September 1996

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Journal of Molecular Recognition

Volume 9, Issue 5-6, Date: September/December 1996, Pages: 528-535

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Digital Object Identifier (DOI)

10.1002/(SICI)1099-1352(199634/12)9:5/6<528::AID-JMR296>3.0.CO;2-Q

Effect of Antibody Orientation on Immunosorbent Performance

Anuradha Subramanian and William H. Velander*

ABSTRACT:
The impact of antibody orientation on immunosorbent efficiency was quantitatively assessed. A pH-dependent murine monoclonal antibody (Mab) against human protein C (hPC), recombinant hPC (rhPC) and two different immobilization chemistries and matrices were used as model systems. The lysyl groups of the rhPC were covalently modified with an acetic acid ester of N-hydroxysuccinimide and this modified rhPC was used as a Fab masking agent (FMA). The FMA was used to mask the antigen binding regions (Fab) of the Mab prior to and during covalent immobilization. Thereafter, the residual active sites of the support were inactivated and the FMA was removed. Mab was immobilizeed at low bead-averaged densities of about 0.4-1.1 mg Mab/mL matrix to minimize local density effects. Immunosorbents made using masked Mab (oriented coupling) gave antigen binding efficiencies (nAgo) f 4248% compared with 18-22% for those made by random coupling. The amount of (Fab), released from pepsin digestion of immuncsorbents was about --fold higher for matrices having been made with FMA-masked Mab relative to unmasked Mab. Thus, the (Fab), accessibility to pepsin correlates well with higher functional efficiency (nAJ and serves as a measure of orientation. In summary, at low Mab density and a 2:l molar rhPC to Mab binding stoichiometry, about 80% or more of the Mab randomly coupled through amino moieties was improperly oriented relative to oriented coupled Mab, which correlated with about 50% of lost Mab functionality upon immobilization.

Keywords: antibody orientation; immunosorbent performance

Introduction

Immunoaffinity-based methods can be used as an efficient and high-resolution step for protein purification (Ta nowsky and Liptak, 1983; Tarnowsky *et* al., 1986). The customized avidity of the parent antibody for a given target molecule can make immunoaffinity chromatography a versatile tool for purifiying closely related proteins in a biologically active form (Orthner *et* al., 1989; Velander *et* al., 1989). However, the use of immunosorbents on a large scale is frequently precluded by high capital costs. These costs can mostly be associated with the low functional activity of immobilized antibodies and the development and production costs of pathogen-free antibody having a desired specificity for therapeutic applications (Orthner *et* al., 1989; Velander *et al.,* 1990). Typically, antigen binding efficiencies of 3-15% are obtained for monoclonal immunosorbents containing 1-10 mg of antibody/mL of support (Eveleigh
and Levy, 1977; Wilchek *et* al., 1984; Hearn, 1986; Strauss *et al.,* 1987; Subramanian *et al.,* 1994). Immunoglobulins (IgGs) are glycoproteins with an approximate molecular weight of 150000Da. The nonantigen binding domain of an IgG is known as the Fc region and has a cell receptor function which is independent of the antigen binding domain and function. IgGs have two identical disulfide-linked, antigen combining sites known as the Fab domains which contain a highly variable sequence to provide antigen binding specificity (Stryer, 1980). lgGs can be enzymatically cleaved by pepsin to give an intact
(Fab), from the Fc domain (Stryer, 1980; Goding, 1983).
Author to whom correspondence should be addressed. The targeted binding by protein A and G to the Fc domain has been utilized to anchor Mabs (Schneider *et* al., 1982). The variable avidity of protein A and G with different IgG subclasses, protein leaching from the matrix and reagent costs make this sandwich anchoring method less amenable to large-scale applications. Alternatively, Fc-specific coupling chemistry which uses hydrazide-based activation to oxydize carbohydrate on the Mab has been developed (Hearn, 1986; O'Shanessey and Hoffmann, 1987; Little *et* al., 1988; Domen *et* al., 1990; Fleminger *et al.,* 1990; Bonde *et al.,* 1991). However, carbohydrate also occurs in the Fab domains and Mabs immobilized on matrices using hydrazide chemistry did not produce significant differences in Mab functional efficiency compared with random immobilization by CNBr chemistry (Orthner *et* al., 1991). The performance of an immunosorbent is strongly dependent on intraparticle transport, adsorption/desorption kinetics of the Mab (Kaster *et al.,* 1993) and on the molecular orientation (Orthner *et* al., 1991; Velander *et al.,*
1992) and local density of the immobilized antibody (Subramanian *et* al., 1994). It has long been hypothesized that optimum binding kinetics for antigen binding requires the right orientation and spacing of the immobilized antibody (Velander *et* al., 1992). Although immunosorbent efficiency is most often characterized on a volume or bead averaged basis, antibody spacing can be a strong function of position and a high local density greatly decreases immunosorbent efficiency (Subramanian *et al.,* 1994). Multipoint attachment of the antibody to the support matrix may also have a negative effect on immunosorbent efficiency and about a 3-1 1-fold increase in immunosorbent activity was observed for immobilized Mabs having reactive NH.

