IDENTIFICATION OF THE FACTORS IMPACTING IMMUNOSORBENT PERFORMANCE

by

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Dissertation submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in Chemical Engineering

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November 1993

Blacksburg, Virginia

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(ABSTRACT)

Immunoaffinity interactions can play a vital role in the purification of therapeutic proteins. In most cases immunoaffinity chromatography is used as the final clean up step to yield a highly pure product. The customized avidity of the parent antibody for a given antigen or hapten can make immunoaffinity chromatography an indispensable tool in the field of bioseparations. However, the use of immunoaffinity chromatography at a large scale is frequently precluded by the high capital costs of antibody columns. This is in part due to the low functional activity of immobilized antibodies and the high cost of pathogen free antibody.

The objective of this research was to detail the impact of orientation, multipoint attachment and high local density upon the functional activity of immobilized antibodies. Immobilization methods were designed to affect changes in both orientation and local density. Both metal and pH dependant murine monoclonal antibodies against human Protein C were used as model

systems. The character of the antigen and antibody were modified to study the impact of orientation, local density and multipoint attachment. For example, modified antigens of various sizes having a single binding epitope were synthesized from polymer-peptide adducts. The lysyl residues of recombinant hPC were chemically modified to reduce chemical reactivity with the matrix while maintaining avidity for the Mab. The synthetic and recombinant antigens were used to mask the antigen binding regions (Fab) of a monoclonal antibody (Mab) prior to covalent immobilization on a porous membrane and beaded supports. In addition, lysyl residues of Mabs against hPC were chemically modified to reduce multipoint covalent attachment.

A 2-step method consisting of permeation and immobilization of antibodies was developed to manipulate local Mab density. Conventional single step immobilization by simultaneous reaction and diffusion produced highly local shell-like density. Immunosorbents made with the 2-Step method at greater than 3 mg Mab / ml gave 2-3 fold higher antigen binding efficiencies and a more uniform distribution of immobilized antibody.

Immunosorbents made from Mabs which were immobilized at low local density having masks consisting of large synthetic or recombinant antigens gave the highest antigen binding efficiency and the most accessible Fab domains to pepsin digest. Mabs at high or low densities containing modified or

unmodified lysyl residues gave no difference in antigen binding efficiencies or accessible Fab domains to pepsin digests. In summary, for Mabs immobilized at low densities "orientation" of the antibody on the support was identified as having the most negative impact on immunosorbent performance with multipoint attachment being less important. At high antibody loadings the functional activity of antibodies is also impacted by high local density superimposed with orientation.

ACKNOWLEDGEMENTS

I wish to acknowledge the generous support and continued guidance of Dr. Bill Velander, which was essential to the completion of my doctoral thesis. I would like extend my gratitude to Dr. Bill Velander for providing me with a challenging yet educative research environment within the Biochemical Engineering Laboratory at Virginia Tech.

I also wish to thank my committee members Dr. W. N. Drohan, Dr. W. G. Glasser, Dr. W. L. Conger and Dr. R. M. Davis for their suggestions and participation. Special appreciation and thanks are also due to my colleagues and friends at 153 Randolph Hall for making my graduate experience a memorable one. I wish to thank Anand for his assistance and patience. A very special thank you to my son Advaith for being such a wonderful child.

Most importantly, I wish to thank my parents for their undivided love and encouragement. This academic experience of mine is a direct result of my father's firm belief in higher education and his confidence in my abilities to undertake devoted graduate research. Their support and trust over the years has meant a lot to me and to them I dedicate this thesis with love.

To

Appa (R. B. Subramanian)

Amma (K. V. Parvathi)

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CHAPTER ONE

BACKGROUND

INTRODUCTION

The recent developments in recombinant DNA technology have enabled the synthesis of valuable therapeutic proteins in bacterial cells as well as in transgenic farm animals. However, the purification of proteins of interest from either the conventional sources, cell culture or novel routes, in a highly purified form necessitates the development of separation techniques capable of recovering proteins from these feed streams in a highly purified form. The purification of therapeutic proteins from biological sources is usually complicated by the presence of endogenous proteins.

The commonly used methods of protein purification on a laboratory scale is summarized in Table-1 (1). Purification methodologies based on ion-exchange or adsorption serve as excellent pre-purification steps but they fail to resolve complex protein mixtures to yield a single purified protein product. Purification techniques based on affinity interactions between molecules (ie. immunoaffinity chromatography) have rapidly evolved using a variety of biological and synthetic ligands.

