Appl. Biochem. Biotechnol.

0273-2289/91/27(1): 55–63/$2.00

Enzyme Modification by MPEG with an Amino Acid or Peptide as Spacer Arms

LUCIANA SARTORE, PAOLO CALICETI, ODDONE SCHIAVON, AND FRANCESCO M. VERONESE*

Dipartimento di Scienze Farmaceutiche (Centro di Chimica del Farmaco e dei Prodotti Biologicamente Attivi del CNR), Università di Padova, via F. Marzolo 5, 35128 Padova, Italy

Received February 3, 1990; Accepted February 14, 1990

ABSTRACT

A method for the modification of enzymes by MPEG carrying an amino acid or peptide as a spacer arm is described and tested with aliphatic or aromatic side chains amino acids. The procedure involves MPEG activation by *p*-nitrophenylchloroformate for the amino acid or peptide coupling that is in turn activated for the protein binding.

The advantage of the method resides in the possibility to introduce proper reporter groups between the polymer and the protein as norleucine for a direct evaluation of the bound polymer chains, tryptophan for structural studies of the polymer-protein adduct, and radioactive amino acid for pharmacokinetic investigations.

The method was positively tested with arginase, ribonuclease, and superoxide dismutase as enzymes of therapeutic value.

Index Entries: Surface protein modifications; monomethoxypolyethylene glycol; amino acid or peptide derivatized MPEG; enzymes for therapy; arginase; ribonucleases and superoxide dismutase.

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

INTRODUCTION

Polyethylene glycol is finding a rapidly expanding use in biochemical and biomedical applications. It recently has been proposed for derivatization of low mol wt drugs and of high mol wt products, such as enzymes of therapeutic value or polypeptide hormones in order to obtain derivatives with more convenient therapeutic properties. Modification with monomethoxypolyethylene glycol (MPEG) of these bioactive substances, is, in fact, reported to largely change their physical, chemical, enzymological, and immunological as well as their pharmacokinetic and pharmacological properties (*1,2*).

Although several methods for MPEG binding to proteins were so far reported, that are adequately reviewed (*3,4*), a suitable procedure for introducing a spacer arm between a polymer and a protein was missing. Here we are reporting a general method to introduce such a spacer composed of proper amino acid or peptide, since we recognized in these structures several advantages:

1. The spacer may contain an unnatural amino acid, such as norleucine, easily detected by an amino acid analyzer, that can be conveniently employed to quantiate directly the number of polymer chains present in the adduct;
2. Using a commercially available radioactive amino acid, a label may be introduced, that will simplify the detection of MPEG-protein in pharmacokinetic or metabolic experiments and will offer a new possibility to evaluate the number of polymer chains in the adduct;
3. It is possible to study the microenvironment at the water-protein interface in close proximity of the polymer using as a reporter a rare amino acid, such as tryptophan, that possesses distinct UV absorption and strong fluorescence; and
4. It may be finally possible to modulate the release of a bound drug by enzymes which are known to cleave at the level of specific amino acids sequences (*5*).

This paper reports the methodology of MPEG functionalization with different arms, the application of the method for the modification of enzymes and finally a pharmacokinetic study with three enzymes of therapeutic value.

MATERIALS AND METHODS

MPEG 5000 and MPEG 1900 were purchased from Aldrich Chemical Co.; triethylamine, 4-nitrophenyl chloroformate, N,N'-dicyclohexylcarbodiimide, *N*-hydroxysuccinimide, and the amino acid were from Fluka.

Yeast superoxide dismutase (SOD, EC 1.15.1.1) was supplied by Debiopharm (CH); ribonuclease-A from bovine pancreas (EC 2.7.7.16),

and bovine liver arginase (EC3.5.3.1) were purchased from Sigma Chemical Company.