moieties protected by dimethylmaleic anhydride (Strauss *et* al., 1987; Hadas *et* al., 1990). We have previously demonstrated that shielding of the Fab domains of Mabs during the immobilization process results in greater relative immunosorbent efficiency and Fab accessibility in a membrane-based, enzyme-linked immunosorbent assay (ELISA) system (Velander et *al.,* 1992). In the present study, we used a lysyl-modified recombinant antigen to mask quantitatively the Mab prior to and during immobilization to chromatographic matrices. Thus, the FMA has been used here as a tool both to quantitatively probe and to engineer Mab orientation in immunosorbents used for purification.

Materials and Methods

Materials

A murine pH-dependent anti-hPC Mab (8861-Mab) was provided by American Red Cross (Rockville, MD). Rabbit antisera against hPC, affinity-purified goat-anti-mouse (whole molecule) and anti-goadanti-mouse immunoglobulins conjugated to horseradish peroxidase (HRP) were purchased from Sigma Chemical (St Louis, MO). Goat antihPC antisera was purchased from American Diagnostics (Greenwich, CT). Emphaze AB 1 biosupport medium was provided by 3M (Minneapolis, MN) and Affiprep polymeric support was purchased from Bio-Rad Laboratories (Anaheim, CA). Immulon I1 microtiter plates were purchased from Fisher Scientific (Pittsburgh, PA). The hPC was provided by American Red Cross. The rhPC was isolated from transgenic porcine whey using immunoaffinity chromatography (Morcol et al., 1994).

o-Phenylenediamine-dihydrochloride (OPD) tablets were purchased from Abbott Laboratories (Chicago, IL). The Nsuccinimide ester of acetic acid and trinitrobenzenesulfonic acid (TNBS) were purchased from Sigma Chemical. All other reagents were purchased from Sigma Chemical as the best grade available. Immunoaffinity separations were performed with Phannacia C-10 columns (15 cmx 1 cm i.d.), a Masterflex peristaltic pump and a Knauer spectrophotometer and a Rainin data acquisition system were used to monitor chromatography. Columns were kept at 4°C with a Lauda Super RMT water cooler.

Synthesis of FMA from recombinant hPC

The reactive amino moieties on the rhPC were covalently modified using an acetic acid ester of N-hydroxysuccinimide (Endo et al., 1987). A 3 mg/mL solution of rhpC in borate buffer (0.05 M borate, 0.14 M NaCl, pH 8.6) was slowly mixed with 10 & of a *200* molar excess of ester in dimethylformamide. The pH of the reaction was maintained at 8.5 by the addition of 1 M NaOH and the reaction was allowed to proceed for 24 h at 4°C. The unreacted ester was quenched by adding a 40 molar excess of glycine using a 0.4 M glycine stock solution at pH 8.5. After 4 h of stirring, the modified protein solution was extensively dialyzed against phosphate-buffered saline (PBS) (50 tn ~sod ium phosphate, 100m NaCl, pH 7.0)' for 24 h to yield a protein-ester conjugate. The degree of modification of the amino residues on rhPC was determined by TNBS assay (Snyder and Sobocinski, 1975).

Preparation of unmasked and FMA-masked Immunosorbent

Affi-prep is a polymeric support with reactive ester groups which react with NH2 groups on proteins to give stable amide linkages. Affi-prep was washed with 10 m~ sodium acetate buffer (pH 4.5) according to the manufacturer's instructions and was suspended as a 50% (v/v) slurry in the coupling buffer, 0.1 M MOPS, 0.15 M NaCl (pH 7.2). Figure 1 shows the preparation of an rhPC-FMA-masked immunosorbent. For the preparation of an FMA-masked immunosorbent, each of two replicate support solutions containing 1 mg of Mab was incubated with 2.4mg of rhPC-FMA (6:l molar ratio) for 2 h at room temperature (RT). In the case of the unmasked immunosorbent, Mab was incubated with coupling buffer for the same period of time.

