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High-performance affinity chromatography of immunoglobulin E on a column of dinitrophenylamino acids covalently bound to a highly cross-linked polymeric micropellicular support

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ABSTRACT

Coupling of different dinitrophenyl (DNP) amino acids to 2.5-μm highly cross-linked polystyrene–divinylbenzene beads was performed, using carbodiimide as catalyst. The binding capacity of affinity-purified monoclonal anti-DNP mouse immunoglobulin E (IgE) antibody to DNP-lysine-coated beads is *ca.* 4 nmols per mg of beads. The structure of the active coupled functional groups was investigated by X-ray photoelectron spectroscopy. The application of these ligand-carrying beads to the high-performance affinity chromatography of IgE antibody was demonstrated and the purity of IgE was confirmed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. This method is also suitable for coupling several other carboxyl compounds to a highly cross-linked polystyrene matrix.

INTRODUCTION

One of the technical problems in high-performance affinity chromatography (HPAC) is establishing 'anchoring functions' on the surface of solid supports which are suitable for the covalent attachment of ligands or ligand-binding proteins. With silica-based supports a large number of alternatives are available [1]. However, with organic supports, such as polystyrene (PS), the choices are more limited. Currently, the following approaches are used for the derivatization of PS for covalent immobilization of various molecules: copolymerization of monomers containing functional groups [2], chemical modification of polymerized products [3] and radiochemical modification of polymerized products by graft polymerization [4] or by radio-derivatization [5]. The basic assumption in all these approaches is that PS products consist of chemically pure PS, which is only reactive under relatively harsh conditions, such

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as those necessary for electrophilic substitutions, to obtain the required anchoring functions. We have recently discovered that highly cross-linked PS beads react with carboxyl-containing compounds in the presence of carbodiimides. This suggests that these beads contain nucleophilic groups on their surface in sufficient numbers to serve as 'anchoring functions' for covalent binding of ligands, such as dinitrophenyl (DNP-amino) acids [6].

Immunoglobulin E (IgE) is one of the subclasses of human immunoglobulins and plays an important role in allergies [7,8].

EXPERIMENTAL

Instrument

The equipment consisted of a high-performance liquid chromatographic (HPLC) gradient system (Model 402; Bio-Rad Labs., Richmond, CA, U.S.A.). Injections were made using 20- and 300-μl sample loops. The γ and β liquid scintillation counters were an LKB Wallac CliniGamma 1272 counter and an LKB Wallac 1215 (Pharmacia, Uppsala, Sweden), respectively.

Column

Highly cross-linked polystyrene–divinylbenzene particles having a rugulose exterior and a mean diameter of 2.5 μm were prepared by emulsion polymerization, using a two-step swelling method with 1-chlorododecane as swelling reagent [9]. The starting 0.9-μm styrene emulsion was produced by the emulsifier-free method [10], modified with regard to ionic strength optimization, which was performed with sodium chloride in order to achieve the required homogeneous size distribution. The derivatized beads were suspended in water. The slurry was sonicated and packed into a 30 × 4.6 mm I.D. stainless-steel column. Water was used as the driving solvent at a starting pressure of 300 bar for 10 min and a final pressure of 500 bar for 30 min by using a Haskel Model MP-110 air-driven fluid pump (Ammann-Technik, Kölliken, Switzerland).

Chemicals

Styrene and divinylbenzene were purchased from Riedel- de Haën (Seelze, F.R.G.). The radiochemicals were obtained from the following suppliers: [2-^{3}H]glycine, 5-hydroxy[G-^{3}H]tryptamine, L-[4,5-^{3}H]lysine and [1,4(N)-^{3}H]putrescine from Amersham (Amersham, U.K.); and [^{3}H]acetic acid, L-[3,4-^{3}H]valine, L-[4-^{3}H(N)]proline and L-[^{3}H(G)]serine from Du Pont (New England Nuclear, Vienna, Austria). DNP-[^{3}H]glycine was prepared from 2,4-dinitrofluorobenzene and [2-^{3}H]glycine according to a published method [6]. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was purchased from Sigma (St. Louis, MO, U.S.A.) and DNP-amino acids were obtained from Fluka (Buchs, Switzerland). A solution containing 1% bovine serum albumin (BSA), 4% PEG 1000 and 0.05% Tween 20 in phosphate-buffered saline (PBS) at pH 7.2 (abbreviated to PBSAT) was used as a binding buffer in the affinity studies.

