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Surface Modification of Proteins by Covalent Binding of Acrylic Polymers

FRANCESCO M. VERONESE,* ROBERTA LARGAJOLLI, CARLO VISCO, PAOLO FERRUTI,† AND ADELAIDE MIUCCI‡

Department of Pharmaceutical Sciences (Centro di Studi per la Chimica del Farmaco e dei Prodotti Biologicamente Attivi del CNR), University of Padua, Padua, Italy

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ABSTRACT

Surface modification of enzymes for a potential use in therapy was obtained with a new type of tailor-made copolymers of *N*-acryloylmorpholine and *N*-acryloxysuccinimide. The first monomer was designed to confer solubility on the polymer, whereas the second was used to give it reactivity toward protein amino groups. The reactivity of polymers of different composition towards amino acid derivatives and model proteins, such as catalase and ribonuclease-A, is described. Water soluble and catalytically active enzyme derivatives were obained using copolymers prepared with a mixture of *N*-acryloxysuccinimide and *n*-acryloylmorpholine in a 1:99 molar ratio. At increasing molar ratio (3:97, 10:90) extensive crosslinking between polymer and enzymes takes place, yielding insoluble adducts.

Index Entries: Protein surface modification, by acryloylmorpholine–acryloxysuccinimide copolymers; ribonuclease-A, and catalase modification by soluble polyacrylic polymers; modification, of therapeutically useful enzymes by polyacrylic polymers; surface modification, of proteins by acrylic polymers; acrylic polymers.

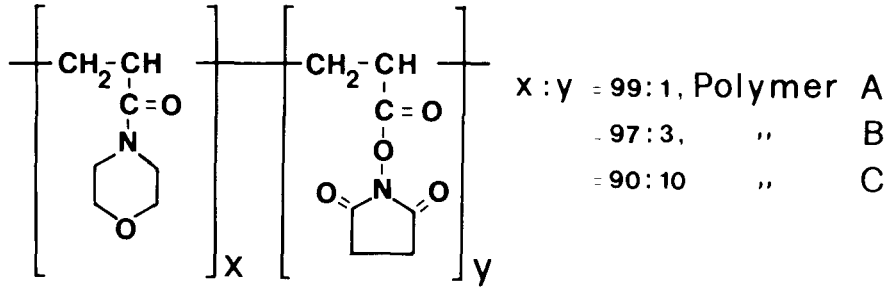
*Author to whom all correspondence and reprint requests should be addressed.
†Faculty of Engineering, University of Brescia, Brescia, Italy.
‡Department of Industrial Chemistry, Chemical Engineering-Polytechnic of Milan, Milan, Italy.

INTRODUCTION

The covalent binding of soluble polymers to the surface of enzymes used in therapy appears to be a quite interesting and useful procedure for improving their pharmacological properties. The modified enzymes often show greater stability, longer clearance time, and reduced immunogenicity than the native enzymes (*1*). Among the polymers so far studied for this purpose, polyvinylpyrrolidones (*2*), dextrans (*3*), and polyethylene glycols (PEG) (*4*) are most often employed. In particular, PEG has been used quite extensively, since this polymer is easily available, is biocompatible, and can be activated easily in order to react with protein functional groups. Thus, several PEG-derivatives of enzymes with improved pharmacological propeties have been already obtained (*5–7*).

The procedures so far described in the literature suffer from the limitation that they imply functionalization of presynthesized polymers. this allows little opportunity to obtain new polymers with tailor-made properties. In addition to reactive groups for protein binding, the inclusion of acidic, basic, hydrophilic, or lipophilic functional groups might well give new biological properties to the modified enzymes. In theory, this goal can be achieved by using copolymers prepared from different monomers, including monomers containing reactive functionalities, by analogy to those copolymers already used for obtaining polymeric derivatives of drugs (*8*).

In this study, we wish to present the results of enzyme modification obtained with copolymers prepared from *N*-acryloylmorpholine and *N*-acryloxysuccinimide in different ratios. These copolymers (Scheme 1) contain the morpholine unit as a water solubilizing functional group and the hydroxysuccinimide ester unit as a reactive function for protein binding, since this active ester reacts with protein amino groups leading to amide bonds between the copolymer and the enzyme. The reactivity of these copolymers with model amino acid derivatives and enzymes of potential therapeutic application has been investigated. A monomeric en-



Scheme 1

zyme, ribonuclease-A (RNase), that has already been proposed as an antiviral and anticancer drug (*9*), and an oligomeric one, catalase, that is a possible agent for treating acatalasemic patients (*10*), have been employed in this study.

