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

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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

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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,

2005). The reagents: allylamine (98%), *N*-(2-aminoethyl)maleimide trifluoroacetate (≥95%), 2-aminoethyl-methacrylate hydrochloride (90%, containing ~500 ppm phenothiazine as stabilizer), 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT, 97%), cysteamine hydrochloride (≥97%), ethylenediaminetetraacetic acid (EDTA, ≥99.4%), 1-mercapto-11-hydroxy-3,6,9-trioxaundecane (97%), *N*-methylmorpholine (NMM, 99%), propargylamine hydrochloride (95%) and (+)-sodium L-ascorbate (≥98%) as well as Dowex® 50WX8 ion exchange resin (hydrogen form, 100-200 mesh) were purchased from Sigma–Aldrich (Finland) and used as received. 1-Azido-11-hydroxy-3,6,9-trioxaundecane and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) were synthesized according to published procedures (Bertozzi & Bednarski, 1991; Kunishima, Kawachi, Monta, et al., 1999). Copper(II) sulfate pentahydrate (puriss.) was obtained from Merck (Germany) and used as received. Acetonitrile (HPLC gradient grade, VWR, Finland) and distilled water were used as solvents. Dialysis was conducted in CelluSep T2 regenerated cellulose tubular membranes (Membrane Filtration Products, U.S.) with a molecular weight cut off (MWCO) of 6000–8000 g/mol. Samples for ^{1}H NMR spectroscopy were prepared in deuterium oxide (D_2O, 99.96% D) and dimethyl sulfoxide-d_6 (DMSO-d_6, 99.80% D) obtained from Euroiso-Top (France).

2.2. Proton Nuclear Magnetic Resonance Spectroscopy (^{1}H NMR)

^{1}H NMR spectra (500.13 MHz) of the samples (5 mg/mL in D_2O/DMSO-d_6 (1:1, v/v) unless otherwise stated) were recorded on a Bruker Avance III 500 spectrometer at 298 K. The chemical shifts are given in parts per million (ppm) and are calibrated relative to the residual solvent protons (HDO: 4.80 ppm). The multiplicities of the signals are indicated by: *s* singlet, *d* doublet, *t* triplet, *p* pentet, *m* multiplet, *br* broad signal and coupling constants (*J*) are expressed in Hz. Degrees of substitution (DS) were calculated from comparing the integrals of the methyl protons (2.2 ppm) of the *N*-acetylglucosamine moiety of HA and distinctive peaks of the introduced side groups and are given in % per 100 disaccharide units.

2.3. Reactions

2.3.1. Amidation of HA in water/acetonitrile

Following a published procedure (Bergman et al., 2007; C. Schanté et al., 2011), HA (752 kg/mol) was coupled with various "clickable" amines. In a typical reaction, HA (1.0 eq., 200.0 mg, 0.50 mmol $-COO^-$) was dissolved in 40 mL water under gentle stirring. The solution was cooled in an ice bath and 27 mL acetonitrile were added dropwise. CDMT (1.0 eq., 87.5 mg, 0.50 mmol) and NMM (1.5 eq., 82 μL, 0.75 mmol) were added to the cold solution and the mixture was subsequently allowed to come to room temperature and was stirred for 1 h. Thereafter the amine compound (1.5 eq., 0.75 mmol) was added and the mixture was stirred at room temperature overnight. The crude mixture was incubated with Dowex® 50WX8 ion exchange resin (*ca.* 2 g) for 1 h, then filtered and dialyzed against aqueous NaCl (0.1 M) for 2 days and against water for 2 days and lyophilized. The product yields were typically around 80% based on the average molecular weight of the disaccharide repeating units considering the degree of substitution.

The pH values of the reaction mixtures were measured after addition of all reactants using a MeterLab PHM 210 standard pH meter (Radiometer, Copenhagen) equipped with a VWR 662-1767 semi-micro pH electrode calibrated in aqueous buffer solutions. The values were converted to real pH values for the water/acetonitrile mixture according to Gagliardi, Castells, Ràfols, Rosés, and Bosch (2007).

^{1}H NMR spectral characteristics of the derivatives:

HA-alkynyl: δ(D_2O/DMSO-d_6 (1:1, v/v)) = 2.12 (–NH–C=O–CH_3, 3.0 H, s), 3.06 (–C≡C*H*, 0.5 H, m), 3.35-4.10 (sugar rings, 12.9 H, br), 4.12 (–CH_2–C≡CH, 0.9 H, d, *J* = 17.7 Hz), 4.25 (–CH_2–C≡CH, 0.8 H, d, *J* = 16.6 Hz), 4.63 (anomeric protons, 1.9 H, br) ppm. DS = 55%.

