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Conjugation of Proteins with Chelating Polymers Via Water-Soluble Carbodiimide and *N*-Hydroxysulfosuccinimide

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ABSTRACT

Water-soluble chelating polymers (CP) based on polylysine and diethylenetriaminepentaacetic acid (DTPA) have been prepared. The effect of the number of DTPA-groups in the polymer chain on the process of CP carbodiimide-mediated coupling to proteins has been studied. CP obtained were conjugated with proteins via carbodiimide and *N*-hydroxysulfosuccinimide (HSSI). The optimal conditions of CP activation were determined using model low-molecular-weight amine. It was shown that the addition of HSSI to an activation mixture increases the coupling efficiency of CP with immunoglobulins by 3–4-fold compared with carbodiimide alone. Possible mechanisms of this phenomenon are discussed.

Index Entries: Polymer carriers, water-soluble; polymers, chelating; immobilization, chemical modification of proteins; antibodies, monoclonal, ^{111}In, immunodiagnostics, carbodiimide, *N*-hydroxysulfosuccinimide.

ABBREVIATIONS

CP, chelating polymer
DTPA, diethylenetriaminepentaacetic acid
caDTPA, cyclic anhydride of DTPA
HSSI, *N*-hydroxusulfosuccinimide
EDC, 1-ethyl-3(3′-dimethylaminopropyl)-carbodiimide

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TME, tryptophan methyl ester
TNBS, trinitrobenzene sulfonic acid
CT, α-chymotrypsin
PL, polylysine
ATEE, *N*-acetyl-L-tyrosine ethyl ester.

INTRODUCTION

For the further development of radioimmunodiagnostics, radioimmunotherapy, and NMR-tomography, it is of great importance to sufficiently increase the number of heavy metal ions bound with an antibody molecule in order to increase the antibody labeling capacity (*1–3*). The modification of monoclonal antibodies with long-chain polymers containing chelating groups in their side-chains has been proposed to solve the problem (*4,5*). This approach might allow 50–100 metal atoms to bind with a single antibody molecule without noticeable loss of its antigen-binding activity. Polymers conjugated with antibodies against components of tumors, thrombi, and necrotic tissues may allow the amount of the antibody preparation required for radioimmunoimaging or radioimmunotherapy to decrease and the concentration of paramagnetic metal label in the target to increase, which is especially important for developing immunocontrasts in NMR-tomography.

Recently several reports have appeared devoted to the synthesis of such conjugates (*4–6*). Polylysine (PL) with molecular weight of 6000–14,000 is usually used as a polymer matrix. PL can be modified with cyclic anhydride of DTPA (caDTPA), as proposed by Hnatovich et al. for proteins (*7*). Free aminogroups of PL are then modified with heterobifunctional reagent SPDP (*N*-succinimidyl-3-(2-pyridildithio)propionate) either for introduction of SH-groups in the polymer chain, or for the conjugation with proteins via PDP-groups of the modified polymer and SH-groups of protein. In such an approach, however, particular attention has to be paid to the possible crosslinking of macromolecules containing SH-groups.

In our previous study (*1*), we proposed the alternative scheme for the conjugation of antibodies with PL-DTPA. Polymer is first activated with water-soluble carbodiimide-1-ethyl-3(3′-dimethylaminopropyl)carbodiimide (EDC), and then reacted with the antibody. As far as free aminogroups in this polymer are succinylated, polymer molecules do not crosslink with each other.

It is desirable to increase the load of the protein with heavy metals, and, consequently, to couple the highest possible number of DTPA-groups with protein via the modified polymer. However, preliminary experiments indicated that polymers with a high content of DTPA-groups demonstrate decreased ability to couple to proteins. Therefore, two questions were

addressed in this study-- (1) What is the influence of the DTPA content of the polymer on the coupling of carbodiimide-activated PL-DTPA-Suc with protein primary aminogroups? and (2) Is it possible to increase the coupling effficiency of primary aminogroups with activated polymer using *N*-hydroxysulfosuccinimide (HSSI)?

MATERIALS AND METHODS

Chemicals

The following chemicals were used: Poly-L-lysine hydrobromide (MW 14000, 18000), EDC, DTPA, succinic anhydride, *N*-acetyl-L-tyrosine ethyl ester (ATEE) and trinitrobenzenesulfonic acid (TNBS) from Sigma Chemical Co., St. Louis, MO; HSSI from Fluka Chemical Co., Switzerland; tryptophan methyl ester (TME) from Reanal, Hungary; $^{59}FeCl_3$ from Amersham, UK; $^{111}InCl_3$ from V/O Isotop. Distilled deionized water (Milli-Q, Millipore, USA) was used. Organic solvents and components of buffer solutions were supplied by Merck, FRG. caDTPA was prepared according to (*7*).