The supernatant was pipetted from the top of the resin and FMA-Mab solution was added to the tubes and the slurry was rotated overnight at 4°C. Upon completion of the coupling reaction, the gel was allowed to settle and the supernatant was pipetted and saved for ELISA. The residual active sites on the resin were blocked with 1.0 M ethanolamine for 1 h at 4°C after which the supernatant was drawn and saved or ELISA. The gel was washed in a column mode with 0.05 M Tris, 0.1 M NaCl (pH 7.0) until the absorbance of the column effluent dropped to zero. The FMA-Mab complex was disrupted with 2 M NaSCN. The column was washed with all the buffer systems to be employed in the actual chromatographic run and the fractions were saved for Mab leakage determination. The columns were stored in the protein coupling buffer at 4°C. Emphaze AB 1 (dp=60 pm) is a polymer-based, rigid and macroporous beaded affinity support for bioseparations (Coleman et al., 1990). Emphaze AB 1 contains an azlactone functionality which readily undergoes a ring-opening reaction with amines to yield stable covalent

linkages. Mabs were immobilized on Emphaze AB1 according to the manufacturer's instructions. Briefly, each replicate treatment consisted of 125 mg of azlactone beads suspended in 1.0 mL of coupling buffer containing 0.5 mg of 8861-Mab and 1.2 mg of rhPC-FMA (6: 1 molar ratio) to yield 1 .*O* mL of affinity sorbent. On completion of the Mab immobilization step, blocking and washing steps similar to those described above were performed.

Determination of Mab bound

The amounts of Mab in feed and coupling step supernatants and washes were determined by the ELISA procedure outlined by Subramanian *et aZ.,* 1994).

Breakthrough Analysis

Breakthrough analysis of FMA-masked and unmasked immunosorbents were performed according to the procedure outlined by Subramanian *et al.* (1994). Each column was subjected to three consecutive chromatographic runs.

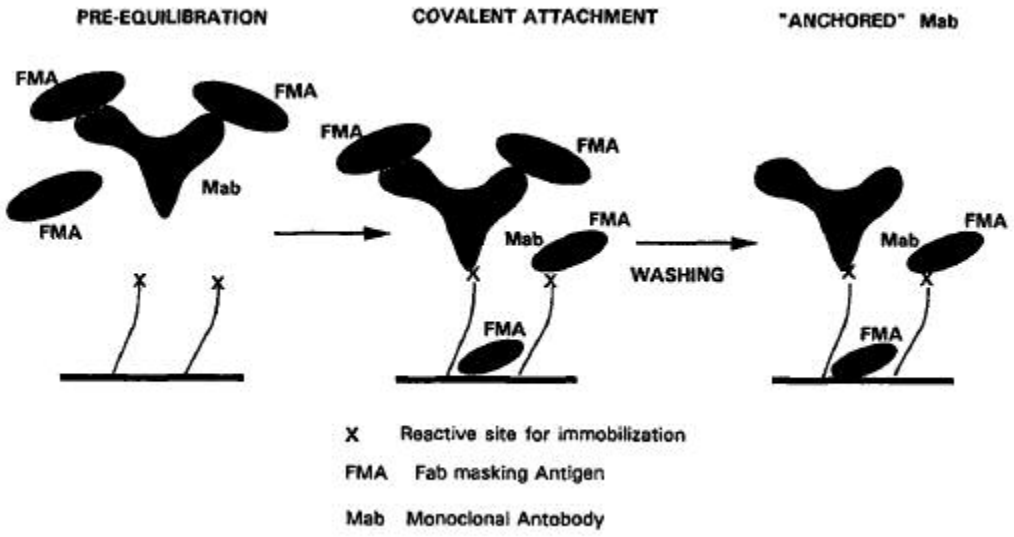

Figure 1.

Preparation of FMA-masked immunosorbent. Mab was incubated with FMA in solution prior to covalent immobilization. FMA-Mab complexes were then incubated with an activated matrix. Unreacted active sites on the matrix were inactivated and the FMA was eluted from the Mab to yield an oriented immobilized Mab.

Determination of protein C in feed and column eluates

The amount of rhPC antigen was determined by the ELISA
procedure outlined by Subramanian *et* al. (1994).

