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Immobilization of monoclonal antibodies for affinity chromatography using a chelating peptide

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ABSTRACT

A procedure was developed for oriented immobilization of monoclonal antibodies on a solid support. The technique involves the specific oligosaccharide-directed covalent modification of the monoclonal antibody (mAb) with the chelating peptide, Lys–Gly–$(His)_6$, in conjunction with immobilized metal ion affinity chromatography. Chelating peptide–mAb conjugates with a molar ratio of 2.2 retained full antigen binding activity. On immobilization of the modified antibodies on a nickel affinity resin, the molar antigen binding ratio was 1.4. The high antigen binding capacity is indicative of oriented immobilization providing maximum access for the antigen. The described method can be used for the preparation of high-capacity immunosorbents for affinity chromatography and it is applicable for all immunoglobulin classes.

INTRODUCTION

Immobilized antibodies are widely used for the efficient purification of a broad spectrum of proteins in biochemistry and biotechnology [1]. For instance, large-scale purification of recombinant interferon α-2a involves affinity chromatography using immobilized monoclonal antibodies directed against human leukocyte interferon [2].

Numerous different procedure have been developed to couple antibodies to solid supports [3,4]. In general, antibodies are cross-linked to the matrix through stable covalent bonds. Most of the methods are based on either random amino acid- or selective oligosaccharide-directed immobilization of antibodies. Random coupling through the amino acid side-chains is often accompanied with a partial or complete loss of antigen binding capacity owing to reduced efficiency of antibody–antigen interactions [5]. However, the decrease in bonding activity can be minimized by oligosaccharide-directed coupling of antibodies to solid supports. Oligosaccharide chains are primarily located on the Fc portion of the immunoglobulin molecules. Thus, antibodies immobilized by their oligosaccharide moieties are oriented in such way that most of the antigen binding activity is retained. Antigen/antibody molar ratios as high as 1.6 have been obtained, corresponding to an increase in binding activity of up to 400% compared with the random coupling [6–8].

Immobilized metal ion affinity chromatography was introduced in 1975 by Porat *et al.* [9]. It takes advantage of the selective interaction between biomolecules containing groups for metal complexation and immobilized metal ions. The nitrilotriacetic acid (NTA) adsorbent, when charged with Ni^{2+} ions, has a high affinity for proteins and peptides containing adjacent histidines [10]. A large number of engineered fusion proteins and protein fragments having a polyhistidine affinity peptide at the C- and/or N-terminus were successfully purified using this Ni^{2+} chelate affinity hromatography [11,12].

In this paper we describe a novel procedure for oriented immobilization of monoclonal antibodies (mAbs). It combines the site-specific covalent modification of the oligosaccharide moiety of immunoglobulins with Ni^{2+} chelate affinity chromatography. A hexahistidine peptide (chelating peptide) is

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chemically conjugated to the aldehyde groups generated on the carbohydrate side-chains of the monoclonal anti-human leukocyte interferon antibody LI-8. The selective interaction of the histidine-containing peptide with the Ni^{2+} ions bound to the NTA resin results in an oriented immobilization of the antibodies which retain most of the antigen binding activity.

EXPERIMENTAL

Materials

Dansylcadaverine, sodium metaperiodate and sodium cyanoborohydride were purchased from Fluka (Buchs, Switzerland). Polyacrylamide supplies, Tween-20, Econo-Pac 10DG desalting columns and coupling buffer were obtained from Bio-Rad Labs. (Richmond, CA, USA). Sephadex G-25 and Sepharose CL-6B were supplied by Pharmacia (Uppsala, Sweden). Coomassie Blue R-250 protein assay reagent was purchased from Pierce (Rockford, IL, USA). C-grade guanidine hydrochloride was obtained from SKW Trostberg (Trostberg, Germany). All other chemicals were of the highest purity available. The chelating peptide Lys–Gly–$(His)_6$ was synthesized by A. Trzeciak, Hoffmann-La Roch (Basle, Switzerland). The following materials were kindly provided by members of Hoffmann-La Roche: NTA resin having a capacity of 9.1 μequiv./ml and monoclonal antibody (LI-8) directed against human leukocyte interferon by A. Schacher, and highly purified recombinant interferon α-2a by U. Ettlin.

