One-Step “Click” Method for Generating Vinyl Sulfone Groups on Hydroxyl-Containing Water-Soluble Polymers

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INTRODUCTION

“Click chemistry” is a concept for chemical synthesis proposed by Sharpless et al. in 2001.1 It requires the reactions to be selective in reactivity while highly efficient. Importantly, the reaction conditions are mild and the procedure is simple. This idea has since received overwhelming responses and has been extended to other disciplines of chemistry. One class of click reactions that involves Michael addition between an electrophilic double bond and a thiol, termed “thiol-Michael addition”,2 has attracted much attention in the field of bioconjugates and in situ gelation. This is because some of its reactions can proceed efficiently at physiological pH and temperature in aqueous conditions, which is required to preserve the labile biomolecules or cells. At physiological pH, thiols are partially deprotonated and become thiol anions (S-, Michael acceptors),3 which can readily react with certain electron-deficient double bonds (Michael acceptor). Among the electron-deficient double bonds, the vinyl sulfone group (VS) is a popular choice because it is effective under mild aqueous conditions.4,5 Various VS-modified polymers, including dextran-VS,6 HA-VS,7 and PEG-VS8−10 have been successfully synthesized and shown to be biocompatible and useful for forming bioconjugates8−9 and in situ hydrogels5,6 with a thiol-containing counterpart. However, most of the methods for producing VS-modified polymers require organic solvents and often involve multiple and lengthy steps. These synthesis schemes are also specific to a particular polymer, which may not be easily adapted to a new material.

We appreciate the ability of VS to participate in “click chemistry” toward thiol, and aimed to take a “click chemistry” approach to generate VS groups in biopolymers. We envisioned that the reaction for generating VS should be simple, efficient, and capable of a controllable degree of modification. To maximize the yield and minimize the potential hazard when applying to living systems, the reaction should also take place in aqueous environment.

Here, we present a one-step reaction method to prepare vinyl sulfone (VS)-functionalized polymers by a simple “click” reaction that fulfills all the proposed requirements. This synthesis method is applicable to all hydroxyl-containing water-soluble polymers in general, including hyaluronic acid (HA), polyethylene glycol (PEG), dextran, alginate and polyvinyl alcohol (PVA). We further show that the VS-functionalized polymers can participate in a subsequent thiol-Michael “click” reaction with a thiol counterpart. This novelty is made possible by our insights of a reaction for the preparation of cross-linked hyaluronic acid (HA) hydrogel, in which the polymer chains were cross-linked by a small molecule, divinyl sulfone (DVS), via the hydroxyl groups of HA.11 Our understanding of the reaction mechanism between DVS and hydroxyl allows us to modify the reaction conditions, essentially by increasing DVS to OH molar ratio and optimizing reaction parameters, including pH and time. The result is that DVS is not used as a cross-linker, but a reagent to modify polymers by a simple “click” reaction. Despite that the changes in the reaction condition seem subtle, the consequences are significant. This simple “click” method enables the polymers to become “clickable” subsequently under physiological conditions. The modified polymers, instead of forming pre-cross-linked hydrogels, generate “clickable” precursors that are suitable for a wide range of biomedical use.

EXPERIMENTAL SECTION

Materials. Hyaluronic acid of molecular weights 8 and 29 kDa was purchased from Shandong Freda Biopharm Co., China. Divinyl sulfone (DVS, 97%), dextran (MW=70 kDa), polyvinyl alcohol (MW 30−70 kDa), and alginate (low viscosity) were purchased from Sigma Chemical Co., U.S.A. Polyethylene glycol (PEG, 7000−9000 Da) for VS modification was purchased from Amresco, U.S.A. PEG for gel filtration chromatography column calibration was purchased from Fluka, U.S.A. All other materials were purchased from Sigma Chemical Co., U.S.A.