MATERIALS AND METHODS

Polymers

N-Acryloxysuccinimide (*11*) and *N*-acryloylmorpholine (*12*) were prepared as described elsewhere (*11*). The copolymerizations were performed at 60°C under a nitrogen atmosphere. A typical procedure was to dissolve the two monomers, in the appropriate ratio, in anhydrous dioxane–anhydrous alcohol-free chloroform (1:1 v/v). The concentration of the sum of the monomers was 25% (w/v). Freshly recrystallized azodiisobutyrronitrile was then added (1% by weight of the monomers) and the reaction mixture carefully purged with nitrogen and maintained at 60 ± 0.5°C for 24 h in a thermostatic bath. The reaction mixtures were then diluted using 20 vol dry ether, and the precipitated polymers were collected, extracted with fresh portions of dry ether, and dried at room temperature and 0.1 torr.

Three polymers were prepared by polymerization of *n*-acryloylmorpholine and *N*-acryloxysuccinimide in the ratio of 99:1, 97:3, and 90:20, for the polymer-A, -B, and -C, respectively. The intrinsic viscosities of the polymers (in chloroform at 30°C) ranged from 0.65 to 0.85 dL/g. Their average molecular weight, measured by osmometry, was in the order of 30,000 daltons. Yields were almost quantitative. Elemental analyses (C, H, N) were in close agreement with theory.

Amino Group Titration

The amino groups were titrated by trinitrophenylation using 2,4,6-trinitrobenzenesulfonate in 0.1*M* borate buffer, pH 9.3, and gave spectrophotometric readings at 420 nm (*13*).

Enzymatic Assays

Bovine pancreatic ribonuclease-A activity was evaluated by the hydrolysis of cytidine-2′,3′-cycle phosphate following the increase in absorbance at 258 nm in 0.1*M*, Tris–acetate buffer, pH7. The activity was also evaluated on the basis of the decrease in absorbance at 300 nm of a ribonucleic acid solution (0.1%) in 0.1*M* Tris–acetate buffer, pH 7.0. Catalase activity was evaluated through the decrease in absorbance at 240 nm of a 0.5% H_2O_2 solution in 0.05*M* phosphate buffer, pH 7.

Gel Filtration of Modified Enzymes

Fractionation of native and of polymer-bound enzymes was performed on a Bio Gel-A 0.5*M* column (1.5 × 30 cm). Calibration of the column was obtained by using protein of known molecular weight. The effluent was assayed by enzymatic activity and by spectrophotometric readings at 280 nm for protein content and at 250 nm for polymer content.

Modification of Amino Acid Derivatives and Proteins by Polymers

Amino acids or their derivatives (1.5–4 mg/mL) were disolved in 0.2*M*, pH 7.8 phosphate or 0.2*M*, pH 8.6 or 9.5 borate buffer and the polymer, usually in a fivefold molar excess calculated on the active ester group over the amino group, was added after being dissolved in the same buffer. The pH was eventually maintained with 0.1*N* NaOH in a pH state. The solutions were kept at room temperature while small aliquots were withdrawn from the reaction mixture for the evaluation of amino groups.

Proteins (5 mg/mL) were reacted in a similar way (the molar excess of reagent was calculated on the basis of the protein free amino groups) and both the decrease in free amino groups and enzymatic activity were evaluated at intervals. Before application to the column of the modified protein, the reactive groups of the polymer were inactivated by adding glycine in a fivefold molar excess over polymer-bound reactive units and leaving the mixture to stand for 30 min.

RESULTS

Following the experimental conditions described in the Experimental section, *N*-acrylolymorpholine–*N*-acryloxysuccinimide copolymers with number-average molecular weights of about 30,000 daltons were obtained.

Since the yields of reaction were almost quantitative, and the two monomers are both of the acrylic type, it is reasonable to presume that the overall composition of the polymers corresponds to that of monomeric mixture. The amount of active *N*-acryloxysuccinimide units with polymers thus prepared can be estimated, on average, as two for polymer-A, six for polymer-B, and 20 for polymer-C.