Proteins

α-chymotrypsin (CT;EC 3.4.21.1) (Koch-Light, UK) was used as a model protein. Monoclonal antibodies (IgG class) designated 9B9 against angiotensin-converting enzyme were kindly provided by S. M. Danilov, USSR Cardiology Research Center.

Preparation and Properties of Chelating Polymers

To prepare CP, the solution of caDTPA in dimethyl sulfoxide was added upon mixing to PL solution (2.5 mg/mL) in 0.1 *M* carbonate buffer, pH 8.0. In 0.5–1 h dry succinic anhydride was added with stirring. pH was continuously adjusted to 8.0 using concentrated NaOH solution. Substitution degree of PL-aminogroups with DTPA was determined following the spectrophotometric titration with TNBS of the polymer prior to addition of succinic anhydride (*8*). The reaction was performed in the presence of 1 *M* NaCl for the elimination of electrostatic effects of the polymer chain.

PL-DTPA-Suc samples were extensively dialyzed against distilled water and lyophilized. If necessary, polymers were labeled with a trace amount of ^{59}Fe or ^{111}In in 0.02 *M* citrate buffer, pH 6.0. Polymer molecular weight was determined by gel-filtration on Sephadex G-100 S (Pharmacia Fine Chemicals, Uppsala, Sweden). For the determination of the polymer heavy metal binding capacity the polymers were labeled with trace amount of ^{59}Fe pre-mixed with the known excess of cold Fe^{3+}. The radio-

activity of individual fractions after column gel chromatography was determined in the gamma counter (Compugamma, LKB, Sweden).

Coupling reaction of CP with the model low-molecular-weight amine (TME) was performed in two steps (*9*)— (1) an appropriate polymer was activated with EDC or with the mixture of EDC and HSSI in water, pH 4.0, for the specified period of time (1–20 min); and (2) a tenfold excess of activated polymer was added to TME solution in 0.05 *M* borate buffer, pH 8.5. The amount of primary amino groups acylated by the polymer carboxyls was determined using TNBS spectrophotometric titration 1.5-2 h after coupling was started.

The activation of polymer in the presence of EDC was monitored as EDC utilization measurement by the optical density decrease of the polymer/water/EDC mixtures (PL concentration—0.25 mg/mL, EDC—1.5 mg/mL) at 247 nm (*9*).

Polymer-Protein Conjugation

To conjugate polymer with protein (CT or monoclonal antibody) PL-DTPA-Suc was activated during the optimal time, previously determined with TME (1 min for EDC-coupling, 5–10 min for EDC/HSSI coupling). The amount of the polymer bound with protein was determined by gel filtration of the reaction mixture obtained after labeling procedure on Sephadex G-100 column (*see* Figs. 6 and 7 for details).

The enzymatic activity of CT was determined in 0.1 *M* KCl, pH 8.0, following the initial rates of ATEE hydrolysis in the Radiometer RTS 822 pH-stat (Radiometer, Denmark) (*10*). pH-optimum of CT activity in conjugates was found to be shifted to the alkaline region as compared with the native CT (results not shown). This shift was taken into account when the inactivation degree of CT after conjugation was determined. ATEE concentration in the pH-stat cell was 10 m*M*.

RESULTS AND DISCUSSION

Synthesis and Properties of Chelating Polymers

We have prepared 5 samples of PL-DTPA-Suc with different modification degrees and molecular weights (*see* Fig. 1 and Table 1). Percent of DTPA-substituted aminogroups calculated from TNBS titration experiments and by the direct determination of DTPA groups according to Fe^{3+} binding differed from each other. This discrepancy is probably connected with the partial inaccessibility of the polymer aminogroups for TNBS.

As caDTPA may act as a crosslinking agent during the polymer modification, increase of caDTPA maximal concentration results in the intercoupling of polymer molecules and raising of the molecular weight values of the polymers prepared (Table 2). Broadening of the polymer molecular