Modification of HA with DVS. Hyaluronic acid was dissolved in 0.1 M NaOH at 2% w/v (corresponding to approximately 200 μmol hydroxyl groups per mL). DVS was added instantly into the vigorously vortexing HA solution in excess at a molar ratio of 1.25× the hydroxyl groups of HA. The reaction was carried out for 10 min and stopped by adjusting the pH to 5 using 5 M HCl. Afterward, the solution was thoroughly dialyzed using Spectra/Por Dialysis Membrane (MWCO: 2000, Spectrum Laboratories, U.S.A.) against deionized (DI) water (pH ~5.3) for 4 days and freeze-dried. 1H NMR spectrometry of the product was performed on 400 MHz spectrometer JNM EX-400 (JEOL, Japan) using D2O as the solvent. The NMR signals of free VS double bonds are at δ = 6.3, 6.4, and 6.9. The degree of modification (DM), defined as the number of vinyl sulfone groups divided by the number of disaccharide repeating units, was calculated from 1H NMR spectra by comparing the integral signals at δ = 6.9 and at δ = 2 (acetyl group of the disaccharide). The molecular weight of unmodified and modified HA was examined by gel filtration chromatography (GFC) with 0.1 M NaNO3 in DI water as the eluent. GFC was performed using TSKgel G3000PWx2 column (Tosoh Biosep, Japan) on Waters 2695 Separation Module connected to a Waters 2414 refractive index

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Modification of Dextran, PEG, PVA, and Alginate with DVS. Dextran, PEG, and alginate were dissolved in 0.1 M NaOH separately at 2% w/v. Divinyl sulfone (DVS) was added instantly into the vigorously vortexing polymer solution at a molar ratio of 1.5× the hydroxyl groups of the polymers. After 3 min, the reactions were stopped by adjusting the pH to 5 with 5 M HCl. The polymers were purified by dialysis using Spectra/Por Dialysis Membrane (MWCO: 6–8000, Spectrum Laboratories, U.S.A.) against DI water (pH = ~5.3). For the modification of PEG, the polymer was dissolved in 0.1 M NaOH at 6% w/v, and DVS in 10× molar excess (to OH of PEG, assuming the MW of PEG is 8000 Da and is homogeneously distributed) was added instantly into the vigorously vortexing polymer solution. The reaction was stopped by adjusting the pH to 5 with 5 M HCl after 10 min, ultrafiltered by Amicon Ultra-15 Ultracel-3k (Millipore, U.S.A.) using DI water, and then freeze-dried. All products were examined by 1H NMR using D,O as the solvent.

Controlling the Degree of VS Modification of HA. For the pH-controlled modification experiment, the HA solution was dissolved in nanopore water and adjusted to different pH values (in the range of 9–10) by dropwise addition of 6 M NaOH. DVS was added instantly into the vigorously vortexing HA solution, at a molar ratio of 1.25× in excess of the hydroxyl groups of HA. The reaction was carried out for 12 h and stopped by adjusting the pH to 5 with 5 M HCl for the time-controlled experiment, HA was dissolved in 0.01 or 0.1 M NaOH solution at 2% w/v. DVS was added instantly into the vigorously vortexing HA solution at a molar ratio of 1.25× the hydroxyl groups of HA. The reactions were stopped at predetermined time points by adjusting the pH to 5 with 5 M HCl. To investigate the changing molar ratio of DVS/OH, HA was dissolved in 0.1 M NaOH and DVS was added instantly into the vigorously vortexing HA solution at a molar ratio of 1.25, 2.5, or 10× the hydroxyl groups of HA. The reactions were stopped at predetermined time points by adjusting the pH to 5 with 5 M HCl.

