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Site-directed immobilization of proteins

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ABSTRACT

To determine if immobilization chemistry can be used to orient antibody on a support so that the bivalent binding potential can be fully utilized, we developed three activated matrices that couple to different functional groups on the molecule. When AminoLink Gel was used to couple antibody randomly through primary amino groups, the molar ratio of immobilized antibody to recovered antigen averaged 1:1. Iodoacetyl groups on SulfoLink Gel couple through sulfhydryls in the hinge region of the antibody molecule, in theory leaving the antigen binding site available. However, the antibody-to-antigen molar ratio was only slightly improved. Hydrazide groups on CarboLink Gel couple to aldehyde groups generated by oxidation of carbohydrate moieties that are located primarily on the Fc portion of the antibody molecule. The molar ratio of immobilized antibody to purified antigen using CarboLink Gel reached the optimum of 1:2. CarboLink Gel is most effective at orienting antibody for better antigen purification capability.

INTRODUCTION

Immobilized antibody has been used extensively as a reagent for the isolation of other biological molecules[1]. This may be of particular value in systems where there is no naturally occurring ligand.

Antibody structure has been characterized using a number of analytical techniques[2]. The immunoglobulin G (IgG) molecule is composed of two light and two heavy chains held together by disulfide bonds (Fig. 1A). There are two antigen sites on each molecule formed by the variable regions of one light and one heavy chain. When the antibody molecule is cleaved enzymatically with pepsin, one major and several smaller fragments are produced (Fig. 1B)[3]. The antigen binding fragment, termed $F(ab')_2$, retains both binding sites. The remainder of the molecule is called Fc for "fragment crystallizable".

A commonly used immobilization chemistry is cyanogen bromide (CNBr) activation[4]. Coupling randomly through primary amino groups scattered throughout the antibody molecule, this matrix has the potential to couple at or near the critical antigen binding regions, blocking those sites[5].

For the present study, three activated matrices were developed that couple to

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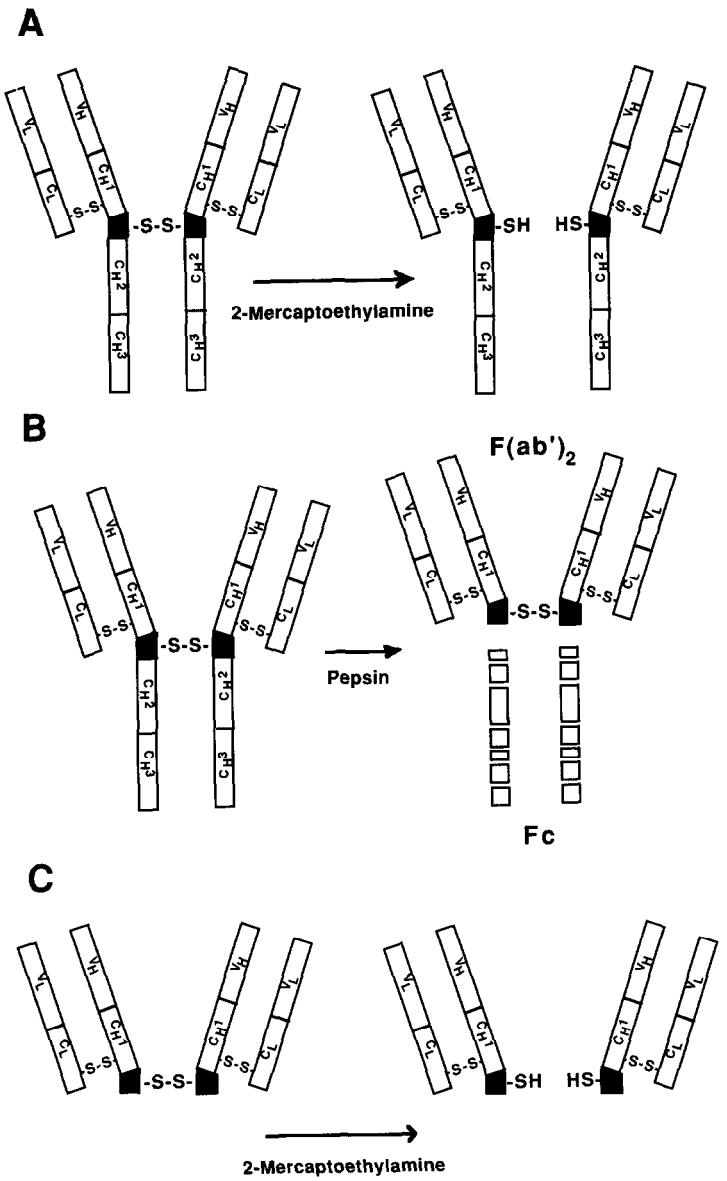

