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Accurate Evaluation Method of the Polymer Content in Monomethoxy(Polyethylene Glycol) Modified Proteins Based on Amino Acid Analysis

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ABSTRACT

To overcome the uncertainty of the colorimetric or fluorimetric method so far employed for the evaluation of monomethoxy(polyethylene glycol) (MPEG) covalently bound to protein, a direct method based on amino acid analysis is proposed. The method exploits the use of MPEG, which was bounded with the unnatural amino acid norleucine (MPEG-Nle). MPEG-Nle was activated at its carboxylic group to succinimidyl ester for the binding to the amino groups of protein.

After acid hydrolysis, the amino acid content is evaluated by conventional amino acid analyzer or by reverse-phase HPLC as phenylthiocarbamyl derivative. The number of bound MPEG chains is calculated from the amino acid composition, since one norleucine residue is released from each bound polymer chain. The method was verified with several proteins in comparison with colorimetric ones, also in the case of proteins that contain chromophores in the visible range, such cytocrome C. It was observed that in most of the cases, the colorimetric methods give an overestimation of the degree of protein modification.

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Index Entries: Monomethoxy(polyethylene glycol); MPEG-modified proteins; protein modification by polymers; MPEG evaluation in modified proteins; Nle-derivatized MPEG.

INTRODUCTION

The covalent binding of monomethoxy(polyethylene glycol) (MPEG) to protein surfaces is a methodology that is acquiring increased application in biotechnology.

This is because MPEG confers to the modified proteins or enzymes new physicochemical properties that can be exploited in fields from biocatalysis to pharmacology. In fact, bound MPEG chains confer to the enzymes unusual solubility in organic solvent where they can carry out specific and stereoselective reactions (*1,2*). On the other hand, MPEG-modified proteins present quite different biological properties in vivo with respect to the native enzyme: longer half-life time in blood, resistance against proteolytic enzymes, as well as reduced immunogenicity and antigenicity (*3–8*). Also, other characteristics, such as binding or crossing of membranes, may be modified (*9,10*).

Despite the increasing interest in MPEG modification, the procedures so far employed to quantify the number of polymer chains bound to the protein are not accurate. Until now, the degree of modification was calculated by colorimetric estimation of the remaining free amino groups in the modified protein as compared to the unmodified one (*3*). Fluorimetric (*11*) or NMR procedure (*12*) were also proposed but they remained so far without significant applications. Fluorescence in fact may be deeply affected by the environment of the fluorophore, whereas NMR requires sophisticated equipment and skilled operators.

To overcome such analytical gaps, the present paper reports a direct method to evaluate the number of MPEG based on amino acid analysis. The method implies the preparation of activated MPEG-Nle, which is then used for the modification of proteins. In principle, this method employs the analysis of norleucine, which was incorporated between protein and MPEG.

Norleucine is an unnatural amino acid that is very stable to acid hydrolysis and easy to resolve and evaluate either in standard amino acid analyzers or in HPLC as phenylthiocarbamyl derivative (*13*).

MATERIALS AND METHODS

MPEG-OH 5000, 2,4,6-trinitrobenzenesulfonic acid (TNBS), *o*-phthaldialdehyde (OPA), triethylamine, 4-nitrophenyl chloroformate, *N,N'*-

dicyclohexyl carbodiimide, *N*-hydroxysuccinimide, and norleucine were purchased from Fluka, (Buchs, Switzerland). Bovine erythrocyte superoxide dismutase (SOD) was supplied by Diagnostic Data Incorporated, (Mountain View, CA), ribonuclease-A from bovine pancreas (RNase), bovine serum albumin (BSA), tyrosinase, and cytochrome C were from Sigma Chemical Co. (St. Louis, MO), *Erwinia Chrysanthemi* asparaginase was from PHLS Centre Porton Down, (Salisbury, UK) and thermolysin was from Boehringer (Mannheim, FRG).

Amino acid analysis was performed in a Carlo Erba instrument or in the case of phenylthiocarbamyl, (PTC), amino acid derivatives in a Pico-Tag Nova Bondapac column operated with a Water HPLC (Milford, MA) was employed.

