Kinetic Analysis and Improvement of the Williamson Reaction for the Synthesis of Poly(ethylene glycol) Propionaldehyde

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ABSTRACT: Poly(ethylene glycol) (PEG) propionaldehyde is an important polymer derivative used in protein modification; it is usually synthesized by the Williamson reaction of 3-chloropropionaldehyde diethyl acetal (CPADA) with PEG alkoxide followed by hydrolysis to deprotect the aldehyde group. However, the side reaction of the Williamson reaction has been a severe drawback leading to a low-aldehyde-content product. In this study, we established a kinetic model to depict the competition of the Williamson reaction and its side reaction. Based on a kinetic analysis, experiments were performed to systemically investigate the influence of the process parameters on the yield of PEG aldehyde, including the reaction solvents, reaction temperature, and molar equivalents of CPADA. The best reaction solvent was determined to be dioxane because the conversion of methoxy pol(ethylene glycol) in dioxane was higher than that in other solvents and because dioxane has low toxicity and is easily handled. The reaction temperature was set as the refluxing point of dioxane to effectively convert PEG into its alkoxide. The equivalents of CPADA were optimized to be 15 to obtain a quantitative yield of mPEG propionaldehyde and avoid wasting the reagent. The quantitative yield of mPEG propionaldehyde was achieved under these optimum conditions. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 111: 1638–1643, 2009

Key words: biological applications of polymers; functionalization of polymers; synthesis

INTRODUCTION

PEGylation, the covalent attachment of methoxy pol(ethylene glycol) to a protein, plays a significant role in the engineering of pharmaceutical proteins to improve drug properties.[1–3] Site-specific PEGylation is preferable for developing reproducible and well-characterized production methods. A general method is to use poly(ethylene glycol) (PEG) aldehyde because the aldehyde is largely selective for the N-terminal α-amine under acidic conditions.[4] The PEGylated version of recombinant human granulocyte colony-stimulating factor (rhG-CSF) prepared in this manner has been approved as a treatment for neutropenia.[5] PEG propionaldehyde, more reactive than PEG benzaldehyde and, unlike the acetaldehyde analog, susceptible to decomposition by aldol condensation,[6] is the common choice among PEG aldehyde derivatives. The compound can be directly prepared by the Williamson reaction of 3-chloropropionaldehyde diethyl acetal (CPADA) with PEG alkoxide followed by hydrolysis. However, this common route possesses some disadvantages due to side reactions, which make it difficult to obtain the PEG propionaldehyde product with high purity. Under the most frequent reaction conditions used for the Williamson reaction, mPEG has a conversion of only 50% or less.[6,7] These conditions work well for the synthesis of mPEG acetaldehyde or mPEG butyraldehyde with fair equivalents of 2-chloroacetaldehyde diethyl acetal[8] or 4-chlorobutyraldehyde diethyl acetal, respectively.[9] Many other routes of synthesis have been developed to obtain higher yield products.[6,7,10–12] For example, Harris[7] reported first converting mPEG to mPEG mercapto and then reacting mPEG mercapto with CPADA to obtain mPEG propionaldehyde linked by thiol ether in a high yield. Rosen[11] developed another way in which mPEG active ester is reacted with 3-aminopropionaldehyde diethyl acetal to obtain mPEG propionaldehyde linked by amide. Although a quantitative conversion can be achieved, these derivation processes have some complexity. In this work, we present an improved protocol for the Williamson reaction by which a quantitative yield of PEG propionaldehyde can be obtained directly. The optimization of this process is based on the reaction mechanism and kinetic analysis of the Williamson

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reaction of mPEG and CPADA under base conditions. The protocol that we have established is applicable to polymers ranging from 5000 to 20,000 Da, and the resultant PEG propionaldehydes of various molecular weights are amenable to modifying proteins.