Fig. 1. Production of antibody fragments. (A) The antibody molecule is composed of two light and two heavy chains held together by disulfide bonds. There are two antigen binding sites on each molecule formed by the variable regions of one light and one heavy chain. (B) Enzymatic cleavage of antibody with pepsin yields one major and several smaller fragments. The antigen binding fragment, termed $F(ab')_2$, retains both binding sites. The remainder of the molecule is called Fc (crystallizable fragment). (A and C) Whole antibody and $F(ab')_2$ fragments treated with 2-mercaptoethylamine are preferentially reduced in the hinge region between heavy chains, yielding two antigen binding fragments.

amino, sulfhydryl or oxidized carbohydrate groups located in unique distribution patterns on the antibody molecule. Functional assays were performed to determine if immobilization chemistry could be used to preferentially bind and position the antibody molecule so that both antigen binding sites are accessible after coupling.

EXPERIMENTAL

Proteins

Bovine serum albumin (BSA), avidin, and trypsin were all obtained from Pierce (Rockford, IL, U.S.A.). Sheep anti-BSA was purchased from Bethyl Labs. (Montgomery, TX, U.S.A.) and thioredoxin was purchased from Calbiochem (San Diego, CA, U.S.A.). Human serum albumin (HSA), collagen (type VI), chorionic gonadotropin, fetuin, ovalbumin, α_1-acid glycoprotein, pepsin, ceruloplasm, aldolase, β-lactoglobin, arginine[8] vasopressin, α_1-antitrypsin, cytochrome *c*, myoglobin, α-lactalbumin, transferrin, hemoglobin, ribonuclease A, lysozyme, bovine IgG, human IgG, and rabbit anti-HSA were all purchased from Sigma (St. Louis, MO, U.S.A.).

Activated matrices

The activated gels and accompanying buffers were all obtained from Pierce. All columns contained 2 ml of activated gel. AminoLink Gel is 4% beaded agarose, periodate oxidized to yield aldehyde groups which react with primary amine groups[6]. SulfoLink Gel is 6% beaded agarose substituted with a 12-carbon spacer arm containing a terminal iodoacetyl group which can react preferentially with a free sulfhydryl group on the ligand[7,8]. CarboLink Gel is 6% beaded agarose substituted with a 23-atom spacer arm containing a terminal hydrazide group which can react with an aldehyde group on the oxidized glycoprotein[9,10].

Sample preparation for AminoLink Gel

The protein (1–20 mg) was dissolved or diluted in 2 ml AminoLink Coupling Buffer (0.1 *M* phosphate, pH 7.0).

Coupling to AminoLink Gel

The sample was added to a 2-ml AminoLink column that was equilibrated with AminoLink Coupling Buffer. The working reducing solution was prepared by dissolving 32 mg sodium cyanoborohydride in 0.5 ml distilled water, and 0.2 ml was added to the protein solution in the column. The column was gently mixed for 2 h at room temperature and then incubated for an additional 4 h at room temperature without mixing. The column was washed with 6 ml of AminoLink Coupling Buffer to remove unbound material.

Blocking of excess reactive sites on AminoLink Gel

The protein-coupled column was equilibrated with AminoLink Quenching Buffer (1.0 *M* Tris–HCl, pH 7.4). An additional 2 ml of AminoLink Quenching Buffer and 0.2 ml of the working reducing solution were added to the column and it was mixed at room temperature for 30 min. The column was then washed sequentially with 16 ml of 1.0 *M* sodium chloride and 16 ml of 0.05% sodium azide.

