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A systematic analysis of DMTMM vs EDC/NHS for ligation of amines to Hyaluronan in water

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ABSTRACT

The activation of carboxyl groups with N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride and N-hydroxysuccinimide (EDC/NHS) for amide formation is the standard method for amine ligation to hyaluronan (HA), and a very well established wide-ranging bioconjugation method. In this paper we compare 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) to EDC/NHS activation chemistry for HA ligation using an array of substrates including small, large and functional molecules. For all the substrates tested DMTMM yields were superior at parity of feed ratio. DMTMM chemistry resulted effective also in absence of pH control, which is essential for EDC/NHS conjugation. Overall our results demonstrate that DMTMM is more efficient than EDC/NHS for ligation of amines to HA and does not require accurate pH control or pH shift during the reaction to be effective. DMTMM-mediated ligation is a new promising chemical tool to synthesize HA derivatives for biomedical and pharmaceutical applications.

1. Introduction

Hyaluronan (HA) is one of the most important bio-polymers in medicine. It is a ubiquitous component of all vertebrates which is fundamental for creating a suitable environment for cells survival, proliferation, motility and differentiation (Laurent & Fraser, 1992). It contributes to connective tissues homeostasis and provides them firmness and mechanical properties. HA-based medical devices and drugs generate substantial sales volumes, about 2.3 billion US dollars per year, with viscosupplementation for osteoarthritis being the largest share. HA can be chemically modified to specific medical applications, some of which are already very well established and commercially successful. For example, crosslinked HA for aesthetic medicine sells millions of syringes every year. Chemically modified HA with specific properties are replacing applications that formerly were prerogative of unmodified HA (e.g., viscosupplementation, ophthalmic surgery medical devices), and pharmaceutical pipelines contain several combinations of HA with drugs, or use of chemically modified-HA-based scaffolds in Tissue Engineering (Collins & Birkinshaw, 2013). Carboxyl group on HA is the most suitable for chemical modification, and allows HA functionalization maintaining its biological properties (Schantè, Zuber, Herlin, & Vandamme, 2011). The formation of amides is particularly convenient for HA ligation through an in vivo non-labile bond. The activation of carboxyl groups with N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride and N-hydroxysuccinimide (EDC/NHS) for amide formation is the standard method for amine ligation to HA, and a very well established method for bioconjugations in general. Activation of HA with EDC leads to the formation an O-acylisourea that rearranges quickly to a stable N-acylurea, which is not reactive towards amines. Bulpitt and Aeschlimann (1999) demonstrated that this quenching of the active intermediate can be prevented by "rescuing" the active O-acylisourea with NHS by formation of a more hydrolysis-resistant and non-rearrangeable active ester intermediate, making the coupling of primary amines to HA possible. Still, this approach has drawbacks. Activation with EDC requires pH between 3.5 and 4.5 to be efficient (Nakajima & Ikada, 1995), and most amines are protonated and non-nucleophile at that pH. Therefore coupling should be carried out at higher pH, and its exact value has to be designed finding a compromise between good amine reactivity and low hydrolysis of the active intermediate. This pH shift during the reaction leads to process complexity and inferior efficiency. Existing alternatives include the use of organic solvents, but in this case the process is unavoidably multi-step, posing issues of cost and waste management.

Similarly to carbodiimides, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium (DMTMM) was initially developed for

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peptide synthesis (Kunishima et al., 1999; Han & Kim, 2004; Falchi, Giacomelli, Porcheddu, & Taddei, 2000). In 2007, the use of DMTMM for derivatization of glucans in water was reported (Farkas & Bystricky, 2007). In the same year Bergman et al. reported a DMTMM precursor (specifically, 2-chloro-4,6-dimethoxy-1,3,5-triazine) for HA ligation in acetonitrile:water 3:2 (Bergman, Elvingson, Hilborn, Svensk, & Bowden, 2007). Nimmo, Owen, and Shoichet (2011) and Yu et al. (2014), Yu, Cao, Zeng, Zhang, and Chen (2013) used DMTMM for preparing furan and tyramine modified HA. However, none of the reported studies have compared efficiency of EDC/NHS vs DMTMM for HA ligation in water, assessing the potential of DMTMM to couple a wide range of molecules to HA.

In this paper, the systematic comparison of EDC/NHS and DMTMM activation chemistry for modifying HA via amide formation in water is performed. Different ligands of interest for HA functionalization have been covered. In particular we grafted HA with

- Adipic acid dihydrazide (ADH), useful to synthesize either hydrazide-bearing HA derivative for further functionalization or covalent HA networks.
- Amino acetaldehyde dimethyl acetal (AADA), useful for creating a protected-aldehyde bearing HA derivative for further ligation.
- Glycine ethyl ester hydrochloride (Gly), a carboxyl protected form of simplest amino acid.
- Bovine serum albumine (BSA), a full length globular protein.
- Doxorubicin (Dox) hydrochloride, an anthracycline antibiotic used as cancer chemiotherapeutic.
- Poly(N-isopropylacrylamide) (pNIPAM), a temperature-responsive polymer.

Besides, N-(1-naphthyl)ethylenediamine dihydrochloride (NED), a UV-active aromatic molecule, was employed to assed the conversion kinetics with different amounts of DMTMM.

The synthesized materials display a wide range of physico-chemical and rheological properties, and hold vast potential in biomedical applications including drug delivery, viscosupplementation, tissue augmentation, adhesion prevention, ophthalmic surgery aids, wound healing and tissue engineering. Results are organized as follows. First, an overview of the DMTMM conjugation chemistry is given, comparing its main features with the EDC/NHS method. The remaining paragraphs are divided by substrate category, describing characterization of the products and comparing reaction yields obtained with EDC/NHS or DMTMM method.