HA-maleimide: δ(D_2O/DMSO-d_6 (1:1, v/v)) = 2.11 (–NH–C=O–CH_3, 3.0 H, s), 3.10–4.20 (sugar rings + others, 11.7 H, br), 4.67 (anomeric protons, 1.3 H, br), 7.08 (–C=O–C*H*=C*H*–C=O–, 0.6 H, s) ppm. DS was calculated to be 1.4 times higher than indicated by the maleimide ring protons at 6.88 ppm, as the spectrum of the *N*-(2-aminoethyl)maleimide trifluoroacetate precursor in D_2O exhibited only 1.5 protons at this position instead of 2. DS = 40%.

HA-methacrylate: δ(D_2O/DMSO-d_6 (1:1, v/v)) = 1.40 (0.3 H, s), 2.09 (–NH–C=O–CH_3, 3.0 H, s), 3.18 (0.4 H, s), 3.20–4.10 (sugar rings + other, 10.0 H, br), 4.33 (–NH–CH_2–CH_2–O–, 1.05 H, br), 4.62 (anomeric protons, 1.0 H, br), 5.91 (–C(CH_3)=CH_2, 0.4 H, s), 6.31 (–C(CH_3)=CH_2, 0.4 H, s) ppm. DS = 41%.

HA-allyl: δ(D_2O/DMSO-d_6 (1:1, v/v)) = 2.13 (–NH–C=O–CH_3, 3.0 H, s), 3.20–4.20 (sugar rings, 12.1 H, br), 4.65 (anomeric protons, 1.3 H, br), 5.37 (–CH=CH_2, 0.6 H, br), 6.03 (–C*H*=CH_2, 0.4 H, br) ppm. DS = 28%.

HA-thiol: δ(D_2O/DMSO-d_6 (1:1, v/v)) = 2.11(–NH–C=O–CH_3, 3.0 H, s), 3.04 (–CH_2–SH, 0.1 H, s), 3.20–4.10 (sugar rings, 13.9 H, br), 4.66 (anomeric protons, 1.8 H, br) ppm. DS = 4%.

2.3.2. Hydrolysis of DMT-MM

The hydrolysis of DMT-MM was followed by ^{1}H NMR of a solution of DMT-MM (~5 mg) in 700 μL D_2O at specific time intervals.

2.4. Active ester formation between HA and DMT-MM

The reaction of HA and DMT-MM to form the active 2-acyloxy-4,6-di methoxy-1,3,5-triazine compound was studied by ^{1}H NMR. To a solution of HA (1.0 eq., 8.7 μmol) in 500 μL D_2O was added a solution of DMT-MM (1.1 eq., 9.6 μmol) in 200 μL D_2O. The mixture was transferred to a NMR tube and spectra were recorded at specific time intervals.

2.4.1. Kinetics of HA amidation in water

The coupling reaction of HA and propargyl amine in water was investigated by ^{1}H NMR. Low molar mass HA (*ca.* 1 kg/mol) was used to facilitate the study. HA (1.0 eq., 8.7 μmol) was dissolved in 500 μL D_2O. To this solution were added stock solutions of propargyl amine (1.1 eq., 9.6 μmol in 100 μL D_2O) and DMT-MM (1.1 eq., 9.6 μmol in 100 μL D_2O). The mixture was transferred into a NMR tube and spectra were recorded at specific time intervals.

2.5. Copper-mediated azide-alkyne-cycloaddition (CuAAC)

In a typical procedure, HA-alkynyl (1.0 eq., 12.5 μmol of propargyl groups, 2.5 g/L) and 1-azido-11-hydroxy-3,6,9-trioxaundecane (1.1 eq., 13.7 μmol) were dissolved in water. The solution was bubbled with argon gas for 15 min to remove oxygen. Solutions of copper(II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$, 0.1 eq., 1.7 μmol, 3.4 g/L) and sodium ascorbate (1.3 eq., 16.8 μmol, 26.6 g/L) in water were prepared separately and also deoxygenated by bubbling with argon gas for 10 min. Subsequently, the copper and ascorbate solutions were added to the first mixture under an inert atmosphere *via* a syringe. The mixture was stirred under constant argon bubbling at room temperature overnight. The cycloaddition product was purified by dialysis of the crude mixture against ethylenediaminetetraacetic acid (EDTA)-containing water for 1 day and against distilled water for 2 days and then lyophilized. The pure product was obtained in 80% yield.

HA-alkynyl-azide click: δ(D_2O) = 2.00 (–NH–C=O–CH_3, 3.0 H, s), 3.23–3.30 (–CH_2–OH, 0.3 H, br), 3.30–3.41 (–(CH_2–O–CH_2)$_3$–, 1.0

H, p, J = 8.4 Hz), 3.41–4.08 (sugar rings, 11.1 H, br), 4.27–4.37 (–N–CH_2–CH_2–O–, 0.3 H, br), 4.44–4.68 (anomeric protons, 2.6 H, m), 8.00 (triazole, 0.2 H, s) ppm.

