NOTE

Influence of Reagent Addition on Carbodiimide-Mediated Amidation for Poly(Ethylene Glycol) Grafting

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INTRODUCTION

Poly(ethylene glycol) is a chemically stable and inert polymer that is soluble in water as well as in many organic solvents,[1,2] hence its use in chemical modification of colloidal particles, including biopolymers.[3–6] The most widely used method for this purpose is carbodiimide-mediated amide binding,[7] especially by 1-ethyl-3-dimethylaminopropyl carbodiimide (EDC). A typical procedure was described by Danishefsky et al.,[8] who applied it to a mucopolysaccharide. Their reaction conditions (i.e., mixing carboxyls, amines, and carbodiimide under pH 4.75) were followed in many studies[5,9,10]; some reports claimed successful formation of amide linkage,[5,8] whereas others reported failure in amide formation.[9,10] Our experience in steric stabilization of cellulose[6] also resulted in scarce amide formation under the Danishefsky's condition.

Recently, the use of *N*-hydroxysuccinimide (NHS) sulfonate, an activating reagent for carboxyls, was found to be effective for the EDC-mediated amide formation.[10] Although the use of NHS for the cellulose-PEG system was effective,[6] we found the efficiency of grafting varied significantly by changing the order of reagent addition. In the present study, we prepared the PEG-grafted microcrystals by four different methods and evaluated the amount of chemically bound PEG by weight increase, decrease in carboxyl content, and pyrolytic gas chromatography with mass spectrometry (Py-GC/MS).

EXPERIMENTAL

Materials

Whatman CF11 powder (Whatman International Ltd., U.K.) was used as cellulose material. Poly(ethylene glycol) aminated on one end (PEG-NH_2, M_w = 1000, Sunbright MEPA-10H) was donated by NOF Corp., Tokyo, Japan. EDC and other chemicals were purchased from Wako Pure Chemicals, Ltd., Japan. NaClO was supplied as aqueous solution of max. 11% content. All chemicals were used without further purification.

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Preparation of Cellulose Microcrystals

Cellulose microcrystal suspension was prepared by HCl treatment and carboxylated as in a previous study.[6] Briefly, 10 g CF11 was hydrolyzed with 100 mL 2.5*N* HCl at 100°C for 15 min, followed by thorough washing with water by filtration. The hydrolysis residue was dispersed in water by a Waring blender for 30 min and centrifuged at 1600 *g* for 5 min to remove coarse particles. Carboxylation was achieved as follows: 500 mL of the suspension containing 0.5–1% cellulose was mixed with 0.5 g 2,2,6,6-tetramethyl-1-pyperidinyloxy radical (TEMPO), and 5 g NaBr and NaClO solution (50% weight against cellulose as solid NaClO), and the mixture was stirred for 4 h at room temperature under pH = 10–11.[6,11,12] By this treatment, the primary alcohol in cellulose was selectively oxidized to carboxyl. The microcrystals were collected as precipitate by adding 1*M* NaCl, followed by successive washing by centrifugation with 0.1*N* HCl and deionized water, then by dialysis. Carboxyl content of the starting and the carboxylated microcrystals were < 10 and 969 mmol/kg, respectively.

Grafting of PEG-NH_2 onto Cellulose Microcrystals

Four methods were examined. In all methods, 100 mL of the carboxylated cellulose suspension (1.41% solid content) was used. Molar ratio of the carboxyl, PEG-NH_2, EDC, and NHS was 0.7 : 1 : 1 : 1. The pH of the mixture was monitored and maintained at the desired level by drop-wise addition of 1*M* HCl or 1*M* NaOH. All reactions were performed at room temperature.

Method A[8]

To the mixture of the carboxylated cellulose suspension and PEG-NH_2, EDC dissolved in 20 mL water was added, followed by stirring for 4 h at pH 4.75.

Method B (adapted from Boccù et al.[4])

To the carboxylated cellulose suspension, EDC and NHS, dissolved together in 20 mL of water, were added. After stirring the mixture overnight, 20 mL PEG-NH_2 aqueous solution was added, followed by overnight stirring at pH 7.5–8.0.

Method C[6,10]

To the carboxylated cellulose suspension, solid PEG-NH_2 was added and the pH was adjusted to 7.5–8.0. To the mixture, 20 mL of aqueous solution of EDC and NHS was added drop-wise and stirred overnight at pH 7.5–8.0.

Method D

To the carboxylated cellulose suspension, solid NHS was added and dissolved completely. To this mixture, solid PEG-NH_2 was added and pH was adjusted to 7.5–8.0. Finally, 20 mL of aqueous EDC solution was added drop-wise and the mixture was stirred overnight at pH 7.5–8.0.