2. Materials and methods

2.1. Materials

HA sodium salt from *Streptococcus equi* was purchased from Contipro Biotech s.r.o. (Czech Republic). Two different batches with weight-average molecular weight 606 kDa and 293 kDa were used.

Amino-terminated poly(N-isopropylacrylamide) (pNIPAM) of number-average molecular weight of 40 kDa was purchased from Polymer Source, Inc. (CA). All other reagents were purchased from Sigma–Aldrich (Switzerland); chemicals were of analytical grade at least, and were used without further purification.

2.2. Syntheses

2.2.1. Overview (all derivatives except HA-pNIPAM and NED)

Syntheses of each derivative (Scheme 1) were performed using DMTMM or EDC/NHS as condensing agent (CA). For both conjugation chemistries the same HA:CA:amine stoichiometric ratio was used. For all EDC/NHS syntheses NHS was used in the same stoichiometric amount to EDC. HA:amine feed ratio was 1:12 for ADH; 1:5 for AADA and Gly; NED:HA ratio was 87.5% w/w; BSA:HA ratio was 19% w/w; Dox:HA was 10% w/w; for pNIPAM different feed ratio to HA were used (see below). Values were chosen according to the solubility of reaction mixtures and isolated products.

2.2.2. General method for DMTMM derivatives

1.08 g of HA sodium salt were dissolved in 45 ml of water. The respective amine for HA ligation was dissolved in 45 ml of water. Besides the listed ligands, a "blank" reaction was performed without addition of any amine in order to check if the DMTMM-activated HA was isolated or if free DMTMM was present in the final product after purification (Supplementary Material, Fig. S1). Solutions were combined, and pH corrected to 6.5 with addition of NaOH or HCl solutions. To this solution, DMTMM in powder was added. For the ADH derivative a 4 fold molar excess of DMTMM was employed; for all other derivatives DMTMM was used stoichiometrically to HA. The reaction was let to proceed at room temperature for 5 days. Products were isolated by precipitation as follows. 270 ml of ethanol 96% were added dropwise to the reaction mixture. The white powder obtained was thoroughly washed with water:ethanol 1:4, ethanol 96% and finally with absolute ethanol. Products were dried under vacuum for 3 days at 38 °C.

2.2.3. General method for EDC/NHS derivatives

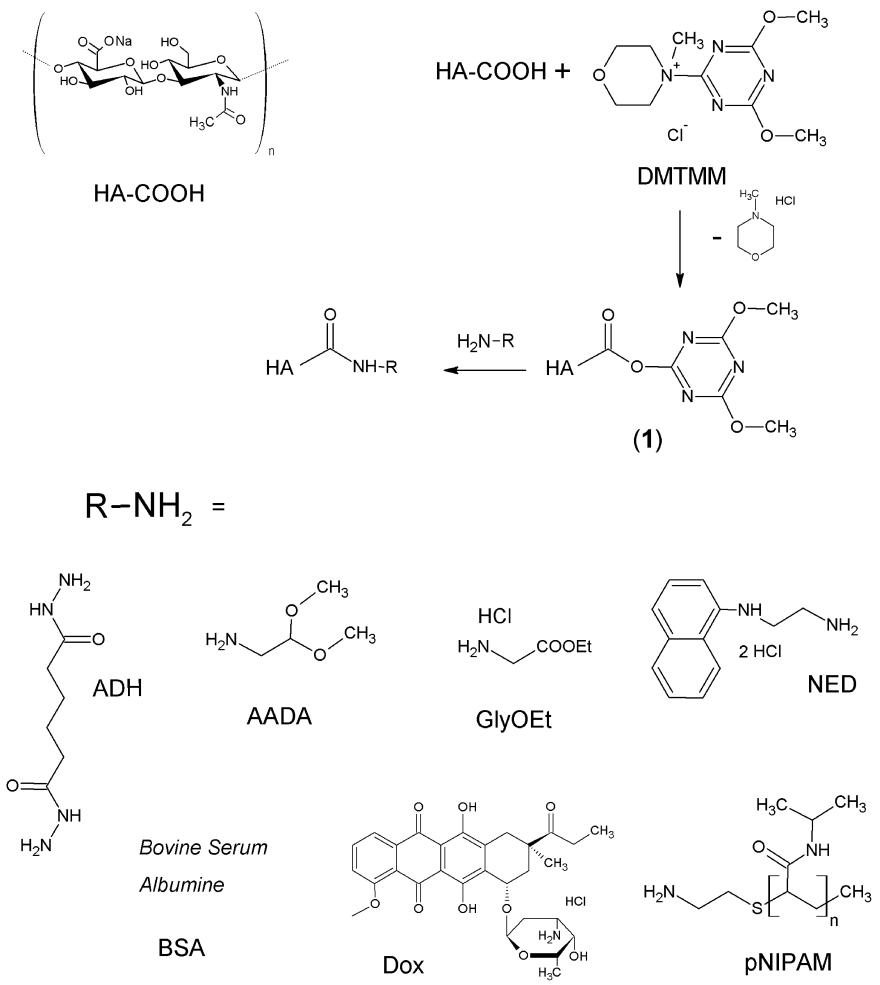
1.08 g of HA sodium salt were dissolved in 45 ml of water. The respective amine for HA ligation was dissolved in 45 ml of water. Solutions were combined, EDC and NHS were added in powder, and pH was corrected to 5.5 ± 0.3 with addition of NaOH or HCl solutions. After 45 min pH was increased to 7.3 ± 0.3 and kept to this value with addition of NaOH or HCl solutions, if necessary. The reaction was let to proceed at room temperature for 5 days. Products were isolated by precipitation as follows. 270 ml of ethanol 96% were added dropwise to the reaction mixture. The white powder obtained was thoroughly washed with a water:ethanol 1:4 blend, ethanol 96% and finally with absolute ethanol. Products were dried under vacuum for 3 days at 38 °C.