Sample preparation for SulfoLink Gel

Whole antibody or $F(ab')_2$ fragments (1–10 mg) were treated with 2-mercaptoethylamine to reduce the disulfide bonds (Fig. 1A and C). The protein was dissolved or diluted in 1 ml of SulfoLink Sample Preparation Buffer (0.1 *M* sodium phosphate, 5 m*M* ethylenediaminetetraacetate, disodium salt, pH 6.0). This solution was added to 6 mg of 2-mercaptoethylamine and incubated at 37°C for 1.5 h. The sample was brought to room temperature and excess reductant was removed using a 15-ml polyacrylamide desalting column (Pierce) equilibrated with SulfoLink Coupling Buffer (50 m*M* Tris, 5 m*M* ethylenediaminetetraacetate, disodium salt, pH 8.5).

Coupling to SulfoLink Gel

The reduced protein solution (2 ml) was added to a 2-ml SulfoLink column that was equilibrated with SulfoLink Coupling Buffer. The column was gently mixed for 15 min at room temperature and then incubated for an additional 30 min at room temperature without mixing. The column was washed with 6 ml of SulfoLink Coupling Buffer to remove unbound material.

Blocking of excess reactive sites on SulfoLink Gel

L-Cysteine–HCl (2 mg) was dissolved in 2 ml of SulfoLink Coupling Buffer and added to the protein-coupled column. The column was gently mixed for 15 min at room temperature and then incubated for an additional 30 min at room temperature without mixing. The column was then washed sequentially with 16 ml of 1.0 *M* sodium chloride and 16 ml of 0.05% sodium azide.

Sample preparation for CarboLink Gel

Carbohydrate moieties on the protein were oxidized to form aldehyde groups. The protein (0.5–10 mg) was dissolved or diluted in 1 ml CarboLink Coupling Buffer (0.1 *M* phosphate, pH 7.0). The sample was added to 5 mg of sodium metaperiodate and allowed to incubate at room temperature for 30 min, protected from light. Excess oxidizing agent was removed on a 5-ml Sephadex G-25 M (Pharmacia, Piscataway, NJ, U.S.A.) desalting column equilibrated with CarboLink Coupling Buffer.

Coupling to CarboLink Gel

The oxidized protein solution (2 ml) was added to a 2-ml CarboLink column that was equilibrated with CarboLink Coupling Buffer. The column was gently mixed for 6 h at room temperature. The column was washed with an additional 6 ml of CarboLink Coupling Buffer to remove unbound antibody and then washed sequentially with 16 ml of 1.0 *M* sodium chloride and 16 ml of 0.05% sodium azide.

Affinity purification

Phosphate-buffered saline (PBS, 0.01 *M* sodium phosphate, 0.15 *M* sodium chloride, pH 7.4) was used to equilibrate all columns and to dissolve the proteins. A 1-ml sample was applied to the columns which were then allowed to incubate at room temperature for 1 h. The columns were washed with 16 ml of PBS and fractions containing bound protein were eluted using ImmunoPure Elution Buffer (Pierce).

Column regeneration

As soon as possible after affinity purification, columns were washed with 16–20 ml of PBS and stored with a degassed solution of 0.05% sodium azide in water.

Antibody fragments

$F(ab')_2$ fragments of intact antibody (Fig. 1B) were generated using ImmunoPure $F(ab')_2$ Preparation Kit (Pierce). Briefly, 10 mg of human IgG were dissolved in 1 ml of digestion buffer (20 m*M* sodium acetate, pH 3.0) and incubated with 0.125 ml of immobilized pepsin for 4 h at 37°C. The digested sample was applied to a 2.5-ml Protein A column to remove undigested IgG and Fc fragments. The $F(ab')_2$ fragments in the eluent were dialyzed against SulfoLink Sample Preparation Buffer.

Coupling efficiency

Coupling efficiency was determined by comparison of the protein concentration in the unbound material to that in the starting sample. All columns were run in duplicate. Standard error was less than 10%. Protein concentration was determined either by absorbancy at 280 nm or by an assay system for protein using bicinchoninic acid as a detection reagent[11] (BCA Protein Assay, Pierce).

RESULTS AND DISCUSSION

AminoLink Gel is agarose, activated to yield aldehydes which react with primary amine groups to form Schiff bases. Reductive amination forms a stable covalent linkage with minimal leakage of the ligand[6]. The data presented in Table I show that AminoLink Gel is able to immobilize a wide variety of proteins. The coupling efficiency of proteins varying in size and isoelectric point (p*I*) averaged 80% when using a 20-mg sample. In general, the higher the p*I* of the protein, the greater the coupling efficiency. The molecular weight of the protein, however, appeared to have no effect. The coupling efficiency of immunoglobulins from a number of species averaged 80–90%.