2.6. Maleimide-thiol Michael addition reaction

To an ice-cold solution of HA-maleimide in water (1.0 eq., 11.2 μmol, 2.5 g/L) was added 1-mercapto-11-hydroxy-3,6,9-trioxaundecane (1.0 eq., 11.2 μmol). The mixture was stirred for 1 h at 0 °C. Subsequently it was warmed to room temperature, dialyzed against water for 2 days and lyophilized to yield 76% of the pure product.

HA-maleimide-thiol click: $\delta(D_2O)$ = 2.04 (–NH–C=O–CH_3, 3.0 H, s), 2.74–2.87 (–S–CH_2–CH_2–O–, 0.4 H, m), 2.95–3.12 (O=C–CH_2–CH(C=O)–S–, 0.6 H, br), 3.20–4.10 (sugar rings + others, 13.9 H, br), 4.11–4.19 (O=C–CH$_2$–CH(C=O)–S–, 0.4 H, br) 4.45–4.69 (anomeric protons, 1.9 H, br) ppm.

3. Results and discussion

3.1. Triazine-mediated amidation of HA with functional amines in water/acetonitrile

We set out first to confirm that, in our hands, the triazine-mediated amidation proceeded well under the conditions reported previously (Bergman et al., 2007; C. Schanté et al., 2011) using five amines ranging in pK_a from 8.2 to 10.8 (see Table 2). The same ratio of [–COO$^-$]:[CDMT]:[NMM]:[–NH$_2$] was used in all reactions. The resulting HA derivatives were purified, isolated, and their DS values were determined by ^{1}H NMR spectroscopy in D_2O/DMSO-d_6 (see Fig. 1). The measurements were conducted in a mixed solvent to ensure the solubility of hydrophobically modified HA-derivatives, thus obtaining the real DS values. However, DS values calculated from pure aqueous solutions were essentially the same. The results of this survey were disheartening (see Table 2): the DS values ranged from 55% at best to 4% at worst. Interestingly, the DS values correlated with the pK_a of the amine: the higher the pK_a, the lower the DS.

To understand the crucial role of the amine basicity on the outcome of the amidation, it is important to review the mechanism and to monitor the kinetics of the triazine-mediated amide bond formation. The mechanism, as described by Kamiński (Kamiński, 1994), is shown in Scheme 1. The first step of the reaction involves the *in situ* generation of the reactive coupling compound 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) starting from 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) and 4-methylmorpholine (NMM) (Kamiński, Paneth, & Rudziński, 1998; Kunishima, Kawachi, Iwasaki, Terao, & Tani, 1999), see Scheme 1a. According to the mechanism suggested by Kamiński (Kamiński, 1994) the 2–acyloxy-4,6–dimethoxy–1,3,5–triazine intermediate, formed from the attack of HA on DMT-MM, is the active species in the coupling reaction (Scheme 1 b). This "superactive" ester, as Kamiński named it, subsequently undergoes aminolysis. The collapse of the tetrahedral intermediate is fast and driven by the tautomeric rearrangement of the triazine leaving group (see Scheme 1 d and e, respectively). Therefore the rate determining step in the amide bond formation is the nucleophilic attack on the carbonyl carbon by the amine, which is only possible if the amine is not protonated. The pH of the reaction mixtures was measured to be 7.61 ± 0.02 at the beginning of the reaction. It decreased to 7.17 ± 0.05 in the course of the reaction, due to the elimination of protons from the coupled amines upon collapse of the tetrahedral intermediate (see Scheme 1 d). Under these conditions, hyaluronic acid and the amines exist as salts (Kunishima et al., 2013). The amines are involved in acid-base equilibria with the liberated *N*-methylmorpholinium and the weakly basic triazine leaving group (Scheme 1c). Thus, as the basicity of the amine increases, the amine exists more and more in its inactive protonated form, and consequently, the yield of the amidation decreases

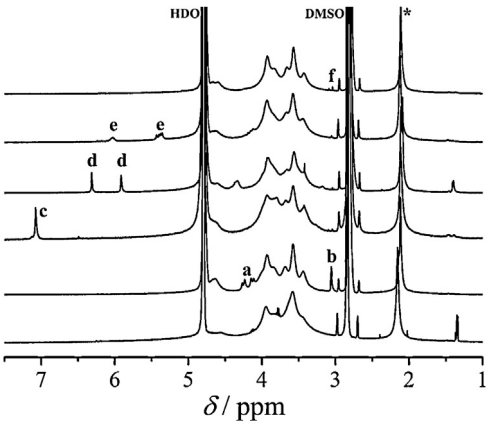


Fig. 1. ^{1}H NMR spectra of sodium hyaluronate and its amidation products (see Table 2) in D_2O/DMSO-d_6 (1:1, v/v). The signals used to calculate DS, are indicated by the letters as follows: (from below) Unmodified HA: methyl protons of the *N*-acetylglucosamine moiety at ~2.2 ppm (*); HA-alkynyl: propargyl protons –CH_2–C≡H at ~4.1 (a) and –C≡CH at ~3.1 ppm (b); HA-maleimide: maleimide ring protons at ~7.1 ppm (c); HA-methacrylate: double bond protons –C(CH$_3$)=CH_2 at ~5.9 and ~6.3 ppm (d); HA-allyl: double bond protons –CH=CH_2 at ~5.4 and ~6.0 ppm (e); HA-thiol: methylene protons –CH_2–SH at ~3.0 ppm (f).

