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Investigating triazine-based modification of hyaluronan using statistical designs

Jue Liang^b, Lulu Cheng^d, and Nathan Ravi^{a,b,c,*}

^aDepartment of Ophthalmology and Visual Sciences, Washington University School of Medicine, Saint Louis, MO, USA

^bDepartment of Energy, Environmental and Chemical Engineering, Washington University in St. Louis, Saint Louis, MO, USA

^cResearch and Development, Veterans Affairs Health Care System, Saint Louis, MO, USA

^dStatistics Technology Center, Monsanto Company, Saint Louis, Missouri, USA

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 NaHCO₃ 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 NaHCO₃, 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.

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)

*Corresponding author at: Department of Ophthalmology and Visual Sciences, Washington University in St. Louis; 660, South Euclid, Campus box 8096, St. Louis 63110, USA. Tel.: +1 314 747 4458; fax: +1 314 747 5073.; ravi@vision.wustl.edu (N. Ravi).

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 pulposus 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; Martinez-Sanz et al., 2011; Martinez-Sanz et al., 2012), cell encapsulation (Coates, Riggan, & 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, NMM, cystamine and NaHCO_3 to COOH were varied at 0.5 and 1.0 for HA. We used water or a 7:5 v:v water:acetonitrile mixture as the solvent. This ratio was determined by testing the solubility of experimental reagents in selected organic solvents. We conducted the experiments in random order to nullify the effect of systematic errors.

2.3. D-optimal response surface design for synthesizing thiol-HA with desired degrees of modification

To investigate the relationship between the significant factors obtained from FF analysis and generate a model for precisely controlling the degree of modification, a two-factor, five-level D-optimal design response surface methodology was utilized (Table 2).

2.4. Amidation of hyaluronic acid

A typical procedure is provided. HA (200 kDa 100 mg, 0.25 meq of carboxylic acids) was dissolved in 7 mL (or 12 mL) of DI water in a 20-mL glass vial equipped with a magnetic stir bar. The mixture was stirred at 22 °C for 20 min yielding a viscous solution. Acetonitrile (5 mL or 0 mL) was added dropwise to the HA solution. Desired amounts of NaHCO_3 , NMM, CDMT, and cystamine dihydrochloride were added sequentially. The mixture was incubated at 22 °C for 20 h yielding a gel or a precipitate. Ten molar excess of dithiothreitol (DTT) compared to cystamine was added to the reaction, and the pH was adjusted to ~7.5 by adding a 1 N NaOH solution (100 or 200 μL depending on the amount of CDMT used). Most of the gels or precipitates were dissolved in 30 min yielding clear solutions. The mixture was incubated at 22 °C for 24 h to fully reduce the disulfide bonds. The excess DTT was removed by dialysis (MWCO = 12,000–14,000) against nitrogen-bubbled HCl solution (1 mM, 8 L $\times$ 5) for 5 days. The products were lyophilized yielding a white solid. The yields were found to be ~70–90%.

2.5. Characterization of thiolated-HA

The percentages of derivatization were measured by a 2-nitro-5-thiosulobenzoate (NTSB) assay. An NTSB stock solution was prepared as described previously (Thannhauser, Konishi, & Scheraga, 1987). This solution was prepared fresh every 2 weeks. A thiolated-HA solution (4 mg/mL, 0.1 mL, 0.5 mg) was mixed with 0.9 mL of the diluted NTSB solution. The mixture was incubated in the dark at 22 °C for 2 h and the absorbance was measured at 412 nm. Cystamine solutions with known concentrations (0.1–1.5 mM) were utilized as standards.

Thiolated-HA was also characterized using ^1H NMR spectroscopy. ^1H NMR spectra were obtained on a Varian Unity Inova 500 MHz (Palo Alto, CA). HA samples were dissolved in D_2O (8 mg/mL) with NaOD (20 μmol). Each sample was scanned 128 times at 25 °C.

