Carbohydrate Polymers 132 (2015) 472–480

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Investigating triazine-based modification of hyaluronan using statistical designs

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ARTICLE INFO

Article history:
Received 2 April 2015
Received in revised form 16 June 2015
Accepted 18 June 2015
Available online 26 June 2015

Keywords:
Hyaluronan
Triazine Coupling
Amidation
Fractional-factorial design
Response surface methodology

Chemical compounds studied in this article:
2-chloro-4,6-dimethoxy-1,3,5-triazine (PubChem CID: 18450)
4-methylmorpholine (PubChem CID: 7972)
Cystamine dihydrochloride (PubChem CID: 5941)

ABSTRACT

Hyaluronan (HA) and its derivatives have been extensively researched for many biomedical applications. To precisely tailor the property of HA by derivatizing it to a pre-determined extent is challenging, yet critical. In this paper, we used 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) and *N*-methylmorpholine (NMM) to derivatize HA via a triazine-based coupling reaction. Using a fractional factorial (FF) design, we observed that water content in the solvent, and molar ratios of CDMT and $NaHCO_3$ to the carboxylate were the significant factors controlling the derivatization. We investigated how the effect of each factor changes as reaction conditions change. Moreover, by altering the amount of CDMT and $NaHCO_3$, we developed a cubic regression model for precise control of the extent of derivatization using a response surface methodology (RSM) with a D-optimal design. No spurious peaks were detected by ^1H NMR spectrum and only 10% decrease of molecular weight of the derivatized HA was determined by GPC. The HA with 6% modification was relatively biocompatible up to 15 mg/mL.

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1. Introduction

Hyaluronan (also called hyaluronic acid, HA) is a non-sulfated glycosaminoglycan consisting of β-1,4-D-glucuronic acid-β-1,3-*N*-acetyl-D-glucosamine disaccharide repeating units (Deen et al., 2014; Oh et al., 2010). HA is widely distributed throughout skin, cartilage, synovial fluid, vitreous body of the eye, extracellular matrix and nucleus pulpous of intervertebral discs (Oh et al., 2010; Shen, Bhargav, Kishen, & Diwan, 2010; Singh et al., 2014). The high viscosity, elasticity, negative charge and biocompatibility of HA have extended its application in biomedical and cosmetic fields. For instance, HA has been used to reduce cytotoxicity in non-viral gene transfer systems (Bahadur, Thapa, & Xu, 2012; Chen et al., 2012; Fan et al., 2013; Feng et al., 2014; He et al., 2013; Sun et al., 2009; Wang et al., 2011; Xu, Quick, & Yeo, 2009). Other HA-based carriers for protein/peptides, drugs and metallic particles have also been investigated (Kwag, Oh, & Lee, 2014; Lee et al., 2012; Li, Yu, Jin, & Yin, 2012; Varshosaz, Ghalaei, & Hassanzadeh, 2014; Xiong et al., 2014). In addition, its use in cataract surgery as a natural lubricant and visco-supplement and intra-articular administration of hyaluronan-based therapeutics for the treatment of osteoarthritis has been approved by the FDA (Atamaz, Kirazli, & Akkoc, 2006; Evans, Kraus, & Setton, 2014; Fraser, Kimpton, Pierscionek, & Cahill, 1993; Goa & Benfield, 1994; Marshall, 2000; Singh et al., 2014; Smith et al., 2008; Wu et al., 2007). Recently, preparations containing crosslinked HAs have been developed to enhance their half-lives in intra-articular applications (Chevalier et al., 2010; Conrozier et al., 2009; Frampton, 2010; Larsen, Dursema, Pollak, & Skrabut, 2012). Another important application of HA is the formation of hydrogels via crosslinking (Allison & Grande-Allen, 2006; Li, Rodrigues, & Tomas, 2012b), which have been investigated for tissue engineering (Hanjaya-Putra et al., 2012; Lee & Kurisawa, 2013;

Abbreviations: FF, fractional factorial; RSM, response surface methodology; HA, hyaluronan or hyaluronic acid; CDMT, 2-chloro-4,6-dimethoxy-1,3,5-triazine; NMM, 4-methylmorpholine.

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http://dx.doi.org/10.1016/j.carbpol.2015.06.067
0144-8617/Published by Elsevier Ltd.

Martinez-Sanz et al., 2011; Martinez-Sanz et al., 2012), cell encapsulation (Coates, Riggin, & Fisher, 2013; Hassan, Dong, & Wang, 2013; Lam, Truong, & Segura, 2014), sustainable drug delivery (Ha et al., 2006; Jung, Park, & Han, 2010; Testa et al., 2009) and vitreous substitution (Nakagawa, Tanaka, & Miyata, 1997).