IMMUNOAFFINITY CHROMATOGRAPHY

Immunoaffinity chromatography is a process in which the binding affinity of a antigen to a parent antibody is utilized as a basis of separation. Antibodies

recognize specific regions of other molecules and bind them by a web of low energy forces (bonds) including ionic, hydrogen and van der waals. Hydrophobic interactions can also contribute to binding energy. The use of monoclonal antibodies (Mab), which display a high degree of avidity for a specific binding site (epitope) on a molecule (antigen and hapten) has greatly impacted immunoaffinity chromatography. The antibody specific to the protein of interest is immobilized onto a rigid solid support to yield an active immunosorbent and a complex mixture of proteins is then passed over the support whereby the antibody captures the protein of interest and the other non-product proteins are washed away in the column fall through. By taking advantage of the metal dependency, pH or chaotropic reagents like NaSCN/Urea, the reversible interaction between the antigen and antibody can be disrupted to yield a highly purified product in the column eluate (2). Due to the customized avidity and specificity, monoclonal antibodies have become indispensable for both protein characterization and purification.

The high costs involved with the production of monoclonal antibodies makes immunoaffinity chromatography a cost-intensive purification technique, both at laboratory and industrial scale. Furthermore, large scale applications of immunoaffinity chromatography have been limited by the low functional efficiency which results when antibodies have been covalently anchored to

supports. Increasing the immunosorbent efficiency would reduce antibody and support matrix costs. It is estimated that buffer costs contribute about 40% of the processing cost in large scale chromatographic purifications. Lowering the operating cost of immunosorbents with higher functional efficiency of immobilized Mab, would also reduce buffer costs by decreasing column-wash and product eluate volumes.

Optimization of the immunosorbent performance requires an understanding of the factors which affect the activity of immobilized antibodies. The aim of this research is to recognize and rank the factors which affect the functional efficiency of immobilized antibodies. A brief description of the factors impacting immunosorbent performance are included in this introductory section:

- Synthesis of Monoclonal Antibodies
- Structure Function Relationship of Antibodies
- Factors Affecting Immunosorbent Performance

SYNTHESIS OF MONOCLONAL ANTIBODIES

Antibodies or immunoglobulins are proteins synthesized by the B lymphocytes (B cells) of an animal in response to the presence of a foreign protein in its blood. By the inherent nature of its synthesis, antibodies possess

specific affinity for the antigen that elicited its synthesis. The antigen forms a reversible non-covalent complex with the antibody through the binding epitope on the antibody, which is similar to the protein which elicited antibody synthesis. An antigen or a protein which is totally foreign to the immunological system of the host animal will elicit antibody synthesis directed against different deterministic sites on its surface. The overall population of antibodies synthesized by a variety B cells is known as polyclonal antisera or antibody. As polyclonal antibodies can bind to an antigen through a variety of epitopes, typically a wide range of binding affinities are found. This multivalent nature of polyclonal antibodies due to their heterogeneous population precludes their wide spread application in affinity bio-separations. Monoclonal antibodies offer a degree of reproducibility that is difficult to achieve with polyclonal antibodies.

The synthesis of monoclonal antibodies by Milstein has speeded the advances in affinity chromatography (3). The unique specificity that a monoclonal antibody displays for an antigen makes it an invaluable tool in affinity chromatography. Monoclonal antibodies are synthesized by the fusion of homologous antibody cell line with a murine myeloma cell (cancerous cell) to yield a hybridoma capable of rapid but sincere replication. Antibodies produced in such a manner exhibit unique specificity for the antigen and are uniform in structure and function.

STRUCTURE FUNCTION RELATIONSHIPS OF ANTIBODIES

Immunoglobulin (IgG) is an approximately 150,000 molecular weight glycoprotein consisting of two identical heavy and light chains. The antigen combining site (antitope or paratope) on the antibody, is found on the N-terminal region of either the heavy or light chains. These antigen binding domains on the antibody are known as the Fab regions. Non-antigen binding sites on the antibody involved in cell receptor functions are known as Fc regions. The association constant for the binding of antigens to antibodies (free to bound) is about 10-8 -10-10 M. The symmetry of the Fab fragments on the antibody molecule predetermines a theoretical antigen binding stoichiometry of 2:1 (molar basis) (4). However, large antigens may interact with only one of the Fab domains, with the second site being inaccessible due to steric hindrance caused by occupation of the first site. Primary amines (lysine residues) are found throughout the antibody molecule whereas CHO moieties are mainly found in the Fc region. Most of the linker chemistries engineer covalent attachment of the antibody via the primary amines or the CHO residues.