Gel permeation chromatography (GPC) was performed with the thiolated-HAs. The GPC system employed a VE 1122 pump with a VE 7510 degasser (Viscotek/Malvern, Houston, TX, USA) that was equipped with a TDA302 triple detector system that measured the refractive index (RI), multi-angle laser light scattering, and viscosity. The column used was a

G5000PWL (Tosoh Biosep, Montgomeryville, PA, USA). Viscotek Omnisec software was used to calculate the RI area, weight-averaged molecular weight, intrinsic viscosity and hydrodynamic radius. Samples (100 μ L) were injected at a concentration of 2 mg/mL. The column buffer (pH 7.6) contained 20 mM of sodium phosphate, 100 mM of NaCl and the flow rate was 0.8 mL/min. The measurements were conducted at 37 $^{\circ}$ C.

2.6. Cytotoxicity of thiol-HA

The cytotoxicity of thiolated-HA was tested by MTT-[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (MTT/Thiazolyl Blue) assay and by cell imaging and morphology. We tested the human microvascular endothelial cell line, adult endodermis (HMVECad) used at passage four. Cells were seeded in 96-well plates at 5000 cells/well. Cell suspensions were plated in 150 μ L of in 131 Medium for HMVECad cells. Cells were allowed to grow for 24 h at 37 $^{\circ}$ C. Thiolated-HA was dissolved in media at the highest concentration to be tested. pH of thiol-HA solution was adjusted to 7–7.5 with sterile 0.5 M NaOH and dilutions were made in tissue culture media; concentrations tested were 5, 10, 12.5 and 15 mg/mL. Media was removed from the cells in the 96-well plate and 150 μ L of each thiolated-HA solution was added to eight wells. After 72 h of incubation, the thiolated-HA solution was removed and the cells were rinsed once with 150 μ L of media. Next, 100 μ L of media containing Thiazolyl Blue at 1 mg/mL was added to each well and cells were incubated an additional 5 h. Media with unincorporated Thiazolyl Blue was then removed and 100 μ L of DMSO was added to each well to dissolve the formed formazan crystals which gives a purple color that is representative of mitochondrial metabolic activity of viable cells. Optical density was measured at the wavelength of 540 nm in a 96—well plate reader. Using the optical density readings, the viability of cells exposed to test samples is expressed as a percentage of viability compared to control cells.

3. Results and discussion

3.1. Effects of the factors on the derivatization of HA

Thiol groups were introduced to HA via an amidation reaction of the side carboxylates with cystamine in the presence of CDMT and NMM, followed by a reducing reaction with DTT (Schemes 1 and 2). NMM reacts with CDMT (reaction (1)) to give an intermediate, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM). The DMT-MM intermediate reacts with a carboxylate (reaction (2)) to give a DMT-activated ester, followed by its reaction with a cystamine (reaction (3)) yielding the desired product (Scheme 2). The major side reactions in this study would be the hydrolysis (S1 and S2) of CDMT and DMT-activated ester (due to the large amount of water in the aqueous solution), and the aminolysis (S3) of CDMT (due to the high reactivity of amine) (Kunishima et al., 2013). Other possible side reactions include the reactions of CDMT and the CDMT intermediates with the hydroxyl groups and carboxylates in HA. Moreover, adding a non-nucleophilic base, such as NaHCO_3 , could help the regeneration of NMM (reaction (4)) to compete with side reactions. Thus, the concentration of water, CDMT, NMM, cystamine and NaHCO_3 could be the key factors to control the extent of amidation.

The pH of HA solutions after derivatization were in the range of 5 to 7.5 (Table 1). Consumption of each CDMT molecule, including hydrolysis, aminolysis and alcoholysis, produces one proton, while amines and NaHCO_3 can neutralize the produced proton. The FF 253 analysis shows that the amount of CDMT is the single significant factor that has the greatest effect on pH (effect 1.19, contribution 64.5%). As expected, NMM (effect 0.56, contribution – 14.5%) and NaHCO_3 (effect 0.44, contribution 8.8%) also influence the pH.

The normal plot (Fig. 1) and Pareto plot (Fig. 2) below show that the most significant factors for the derivatization percentage are CDMT, water, and NaHCO_3 . Higher concentration of water led to more hydrolysis, so the percentages of derivatization were lower in water. Due to the CDMT-related side reactions (S1–S3), it is as expected that the amount of CDMT was one of the important factors controlling the derivatization of HA. Since CDMT is the limiting factor in most runs, minimizing side reactions, and thus enhancing the efficiency of CDMT is critical in this reaction. In the following discussion, the efficiency of CDMT will be used as a response for interpretation.

