC-MALLS, size exclusion chromatography coupled to multi-angle laser light scattering; SEM, scanning electron microscopy; SLP, stereolithography; SpHyl, Streptococcus pneumoniae hyaluronate lyase; t_{exp}, irradiation time; THX-H, thioxanthone acetic acid; THX-Na, sodium thioxanthone acetate; TOCSY, total correlation spectroscopy; UV/vis, UV–vis spectroscopy; w/v, weight per volume; δ, chemical shift; λ_{em}, maximum emission wavelength; λ_{max}, maximum absorption wavelength.

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http://dx.doi.org/10.1016/j.carbpol.2017.01.009
0144-8617/

gelatin (Nichol, Koshy, Bae, Hwang, Yamanlar, & Khademhosseini, 2010) and chitosan (Fukuda et al., 2006).

Methacrylates are the most common reactive groups used in radical polymerizations, because they react rapidly with radicals. Fortunately, the HA backbone presents several groups for methacrylation (Burdick & Prestwich, 2011). The simplest route is the acylation with methacrylic anhydride (Burdick, Chung, Jia, Randolph, & Langer, 2005; Smeds & Grinstaff, 2001). This approach works in aqueous conditions, but its efficiency is significantly lowered by a competitive hydrolysis of both reagents and products. Therefore a large excess of methacrylic anhydride (~20-fold excess) is required to obtain a satisfactory degree of methacrylation (5–15%).

An alternate method for methacrylation is alkylation with glycidyl methacrylate (Leach, Bivens, Patrick, & Schmidt, 2003; Leach & Schmidt, 2005). Although this way affords high DM (up to 90%) and hydrolytically stable ether conjugates, it also suffers from several drawbacks such as a large excess of glycidyl methacrylate (50–100 fold) and triethylamine (20–30 fold), strong basic pH (11–13), presence of an organic solvent (toxic DMF), long reaction times (5–10 days) and peeling reactions leading to substantial polymer degradation. We think, that all above mentioned limitations have a negative impact on biological, physical and mechanical properties of methacrylester-HA hydrogels.

Therefore, we prepared hydrolytically stable methacrylhydrazide-HA (MAHA) under mild reaction conditions, which readily photopolymerized to densely crosslinked hydrogels. We also systematically studied the effects of macromer molecular weight (M_w), degree of methacrylation (DM), concentration and irradiation time (t_{exp}) on physical (swelling, degradation) and mechanical properties (stiffness, toughness) of resulting MAHA hydrogels. In addition, we demonstrated potential applications of photolithography and light-mediated micromolding for 3D fabrication and surface patterning of MAHA hydrogels.

2. Materials and methods

2.1. Chemicals and reagents

Hyaluronan (M_w 116–2100 kDa) was provided by Contipro a.s. 1-Hydroxybenzotriazole monohydrate (HOBt, 97%, Aldrich), 1,4-dioxane (99%, Lach-ner), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 98%, Aldrich), CellTiter-Glo® Luminescent Cell Viability Assay (Promega), ethyl acetate (99.7%, Lach-ner), glucose (99.5%, Aldrich), glutamine (99%, Aldrich), hydrazine monohydrate (98%, TCI), methacrylic anhydride (94%, Aldrich), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC, 98%, Aldrich), penicillin/streptomycin (Aldrich), phenoxyacetic acid (98%, Aldrich), propan-2-ol (99.8%, Brenntag), sodium bicarbonate (100%, Lach-ner), sodium hydroxide (98%, Lach-ner), sulfuric acid (95%, Lach-ner), tetrahydrofuran (THF, 99.8%, Lach-ner), thiosalicylic acid (97%, Aldrich), triethanolamine (99%, Aldrich), Triton X-100 (Aldrich) and trypsin/EDTA (Aldrich) were used as obtained. $CDCl_3$ (99.8%), D_2O (99.9%) and DMSO-d_6 (99.9%) were purchased from CortecNet. Deionized water or PBS (pH 7.4, c 0.9% w/v) were used in all experiments. One equivalent (eq) of reagents used in the study means, 1 mol of a reagent to 1 mol of HA dimer.