EXPERIMENTAL

Materials

mPEG 5K was obtained from Polysciences, Inc. (Warrington, Pennsylvania) mPEG 10K and mPEG 20K were obtained from Bio Basic, Inc. (Ontario, Canada). All PEG materials were dried azeotropically before use. CPADA was obtained from Alfa Aesar (Tianjin, China). All other reagents and solvents were obtained from Beijing Chemical Reagents Co. (Beijing, China).

Carbonyl-free methanol was prepared from commercial methanol as described in the literature.[13]

rhG-CSF was obtained from genetically engineered *Escherichia coli* according to a reported method.[14]

NMR spectroscopy

^{1}H-NMR spectra were recorded on an Avance Bruker (Fällanden, Switzerland) 600-MHz spectrometer with deuterated dimethyl sulfoxide (DMSO-d_6) as the solvent separately in 50-mm NMR tubes. In all the spectra, tetramethylsilane was used as the internal reference.

2,4-Dinitrophenylhydrazine (DNPH) determination of mPEG aldehyde

The aqueous test solution (2 mL) was added to 2 mL of the reagent, DNPH (0.1% w/v), in carbonyl-free methanol containing 0.4% (v/v) concentrated hydrochloric acid by pipetting on the wall of the tube above the level of the DNPH solution. After it stood for 0.5 h at 20°C, 10 mL of methanolic KOH was added (160 g of KOH pellets, dissolved in 80 mL water was diluted to 400 mL, with carbonyl-free methanol, and after it stood overnight, the carbonate was centrifuged off). It was mixed and read immediately at 440 or 530 nm.

Preparation of mPEG propionaldehyde acetal

In a typical preparation, 2 g of mPEG 20K (0.1 mmol) was dissolved in toluene. Some of the solvent was distilled to azeotropically remove water. The solution was then cooled to room temperature, and 270 μL (1.5 mmol) of CPADA was added under N_2. Powdered NaOH (240 mg, 6 mmol) was then added, and the resultant suspension was stirred vigorously under refluxing for 48 h. After cooling to room temperature, the suspension was filtered, and the filtrate was evaporated to dryness *in vacuo*. The residue was dissolved in water, and the pH was adjusted to 7.0 with dilute HCl. The solution was extracted with three portions of CH_2Cl_2 (45 mL). The combined extracts were dried over Na_2SO_4 and evaporated *in vacuo* to about 10 mL. Cold ethyl ether was added, and the resultant precipitate was collected by vacuum filtration and dried *in vacuo* to obtain the product as a white powder.

^{1}H-NMR (DMSO-d_6, δ ppm): 3.24 (CH_3O—, 3H), 3.51 (PEG main chain, 1982H), 1.10 (CH_3CH_2O—, 6H), 1.75 [$OCH_2CH_2CH(OCH_2CH_3)_2$, 2H], 4.55 [$OCH_2CH_2CH(OCH_2CH_3)_2$, 1H].

Preparation of mPEG propionaldehyde

mPEG propionaldehyde acetal was dissolved in a sodium phosphate buffer (phosphate-buffered saline; pH 2). The solution was stirred at 50°C for 2 h under N_2. After it cooled to room temperature, the pH of the aqueous solution was adjusted to pH 7 with a 5% $NaHCO_3$ (w/v) solution. The resultant solution was saturated with NaCl and then extracted with three portions of CH_2Cl_2 (45 mL). The combined extracts were dried over Na_2SO_4 and evaporated *in vacuo* to about 10 mL. Cold ethyl ether was added, and the resultant precipitate was collected by vacuum filtration and dried *in vacuo* to obtain the product as a white powder.