3.2. Effects of the factors on the efficiency of CDMT for the amidation of HA

For the efficiency of CDMT for amidation of HA, the normal probability plot (Fig. 3) and Pareto plot (Fig. 4) below show that the most significant factors are water and NaHCO_3 followed by the inter action between water and NaHCO_3 , and the interaction between CDMT and NMM. In addition, CDMT, the interaction between CDMT and NaHCO_3 , and the interaction between NaHCO_3 and cystamine were important but less significant.

An ANOVA model was conducted to evaluate the selected potential factors from the screening plots (Table 3). Five main effects, the interaction between water and NaHCO_3 , the interaction between CDMT and NMM, the interaction between CDMT and NaHCO_3 , and the interaction between NaHCO_3 and amine were put in the ANOVA model for evaluation. The fitted model is highly significant with F value = 23.45, and p -value = 0.0005. The determinant of coefficient $R^2 = 0.97$ and the adjusted $R^2 = 0.92$. The strongly significant effects (p -value < 0.05) were from water, CDMT, NaHCO_3 , interaction between water and NaHCO_3 , interaction between CDMT and NMM, and interaction between CDMT and NaHCO_3 .

The FF analysis of the 16 runs gives an overall effect of each factor within the selected range of value. However, the reactants behave differently when the solvent composition is changed, particularly for the reactions involving charges. Therefore, the effects of NaHCO_3 , NMM and cystamine on the efficiency of CDMT should be dissimilar in different solvents. In addition, cystamine reacts not only with the DMT-HA ester as desired, but also with CDMT, which decreases the efficiency of CDMT. The presence of other bases in this reaction, such as NaHCO_3 and NMM, can enhance the reactive non-protonated portion of cystamine as well. Thus, the effects of NaHCO_3 , NMM, and cystamine may be different under conditions where their amounts are greater or less than that of CDMT. To better understand the interactions of these factors, and to investigate how the effects of NaHCO_3 , NMM and cystamine vary under different conditions as mentioned above, the 16 runs are divided into 4 groups: (1) factor $A = 100$ v% of water and factor B (the relative amount of

CDMT) = 0.5; (2) factor $A = 100$ v% of water and factor $B = 1.0$; (3) factor $A = 58$ v% of water and factor $B = 0.5$; (4) factor $A = 58$ v% of water and factor $B = 1.0$ (Table 4).

There are four runs in each group. The impacts of increasing the molar ratio of NaHCO_3 NMM and cystamine from 0.5 to 1 on the efficiency of CDMT in each group, $e_{\text{NaHCO}_3}^{0.5-1}$, $e_{\text{NMM}}^{0.5-1}$, $e_{\text{cystamine}}^{0.5-1}$ were calculated by comparing the equations (Equation 1) generated from the four runs. A maximum efficiency of CDMT (Max. E.), when the molar ratios of all reactants are in the range of 0.5 to 1, is also calculated based on the values of $e_{\text{NaHCO}_3}^{0.5-1}$, $e_{\text{NMM}}^{0.5-1}$, $e_{\text{cystamine}}^{0.5-1}$ in each group (Table 5).

Equation 1—The equation for calculating the effect of each reactant on the efficiency of CDMT in the amidation of HA.

i : the run number

$E_{0.5}$: the efficiency of CDMT when Factor C , D , E are all at 0.5 E_i : the efficiency of CDMT at run number i

$e_{\text{NaHCO}_3}^{0.5-1}$, $e_{\text{NMM}}^{0.5-1}$, $e_{\text{cystamine}}^{0.5-1}$: the impact of increasing of the molar ratio of NaHCO_3 NMM and cystamine from 0.5 to 1 on the efficiency of CDMT

$$E_{0.5} + \frac{\text{Fact. } C_i - 0.5}{0.5} e_{\text{NaHCO}_3}^{0.5-1} + \frac{\text{Fact. } D_i - 0.5}{0.5} e_{\text{NMM}}^{0.5-1} + \frac{\text{Fact. } E_i - 0.5}{0.5} e_{\text{cystamine}}^{0.5-1} = E_i$$