2.2.4. HA-pNIPAM derivatives

A first set of experiments with pNIPAM:HA = 1.4, 1.5, 2.7, 3.4 w/w was performed using HA:CA = 1:1. In these conditions DMTMM gave significant substitution (Fig. 5), while with EDC/NHS grafting was nearly undetectable. Therefore a second set of syntheses was performed with HA:EDC = 1:4 in moles, and pNIPAM:HA = 0.6, 1.2, 1.7 (see paragraph 3.6). Syntheses were performed according to the general recipes above until the isolation step. HA-pNIPAM derivatives were purified via dialysis with 50 kDa MWCO tubes for 5 days changing the water twice per day, followed by freeze-drying to obtain the co-polymers as white spongy lyophilized.

2.2.5. HA-NED derivative

2.18 g of HA sodium salt were dissolved in 65 ml of water. 1.24 g of NED were dissolved in 65 ml of water. Solutions were combined, and pH corrected to 6.55 with addition of NaOH or HCl solutions. To this solution, DMTMM in powder was added. Reaction was performed using DMTMM/HA = 0.6 and 0.2 in moles. At pre-determined time points an aliquot of 25 ml of reaction solution was withdrawn and HA-NED isolated by precipitation as follows. 75 ml of ethanol 96% were added dropwise to the reaction mixture. The white powder obtained was thoroughly washed with water:ethanol 1:4, ethanol 96% and finally with absolute ethanol. Products were dried under vacuum for 3 days at 38 °C. Molar degree of substitution (DS_{mol}) was assessed via UV spectrophotometry as described below.



Scheme 1. Top: representation of the DMTMM activated amidation of HA including the s-triazine HA-active ester intermediate (**1**). Bottom: moieties grafted to HA.

2.3. Characterization

Fourier transform infrared spectroscopy (FT-IR) analysis was performed on a Bruker Tensor 27 spectrophotometer equipped with a single reflection diamond attenuated total reflection (ATR) accessory. Derivatives freeze-dried or in powder were placed directly on the accessory. Spectra were acquired between 4000 and 500 cm^{-1} averaging 32 to 128 scans (depending on the signal to noise ratio) and processed with the Opus 6.5 software.

^1H NMR analysis was performed on a Bruker Avance AV-500 NMR spectrometer using as a solvent deuterium oxide containing 220 U/ml of hyaluronidase, corresponding to 0.4 mg/ml. Polymeric samples were dissolved no less than 48 h before spectra recording and kept at room temperature. A spectrum of the solvent (D_2O + hyaluronidase 0.4 mg/ml) was recorded in order to evaluate the contribution of hyaluronidase to the integrals of the polymers to analyze. Overall, hyaluronidase contribution was less than 0.4%, well below the accuracy of NMR peaks integration, and therefore negligible. Chemical shifts were calibrated using 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt as internal standard, and processed with Mestrenova 6.2.1 software. For HA-ADH derivatives polymeric samples were dissolved using 300 U/ml of hyaluronidase at 37 °C.

Rheological measurements were acquired on an Anton Paar MCR-302 rheometer equipped with Peltier temperature control device and thermostatic hood. Cone-plate geometry of 25 mm diameter was used; gap was set at 49 μm, correspondent to the cone truncation. For each sample an amplitude sweep was measured at 10 rad/s and 20.00 ± 0.02 °C. Frequency sweep (also said mechanical spectrum) was performed between 0.1 and 100 rad/s with oscillation amplitude within the linear viscoelastic range; temperature was set at 20.00 ± 0.02 °C. Flow curves were recorded at shear rate between 0.1 and 100 s^{-1} at 20.00 ± 0.02 °C. These measurements have been performed on solutions of the co-polymers at the concentration of 20 mg/ml in phosphate buffer saline (PBS). For HA-pNIPAM viscoelastic shear moduli were measured as function of the temperature between 20 and 40 °C; frequency was set at 1 Hz at amplitude within the linear viscoelastic range; concentration of the co-polymer was 10% w/w in PBS. Measuring chamber was saturated with water vapour to avoid evaporation at the meniscus interface.

UV spectra were recorded on a Thermo Scientific Multiskan™ Go single-beam spectrophotometer in cuvette modality with 2 nm resolution. DS_{mol} assessed via UV were determined quantitating the respective chromophores absorption with the calibration curves of the pure standards, and comparing with the weight concentration of the co-polymers. Water was used as solvent. BSA content was calculated via UV through calibration curve at 279 nm. Extinction coefficient obtained ε = 42,447 M^{-1} cm^{-1} was in good agreement with literature data (43,824 $M^{-1}cm^{-1}$ at the same wavelength (Peters, 1975)).

For all derivatives except HA-pNIPAM the DS_{mol} is expressed as ratio between moles of grafted moiety and moles of HA repeating units. For HA-pNIPAM substitution is expressed as Cumulative Grafting (CG), defined as ratio between moles of pNIPAM monomers and moles of HA repeating units. CG was chosen in

order to be independent of the molecular weight distribution of the polymeric moiety; moreover rheological properties and temperature-induced sol–gel transition of the co-polymer show a good correlation with CG. DS_{mol} was calculated from ^{1}H-NMR integration for AADA, ADH, NED, Gly and pNIPAM derivatives. For derivatives with BSA, Dox and NED the DS_{mol} was determined from UV spectra.