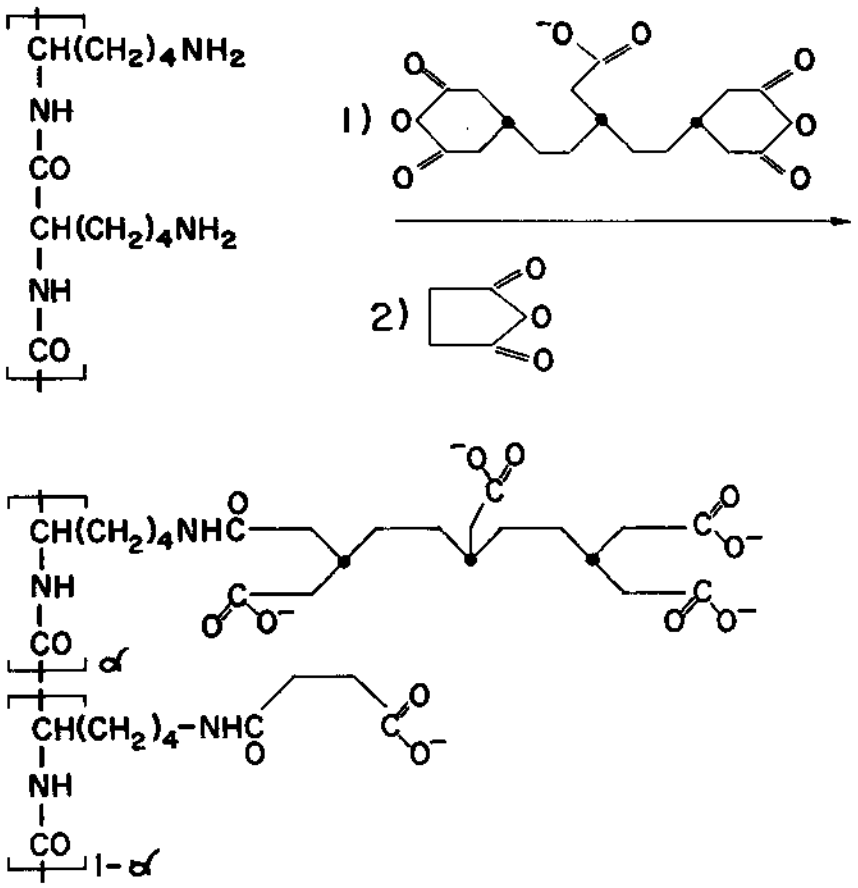

Fig. 1. The scheme of PL-DTPA-Suc synthesis.

Table 1
Synthesized Samples PL-DTPA-Suc

Polymer designation	Modification degree		Molecular weight
	TNBS analysis	Fe^{3+} binding	
PL-DTPA$_{24}$-Suc	0.24	0.16	50000
PL-DTPA$_{53}$-Suc	0.53	not determined	50000
PL-DTPA$_{75}$-Suc	0.75	0.31	50000
PL-DTPA$_{92}$-Suc	0.92	not determined	50000
PL-DTPA$_{36}$-Suc	0.36	not determined	25000

Table 2
The Effect of caDTPA Concentration During PL Modification on the Molecular Weight of PL-DTPA-Suc

Polymer	Starting polymer concentration, mg/mL	Maximal caDTPA concentration in PL solution, mg/mL	Molecular weight of starting PL	Molecular weight of PL-DTPA-Suc
PL-DTPA$_{24}$-Suc	2.5	1	14000	50000
PL-DTPA$_{53}$-Suc	2.5	2	14000	50000
PL-DTPA$_{36}$-Suc	2.5	0.1	18000	25000

weight distribution is also observed (data not shown). These undesirable effects can be diminished if PL concentration in the reaction mixture is low and the bifunctional chelate is added to PL solution in small aliquots with vigorous stirring.

Activation of Chelating Polymers with Carbodiimide and Binding of Tryptophan Methyl Ester

To develop a reliable EDC-mediated polymer coupling technique to aminogroups of proteins, model experiments were performed with tryptophan methyl ester (TME) as the low-molecular-weight model amine. TME was chosen because of its high solubility and absence of carboxyls and other functional groups that could cause side reactions with carbodiimide.

Coupling was performed according to the two-step procedure (*9*) (*see* Methods). Briefly, the polymer possessing carboxy groups, which contained no primary aminogroups, was activated at mild acidic pH with EDC. After a specified period of time, amine was added in the borate buffer and coupling (aminogroup acylation) step performed at pH 8.5. This scheme allows one to perform both steps at optimal conditions and to avoid undesirable crosslinking of the protein molecules.

Presence of the increasing number of DTPA residues on the polymer backbone results in the decrease of TME binding to the polymer (*see* Fig. 2). The optimal time of activation of the polymer with EDC is also decreased when PL-DTPA-Suc is used for coupling instead of PL-Suc.

It is important to compare these results with the rate of EDC utilization during the polymer activation with EDC (*see* Fig. 3). It may be seen that these rates decrease as a modification degree of PL with DTPA groups increase; it is also seen that the activation rate of PL-Suc is even less than for PL-DTPA$_{53}$-Suc. Consequently, the greater rate of O-acylisourea formation in case of PL-Suc reaction with EDC is not sufficient for the explanation of the Fig. 2 data. One may suppose that O-acylisourea of PL-DTPA-Suc carboxy groups is more susceptible to side reactions than in case of PL-Suc carboxyls. These side reactions may take place both at the first step of the coupling at acidic pH, and at the second step, result-