The enzymatic activity of RNase was evaluated using cytidine 2′:3′-phosphate as a substrate according to Crook et al. (*6*). The method for evaluation of SOD enzymatic activity was based on the enzyme inibition for $NADH^+$ reduction detected at 340 nm (Paoletti et al. (*7*)). The extent of protein modification was determined on the basis of unreacted amino groups evaluated according to the trinitrophenylation method (TNBS) of Snyder and Sabocinsky (*8*). The spectrosopic detection of MPEG was performed at 535 nm after iodine reaction (*9*).

Preparation of Activated MPEG with an Amino Acid or Peptide Spacer Arm

To 2 m*M* of MPEG–OH (5000 or 1900 D), dissolved in 50 mL of anhydrous methylene chloride, 4 mM of triethylamine (TEA) and 4 m*M* of 4-nitrophenyl chloroformate were added under stirring while the pH was adjusted at 7.5–8.0 with TEA. After reacting for 4 h at room temperature, the mixture was concentrated under vacuum to about 10 mL and was then dropped into 200 mL of stirred diethyl ether. The precipitate (MPEG–O–CO–O–C_6H_4–NO_2) was collected by filtration and crystallized twice from hot ethyl acetate.

The amino acid or peptide, 20 m*M*, were dissolved in 20 mL of water or acetone-water (1:1), the pH was brought to 8.0–8.3 with NaOH, and 2 m*M* of MPEG–O–CO–O–C_6H_4–NO_2 were added under stirring while the pH was maintained with NaOH. After standing overnight at room temperature, the solution was cooled at 0°C and brought to pH 3 with 2*N* HCl and was then extracted three times with $CHCl_3$. The chloroform was washed with water, dried with Na_2SO_4, concentrated, precipitated with diethyl ether as above reported, and the product, MPEG–O–CO–(AA or peptide)–OH, was recrystallized from ethanol.

MPEG–O–CO–(AA or peptide)–OH, 2m*M*, was dissolved in 50 mL of anhydrous methylene chloride, cooled to 0°C, and 4m*M* or *N*-hydroxysuccinimide and 4 m*M* of N,*N*′-dicyclohexylcarbodiimide were added under stirring. The stirring was continued for 4 h, while the temperature was raised to 20°C. The precipitated dicyclohexylurea was removed from the reaction mixture by filtration and the solution was concentrated under vacuum, whereas the product, MPEG–O–CO–(AA or peptide)–OSu, was precipitated with ether and recrystallized from hot ethyl acetate.

Enzyme Modifications with Amino Acid or Peptide Derivatized MPEG

Superoxide Dismutase Modification

Yeast superoxide dismutase (100 mg) was dissolved in 10 mL of borate buffer 0.2*M*, pH 8, and 650 mg of MPEG 5000–(AA or peptide)–OSu were

added at room temperature under vigorous agitation, whereas the pH was maintained. The mixture was left standing for 30 min.

The excess of polymer was removed by repeated ultrafiltration on a PM 10 Amicon membrane. The concentrated enzyme was chromatographed on a Bio-Gel A 0.5 m column. The elution profile of the column was followed by UV absorption, iodine reaction for MPEG and enzymatic activity. The protein peak fractions were collected and lyophilized after membrane ultrafiltration. The dry modified protein, MPEG 5000-(AA or peptide)-SOD, could be stored at 0°C in a desiccator without activity loss.

The same procedure was followed to obtain MPEG 1900-AA-SOD.

Arginase Modifications

Bovine liver arginase, 20 mg, purified as previously described to give a specific activity of 1900 IU/mg, (*10*) was dissolved in 15 mL of carbonate buffer pH = 8.5, 0.2*M* and 800 mg of MPEG 5000-(Gly or Trp)-OSu were added under vigorous stirring while the pH was maintained by a pH-stat with NaOH 0.1*N* in a microburette. After 30 min, the solution was diluted to 50 mL with water and ultrafiltered at 4°C with an Amicon PM 10 ultrafiltration membrane to reduce the volume to about 5 mL. The modified arginase sample was further purified from excess reagent and by products of the reaction through column chromatography.