3. Results and discussion

3.1. Overview of the DMTMM conjugation chemistry

DMTMM is soluble and stable in water for an extended period of time (Raw, 2009). This feature is quite unique compared to classic coupling reagents such as N,N′-dicyclohexylcarbodiimide, N,N′-diisopropylcarbodiimide, N-acylimidazoles. EDC, the archetype of water-soluble CA, has a half-life of 3.9 h in water at pH 5.0 (Gilles, Hudson, & Borders, 1990). By contrast, DMTMM does not display degradation in water at room temperature, with 100% recovery after 3 h (Kunishima et al., 1999). Use of DMTMM chloride in organic solution is limited by its instability, as it undergoes self-immolative degradation. Its half-life is 120 min in DMSO, 15 min in DMF, and even shorter in Chloroform (Raw, 2009). Self-immolative degradation of DMTMM in organic medium is drastically decreased if chloride is substituted by non-nucleophilic counterions (Kaminski et al., 2005).

DMTMM-mediated amidation of HA proceeds through an aromatic substitution forming an intermediate s-triazine ester of HA reactive towards amines (compound **1**, Scheme 1). Use of DMTMM for bioconjugations has been reported at both alkaline (Pelet & Putnam, 2011) and acidic pH (Nimmo et al., 2011; Yu et al., 2014).

One of the main limitations of the EDC/NHS conjugation to HA is the necessity of an accurate pH control. The generation of the NHS ester of HA requires acidic pH, while the nucleophilic attack of the amine is only efficient at neutral or alkaline pH, where the NHS ester of HA is hydrolytically labile (Schantè et al., 2011; 2013b). The optimal conditions of coupling are therefore a non-trivial compromise. A pH shift during the reaction is inconvenient at the lab scale, and difficult to scale up with the robustness required for industrial productions, especially in the biomedical field. DMTMM conjugation does not require such undesirable pH control. For all DMTMM syntheses reported in this study pH of the reaction was corrected to 6.5 ± 0.2 at the beginning of the reactions and left untouched until their end. No buffer was used. This protocol was adopted in order to verify the efficacy of DMTMM-mediated HA amidation with minimal handling. Therefore reaction yields obtained might be improved with substrate-specific pH control. Interestingly HA can self-buffer its solutions. As poly-carboxylic acid, HA and can act as "protons sponge" being able to both accept and release protons.

The non-necessity of accurate pH control or buffers makes the DMTMM chemistry more practical, reduces the quantity of chemicals to be used and disposed, and simplifies the purification steps. These aspects are a clear advantage for lab and industrial scale production of HA derivatives.

Spectra of the products obtained did not display the absorption maximum at 236 nm typical of DMTMM, confirming its elimination after the reaction. As additional proof, a control reaction was carried out reproducing the standard conjugation in absence of any amine. Spectrum of the obtained product is devoid of the absorption pattern of DMTMM (Supplementary Material, Fig. S1). Therefore, the active intermediate (**1**) HA-DMTMM does not leave residues, and is not isolable under these conditions. This is also a demonstration that the purification protocol does not leave any unbound DMTMM.

Table 1
Summary of different derivatives prepared with both DMTMM and EDC/NHS method, reporting the mole ratio of the Condensing Agent (CA) versus HA and the DS_{mol}. For BSA, NED and Dox derivatives, the efficiency of coupling was measured via UV. For AADA, ADH, Gly and pNIPAM substitutions are calculated from NMR.

Moiety	CA:HA	DS_{mol} DMTMM	DS_{mol} EDC/NHS
ADH	4:1	35%	18%
AADA	1:1	65%	49%
Gly	1:1	53%	22%
BSA	1:1	0.63% (UV)	0.16% (UV)
Dox	1:1	2.62% (UV)	2.24% (UV)
NED	0.2:1; 0.6:1	14%; 36%	/
pNIPAM	1:1	See Fig. 5	

Table 1 compares the degree of substitution of different derivatives prepared using DMTMM and EDC/NHS. With the exception of ADH, for all derivatives the CA was used in stoichiometric amount to HA disaccharide units. For ADH a four-fold molar excess was used in order to reproduce the conditions reported by Bulpitt and Aeschlimann (1999). The viscoelastic behaviour of HA-ADH allowed us to compare DS_{mol} measurement via ^{1}H-NMR with the rheological profile to measure the crosslinking efficiency of the two CAs. DMTMM displayed systematically higher DS_{mol} for all the substrates examined. Characterization and specific features of each compound are discussed in the following paragraphs.

3.2. Adipic acid dihydrazide

Adipic acid di-hydrazide (ADH) derivative of HA has been extensively investigated for pharmaceutical and regenerative medicine applications (Pouyani, Harbison, & Prestwich, 1994; Pouyani & Prestwich, 1994; Schantè et al., 2011). Mono-condensation of ADH introduces terminal nucleophilic hydrazides suitable for further HA modification that would not be possible on the carboxyl moiety. Hydrazides have also the advantage of being non-protonated in mild acidic conditions, making the functionalization of HA possible at lower pH. ADH is a homo-bi-functional moiety, and therefore if reacted under proper conditions, it has the ability to crosslink HA to obtain molecular networks for the preparation of biomaterials such as scaffolds or hydrogels (Su, Chen, & Lin, 2010). We performed the conjugation of HA and ADH with 4 fold molar excess of activator and large excess (12 fold) of ADH (Bulpitt & Aeschlimann, 1999). Under these conditions HA was cross-linked.

