Carbohydrate Polymers 116 (2015) 42–50

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Optimized triazine-mediated amidation for efficient and controlled functionalization of hyaluronic acid

Tina Borke[a], Françoise M. Winnik[a,b,c], Heikki Tenhu[a], Sami Hietala[a,*]

[a] Laboratory of Polymer Chemistry, Department of Chemistry, P.O. Box 55, 00014 University of Helsinki, Finland
[b] Faculty of Pharmacy and Department of Chemistry, Universite de Montreal, CP 6128 Succursale Centre Ville, Montreal, QC H3C 3J7, Canada
[c] World Premier International (WPI) Research Center Initiative, International Center for Materials Nanoarchitectonics (MANA), National Institute for Materials Science (NIMS), 1-1 Namiki, Tsukuba, Ibaraki 305-0044, Japan

ARTICLE INFO

Article history:
Available online 19 April 2014

Keywords:
Hyaluronic acid
Triazine
Carbodiimide
Maleimide-thiol
Azide-alkyne
Click reaction

ABSTRACT

Triazine-based coupling agents have the potential to replace carbodiimides in the functionalization of hyaluronic acid (HA) giving derivatives with high degrees of substitution (DS) under mild conditions with excellent efficiency. Kinetics of the triazine-mediated amidation of HA in aqueous solution were investigated to understand the reaction mechanism and the role of the amine reagent. The DS decreased with increasing basicity of the amine. The water soluble coupling agent was stable under the reaction conditions ($t_{1/2}$ = 10 days) in the absence of amines. The activation of HA proceeded quantitatively. The stoichiometry of amine was the limiting factor in the substitution. Functional HA derivatives with DS up to 55% were obtained by the triazine-mediated amidation. They were used successfully to prepare well-defined HA conjugates *via* the maleimide-thiol and the azide-alkyne "click" reactions.

1. Introduction

Hyaluronic acid (HA), a naturally occurring glycosaminoglycan found ubiquitously in the extracellular matrix, is an attractive scaffold for various medical applications. It is readily modified to form hydrogels of unique viscoelastic and hygroscopic properties useful for applications in tissue engineering or drug delivery (Coviello, Matricardi, Marianecci, & Alhaique, 2007; Garg & Hales, 2004; Huerta-Angeles et al., 2012; Owen, Fisher, Tam, Nimmo, & Shoichet, 2013; Xu, Jha, Harrington, Farach-Carson, & Jia, 2012). The chemical modification of HA poses several obstacles related to its solubility and reactivity characteristics. Sodium hyaluronate is soluble readily in water, but it scarcely dissolves in most solvents commonly used in synthesis. Also, HA is prone to degradation and depolymerization under harsh reaction conditions, such as strongly alkaline, acidic or oxidative solutions, excessive heat, shear, and ultrasound or microwave irradiation (Dřímalová, Velebný, Sasinková, Hromádková, & Ebringerová, 2005; Maleki, Kjøniksen, & Nyström, 2008). To preserve the chemical integrity and the molar mass of HA, it is best to select reactions that proceed under mild conditions, and ideally in neutral aqueous solution at room temperature.

When designing a particular HA derivative the targeted degree of substitution (DS) is dictated by the envisaged application. For applications in the area of cell growth within a hydrogel scaffold (Seidlits et al., 2010) or targeted drug/gene delivery vehicles (Takei et al., 2004), low degrees of substitution are needed to maintain the biological functionality of HA. Oh et al. observed that HA derivatives with DS less than 25% could be efficiently taken up by cells *via* receptor-mediated endocytosis (Oh et al., 2010). In contrast, if a derivative with long residence time in the body, such as a hydrogel for tissue augmentation (Oh et al., 2008; Yeom et al., 2010), is designed the carboxylic acid groups are preferentially masked by a high degree of substitution to inhibit the recognition by hyaluronidase and thus slow down the degradation rate (C. Schanté, Zuber, Herlin, & Vandamme, 2011; Yeom et al., 2010).

Chemical modification of HA can be accomplished by amidation or esterification of the carboxylic acid substituent of the D-glucuronic acid moiety of the HA disaccharide repeating unit (Bulpitt & Aeschlimann, 1999; Crescenzi, Cornelio, Di Meo, Nardecchia, & Lamanna, 2007; Di Meo et al., 2006; Dong et al., 2012) as well as by substitution of the primary alcohol of the *N*-acetyl-glucosamine (see a review by C.E. Schanté, Zuber, Herlin, & Vandamme, 2011). Several methods have been devised to facilitate the amidation of the HA carboxylate groups (see Table 1). They usually involve "activation" of the carboxylate

* Corresponding author. Tel.: +358 9 191 50333; fax: +358 9 1915 0330.
E-mail addresses: tina.borke@helsinki.fi (T. Borke), francoise.winnik@umontreal.ca (F.M. Winnik), heikki.tenhu@helsinki.fi (H. Tenhu), sami.hietala@helsinki.fi (S. Hietala).

http://dx.doi.org/10.1016/j.carbpol.2014.04.012
0144-8617/

Table 1
Representative examples of procedures for the coupling of sodium hyaluronate with amines in aqueous solution.

