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Hyaluronic acid auto-crosslinked polymer (ACP): Reaction monitoring, process investigation and hyaluronidase stability

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ABSTRACT

Hyaluronic Acid (HA) is a non-sulphated glycosaminoglycan that, despite its high molecular weight, is soluble in water and is not resistant to enzymatic degradation, the latter of which hinders its wider application as a biomedical material. Auto-crosslinked polymer (ACP) gels of HA are fully biocompatible hydrogels that exhibit improved viscoelastic properties and prolonged *in vivo* residence times compared to the native polymer. Crosslinking is achieved through a base-catalysed reaction consisting of the activation of HA carboxyl groups by 2-chloro-1-methylpyridinium iodide (CMPI) and subsequent nucleophilic acyl substitution by the hydroxyl groups of HA in organic solvent.

In this study, a number of ACP hydrogels have been obtained via reactions using varying ratios of CMPI to HA. The crosslinking reaction was monitored by rheological measurements in organic solvents during CMPI addition to the reaction mixture. The ACP intermediates, powders and hydrogels were characterized, helping to elucidate the crosslinking process. A two-step mechanism was proposed to explain the observed trends in viscosity and particle size. Syntheses were carried out by varying the reaction temperature, respectively at 0 °C, 25 °C and 45 °C in N-Methyl-2-Pyrrolidone (NMP), as well as the solvent respectively in NMP, DMSO and DMF at 25 °C. Interestingly, varying these parameters did not substantially affect the degree of crosslinking but likely did influence the intra/inter-molecular crosslinking ratio and, therefore, the viscoelastic properties. A wide range of crosslinking densities was confirmed through ESEM analysis.

Finally, a comparative hyaluronidase degradation assay revealed that the ACPs exhibited a higher resistance toward enzymatic cleavage at low elastic modulus compared to other more chemically resistant, crosslinked HAs. These observations demonstrated the importance of crosslinking density of matrix structures on substrate availability.

1. Introduction

Hyaluronic acid (HA) is a linear anionic polysaccharide comprising repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine. HA is a biodegradable and biocompatible polymer with unique viscoelastic and rheological properties, which, along with its high capacity for lubrication, makes it a valuable component of many tissues in the body [1]. Unfortunately, the low residence lifetime of HA, due to the action of the enzyme hyaluronidase and free radicals [2], often restricts its application as a biomaterial. While HA hydrogels are mechanically too weak to provide sufficient support when used in the body [3], chemical crosslinking has been shown to be an effective method for enhancing the residence time of the polymeric structure of HA.

Presently, two strategies can be described for introducing crosslinks into HA molecules: using a bifunctional crosslinking agent and using a “zero-length” crosslinking agent.

As bifunctional agent can be used a highly reactive crosslinker, with examples being glutaraldehyde [4], divinyl sulphone [5], diepoxide (e.g., 1,4-butanediol diglycidyl ether) [6] and 1,2,7,8-diepoxyoctane, epichlorhydrin [7], or a bifunctional linker, such as diamine (e.g., hexamethylenediamine) [8] or dihydrazines [9]; that require activation of the HA carboxyl group with a coupling reagent. HAs that are crosslinked using these derivatives generally exhibit high biostabilities; however, the crosslinking agents themselves may be toxic, and their binding to biopolymers can modify the HA biocompatibility. The “zero-length” crosslinking agent is a coupling reagent, such as 2-chloro-1-methylpyrinium

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iodide (CMPI) [10,11] or 1-ethyl-(3,3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) [12], that promotes the formation of intra- and intermolecular ester bonds between the carboxyl and the hydroxyl groups of the HA chains. CMPI facilitated the synthesis of HA films with networks of higher crosslinking density and superior resistance against *in vitro* hydrolytic degradation than those crosslinked by EDC [12]. Moreover, using the CMPI reagent avoided any foreign bond incorporation into the crosslinked matrix compared to the EDC-crosslinked HA films, which resulted in the introduction of N-acylurea groups. These so-called HA auto-crosslinked polymers (ACP), compared to those crosslinked using bifunctional agents, show no risk of toxicity, higher biocompatibility and water solubility, but generally exhibit lower biostability [3]. ACPs have been widely used for different medical applications, such as for prevention of post-surgical adhesion [13] or as dermal fillers [14], and have also been widely proposed for tissue engineering applications [15].

For this process, many variables play a determinant role on both physical and structural features, making esterification hard to control when defined hydrogel performances are required [15]. Gamini et al. [16] investigated the importance of matrix homogeneity on a wide range of ACP hydrogels, proving that, apart from the degree of crosslinking, the rheological properties of the final products are strongly influenced by the crosslinking density. Morphological differences are ascribed to the regioselectivity of the esterification reaction, which may involve groups from the same polymer chain (intramolecular), as well as from different chains (intermolecular).