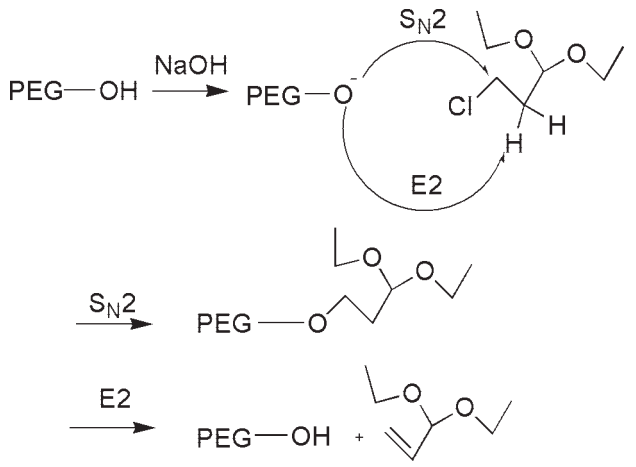
^{1}H-NMR (DMSO-d_6, δ, ppm): 3.24 (CH_3O—, 3H), 3.51 (PEG main chain, 1992H), 9.64 (OCH_2CH_2CHO, 1H), 2.60 (OCH_2CH_2CHO, 2H).

PEGylation of rhG-CSF

In a typical preparation, the PEGylation of rhG-CSF with 5-, 10-, or 20-kDa mPEG aldehyde was performed for 2 h at room temperature with a 0.1*M* sodium phosphate buffer (phosphate-buffered saline; pH 4.0) in the presence of 20 m*M* $NaBH_3CN$. The PEG-to-protein molar ratio was 5 or 10.

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis was performed on a Bio-Rad Mini-Protein apparatus. Samples were diluted with a reducing buffer solution. Gels were prepared discontinuously by stacking and running [4.5% (w/v) and 13.5% (w/v) polyacrylamide, respectively]. Electrophoresis was carried out in a constant-voltage mode at 200 V with a Bio-Rad power supply in a trisglycine/sodium dodecyl sulfate buffer. A silver stain was used to visualize the protein.



Scheme 1 Reaction mechanism of PEG and chloropropionaldehyde acetal.

REACTION MECHANISM AND KINETIC MODEL

The Williamson reaction of PEG and CPADA under base conditions is the process in which the halide undergoes nucleophilic substitution of PEG alkoxide and forms an ether chain. The conditions used for substitution reactions very often lead to elimination. In our case, elimination producing acrolein acetal competes with substitution producing PEG propionaldehyde diethyl acetal (Scheme 1). A kinetic analysis was performed to depict the competitive relationship between these two parallel reactions. It was essential to simplify these processes when we established the kinetic model. The preconditions to the kinetic model can be summarized as follows:

1. The base is greatly excessive, so all the end groups of PEG are converted into anions in the presence of the excessive base. This means that all PEG molecules in the reaction system exist as PEG alkoxide forms.
2. The substitution and elimination reactions are treated as a bimolecular elementary reaction, which is a reasonable assumption because 1° halides under a strong base produce only bimolecular substitution (S_N2) and elimination (E2), especially in polar aprotic solvents.

On the basis of these preconditions, the rates of substitution and elimination are

$$\frac{d[\mathrm{S}]}{dt} = k_s[\mathrm{PEG}][\mathrm{CPADA}] \tag{1}$$

$$\frac{d[\mathrm{E}]}{dt} = k_E[\mathrm{PEG}][\mathrm{CPADA}] \tag{2}$$

where $t = 0$ is the time, $[\mathrm{S}] = 0$ is the concentration of the substitute product (i.e., PEG propionaldehyde diethyl acetal), $[\mathrm{E}] = 0$ is the concentration of the elimination product (i.e., acrolein acetal), k_S is the substitution reaction rate coefficient, and k_E is the elimination reaction rate coefficient. Equation (1) is divided by eq. (2) and then integrated to obtain the following:

$$\frac{d[\mathrm{E}]}{d[\mathrm{S}]} = \frac{k_E}{k_s} \rightarrow \frac{\int_0^t d[\mathrm{E}]}{\int_0^t d[\mathrm{S}]} = \frac{k_E}{k_s} \rightarrow \frac{[\mathrm{E}]}{[\mathrm{S}]} = \frac{k_E}{k_s} \tag{3}$$

Equation (3) demonstrates that the ratio of the substitution product to the elimination product is constant for the whole duration of the reaction; this is defined as K. Obviously, the degree of side reaction occurrence is proportional to the value of K. The factors influencing the value of K include the molecular structure of CPADA, nucleophilicity degree of PEG alkoxide, reaction solvent, and reaction temperature. Given unchangeable reactants, we can reduce the value of K only by optimizing the reaction solvent and reaction temperature.