IMMUNOSORBENT PERFORMANCE

The performance of an immunosorbent is dependent on physical characteristics of the matrix, surface density of the antibody and "orientation"

of Mab (5). A high ratio of reactive coupling ligands per unit surface area of the matrix may lead to clustering of antibodies close to the surface of the matrix thereby reducing the antigen binding capacity by steric hinderance. It is imperative for the immobilized antibody to be in the right "orientation" to specifically interact with the antigen in solution. For the immunosorbent to retain maximum theoretical antigen binding capacity, the Fab domains should be shielded during the immobilization process. This will ensure that the Mab is not anchored to the matrix via the -NH2 moieties found on the Fab region thus rendering the antigen binding site inactive. Multipoint attachment of the antibody to the matrix may also lead to a loss in immunosorbent performance. Distortion in the structure of the antibody may occur due to improper orientation or multipoint attachment, thus affecting the affinity for the antigen. Various methodologies have been developed to enhance the antigen binding activity of immobilized antibodies and these will be discussed in the sections to follow.

SUPPORT CHARACTERISTICS

The immunosorbent performance is dependent on the support matrix upon which the Mab is immobilized. Efficient immunosorbents should possess mechanical/physical stability, good flow properties, acceptable pressure drop, minimal non-specific binding, surface area for Ab-Ag interactions and chemical

stability. Polymeric and/or agarose based supports are extensively used in affinity chromatography. Both of above mentioned supports lend themselves to a variety of conjugation chemistries and offer reasonable physical/mechanical properties and are resistant to the various solvent systems used in affinity chromatography. One of the ways to enhance the immunosorbent performance is to engineer the support matrix such that maximum antibody activity is retained. The functioning of an immunosorbent column is dependent on the activation chemistry used couple the antibody to the matrix.

SURFACE DENSITY

The effect of surface density (Mab/ml of resin) on the antigen binding characteristics of the antibody has been investigated in detail. The performance of the immunosorbent was found to be dependent on the amount of Mab immobilized. A decrease in the immunosorbent activity was observed at very high levels of antibody density. At high surface concentration of immobilized Mab, the antigen binding sites of the antibodies may be in close proximity thereby causing steric hinderance as well as restricting the access of antigen to the binding epitope on the Mab (6-13).

In order to reduce the deleterious impact of surface density, antibodies were immobilized onto a variety of matrices using spacer arms or molecular

extenders (14). Immobilization of ligand at a distance from the matrix reduces of surface density by minimizing the ligand concentration per unit surface area of the matrix. Mabs against Protein S and Protein Z when immobilized on beaded supports using molecular extenders failed to show any appreciable improvement in functional efficiency.

ORIENTED IMMOBILIZATION (VIA Fc REGION OF THE Mab)

Maximum antigen binding can be attained by immobilizing the Mab via the Fc regions. One method of accomplishing such an "oriented" coupling is to utilize the affinity of protein A to bind Mabs via their Fc domains. In this method protein A is first covalently anchored to a matrix, whereupon the antibody of interest is coupled with protein A followed by cross-linking of the complex thus generating an active immunosorbent. Monoclonal antibodies anchored in this fashion were used to isolate transferrin receptor and leukemia-associated antigens from cell lysates (15). The potential of protein A leakage in the product coupled with varying affinity of protein A for Mabs from different species makes this technique very tedious. Recently protein G has been employed to co-anchor Mab to the matrix.

Recent efforts have been focused on the immobilization of Mabs via their Fc region by using "Hydrazide Chemistry". Most monoclonal antibodies used

in immunoaffinity chromatography belong to the family of glycoproteins, whose -CHO moieties are located at sites distant from the Fab domains and commonly found in the Fc domains. Chemical modification of -CHO moieties on the Fc domain of the Mab will not affect its antigen binding activity. Immobilization of the Mab via CHO moieties requires derivatization of agarose based matrices with hydrazine hydrate to yield reactive hydrazido (NHNH2) functionality. The CHO moieties on Mabs are oxidized in the presence of sodium metaperiodate to yield aldehyde groups. Oxidized Mab interacts with the activated support to yield stable hydrazone linkages (16). A variety of monoclonal and polyclonal antibodies were immobilized on beaded supports via "hydrazide chemistry" (17-21). An 2-10 fold enhancement in immunosorbent performance was observed for immobilized Mabs (17-21). No significant improvement in the performance of immobilized polyclonal antibodies were observed (17).