Pepsin digestion of immobilized Mab

A 200pL of a *50%* (v/v) slurry of FMA-masked or unmasked immunosorbent was pipetted into screw-capped tubes in replicates to yield a final immunosorbent volume of 100 pL. The immunosorbents were washed four times with 0.1 M sodium citrate (pH 4.2) and suspended in 100 pL of citrate buffer. A 10 pL volume of a 10 mg/mL solution of pepsin in 0.1 M acetate buffer (pH 4.5) was added to the reaction tubes and digestion was carried out at 37°C for *5* h. Samples were taken at various time intervals ranging from 0 to 360 min and digestion was terminated with an addition of 12 pL of 1 M Trisbase. The reaction mixture was centrifuged for 1Omin at 2000g and the Supernatant was subjected to total (Fab), determination. In a parallel experiment, pepsin-assisted enzymatic digestion was carried out with solution-phase Mab as a control (Johnstone and Thorpe (1987).

Determination of (Fab)2 in pepsin digest

Immulon I1 microtiter plates were coated with 100 pL per well of *5* pg/mL diluted Fab specific murine antisera in 0.1 M NaHC03 (pH 9.3) for 24 h at 4°C. The wells were washed with TBS (0.05 M Tris-O.1 M NaCl) 0.05% Tween and the residual reactive sites were blocked with TBS-O. 1% polyethylene glycol (PEG) (MW 17 *OOO)* for 10 min at RT. Various dilutions of (Fab), standard and supernatants from pepsin digest in TBS-O.176 PEG were added to the wells, Following incubation the wells were washed four times and 1000-fold diluted HRP conjugated goat-anti-mouse IgG was added to the wells and incubated for 20 min at 37°C. 100 µL in each well, and incubated for 20 min at 37°C. The wells were washed four times and 100 pL of OPD substrate were added to each well. The colorimetric reaction was stopped after 3-5 min by the addition of la0 pL of 1.5 M H2S04 to each well. Bound chromophore was detected at 490nm using a Bio-Tek EL308 microplate reader.

Results

Modification of the reactive primary amines

The number of available reactive primary amines on rhPC were modified by covalent and irreversible reaction with an ester. The extent of covalent modification was determined by the ability of modified rhPC to react with TNBS. The primary amines on proteins react with TNBS to yield a TNBS-protein complex which absorbs at 420 nm and the absorbance is directly proportional to the available reactive primary amines on the protein. Figure 2 shows the linear range of rhPC (native)-TNBS and modified rhPC-TNBS assay signals. The extent of modification was estimated from the ratio of the slopes of the modified rhPC to that of rhPC (native). Thus, the extent of covalent modification was estimated to be about 60+2%.

Antigen binding experiments

The relative binding stabilities *of* immunocomplexes formed in solution from unmodified and modified rhPC and native hPC from plasma with 8861-Mab were screened by ELISA (Subramanian *et* al., 1994). The rhPC was immunopurified to 95% purity by randomly coupled 8861-Mab (Morcol *et al.,* 1994). Figure 3 presents the ELISA signal of immunosorbed rhPC-8861 -Mab (native or modified) complex which had been first formed in solution and then immunocaptured. In general, the ELISA signal of unmodified rhPC- and hPC-8861-Mab complexes showed Immobilization of Mab in the presence of FMA gave lower

