0273-2289/93/4202-03—0127$03.00

Covalent Attachment of Poly(ethyleneglycol) to Peptides and Proteins

Reevaluation of the Synthesis, Properties, and Usefulness of Carbonylimidazol-1-yl-Methoxypoly(ethyleneglycol)

JEANNE BRYGIER,[1] MICHEL GELBCKE,[2] CLAUDE GUERMANT,[1] MICHELLE NIJS,[1] DANIELLE BAEYENS-VOLANT,[1] AND YVAN LOOZE*,[1]

[1]*Protein Chemistry Unit, Faculty of Medicine, University of Brussels; and* [2]*Department of Pharmaceutical Organic Chemistry, Institute of Pharmacy, University of Brussels*

Received January 15, 1993; Accepted March 26, 1993

ABSTRACT

Carbonylimidazol-1-*yl*-mPEG is obtained quantitatively by reacting methoxypoly(ethyleneglycol) (mPEG) with 1.1 Eq of *N,N'*-carbonyldiimidazole in the presence of a stoechiometric amount of 4-dimethyl-aminopyridine used as hypernucleophilic acylation catalyst. Carbonylimidazol-1-*yl*-mPEG is quite stable in aqueous solutions with half-lives up to 70 h in pHs ranging from 6.0 to 7.0 at 25°C. From reactivity studies toward amines with various nucleophilic strengths, it is suggested that carbonylimidazol-1-*yl*-mPEG may be best used to modify α-amino terminal function of proteins selectively or to introduce amino function on PEG chains.

*Author to whom all correspondence and reprint requests should be addressed.

Index Entries: Poly(ethyleneglycol); acylation; covalent attachment; methoxyPEG; carbonylimidazol-1-yl-PEG; aminoPEG; pegylation.

INTRODUCTION

Since its introduction by Abuchowski (*1*), the covalent attachment of poly(ethyleneglycol) (PEG) chains to peptides and proteins has been increasingly used. The properties conferred by PEG chains to the modified proteins include: increased resistance to proteolysis, decreased antigenicity and immunogenicity, and decreased rate of clearance from plasma (*2,3*). These advantages are particularly important for enzymes used for therapeutic purpose, such as adenosine deaminase, ribonuclease, uricase, superoxide dismutase, and asparaginase, among others (*3*).

In this context, covalent attachment of PEG chains has been carried out to rectify some vulnerable points of recombinant proteins technology, for example, because of lack of glycosylation. Interleukin-2, streptokinase, *N*-acetyleglin, granulocyte colony-stimulating factor, and various other polypeptide chains produced by the recombinant technology have all been subjected to evaluation after such a treatment (*4–8*). Other clinical evaluations involving covalently bound PEG chains to allergens (honey bee venom, ragweed pollen, house dust mite allergen) for treating allergic patients have also outlined the real benefit obtained by chemically modifying these allergens prior to carry out the hyposensibilizing treatment (*9–11*). Modification with PEG chains has also been found useful in the treatment of experimental myasthenia in the rabbit. Such a treatment involved injection of the major epitope of the acetylcholine receptor, a peptide containing 24 amino acids, obtained by chemical synthesis and modified at its N-terminal α-amino function with a PEG chain. Immunotolerance toward this peptide could be induced in the rabbit at a rate of 85% (*12*).

Thus, this technique, which consists of chemically linking PEG molecules to peptides and proteins, seems very promising in various medical fields. In the near future, it is expected that this technology could take a place of choice among the technologies of protein engineering. However, to reach this aim, we obviously need better control of the chemical linking itself by obtaining further information concerning the PEG derivatives which are used to link PEG chains covalently to peptides and proteins. The purpose of the present article is to reevaluate the synthesis and some properties of Carbonylimidazol-1-yl-methoxypoly(ethyleneglycol) (CI.mPEG), a very popular PEG derivative introduced by Beauchamp et al. (*13*). CI.mPEG was chosen here because of its relative low reactivity toward amines, as compared to most PEG derivatives described so far (*14*). It was speculated that such a low reactivity would give the advantage of an extended level of selectivity.