3.2. Triazine-mediated amidation of HA in water

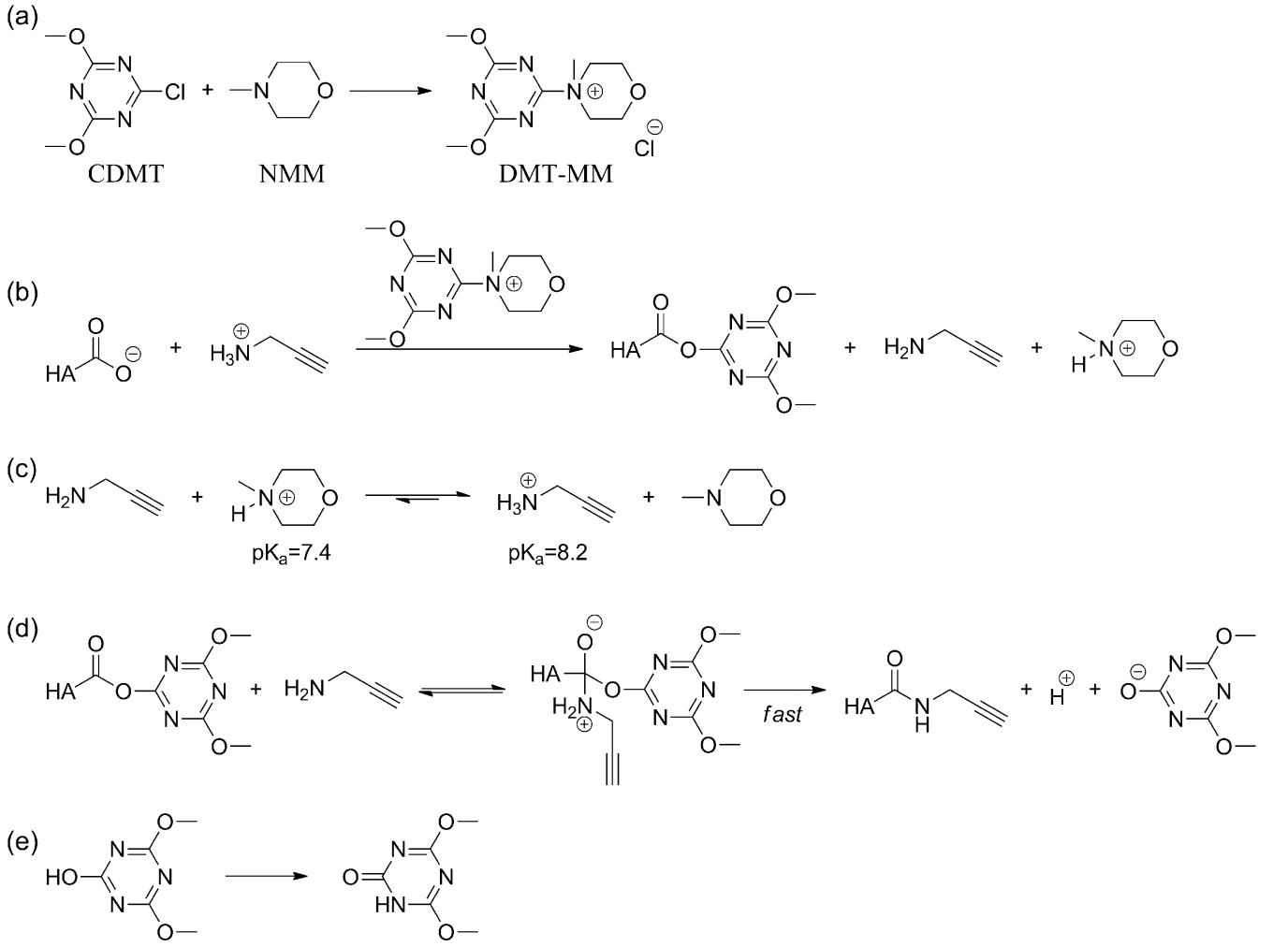
3.2.1. Kinetics of activated HA in water

Having established the validity and limitations of the published procedure, we set out to improve it in order to provide a viable alternative method to the EDC/NHS mediated amidation. First, we addressed the mixed solvent issue. It is necessary to use a mixed solvent system of water and acetonitrile in the published procedures to ensure the dissolution of the CDMT precursor. It is interesting to note that DMT-MM, which is water-soluble, can be prepared and isolated in high yields (Kunishima, Kawachi, Monta, et al., 1999). We envisaged the possibility of using DMT-MM obtained *ex situ*, as activating agent for amidation in water in the absence of co-solvent. To check the feasibility of the approach, we monitored by ^{1}H NMR spectroscopy the kinetics of DMT-MM hydrolysis in water and in an aqueous HA

Table 2
Degree of substitution of "clickable" hyaluronic acid derivatives prepared from amines with different basicity.