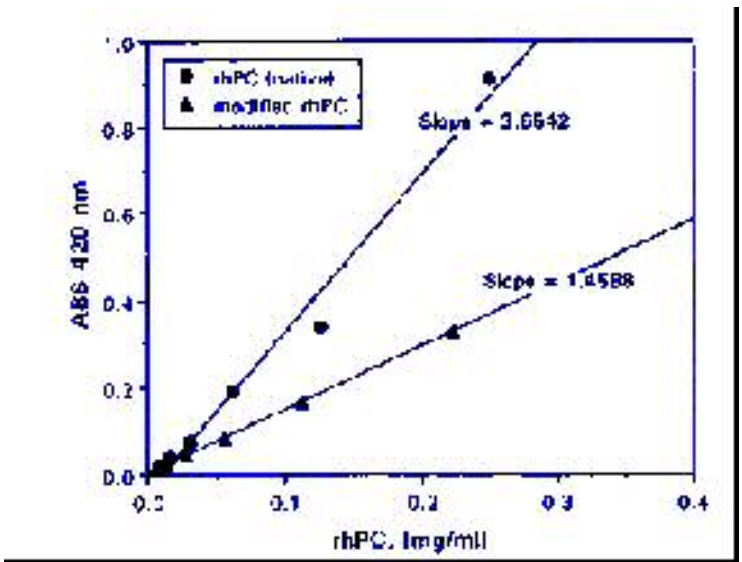

Figure2. Reactivity of rhPC (native) and modified rhPC with TNBS. The extent of modification of the amino residues on rhPC was determined by TNBS assay (Velander *et a/.,* 1992). Serial dilutions of native and modified rhPC were incubated with 0.03 M TNBS for 30 min at RT. Absorbances of the samples were read at 420 nm againt a reagent blank (assay buffer alone). The percentage modification was determined from the ratio of the slopes of the respective calibration graphs.

similar increases with increasing antigen concentration. The ELISA profile of the modified rhPC showed a slightly higher signal than unmodified rhPC and hPC at antigen to Mab molar stoichiometries of 1:l and 2:l. The profile was otherwise similar for modified rhPC (FMA), rhPC and hPC with a sharp maximum at a 6:l molar ratio of antigen to Mab (Subramanian et al., 1994).

Effect of FMA on immobilization of Mab

Table 1 gives the coupling efficiencies obtained for masked and unmasked immunosorbents using Affi-prep or Emphaze AB1. Masking of the Mab was achieved with FMA (modified rhPC). Prior to covalent immobilization, FMA was incubated with 8861-Mab at a molar ratio of 6:l.

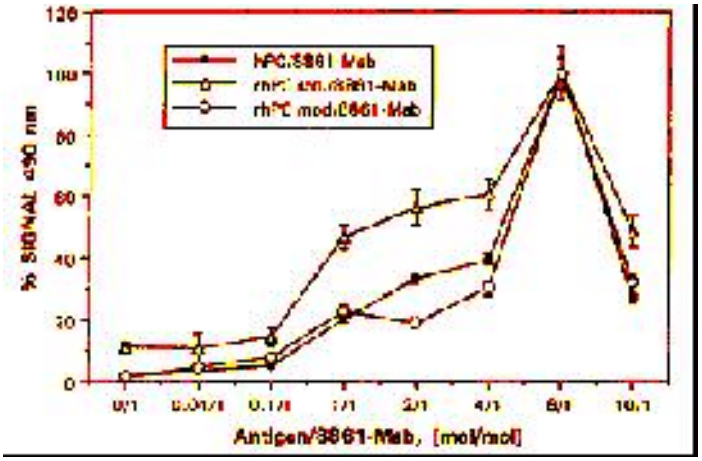

Figure 3. Antigen binding experiments. Increasing concentrations of hPC and rhPC were incubated with 8861-Mab at 1 h at 25°C and then added to rabbit anti-hPC-coated wells. After 1 h of incubation at 37"C, goat-anti-mouse IgG HRP conjugate was added to the wells and incubated for 1 h. The hPC or rhPC- 8861-Mab complex was detected and quantified with OPD and reading at 490 nm.

coupling efficiencies than Mab immobilized in the absence of FMA on both Affiprep and Emphaze AB1 (Table 1). These masked Mabs were incubated at 2-3-fold higher
concentrations to achieve similar average densities. Immobilization of unmasked and masked 8861-Mab on Affi-prep gave absolute Mab coupling efficiencies of 60-t

4% and 51 *3%, respectively, yielding 0.4 mg Mab/mL of support. Immobilization of unmasked 8861-Mab on Emphaze AB1 gave Mab coupling efficiencies of 9722% and 94i3%,
yielding 0.5 and 1.0 mg Mab/mL of support, respectively. Immobilization of masked 8861-Mab on Emphaze AB1 gave Mab coupling efficiencies of 6624% and 52i3%, yielding 0.7 and 1.1 mg Mab/mL of support, respectively. In the calculation of relative coupling efficiencies, a 100% value was assigned to unmasked immunosorbents. Thus, the presence of the FMA reduced the relative coupling efficiency of the Mab by about 15% for Affi-prep and 3045% for Emphaze AB 1.

Leaching of Mab

Prior to testing the immunosorbent capacity, three proteinfree but otherwise identical sequences of buffers used in the course of immunosorption chromatography were passed columnwise and all buffer streams were assayed for leached Mab and rhPC by EIAELISA. All product peaks from immunosorbent chromatography were assayed for leached Mab by EIA. Less than 0.3 ng Mab/mg rhPC was detected in product eluates from unmasked or masked, EMPHAZE AB1 or Affiprep immunosorbents.