Fig. S2 (Supplementary Material) displays FT-IR spectra of HA-ADH prepared via DMTMM, EDC/NHS and pristine HA. Carbonyl C=O stretching zone around 1650 cm^{-1} gave a broad peak resulting from the superimposition of several signals: carboxylate (free carboxylic groups on HA, with or without hydrogen bonding), N-acetyl on HA, hydrazide C=O on ADH. Still, the new hydrazide formed upon HA ligation was visible as distinct peak at 1709 cm^{-1} (C=O stretching). The signal at 1556 cm^{-1}, giving a small shoulder on pristine HA, became a separate band for HA-ADH. These spectral modifications were more evident for the DMTMM rather than EDC/NHS derivative, suggesting higher substitution for DMTMM. Rheological analysis was used to compare the degree of crosslinking of the derivatives obtained with DMTMM and EDC/NHS, and the efficiency of coupling as consequence. Oscillatory analysis showed a significant difference for the derivatives obtained with the 2 different methods. Complex viscosity (Fig. S3, Supplementary Material) at 0.1 rad/s is about 300 times higher for the DMTMM derivative (225 Pa s against 0.708 Pa s). Moduli (Fig. 1) are higher all over the frequency range. EDC/NHS derivative displays loss modulus higher than storage modulus in the whole range of frequency analyzed, indicating a scarcely or non-crosslinked polymer. DMTMM derivative displays storage modulus G′ always over G″ and a significant decrease of complex viscosity (from 225 Pa s at

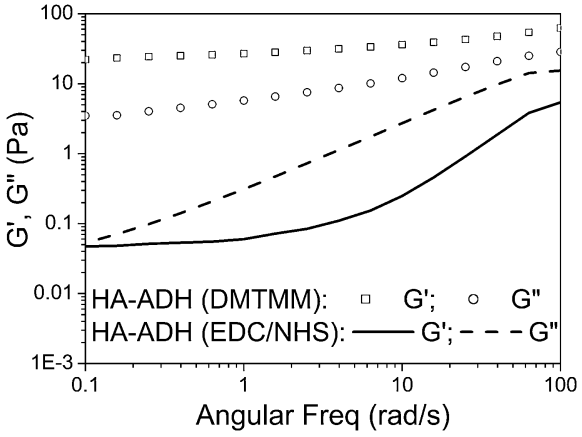


Fig. 1. Mechanical spectra of HA-ADH prepared with EDC/NHS or DMTMM method.

0.1 rad/s to 0.69 Pa s at 100 rad/s). Both characteristics are distinctive of a covalently crosslinked network. NMR spectra of HA-ADH derivatives are displayed on Figs. S4 and S5 (Supplementary Material). DS_{mol} was calculated comparing the peaks at 2.01 ppm and 1.65 ppm. The former arises from the superposition on the N-acetyl group of HA (3H) and one methylene group of ADH (2H), while the signal at 1.65 pm is originated by one methylene group of ADH (2H). DMTMM derivative yields a DS_{mol} of 35% against 18% for EDC/NHS.

Summarizing, results of FT-IR, NMR and rheological analyses are in good agreement indicating a higher efficiency of ligation for DMTMM-mediated coupling.

3.3. Amino acetaldehyde dimethyl acetal

Aldehydes are versatile compounds in organic synthesis. Insertion of a protected aldehyde group is a convenient route to create an isolable off-the-shelf HA intermediate. This HA modification has been pursued with different routes, including direct reduction of carboxyl groups (Cha, 1989), and periodate oxidation of the polysaccharide backbone. The insertion of an aldehyde group protected as acetal is preferable (Bulpitt & Aeschlimann, 1999; Mero, Pasqualin, Campisi, Renier, & Pasut, 2013). It does not imply opening the saccharides rings with demolition of the HA backbone and the protection can be easily removed with a mild acidic treatment.

Coupling of AADA was performed using a stoichiometric quantity of CA, and 5 fold excess on AADA. Use of DMTMM gave a conjugate with DS_{mol} = 65%, while the EDC/NHS method gave DS_{mol} = 45%. ^{1}H-NMR spectrum of the conjugate in Fig. 2 shows a sharp singlet at δ = 3.43 ppm from the O-methyl groups superimposed to the HA spectral features, as expected. Spectrum of the derivative from EDC/NHS is reported on Fig. S6 (Supplementary Material).

Mechanical spectrum of HA-AADA (not shown), similarly to pristine HA, shows loss modulus G″ over the storage modulus G′ in the whole frequency range investigated. Moduli display pronounced frequency dependence. Both features are clear signatures of the non-crosslinked nature of the derivative. The viscosity curve of HA-AADA and underivatized HA are shown in Fig. S7 (Supplementary Material). HA-AADA has a reduced shear-thinning compared to the pristine HA, giving a viscosity fairly stable over a wide range of shear rates. Shear thinning is caused by progressive disentanglement and alignment of entangled polymer coils under shear, decreasing the flow resistance. Therefore HA-AADA is less entangled than pristine HA in PBS solution at the concentration of 20 mg/ml. A possible reason for the decreased entanglement is the significant perturbation in the architecture of interactions between the HA chains generated by AADA ligation. The relatively high DS_{mol} achieved makes this effect clearly visible. A similar effect has been reported for alkylated HA (Lapcik, Benesova, Lapcik, De Smedt, & Lapcikova, 2010). Viscosity curves of the HA-AADA derivatives prepared via DMTMM or EDC/NHS activation are very similar, reflecting the high derivatization degree obtained with both methods (Supplementary Material, Fig. S7).