The procedures described above can be summarized as follows:

Method A: [—COOH + *R*-NH_2] + EDC, pH 4.75, 4 h

Method B: [—COOH + EDC + NHS] (overnight) + *R*-NH_2, pH 7.5–8.0, overnight

Method C: [—COOH + *R*-NH_2] + [EDC + NHS], pH 7.5–8.0, overnight

Method D: [—COOH + NHS] + *R*-NH_2 + EDC, pH 7.5–8.0, overnight

After the reaction, the mixture was made acidic (pH 1–1.5) by HCl to make the remaining carboxyl groups a free-acid form. Remaining PEG-NH_2, EDC, NHS, and possible byproducts were removed from the suspension by dialysis by using a standard cellulose tubing (nominal pore size 24 Å). The PEG-grafted products prepared by methods A–D are denoted as samples A–D, respectively.

Characterization

Weight increase of the microcrystal by PEG grafting was determined by the total weight of suspension and its solid concentration determined gravimetrically by drying at 55°C. Carboxyl content was determined by conductometric titration [6,13] and expressed below in mmol/kg dry cellulose (weight increase by PEG binding corrected).

Py-GC/MS was performed by a Shimadzu GC-17A/QP-5000. Analysis of the mass spectra was done by Shimadzu Class-5000 software. The freeze-dried sample measuring100–150 μg was packed in a platinum sample pot and pyrolyzed by dropping it into a furnace of 500°C under argon flow. The degradation product was injected at 230°C to a capillary column (NeutraBond-I, GL Science Inc., 30 m long, 0.25 diameter) under 1 mL/min argon flow. Column temperature was increased from 60 to 200°C at 5°C/min, followed by increases up to 250°C at 10°C/min. The calibration for PEG-specific peaks was obtained by using a series of mixtures of carboxylated cellulose and PEG-NH_2.

For stability test, samples A–D were mixed with NaCl solution to form 0.4% (w/v) cellulose suspension in 0.5*M* NaCl. The samples were shaken vigorously in vials and allowed to stand at room temperature up to 1 month.

RESULTS AND DISCUSSION

Figure 1 shows a typical pyrolytic gas chromatogram (sample C). A peak at 4.88 min (column temperature

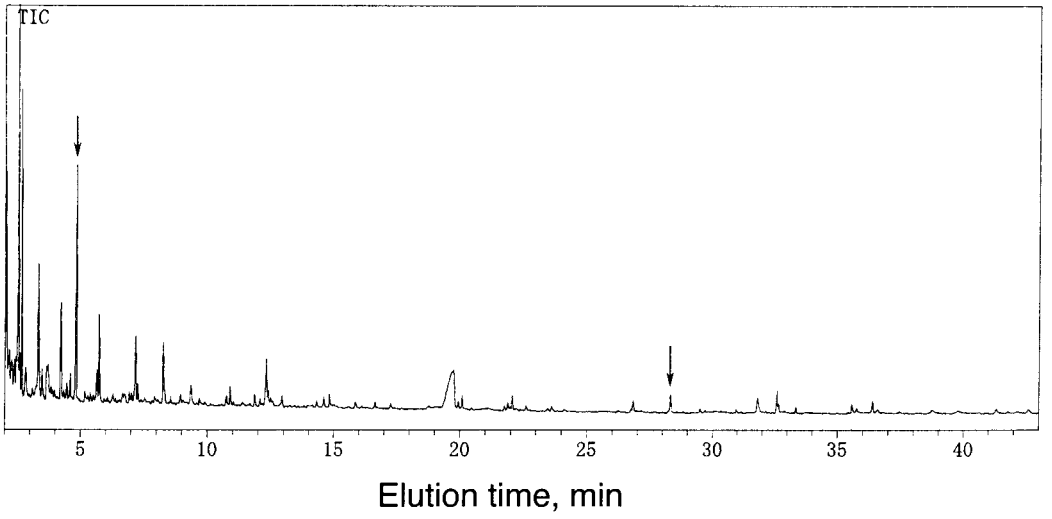

Figure 1 Pyrolytic gas chromatograms of sample C. Peaks at 4.88 and 28.3 min are shown by arrows.

84.4°C) appears in the chromatogram of cellulose, but is absent in that of PEG-NH_2. From mass spectrometry, this peak was identified as 3-methylcyclopentanone, a typical pyrolytic product of cellulose.[14] On the other hand, a peak at 28.3 min (column temperature 203°C) appeared from PEG-NH_2. The peak was identified as either 2-2-(2-methoxyethoxy)ethoxyethanol, or 1,1′-oxybis 2-ethoxy-ethane, which have been detected as pyrolysis products of a PEG-containing polymer.[15] The ratio of these peak heights, $I_{28.3}/I_{4.88}$, was determined for a series of cellulose–PEG mixtures. The result (Fig. 2) shows a good linear correlation between $I_{28.3}/I_{4.88}$ and PEG content to give a calibration graph, although in the narrow ranges of PEG content (10–40%).