We have also defined the initial ratio of CPADA to PEG (M) and the yield of mPEG propionaldehyde diethyl acetal (Y). According to stoichiochemistry, we have

$$\begin{aligned}[\mathrm{PEG}] &= [\mathrm{PEG}]_0(1-Y)]_0(1-Y) \\ [\mathrm{CPADA}] &= [\mathrm{CPDAD}]_0 - [S] - [E] \\ &= [\mathrm{PEG}]_0(M-(1+K)Y)\end{aligned}$$

We can combine the result with eq. (1) to obtain

$$\begin{aligned}&\frac{dY}{dt} = k_s(1-Y)(M-(1+K)Y) \\ &Y_{t=0} = 0\end{aligned} \tag{4}$$

After integrating, we can conclude that

$$Y = \frac{1-e^{-k_s(M-(1+K))t}}{1-\frac{K+1}{M}e^{-k_s(M-(1+K))t}} \tag{5}$$

As the reaction approaches its end, Y approaches two different limits:

$$M < K+1, Y \rightarrow M/(K+1) \tag{6}$$

$$M \geq K+1, Y \rightarrow 1 \tag{7}$$

A glance at the kinetic analysis shows that a quantitative conversion can be obtained only once M, the molar equivalent of CPADA, exceeds a threshold value that varies with K. The lower the value of K is, the lower the value of M is that is necessary to attain a quantitative conversion. On the basis of these results, we could perform systematic studies on factors such as the reaction temperature, reaction

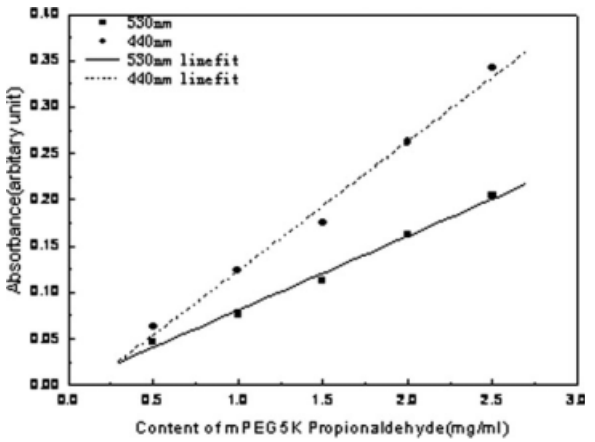


Figure 1 Calibration curve of mPEG propionaldehyde by the DNPH photometric method.

solvent, and molar ratio of CPADA to mPEG that influence the yield of mPEG aldehyde. More specifically, the kinetic analysis demonstrates two ways of optimizing this reaction: (1) reducing K by optimizing the reaction conditions, that is, the reaction solvent and temperature, and (2) optimizing M to obtain a quantitative conversion and avoid wasting reagents.

RESULTS AND DISCUSSION

Our initial attempt to synthesize PEG propionaldehyde following the straightforward route using PEG alkoxide and CPADA resulted in a low aldehyde content of PEG. A similar phenomenon was reported by Harris, who postulated that an extensive elimination reaction accompanying the desired reaction accounted for the low yield of PEG propionaldehyde.[6,7,10] On the basis of this assertion, we established a kinetic model to describe the competition between the substitution and elimination reactions. According to the results of the kinetic analysis, we performed a series of experiments to optimize the reaction and verify our model.