Oligosaccharide-directed modification of mAb (LI-8)

The monoclonal anti-human leukocyte interferon antibody [13] was site-specifically modified on the oligosaccharide moiety with either dansylcadaverine or the chelating peptide, Lys–Gly–$(His)_6$. Prior to the oxidation of the oligosaccharides on the Fc region, the storage buffer of the antibody [0.1 *M* potassium phosphate (pH 7.2)–0.1 *M* NaCl] was exchanged with coupling buffer (pH 5.5) using Econo-Pack 10DG columns. Sodium metaperiodate stock solution (21 mg/ml)m was added to the mAb solution (4 mg/ml) at one tenth the final volume. After shaking for 1 h in the dark at room temperature, the mixture was passed through an Econo-Pack 10DG column equilibrated with coupling buffer (pH 5.5) in order to remove the excess of sodium metaperiodate. The oxidized antibody was incubated with dansylcadaverine at various molar ratios or with a 270-fold molar excess of chelating peptide for 1 h at room temperature. After addition of sodium cyanoborohydride to a final concentration of 10 m*M*, the incubation was continued for up to 20 h. The modified mAb was recovered by passing the reaction mixture through a Sephadex G-25 column (20 × 2.6 cm I.D.) equilibrated with 0.1 *M* Tris–HCl (pH 7.0)–1 *M* NaCl.

Chromatography

The NTA ligand and Ni^{2+} ion content of the metal chelate affinity resin [10] used in this study was 9.1 μequiv./ml of packed resin. The mAb modified with the chelating peptide and dissolved in 0.1 *M* Tris–HCl (pH 7.0)–1 *M* NaCl buffer was loaded on to a 1–2-ml Ni^{2+}–NTA affinity column equilibrated with the same buffer at a flow-rate of 0.5 ml/min. After extensive washing with loading buffer in order to remove unmodified antibody molecules, the column was equilibrated with 0.1 *M* sodium phosphate (pH 8.0)–0.2 *M* NaCl buffer and the antigen, interferon α-2a, was applied at a flow-rate of 0.1 ml/min. The bound interferon α-2a was eluted with 0.1 *M* sodium phosphate (pH 7.0)–0.2 *M* NaCl buffer containing 2 *M* guanidine hydrochloride and 0.1% Tween-20. The immobilized antibody could be eluted by washing the column with 0.1 *M* acetic acid (pH 4.0)–0.15 *M* NaCl.

Sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

Modified mAb and samples from the metal affinity chromatography were analysed by SDS-PAGE. Proteins were electrophoresed on 12.5% SDS polyacrylamide gels by the method of Laemmli [14]. The gels stained with Coomassie Blue R-250 were scanned using a Hirschmann Elscript 400 densitometer.

Protein determination

The protein content was determined according to Bradford [15] with bovine serum albumin as a standard or by measuring the UV absorption at 280 nm using a molar absorptivity of 1.4 for mAb (LI-8) and 1.0 for interferon α-2a.

Fluorescence measurement

Coupling of dansylcadaverine to the oxidized mAb was monitored by fluorescence spectrometry using a Kontron (Zurich, Switzerland) spectrofluorimeter. Samples containing dansylcadaverine in phosphate-buffered saline were excited at 350 nm and the emission was measured at 550 nm.

Amino acid analysis

For the determination of the amount of chelating peptide coupled to the antibody, the conjugates were assessed by amino acid analyses. Amino acid analysis was performed according to a modified method of Spackman *et al.* [16]. The instrumentation consisted of a Kontron Liquimat II amino acid analyser.

Assay of antigen binding activity

Antigen binding activity was measured by an enzyme immunoassay as described [17].

RESULTS AND DISCUSSION

Labeling of mAb with fluorescent dansylcadaverine

Dansylcadaverine [N-(5-aminophenyl)-5-dimethylamino-1-naphthalensulphonamide] was used as a model agent for the development of a suitable coupling procedure. Dansylcadaverine is a fluorescent amine [18]. Incorporation of the primary amine of dansylcadaverine into the monoclonal anti-human leukocyte interferon antibody (LI-8) can be quantified easily and rapidly by measuring the fluorescence. Therefore, coupling conditions for the chelating peptide were optimized with this fluorescent compound. The procedure involved oxidation of the oligosaccharides of the mAb and subsequent condensation of the generated aldehydes with the primary amines. The resulting Schiff base was reduced by cyanoborohydride to stabilize the linkage. After separation of residual free fluorescent label by gel filtration, the incorporation of dansylcadaverine was assayed spectrofluorimetrically. The extent of antibody cross-linking which can occur by reaction of the aldehyde groups with primary amines of the antibody was assessed by SDS-PAGE. The results of these studies are summarized in Table I.