Cross-Linking of VS-Functionalized Polymer with Dithiophreitol and Dynamic Mechanical Analysis (DMA). VS-modified HA (HA-VS, MW = 29 kDa, DM = 44%) and VS-modified dextran (dextran-VS, DM = 12%) were dissolved in 0.1 M phosphate buffer (PB, pH 7.4) at 7% w/v. The cross-linker, dithiophreitol (DTT), was dissolved in the same buffer at 0.1 mg/µL and added to the polymer solution with SH/VS at a molar ratio of 1:1. To characterize the mechanical properties of the hydrogel, the storage modulus (G′), loss modulus (G″), and tangent delta were measured by ARES Rheometer (TA Instruments, U.S.A.). The VS-functionalized polymer was first mixed with DTT in a microcentrifuge tube. Immediately afterward, 50 µL of solution was transferred to an 8 mm parallel plate transducer (TA Instruments 708.01517, DE, U.S.A.). The dynamic time sweep tests (10 rad/s with 50% strain) were performed once the mixture was loaded. The gap between the plates was set at 1 mm. Mixing and loading took about 1 min before the start of the DMA measurement.

Conjugation of Glutathione on VS-Functionalized Polymer. HA-VS (MW = 29 kDa, DM = 40%) and dextran-VS (dextran-VS, DM = 20%) were dissolved in 0.1 M PB at pH 7.4. Glutathione (GSH), a thiol-containing peptide, was added to the polymer solution at a molar ratio of 3× the respective VS groups. The reaction was carried out for 2 h at room temperature. Afterward, the conjugate HA-VS-GSH was purified by ultrafiltration using Amicon Ultra-15 Ultracel-3k (Millipore, U.S.A.) with DI water and freeze-dried. Another conjugate, dextran-VS-GSH, was purified by repeated precipitation using methanol in excess. The solvent was evaporated before characterization. 1H NMR was performed to confirm the successful conjugation and the elimination of double bonds.

RESULTS AND DISCUSSION
Modification of Hyaluronic Acid. Hyaluronic acid (HA) is a polysaccharide composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine.12 In recent decades, this material has attracted much attention in tissue engineering and drug delivery because of its unique properties. First, HA is produced endogenously in human as part of the extracellular matrix, and HA-based material can support the proliferation and differentiation of various types of cells.13,14 Second, commercially manufactured HA has been shown to be biocompatible, degradable and nonimmunogenic to human.15 Third, HA can interact with HA receptors, which are overexpressed in certain types of cancer cells.16 We envision that VS-modified HA would be a suitable candidate for preparing tissue engineering scaffolds, controlled drug delivery depots, polymer–drug conjugates, and as a material for other biomedical uses.

Pioneered by Balazs' work in the 1980s, it is well established that DVS is reactive toward HA, which has four OH per disaccharide unit, at high pH (e.g., 0.2 M NaOH, pH = 13.3).17,18 Because the pKₐ of hydroxyl groups in saccharides is about 12~13,19 at such high pH environment, most of the hydroxyl groups are deprotonated and become an alkoxide ion (RO−). Being an active nucleophile, it can form a covalent linkage with the electrophilic double bond of VS by 1,4-nucleophilic conjugate addition (Michael-type addition).20 Balazs and co-workers, as well as other groups,21,22 have utilized this reaction to cross-link HA with DVS. In these reports, the molar ratio of DVS to OH is low (e.g., 1:4) such that both VS groups of each DVS react with OH groups of HA and cross-link the HA chains.