In this study, a number of ACP hydrogels were synthetized using different CMPI/HA ratios and the crosslinking reactions monitored by rheological measurements during CMPI addition. The syntheses were carried out at varying temperatures (e.g., 0 °C, 25 °C and 45 °C in NMP) as well as in different reaction solvents (e.g., NMP, DMSO and DMF at 25 °C). The obtained products were precipitated with ethanol to afford a fine white powder, then vacuum-dried and hydrated at fixed concentrations in water or PBS to obtain the hydrogels. The ACP gels were sterilized by heat and characterized by rheological and particle size analysis. Furthermore, the insoluble HA fraction and degree of esterification were determined. All of these parameters were correlated with the CMPI/HA ratio and the effect of solvent and temperature were evaluated and correlated with the crosslinking density network, also by Environmental Scanning Electron Microscopes (ESEM) analysis. Finally, a hyaluronidase degradation assay was performed, wherein ACP hydrogels were compared with two other classes of HA-based hydrogels, HBC and HDC, which are HA crosslinked with 1,4-butanediol-diglycidyl-ether (BDDE) and divinyl sulphone (DVS) difunctional agents, respectively.

2. Materials and methods

2.1. Materials

Hyaluronic acid sodium salt (HA) and tetrabutyl ammonium salt of HA were provided by Fidia Farmaceutici S.p.A. Bovine testes hyaluronidase (BTH; EC 3.2.1.35) was purchased from EDQM. All other reagents were supplied by Sigma and used without further purification.

2.2. Synthesis and characterization of auto-crosslinked polymer (ACP)

For this study, 3.75 g (on a dry basis) of tetrabutyl ammonium salt of HA (HATBA; 200 kDa) was dissolved in 150 mL of N-Methyl-2-Pyrrolidone (NMP) at 0 °C. As a representative example, to form ACP 5.2%, 0.080 g of 2-chloro-1-methylpyridinium iodide (CMPI) in 80 mL of NMP was added dropwise. The ACP % is expressed as [mol of CMPI/mol of HA repeat unit (r.u.)] x 100; the MW of HA-tetrabutyl ammonium salt r.u. is 620.8 Da. Concurrently, 0.4 mL of triethylamine (TEA) in 20 mL of NMP was added dropwise.

To obtain ACP 9%, 13%, 17%, and 22%, 0.14, 0.20, 0.26, and 0.34 g of CMPI, was added, respectively, in the presence of TEA. At different time points during the CMPI addition, 2 g of the reaction mixture was collected and stored at 4 °C for a maximum of 2 h until rheological analyses were performed as described below. After addition of a saturated NaCl solution, the products were precipitated into acetone, filtered, washed with a mixture of 5:1 v/v acetone/water, and finally vacuum-dried at 40 °C. The ACP gels were formulated at 20 mg/mL in PBS buffer (pH 6.4) or at 30 mg/mL in saline (final pH 6.5). All formulations were sterilized by autoclaving (Table 1). The same synthetic procedure was performed at different temperatures (25 °C and 45 °C) in NMP, as well as in different organic solvents (DMSO and DMF) at 25 °C to obtain the ACP derivatives (22%).

2.3. Rheological analysis

Approximately, 1 g of each ACP hydrogel sample and 1 g of each ACP reaction intermediate, collected during the reaction, were analysed using a Thermo Haake Mars II Rheometer at 25 °C. The G′ (elastic modulus) and G″ (viscous modulus) were measured (in Pa) from 0.07 to 90.0 rad/s at a fixed strain value of 10% (an initial strain sweep with an oscillatory shear strain of increasing amplitude, γ, at a constant frequency of $\omega = 1$ Hz was applied to determine the region of linear response of the sample: at 10% the viscoelastic range is linear). From the dynamic moduli, the complex viscosity is given by: $|\eta^*| = [(G'/\omega)^2+(G''/\omega)^2)]^{1/2}$.

All of the analysed samples were processed with Haake Rheowin Job Manager 4.0 software and the collected data were reprocessed using Origin 8SR4 and Microsoft Excel.

2.4. Determination of the degree of esterification of ACP products

The esterification degree (ED) of the ACP products was determined by spectrophotometric analysis of ferric hydroxamate complex formation, based on the method described by McComb et al. [17]. The colorimetric method is based on the conversion of ester bonds to hydroxamic acids and subsequent iron complexation. The results are tabulated in Table 1.

2.5. Water-insoluble HA fraction

Each ACP gel (diluted to 0.5 mg/mL in PBS at pH 7.2) was stirred for 6 h at 37 °C. The solution was filtered using a 0.2 μm nylon syringe filter and the soluble HA fraction was quantified by means of a Viscotek TDA Max 302 system, equipped with a triple detector (RI, LALS-RALS and a Differential Viscometer). Two Viscogel GMPWxl columns were eluted with a buffer (pH 7) comprising 0.1 M $NaNO_3$ and 3.0 mM NaN_3 (40 °C; flow rate: 0.6 mL/min; injection loop: 100 μL). All the acquired chromatograms were processed with OmniSec 4.5 software using a refractive index increment (dn/dc) of 0.155. The water-insoluble HA fraction was determined by the mass difference between the initial HA amount and the water-soluble fraction, expressed as % w/w.

2.6. Particle size analysis

The particle size measurements of the HA clusters in the ACP gels (30 mg/mL in saline, sterilized by heat for 121 °C for 15 min) were made using a Mastersizer 4000 (Malvern) [18]. The gels were