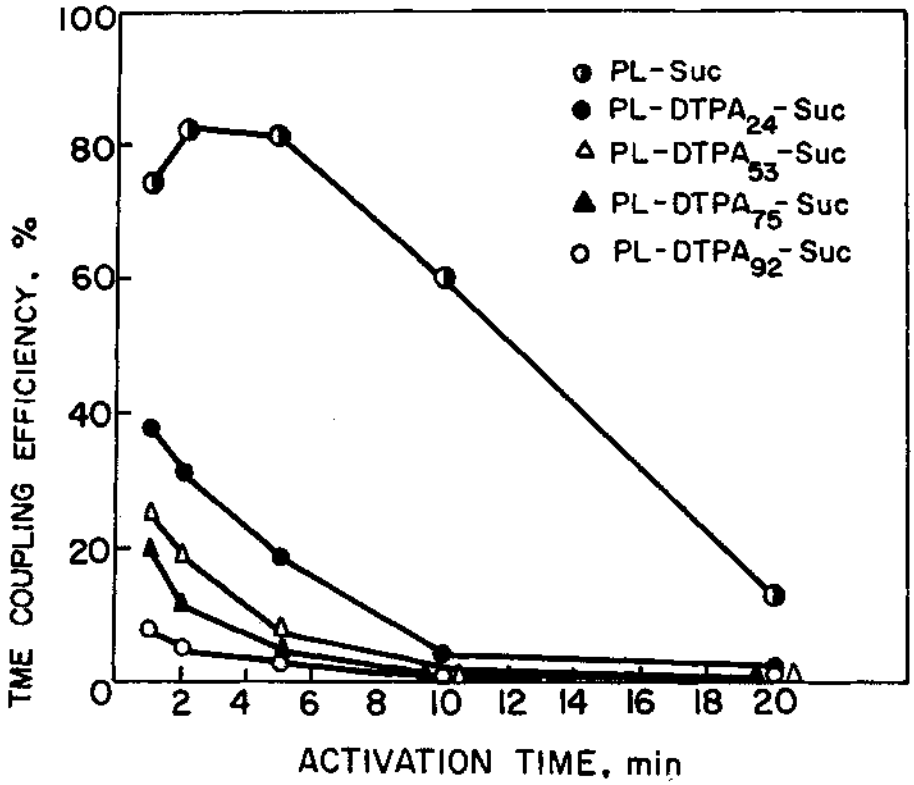

Fig. 2. The effect of the time of polymer activation with EDC on the coupling efficiency of TME with PL-Suc or PL-DTPA-Suc with different modification degrees (md; DTPA substitution degree). (Polymer) = 1.5 mg/mL; [EDC] = 1.0 mg/mL.

ing in decrease of TME aminogroups acylation yield. This assumption is confirmed by the shift of the optimal time of activation with EDC, which is equal to 5–10 min for PL-Suc compared to 1 min in the case of PL-DTPA-Suc. Optimal activation time corresponds to O-acylisourea maximal concentration and, consequently, to the maximal efficiency of the polymer coupling to TME aminogroups.

Increase of TME Coupling Efficiency with Chelating Polymers, Activated by EDC in the Presence of HSSI

Recently it was shown that the addition of HSSI to the carboxylic compounds in the presence of EDC results in the formation of hydrophilic *N*-hydroxysulfosuccinimide esters (*11*). These esters are known to interact effectively with aminogroups; at the same time they are more resistant to hydrolysis compared to corresponding O-acylisourea. Staros et al. have shown that application of HSSI in the coupling of glycine to hemocyanine via EDC resulted in 15-fold increase of glycine coupling yield (*11*).