Antigen binding efficiency (qAJo f immobilized Mabs

Antigen binding efficiencies of 8861-Mab immobilized on either Emphaze AB1 or Affiprep were evaluated by breakthrough analysis. Figure 4 shows typical breakthrough and elution profiles at 280nm for rhPC loaded on both masked and unmasked 8861 -Mab immunosorbent columns on either Emphaze AB1 or Affiprep. The shapes of the breakthrough profiles and 2 M naSCN elution peaks were similar for FMA-masked and unmasked 8861-Mab immunosorbents. The peak area under 2~ NaSCN eluate
fractions for FMA-masked immunosorbents were 2-3-fold higher than those for immunosorbents made with unmasked 8861-Mab at comparable Mab densities. The η_{Ag} values of immobilized 8861-Mab on Emphaze ABl and Affiprep based on the amount of antigen in the product eluents from breakthrough loading are presented in Table 2. Immobilization of unmasked and masked 8861-Mab on
Affiprep at 0.4 mg Mab/mL of gel gave η_{Ag} o f 18±3% and 48 *2 5%,* respectively. Immobilization of 'unmasked 8861-Mab on Emphaze AB1 at 0.5 and 1.0 mg Mab/mL of support gave (n,) of 2022% and 1923%, respectively. Immobilization of masked 8861-Mab on Emphaze AB1 at 0.7 and 1.1 mg Mab/mL of support gave (η_{Ag}) of 44±4% and 42±5%, respectively.

ent trials. Immobilization of masked 8861-Mab on Affi prep gave 0.11±0.05 μg of (Fab)$_2$/mg of immobilized 8861-Mab whereas unmasked 8861 on Affi-prep gave 0.023±0.001 μg of (Fab)$_2$/mg of immobilized 8861-Mab. Immobilization of masked 8861 Mab on Emphaze AB1 gave 0.036±0.006 μg of (Fab)$_2$/mg of immobilized 8861-Mab whereas unmasked 8861 on Emphaze AB1 gave 0.011±0.003 μg of (Fab)$_2$/mg of immobilized 8861-Mab.

(Fab)$_2$ Release by pepsin treatment of immunosorbents

of (Fab)$_2$ from unmasked and masked 8861-Mab immunosorbents at a Mab density of 0.5 mg/mL in the presence of pepsin. The kinetic profile of (Fab)$_2$ release from both FMA masked and unmasked immunosorbents was similar but 2–3-fold higher amounts of Fab$_2$ were released from immunosorbents made with FMA-masked Mab relative to immunosorbents made with unmasked 8861-Mab at each time point studied. Table 2 gives the total amount of (Fab)$_2$ detected per mg of immobilized Mab after 750 min of pepsin treatment. For all immobilizations studied, masked Mab released about 3–4-fold greater amounts of (Fab)$_2$/mg immobilized Mab in the pepsin digest than with immobilizations performed with unmasked Mab. The standard error was calculated from triplicate application of three independ-

Under acidic conditions, pepsin enzymatically cleaves an intact IgG molecule at the hinge region to yield a (Fab), and Fc fragment. Figure 5 represents the time-dependent release of (Fab)$_2$

To gain a better understanding of the impact of the matrix and activation chemistries upon orientation, we evaluated the performance of 8861-Mab immobilized on Affi–prep and Emphaze AB1 via random (unmasked) and oriented (masked) coupling methods. The lower coupling efficiencies seen with immobilizations to either matrix type in the

Discussion

In previous studies we used masks consisting of synthe polymer–peptide adducts to mask Mab prior to coval immobilization (Velander *et al*, 1992). Using an enzy immunoassay format on covalently activated synthe membranes, we demonstrated that higher relative immu sorbent efficiencies were obtained using masks which w larger than about 10 kDa (Velander *et al.*, 1992). The use the rhPC as a mask both provides the necessary large s and is a facile source of mask. The mask was covalen modified with chemistry which primarily targets the m reactive of rhPC lysyl moieties in order to minim coupling of the mask to the support. However, the degree modification of lysyl groups was made such that the ma retained an avidity similar to that of the precursor antig A sharp increase in the ELISA signal at ratio of 6:1 for rh or modified rhPC to 8861-Mab suggests that the second antigen binding site of the Mab possess lower avidity th the first site (Subramanian *et al.*, 1994). Therefore, shielding of the secondary site would require a hig concentration driving force to drive FMA on to that si Thus, a FMA to Mab ratio of 6:1 was used in the orientati experiments of this work to promote more compl masking of both sites in the divalent Fab domains. T effects of masking on Mab–matrix linkage stability, int particle transport to Mab sites and orientation effects at l Mab density on the adsorption/desorption kinetics of immunosorbent are discussed below.