Preparation of Activated MPEG-NLe

To 5 g (1 m*M*) of MPEG 5000 dissolved in 20 mL of anhydrous methylene chloride, 0.28 mL (2 m*M*) of triethylamine (TEA), and 0.40 g (2 m*M*) of 4-nitrophenylchloroformate were added under stirring while the pH was adjusted to 8 with TEA. The reaction mixture was maintained at room temperature for 4 h. The mixture, concentrated under vacuum to about 10 mL, was dropped into 100 mL of stirred diethyl ether. The precipitate was collected by filtration and crystallized twice from ethyl acetate. The yield of MPEG-*p*-nitrophenylcarbonate, (MPEG-OCO-O-$C_6H_4NO_2$), calculated spectrophotometrically on the basis of the released 4-nitrophenol absorption in alcaline media was 98%.

Norleucine, 0.52 g (4 m*M*), was dissolved in 20 mL of water at pH 8.0–8.3 and 4 g (0.8 m*M*) of MPEG-OCO-O-C_6H_4-NO_2 were added in portions for 3 h while the pH was maintained at 8.3 with NaOH. After overnight stirring at room temperature, the solution was cooled at 0°C and brought to pH 3 with 2*N* HCl. Side reaction product, 4-nitrophenol, was extracted with diethyl ether. The product, MPEG-O-CO-Nle-OH, was extracted three times with chloroform, dried, concentrated, precipitated with diethyl ether, and finally recrystallized from ethanol. The yield, calculated by —COOH titration of the product or by Nle evaluation by amino acid analysis after acid hydrolysis, was 95%.

MPEG-Nle-OH, 3.5 g (0.7 m*M*) was dissolved in 10 mL of anhydrous methylene chloride, cooled to 0°C, and 0.16g (1.4 m*M*) of *N*-hydroxysuccinimide and 0.29g (1.4 m*M*) of *N,N*-dicyclohexylcarbodiimide were added under stirring. The temperature was raised to 20°C and the reaction mixture was stirred for 4 h. The precipitated dicyclohexylurea was removed by filtration and the solution was concentrated and precipitated with diethyl ether. The product (MPEG–Nle–OSu) was recrystallized from ethyl acetate. The yield of esterification, calculated from the UV hydroxysuccinimide absorption, was over 95%.

Enzyme Modification with MPEG–NLe–OSu and Purification of Polymer Protein Adducts

Proteins were dissolved in borate buffer 0.2*M*, pH 8 at a concentration of about 3 mg/mL, and MPEG-Nle-OSu was added at room temperature under vigorous stirring. The amount of polymer used for the modification was calculated on the basis of the available amino groups of the proteins. MPEG–Nle–OSu/$-NH_2$ molar ratios of 0.5:1, 1:1, 2.5:1, and 5:1 were used to obtain, for the same protein, different modification extents.

After standing for 1 h, the product was ultrafiltered twice and concentrated to a small volume in an Amicon system with a PM 10 membrane (cut off 10000) to eliminate the excess MPEG and side products. The concentrated solution was further purified by gel-filtration chromatography in a Pharmacia Superose 12TM column operated by an FPLC instrument. The modified protein was collected, concentrated, and ultrafiltered to eliminate the buffer salt.

Colorimetric Evaluation of Protein Amino Groups and Protein Concentration

The evaluation of free protein amino groups was performed by TNBS according to the Habeeb procedure (*14*) or to the more recent Snyder and Sabocinsky modification (*15*), and in some cases, also by the OPA method based on spectroscopic readings of the 1-alkylthio-2-alkylisoindoles adducts (*16*).