TABLE I

COUPLING EFFICIENCY OF PROTEINS USING AMINOLINK GEL

A 20-mg amount of each protein was applied to 2 ml of AminoLink Gel as described in Experimental.

Protein	*pI*	*Molecular weight*	*Coupling efficiency (%)*
Ovalbumin	4.7	45 000	52
α_1-Antitrypsin	4.0	–	66
Fetuin	3.3	–	74
IgG (bovine)	–	150 000	79
Cytochrome *c*	9.0–9.4	11 700	79
Myoglobin	6.8–7.8	16 900	84
α-Lactalbumin	5.2	35 000	84
Transferrin	5.9	85 000	88
Hemoglobin	6.8	64 500	91
Ribonuclease A	9.5	13 700	92
Lysozyme	11.0	14 000	96

On the antibody molecule, primary amine groups are not localized, but occur throughout the molecule. Therefore, antibody can be immobilized near the antigen binding region as well as the Fc end, resulting in either total or partial loss of antigen binding capability.

Fig. 2 shows an affinity chromatographic profile using an AminoLink column coupled with 6 mg of anti-HSA. Another AminoLink column, without immobilized antibody but with reactive sites blocked, was included as a control to determine any non-specific binding. All columns were loaded with 5 mg of HSA. Fig. 2 shows that there is minimal non-specific binding to the blank control column. Approximately 2 mg of HSA were recovered from the antibody-coupled columns. The molar ratio of immobilized antibody to bound antigen never exceeded 1:1, indicating that half of the antigen binding sites on the molecule were not accessible. This supports the concept that coupling through amino groups results in random orientation of the molecule.

However, AminoLink chemistry offers an advantage over CNBr activation chemistry. The leakage of immobilized proteins from CNBr-activated Sepharose 4B is well documented[6,12–15]. The stable covalent bond formed with AminoLink Gel results in minimal leakage of the ligand, ensuring reuse of the column and low contamination of the purified sample.

Iodoacetyl groups on SulfoLink Gel react with free sulfhydryl groups to form a stable thio ether linkage. Antibody treated with 2-mercaptoethylamine is preferentially reduced in the hinge region between heavy chains, yielding two antigen binding fragments (Fig. 1)[16]. Immobilization at the hinge region sulfhydryls should increase the potential of leaving the antigen binding site unobstructed. Table II shows that reduced human IgG, either whole molecule or $F(ab')_2$ fragments, had an average coupling efficiency to SulfoLink Gel of 90%.

Fig. 3 shows an affinity chromatographic profile using SulfoLink Gel coupled with 5 mg of anti-HSA. A 2-ml SulfoLink column, without antibody but with reactive

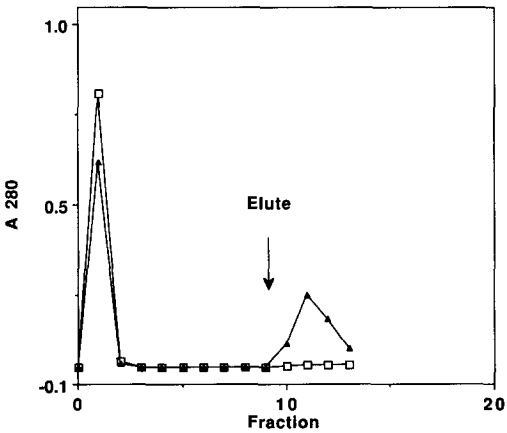

Fig. 2. Affinity purification of HSA using AminoLink Gel. A column (▲) containing 2 ml AminoLink Gel was coupled with 6 mg of anti-HSA as described in the Experimental section. Another AminoLink column (□), without immobilized antibody but with reactive sites blocked, was included as a control to determine any non-specific binding. For affinity chromatography, all columns were loaded with 5 mg of HSA. Columns were eluted and 2-ml fractions were collected.

TABLE II

IMMOBILIZATION OF IgG TO SULFOLINK GEL

Reduced human IgG (1–10 mg) and 4 mg of reduced $F(ab')_2$ fragments were applied to 2 ml of SulfoLink Gel according to the protocol described in Experimental.