3.4. Glycine ethyl ester hydrochloride, bovine serum albumine, doxorubicin

Modification of HA with drugs and biological drugs is a major trend, with applications spanning from disease treatment to tissue

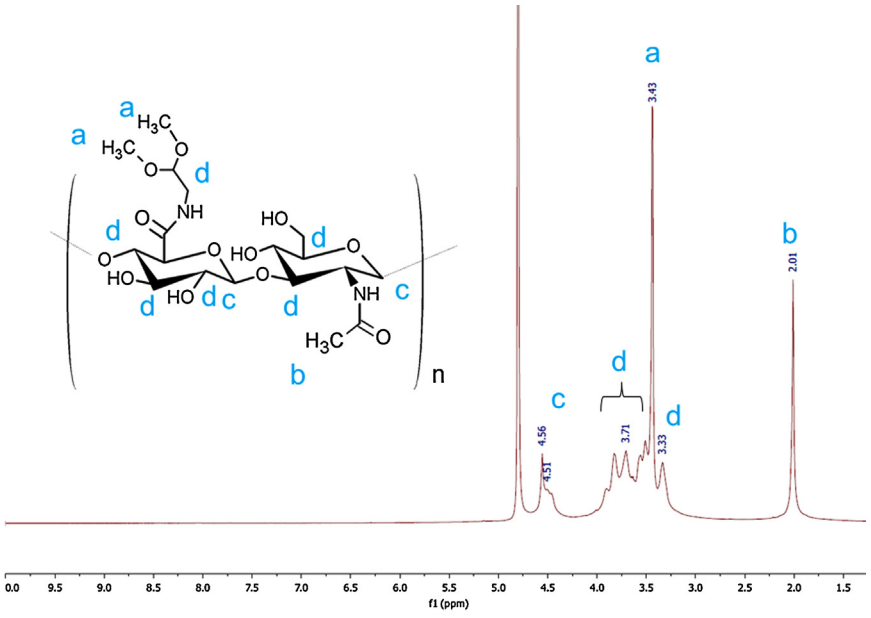


Fig. 2. NMR spectrum of HA-AADA synthesized via DMTMM. Spectrum of HA-AADA synthesized via EDC/NHS is Fig. S6 (Supplementary Material).

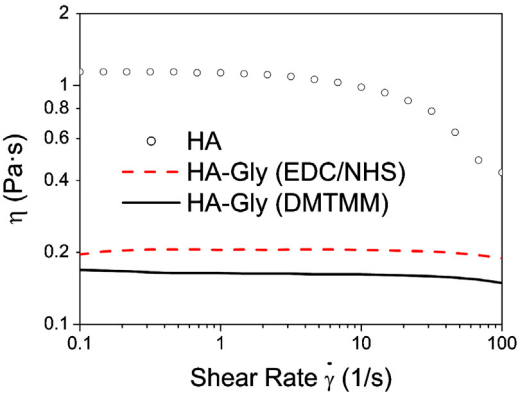


Fig. 3. Viscosity curve of HA-Gly prepared via DMTMM and EDC/NHS compared to pristine HA.

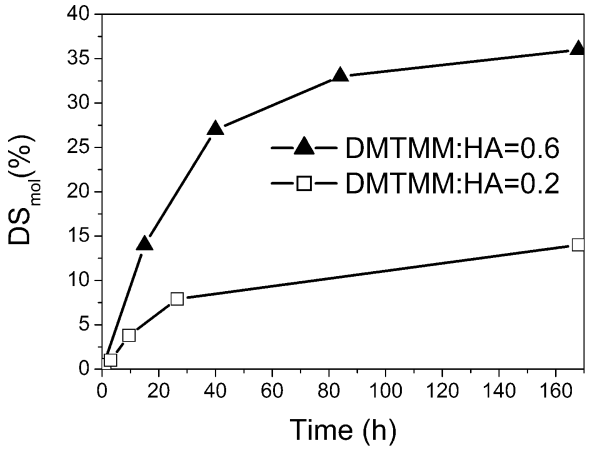


Fig. 4. Kinetics of HA-NED formation for synthesis performed with DMTMM:HA stoichiometric ratio of 60% (▲) and 20% (□).

regeneration. Glycine is the simplest amino acid, and therefore a convenient model of poly-functional molecule bearing the same functional groups of more complex peptides and proteins. In order to avoid self-condensation, the ethyl ester of glycine was used in this study.

The conjugation of Gly to HA with DMTMM gave a DS_{mol} of 53%, while with EDC/NHS we obtained 22% under the same stoichiometric ratio HA to CA to amine (NMR Spectra on Figs. S8 and S9, Supplementary Material). Rheological analysis revealed features very similar to HA-AADA. Mechanical spectrum confirms the non-crosslinked nature of HA-Gly (Fig. S10, Supplementary Material). Viscosity curve (Fig. 3) displays the elimination of the shear-thinning seen for pristine HA. Also in this case, behaviour can be attributed to the disruption of the interactions between HA chains caused by chemical modification. At every shear rate, viscosity follows the order: η (pristine HA) $\gg \eta$ (HA-Gly EDC/NHS) $> \eta$ (HA-Gly DMTMM) which is in good agreement with the DS_{mol}.

After assessing the superiority of DMTMM for HA ligation to the simplest amino acid, a full length globular protein (BSA) was linked to HA. UV absorption maximum of BSA (280 nm) is well separated from HA (210 nm) and DMTMM (235 nm) absorption, and was therefore used to calculate ligation efficiency. Substitution degree was 0.63% for DMTMM, and 0.16% for EDC/NHS mediated ligation. This low value is expected for a large moiety, which has to overcome significant steric hindrance in order to reach the reaction sites.