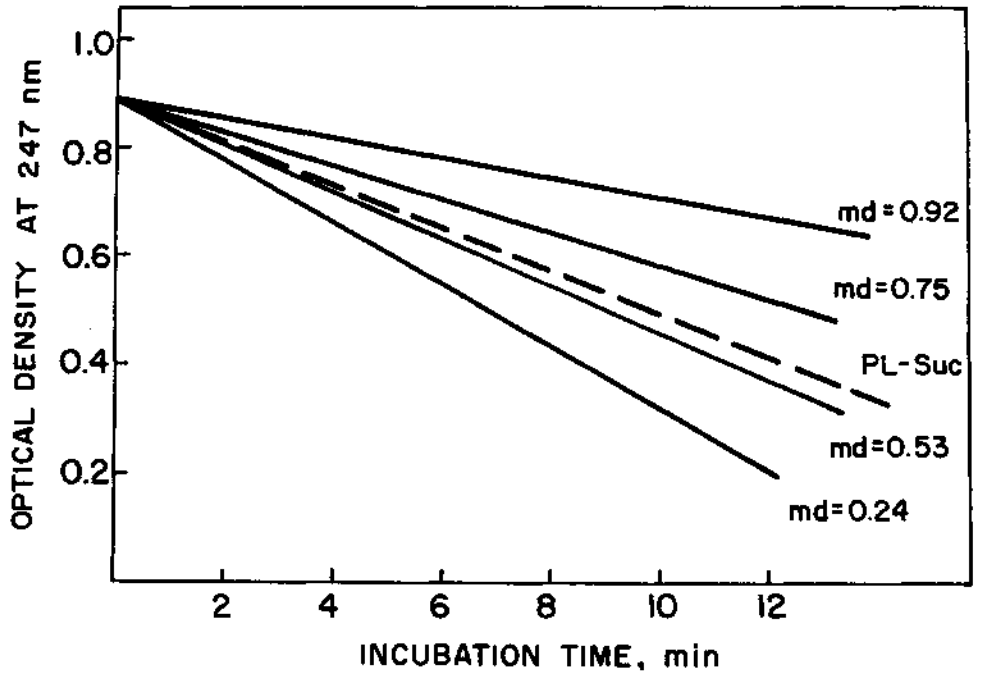

Fig. 3. The time course of optical density decrease at 247 nm after EDC addition to water solution of PL-Suc or PL-DTPA-Suc with different modification degrees (md). (Polymer) = 0.25 mg/mL; (EDC) = 1.0 mg/mL.

In the procedure proposed in (*11*), however, all the compounds (EDC, HSSI, protein, and carboxyl-containing modificator) were present in the reaction mixture simultaneously. Application of this approach to our system may result in the formation of significant amounts of side products, for example, crosslinked protein molecules. Therefore, we used the two-step scheme of carbodiimide coupling (*9*) (*vide supra*) with the addition of HSSI at the first step (during activation of COOH-containing polymer with carbodiimide).

Addition of HSSI to the activation mixture resulted in an increase of the coupling yield of low-molecular-weight amine (TME) with PL-DTPA-Suc. Increase of the optimum activation time is also observed. Two curves that illustrate the time dependence of TME coupling efficiency with PL-$DTPA_{36}$-Suc, activated either with EDC alone, or with the mixture of EDC and HSSI, are presented in Fig. 4. Shape of the upper curve in Fig. 4 (HSSI/EDC activation of PL-DTPA-Suc) is similar with that of the upper curve in Fig. 2 (EDC activation of PL-Suc). Maximal yield of TME-aminogroups acylation with HSSI/EDC activated polymer is equal to 90%. This value is approximately threefold higher than the corresponding value for polymer activated with EDC alone. The optimal time of PL-DTPA-Suc activation is 5–10 min as compared to 1 min for traditional EDC activation route.