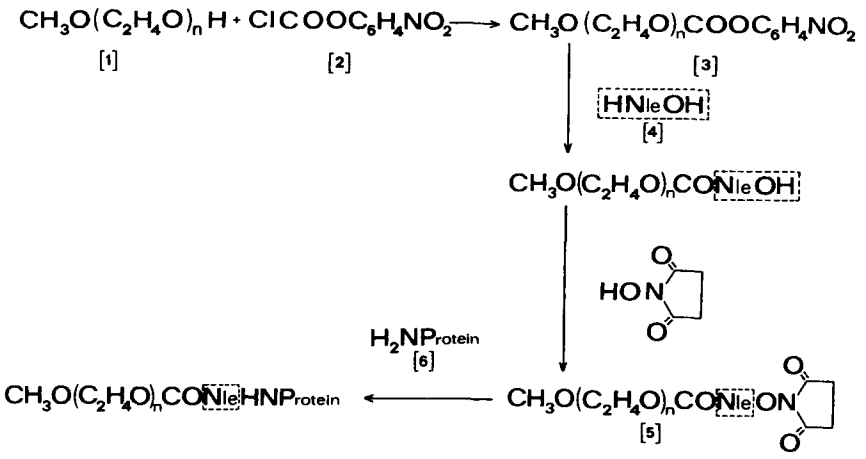
The protein concentration was evaluated colorimetrically by the biuret method (*17*), using the native protein as reference standard.

Amino Acid Analysis

Samples of purified MPEG-protein, diluted with equal volume of concentrated HCl were hydrolyzed under vacuum at 110°C for 24 h. The amino acid composition was evaluated using the standard ion-exchange analyzer (*18*).

Alternatively, the amino acid composition was evaluated by reverse-phase HPLC after derivatization with phenylthiocarbamyl. In this case the hydrolysis was performed in 6*N* HCl at 150°C for 1 h (*13*).

The protein concentration in the modified protein was calculated on the basis of the protein amino acid composition. The most important character of our method is that, it also gives directly the degree of modification on the basis of norleucine amount in comparison to other stable amino acid residues (usually Phe, Leu, Ala). From the MPEG–Nle–protein adduct composition for each mole of polymer one mole of norleucine is released. The released MPEG is eluted with the void volume of the column and do not interfere with the amino acid analysis.



Scheme 1

RESULTS AND DISCUSSION

Preparation of the Modified Protein Samples

We recently reported a method to introduce amino acids or peptides as suitable spacer arm in the MPEG derivatization of proteins (*19*). This method is based on the MPEG [1] reaction with 4-nitrophenylchloroformate [2] to give MPEG-4-nitrophenylcarbonate [3]. The reaction was proceeded in aqueous solution with the free, unprotected amino acid or peptide [4], followed by the activation of its carboxylic group as the succinimidyl ester [5] for reaction with protein amino groups [6] (*see* Scheme 1). The procedure reported in this paper for norleucine is such to assure that no trace of the reactive MPEG-4-nitrophenyl carbonate is present in the final product. At the pH of amino acid coupling (pH 8.3), the MPEG-4-nitrophenyl carbonate is totally decomposed by water; its half-time of decomposition by water is 3.5 h, whereas the reaction with the amino groups is much faster (*19*). At the end of the preparation, MPEG–Nle–OSu will be the only reactive polymer species. Trace amounts of unreactive MPEG-OH may be present, but this impurity has no effect on protein modification.

The purification of MPEG–protein adduct by chromatographic step must be performed with great care to avoid contamination by excess reagent, since Nle would be generated by acid hydrolysis.

Several proteins with different molecular weight, some with chromophores in the visible range as cytocrome C, were reacted with MPEG- Nle–OSu using different molar ratios of activated polymer to protein amino groups to give adducts with different degree of modification (*see* Table 1).

Table 1
Evaluation of Degree of Modification
in MPEG-Nle-Protein by Different Methodologies

Proteins	Modification conditions[a]	Modified-NH_2, %			
		TNBS[b]	TNBS[c]	OPA[d]	Nle Anal[e]
Albumin	1	16.0	-	-	11.3
	2	28.6	-	-	14.7
	3	48.2	-	-	25.9
	4	50.3	47.2	60.2	39.2
Asparaginase	4	87.1	60.9	75.2	62.1
Cytocrome-C	2	45.8	-	-	46.7
	3	78.7	-	78.0	71.2
Ribonuclease A	1	21.7	-	-	21.1
	2	43.7	-	-	45.6
	3	80.5	75.6	82.3	71.3
SOD	1	26.6	-	-	20.0
	2	44.0	30.2	-	26.6
	3	64.6	49.2	55.4	45.0
	4	80.0	-	-	61.3
Termolysine	3	48.5	48.1	49.2	33.7
Tyrosinase	2	21.3	-	-	21.1
	3	42.6	40.1	-	38.2