Signal integration of the ^{1}H-NMR spectra can be used to quantify the conversion of mPEG.[15] In the ^{1}H-NMR spectra of mPEG aldehyde, the proton of the aldehyde group (C*H*O) shows a triplet peak at 9.64 ppm, and the protons of methylene (CH_2CHO) adjacent to the aldehyde group show a quintet at 2.60 ppm. Therefore, the intensity ratios of CH_3O— and the C*H*O or CH_2CHO signals in the ^{1}H-NMR spectra of mPEG aldehyde can be used to determine the yield of mPEG aldehyde. As compensation for the ^{1}H-NMR method, we also developed a direct photometric analysis to determine the conversion of PEG propionaldehyde with DNPH. DNPH readily reacts with PEG propionaldehyde to form dinitrophenylhydrazone. The direct analysis was performed in alkaline solutions to eliminate the interference of excess reagents, in which weakly acidic DNPH would not be ionized, whereas dinitrophenylhydrazone would be. Because the anions absorbed at much longer wavelengths (440 or 530 nm) than neutral DNPH (314 nm), direct analysis was possible.[13] Figure 1 demonstrates the calibration curve developed with one of our samples, which had 60% aldehyde content as precisely determined by ^{1}H-NMR. Combining the NMR and photometric methods, we used mPEG 5K as a model to optimize the reaction of mPEG and CPADA.

In the kinetic model section, we determined that the quantitative conversion of mPEG can be achieved only when M attains the value of $K + 1$. Therefore, the first essential step to optimizing the reaction is to determine the best value of K. The factors that influence the value of K include the molecular structure of CPADA, nucleophilicity degree of PEG alkoxide, reaction solvent, and reaction temperature. In our reaction, the value of K can be tuned only by screening of the reaction solvent and temperature. We selected five solvents commonly used for the Williamson reaction and set two temperature levels to optimize our reaction under 5 equiv of CPADA. Figure 2 shows the yield of PEG aldehyde under different conditions that we screened. The apparent result is an unfavorable yield of PEG aldehyde at a lower temperature with all the solvents. There are two possible reasons accounting for the low yield of mPEG aldehyde at a low temperature. First, mPEG cannot be effectively converted to its alkoxide, which is believed to be more nucleophilic than neutral mPEG. The other reason is the sacrifice of the reaction velocity at a lower temperature. Figure 2 also shows that the yield of PEG aldehyde at a higher temperature is higher than that at a low

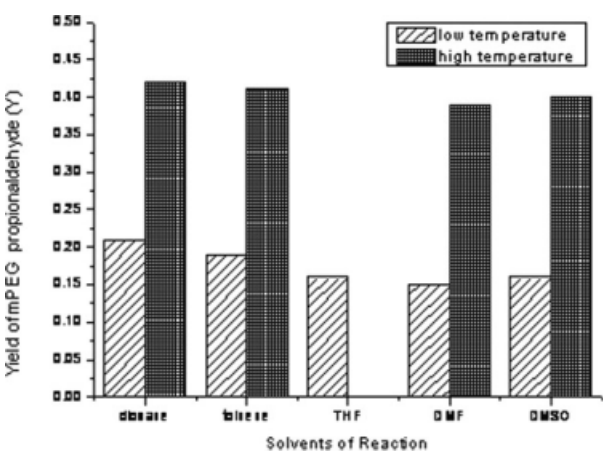


Figure 2 Effect of the reaction system on the yield of mPEG propionaldehyde (low temperature level: refluxing point for tetrahydrofuran (THF) and 60°C for other solvents; high temperature level: 100°C for all solvents).

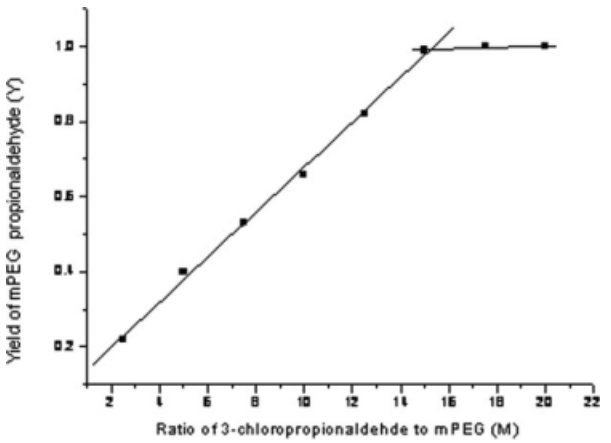


Figure 3 Effect of the molar equivalents of CPADA on the yield of mPEG propionaldehyde (dioxane and refluxing conditions).