Antibody

Affinity-purified monoclonal anti-DNP mouse IgE antibody, IgE(aDNP), clone SPE-7, was obtained from Sigma. Iodination of IgE(aDNP) was carried out by the Enzymobead method (single reaction, Bio-Rad Labs.) following the manufacturer's directions. The iodinated product was purified on a 50 × 10 mm I.D. Sephadex G-25 column, equilibrated with 0.2 *M* phosphate buffer at pH 7.2. Radioactivity of the collected fractions was measured in a gamma counter. The purity of IgE(aDNP) was checked by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions [11].

Coupling of DNP-[^{3}H]Gly to polystyrene beads

Five different concentrations of DNP-Gly were prepared in a coupling solution (0.1 *M* NaCl, adjusted to pH 3.0 with HCl). An amount of $6 \cdot 10^5$ cpm of DNP-[^{3}H]Gly was added to 250 μl of 10 m*M* DNP-Gly. From this solution, four other standards were prepared by sequential three-fold dilutions. A 10-mg amount of 2.5-μm highly cross-linked polystyrene beads was suspended in 250 μl of the coupling solution and 25 μl (containing 1 mg of beads) were pipetted into two series of ten Eppendorf vials each, one series without EDC ('O') and one with EDC ('E'). From each of the standard solutions, 25 μl were added to both series. A 50-μl volume of coupling solution was added to the 'O' and 50 μl of a 4-mg/ml solution of EDC in the coupling solution to the 'E' series. The suspensions were mixed by vortexing and incubated at room temperature for 1 h on a shaking table. Following centrifugation at 13 000 rpm for 3 min in a microfuge, the beads were first washed five times with 1 ml of 90% ethanol, then once with 1 ml of 0.1 *M* sodium hydrogencarbonate and 1 ml of water, with centrifugation at each step. The tips of the vials were cut off with a red-hot razor blade and counted in the beta counter with 2 ml of Biofluor emulsifier cocktail (New England Nuclear, Boston, MA, U.S.A.). About $5 \cdot 10^6$–$1 \cdot 10^7$ cpm of DNP-[^{3}H]Gly were coupled with 100 mg of the beads, as described above.

The stability of the bond was checked by the following procedure. The DNP-[^{3}H]Gly-coated beads were resuspended in 5 ml of water, and 100-μl aliquots were centrifuged at 13 000 rpm of 3 min. A 50-μl volume of the reagents listed in Table I was added each individual pellet of beads and incubated on the shaking table for 5 h. After adding 1 ml of water and recentrifugation, the beads were washed three times with 0.1 *M* sodium hydrogencarbonate and transferred quantitatively to the counting tubes with 0.5 ml of 0.5% Tween 20. The uptake of radioactivity was compared with the untreated bead.

Coupling of different radiochemicals to the beads

An amount of $1 \cdot 10^6$ cpm per 250 μl of each of the compounds listed in Table II was diluted with water to give 250 μl, then mixed with another 250 μl of 20 m*M* of the corresponding unlabelled standard. A 200-mg amount of the beads was suspended in 10 ml of coupling solution and 100-μl aliquots were used for coupling.

Binding of IgE(aDNP) to beads coated with different DNP-amino acids

A 1-ml volume of 5 m*M* DNP-amino acids listed in Table III was coupled with 10 mg of the beads in Eppendorf vials following the procedure given in the preceding paragraph, then 500 μl of $5 \cdot 10^5$ cpm [^{125}I]IgE(aDNP) were added and the mixture

was incubated at room temperature for 4 h on a shaking table. The beads were washed eight times with 1 ml of PBSAT and the radioactivity was measured in a gamma counter. As DNP-lysine turned out to be the best ligand for IgE, the binding capacity of PS–DNP-lysine was then determined. A $6 \cdot 10^5$ cpm amount of [^{125}I]IgE (aDNP) was mixed with 50 μl of monoclonal anti-DNP mouse IgE (50 μg) and diluted to 300 μl with PBSAT. Again, four other standards were prepared by sequential three-fold dilutions. DNP-lysine was coupled with 12 mg of the beads. The coated pellet was resuspended in 1.2 ml of PBSAT and 100-μl aliquots were used for capacity measurements.

Atomic composition of highly cross-linked micropellicular PS beads

The atomic composition of the highly cross-linked PS beads was determined by X-ray photoelectron spectroscopy (XPS) using the method of Bertoti *et al.* [12]. The spectra were recorded with a Kratos XSAM 800 spectrometer, operated in the fixed retarding ratio mode, using Mg Kα radiation (1253.6 eV). Spectra were referenced to the C 1*s* line of adventitious carbon, fixed at a binding energy of 284.6 eV. The gold decoration method was used to check the applicability of this method. Ion etching was performed with 2.5-keV Ar^+ ions with an ion current density of 3.4 μA cm^{-2}. A detailed description of the experimental conditions can be found elsewhere [13]. Wide-scan spectra in the 50–1250 eV kinetic energy range were recorded for all samples. Detailed spectra of the C 1*s*, O 1*s*, Si 2*p*, Na 2*s*, Al 2*p*, Cl 2*p* and K 2*p* lines were recorded after each etching step. The effective sampling depths for these lines were 2–4 nm. For quantitative analysis the method and experimental cross-section values of Evans *et al.* [14] were applied.