Figure 1A shows the time course of the reaction of polymer-A with the model peptide glycylglycine at different pHs. The reaction has been carried out in buffered solution at the indicated molar excess of reactive ester with respect to amino groups. The rate of reaction was determined following the decrease in free amino groups using the colorimetric trinitrophenylation reaction *(13)*. Since the reaction involves acylation of the α-amino group of the dipeptide, a higher rate of reaction is observed

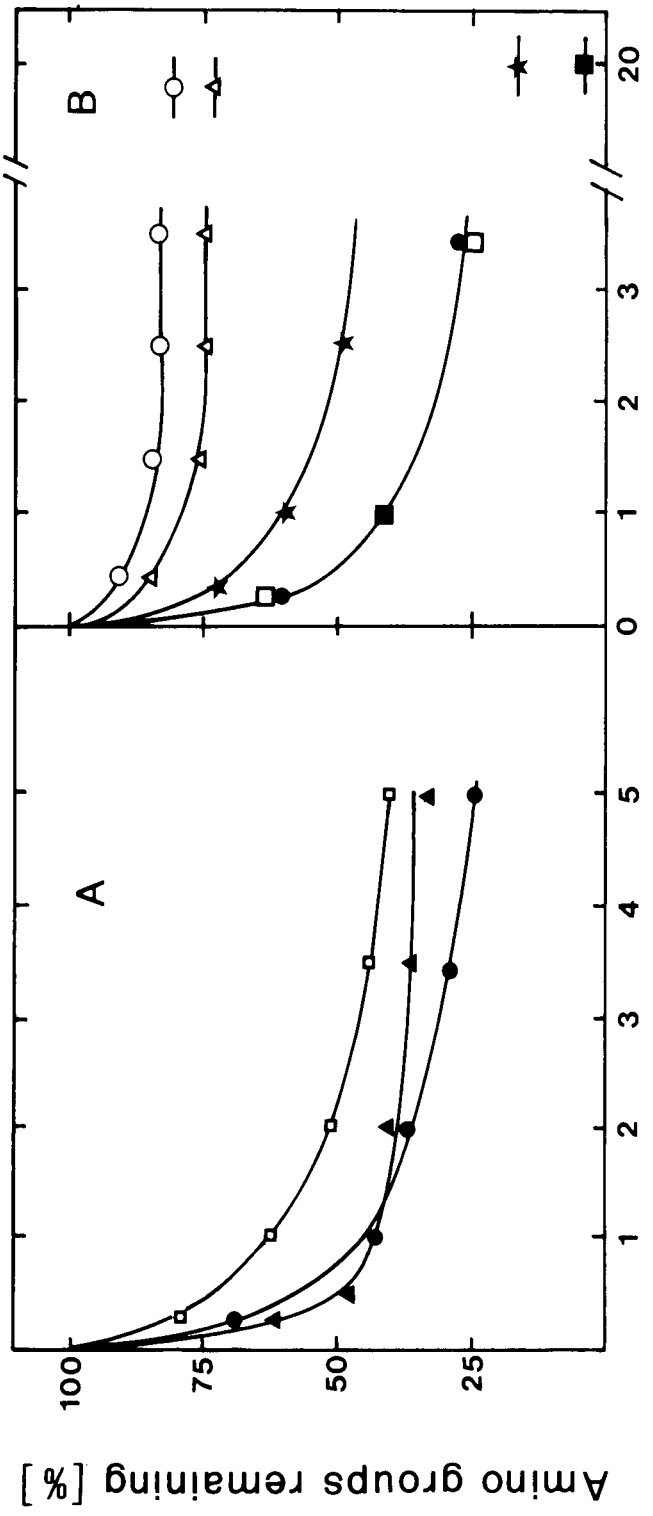


Fig. 1A. Rate of glycylglycine modification by polymer-A at pH 7.8, 0.2*M* phosphate buffer (□) or pH 8.6 (●), and 9.5 (▲) 0.2*M* borate buffer. The ratio of active ester groups in the polymer to α-amino groups in the dipeptide was 5. 1B. Rate of amino group modification at pH 8.6, 0.2*M* borate buffer of glycine (●), glycylglycine (□), α-carbobenzoxy-lysine (*), RNase (○), and catalase (△).

at mildly alkaline pH. However, at pH 9.5, the reaction is initially fast and then is rapidly slowed down; this is probably related to a concomitant hydrolysis of the active ester.