We reason that cross-linking can be avoided if DVS in such a reaction is in excess to hydroxyl groups. Stoichiometrically, only one VS group of each DVS molecule will react with the OH group of HA, and the other VS group will remain unreacted. The remaining VS groups can then be used as in situ cross-linking points or protein conjugation points in a subsequent click reaction. Initially, we performed the reaction in 0.2 M NaOH while adding 10× molar excessive amount of DVS. However, the reaction did not generate useful products, and instead, precipitation occurred shortly after the reaction started. We attribute the reduction of solubility to ethyl vinyl sulfone being more hydrophobic than unmodified hydroxyl, so that precipitation occurs when DM is too high. Undesirably high DM occurs when the reaction rate is too high, which can be contributed to the reaction pH that is higher than the pKₐ of hydroxyl, turning most hydroxyl to alkoxide reactive to DVS, and the higher concentration of DVS. We have confirmed, in a later experiment, that indeed HA-VS at DM > 90% is poorly soluble in aqueous solution. Thus, the key to the preparation of soluble HA-VS is to control the availability of nucleophilic alkoxide ion and amount of DVS during reaction. We reduced the reaction pH to reduce the amount of alkoxide and, in the meanwhile, reduce the molar excess of DVS to OH, so that the HA-VS generated contained enough unreacted hydroxyl groups to impart solubility. Indeed, when HA was dissolved in 0.1 M NaOH and the DVS to OH molar ratio was reduced to 1.25, successful modification was achieved in 10 min and the resulting polymer remained soluble. The degree of modification, calculated by comparing the integral of signals at δ = 6.9 and 2, was found to be 48% (Figure 1).

To examine whether 1.25 molar excess is enough to prevent cross-linking of HA and to examine whether there would be degradation of HA at the relatively high reaction pH, gel filtration chromatography (GFC) was performed to measure the molecular weight of 8 kDa HA before and after modification. The theoretical increase in molecular weight is 14% when HA is converted to HA-VS at a DM of 48%. The
weight averaged molecular weight \((M_w)\) for unmodified and modified HA was found to be 8550 (±117) and 9845 (±572) Da (≈15% increase in mean \(M_w\)), which indicates the absence of cross-linking or degradation (Supporting Information, Figure 1).

The proposed reaction scheme is shown in Scheme 1. The first step of the reaction is the deprotonation of hydroxyl at alkaline condition. Each deprotonated OH or alkoxide ion will then react with one DVS, such that for each DVS conjugated to HA there will be one free VS group.

Modification of Other Dextran, PEG, Alginate, and PVA. The attractive properties of the electrophilic double bond of VS has attracted researchers to synthesize other VS-modified polymers for various biomedical applications, including polymer–drug conjugates,\(^7\,^9\) nanoparticle biofunctionalization,\(^8\) in situ gelation,\(^5\,^6\) and encapsulation of labile biomolecules or cells.\(^23\) However, most of the modifications require organic solvents and often involve multiple and lengthy steps. The complicated reaction schemes may hinder the application of such modification method to a wider audience. Moreover, because most modifications described in the literature are specific, it may be difficult to choose the appropriate scheme to introduce VS groups to a different polymer.

Because the polymers used in many of these studies are hydroxyl-containing polymers (e.g., dextran), and the reaction scheme we proposed for HA modification simply involves the deprotonation of hydroxyl and its subsequent reaction with DVS, we went on to examine whether this simple method can be applied to other hydroxyl-containing, water-soluble polymers.

Four representative polymers, dextran, PEG, PVA, and alginate, were chosen to be modified with DVS by our method. For all of these polymers, we dissolved them in 0.1 M NaOH, added an excessive amount of DVS, and reacted for several minutes. \(^1\)H NMR spectra shows that all four polymers were easily modified based on our reaction method (Figure 2), suggesting that this protocol can be applied to other hydrophilic polymers containing hydroxyl groups.

Controlling the Degree of Modification of HA. One important issue in polymer modification is the ability to control the degree of modification, because the required degree of modification may be different in different applications. We therefore assessed the level of control using HA as a model. The degree of modification is related to the rate of VS formation and the reaction time. According to the proposed reaction scheme (Scheme 1), the rate of VS formation should follow second order reaction kinetics and is related to the concentration of alkoxide ion and DVS, as shown below:

\[
\frac{d[HA - VS]}{dt} = k[O^-][DVS]
\]

where \(k\) is the reaction rate constant between alkoxide and DVS.