For the amidation of HA, $e_{\text{NaHCO}_3}^{0.5-1}$, $e_{\text{NMM}}^{0.5-1}$, $e_{\text{cystamine}}^{0.5-1}$ are all negative when the molar ratio of CDMT is 0.5 in water (group 1). This is due to the excess amounts of NaHCO_3 NMM and cystamine (relative to CDMT) causing an increase in the aminolysis of CDMT (S3) over the increase of the desired reactions (1), (3) and (4). By increasing the molar ratio of CDMT to 1 (group 2), the most significant change is that $e_{\text{NMM}}^{0.5-1}$ was increased from -9.41 to 8.35 . This suggested that when – NMM is less than CDMT, increasing NMM from 0.5 to 1 led to an increase of the desired reactions (1), (3) and (4) over the increase of reaction S3. Similarly, $e_{\text{NaHCO}_3}^{0.5-1}$ was also increased from -6.41 to -1.45 in group 2. When water was used as a solvent, the predicted maximum efficiency of CDMT is 30% in both groups 1 and 2. In the solvent containing only 58 v% of water (groups 3 and 4), the dielectric constant is lower and negatively charged bicarbonate is more likely to be neutralized. Thus, the basicity of bicarbonate is higher in a water:acetonitrile mixture than in only water. Meanwhile, cystamine and NMM are less likely to be protonated and form positively charged ammoniums in a solvent with a lower dielectric constant, as such, they are less basic in a water:acetonitrile mixture than in water. Therefore, the reaction below, which neutralizes two charges, is much more favorable in a medium with a more aprotic component.

As a result $e_{NaHCO_3}^{0.5-1}$ became highly negative and was the most dominating factor in the runs conducted in a water:acetonitrile mixture. By comparing $e_{NaHCO_3}^{0.5-1}$, $e_{NMM}^{0.5-1}$ and $e_{cystamine}^{0.5-1}$ in group 3 and 4, the effects $NaHCO_3$, NMM and cystamine became more negative when their amounts were higher than that of CDMT. This was caused by the greatly enhanced aminolysis of CDMT as mentioned in the discussion of groups 1 and 2. Moreover, the predicted maximum efficiency of CDMT was 42.1 and 40.9% in groups 3 and 4, respectively, which are higher than the 30% predicted for groups 1 and 2. This is due to the reduced hydrolysis of CDMT in the mixed solvent. In comparison, the maximum efficiency of EDC in the EDC/NHS-mediated amidation was only 19.5% when excess amine was used (Santhanam et al., In Press).

3.3. Analysis of response surface design

An important follow-up study to screening the significant factors using the FF design is to optimize the process by investigating the factor level combinations that give targeted response levels with minimum undesirable effects. (RSM) is a mathematical and statistical tool to develop a relationship between a response of interest and several associated factors. This relationship is generally presented by a polynomial equation, which can fit the experimental data set and make predictions. Using the developed polynomial equation, one can find the best set of experimental conditions with optimized levels of variables to achieve a desired overall result. Based on the result from FF design, the most significant factors for the amidation of HA are the solvent content, and the amounts of DMT and $NaHCO_3$. Since this reaction was much more efficient and had less hydrolysis in a less protic solvent, we decided to keep the solvent content constant for the RSM design. Consequently, we chose to use the amounts of CDMT and $NaHCO_3$ as the varying factors, to optimize the efficiency of CDMT. Meanwhile, the other two reagents, NMM and cystamine were kept at a 1:1 ratio to CDMT in the RSM design. The molar ratio of each reactant to COOH was varied at five levels: 0.1, 0.325, 0.55, 0.775 and 1.00. This resulted in 18 combinations of experimental conditions (Table 2). The obtained HAs had modification degrees in the range of 1.8–47.5%.