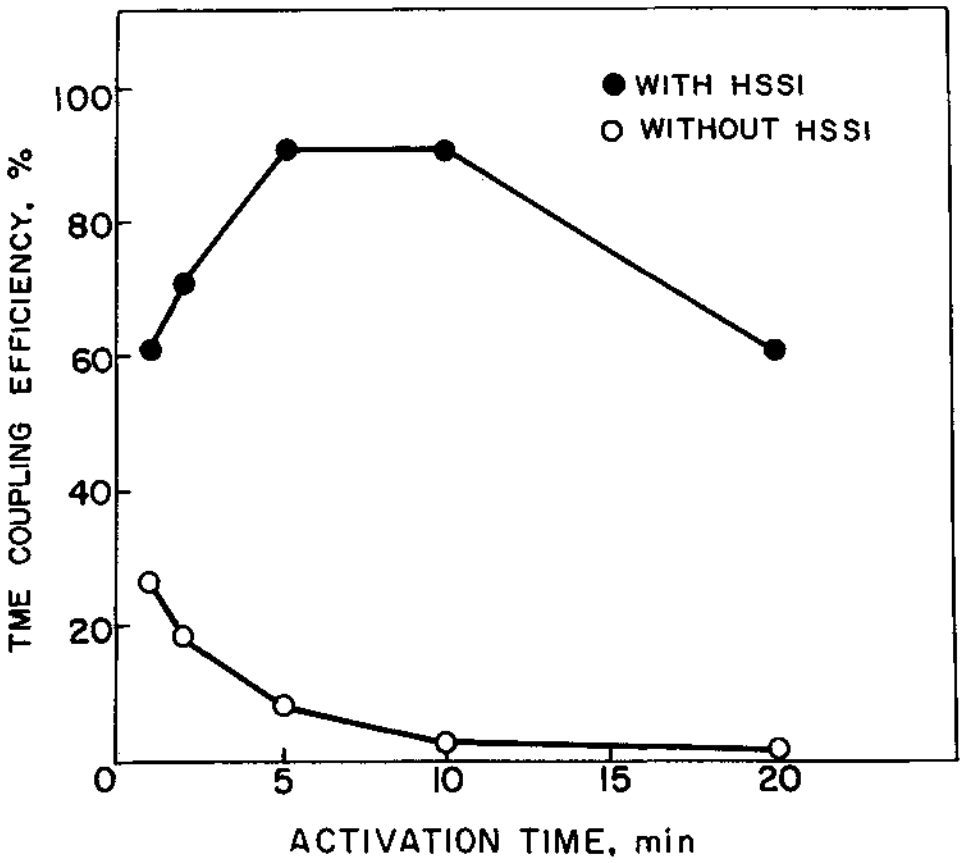

Fig. 4. The effect of the time of polymer activation on the TME coupling efficiency with PL-DTPA$_{36}$-Suc, activated either with EDC, or with EDC/HSSI. (Polymer) = 1.5 mg/mL; (EDC) = 1.5 mg/mL; (HSSI) = 2.4 mg/mL.

The hypothetic scheme of possible reactions in the system described (*11,12*) is presented in Fig. 5. The role of O-acylisourea side-reactions in the case of EDC/HSSI-mediated coupling is likely to decrease owing to preferential proceeding of the reactions distinguished at Fig. 5 by the thick arrows.

Conjugation of Chelating Polymers with Proteins

The relationships observed for low-molecular-weight model amine TME coupling to carboxylic polymers were confirmed for conjugation of PL-DTPA-Suc with proteins.

α-chymotrypsin (CT) is the usual enzyme applied as a model protein for immobilization studies because its properties are known in detail and its activity can be determined by a simple and sensitive procedure using a pH-stat.

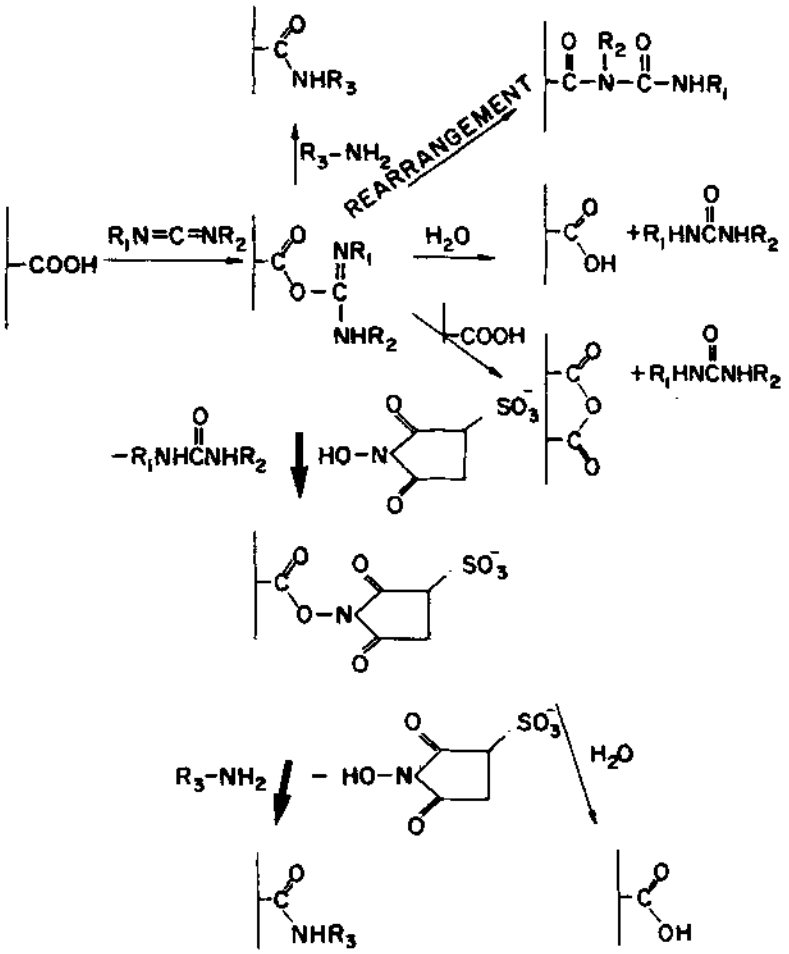

Fig. 5. The scheme of possible reaction pathways during the coupling of chelate polymer to protein via EDC.

Crosslinking of PL-DTPA-Suc with CT was performed in the manner similar to TME coupling. Polymer was activated with EDC for 1 min and then reacted with CT (1:1 initial polymer/CT molar ratio). Coupling efficiency of the polymer to CT decreases with the increase of the number of DTPA groups in the polymer molecule. About 95% of CT added was conjugated with PL-DTPA$_{53}$-Suc as estimated by Sephadex G-100 gel filtration, *see* Fig. 6. Final conjugate contained equimolar amounts of CT and polymer. In the similar conditions, about 100% and 70% of CT were conjugated with PL-DTPA$_{24}$-Suc and PL-DTPA$_{75}$-Suc, respectively. Besides this influence on the coupling yield, the increase of the number of DTPA residues in the polymer (and, consequently, increase of the negative charge density of the polymer chain) resulted in some decrease of the CT enzymatic activity in the conjugate (Table 3).