Human IgG	*Coupling efficiency (%)*
Reduced whole molecule	90
Reduced $F(ab')_2$	88

sites blocked, was used to test for non-specific binding. All columns were loaded with 5 mg of HSA. Fig. 3 shows that, with this matrix also, there is minimal non-specific binding. Approximately 2.6 mg of HSA were bound and recovered from the antibody-coupled columns. The molar ratio of immobilized antibody (whole molecule) to recovered antigen ranged from 1:1 to 1:1.2. Restated, 50–60% of the antigen binding sites on the reduced antibody molecules were able to bind antigen.

Although polyacrylamide gel electrophoresis reveals that the predominant species following reduction with 2-mercaptoethylamine is the half molecule containing an antigen binding site, the presence of single light and heavy chains is also found. This may account, in part, for the less than optimum binding capacity.

Table III shows the coupling efficiency of several sulfhydryl- and/or disulfide-

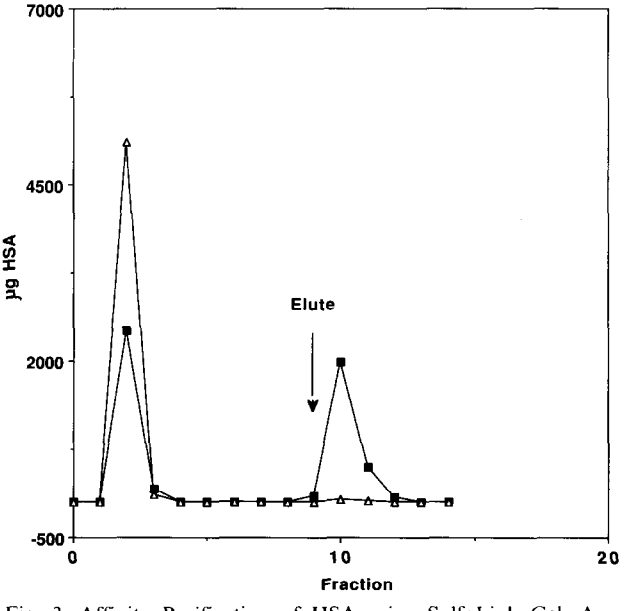

Fig. 3. Affinity Purification of HSA using SulfoLink Gel. Approximately 5 mg of anti-HSA were immobilized to 2 ml of SulfoLink Gel. (■) Another SulfoLink column, (△), without immobilized antibody but with reactive sites blocked, was included as a control to determine any non-specific binding. All columns were loaded with 5 mg of HSA for affinity chromatography. Columns were eluted and,2-ml fractions were collected.

TABLE III

COUPLING EFFICIENCY OF PROTEINS TO SULFOLINK GEL

Protein[a]	*Molecular weight*	*–SH group per molecule*[b]	*S–S group per molecule*[b]	*Coupling efficiency (%)*
Ceruloplasm	150 000	1–3	–	75
Aldolase	147 000	7–28	–	87
BSA	66 000	0.7	17	25
HSA	66 000	0.7	17	72
Ovalbumin	45 000	3–4	1	40
β-Lactalbumin	36 000	2	2	63
Trypsin	24 000	0	6	13
Thioredoxin	11 700	2	1	20
Arginine[8] vasopressin	1084	0	1	90

[a] A 2-mg amount of the reduced form of the peptide, arginine[8] vasopressin, and 8 mg of reduced protein were applied to 2 ml of SulfoLink Gel as described in Experimental.

[b] Values for sulfhydryl content reported in the literature[17,18].

containing proteins of varying size. All proteins were reduced prior to immobilization. Coupling efficiency cannot be strictly correlated with the number of potential free sulfhydryls in the reduced form of the protein. The accessibility of the sulfhydryls would be a contributing factor. As was seen with AminoLink Gel, there is no correlation with the size of the molecule although the best coupling efficiency was seen with the peptide arginine[8] vasopressin.

The coupling chemistry of SulfoLink Gel is also suitable for the purification of anti-peptide antibodes. Most peptide sequences are synthesized with a terminal cysteine residue added for immunogen conjugation. The free sulfhydryl provides a reactive group with which to couple the peptide to a carrier protein for immunization. This same reactive group can be used to couple the peptide to SulfoLink Gel in the same orientation as that used for the immunogen conjugate[19]. The spacer arm on the matrix will also reduce steric hindrance and facilitate the isolation of peptide-specific antibodies.