Table 1. Effect of recombinant antigens on map coupling efficiency[a]

Support	Molar Ratio of FMA to Mab	Mab coupling efficiency (%)	Normalized Mab coupling efficiency (%)	Mab density (mg Mab/mL of gel)
Affiprep	0:1	60±4	100±7	0.4
	6:1	51±3	85±4	0.4
Emphaze AB1	0:1	97±2	100±3	0.5
	0:1	94±3	100±4	1.0
	6:1	66±4	69±5	0.7
	6:1	52±3	55±4	1.1

[a] FMA-masked and unmasked 8861-Mab were immobilized on both Affiprep and Emphaze AB1 as described in the Methods section. Mab coupling efficiency was determined as a ratio of total Mab bound to total Mab challenge. The coupling efficiency obtained for unmasked Mab was assigned a maximum value of 100% and coupling efficiencies obtained with masked Mab are reported as a percentage fraction of the maximum.

presence of FMAs are consistent with an appreciable stearic shielding of reactive lysyl groups on the Mab by the FMA. The general inertness of the FMA was indicated by both the nearly quantitative recovery of the treated rhPC after immobilization and the lack of rhPC release from matrix during subsequent conditioning washes, which included harsh conditions such as 2 M NaSCN. The stability of the Mab-matrix linkages formed from unmasked and masked Mab was not appreciably different as judged by the similarly minimal release of Mab from either unmasked or masked immunosorbents into past-coupling conditioning washes or product eluates. The lower reactivity of the masked Mab may have allowed greater permeation of the Mab into the matrix before coupoling. Therefore, a greater spatial distribution of Mab could have resulted throughout the matrix and thus altered the intraparticle transport phenomena relative to that of unmasked Mab. Analysis of rhPC breakthrough curves on immunosorbents made with masked or unmasked Mab

Table 2. Effect of recombinant antigens on immunosorbent efficiency[a]

Ratio of FMA to Mab	Immobilized Mab density	η_{Ag} (%)	$\Delta\eta_{Ag}$(%)	µg $(Fab)_2$/mg of immobilized Mab	$\Delta(Fab)_2$(%)
Affiprep					
0:1	0.4	18±3	100±4	0.023±0.001	100±4
6:1	0.4	48±5	267±7	0.11±0.05	455±55
Emphaze AB1					
0:1	0.5	22±2	100±2	0.012±0.001	100±8
0:1	1.0	19±3	100±4	0.010±0.001	100±9
6:1	0.7	44±4	220±5	0.039±0.006	352±38
6:1	1.1	42±5	210±6	0.033±0.005	321±25

[a] Affiprep and Emphaze AB1 immunosorbents described in Table 1 were challenged with pure rhPC at a concentration of 0.8–1.0 mg/mL. Pure rhPC was loaded until the breakthrough front levelled off. Bound antigen was eluted with 2 M NaSCN. The eluate fractions were assayed for rhPC by ELISA as described in the Methods section. Antigen binding efficiency was calculated assuming a 2:1 binding stoichiometry of antigen with antibody. Immunosorbents prepared with unmasked and masked antibody were subjected to pepsin digestion as described in the Methods section. The total amount of $(Fab)_2$ released was measured by ELISA. Total $(Fab)_2$ and (η_{Ag}) obtained for unmasked Mab were assigned a maximum value of 100% and (η_{Ag}) and total $(Fab)_2$ obtained with masked Mab are reported as a percentage fraction of the maximum.