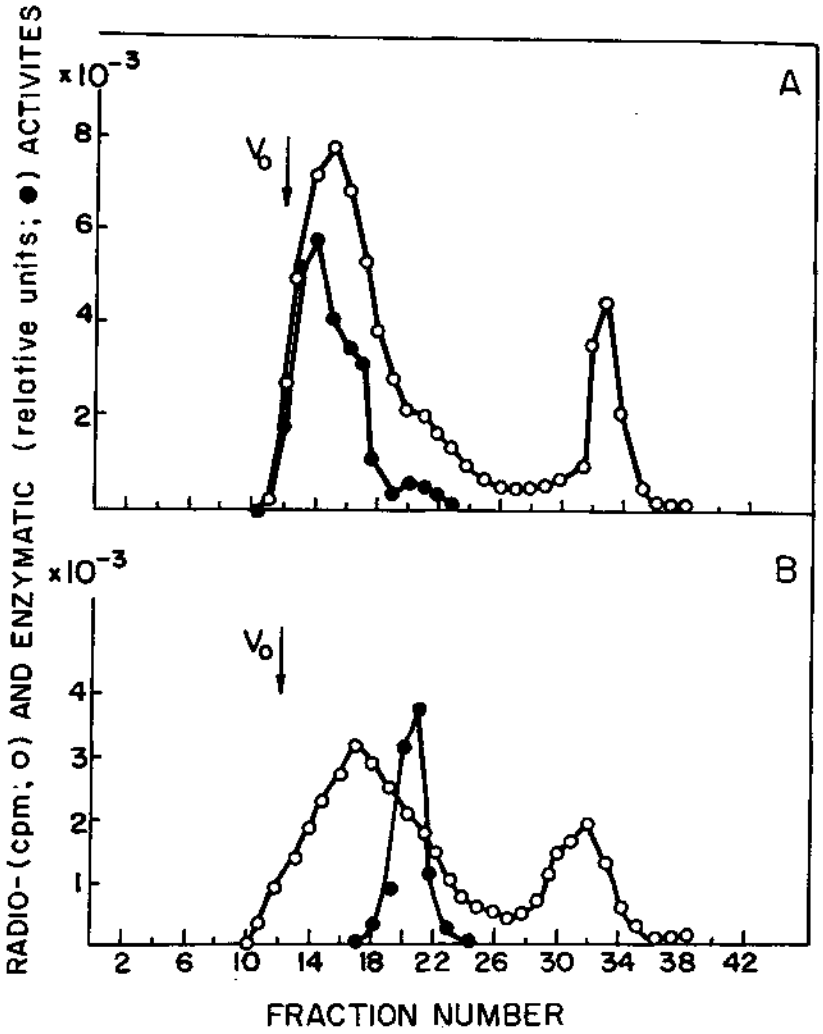

Fig. 6. Gel-filtration of ^{59}Fe-labeled conjugate CT-PL-DTPA$_{53}$-Suc (a) and free CT and ^{59}Fe-labeled polymer (b). Sephadex G-100-superfine column (1.6*30 cm). V_{fr}=2.5 mL. Eluent, 0.05 *M* acetate+0.15 *M* NaCl, pH 6.5.

Table 3
The Effect of the DTPA Content in the Polymer on the Chymotrypsin Coupling Yield and Enzymatic Activity in the Conjugate

Polymer	Coupling yield of chymotrypsin, %	Enzymatic activity in the conjugate, % of initial
PL-DTPA$_{24}$-Suc	100	80
PL-DTPA$_{53}$-Suc	95	66
PL-DTPA$_{75}$-Suc	70	65