A high concentration of dansylcadaverine was needed to quench antibody cross-linking. A molecular excess of less than 150-fold resulted in almost complete antibody cross-linking (data not shown). Using a 270-fold molar excess of the reporter molecule, we found a molar coupling ratio of 1.9, whereas Rodwell *et al.* [19] attached an average number of five chelating agent molecules to the mouse monoclonal anti-phosphocholine immunoglobulin M under slightly different conditions. The attachment of dansylcadaverine was dependent on prior oxidation of the antibody with sodium periodate and it was drastically affected by reducing the reaction time. Surprisingly, increasing the molar ratio of dansylcadaverine to mAb did not result in a higher degree of modification. The fraction of cross-linked antibody molecules, however, was reduced. The issue of antibody cross-linking is addressed in the following section.

Taken together, these observations indicate that conjugates with the required chelating peptide/mAb molar ratio of 1–2 can potentially be obtained by

TABLE I

EFFECT OF VARYING EXPERIMENTAL CONDITIONS ON THE MODIFICATION OF mAb WITH DANSYLCADAVERINE

The amount of coupled dansylcadaverine was determined by measuring the fluorescence. The increase in fluorescence intensity (excitation at 350 nm and emission at 550 nm) was linear in the range 1.5–60 nmol/ml dansylcadaverine. The percentage of mAb cross-linked during the modification reaction was determined by SDS-PAGE followed by densitometry.

Oxidation with periodate	Dansylcadaverine (molar excess)	Reduction with cyanoborohydride (time)	mol dansylcadaverine / mol mAb	Cross-linked mAb (%)
+	270	Overnight	1.9	41
–	270	Overnight	0	0
+	1000	Overnight	1.8	28
+	270	4 h	0.1	28

TABLE II

CHARACTERIZATION OF CHELATING PEPTIDE–mAb CONJUGATES

Chelating peptide–mAb conjugates were prepared as described under Experimental. The extent of coupled chelating peptide was determined by amino acid analysis, the percentage of the cross-linked mAb fraction by densitometry of SDS polyacrylamide gels stained with Coomassie Blue R-250 and the antigen binding activity by enzyme immunoassay. Each result is the mean ± standard deviation of three independent experiments. IBU, interferon binding units.

Species	mol peptide / mol mAb	Cross-linked mAb (%)	Antigen binding activity (IBU/μg)
Conjugates	2.20 ± 0.08	43.1 ± 3.1	11019 ± 975
mAb	–	–	9892

incubating the periodate-oxidized mAb overnight with about a 300-fold molar excess.

Chelating peptide coupling

The results in Table II show that under the experimental conditions identified for the dansylcadaverine coupling, a similar number of the chelating peptide Lys–Gly–$(His)_6$ could be coupled to mAb LI-8. Further, SDS-PAGE of the modified mAb also revealed a similar fraction of cross-linked antibodies (Fig. 1). The main species of the high-molecular-mass fraction migrated on the SDS polyacrylamide gel with an apparent molecular mass of about 200 000. This band probably represents cross-linked heavy chains resulting from inter- and/or intramolecular Schiff base formation, as the relative amount of the heavy chain in the conjugate fraction is reduced compared with the control antibody. A solid-phase immunoassay based on the "sandwich" principle [17] was used to determine whether cross-linking and incorporation of the chelating peptide were paralleled by a decrease in the antigen binding activity of the antibody. The conjugated mAb exhibited the same ability to bind interferon α-2a as the parental molecule (Table II), thereby demonstrating that neither the attachment of the peptide to the carbohydrate residues nor antibody cross-linking during the coupling reaction affected the accessibility of the antigen binding site. Therefore, it is very likely that the linkage sites for the formation of cross-linked antibody molecules are not located in the regions near or at the antigen binding site.

The data in Table II are also in good agreement with several reports showing that oligosaccharide-directed covalent modification of antibodies with reporter molecules has no measurable effect on the antigen binding activity [19–21]. In these studies, a wide range of different reporter molecules, including liposomes [20], vitamins [21], chelating agents [19], chlorin e_6 coupled to dextran molecules [22] containing either amines [19,22] or hydrazides [20,21] as functional groups, were used. The potential applications of such carbohydrate-labelled antibodies in basic research, diagnostics or immunotherapy were reviewed by O'Shannessy and Quarles [23].