The RSM was analyzed with Design-Expert software. The analysis of variance suggested that the cubic regression model was most appropriate for the data among all polynomial regression models. First, the sequential model sum of squares indicate the cubic regression is the highest order where the additional terms are significant while the model is not aliased (F -value = 8.87 and p -value = 0.0049 when comparing the cubic regression to the quadratic regression). Second, the lack of fit F -value = 0.70 and p -value = 0.5326 implies no significant lack of fit relative to the pure error, while lower models all indicate significant lack of fit. Thus the cubic regression was used in this analysis. The cubic regression equation is listed in Equation 2:

Equation 2—The cubic regression equation obtained from RSM study for predicting the percentage of derivatization of HA by using CDMT as the activating agent.

$$\text{Deri.}\% = 0.86 - 12.87 \times NaHCO_3 + 73.47 \times CDMT - 36.63 \times NaHCO_3 \times CDMT + 15.48 \times NaHCO_3^2 - 14.88 \times CDMT^2 - 8.60 \times NaI$$

This cubic model was graphically presented in Fig. 5 by the three dimensional response surface. The model is statistically significant, with a model F -value = 1273.64 and p -value < 0.0001. The coefficient of determination $R^2 = 0.9993$, and adjusted $R^2 = 0.9985$ indicating a good fit; the predicted $R^2 = 0.9950$ is in a reasonable agreement with the adjusted R^2 , suggesting the cubic regression model also has great prediction ability to new observations.

The normal probability plot in Fig. 6 (A) shows points close to the diagonal, which means a normal pattern was observed for the regression residuals. The residual plot in Fig. 6 (B) shows no outlier or special pattern for the data. Thus, there were no problems detected from the model diagnostics. Generally, the extent of modification of HA can be increased by increasing the amount of CDMT or decreasing the amount of sodium bicarbonate according to the model graph, which is consistent with the finding from FF design. More importantly, by using the equation, one can precisely predict and control the extent of modification of HA and the properties of the final material. Another important value of this model is that one can change the value of one factor within a certain range while maintaining the same extent of modification by changing the other factor in the basis of the equation. Therefore, the reaction condition can be tailored to meet specific requirements or limitation.

3.4. Purity and stability of the thiolated-HA

As we reported previously, one of the drawbacks of using EDC as an activating agent for amidation of HA is the difficulty of removing the *N*-acylurea side product from the HA backbone.⁴⁴ An advantage of using CDMT instead of EDC is that all of the side products can be removed to obtain a pure product. The pure product was demonstrated with a ^1H NMR spectrum (Fig. 7).

It is also important to determine the stability of the derivatized HA through the synthesis and purification process. The product yields were all above 95%, which suggested that the formation of small fragments which could be lost by dialysis was insignificant. In addition, three representative thiolated-HAs with modification from 6.44 to 46.05 mol% were characterized with GPC (Table 6). Two native HAs (1), the original HA as received, (2) and one stirred in the mixed solvent and dialyzed in DI water were also characterized with GPC as controls. The molecular weights of controls 1 and 2 are almost identical indicating that the process of synthesis and purification does not cause any degradation. The three modified HA samples have very close molecular weights to each other and slightly lower than the controls.

3.5. Cytotoxicity of the thiol-HA

Cytotoxicity of a 6 mol% derivatized thiol-HA was tested at 5, 10, 12.5 and 15 mg/mL since 2-6 mol% derivatized thiol-HA would be used for making hydrogel and for coating gold nanoparticles in our future research as demonstrated by other groups (Kafedjiiski et al., 2007; Li et al., 2012a). HMVECad adult Endothelial Cells (Life Technologies, USA) was utilized as a model cell line for assessing the cytotoxicity of thiol-HA. Thiol-HA was found to be relatively non-toxic up to 15 mg/mL by MTT assay (Fig. 8 (a)). When observing the morphology of the cells (Fig. 8b), there was a small increase in rounded cells between the control cells and the cells exposed to the copolymers.