Figure 1B shows the rate of reaction, observed at pH 8.6, of polymer-A with glycine, glycylglycine, α-carbobenzoxy-lysine, RNase, and catalase. It is seen that α-carbobenzoxy-lysine reacts at a lower rate

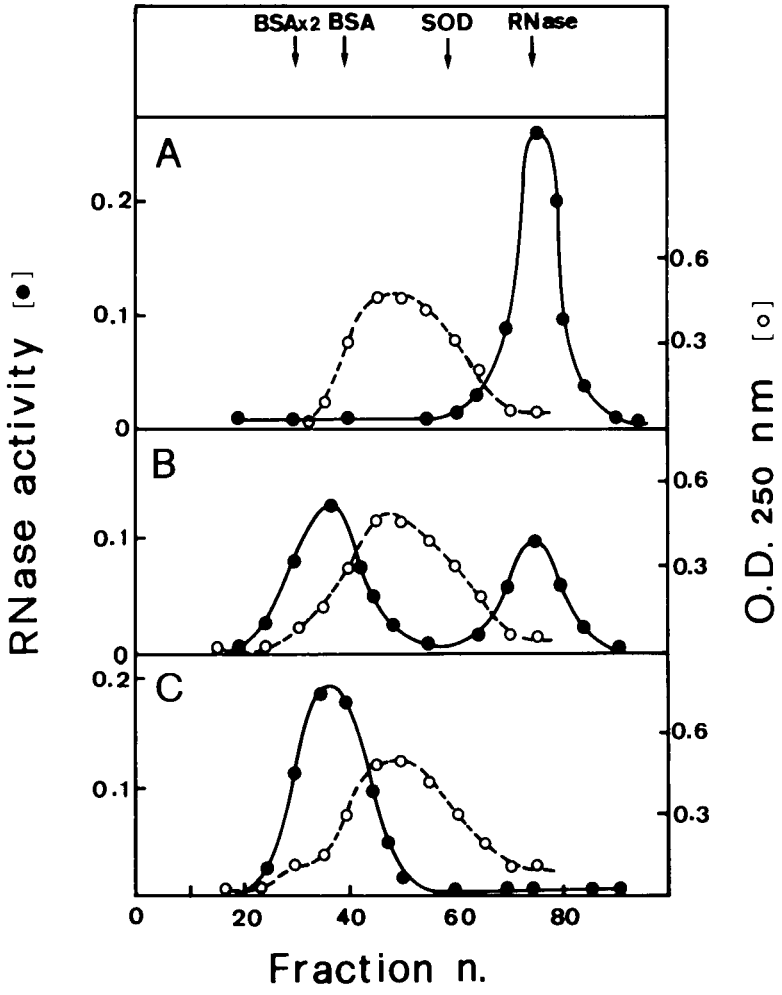


Fig. 2. Elution profile of unmodified RNase and following reaction with polymer-A at different reaction times evaluated by enzymatic activity toward cytidine-2′,3′-cyclic phosphate (●). The elution volumes of standard proteins: bovine pancreas RNase, 13,700; human erythrocyte superoxide dismutase, 32,000; bovine serum albumin, 66,200 and its dimer, 132,400, are indicated. The elution of polymer-A is evaluated by its absorbance at 250 nm (○). A, The elution of RNase incubated with polymer-A previously deactivated by the reaction between its active esters and excess glycine. B, The elution of RNase reacted for 10 min with polymer-A. C, The elution of RNase after 30 min reaction with polymer-A. The reaction was at pH 9.5.

than glycine or glycylglycine. This can be explained by the higher pK_a of the ϵ-amino group of lysine and thus by its lower nucleophilicity at the pH of the reaction. On the other hand, ϵ-amino groups of the proteins investigated react at a lower extent (only 19% of the available RNase amino groups and 25% of the catalase amino groups). This is likely to be caused by the steric hindrance of the bulky polymer and protein.

The separation of unreacted RNase from the polymer-modified RNase has been achieved by gel filtration chromatography (Fig. 2). As expected, the polymer-modified RNase is eluted earlier from the column than the native enzyme (Fig. 2B and C). In addition the chromatogram shown in Fig. 2A indicates that there is no aggregation between RNase and polymer-A reacted with excess glycine in order to quench the active ester.