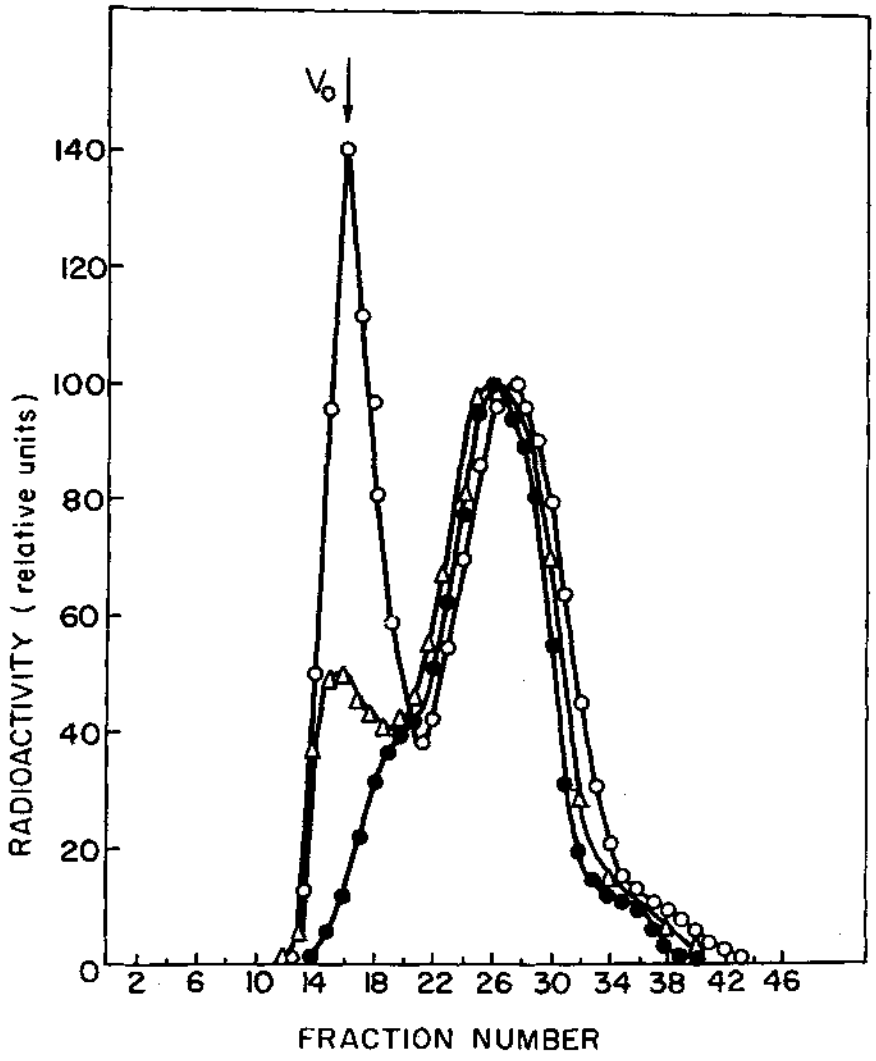

Fig. 7. Gel-filtration of free ^{111}In-labeled PL-DTPA$_{36}$-Suc (●), and conjugates of PL-DTPA$_{36}$-Suc with 9B9 monoclonal antibody, polymer activation-being performed either with EDC (△), or with EDC/HSSI mixture (○). Sephadex G-100 superfine column (1.6*30 cm). V_{fr}=2.0 mL. Eluent, 0.05 *M* acetate+0.15 *M* NaCl, pH 6.5.

Coupling of the mouse monoclonal immunoglobulin to EDC-activated polymer was different significantly from that of model protein (CT). Coupling efficiency of the chelating polymer (PL-DTPA$_{36}$-Suc; molecular weight, 25000) with this IgG reduced drastically as compared with CT. (In this case, using GPC on Sephadex G-100 we determined only the inclusion percent of polymer in the conjugate because the change of IgG molecular weight was too insignificant). When the initial molar ratio PL-DTPA$_{36}$-

Suc/IgG is equal to 2:1 and PL-DTPA$_{36}$-Suc is activated with EDC alone, then the polymer coupling efficiency is equal to 8%. It corresponds to 16 percent content of the modified IgG molecules at the average. The most probable reason of such difference between coupling efficiencies for IgG and CT is, by our opinion, the difference between isoelectric points for these proteins: at the coupling reaction pH (8.5) CT molecule carries slightly positive charge while IgG molecule carries a negative charge. Consequently, the interaction with negatively charged polymer is facilitated in case of CT and inhibited in case of IgG.

In order to increase the IgG-polymer coupling yield we have performed activation of PL-DTPA$_{36}$-Suc with EDC in the presence of HSSI. The inclusion percent of polymer in the conjugate increases up to 25% (i.e., more than threefold as compared to activation in absence of HSSI). Consequently, the average percent of modified IgG molecules raises to 50%. The effect described is illustrated in Fig. 7, which presents chromatogramms of ^{111}In-labeled conjugates and free polymers on Sephadex G-100.

In summary, the method proposed for the conjugation of proteins with polycarboxylic chelating polymers via EDC and HSSI may prove useful for the further development of noninvasive diagnostics and radioimmunotherapy methods based on monoclonal antibodies. We observed minor reduction of chymotrypsin enzymatic activity after its conjugation with PL-DTPA-Suc. Therefore, one might expect that conjugation of specific monoclonal antibodies with PL-DTPA-Suc would not result in drastic reduction of their immunological activity. However, antibody yield achieved for IgG coupling with chelating polymers is about 50%. For the practical application it is necessary to increase the yield further. Work in this direction is currently being carried out in our laboratory.

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