Entry[a]	Coupling agent CA_1/CA_2	Amine	Eq.[b] of CA_1	Eq.[b] of CA_2	Eq.[b] of Amine	DS [%]	Method to determine DS[c]	Ref.
1	EDC/NHS	Spermidine·3HCl	1.5	1.5	7.9	31	^{1}H NMR, DOSY[d]	(Di Meo et al., 2006)
2	EDC/NHS	L-Alanine ethyl ester	4.0	4.0	8.0	13	^{1}H NMR	(C. Schanté et al., 2011)
3	EDC/NHS	Propargyl amine	5.0	5.0	7.6	21	^{1}H NMR	(Crescenzi et al., 2007)
4	EDC/HOBt	Cysteamine·HCl	10.0	30.0	30.0	64	^{1}H NMR	(Bencherif, Washburn, & Matyjaszewski, 2009)
5	EDC/HOBt	Tyramine·HCl	4.0	4.0	21.6	9	^{1}H NMR	(Kurisawa, Chung, Yang, Gao, & Uyama, 2005)
6	EDC/HOBt	$(LS)_4$-peptide	2.5	3.5	0.06	6	Elemental analysis	(Elder, Dangelo, Kim, & Washburn, 2011)
7	CDMT/NMM	L-Alanine ethyl ester	3.0	4.5	4.5	50	^{1}H NMR	(C. Schanté et al., 2011)
8	CDMT/NMM	1-Propanamine	0.5	1.0	1.0	20	^{1}H NMR	(Bergman et al., 2007)
9	DMT-MM	Furfuryl amine	4.0	–	2.0	75	^{1}H NMR	(Nimmo, Owen, & Shoichet, 2011)
10	DMT-MM	Furfuryl amine	2.0	–	1.0	61	^{1}H NMR	(Nimmo et al., 2011)
11	DMT-MM	Furfuryl amine	1.0	–	0.5	49	^{1}H NMR	(Nimmo et al., 2011)

[a] Color code: white = Sum of $Equivalents_{(CA1+CA2+Amine)} < 5$; light gray < 15; medium gray < 30; dark gray > 30
[b] Compared to amount of HA-carboxylic acid groups.
[c] NMR spectra were measured in deuterium oxide.
[d] Diffusion-ordered NMR spectroscopy.

groups with reagents, such as *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) or 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) (C.E. Schanté et al., 2011). EDC reacts with HA carboxylic acid groups to form an *O*-acylisourea intermediate, which can then be attacked by the nucleophilic amine. Unfortunately this intermediate is prone to rapid rearrangement to an unreactive *N*-acylurea group linked covalently to HA (Kuo, Swann, & Prestwich, 1991; Nakajima & Ikada, 1995). This side reaction can be reduced by activation of the carboxylates with *N*-hydroxysuccinimide (NHS) or 1-hydroxybenzotriazole (HOBt) to form active esters with HA, which undergo further aminolysis (Bulpitt & Aeschlimann, 1999). Nevertheless the efficiency of carboxylic acid activation by carbodiimides is low and the DS are poorly controlled. The coupling reagents, additives and amines have to be applied in large molar excess to achieve DS ranging typically from 5 to 30 per 100 disaccharide units causing high cost and waste production (see Table 1).

The triazine-mediated amidation of polysaccharides has been reported more recently. It was shown to proceed with high efficiency and without side reactions (Bergman, Elvingson, Hilborn, Svensk, & Bowden, 2007; Farkaš & Bystrický, 2007; C. Schanté et al., 2011). Here, HA is activated by formation of an acyloxytriazine (Kamińska, Kamiński, & Góra, 1999; Kunishima, Kawachi, Hioki, Terao, & Tani, 2001), which is subsequently attacked by the amine to form a tetrahedral intermediate (Kamiński, 1994). The latter preferentially forms the amide derivative. By this method, it is possible to obtain HA amide derivatives with a high DS, up to 75%, with reduced amounts of coupling reagents (see Table 1). These results represent a great improvement compared to the EDC-mediated substitution, in view of the reagent amounts needed, the mild conditions, and the minimal degradation of the polysaccharide chain (Bergman et al., 2007).

In addition, triazine coupling agents are milder than carbodiimide compounds, less allergenic, and safer (Kamiński, 1985; Rayle & Fellmeth, 1999). They are easily synthesized from inexpensive commodity chemicals, such as cyanuric acid (Cronin, Ginah, Murray, & Copp, 1996). These excellent features motivated us to investigate in detail the use of the triazine-mediated amidation to functionalize HA. The results of this study are presented here. We start with a series of experiments aimed at understanding the amidation mechanism, in particular the effect of the pK_a of the amine. Although ratios of coupling agent and amine to HA-carboxylate groups are commonly varied to obtain different DS (see Table 1), little is known on the specific influence of the amine concentration or the carboxylate concentration on the outcome of the reaction. To further the understanding of the triazine mediated amidation of HA, we investigated the kinetics of the steps involved in the reaction and monitored the stability of the coupling agent under various reaction conditions. The amidation procedure was validated with five functional amines selected with a view on further modifications of HA. Each amine carries a functional group, stable under the conditions imposed by the amidation, but reactive upon implementation of common "click" coupling reactions. We illustrate the use of HA derivatives obtained *via* triazine-mediated amidation as starting materials for azide-alkyne and maleimide-thiol "click" reactions.

2. Experimental

2.1. Materials

High molar mass sodium hyaluronate (HA, 752 kg/mol according to manufacturer, research grade) was obtained from Lifecore Biomedical (U.S.) and used as received. Low molar mass HA (~1 kg/mol) was obtained by enzymatic hydrolysis of the former according to a published procedure (Yang, Kataoka, & Winnik,