Derivative	Amine reagent	pK_a ofAmine	DS[a] [%]	Ref. for pK_a values
HA-alkynyl	Propargyl amine·HCl	8.2	54.5 ± 4.4	(Jarman et al., 1993)
HA-maleimide	*N*-(2-Aminoethyl)-maleimide·CF_3COOH	8.4[b]	39.5 ± 2.6	–
HA-methacrylate	2-Aminoethyl- methacrylate·HCl	8.8	41.3 ± 6.1	(Riauba, Niaura, Eicher-Lorka, & Butkus, 2006)
HA-allyl	Allylamine	9.5	28.2 ± 2.2	(Thompson, Read, & Armes, 2008)
HA-thiol	Cysteamine·HCl	10.8	3.7 ± 0.7	(Houson, 2011)

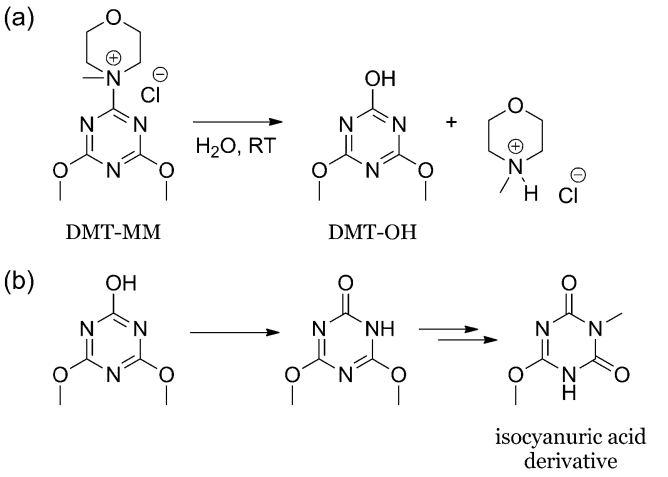
[a] Number of functionalized side groups per 100 disaccharide units as determined by ^{1}H NMR. Average value of three integrations ± standard deviation.
[b] Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994–2013 ACD/Labs).



Scheme 1. Mechanism of the triazine-mediated coupling of hyaluronic acid and propargyl amine in aqueous solution.

solution (see Fig. 2 and Fig. S1 in SI). The overall half-life ($t_{1/2}$) of DMT-MM in D_2O at room temperature was found to be 10 days. The decomposition of DMT-MM involved several steps. DMT-MM was first hydrolyzed to *N*-methylmorpholinium (NMM-H^+) and 2-hydroxy-4,6-dimethoxy-1,3,5-triazine (DMT-OH), which rearranged to its tautomer, 4,6-dimethoxy-1,3,5-triazin-2-one, and finally underwent isomerization to 6-methoxy-3-methyl-1,3,5-triazine-2,4-dione by *O*→*N* migration (see Scheme 2a and b) (Kamiński, 2000; Tosato, 1979). Isomerization of the triazine by-product was previously observed when the reaction was carried out at 100 °C in an organic solvent. In this case, both methyl groups migrated during the rearrangement (Kamiński, 2000). The isocyanuric acid derivative obtained here results from the migration of a single methyl group. It is stable in water at room temperature. De-methylation and formation of chloromethane was reported to occur when the reaction is carried out in organic solvents (Kunishima, Kawachi, Iwasaki, et al., 1999) It was not observe in this study, as indicated by the constant total intensity, over as long as 63 days, of the $-N^+-CH_3$ signals originating from DMT-MM (δ 3.54 ppm) and NMM-H^+ (δ 2.96 ppm), respectively (see Fig. S2 in SI). All hydrolysis products as well as the coupling agent remained water-soluble. They were easily removed by dialysis.

Next, we monitored the consumption of DMT-MM in the presence of HA, [COO^-]:[DMT-MM] = 1.0:1.5, which should lead to the formation of the "superactive" ester. The disappearance of DMT-MM in the presence of HA was found to be about 4 times faster ($t_{1/2}$ = 2.7 days) than the hydrolysis of DMT-MM alone (Fig. 2d). The formation of the ester was indicated by a shift in the ^1H NMR signal



Scheme 2. Slow hydrolysis of DMT-MM in aqueous solution to DMT-OH and NMM-H^+ ($t_{1/2}$ = 10 days). DMT-OH rearranges and then isomerizes by *O*→*N* migration of the methyl group.