Affinity chromatography of monoclonal anti-DNP mouse IgE

After coupling as outlined above, the PS–DNP-lysine beads were packed into a HPLC column (see above). The column was equilibrated with PBSAT at 0.02 ml/min. A $2 \cdot 10^5$ cpm amount of [^{125}I]IgE was introduced via a 20-μl injection loop. A flow-rate of 0.2 ml/min was maintained for 100 min and then increased to 0.5 ml/min. Fractions of 1 ml were collected until no radioactivity was observed above the background. In order to test the strength of affinity interaction, the bound antibody was eluted with two eluents at 0.5 ml/min, *viz.*, 1 *M* ammonium thiocyanate and 2.5 m*M* DNP-lysine. The purity of each fraction was confirmed by SDS-PAGE. The binding capacity of the column was determined as described above, using $5 \cdot 10^5$ cpm of the labelled IgE and eluting with 2.5 m*M* DNP-lysine. Labelled IgE in the fractions was subjected to SDS-PAGE and using 'cold' (*i.e.*, unlabelled) IgE as the control.

Applications

Crude IgE from two types of cell suspensions were iodinated as above and introduced directly into the column via a 320-μl injection loop, using 1 *M* ammonium thiocyanate first, followed by 2.5 m*M* DNP-lysine. The fractions were also subjected to SDS-PAGE.

RESULTS AND DISCUSSION

In Fig. 1 the amount of DNP-[^{3}H]glycine that is attached to the beads is depicted as a function of ligand concentration. If the coupling procedure is performed without EDC as a catalyst, no significant coupling of DNP occurs. The radioactivity remaining after treatment with acids, oxidizing agents and bases is given in Table I. The bond between the ligand and the support is resistant to detergents such as Tween 20 and to high salt concentrations (2 *M* KCl) at low pH, whereas bases and oxidizing agents break the bond between the ligand and the matrix. The sensitivity towards acids and oxidizing agents may vary according to the reagent used. There is no significant leakage of DNP during incubation with the buffer used as the mobile phase for the antibody-binding studies in this work. These investigations suggest the bond between DNP-[^{3}H]glycine and the polymeric support is covalent in nature. To compare the effect of functional groups on coupling, different radioactively labelled compounds were used at pH 6 and 3. Generally, coupling was superior at pH 6 except with DNP-glycine (which showed optimum coupling at pH 3) (Table II).

The nature of the bond formed between PS and carboxyl compounds is unknown. Carboxyls can react in the presence of carbodiimides with a variety of nucleophiles in addition to amino groups [15]. According to the atomic composition of the beads, obtained by XPS, the polymer contains *ca.* 174 O atoms per 1000 C atoms. This is equivalent to 1.4 O atoms per vinyl monomer. These oxygens, in various forms (aliphatic, aromatic, hydroxyls, peroxides, etc.), may serve as reactive groups on the surface of PS beads. The sensitivity of the bond to bases is indicative of ester-type linkages between DNP-amino acids and PS. The possible nucleophile is the hydroxyl group of poly(vinyl alcohol) (PVA). PVA was used as a stabilizer during the polymerization step and it is known to be incorporated into the surface of micropellicular PS [16]. A recent report demonstrated that the adsorption of PVA at a polystyrene latex surface yields layers of controlled thickness [17], useful as a reactive coating on the polymer surface. The reaction scheme proposed for the coupling mechanism is shown in Fig. 2. The presence of about 0.5% of acetate groups in PVA [17] may make the particles reactive with amino-containing compounds, such as putrescine and histamine, in the presence of carbodiimide (see Table II).

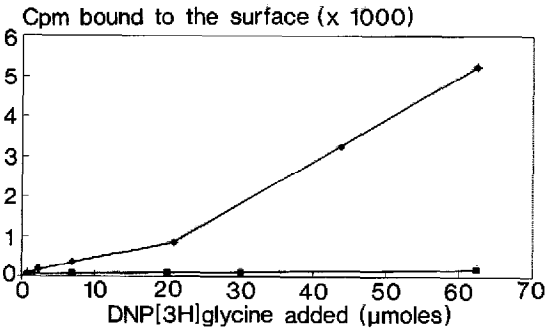

Fig. 1. Coupling of DNP-[^{3}H]glycine to highly cross-linked polystyrene sorbent (●) with and (■) without EDC. The calculated maximum coupling capacity is 780 pmol of DNP-glycine per mg of PS beads.