According to the reaction rate equation, we proposed that we can control the final degree of modification (DM) by three parameters: pH (which determines the concentration of alkoxide ion \([O^-]\)), reaction time, and DVS to OH molar ratio. We first demonstrated the effect of reaction pH on DM. The DVS to OH ratio was kept at 1.25 for this set of experiments. We found that if the reaction was carried out in Nanopure water (pH ~ 5.3),\(^24\) no DVS was conjugated to HA even after 3
days of reaction, which also provides experimental evidence that we can stop the reaction by lowering it to this pH. And as we increased the pH to 9 and 10, after 12 h, the modification was successful and DM was found to be 3 and 55% (Table 1), respectively. We conclude that we can control the DM by varying the reaction pH.

Table 1. Controlling the Degree of Modification of HA by Varying pH or Reaction Time

<table>
<thead>
<tr>
<th>pH-Controlled Modification</th>
<th>reaction pH</th>
<th>5</th>
<th>9</th>
<th>10</th>
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<tr>
<td>degree of modification</td>
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<td>0%</td>
<td>3%</td>
<td>55%</td>
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<tr>
<th>Time-Controlled Modification (0.01 M NaOH)</th>
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<th>2 min</th>
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<th>15 min</th>
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</thead>
<tbody>
<tr>
<td>degree of modification</td>
<td></td>
<td>5%</td>
<td>13%</td>
<td>25%</td>
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<table>
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<tr>
<th>Time-Controlled Modification (0.1 M NaOH, 1.25× DVS)</th>
<th>reaction time</th>
<th>1 min</th>
<th>4 min</th>
<th>8 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>degree of modification</td>
<td></td>
<td>12%</td>
<td>25%</td>
<td>38%</td>
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</table>

<table>
<thead>
<tr>
<th>Time-Controlled Modification (0.1 M NaOH, 2.5× DVS)</th>
<th>reaction time</th>
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<th>4 min</th>
<th>8 min</th>
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<tbody>
<tr>
<td>degree of modification</td>
<td></td>
<td>24%</td>
<td>48%</td>
<td>70%</td>
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</table>

<table>
<thead>
<tr>
<th>Time-Controlled Modification (0.1 M NaOH, 10× DVS)</th>
<th>reaction time</th>
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<th>6.5 min</th>
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<tbody>
<tr>
<td>degree of modification</td>
<td></td>
<td>91%</td>
<td>160%</td>
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For pH-controlled modification, reaction time was 3 days for the pH 5 sample and 12 h for the other two samples. For time-controlled modification, the reaction was done in 0.01 and 0.1 M NaOH solution. The effect of a different molar excess of DVS to OH to the degree of modification was also examined. Degree of modification is calculated by dividing the number of VS groups by the number of repeating (disaccharide) units.

Reaction time also has an effect on the DM. For reactions that proceeded in 0.01 M NaOH (pH ≈ 12) and 0.1 M NaOH (pH ≈ 13), degree of modification went up from 5 to 25% and 12 to 38%, respectively, as the reaction time increased (Table 1). As implied from the previous experiment on pH control, achieving the same DS requires a much shorter time when the reaction proceeds in NaOH of higher concentration. Here, we dissolved the polymer in a known concentration of NaOH instead of adjusting the reaction pH to enhance reproducibility.

For DVS/OH molar-ratio-controlled experiments, we varied the ratio of DVS to the hydroxyl groups on HA to three values: 1.25, 2.5, and 10. The reaction was stopped at predetermined time points. We observed that, for the same time point, DM is higher when the DVS/OH ratio is higher. Moreover, if we plotted DM against reaction time for samples containing DVS/OH at 1.25 and 2.5, we found a linear increase, and for the same time point, DM is doubled when the DVS/OH is doubled (Figure 3). The linear increase suggests that the reaction rate is constant during the examined period of the reaction, which means the reaction rate constant, the concentration of alkoxide ion, and the concentration of DVS are constant. Because, at a constant pH, the concentration of the alkoxide ion and the reaction rate constant k should be constant, this experiment demonstrated that DVS, at either 1.25× or 2.5× the amount of OH, is indeed in excess to the alkoxide ion. The concentration of DVS appears constant because the consumption of DVS is negligible compared to the initial amount of DVS. By increasing the DVS concentration to 10× the hydroxyl, we can achieve DM of up to 160% within 10 min, which to our knowledge is the highest degree of modification of HA. At this extremely high DM, however, the polymer has low solubility.