For many biomedical applications, the properties of HA need to be tailored or enhanced by precise chemical modification. The incorporated functional groups can be used for crosslinking (Eng, Caplan, Preul, & Panitch, 2010; Hassan et al., 2013; Lee & Kurisawa, 2013; Testa et al., 2009), active targeting (Kwag et al., 2014) and conjugation with metallic particles (Lee et al., 2012; Xiong et al., 2014), drugs (Li et al., 2012a; Varshosaz et al., 2014), or proteins (Kwag et al., 2014). Thiol-modified HAs are of particular interest due to their ability for crosslinking (Eng et al., 2010; Hassan et al., 2013), coating gold nanoparticles (Lee et al., 2012), inhibition towards peptidase and enhanced mucoadhesion (Kafedjiiski et al., 2007; Li et al., 2012a). The well-known carbodiimide chemistry has been extensively used to functionalize HA (Eng et al., 2010; Kafedjiiski et al., 2007; Kuo, Swann, & Prestwich, 1991; Li et al., 2012a; Xu et al., 2009b). We have previously investigated the factors controlling derivatization of HA through the carbodiimide reaction and determined a polynomial model to obtain HAs with desired degrees of amidation (Santhanam, Liang, Baid, & Ravi). Although the carbodiimide method is well established, there is room for significant improvement to overcome the low efficiency of carbodiimide and inevitable *N*-acylurea side product on the HA backbone. A triazine-based coupling method, which postulates the concept of a "superactive ester" intermediate, has gained much attention (Kaminski et al., 2005; Kunishima et al., 2012, 2013). Because of its excellent efficiency and low cost, the triazine-based coupling method has been used for synthesizing or modifying peptides (Higashibayashi et al., 2004; Tachibana, Monde, & Nishimura, 2004), nucleic acids (Chhabra, Sharma, Liu, & Yan, 2006; Gartner, Kanan, & Liu, 2002; Li, Gartner, Tse, & Liu, 2004; Liu et al., 2008) and polysaccharides (Aoki, Arai, & Hattori, 2004; Aoki, Kinoshita, Mikuni, Nakanishi, & Hattori, 2007; Farkas & Bystricky, 2007; Worthen & Lapitsky, 2011).

In this paper, amidation of HA using 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) and *N*-methylmorpholine (NMM) as the activating agents is investigated. A diamine, cystamine, was used as the reactive amine. The yielded cross-linked HA was reduced by dithiothreitol to produce partially derivatized *N*-(2-thioethyl)-hyaluronamide (referred as thiolated-HA below), which will be used to make hydrogels and to coat gold nanoparticles. A fractional factorial design was carried out as the first step to screen the important factors producing the most significant main effects and interactions. Besides the reactive amine, cystamine, and the activating agents CDMT and NMM, a proton-capture agent $NaHCO_3$ was also used in this reaction. This reaction was carried out in water or a water/acetonitrile mixture to provide solubility of HA. Consequently, the five factors, which may affect the amidation, including the water content in the solvent and the mole ratios of CDMT, NMM, cystamine and $NaHCO_3$ to COOH, were chosen for this study. We also investigated the change of effect on amidation of each factor under different reaction conditions. Next, a D-optimal design of response surface methodology (RSM) was applied to the important factors selected from the fractional factorial design to learn how they affect the derivatization of HA, and thus to build a model for precise control of the derivatization extent of HA.

2. Experimental

2.1. Materials

Sodium hyaluronate (M_w = 200 kDa, cat# HA-200K-1) was purchased from Lifecore Biomedical and used as received. Cystamine dihydrochloride (98%, cat# 30050), 2-chloro-4,6-dimethoxy-1,3,5-triazine (97%, cat# 375217), acetonitrile (99.8%, cat# 271004), 4-methylmorpholine (98%, cat# 67870), sodium sulphite (98%, cat# S0505), 5,5′-dithiobis(2-nitrobenzoic acid) (98%, cat# D8130) and dithiothreitol (98%, cat# 43819) were purchased from Sigma-Aldrich and used as received. Glycine (tissue culture grade, cat# BP381-1) and ethylenediaminetetraacetic acid disodium salt (electrophoresis grade, cat# BP120-500) were purchased from Fisher and used as received.

2.2. Fractional factorial design for screening the significant factors

The experimental design and statistical analysis were carried out using Design-Expert software 7.0.0. Five factors, including the water content in the solvent and the mole ratios of CDMT, NMM, cystamine and $NaHCO_3$ to COOH, were chosen and varied at two levels to investigate the factors and screen for the significant variables affecting the responses (Table 1). A normal design using five factors at two levels would require 2^5 (32 experiments); however, as we were initially interested in only the effect of the main factors and not their higher order interactions, we chose a FF design of resolution V in which the main effects were unconfounded, while two-factor interactions were confounded with three-factor interactions in 16 experimental runs ($2^{(5-1)}$). The mole ratios of CDMT,

Table 1
The experimental conditions (factors) and responses of each CDMT-mediated amidation of HA using an FF design.

Run	Fact. A: water V%	Fact. B: CDMT*	Fact. C: $NaHCO_3$*	Fact. D: NMM*	Fact. E: 2× cystamine*	Resp. 1: Final pH	Resp. 2: Deriv. %	Resp. 3: Effic. of CDMT**
1	100	0.5	0.5	0.5	0.5	6.5	15.1	30.2
2	100	1	1	1	1	6.5	24.6	24.6
3	58	1	0.5	0.5	0.5	5	36.0	36.0
4	58	1	1	1	0.5	6	27.4	27.4
5	58	1	0.5	1	1	6	40.9	40.9
6	100	1	0.5	1	0.5	6	30.0	30.0
7	58	0.5	1	1	1	7.5	8.64	15.3
8	100	0.5	0.5	1	1	7	9.00	18.0
9	100	1	0.5	0.5	1	5.5	17.7	17.7
10	58	0.5	1	0.5	0.5	7.5	12.3	24.6
11	100	0.5	1	0.5	1	6.5	10.5	21.0
12	100	1	1	0.5	0.5	6	20.2	20.2
13	58	0.5	0.5	1	0.5	7.5	18.7	37.4
14	58	1	1	0.5	1	6	26.4	26.4
15	100	0.5	1	1	0.5	7.5	7.19	14.38
16	58	0.5	0.5	0.5	1	6.5	18.8	37.6

* The numbers stand for the ratio of the moles of each reactant to the moles of COOH.
** This was calculated by the following formulation: response 3 = response 2/factor B.