TABLE I

PERCENTAGE OF DNP-[^{3}H]GLY REMAINING BOUND TO THE BEADS AFTER TREATMENT WITH VARIOUS REAGENTS

Reagent	Bound (%)	Reagent	Bound (%)
Untreated	100	NaOH, 1.00 *M*	15
H_2SO_4, conc.	42	NaOH, 0.10 *M*	27
HCl, conc.	45	NaOH, 0.01 *M*	22
HNO_3, conc.	88	$NaHCO_3$, 0.1 *M*	76
$HClO_4$, 70%	37	$KMnO_4$, 5%	25
CH_3COOH, 100%	78	KCl, 2 *M*–glycine, 0.2 *M* (pH 3)	94
Acetic anhydride	87	Binding buffer (PBSAT)	94

The binding capacities of different DNP-amino acids as affinity ligands for IgE were examined by coupling them to the beads and the uptake of [^{125}I]IgE(aDNP) was compared with that of DNP-glycine (see Table III). DNP-lysine-coupled beads showed the highest capacity for IgE(aDNP). When [^{125}I]IgE(aDNP) was added to the DNP-lysine coupled beads in increasing concentrations, saturation-type binding occurred. The capacity calculated from a double reciprocal plot is *ca.* 4 nmol of IgE(aDNP) per mg of beads.

The PEG present in PBSAT increases the stability of antibody–ligand complexes [18,19] and the BSA minimizes non-specific interactions. Addition of the surfactant (Tween 20) reduces 'non-specific' hydrophobic and van der Waal's interactions [20]. Of the injected IgE, 57% was adsorbed to the stationary phase; this was first eluted with ammonium thiocyanate as a non-specific eluent, followed by DNP-lysine as a competitive eluent [20,21]. Fig. 3 shows the chromatogram of the adsorbed and eluted IgE. Owing to the different tertiary structures possible, two IgE peaks are obtained, which exhibit different binding properties. Fig. 4 shows the SDS-PAGE of the collected fractions. The fractions which were eluted by ammonium thiocyanate and DNP-

TABLE II

COUPLING OF DIFFERENT RADIOCHEMICALS TO THE BEADS AT pH 3.0 AND 6.0

Radiolabelled chemicals	Amount coupled (pmol/mg PS)	
	pH 3.0	pH 6.0
DNP-glycine	21.71	9.74
L-Proline	3.14	6.16
Acetic acid	1.48	3.01
L-Valine	1.86	11.20
L-Serine	0.89	2.06
5-Hydroxytryptamine	2.08	36.21
L-Lysine	2.24	25.10
Putrescine	2.29	73.68
Glycine	1.43	7.38

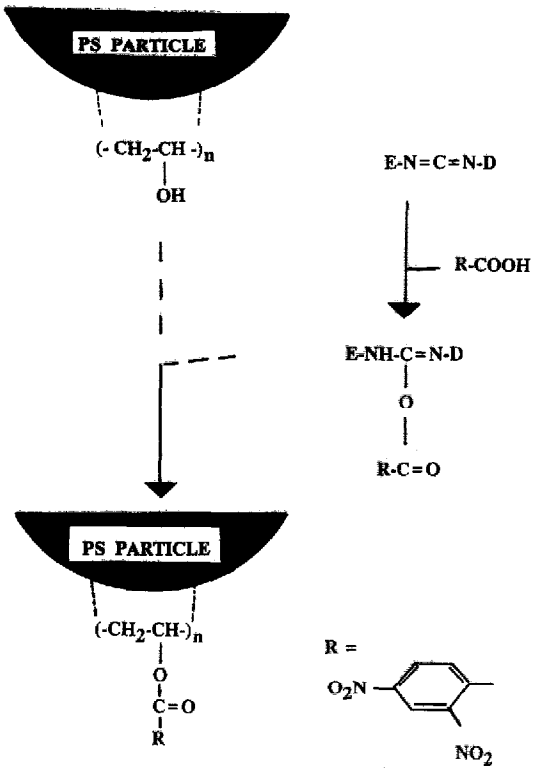

Fig. 2. Proposed reaction scheme. The available nucleophile on the surface of the polystyrene particle is believed to be the hydroxyl group of poly(vinyl alcohol) which is used as a stabilizer in the polymerization step [16].