In summary, using HA as a model polymer, we have validated the reaction scheme proposed, and we can control DM over a wide range from 3 to 160% by simply varying the pH, reaction time, and DVS to OH ratio.

VS-Functionalized Polymer Used for Subsequent Thiol-Michael “Click” Reaction. To determine whether the VS groups grafted on polymer using the present method can participate in thiol-Michael “click” reaction, we used HA-VS and dextran-VS as examples of polymer precursors to form in situ chemically cross-linked hydrogel and bioconjugate.

For hydrogel formation, dithiothreitol (DTT), which contains two thiols per molecule, was used as a model cross-linker. After mixing DTT with HA-VS or dextran-VS at pH 7.4 and room temperature, gels were formed within minutes (Figure 4). To characterize the mechanical properties of the hydrogel, we monitored the change of storage modulus (G’), loss modulus (G”), and the loss angle (tan δ) over time after mixing the polymer and the cross-linker. The start of gelation is indicated when tan δ < 1, meaning that the elastic property (as measured by G’) dominates over viscous property (as measured by G”). For both HA-VS and dextran-VS mixed with DTT, gelation starts shortly after the initiation of the DMA measurement. Nevertheless, the mixture remains injectable through a 30-gauge needle for about 5 min, which is a reasonable time frame for biomedical application. The storage modulus at the end of our examination is about 10 kPa for HA-VS gel and 1 kPa for dextran-VS gel, which are at least 3–4 orders of magnitude higher than the value before cross-linking, supporting that strong chemically cross-linked hydrogels have been formed (Figure 4C,D).

For bioconjugation experiments, we chose reduced glutathione (GSH), which is a tripeptide containing a thiol group, as a model compound. We simply mixed the peptide with each of the two polymers at room temperature and physiological pH for 2 h, and the peptides were conjugated. This was confirmed by 1H NMR spectrum showing the presence of GSH signals and the elimination of the double bond (Figure 5).

These studies demonstrated that the VS groups on the functionalized polymer remain active and readily react with thiol groups under mild conditions. These VS-functionalized polymers are good candidates for in situ gel formation and bioconjugation.
CONCLUSIONS

We have successfully synthesized various kinds of VS-functionalized polymers, all by one simple “click” chemistry method. We demonstrate that we can control the degree of modification by varying the pH, reaction time, and DVS to OH molar ratio. When an appropriate condition was chosen, the desired degree of modification can be achieved within minutes. Furthermore, VS-grafted polymers can effectively react with thiols at pH 7.4 in aqueous phase, making them excellent candidates to prepare in situ hydrogels and bioconjugates.

ASSOCIATED CONTENT

Supporting Information

Example GFC traces of 8 kDa HA and HA-VS. This material is available free of charge via the Internet at http://pubs.acs.org.

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REFERENCES


Figure 4. Gelation of HA-VS and dextran-VS by DTT. The physical appearance of (A) HA-VS-based gel and (B) dextran-VS-based gel. (C) The mechanical properties of HA-VS cross-linked with DTT, showing the change of storage modulus (−), loss modulus (+), and tan δ (●) over time. (D) The mechanical properties of dextran-VS cross-linked with DTT, showing the change of storage modulus (−), loss modulus (+), and tan δ (●) over time.

Figure 5. 1H NMR spectrum of HA-VS (A), HA-VS-GSH (B), dextran-VS (C), and dextran-VS-GSH (D). The double bonds were eliminated and the new corresponding peaks were present for both polymers.


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