The conjugation of HA with anticancer drugs is of paramount importance in HA science, and several of these combinations are under investigation at the moment (Bassi et al., 2011; Gibbs et al., 2009, 2011; Meléndez-Alafort et al., 2009; Rosenthal et al., 2005; Tringali et al., 2012; Udabage, Brownlee, Nilsson, & Brown, 2005), including phase III trials (2013a). HA is recognized by CD44, a cellular receptor expressed in many solid tumours. Therefore HA can be used for the active targeting of antineoplastic agents to the tumour site. The association avoids systemic spreading, improves tolerability and minimizes the toxicity, which is the dose limiting factor. Dox in particular is a strong vesicant and therefore not suitable for subcutaneous injection. Moreover it is characterized by dose-dependent cardiotoxicity. Pure Dox displays a maximum of absorption at 488 nm which is not altered upon conjugation, and can be used to measure with good accuracy the degree of modification. Dox is a non-trivial target for HA modification, and in order to increase the grafting up to a therapeutic significant threshold a spacer arm strategy was recently reported for HA conjugation (Cai et al., 2010). Being our focus the comparative assessment of the CA efficiency, we performed a direct conjugation. Use of DMTMM activation gave a conjugate with DS_{mol} = 2.62% against 2.24% for EDC/NHS. Results compare well with the 4% obtained with the spacer arm (Cai et al., 2010) reported a DS of 5.2% w/w, corresponding to DS_{mol} = 4%). At parity of other conditions, DMTMM improves the ligation efficiency of 17% compared with EDC/NHS.

To summarize, superiority of DMTMM towards EDC/NHS for conjugation of simple moieties to HA was confirmed also for multifunctional moieties, full length proteins and complex drugs.

3.5. HA-NED and coupling kinetics

Modification of HA with aryl derivatives gives rise to semi-synthetic polymers used as hydrogels, scaffolds, (non-)woven fabric used as pharmaceutical ingredients and biomaterials (Caravaggi et al., 2003; Revell et al., 2007; Turner, Kielty, Walker, & Canfield, 2004). Since its high absorptivity in a spectral range not overlapping to pristine HA, NED has been selected as probe for measuring the ligation kinetics to HA.

Grafting advancement over time was studied at room temperature using 20% and 60% mole of DMTMM compared to HA disaccharides units. In Fig. 4 the DS_{mol} is reported as a function of the reaction time. DS_{mol} has almost reached a plateau after 6 days of reaction. Interestingly, the higher DMTMM concentration gave higher DS_{mol}, but the reaction was not faster. Therefore, the rate determining step does not depend on the concentration of active s-triazine substituted carboxyl on HA (**1**), but on the ability of NED to reach the active sites. Further studies are necessary to confirm this hypothesis.

The coupling kinetics is substrate-dependent, and therefore the results obtained for NED cannot be generalized. Still, the findings indicate that the coupling reaction might take a few days to go to completion at room temperature. Consistently with the chemical stability of the species involved and eventual side-reactions, an increase of reaction temperature would be advisable for faster kinetics. Usually HA amidation is assumed to be completed overnight or in two days. To the best of our knowledge, no study is reported comparing the degree of substitution at early and later time points for DMTMM amidation of HA. In our study MW of HA is higher compared to other published studies, and this might slow down the reaction.

Products isolated after one week of reaction where analyzed by ^{1}H-NMR. Spectra (Figs. S11 and S12, Supplementary Material) show features of both HA and NED, as expected. DS_{mol} calculated from ^{1}H-NMR spectra gives 10% (20% DMTMM) and 32% (60% DMTMM), against 14% and 36% obtained from UV, respectively. Viscosity of the HA solutions brings to a widening of the bandwidth in NMR

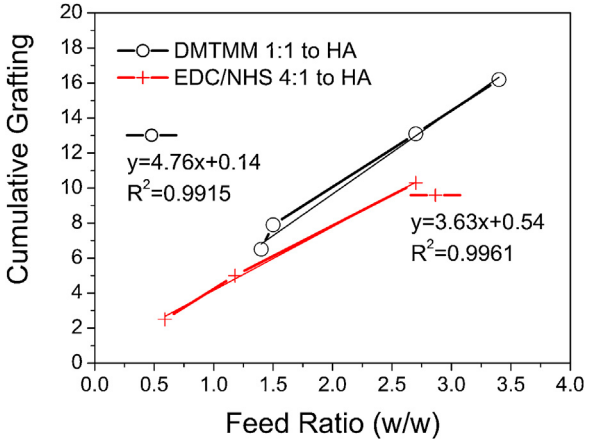


Fig. 5. Cumulative grafting (as defined in the main text) against feed ratio for different HA-pNIPAM derivatives prepared with EDC/NHS activation (red curve with "+" symbol) where EDC is used in a 4 fold molar excess, and of DMTMM activation (dark curve with hollow spheres), where DMTMM is used 1.1/1 molar ratio to HA.

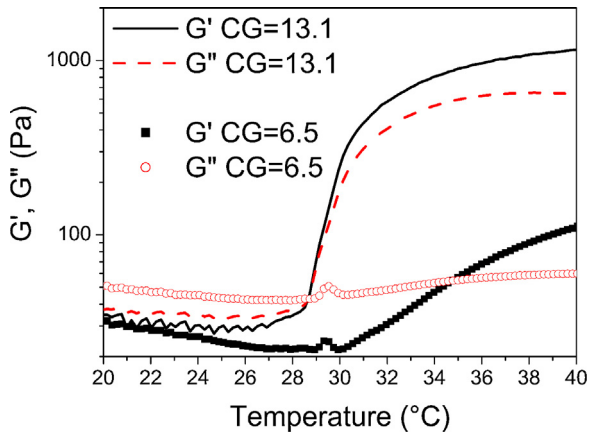


Fig. 6. Temperature dependence of viscoelastic shear moduli of HA-pNIPAM derivatives with CG = 6.5 and CG = 13.1.

which reduces the accuracy achievable, especially for low DS_{mol} values. Therefore, the agreement attained is reasonable.