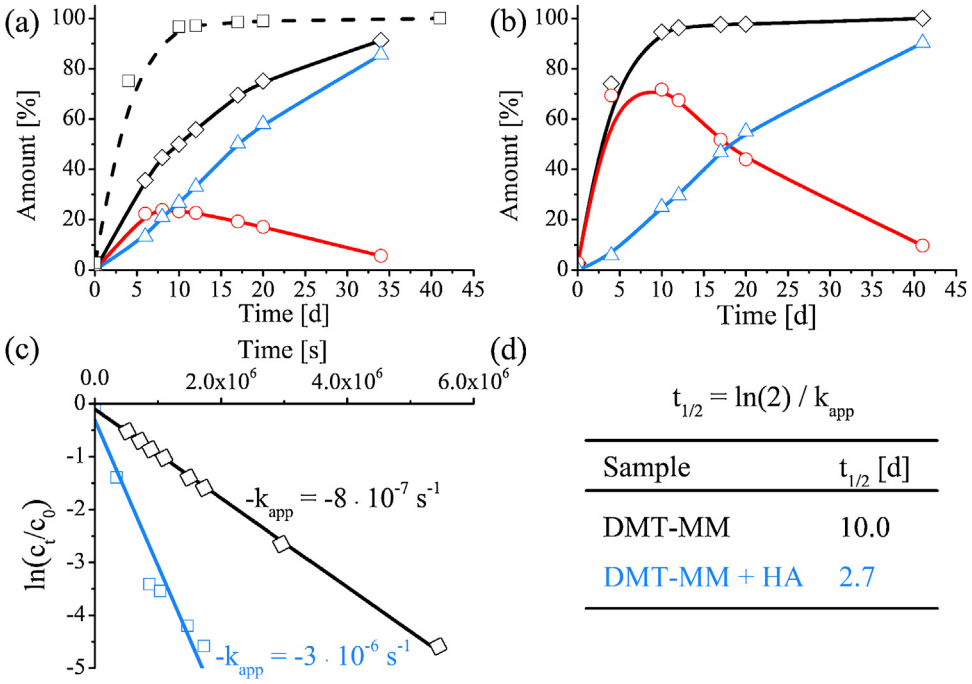


Fig. 2. (a) Hydrolysis of DMT-MM in D_2O over time followed by ^1H NMR. Diamonds: Consumption of DMT-MM; circles: appearance of hydrolysis product, DMT-OH; triangles: formation of isocyanuric acid derivative by *O→N* migration of one methyl group; squares: for comparison, consumption of DMT-MM in presence of HA. (b) Active ester formation between HA and DMT-MM in D_2O and hydrolysis over time followed by ^1H NMR. diamonds: Consumption of DMT-MM by reaction with HA and hydrolysis; circles: appearance of active ester and hydrolysis product, DMT-OH; triangles: formation of isocyanuric acid derivative from isomerization of DMT-OH. (c) First-order kinetic plot of DMT-MM consumption [$\ln(c_t/c_0) = -k{\cdot}t$] by hydrolysis (diamonds) or in presence of HA (squares) with respective apparent rate constants determined from the slope of the curves. (d) Formula of the half-life ($t_{1/2}$) of a reaction following first order kinetics and $t_{1/2}$ of DMT-MM in aqueous solution and in presence of HA.

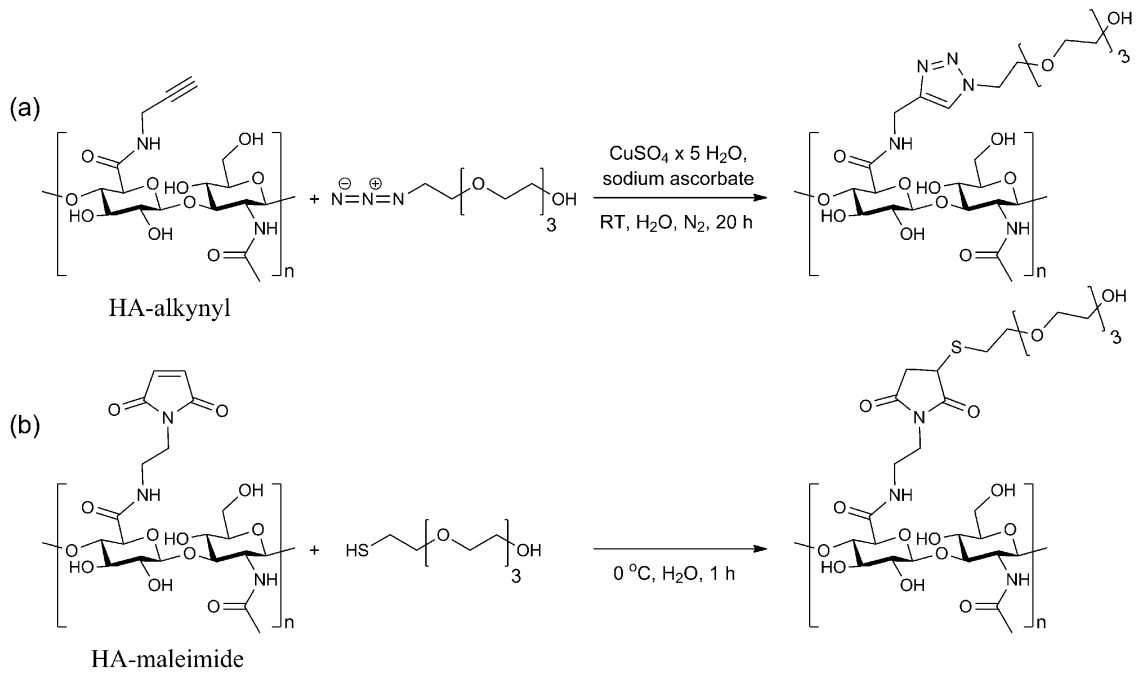
of the methoxy protons of DMT-MM from 4.15 to 4.02 ppm. The intensity of the new signal reached a plateau value of 70% after 4 days. This value corresponds to a near quantitative reaction of DMT-MM and HA, given that the initial ratio of [COO^-]:[DMT-MM] was 1.0:1.5 (see Fig. 2b, Tab. S1 in SI). Unfortunately the methoxy signals of the "superactive" ester and of the hydrolysis product, DMT-OH, have the same chemical shift (4.02 ppm) and cannot be distinguished by ^1H NMR spectroscopy. Nonetheless, we can conclude from these experiments that the kinetics of ester formation are much faster than the hydrolysis of DMT-MM. Also, the intensity of the methoxy signal in the ^1H NMR spectrum of the mixture remained constant for at least 10 days, which suggests the presence and stability of the "superactive" ester. This has also been observed by Kunishima, Kawachi, Monta, et al. (1999), who suggested earlier that the 2-acyloxy-4,6-dimethoxy-1,3,5-triazine derivative is more stable than the quaternary ammonium compound, DMT-MM. In addition, it was shown here that the "superactive" ester is more stable than the hydrolysis product, DMT-OH, which does not accumulate in the reaction mixture, but quickly isomerizes into the partially saturated isocyanuric acid derivative (compare Fig. 2a, circles and triangles).