MATERIALS AND METHODS

Monomethoxypoly(ethyleneglycol) (mol wt: 5 kDa; mPEG), *N,N'*-carbonyldiimidazole, aminoacetonitrile, chloroform-*d*, Tris, 4-dimethylaminopyridine, hydroxylaminesulfate, and ethylenediamine were provided by Aldrich. *N*-α-acetyl-L-lysine, glycyl-glycine, and glycyl-α-valine were purchased from Sigma Chemical Co. All the other chemicals as well as the solvents were of the highest grade available. Solvents were usually dried and maintained on molecular sieves, 3 A, 4–8 mesh (Janssen Chimica).

t-Butyloxycarbonylimidazole was obtained from anhydrous *t*-butanol and *N,N'*-carbonyldiimidazole according to Klee and Brenner (*15*). ^{1}H-NMR spectra were obtained from a Brucker WP-250 Fourier transform spectrometer operating at 250.13 MHz. Chemical shifts were expressed in ppm relative to TMS used as internal reference. Coupling constants were measured in Hertz. The infrared spectra (KBr pellets) were obtained from a Shimadzu IR-470 spectrophotometer. Absorbances and absorption spectra were recorded on a Cary model 118 spectrophotometer.

RESULTS AND DISCUSSION

Synthesis and Characterization of *t*-butyloxycarbonylimidazole

t-Butyloxycarbonylimidazole (BCI) was obtained with a yield of 81% after reacting with stoechiometric amounts of *N,N'*-carbonyldiimidazole and *t*-butanol in refluxing hexane as described previously (*15*). After recrystallization from *n*-pentane, BCI melted at 46–47°C. The infrared spectrum of BCI showed the presence of an absorption band at 1744 cm^{-1}, in a range where the carbamate function was known to absorb while *t*-butanol and imidazole were both transparent. The structure and the purity of BCI were confirmed by ^{1}H-NMR spectroscopy:

^{1}H-NMR ($CDCl_3$) (δ in ppm): 1.62 (s, 9H, CH_3); 7.04 (dd, 1H, J = 1.52 and 0.85 Hz, H-4); 7.37 (dd, 1H, J = 1.52 and 1.30 Hz, H-5); 8.07 (dd, 1H, J = 0.85 and 1.30 Hz, H-2)

UV absorption spectra of BCI were obtained from freshly prepared solutions of BCI in HPLC-grade acetonitrile, which were immediately diluted in water before recording the UV spectra. By this way, an ε value at 231 nm (ϵ_{231}) of $3590 M^{-1} \cdot cm^{-1}$ was determined.

Aqueous solutions of BCI were quite unstable. On hydrolysis of BCI, the UV absorption band at 231 nm disappeared, and the UV absorption spectrum after completed hydrolysis was identical to that of imidazole (λ_{max}: 207 nm; $\epsilon_{207} = 5050 M^{-1} \cdot cm^{-1}$; solvent: H_2O). The half-life of BCI (225 m*M*) in aqueous solutions (pH: 6.5) was found to be 1 h. Hydrolysis of BCI proceeded according to an apparent first-order mechanism.

Reexamination of Carbonylimidazol-1-yl-mPEG Synthesis

Beauchamp et al. (*13*) have obtained CI-mPEG after reacting mPEG (5 m*M*) with *N,N'*-carbonyldiimidazole (50 m*M*) in dry 1,4-dioxane for 2 h at 37°C. CI-mPEG was then purified by dialysis against H_2O and lyophilized. The authors, like others more recently (*3,7*), obtained nearly quantitative reaction if calculated from the weight of recovered lyophilized powder. These results are quite understandable. Indeed, mPEG, with a mol wt of 5 kDa, has been shown by us and by others (*14*) to be quantitatively retained in dialysis bags with a theoretical cutoff of 12–14 kDa.

The purity of CI-mPEG was estimated by Beauchamp et al. on the basis of elemental analysis. Considering CI-mPEG with a mol wt of 5 kDa, thus responding to the formula (C_{231} H_{458} N_2 O_{115}), the elemental analysis would theoretically lead to C: 54.36, H: 9.05, N: 0.55, and O: 36.05%. The particularly low percentage of nitrogen in this kind of PEG derivative does not favor accurate estimations of its purity. In the absence of reliable information about yields and purity of CI-mPEG samples, a reexamination of these problems was undertaken on the basis of spectrophotometric analysis using the UV band at 231 nm, a UV range where mPEG and imidazole were both transparent.