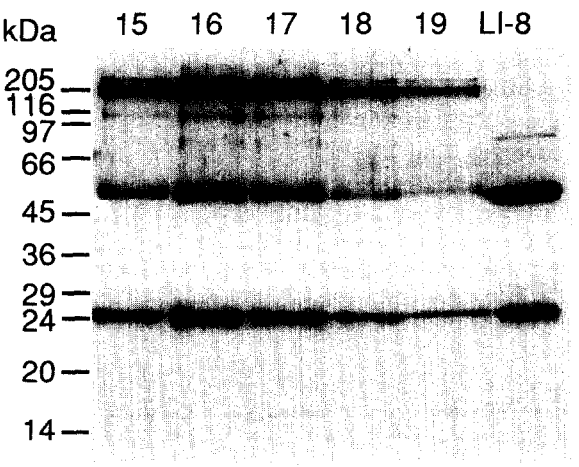

Fig. 1. Electrophoretic analysis of His–mAb conjugates. mAb was modified with the chelating peptide as described under Experimental. The conjugates present in the fractions (Nos. 15–19) eluting from the Sephadex G-25 gel filtraton column were separated on a 12.5% SDS polyacrylamide gel and stained with Coomassie Blue R-250. Positions of Sigma molecular mass markers are indicated on the left-hand side. LI-8, unmodified monoclonal antibody. kDa = kilodalton.

Immunoaffinity chromatography

To prepare an immunoaffinity absorbent for interferon α-2a, we made use of the high-affinity binding of neighbouring histidine residues to immobilized Ni^{2+} ions. The mAb modified on the oligosaccharides with Lys–Gly–$(His)_6$ peptide (His–mAb) was applied to the Ni^{2+}–NTA resin. Using 0.1 *M* Tris–HCl (pH 7.0)–1 *M* NaCl as buffer system, over 92% of the His–mAb conjugates bound to the Ni^{2+} chelate matrix, whereas mAbs lacking the histidine peptide were completely recovered in the flow-through fraction (Fig. 2). These results indicate that at least one chelating peptide is attached to more than 90% of the monoclonal antibody molecules and that the immobilized Ni^{2+} ions selectively interact with the hexahistidine label. In addition, even if the mAb contained surface-accessible metal ion binding sites, no stable complexes were formed under these conditions. The interaction of the hexahistidine peptide with immobilized Ni^{2+} ions has been found to be extremely stable; even harsh conditions such as the presence of chaotropic agents (6 *M* guanidine hydrochloride or 8 *M* urea

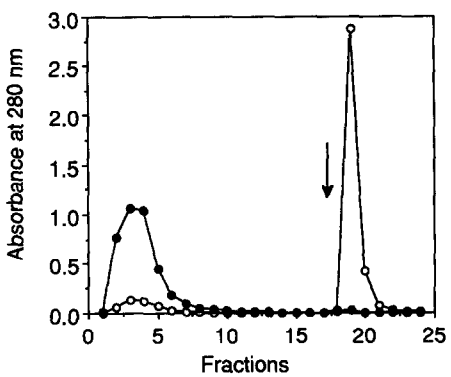

Fig. 2. Characteristics of the binding to and elution from the Ni^{2+}–NTA matrix of His–mAb conjugates and mAb. Modified mAb (10.7 mg) and control mAb (8.7 mg) in 0.1 *M* Tris–HCl (pH 7.0)–1 *M* NaCl buffer were applied to identical 2-ml Ni^{2+} chelate affinity columns. The columns were washed with the same buffer followed by elution of the bound protein with 0.1 *M* acetic acid (pH 4.0)–0.15 *M* NaCl. Absorbance measured at 280 nm of the collected fractions (3.5 ml) from (○) His–mAb conjugate and (●) mAb runs. Arrow indicates the position of the buffer change. The differences in peak height and width for the modified mAb and the control mAb might be due to non-specific interactions of the control mAb with the absorbent at pH 7. These interactions seem not to be manifest for the modified mAb at pH 4.

[11,12] did not release the histidine peptide from the metal complex. Further, equilibrium binding analyses showed that the complex of the dihydrofolate reductase fusion protein bearing a C-terminal hexahistidine peptide extension with Ni^{2+} ions immobilized on the NTA resin had an apparent dissociation constant (K_d) of $0.7 \cdot 10^{-6}$ *M* under identical experimental conditions (not shown).