3.3. Kinetics of HA amidation in water

The kinetics of HA amidation with propargyl amine in D_2O in the presence of DMT-MM ([$-COO^-$]:[$-NH_2$]:[DMT-MM] = 1.0:1.1:1.1) were monitored by ^1H NMR spectroscopy. The consumption of the coupling agent, monitored *via* the signal at 4.15 ppm, the degree of substitution, monitored by the appearance of a signal at 2.70 ppm, and the liberation of the triazine leaving group, DMT-OH, monitored by the signal at 4.02 ppm, proceeded linearly and with equal rates, during the first 10 h of the reaction (see Fig. 3a). The apparent rate constant of DMT-MM consumption in the presence of HA and amine ($k_{app} = 5 \times 10^{-6}\ s^{-1}$) was comparable to the rate of consumption of DMT-MM in presence of HA only ($k_{app} = 3 \times 10^{-6}\ s^{-1}$), Figs. 3b and 2c, respectively, suggesting that the amine has no significant effect on the formation of the "superactive" ester. Some researchers have reported an acceleration of the reaction between DMT-MM and the acid in the presence of an amine (Hasani & Westman, 2007; Kamiński et al., 2005; Kunishima et al., 2013); this effect was not observed in this study (see Tab. S1 in SI). The formation of the 2-acyloxy-1,3,5-triazine intermediate is only possible if the carboxylic acid is deprotonated. Hence, commercial sodium hyaluronate can be used, without special pre-treatment.

After about 1 day, the amidation of HA by propargyl amine slowed down. The decrease in reaction rate may be due to the shift of the acid-base equilibrium towards the more protonated amine species as a result of the pH change in the reaction mixture. It could be due also to the increased bulkiness of HA as a consequence of the amidation or the formation of hydrogen bonds between the newly formed amide groups and the carboxyl groups of the unreacted D-glucuronic acid moieties. Eventually, the reaction levels off when the DS reached 40%.

The DS value obtained during the kinetics studies is lower than the value we obtained in our initial assessment of the triazine-activated amidation with *in situ* generation of DMT-MM in water/acetonitrile mixture (see Section 3.1). It is difficult, based on the kinetic studies, to compare the two experimental conditions. The reaction in water/acetonitrile was conducted with high molar mass HA and 1.5 molar excess of propargyl amine to [$-COO^-$] under stirring for 20 h. The kinetics study was done in D_2O with low molar mass HA and a 1.1 molar excess of amine. The coupling efficiency however was the same in both cases, *viz.* 36.7 and 36.4% DS per molar equivalent of amine to carboxylate group, respectively, which implies that the amine stoichiometry is the limiting factor controlling the ultimate DS of HA.



Scheme 3. Model "click" reactions conducted on the HA-derivatives: (a) copper-mediated azide-alkyne click reaction; (b) maleimide-thiol Michael addition reaction.