4. Conclusion

By using design of experiment including FF and RSM, we investigated the synthesis of thiol-HA with a triazine coupling method and successfully developed a model for modifying HA with a pre-determined modification degree. We identified that the significant factors that influence the modification are CDMT, sodium bicarbonate and water content in the reaction solvent. The effect of each factor behaved differently under different reaction conditions or formulations. It was determined that the triazine-based activating agent CDMT is much more efficient than the carbodiimide activating agent EDC, especially in a water/acetonitrile mixed solvent. We also determined a cubic polynomial model for predicting the optimal reaction formulations to precisely control the degree of derivatization while minimize any undesirable result. Our result suggested a complete removal of side products and a good stability of HA in the described protocol. We also found that the 6 mol% thiol-HA was relatively biocompatible up to 15 mg/mL.

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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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Fig. 1.

Normal probability plot of the effects of the factors and their interactions on the derivatization percentage. Effect of single factors is represented by A, B, C, D and E; their interactions with other variables are represented by AB, etc. In this figure, A represents water content, B represents CDMT, C represents NaHCO_3 . The orange marks represent positive effects; whereas the blue marks represent negative effects. The graph aids selecting the dominant factors that are highlighted and labeled in plots; other factors are considered as errors. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

Pareto chart representing the effect of each factor on derivatization percentage in the descending order from greatest to lowest contribution for FF design. Bonferroni limit is the threshold where the effects emerging above this limit are significant. T limit is the threshold where the effect emerging above this but below Bonferroni limit may possibly be significant. The effect of B-CDMT is above the Bonferroni limit which indicates it is strongly significant, while A-water and C-NaHCO₃ are above the *t* limit indicating possible significant effects.

Fig. 3.

Normal probability plot of the effects of the factors and their interactions on the efficiency of CDMT. In this figure, A represents water content, C represents NaHCO_3 , AC represents two factorial interaction of water content and NaHCO_3 , BD represents two factorial interaction of CDMT and NMM. The orange marks represent positive effects; whereas the blue marks represent negative effects. The graph aids selecting the dominant factors that are highlighted and labeled in plots; other factors are considered as errors.

Fig. 4.

Pareto chart representing the effect of each factor on efficiency of CDMT in the descending order from greatest to lowest contribution for FF design. Bonferroni limit is the threshold where the effects emerging above this limit are significant. T limit is the threshold where the effects emerging above this but below Bonferroni limit may possibly be significant. Effects of C- NaHCO_3 and A-water content are above the Bonferroni limit which indicates they are strongly significant, while the interaction between A-water and C- NaHCO_3 , and the interaction between B-CDMT and D-NMM are above the t limit indicating possible significant effects.

Fig. 5.

Model graph depicting the nature of interaction between factors HA:CDMT and HA:NaHCO₃ in response to thiol derivatization at constant values of other factors of CDMT:NMM:amine = 1:1:1 (mole ratio), and solvent = 58 v% of water in water/CH₃CN mixed solvent. This model, developed from the D-optimal design of RSM, has a cubic fit and a non-significant lack of fit with a p value < 0.0001. represents the gradient of colors with increasing thiol content from 1.73 to 47.48. ● indicates design points above the predicted value; ○ indicates design points below the predicted value. This model is significant, with log transformation as recommended by the Box-Cox plot of power transforms.

Fig. 6. Residual analysis of the cubic regression model (A) Plot of normal probability of residuals and (B) Plot of internally standardized residuals with predicted values. represents the gradient of colors with increasing thiol content from 1.73 to 47.48.

Fig. 7.
A characteristic ^1H NMR spectrum of thiolated-HA.

Fig. 8.

(A) The cytotoxicity test of thiolated-HA by MTT assay. (B) Images of HMVEC cells (5000 cells/well initial) exposed to 6 mol% derivatized thiolated-HA at 5, 10, 12.5 and 15 mg/mL, after 72 h of exposure.

Scheme 1.

The reaction scheme of amidation of HA using CDMT and NMM as activating agents.

Scheme 2.

A summary of possible reactions involved in the CDMT-based amidation. The desired reactions (drawn in red) are labeled with numbers 1–4. The major side reactions (drawn in black) are labeled with numbers S1–S3. (For interpretation of the references to color in this scheme legend, the reader is referred to the web version of this article.)

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.

Table 2

The experimental conditions (factors) of each CDMT-mediated amidation of HA using an RSM design.