In a first set of experiments, CI-mPEG was synthesized according to Beauchamp et al. (*13*). Dialysis was omitted, and precipitation of CI-mPEG (together with possible unreacted mPEG) in diethylether was preferred. CI-mPEG was collected by filtration, washed thoroughly with ether, and dried under vacuum in the presence of P_2O_5. Aqueous solutions of known concentration were then prepared, and their final CI-mPEG concentrations determined spectrophotometrically using the value $\epsilon_{231} = 3590 M^{-1} \cdot cm^{-1}$ as measured previously for BCI.

By using this test, the yield for CI-mPEG was estimated to be at most 42%. This value did not take into account the expected further drop of yield resulting from hydrolysis of CI-mPEG in the course of the exhaustive dialysis performed by Beauchamp et al. Since this yield was considered as unsatisfactory, various parameters were studied and adjusted in a second set of experiments, so as to improve it. The following procedure led to a 98% yield: mPEG (25 g; 5 mmol) and 4-dimethylaminopyridine (611 mg; 5 mmol) were dissolved in toluene (300 mL). Traces of water present in mPEG (0.4% as measured by the Karl Fisher method) were removed by an azeotropic distillation of 150 mL of solvent. To the mPEG solution, cooled to room temperature, *N,N'*-carbonyldiimidazole (5.5 mmol) was added. The reaction was allowed to proceed at 25°C for 20 h. The reaction mixture was then filtered, and the clear filtrate added to 850 mL of diethylether under vigourous stirring. CI-mPEG was collected by filtration, washed with ether, and dried under vacuum in the presence of P_2O_5.

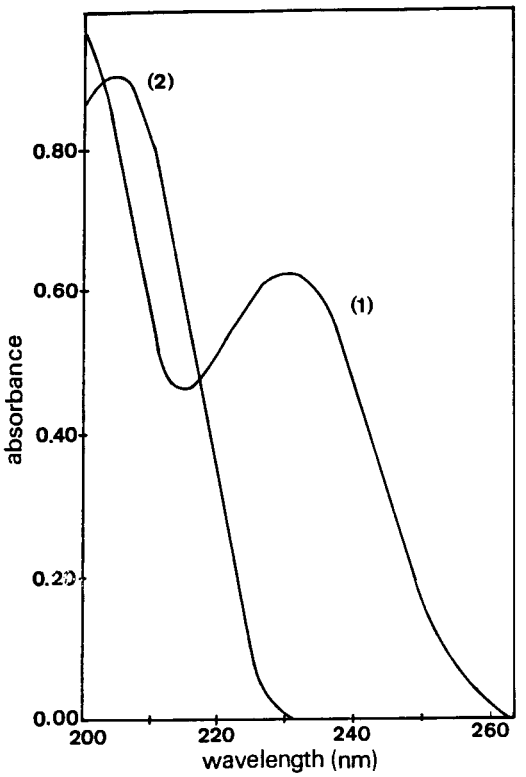

Fig. 1. UV absorption spectrum of CI-mPEG (178 m*M*, 25°C, pH: 7.0; solvent: H_2O) before (1) and after (2) hydrolysis.

The UV absorption spectrum of CI-mPEG is shown in Fig. 1. Our preparations were usually characterized by ϵ_{231} values higher than $3520M^{-1}\cdot cm^{-1}$, which indicated CI-mPEG contents higher than 98%. On hydrolysis, the UV absorption band at 231 nm decreased. When hydrolysis was completed, the UV spectrum was identical to that of imidazole. The infrared spectrum of CI-mPEG confirmed the presence of the carbamate function (absorption band at 1760 cm^{-1}).

pH-Dependence of the Rate of Hydrolysis of CI-mPEG

The rates of hydrolysis of CI-mPEG were measured at 25°C by following the decrease of the absorbance at 231 nm (A_{231}) of CI-mPEG (0.2 m*M*) in buffered aqueous solutions. Solutions containing 10 m*M* acetic acid-sodium acetate, 50 m*M* potassium phosphate, and 50 m*M* sodium borate were used in the pH ranges 4.0–5.0, 5.5–7.5, and 8.0–10.5, respectively. Figure 2 illustrates the pH dependence of the half-life of CI-mPEG aqueous solutions. CI-mPEG was found to be remarkably stable. Within the pH range of 6–7, half-lives up to 70 h were measured (25°C). Under