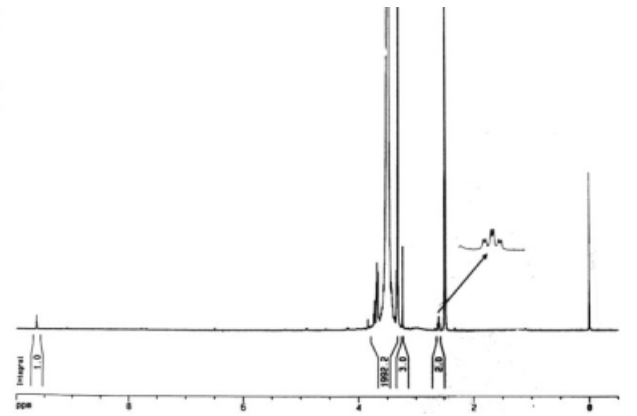


Figure 4 ^{1}H-NMR spectrum of mPEG 20K propionaldehyde.

temperature for each solvent that we screened. However, the yield of mPEG aldehyde shows no significant difference between these solvents. These results indicate that K is mainly determined by the structure of CPADA and the poor nucleophilicity of PEG alkoxide. This means that the value of K cannot be further decreased by optimization of the reaction conditions. Accordingly, we leveled down the criteria for determining reaction solvents to low toxicity and easy handling. Therefore, dioxane was selected for further study, and the reaction temperature was the refluxing point of dioxane. Indeed, the yield of mPEG propionaldehyde in dioxane is higher than that in other solvents.

Our kinetic analysis determined that when M exceeds a certain threshold value, the quantitative conversion of mPEG can be accomplished. In the aforementioned experiment for the optimization of the reaction conditions, 5 equiv of CPADA was used, and the best yield of PEG aldehyde was only 43%. To further address the influence of M on the yield of PEG aldehyde, experiments under the same reaction conditions were performed with a series of M values ranging from 2.5 to 20. The conversions of PEG aldehyde at different values of M are shown in Figure 3. M affects the conversion in two ways. First, a rise in M increases the yield of PEG aldehyde proportionally; this means that a doubling of M will lead to a doubling of Y. When M is equal to 15, a quantitative yield of PEG aldehyde is achieved. M becomes saturated above 15, and a further increase in M leads to a marginal change in Y. The profile of Y is in excellent agreement with the results of the kinetic analysis. The value of K under the selected conditions is 14, as evaluated from the slope of the line ($0 < M < 15$); this means that the rate of the side reaction is 14 times that of the main reaction. The severe degree of the side reaction accounts for the low yield of PEG aldehyde with the straightforward synthesis route.

Based on the kinetic analysis and the experiments, a protocol to obtain a quantitative yield of mPEG propionaldehyde was established. Dioxane was selected as the reaction solvent because the conversion of mPEG in dioxane was higher than that in other solvents and, more importantly, it has low toxicity and is easily handled. The reaction temperature was set as the refluxing point of dioxane to effectively convert PEG to its alkoxide and to avoid a reduction of the reaction velocity. The same conditions were also used in the synthesis of mPEG acetaldehyde, and a high yield of PEG acetaldehyde was obtained for the reaction of mPEG and 2-chloroacetaldehyde acetal unaccompanied by a severe side reaction. A reference report combined with our