CarboLink Gel is agarose, activated to contain hydrazide groups which react with oxidized carbohydrate moieties. On the antibody molecule, carbohydrate residues are located primarily on the Fc end. The coupling efficiency of immunoglobulins to CarboLink Gel was variable and slightly lower than that found with the other activated matrices, averaging 70%. One possible reason is that the degree of glycosylation and the composition of the carbohydrate residues are not consistent within a polyclonal response and yield a varying number of reactive aldehyde groups following periodate oxidation[9,20]. Table IV shows that the coupling efficiency of several other glycoproteins to CarboLink Gel was also variable but generally good.

Coupling to antibody through the Fc portion of the molecule should increase the chances of retaining full antigen binding ability following coupling. This is supported by the data shown in Fig. 4. Approximately 0.5 mg of anti-BSA were coupled to 2 ml of gel, and 2 mg of BSA were applied for affinity purification. As a control for non-specific binding, a 2-ml column containing no antibody was also loaded with BSA. The elution profile shows that, with this matrix also, there is minimal

TABLE IV

COUPLING EFFICIENCY OF PROTEINS USING CARBOLINK GEL

A 2-mg amount of protein was applied to 2 ml of CarboLink Gel as described in Experimental.

Protein	*Molecular weight*	*Coupling efficiency (%)*
Collagen (type VI)	163 000	63
Human IgG	150 000	74
Avidin	66 000	95
Chorionic gonadotropin	59 000	91
Fetuin	48 700	87
Ovalbumin	45 000	86
α_1-Acid glycoprotein	44 100	90
Pepsin	34 000	47

non-specific binding. Approximately 0.5 mg of BSA were bound and recovered from the antibody-coupled column. This matrix consistently demonstrated the optimum molar ratio of immobilized antibody to bound antigen of 1:2. The increased efficiency in antigen binding by antibody immobilized through carbohydrate moieties is supported by the work of others[21].

Immobilized antibody has become a commonly used reagent for the isolation and purification of other molecules. However, antibody with specificity for the particular antigenic determinant(s) of the molecule of interest must be generated. In some cases, only small amounts of antibody are produced. For example, there may be a limited supply of purified antigen, antigenic fragments or synthetic peptides with which to stimulate an immune response, resulting in a low yield of polyclonal

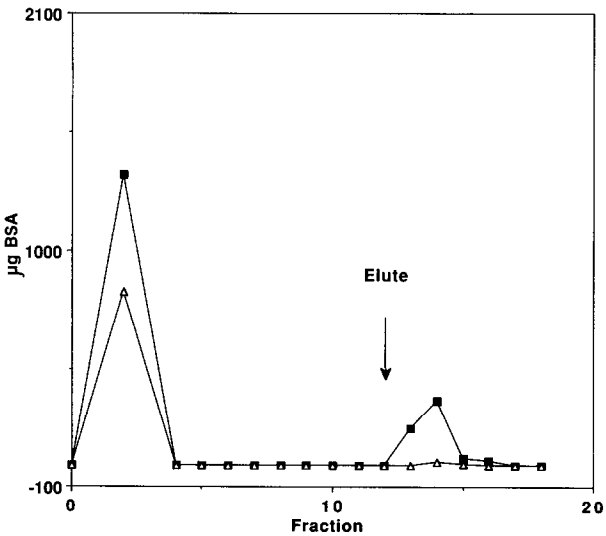

Fig. 4. Affinity Purification of BSA using CarboLink Gel. Approximately 0.5 mg of anti-BSA were coupled to 2 ml of CarboLink Gel, and 2.0 mg of BSA were applied for affinity chromatography (■). Control columns (△) containing no antibody were also loaded with BSA. Columns were eluted and 1-ml fractions were collected.

antibody. Adequate quantities of monoclonal antibody may not be available due to the loss or mutation of the hybridoma. In these circumstances in particular, it is desirable to take advantage of the full binding capability of the antibody by using a matrix capable of correctly orienting the molecule.

Equally important is the ability to reuse the support. All of the matrices provided stable linkage as demonstrated by the fact that they were used and regenerated ten times with minimal loss of affinity purification capability (data not shown).

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