Ribonuclease Modification

Ribonuclease A from bovine pancreas was modified and purified as was reported for SOD using for the modification MPEG-(AA or peptide)-OSu at a molar ratio of 2.5/1 calculated on the available amino groups of the enzymes. In the preparation of MPEG-^{3}HGly-RNase, the ^{3}H labelled polymer had a specific radioactivity of 5×10^{-5} mCi/mg.

Pharmacokinetic Evaluation of Unmodified and MPEG-Modified Enzymes

Unmodified yeast superoxide dismutase 5.5 mg or equimolar amount of SOD modified with MPEG 5000-Gly or MPEG 1900-Gly were injected into the tail vein of Wistar albine male rats.

On a scheduled time, the blood was removed by puncturing the heart with a heparinized syringe and SOD evaluated in plasma by enzymatic activity. Before activity evaluation, the enzyme was purified from interferences present in the plasma by CM cellulose and Sephadex G25 column chromatography as recently reported (*11*).

The clearance of arginase was evaluated in the blood by enzyme activity as previously reported (*10*).

The concentration of MPEG-AA modified ribonuclease in the plasma was evaluated by radioactivity measurements using ^{3}H Gly as the amino acid spacer arm.

Table 1
Degree (%) of MPEG 1900 and 5000
Functionalization with Different AA or Peptides

$MPEG_{5000}$-O-CO-O-Ø-NO_2	$MPEG_{5000}$-O-CO-AA-OH		$MPEG_{5000}$-O-CO-AA-OSu
92 a	Gly	82 b	82
	Gly-Gly	80 b	79
	Gly-Ala-Gly	78 b	76
	Phe	80 b 76 c	80
	Trp	75 b 70 c	73
	n-Leu	86 b 82 d	83
$MPEG_{1900}$-O-CO-O-Ø-NO_2	$MPEG_{1900}$-O-CO-AA-OH		$MPEG_{1900}$-O-CO-AA-OSu
95 a	Gly	90 b	90
	Phe	85 b 83 c	84

Evaluated spectrophotometrically by the release of *p*-nitrophenol in alkaline medium (a). The linked amino acid or peptide was evaluated by potentiometric titration (b), by UV spectra (c) or by amino acid analysis (d). The degree of succinimide esterification was evaluated spectrophotometrically by the release of hydroxysuccinimide in alkaline solution.

RESULTS

To bind MPEG with an amino acid or peptide as a spacer arm to a protein, the polymer was first activated by *p*-nitrophenyl chloroformate in an organic solvent. Then the amino acid or peptide was bound to the MPEG in an aqueous medium. Finally, the purified product was activated as succinimidil ester in an organic solvent.

The extent of MPEG 1900 and MPEG 5000 functionalization, at any step and with different amino acids or peptides, is reported in Table 1. The amount of bound amino acids or peptides was evaluated in all cases by potentiometric titration of the amino acids carboxylic group, and the aromatic amino acid were also evaluated by their absorption spectra. In the case of norleucine, the degree of binding was also evaluated by amino acid analysis. The degree of esterification of the amino acid or peptide,

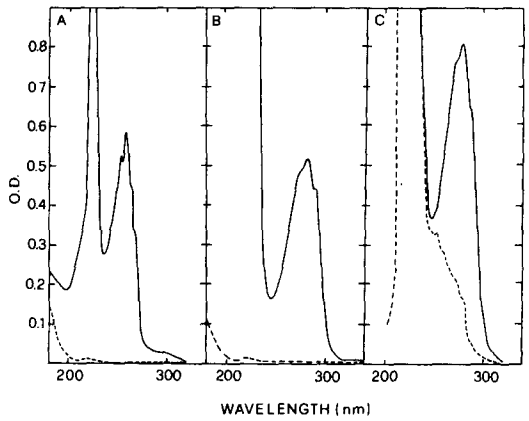

Fig. 1. A. MPEG-Phe-OH (———) and MPEG-OH (– – –); B. MPEG-Trp-OH (———) and MPEG-OH (– – –); C. MPEG-Trp-SOD (———) and MPEG-Gly-SOD (– – –) in phosphate buffer 0.05 M pH=7.

was evaluated spectrophotometrically by the release of hydroxysuccinimide in alkaline solution when an aliphatic amino acid was used. In the case of phenylalanine and tryptophan derivatives, the MPEG-AA and hydroxysuccinimide obtained in the hydrolysis were separated by HPLC before hydroxysuccinimide evaluation. The results show that while a 10% loss occurs in the spacer arm binding to MPEG the final reaction of esterification is nearly quantitative.