[a]Different extent of protein modification were obtained changing the ratio of MPEG-Nle-OSu to protein amino groups (1) 0.5:1, (2) 1:1, (3) 2.5:1, and (4) 5:1.
[b]The degree of MPEG binding was evaluated colorimetrically by TNBS according to Snyder and Sabocinsky, and the value expressed as percentage of polymer modified amino groups with respect to the total present in the protein.
[c]The degree of MPEG binding was evaluated colorimetrically by TNBS according to Habeeb and expressed as in (b).
[d]The degree of MPEG binding was evaluated by the OPA method and expressed as in (b).
[e]The degree of MPEG binding was evaluated from the amount of Nle after amino acid hydrolysis and expressed as in (b).

Evaluation of the Degree of Bound Polymer Chains

The number of bound polymer chains was calculated on the basis of Nle content evaluated by amino acid after acid hydrolysis. Nle was evaluated either as such in standard amino acid analyzers or, better, as a PTC derivative using HPLC chromatography. Following the latter procedure, a good separation of Nle is achieved as shown in Fig. 1. The advantage of this method resides in the extremely low amount of protein sample necessary for the analysis.

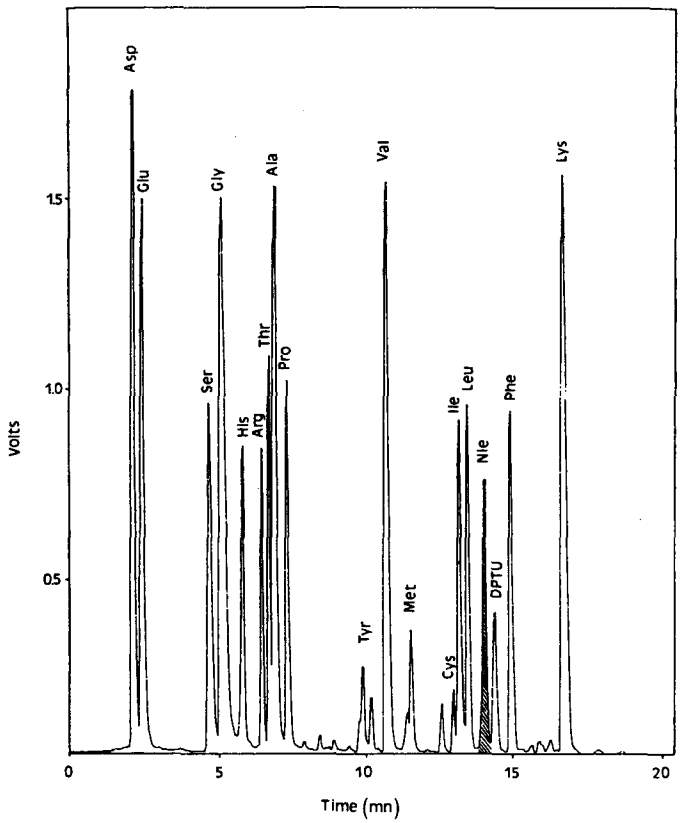


Fig. 1. FPLC pattern of MPEG-Nle-SOD (sample 3 of Table 1) after acid hydrolysis and derivatization with phenylisothiocyanate. The separation was obtained by reverse-phase chromatography according to Bidlingmeyer et al. (*13*). The Nle peak is shaded. DPTU corresponds to the diphenylthiourea elution.

In Table 1, the results were compared with the data estimated by colorimetric methods (TNBS following two different procedures (*14,15*) and in some cases by OPA (*16*)).