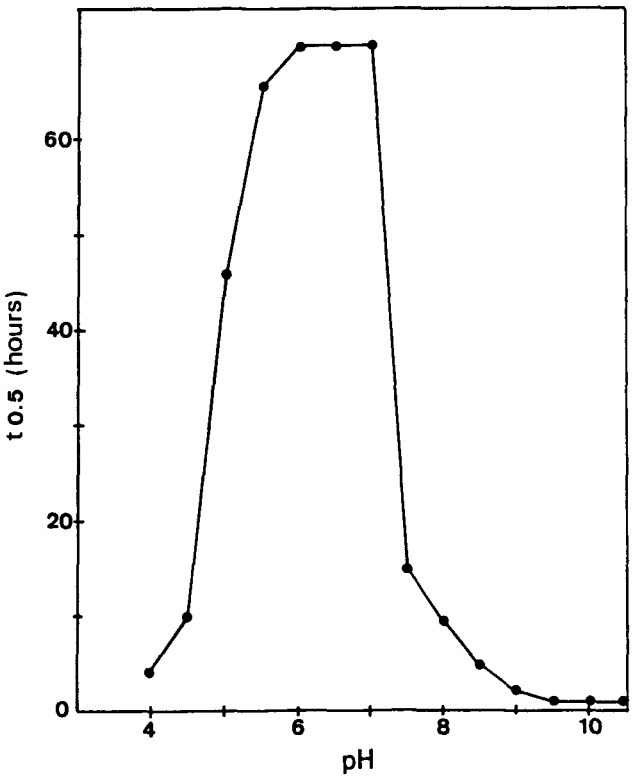

Fig. 2. pH-dependence of the rate of hydrolysis of CI-mPEG. Hydrolysis was followed at 25°C by the decrease of A_{231} of CI-mPEG (0.2 m*M*) in buffered aqueous solutions. Solutions containing 10 m*M* acetic acid solution, 50 m*M* potassium phosphate, and 50 m*M* sodium borate were used in the pH ranges 4.0–5.0, 5.5–7.5, and 8.0–10.5, respectively.

similar conditions, BCI showed half-lives of only 60 min. On the other hand, at pHs 8.5 and 9.0, which are the recommended values in the literature (*7*) for the pegylation of proteins with this PEG derivative, CI-mPEG still resists hydrolysis for several hours.

Estimation of the Reactivity of CI-mPEG toward Amines

The reactivity of CI-mPEG toward amines was estimated from the decreasing absorption rates (A_{231}) of CI-mPEG (0.2 m*M*) solutions resulting from the addition of various amines. Reactions proceeded at pH 7.0 and 25°C in the thermostated cell of the spectrophotometer.

A set of amines with different pK_as and various nucleophilic strengths were compared. Figure 3 illustrates some kinetic patterns observed in the course of this study. It shows that imidazole is rapidly displaced from CI-mPEG by powerful nucleophiles, such as hydroxylamine and ethylenediamine. When hydroxylamine is used in (100-fold) excess, the reaction proceeds according to an apparent first-order kinetic. Measurable rates of