In Fig. 1a and 1b, the spectra of MPEG-Phe and MPEG-Trp are respectively reported. No significative variations are observed in the wavelength absorption of both amino acid after MPEG coupling.

Table 2 reports the extent of modification and the residual activity of three enzymes modified using a molar ratio of activated MPEG-(AA or peptide)/-NH_2 protein groups of 2.5/1. Whereas the extent of amino groups modification was calculated by using only the TNBS method, in the case of MPEG-*n*-Leu, it was also calculated by amino acid analysis after acid hydrolysis since for each mole of bound MPEG one mole of *n*-Leu is expected. It is of interest to note that similar degrees of protein modification, evaluated by TNBS method, and enzyme activity loss was observed when the same enzymes were modified in our laboratory by MPEG without the spacer arm (*10,12*).

In Fig. 1c, the spectrum of SOD modified with tryptophan as a spacer arm is reported in comparison with the MPEG-Gly-SOD spectrum.

Figure 2 reports the fluorescence spectra of tryptophan, MPEG-Trp and MPEG-Trp-SOD. A shift to longer wavelengths was observed in the fluorescence emission maximum of tryptophan coupled to the polymer ($\lambda max = 352$ nm for Trp and 354.5 nm for MPEG-Trp). A negligible blue shift in the emission maximum occurs when MPEG-Trp is bound to a protein ($\lambda max = 349.5$ nm). Superoxide dismutase, an enzyme devoid of tryptophan, was an useful model to reveal these effects.

Table 2
Extent (%) of Protein Amino Groups Modification (A)
and Residual Enzymatic Activity (B)

Activated MPEG	RNase		SOD		Arginase	
	A	B	A	B	A	B
PEG 5000-Gly	90	80	85	78	50	95
PEG 1900-Gly	90	70	88	70		
PEG 5000-Phe	94	74	82	75		
PEG 1900-Phe	90	72	84	70		
PEG 5000-Trp			85	80	55	90
PEG 5000-n-Leu			80	85		
PEG 5000-GlyGly	85	80				

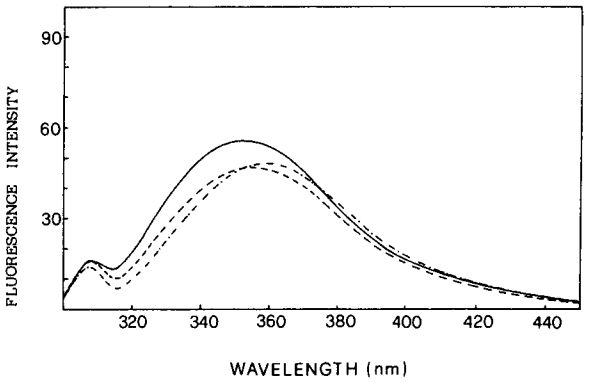

Fig. 2. Uncorrected fluorescence spectra of Trp (———), MPEG-Trp (—·—·—) and MPEG-Trp-SOD (———) in phosphate buffer 0.02 M pH=7.

The pharmacokinetic behavior of native and modified enzymes is reported in Table 3 as the time to reach the 50% of clearance. There are also reported, when available, the values obtained with the same modified proteins without amino acid arm. Whereas the pharmacokinetic behavior of superoxide dismutase and arginase were evaluated in blood by enzymatic activity, the one of ribonuclease was evaluated by radioactivity after modification with MPEG-^{2}HGly. On the other hand, the clearance of unmodified ribonuclease was studied using an ^{3}H labeled sample prepared with a procedure that will be reported elsewhere (*13*).