3.4. Model "Click" reactions

The HA derivatives prepared can be reacted with various biomolecules, drugs, polymers or probes *via* "click" reactions for the formation of bioconjugates. Reactions of the "click family" proceed under mild conditions in an orthogonal, fast and quantitative manner giving stable, regiospecific linkages in high yields without appreciable amounts of side products (Lutz & Börner, 2008; Nandivada, Jiang, & Lahann, 2007). Besides the well-known copper-mediated azide-alkyne click (CuAAC) reaction, based on the Huisgen 1,3-dipolar cycloaddition, several thiol-ene addition reactions have proven to be successful. They typically employ allyl-, (meth)acrylate- or maleimide-derivatives to which thiols easily attach *via* either free radical or nucleophilic reaction (Dong et al., 2012; Jing et al., 2013; Pounder, Stanford, Brooks, Richards, & Dove, 2008; Yu, Chan, Hoyle, & Lowe, 2009). To demonstrate the utility of the aziridine mediated amidation method, two model "click" reactions, *i.e.* the CuAAC and the maleimide-thiol Michael addition (Scheme 3), were conducted starting from modified HA bearing, respectively, propargyl and maleimide moieties. Michael addition of 1-mercapto-11-hydroxy-3,6,9-trioxaundecane to the maleimide groups of HA proceeded rapidly in water at 0 °C reaching completion after 1 h as judged by NMR spectroscopy (see Fig. S3 in SI). The CuAAC reaction of 1-azido-11-hydroxy-3,6,9-trioxaundecane with HA-alkynyl, carried out in water in the presence of Cu(II) ions and ascorbic acid, was complete after 20 h (see Fig. S3 in SI). The degree of modification of HA-alkynyl, estimated from the integral of the ^{1}H NMR signal of the triazinyl proton, was ~23%, which is significantly lower than the DS of HA-alkynyl (55%), possibly due to the poor solubility of 1-azido-11-hydroxy-3,6,9-trioxaundecane in water. These initial results confirm that the products of the triazine-mediated amidation of HA serve their intended purpose and that they are poised to enable the preparation of well defined HA-conjugates.

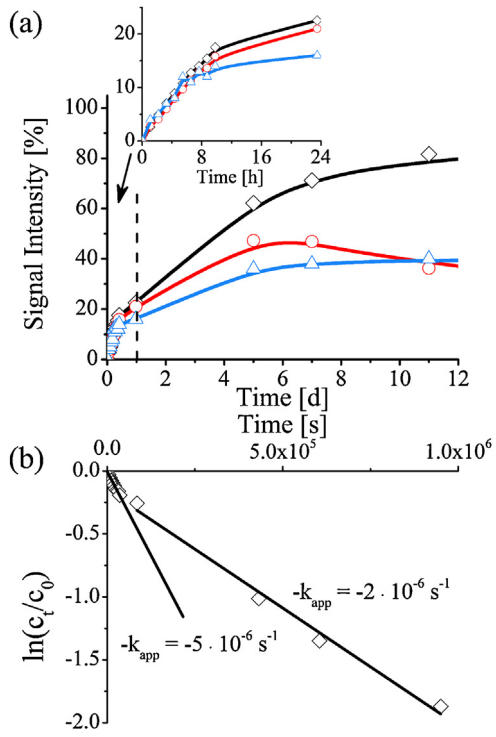


Fig. 3. (a) DMT-MM mediated coupling reaction between hyaluronic acid and propargyl amine in D_2O at room temperature followed by ^{1}H NMR spectroscopy. Diamonds: Consumption of DMT-MM; circles: formation of the "superactive" ester or triazine leaving group, DMT-OH, respectively; triangles: degree of amidation of HA. (b) First-order kinetic plot of DMT-MM consumption [$\ln(c_t/c_0) = -k \cdot t$]. Reaction slows down after about 10 h as indicated by different slopes of the fitted lines.

4. Conclusions

The triazine-based coupling agent, DMT-MM was used to carry out the amidation of HA in aqueous solution under mild conditions with excellent efficiency. Compared to the commonly used EDC/NHS coupling reaction, the method described here is more economical, yields higher degrees of substitution (up to 55%) while using almost stoichiometric amounts of reagents. The activating agent, DMT-MM, was prepared *ex situ* and used on demand. It

was stable upon storage in the dry form at ambient conditions and in aqueous solution of neutral pH. Kinetic studies showed that DMT-MM activates the carboxylic acid groups of HA fast and quantitatively, yielding a stabilized intermediate. Furthermore the amine basicity and stoichiometry were found to be the limiting factors for controlling DS. During the reaction the coupling reagent is converted into soluble by-products that can be removed easily. 6-Methoxy-3-methyl-1,3,5-triazine-2,4-dione, an isocyanuric acid derivative with one migrated methyl group, was found to be the stable by-product in aqueous solution at RT. Understanding the role of the amine was critical for establishing effective synthetic parameters. The triazine-mediated synthesis of functional HA derivatives followed by conjugation by "click" chemistry of suitably functionalized molecules provides a mild and efficient route towards well-defined bioconjugates.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.04.012.

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