NON-SPECIFIC PROTECTION OF REACTIVE PRIMARY AMINES

A more recent effort to enhance the bound activity of both polyclonal and monoclonal antibody is to reversibly but non-specifically protect the reactive NH2 groups on the antibody molecule by dimethyl maleic anhydride (DMA) prior to covalent immobilization (22). Mabs reversibly but non-specifically protected with DMA gave 2-5 fold higher immunosorbent performance (23).

Previous studies did not quantitatively correlate antigen binding efficiency to the effects of orientation, local density and multipoint attachment of the immobilized antibody. In the present study, we use modified antibodies and antigens to quantitatively probe the impact of these effects upon antigen binding efficiency.

REFERENCES

1 Chase, H. L., (1983). Chemical Engineering Science, 39 (No. 7/8), 1099-1125.

2 Velander, W. H., (1989), Bio/Technology, 5(No. 3), 119-124.

3 Milstein, C., (1980). Scientific American, 234 (No. 4), 66-70.

4 McCoy, M. A., and Liapis, A. I, J. Chromatogr., 548 (1991) 25-34.

5 Velander, W. H., Subramanian, A., Madurawe, R. D., and Orthner, C. L., (1992). Biotechnology and Bioengineering, 39, 1013-1023.

6 Eveleigh, J. W., and Levy, D. E, J. Solid-Phase Biochem., 2 (1977) 45-77.

7 Fowell, S. L., and Chase, H. A, J. Biotechnol., 4 (1986) 1-13.

8 Tharakan, J. P., Clark, D. B., and Drohan, W. N, J. Chromatogr., 522 (1990) 153-162.

9 Katoh, S, TIBTECH, 5 (1987) 328-331.

10 Weston, P. D., and Scorer, R, Affinity Chromatography, (1978) 207-210.

11 Narayanan, S. R., and Crane, L. J, TIBTECH, 8 (1990) 12.

12 Sportsman, J. R., and G. S. Wilson, Anal. Chem., 52 (1980) 2013.

13 Wu, D., and Walters, R. R, J. Chromatogr., 458 (1988) 169.

14 Strauss, W. M., Broze, G. J., Milteich, J. P, and Null, H. R., (1988),

Applied Biochemistry and Biotechnology, 9, 462.

15 Schneider, C., Newman., R. A., Sutherland, R., Asser, U., and Greaves, M. F., (1982). Journal of Biological Chemistry, 257 (18), 10766-10769.

16 O'Shanessey, D. J., and Hoffman, W. L., (1987). Biotechnology and Applied Biochemistry, 9, 488-496.

17 Little, M. C., Siebert, C. J., and Matson, R. S., (1988). BioChromatography, 3 (4), 156-160.

18 Fleminger, G., Hadas, E., Wolf, T., and Solomon, B., (1990). Applied Biochemistry and Biotechnology, 23, 123-137.

19 Domen, P. L., Nevens, J. R., Mallia, A. K., Hermanson, G. T., and Klenk, D. C., (1990). Journal of Chromatography, 510, 293-302.

20 Bonde, M., Pontoppidan, H., Pepper, D. S., (1991). Preparative Chromatography, 1 (3), 269-277.

21 Orthner, C. L., Highsmith, F. A., Tharakan, J., Madurawe, R. D., Morcol, T. M., and Velander, W. H, (1991). Journal of Chromatography, 558, 55-70.

22 Endo, N., Umemoto, N., Kato, Y., Takeda, Y., and Hara, T., (1987). Journal of Immunological Methods, 104, 253-259.

23 Hadas, E., Koppel, R., Schwartz, F., Raviv, R., and Fleminger, G., (1990). Journal of Chomatography, 510, 303-309.

TABLE 1

METHODS OF PROTEIN PURIFICATION

METHOD	DEGREE OF RESOLUTION OF PROTEINS	EASE OF SCALE-UP
Ultrafiltration	low	easy
Precipitation	low/medium	easy
Molecular exclusion	Electro-methods	medium
Electrophoresis		
Electrofocusing	medium/high	difficult
Isotachophoresis		
Adsorption Methods		
Adsorption	medium	medium
Ion-exchange	medium	medium
Affinity	high	medium

Adapted from (1).