DISCUSSION

Amino acids or peptides as spacer arm between MPEG and protein, have unique advantage for the possibilities offered by this class of molecules to introduce into the adduct a variety of useful labels, reporter groups,

Table 3
Clearance Time of Native and Modified Enzymes with (A) and without (B) Amino Acid Spacer Arm Administered to Rats by IV

Native and MPEG modified enzymes	T 1/2 Clearance	
	A	B
SOD	6 min.	
MPEG 1900-SOD	15 h	
MPEG 5000-SOD	28 h	25 h
Arginase	1.5 h	
MPEG 5000-Arginase	10 h	8 h
RNase	45 min.	
MPEG 5000-RNase	34 h	

or tracers. These can be conveniently employed in chemical, biological or medical problems connected with the characterization and use of this polymer-protein adducts.

The methodology of synthesis proposed here involves

1. First MPEG activation as *p*-nitrophenylcarbonate to bind the spacer arm; and
2. *N,N'*-dicyclohexylcarbodiimide and *N*-hydroxysuccinimide to activate the spacer.

The interest of this methodology is in the fact that unnatural or potentially toxic coupling reagents are avoided, since a simple C=D group remains between polymer and the next amino acid. Furthermore, the activated MPEG which eventually does not react with the spacer amino acid or peptide arm during the synthesis and that, as known from literature (*14*), is very difficult to purify from the product with conventional methodology, and is decomposed in the aqueous conditions of the reaction. In addition, the aqueous condition of the reaction allows the direct coupling of unprotected amino acids or peptides. With such a strategy of differential activation of MPEG and MPEG spacer arm, the possibility of linking to the protein MPEG with unbound arm is therefore avoided. The eventually unreacted MPEG-OH will be removed from the modified protein in the final step of protein purifications that is performed by ultrafiltration, or better, by column chromatography, together with the excess of MPEG-AA.

MPEG that is transparent by light, and that is usually revealed by an iodine reaction, characterized by low accuracy, may be evaluated spectrophotometrically once an aromatic amino acid is bound. This allows a great simplification in the characterization of the polymer. MPEG-Trp may also be revealed with high sensitivity for its fluorescence.

The use of Phe or Trp as spacer arm has advantage in the protein adduct purification also, since the complete removal of excess polymer from the reaction mixture is easily verified following the amino acid absorption.

Using a protein devoid of tryptophan, yeast superoxide dismutase, it was possible with Trp as spacer arm, to have preliminary informations on the nature of the environment at the polymer-protein interface: the fluorescence emission spectrum of MPEG-Trp-SOD adduct ($\lambda max = 349.5$ nm) was found very close to the one of free tryptophan ($\lambda max = 352$ nm) and of MPEG-Trp ($\lambda max = 354.5$ nm). These values indicate that, in both cases, tryptophan is in an exposed aqueous environment, since blue shifted maxima of emission in the range of 320–330 are observed for completely buried tryptophan residues (*15*).

The protein modification with the MPEG-AA as spacer arm was verified with superoxide dismutase, arginase and ribonuclease; as expected the extent of modification and the effect on the enzyme activity reported on Table 2 were similar to those obtained with already reported methods (*10,12*). On the other hand, the use of *n*-Leu as spacer arm offers the unique possibility of a direct evaluation of the number of polymer chains bound to the enzyme by amino acid analysis after acid hydrolysis. Both protein concentration and number of MPEG chains are obtained in the same analysis. This evaluation was performed on a sample of modified superoxide dismutase and, to our surprise, the extent of modification was significantly lower than that obtained by the colorimetric assay: the chains of bound polymer were found to be 14 with the *n*-Leu method, whereas 18 with the colorimetric one. A systematic investigation with proteins of different structure modified with MPEG-*n*-Leu at different extents is now under way. Previous results showed that large differences were found in several cases and are suggesting a revision of many data on MPEG modification of enzymes reported in literature (Sartore, personal communications). We may suggest that the higher value found by the colorimetric method may reside in the difficulty for TNBS to react with some free amino groups of the polymer modified protein, due to the steric hindrance of the bound polymer chains.