To demonstrate that the immobilized antibodies were oriented in a way that allowed maximum interaction with the antigen, an excess of highly purified interferon α-2a was loaded on to the immunometal chelate affinity column. As shown by the data in Table III, an interferon α-2a/mAb molar ratio of 1.43 was obtained in the first binding–elution cycle. Importantly, these results showed that immobilization of the modified mAb on the solid support had a minimal effect on the antigen binding activity. Most of the activity was retained, indicating that the antigen binding F_{ab} regions were oriented away from the matrix, allowing efficient recognition and capture of the antigen. As the Ni^{2+}–histidine complexes are acid sensitive (Fig. 2), the most commonly employed acid-elution strategy in immunoaffinity chromatograpy cannot be applied to elute specifically the antigen from the antibody column. The empirically determined solvent for the recovery of bound interferon α-2a consisted of a phosphate buffer (pH 7.0) containing 2 *M* guanidine hydrochloride and 0.1% Tween-20. While 75% of the interferon α-2a were released under these conditions, the remaining 25% were retained on the immunosorbent in the first cycle (Table III). As verified by SDS-PAGE, there was no detectable leakage of His–mAbs or His–mAb–interferon complexes during the elution with the chaotropic agent (Fig. 3). Subsequent binding–elution cycles showed that whereas the binding capacity decreased to an antigen/mAb molar ratio of 1 and stabilized at this level, the amount of recovered interferon α-2a increased to 90%. These results indicate that the observed reduction in antigen binding activity is not due to denaturation of the immobilized mAb, but rather to the blocking of potential binding sites by non-eluted interferon α-2a. Increasing the guanidine hydrochloride concentration to 4 *M* for a higher recovery of the antigen destroyed about 70% of the mAb activity. Preliminary experiments showed that the developed immuno-metal chelate affinity

TABLE III

ANTIGEN BINDING AND ELUTION PROPERTIES OF HIS–mAb CONJUGATES IMMOBILIZED ON THE Ni^{2+}–NTA RESIN

The mAb modified with the chelating peptide was immobilized on a 1-ml Ni^{2+}–NTA column. A four-fold molar excess of highly purified interferon α-2a in 0.1 *M* sodium phosphate (pH 8.0)–0.2 *M* NaCl was applied to the column. After extensive washing, the bound protein was eluted with 0.1 *M* sodium phosphate (pH 7.0)–0.2 *M* NaCl–2 *M* guanidine hydrochloride–0.1% Tween-20. This cycle was repeated three times. The amount of immobilized His–mAb conjugates was 9.7 mg.

Cycle	Interferon bound (mg)	mol interferon / mol mAb	Interferon eluted	
			mg	%
1	1.81	1.43	1.34	74
2	1.30	1.03	1.13	87
3	1.15	0.91	1.06	92

sorbent can also be applied for the purification of recombinant interferon α-2a from a crude bacterial extract (data not shown).

The concept of oriented immobilization of antibodies on solid supports through a "binding protein" has been described for the avidin–biotin system, which exploits the strong binding forces exhibited between the protein and the vitamin [24–26]. In this procedure, biotin hydrazide is selectively coupled to the oxidized carbohydrate residues of mAbs [21], and the biotinylated antibodies are then immobilized on glass beads coated with streptavidin. Unfortunately, no quantitative binding and elution data were reported.

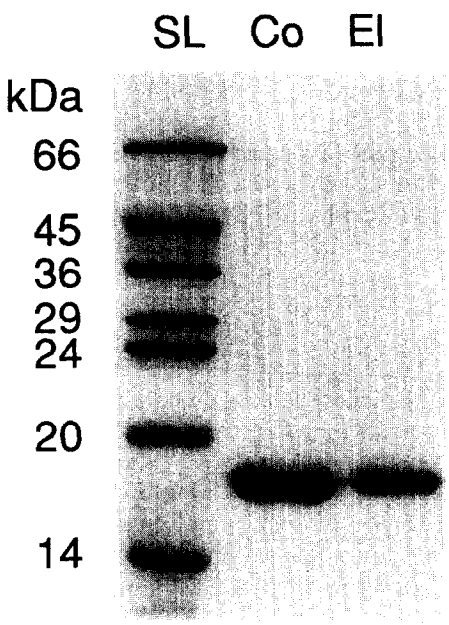

Fig. 3. SDS-PAGE of interferon α-2a recovered from the immuno-metal chelate affinity column. Interferon α-2a was bound to and eluted from the immunosorbent as described in Table III. The eluted protein was separated on a 12% SDS polyacrylamide gel and stained with Coomassie Blue R-250. The lanes represent samples of control interferon α-2a (Co), of interferon α-2a eluted from the immunoaffinity column (EI) and of the low-molecular-mass marker from Sigma (S_L).

In summary, although the inability to recover the bound antigen by acid elution might limit the potential of the described method to some extent, the possibility of differential elution of the antigen, antigen–antibody complex and antibody may be of general use in the wide variety of immuno-chromatographic separation techniques.

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