It is worthwhile to recall that by our method, the amount of bound polymer per protein molecule is obtained directly from norleucine content. In other words, the relative amount of MPEG is obtained from the amount of Nle, whereas the relative amount of protein is given from the amount of any other amino acid. Our method avoids the separate evaluation of protein and polymer for the estimation of the degree of modification. It should be noted that there is always concern that attached MPEG will affect the colorimetric method of determining proteins concentration.

Also note the differences among the methods of evaluation and the fact that the values obtained by the norleucine procedure are usually lower than those obtained by the colorimetric ones. This is not surprising if one takes into account the intrinsic limitation of any colorimetric evalu-

ation applied to the protein molecules. A literature examination of the well-studied TNBS procedure for the evaluation of amino groups in proteins indicates that there are several parameters that may influence the results (*14,15,20,21*). These include the importance of the temperature of reaction, the presence of detergent that reduce the reactivity of amino groups at some pH, the properties of the protein under examination, the wavelength of reading, the different color yield of the amino acids, and the different reactivity of buried amino acids residues. These parameters, studied in detail with unmodified proteins, must play some role also in the rate of reaction and accessibility on the MPEG–protein adducts, but they have so far not been taken into consideration.

Furthermore, new source of errors in the colorimetric evaluation of polymer adducts may reside in the MPEG chains steric hindrance to the approach of TNBS toward the amino groups, or in changing of extinction coefficient of the TNBS–adduct in the new polymer–protein environment.

In this regard, it may be useful to recall similar problems found in the spectrophotometric evaluation of tryptophan in proteins (*22*), an amino acid not easily evaluated by standard methods based on acid hydrolysis for its acid instability. Similar problems are encountered as well in the evaluation of cysteine by —SH-specific reagents (*23*). In both cases, the problems come from the different molar extinction coefficient of the chromophore as verified in the environment of different proteins or in different position of the same molecule (*24*). In the case of tryptophan, both wavelength and intensity of UV absorbtion and fluorescence were used as probes of their environment (*24*).

For all of the above-mentioned reasons, we are now much more confident in the direct method of polymer evaluation based on norleucine content.

The table shows that the values, obtained by the norleucine method, are closer to those obtained with TNBS according to the Habeeb procedure (*14*) than to those given by the more recently proposed Snyder and Sabocinsky modification (*15*), or by the OPA method (*16*). This is probably owing to the higher temperature of the TNBS reaction in the Habeeb procedure that, increasing the flexibility of the MPEG chains reduces the steric hindrance offered to the approach of the reagent. PEG is also less well hydrated at higher temperatures, as shown by its cloud-point phenomenon (*25*).

It is of interest to note that with protein containing chromophores, the direct norleucine procedure avoids the problems related to a correct blank calculation. The interference resulting from the chromophore absorbance may be very significant, for instance, the absorbance at 420 nm (the wavelength used in the Snyder and Sabocinsky procedure) of native cytocrome C corresponds to the absorbance value of about nine amino groups reacted with TNBS.

As a further observation, it is worthwhile to point out that with TNBS or OPA, and also with the fluorescence procedure, only polymer chains

bound to amino groups are evaluated. With the norleucine method chains bound to other potentially reactive groups are also revealed. In this regard, we note that the particular protein environment and the proximity of proper amino acid residues may change deeply the nucleophilicity and reactivity of groups such as —OH of tyrosine, serine, or threonine, or nitrogen of histidine, and so on, to which the activated polymer may bound. Several of such overreactivity of these amino acid residues in proteins have been reported (*26*). A question that can be raised is the biological behavior of the MPEG-Nle-modified proteins with particular regard to clearance and immunogenicity. Using proteins SOD and RNase as model, we already found no difference in blood residence time between the protein modified with norleucine arm or with MPEG directly bound to protein prepared with standard methods (*3,7*). Immunogenicity was not tested, however, it is unlikely that it would increase for the introduction of norleucine, since this amino acid is deeply buried between the protein molecule and the bound MPEG chains.

As a final consideration it must be noted that an exact characterization of any new biotechnological product (in particular when devised for human therapy) is necessary to obtain approval for marketing.

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