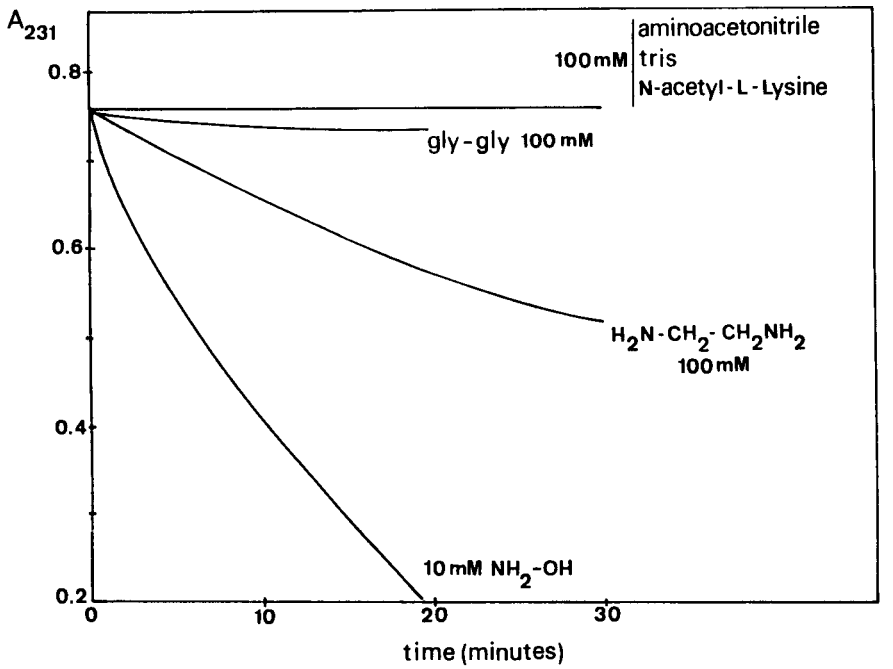

Fig. 3. Kinetic patterns characterizing aminolysis of CI-mPEG by various amines. CI-mPEG (0.21 m*M*) was reacted at pH 7.0 (50 m*M* phosphate buffer) with different amines. Reactions were performed at 25°C in the thermostated cell of the spectrophotometer. The decay of A_{231} was recorded as a function of time.

aminolysis of CI-mPEG were also observed with the α-NH_2 function of dipeptides, such as glycyl-glycine and glycyl-α-valine. On the other hand, in the interval of time considered in Fig. 3, no reaction could be observed with the ϵ-NH_2 function of N-α-acetyl-L-lysine, nor with other less nucleophilic amines, such as Tris or aminoacetonitrile.

The foregoing results allow us to conclude that CI-mPEG would be an excellent reactant to modify the α-aminoterminal function of peptides and proteins selectively. Hen egg white lysozyme could indeed be selectively acylated at the level of its α-NH_2 terminal function (*16*).

Furthermore, CI-mPEG is an excellent intermediate to introduce quantitatively an amino function onto a PEG chain. For example, when solid CI-mPEG (10 g: 2 mmol) is dissolved, at room temperature, in a solution of ethylenediamine (2*M*) preadjusted to pH 7.5 with 12*N* HCl, the displacement of imidazole reaches completion within 30 min. The reaction mixture is then exhaustively dialyzed against distilled water and lyophilized to yield amino PEG.

The purity of the amino PEG derivative is found to be 100% on the basis of microtitration with standardized NaOH solutions. The schematic route to amino PEG reported here is found to be quite easy to handle as compared to more sophisticated methods described previously (*17,18*) and summarized in Scheme 1.

SCEME 1 : SCHEMATIC ROUTES TO AMINO-m PEG

1) According to Rajasckharan - Pillai et al (17).

m PEG —(p-toluene sulfonyl chloride; pyridine in CH_2Cl_2; 24 hours - room temperature)→ m PEG - O - S(=O)(=O) - C$_6$H$_4$ - CH_3

—(potassium phtalimide; in DMF; 4 hours - under reflux)→ m PEG - N(C=O)$_2$C$_6$H$_4$

—($H_2N - NH_2$; in Et OH; 12 hours - under reflux)→ m PEG - NH_2 (overall yield : 64%)

2) According to Bückmann et al (18).

m PEG —($SOBr_2$ and $(CH_3)_3N$; in toluene; 1 hour at room temperature plus 1 hour under reflux)→ m PEG - Br

—($H_2N - (CH_2)_n - NH_2$; in Et OH; 24 hours - under reflux)→ m PEG- NH - $(CH_2)_n$ - NH_2 (overall yield : 66%)

3) This work

m PEG —(N,N' - Carbonyldiimidazole and 4 - dimethylaminopyridine; in toluene; 24 hours - room temperature)→ m PEG - O - C(=O) - N(C$_3$H$_3$N)

—($H_2N - CH_2 - CH_2 - NH_2$ in water - pH 7.5; 30 minutes - room temperature)→ m PEG - O - C(=O) - NH - CH_2 - CH_2 - NH_2 (quantitative)

ACKNOWLEDGMENTS

The authors thank their colleagues Professor A. G. Schnek, Dr. C. Paul, Dr. J. Vincentelli, and Dr. G. deVries-Petiau for helpful discussions. Nicole Smolders is gratefully acknowledged for her expert technical assistance.

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