In the case of SOD and arginase modified with MPEG-Gly-OSu, the pharmacokinetic behavior following IV administration to rats was evaluated and compared with that obtained when MPEG was bound to protein without the arm. The very similar results obtained with the two methods (see Table 3) demonstrate the stability "in vivo" of the bounds involved in coupling the spacer arm both to water hydrolysis and towards proteolytic enzymes. Most probably a strong steric hindrance exists at the level of the MPEG-protein bounds. This demonstrates that the use of a radioactive amino acid in the arm could be safely employed in the pharmacokinetic of the MPEG-protein adducts. This method was proved, in fact, quite convenient with ^3H glycine to study the clearance of modified RNase.

The methodology of binding the spacer arm described here may be suitable for application to other -OH terminating polymers. Promising

results are already obtained in the functionalization of polyvinylpyrrolidone ending with single hydroxyl group that was prepared by radical polymerization of *N*-vinylpirrolidone in the presence of mercaptoethanol as chain-transfer agent (*16*).

The data obtained in this paper may also suggest an application of this methodology in the modification of non peptide-drugs carring $-NH_2$ groups since proper amino acid may be introduced to direct the drugs to specific cells or to release them intracellularly when amino acid sequences recognized by lysosomal enzymes are used (*5,17*).

ACKNOWLEDGMENTS

This research was partially supported by Ministero della Pubblica Istruzione.

REFERENCES

1. Abuchowski, A. and Davis, F. F. (1981), in *Enzymes as Drugs*, Holcenberg, J. S. and Roberts, J., ed., Wiley & Sons, pp 367–384.
2. Conforti, A., Franco, L., Milanino, R., Velco, G. P., Boccu, E., Largajolli, R., Schiavon, O. and Veronese, F. M. (1987), *Pharmacol. Research Comunic.* **19,** 287.
3. Veronese, F. M. (1989), *Chimica Oggi* **7,** 53.
4. Yoshinga, K. and Harris, J. M. (1989), *J. Bioactive Compatible Polymers* **4,** 17.
5. Kopecek, J. and Duncan, R. (1987), *J. Controlled Release* **6,** 315.
6. Crook, E. M., Mathias, A. P. and Rabin, B. R. (1960), *J. Biochem.* **74,** 234.
7. Paoletti, F., Aldinucci, D., Mocali, A. and Caparrini, A. (1986), *Anal. Biochem.* **154,** 536.
8. Snyder, S.L. and Sabocinsky, P. Z. (1975), *Anal. Biochem.* **64,** 248.
9. Sims, G. E. C. and Snape, T. J. (1980), *Anal. Biochem.* **107,** 60.
10. Visco, C., Benassi, C. A., Veronese, F. M., and Miglioli, P. A. (1987), *Il Farmaco* **42,** 549.
11. Caliceti, P., Schiavon, O., Mocali, A., and Veronese, F. M. (1989), *Il Farmaco* **44,** 711.
12. Veronese, F.M., Caliceti, P., Pastorino, A., Schiavon, O., Sartore, L., Banci, L., and Monsu Scolaro, L. (1989), *J. Controlled Release* **10,** 145.
13. Schiavon, O. et al. in preparastion.
14. Joppich, M., and Luisi, P. L. (1979), *Makromol. Chem.* **180,** 1381.
15. Brand, L. and Whithold, B. (1967), *Methods Enzymol.* **11,** 776.
16. Veronese, F. M., Sartore, L., Caliceti, P., Schiavon, O., Ranucci, E., and Ferruti, P. (1989), *J. Bioactive Compatible Polymers* (in press).
17. Hemant, N. J. (1988), *Pharmaceutical Technology* **12,** 118.