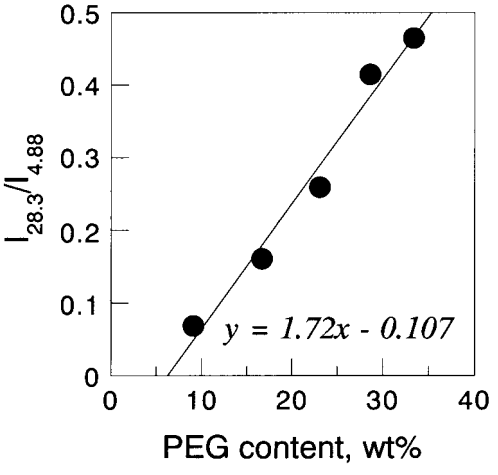

Figure 2 Ratios of the height of peaks at 4.88 and 28.3 min plotted against PEG content of the calibration sample.

Table I summarizes the amount of grafted PEG determined by various methods. Standard deviation of weight increase values and carboxyl consumption was estimated as ~ 10–12% from repeated preparations of Sample D. Here the values from conductometric titration are based on the assumption that the decrease in carboxyl is totally due to the PEG binding. These values are remarkably greater than those of the other methods. This discrepancy is considered to arise from consumption of carboxyl groups by a side reaction (i.e., the formation of *N*-acylurea).[6,9,10]

The aqueous suspensions of ungrafted sample and samples A–D were all stable and showed flow birefringence. When 0.5*M* NaCl was added, the ungrafted sample and samples A and B lost flow birefringence immediately and precipitated after 1 day. In contrast, samples C and D retained flow birefringence and did not precipitate [Fig. 3(a)]. Sample C, however, formed a loose precipitate after 2 weeks [Fig. 3(b)]. Sample D was stable over 1 month under 0.5*M* NaCl. Thus the degree of PEG grafting was not enough for samples A and B to be sterically stabilized. The grafting was apparently more extensive for samples C and D. The

Table I The Amount of Grafted PEG (grams per 1 kg cellulose) Estimated by Various Methods

Samples	Weight Increase	Conductometric Titration	Py-GC/MS
Sample A	84	207	95
Sample B	112	138	112
Sample C	231	401	123
Sample D	203	614	169

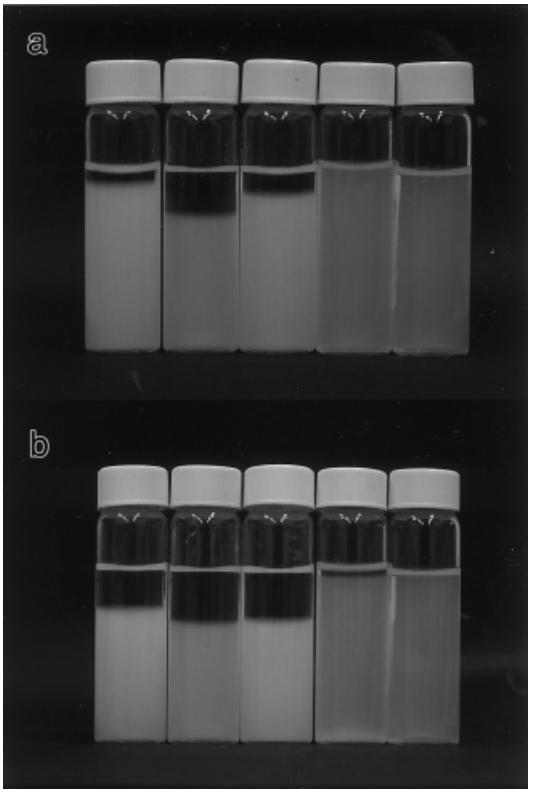

Figure 3 Appearances of the samples dispersed in 0.5*M* NaCl. From left to right, the ungrafted cellulose microcrystals, samples A, B, C, and D. (a) After standing 1 day and (b) after standing 2 weeks.

order of steric stabilization agrees well with the amount of grafted PEG determined by weight increase, considering the deviation stated above (Table I).

Though the values in Table I should be taken as semiquantitative because of experimental difficulties, they show an overall consistency. The results clearly indicate that the order of reagent addition significantly affects the efficiency of intended amide formation. Method A, originally described by Danishefsky,[8] does not employ the activation of carboxyl by NHS. This method gave the lowest PEG binding in the four methods, presumably because of significant byproduct formation, *N*-acylurea derivative.[6,9,10]

The use of NHS is supposed to give the active ester as intermediate, which subsequently undergoes amide formation with PEG-NH_2 mediated by EDC. In fact, methods B–D, all using NHS, gave better results than method A. However, the results in Table I and dispersion stability test show that method B is not effective enough, whereas methods C and D are more effective. Furthermore, the superiority of method D over method C means that the addition of EDC to the mixture of activated carboxyl and PEG-NH_2 is the best way to form the desired amide linkage. A possible reason is that in method C a certain amount of EDC binds to carboxyls prior to the binding of NHS because of the simultaneous addition of these reagents. This would have resulted in more consumption of carboxyls in sample C than that in sample D and caused a difference in the long-term stability of suspension under the presence of electrolyte.

Because our results showed certain extents of PEG binding by all the methods tested, it is difficult to give a clear-cut explanation for the underlying mechanisms. Our conclusion here is that method D is the best way for EDC-mediated PEG grafting to carboxylated cellulose, and perhaps the same is the case for other systems employing amide formation for chemical modification of colloidal particles.

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