CHAPTER TWO

POLYOXAZOLINE-PEPTIDE ADDUCTS WHICH RETAIN ANTIBODY AVIDITY

SYNTHETIC ADDUCTS RETAINING ANTIBODY AVIDITY

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ABSTRACT

Synthetic polymers have long been used to modify various properties of proteins such as activity and solubility. Polyethylene glycol (PEG) has been widely used to form adducts with enzymes and antibodies. In this study, the polyoxazoline family of water-soluble polymers were used to synthesize adducts containing a synthetic peptide recognized by a monoclonal antibody (Mab) directed against human protein C (hPC). This is the first application of direct conjugation of unterminated or "living" polymer to a peptide. The avidity of the antibody for the various adducts was characterized with respect to size and hydrophilicity of methyl- and ethyl-substituted polyoxazoline polymers (POX). Avidity of the adducts was not found to be dependent upon the hydrophilicity and was slightly decreased due to polymer modification. The methyl-POX-peptide adducts were found to be highly water soluble while the ethyl-POX-peptide adducts showed sporadic problems with aqueous solubility. Because the polymer-peptide adducts retained avidity for the antibody, polyoxazoline polymers may have potential application to protein-adduct chemistry.

INTRODUCTION

The synthesis of protein-polymer adducts has been reported for antibodies (3), enzymes (1,2,4,5,7,8,11,13,15,18,22,), and peptides (6). Most often, these chimeric macromolecules have modified biochemical activity relative to that of the parent protein. This activity could be of an enzymatic, antigenic or of a receptor-ligand nature. In some instances, chimeric polymer-peptides have an increased immunogenicity relative to the smaller parent peptide (6). The covalent addition of a polymer to a protein can also change the solubility of the parent protein (8). The degree to which any of the above properties have been changed has varied with molecular weight of the polymer and the number of modification sites on the protein (4,11).

Polymer derivatization of proteins has been shown to increase circulation half-lives in animal models (5,15) as well as in humans. Water soluble polymers such as polyethylene glycol (PEG) are often used in these studies because PEG is amenable to conjugation chemistry and is generally considered to be non-antigenic. PEG has been conjugated to recombinant-human tissue plasminogen activator (5), streptokinase (11,15), urokinase (18), and albumin (8) by reagents such as 1,1'-carbonyldiimidazole. A range of functional activities has been found for these adducts (11,15). A higher substitution of amino groups by polymer on the parent protein has been found to result in

lower functional activity (11). In other experiments, the inhibitory activity of α_2-macroglobulin was found to be reduced by modification with PEG (4), whereupon decreased α_2-macroglobulin activity was found as the molecular weight of the PEG was increased.

Avidity between antibody and respective antigen is an example of another protein interaction which can be affected by polymer modification. The relationship between avidity and the size of the polymer which is used to derivatize the antigen has been evaluated for streptokinase derived from hemolytic streptococci (11). These studies found that agglutination reactivity was reduced as the amount of covalent linkages to streptokinase with PEG of Mr = 5000 was increased. It was presumed that steric hindrance masked the binding of the modified protein to the immunoglobulins.

In this work, we have sought to characterize the effect of polymer size and hydrophilicity upon the avidity of a synthetic peptide for a monoclonal antibody (Mab) against hPC. This peptide, HCPC[6-17], corresponds to residues 6-17 of the heavy chain of hPC and has been shown to contain the binding site (epitope) for the monoclonal antibody referred to as HPC4-Mab (20). Both hPC as well as the HCPC[6-17] peptide bind to HPC4-Mab in a Ca^{2+}-dependent manner. The polymers used to modify HCPC[6-17] are

members of the polyoxazoline (POX) family of water-soluble polymers (9,10,12,16,17). We have discussed the preliminary findings of this work at the ACS Annual Meeting, Miami (September 1989). This is the first study of POX-adduct chemistry which uses unterminated or "living" polymer for direct coupling to lysyl-amino groups of a peptide. More recently, terminated POX polymers have been derivatized with carbodiimide and then coupled to catalase (12). In the present study, POX polymers which varied in both size and hydrophilicity were synthesized. Hydrophilicity was varied by the polymerization of a 2-ethyloxazoline versus a less hydrophobic 2-methyloxazoline monomer. The retention of avidity for the HPC4-Mab by the HCPC[6-17] after polymer modification demonstrates the potential that POX-polymers may have for applications to protein-adduct chemistry.