Run	NaHCO ₃ *	CDMT, NMM and cystamine *	Derv. %
1	0.10	0.10	6.44
2	1.00	0.10	1.83
3	0.55	0.55	23.68
4	0.10	0.10	6.55
5	0.325	0.775	36.39
6	0.55	0.55	23.06
7	0.10	1.00	46.05
8	0.55	1.00	37.76
9	0.55	0.10	3.09
10	1.00	1.00	27.24
11	0.775	0.775	27.22
12	0.10	0.55	32.93
13	1.00	0.10	1.73
14	0.10	1.00	47.48
15	1.00	1.00	28.30
16	1.00	0.55	16.36
17	0.55	0.55	24.02
18	0.55	0.325	13.60

* The numbers stand for the ratio of the moles of each reactant to the moles of COOH.

Table 3

The ANOVA analysis of the factors on the efficiency of CDMT for the amidation of HA in FF design.

Term	Sum of squares	<i>F</i> ratio	Prob> <i>F</i>
Fact. A: water V%	302.06	61.54	0.0002*
Fact. B: CDMT	38.19	7.78	0.0316*
Fact. C: NaHCO ₃	341.51	69.58	0.0002*
Fact. D: NMM	2.04	0.42	0.5425
Fact. E: Amine	21.81	4.44	0.0796
Fact. A: water V% × Fact. C: NaHCO ₃	112.78	22.98	0.0030*
Fact. B: CDMT × Fact. C: NaHCO ₃	30.03	6.12	0.0482*
Fact. B: CDMT × Fact. D: NMM	162.05	33.02	0.0012*
Fact. C: NaHCO ₃ × Fact. E: Amine	25.30	5.16	0.0636

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Table 4

The divided four groups of the amidation of HA in the FF design.

Group	Run	Fact. A: water V%	Fact. B: CDMT	Fact. C: NaHCO ₃	Fact. D: NMM	Fact. E: 2 × cystamine	Resp.: Effic. of CDMT
1	1	100	0.5	0.5	0.5	0.5	30.2
	8	100	0.5	0.5	1	1	18
	11	100	0.5	1	0.5	1	21
	15	100	0.5	1	1	0.5	14.38
2	2	100	1	1	1	1	24.6
	6	100	1	0.5	1	0.5	30
	9	100	1	0.5	0.5	1	17.7
	12	100	1	1	0.5	0.5	20.2
3	7	58	0.5	1	1	1	15.3
	10	58	0.5	1	0.5	0.5	24.6
	13	58	0.5	0.5	1	0.5	37.4
	16	58	0.5	0.5	0.5	1	37.6
4	3	58	1	0.5	0.5	0.5	36
	4	58	1	1	1	0.5	27.4
	5	58	1	0.5	1	1	40.9
	14	58	1	1	0.5	1	26.4

Table 5

The effects of NaHCO_3 , NMM and cystamine and the maximum efficiency of CDMT for amidation of HA under different conditions.

Group	Fact. A: water V%	Fact. B: CDMT	$c_{\text{NaHCO}_3}^{0.5-1}$	$c_{\text{NMM}}^{0.5-1}$	$c_{\text{cystamine}}^{0.5-1}$	Max. E.
1	100	0.5	-6.41	-9.41	-2.79	30.2 (0.5, 0.5, 0.5) *
2	100	1	-1.45	8.35	-3.95	30.0 (0.5, 1, 0.5)
3	58	0.5	-17.55	-4.75	-4.55	42.1 (0.5, 0.5, 0.5)
4	58	1	-11.55	2.95	1.95	40.9 (0.5, 1, 1)

* (x, y, z): The predicated Max. E. can be achieved when Fact. C = x, Fact. D = y, Fact. E = z.

Table 6

Molecular weights of native and derivatized HAs.

HA	Deriv. %	M_n (Da)	M_w (Da)
Placebo 1	0	139.5 k	201.0 k
Placebo 2	0	130.1 k	198.2 k
RSM-1	6.44	115.9 k	178.0 k
RSM-7	46.05	124.4 k	178.2 k
RSM-15	28.30	127.8 k	179.4 k