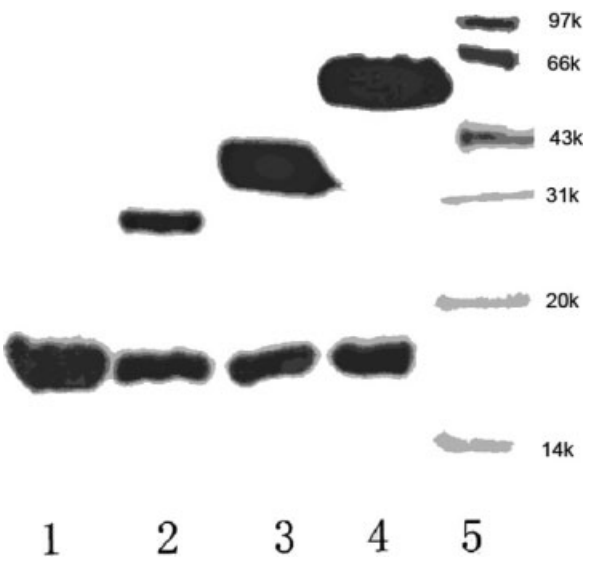


Figure 5 Sodium dodecyl sulfate gel electrophoresis: (1) native rhG-CSF, (2) G-CSF/PEG (5 kDa), (3) G-CSF/PEG (10 kDa), (4) G-CSF/PEG (20 kDa), and (5) low-molecular-weight standards.

experimental results implied that the structure of CPADA was the predominant factor affecting the competition of substitution and elimination, that is, the values of *K*. Because the value of *K* could not be decreased further by optimization of the reaction conditions, a high equivalent of CPADA was necessary to obtain a quantitative conversion of PEG aldehyde. The experimental results determined the threshold of *M* for the quantitative conversion of mPEG to be 15. Under the determined equivalent of CPADA, we obtained a quantitative yield of mPEG propionaldehyde and avoided wasting the reagent. Because the product of mPEG aldehyde had high purity, the difficult task of separating mPEG aldehyde from mPEG could be avoided, and this may balance the cost of synthesis due to high equivalents of the reagent.

Furthermore, we extended the protocol to high-molecular-weight mPEG. Figure 4 shows a representative ^{1}H-NMR spectrum for mPEG 20K propionaldehyde. In Figure 4, the single peak at 3.24 ppm, ascribed to protons of the methoxyl group (CH_3O—), is set as a reference. The integration of the triplet peak at 9.64 ppm, ascribed to the proton of the aldehyde group (C*H*O), or the quintet at 2.60 ppm, ascribed to the protons of methylene (CH_2CHO), provides evidence for the quantitative yield of mPEG. These results demonstrate that our established method can be used for mPEG of a high molecular weight. The general application of the protocol is likely due to the similarity in the degree of nucleophilicity between mPEGs of different molecular weights.

As an example of an application, the resultant mPEG propionaldehydes with molar weights of 5, 10, and 20 kDa were used to modify rhG-CSF by reduction amination. The conjugation of granulocyte colony-stimulating factor (G-CSF)/PEG was characterized with sodium dodecyl sulfate gel electrophoresis (Fig. 5). The single band of each conjugation indicated monomodification of the protein by mPEG aldehyde. The position of the conjugations did not reflect the sum of the molecular weights of G-CSF and the polymer because, as we know, PEG has a higher hydrodynamic volume than a protein with the same molecular weight. The molecular weight of the covalent conjugations could be further confirmed by matrix-assisted laser desorption/ionization time-of-flight or other mass spectroscopy.

CONCLUSIONS

We have demonstrated the possibility of getting a high yield of PEG propionaldehyde by the Williamson reaction with a severe side reaction level. A kinetic model of the competition of the substitution and elimination reactions has been established. On the basis of the kinetic analysis, a quantitative conversion of PEG aldehyde can be achieved by optimization of the reaction conditions and molar equivalents of CPADA. A profile of the yield versus the molar equivalents of CPADA has also confirmed the validity of our kinetic model and the rationality of its presumptions. Because CPADA is an important intermediate for introducing the propionaldehyde moiety into polymers or surfaces, the kinetic analysis that we have performed is applicable to others reactions involving CPADA.

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