3.6. Stimuli-responsive polymers

The coupling of polymeric brushes to HA is of particular interest for the preparation of stimuli-responsive materials. Thermoresponsive HA derivatives can be used for example for bio-printing, or for the preparation of injectable solutions that display gelation upon heating from room to body temperature (D'Este, Alini, & Eglin, 2012; Mortisen, Peroglio, Alini, & Eglin, 2010). Thermoresponsive HA hydrogels are particularly attractive as cell carriers for tissue engineering applications (D'Este & Eglin, 2013; Peroglio et al., 2012; Peroglio, Eglin, Benneker, Alini, & Grad, 2013).

Poly(N-isopropylacrylamide) (pNIPAM) is the most researched temperature-responsive polymer. Pure pNIPAM displays a Low Critical Solution Temperature of 32 °C in water, and this value can be adjusted including different monomers within pNIPAM primary structure, or conjugating pNIPAM with other (macro)molecules. Sharpness and physiologically relevant range of its transition make pNIPAM particularly suited for biomedical applications. Hence, we have compared the DMTMM and the EDC/NHS activation chemistry for binding thermoresponsive brushes of pNIPAM to HA.

A first set of experiments was carried out using a 1:1 stoichiometric amount of CA to HA repeating units. In these conditions EDC/NHS attained substitution undetectable by NMR. Therefore we performed a new set of syntheses using a 4:1 molar excess. By contrast DMTMM in 1:1 stoichiometric ratio to HA repeating units was sufficient to give significant substitution. All the results shown in this paragraph refer to the use of EDC/NHS in 4:1 molar excess, while DMTMM was used 1:1 to HA.

The CG (cumulative grafting, defined as pNIPAM:HA repeating units molar ratio in the obtained copolymer) as function of the reaction feed ratio (pNIPAM:HA weight ratio used for the reaction) and the linear interpolation for each curve are reported in Fig. 5. Even though DMTMM was used in much lower amount (1:1 molar ratio to HA against 4:1 for EDC/NHS), at equivalent feed ratio DMTMM gives higher degree of substitution. For a pNIPAM:HA feed ratio of 2.7 w/w, EDC/NHS (4:1 in moles to HA) gives CG = 10.3, while DMTMM (1:1 to HA) gives CG = 13.1. Results in Fig. 5 show how the degree of substitution of pNIPAM into HA can be easily controlled through the pNIPAM:HA feed ratio. For both activation chemistries a linear relationship ($R^2 > 0.99$) was found (Fig. 5). The slope of the linear interpolation line is 30% higher for DMTMM (4.76 against 3.63). The increased gradient is a further indication that HA activated with DMTMM is more prone to react with increasing substrate concentration and suggests higher reactivity for HA activated with DMTMM rather than EDC/NHS. The 4 fold reduction of CA amount to obtain similar substitution is a clear advantage in terms of reaction efficiency, chemical waste production and disposal, and cost saving.

The CG of pNIPAM to HA has dramatic consequences on the rheological properties of the obtained hydrogels. Derivatives with CG = 6.5 do not display an appreciable temperature-induced sol–gel transition (Fig. 6). By increasing the feed ratio to 2.7 w/w CG goes over 13. The corresponding derivative does display a sharp transition in a small temperature range, increasing its storage modulus more than 25 times, from 38 to 10^3 Pa between room and body temperature (Fig. 6). Previous studies demonstrated that higher degrees of substitution are not desirable, because the resulting material would display shrinking upon transition (Mortisen et al., 2010); moreover the higher pNIPAM content could compromise the biocompatibility of the system. ^{1}H-NMR spectrum of HA-pNIPAM displays all the signals expected (Supplementary Material, Fig. S13). The sharpness of the transition within a physiologically relevant temperature range makes the material suitable for minimally invasive surgery procedures. The low viscoelasticity at room temperature allows injection, and the sharp transition to a stiffer state avoids the washing out and the dislocation of the hydrogel from the implantation site. This in situ setting feature is particularly useful for using the material as cell carrier, because a high viscoelasticity at room temperature during injection would generate a mechanical shear detrimental for the cell payload.

4. Conclusion

In this paper we have systematically compared DMTMM and EDC/NHS activation for the coupling of water-soluble amines to HA in water. An array of moieties with different physico-chemical properties was conjugated, covering the major categories of interest for HA modification.

The data indicate that use of DMTMM is superior to EDC/NHS for grafting HA in water. The main benefits of DMTMM activation are

- No need of pH shift during the reaction.
- Improved yields at equivalent substrate/HA stoichiometric ratio.
- Reduced quantity of coupling agent necessary.
- No need for buffering the reaction mixture.

Of course we cannot assume that this superiority applies for every ligand. For example, some amines might cross-react with DMTMM impeding further functionalization. The materials synthesized display a wide range of physico-chemical and rheological properties, and are suitable for a plethora of different applications in the biomedical field. These findings encourage further studies to explore the effect of other coupling parameters, use of DMTMM as ligation agent for bioconjugations besides HA, and the limitations of this approach.

The advantages of DMTMM activation are particularly useful for the scale-up of preparations from the lab to the industrial scale, which is a critical step for the translation of carbohydrate polymers, biomaterials, drug–device combinations and controlled release systems in the clinical use.

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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.2014.02.070.

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