TABLE III

BINDING EFFICIENCY OF DIFFERENT DNP-AMINO ACID-COUPLED BEADS TO [^{125}I]IgE (aDNP)

DNP-amino acid coupled to the beads	IgE bound at pH 3.0 (% of total IgE added)
Lysine	95.56
Alanine	6.61
Proline	9.87
Ornithine	10.75
Asparagine	12.96
Valine	15.12
Aspartic acid	12.30
Glycine	8.48

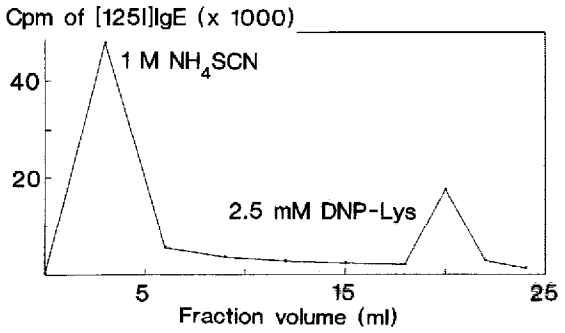

Fig. 3. Chromatogram of [^{125}I]IgE(aDNP) with 1 *M* ammonium thiocyanate and 2.5 m*M* DNP-lysine as eluents.

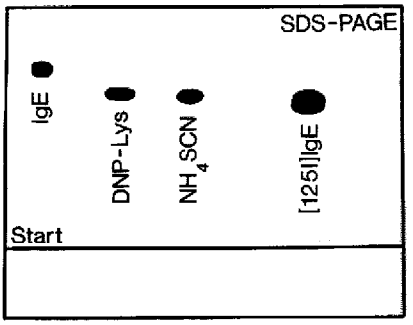

Fig. 4. Electropherogram of IgE from different fractions in Fig. 3.

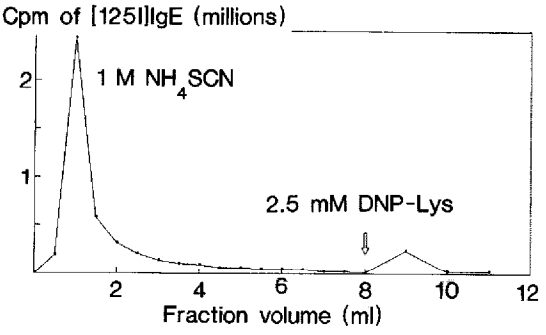

Fig. 5. Chromatogram of immunoglobulin E from crude B 4 cell suspension.

lysine show the same electrophoretic mobilities as the [^{125}I]IgE(aDNP) standard. A chromatogram of iodinated crude IgE from one type of B 4 cell suspension (see Fig. 5) also shows two peaks. Fig. 6 shows an electropherogram of the ammonium thiocyanate fractions of crude IgE from B 4 and B 142 hybridoma cultures, both having the same mobility. PVA-coated polystyrene beads have been shown to be useful for

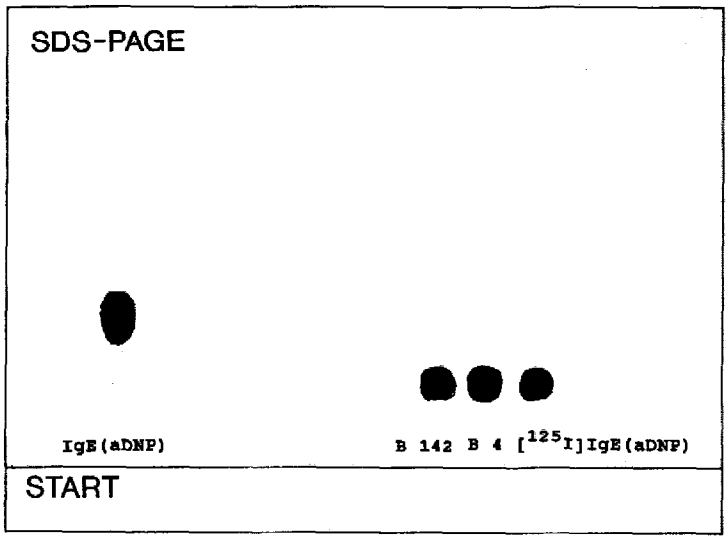

Fig. 6. Electropherogram of labelled IgE (ammonium thiocyanate fraction) from two types of cell suspensions (B 4 and B 142).

various further derivatizations by covalent bonding of affinitiy ligands. Such beads in the DNP-lysine-coated form have been used successfully for resolving immunoglobulins, showing high selectivity and binding capacity. This type of affinity column can readily be employed for the preparative one-step purification of immunoglobulin E from hybridoma culture supernatants.

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