2.2. NMR spectroscopy

Solution-state NMR spectroscopy was carried out on Bruker Avance III 500 MHz spectrometer. Spectra were acquired and elaborated by Bruker 2.1 Topspin software. The samples were dissolved in D_2O, $CDCl_3$ or DMSO-d_6 (0.8 mL) and transferred into 5 mm NMR quartz tubes.

2.2.1. Degree of methacrylation determination

The DM of MAHA was determined by proton NMR. The DM was defined as a molar ratio of vinyl protons at 5.63 ppm or 5.88 ppm and two protons of a HA dimer (H1, GlcA), (H1′, GlcNAc) at 4.47 ppm and 4.55 ppm multiplied by 100.

2.3. Molecular weight determination

SEC-MALLS was performed using a Waters Alliance liquid chromatograph (Model e2695) equipped with refractive index detector (Model 2414, Waters) and a TREOS multi-angle laser light scattering photometer (Wyatt Technology Corporation). The separation of MAHA was carried out using columns (PL aquagel-OH 20, 40 and 60) connected in series. The mobile phase was 0.1 M PBS + 0.05% NaN_3 at a flow rate 0.8 mL.min^{-1}. Data acquisition and M_w calculations were performed using ASTRA V software (Wyatt Technology Corporation, USA). The specific refractive index increment dn/dc of 0.155 mL g^{-1} was used for the determination.

2.4. UV/Vis spectroscopy

UV/Vis spectroscopy was performed on Shimadzu UV-2401PC spectrometer in a range of 200–600 nm. UV spectra were processed by software UV Probe version 2.00.

2.5. LC–MS spectroscopy

An Acquity UPLC chromatographic system connected to Synapt G2-Si mass spectrometer (Waters) was used for structural analysis of MAHA (1 mg mL^{-1}) after SpHyl degradation (3 IU mg^{-1}, 37 °C, 2 h). Separation was performed on a Waters Acquity BEH C18 column at 40 °C. The chromatographic eluent consisted of a binary phase of 0.1% formic acid in water and acetonitrile pumped at 0.4 mL min^{-1} with the gradient mode.

The mass spectrometer was equipped with an electrospray ionization source operating in negative resolution mobility mode. Sodium formate (0.5 mM in water: propan-2-ol = 10: 90, v/v) was used for instrument calibration in the m/z range 50–1850. The effluent was introduced into an electrospray source with an UPLC pump directly after the column separation. Instrumental parameters for full MS spectra acquired in the mass range 50–1850 were as follows: spray capillary voltage 2.7 kV, source temperature 100 °C, desolvation temperature 500 °C, sampling cone voltage 140 V, source offset 80 V, cone gas flow (N_2) 50 L h^{-1}, desolvation gas flow (N_2) 850 L h^{-1}, nebulizer gas flow (N_2) 6.0 bar, trap collision voltage 4.0 V, transfer collision voltage 2.0 V, trap gas flow (Ar) 2.0 mL min^{-1}, helium cell gas flow (He) 180.0 mL min^{-1}, ion mobility spectrometry (drift gas) flow (N_2) 90 mL min^{-1}, wave height 40.0 V and wave velocity 500 m s^{-1}. MS/MS spectra were acquired with the low mass resolution for precursor ion selection in quadrupole 10, and the collision energy of 50 V applied in the transfer cell. Data were collected at 0.5 scans s^{-1} and elaborated using the MassLynx 4.1 software.

2.6. Scanning electron microscopy (SEM)

The surface morphology of the freeze-dried gels was analysed by a scanning electron microscope Zeiss Ultra Plus with the highest resolution 0.8 nm and beam voltage 0.1–30 kV. The samples were whipped with Au by a Mini Sputter Coater SC7620 (Quorum Technologies, UK) in approximately 10–20 nm thickness. The samples