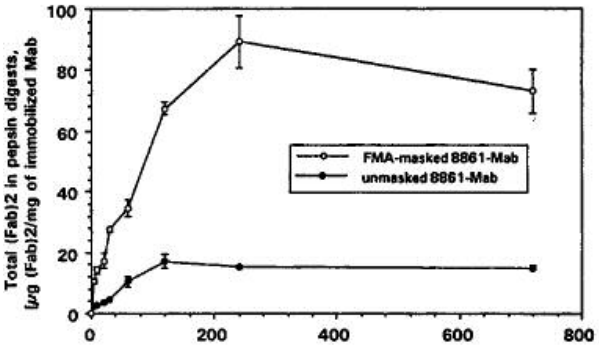

Figure 5. Enzymatic digestion of unmasked and FMA-masked immunosorbents. 100 pL of immunosorbents in 0.1 M sodium citrate (pH 4.2) were incubated at 37°C with 80 pg of pepsin. Samples were taken at the time intervals indicated and quenched with 1 M Tris base (pH 8.6). Supernatants were assayed for total [Fabl by ELISA.

did not exhibit any discernible differences in the breakthrough loading or elution profiles and thus mass transport phenomena. Further, although we did not directly investigate the intraparticle immobilization profile resulting from masked Mab, our previous work with the controlled
intraparticle distribution of unmasked Mab on Emphaze AB 1 indicated that no significant mass transfer differences occur for a broad range of intraparticle Mab spatial
distributions at immobilized loadings of about 1-12 mg Mab/mL matrix (Subramanian et *al.,* 1994). The low bead average density of Mab used here showed little impact on (f)o r couplings with unmasked Mab and is consistent with a density regime where Mab crowding is not a chief cause of the loss of activity (Tharakan *et al.,* 1990; Velander *et al.,* 1992; Subramanian *et al.,* 1994). However, it is possible that the presence of the FMAshielding could also affect local Mab crowding under conditions where the local Mab densities would be higher. We are continuing to study these combined effects of locally high Mab density and improper orientation. The higher amount of (Fab),/mg immobilized Mab released by pepsin treatment of immunosorbents prepared with masked Mab relative to unmasked Mab correlated well with the respective increases in (η_{Ag})B. oth Emphaze AB1 and Affiprep immunosorbents prepared using FMA-masked Mab gave a 2-3-fold higher (η_{Ag})a nd a 34fold higher total pepsin-mediated (Fab), release relative to immunosorbents prepared with unmasked Mab. Furthermore, the similarity of the kinetic profiles of the time-dependent release of (Fab), for either masked or unmasked Mab is consistent with oriented Mab acting as a pepsin substrate and independent of whether the orientation resulted from a random or directed coupling process. The similar adsorptiorddesorption kinetic

signatures detected by breakthrough antigen loading and elution profiles between random and directed coupling processes indicate that increases in (η_{Ag}) re probably not attributable to allosterically created conformations resulting from the presence of the mask in the binding sites of the (Fab), domain during coupling. This lack of difference in kinetic signature shown in Fig. 3 includes both antigen association under loading conditions and dissocation under washing and elution conditions. In terms of kinetics, the adsorption step of the immunosorption process for the oriented antibody can be interpreted as having a much larger association rate relative to the unoriented Mab. Properly oriented Mab may be found in either unmasked or masked produced immunosorbents, but to a much lesser extent in immunosorbents made from unmasked Mab. In summary, the kinetics of the unoriented Mab appear to be so slow as to be turned off relative to an oriented Mab, Estimation of the sizes of FMAs studied in this and our past work suggest that effective masks should approach the size of the antigen combining site on the Fab fragment of an IgG molecule. Our previous work with synthetic FMA showed that larger masks of about 5-12kDa gave higher increases in relative efficiency and a greater correlation with the accessibility of Fab domains (Eveleigh and Levy, 1977). Assuming similar globular behavior, the size of the Fab domain on an IgG molecule (45kDa) and rhPC-FMA (62 ma) can be expected to be similar on a molecular level, which is about 2-3 nm (Sarma el *af.,* 1971; Novotny *et al.,* 1983).

Conclusions

Our exepriments have shown that a greater degree of random coupling of the protein to the matrix occurs when the Mab is unmasked and that oriented coupling occurs when the Mab is masked with FMA. We have shown quantitatively that orientation plays a primary role in immunosorbent activity where orientation phenomena may be impacting about 50% of the immobilized Mab functionality. FMAs can be used to increase the percentage of functional antibody relative to that obtained using random
covalent attachment without altering the Mab or the support matrix. The orientation phenomena achieved by masking the immunoglobulin prior to immobilization appear to be independent of the surface activation chemistry and support matrix.

Acknowledgements

This work was supported by NSF grant BCS-9011098. We sincerely appreciate the comments and suggestions of Dr H. Lubon with the American Red Cross (Rockville, MD). The technical assistance of K. Hess *is* greatly appreciated. The gift of 8861-Mab and 8861-Mab immunopurified hFC from the ARC is greatly appreciated.

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