At pH 9.5, the enzyme rapidly reacts with polymer-A, since after 10 min only 60% of the enzyme is modified, whereas after 30 min the modification is complete.

The modified enzyme is eluted from the gel filtration column in a peak broader than that expected for the elution of a single protein species. This suggests that the derivatized protein has some degree of heterogeneity.

The influence of pH on the rate of RNase modification by polymer-A was studied after separation and evaluation of the modified and unmodified species by gel filtration. The results reported in Table 1 indicate that, by analogy to the behavior of glycylglycine (Fig. 1), more effective binding of polymer to RNase is achieved at a more alkaline pH.

Polymer-modified RNase and catalase maintain essentially full enzymatic activity, as assessed for RNase with ribonucleic acid as well as with a low molecular weight substrate, cytidine-2′3′-cyclic phosphate and for catalase with H_2O_2.

TABLE 1
Ribonuclease Modification by Polyacrylic Polymer-A[a]

Reaction Conditions		Unmodified RNase,	Polymer-bound
pH	Time	%	RNase, %
7.8	30 min	88	12
	3 h	61	39
	7 h	55	45
8.6	30 min	62	38
	3 h	35	65
	7 h	25	75
9.5	10 min	40	60
	30 min	0	100

[a]After the scheduled reaction time, the unreacted polymer-A was inactivated by excess glycine before application to the column. The ratio of unmodified to modified enzyme was obtained from the area of the activity peaks after separation by gel filtration, as reported in Fig. 2.

Polymers-B and -C were found to modify amino groups of glycine and α-carbobenzoxy-lysine essentially at the same rate as polymer-A. In the case of RNase and catalase, molecules possessing several reactive amino groups per mole, extensive crosslinking occurred, as was verified by the precipitation of protein–polymer conjugate in the form of a gel. In these two cases, in order to evaluate the degree of modification by trinitrophenylation, modified proteins were first solubilized in guanidine hydrochloride solution and then reacted with 2,4,6-trinitrobenzenesulfonate (*see* Experimental). The immobilized RNase and catalase, obtained after a 1-h reaction at pH 8.6 with polymer-B and -C, were still enzymatically active, as verified after removal of the unbound enzyme by filtration of the gel with a Millipore membrane.

DISCUSSION

Polymer-A, composed of *N*-acryloylmorpholine–*N*-acryloxysuccinimide in a molar ratio of 99 to 1, with an approximate molecular weight of 30,000, reacts with amino groups of model peptides at mildly alkaline pH. The rate depends on the nucleophilicity of the amine up to pH 9.5, where the decomposition of the active ester becomes a competitive reaction. Also with the model proteins, RNase and catalase, the polymer reacts at a pH-dependent rate. In this case, however, only a limited amount of the available amino groups are modified, most probably for the steric hindrance of both reactants. Soluble polymer–protein adducts are thus obtained without noncovalent aggregation.

The polymer-derivatized proteins completely maintain their catalytic activity towards H_2O_2 in the case of catalase, and both towards the small molecular weight substrate cytidine-2′,3′-phosphate cyclic as well as toward ribonucleic acid for RNase. This is of interest since RNase modified with a different polymer, monomethoxypolyethylene glycol activated with phenylchloroformate (*14*), rapidly loses its activity towards the macromolecular substrate. This difference can be explained either by the different properties of the polymers or by the lower accessibility of the more hindered polymer-A to the cleft of the RNase active site.

The results obtained with proteins described here open the way to the use of this new class of soluble polymers, with tailor-made properties, for the surface modification of proteins, complementing the use of presynthesized polymers already reported in literature. The acrylic polymer here described is similar to those already used for preparing polymeric derivatives of drugs (*8*), which, on the contrary, contain a high proportion of binding sites in order to ensure a reasonably high loading of drug molecules. These last polymers are not suitable as such for protein modification, since the presence of several reactive groups in both the polymer and the protein would result in extensive crosslinking. This occurred in the modification of RNase and catalase with polymer-B and,

to a greater extent, with polymer-C, which have six and 20 active units, respectively, per polymer chain and gave insoluble, although still enzymatically active, protein derivatives. This enzyme crosslinking effect appears to be quite promising as a new strategy for enzyme immobilization and needs further investigation.

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