MATERIALS AND METHODS

Monomeric 2-ethyloxazoline and 2-methyloxazoline were purchased from Aldrich Chemical Co. (Milwaukee, WI.) A peptide corresponding to residues 6-17 of the heavy chain of human Protein C (HCPC[6-17]) was made by t-boc synthesis. The sequence of HCPC[6-17] is given in Figure 1. The methyl benzhydrylamine-HCL anchoring resin for peptide synthesis was purchased from Colorado Biotechnology Associates. (Boulder, CO.) Murine monoclonal antibody to hPC, HPC4-Mab, was provided by Dr. Charles T. Esmon of the Oklahoma

Medical Research Foundation. (Oklahoma City, OK.) Rabbit antisera against human Protein C was purchased from American Bioproducts. (Parsippany, NJ.) Affinity purified goat, anti-mouse immunoglobulins conjugated to horseradish peroxidase (HRP) were purchased from Sigma. (St. Louis, MO.) O-phenylenediamine-2HCl tablets were purchased from Abbott Labs. (Chicago, IL.) Immulon II microtiter plates were purchased from Dynatech Laboratories Inc. (Chantilly, VA.) All reagents were of the best commercial grade available. Human Protein C (hPC) was isolated by immunoaffinity methods as described by Orthner et al. (14).

Synthesis of Polymer-Peptide Adducts

Synthesis of "living" polyoxazolines.

Polymer sizes of 1K, 5K, and 10K poly(2-ethyloxazoline) were made using the method described by Sinai-Zingde et al. (19). Polymer sizes of 1K, 5K, and 10K poly(2-methyloxazoline) were also made using this same method. To explain the procedure in brief, benzyl iodide was used to initiate the polymerization reaction with either 2-methyloxazoline or 2-ethyloxazoline at 90°C in acetonitrile. A specific molar ratio of initiator to monomer was reacted whereupon the amount of monomer charged was stoichiometrically determined by the desired polymer chain length. For example, a 50 molar excess of initiator to monomer was used in the synthesis of 5 K POX and a 100 molar excess

was used in the synthesis of 10 K POX (19). The propagation reactions were monitored by proton nuclear magnetic resonance spectroscopy (pNMR). Typical batch sizes were 5 ml of a 250 mg/ml "living" polymer solution. "Living" polymer in acetonitrile was apportioned into 200 microliter aliquots (typically containing 5 to 50 mg polymer) into tapered 1.5 ml Eppendorf plastic microtubes using an Eppendorf pipette with plastic disposable pipette tips. The dry weight of the "living polymers" were determined after vacuum-drying at 0.1 torr overnight using a Savant centrifugal Speedvac Concentrator.

Activation of Synthetic Peptide HCPC[6-17].

The epsilon amino roup of the carboxy-terminal lysine residue of the HCPC[6-17] peptide was activated using the following procedure. One ml of 30 mg per ml aqueous, dibasic sodium phosphate, at either pH 10.5 or pH 12.4 at 0°C was added to 15 mg peptide. This is approximately a three-fold molar excess of phosphate for a theoretical maximum of 9 titratable protons present in the peptide. This mixture was quickly mixed by vortex and frozen at -90°C for 5 minutes to minimize hydrolysis of the peptide. The frozen mixture was then lyophilized at 0.1 torr using a Savant centrifugal Speedvac concentrator for 3 hours. The titrated peptide-phosphate solid mixture was extracted with 1 ml of cold methanol by vortex mixing followed by centrifugation at 1000 x g for 10 minutes. An essentially quantitative extraction of the peptide from the

phosphate solids was achieved using this method as judged by competitive enzyme-linked immunosorbent assay (ELISA). This ELISA procedure is given below. The titrated stock solution was stored in methanol at -25°C.

Termination of the "Living" Polyoxazoline Using Activated HCPC[6-17].

Methanol was found to solubilize both polymer and peptide and did not appear to terminate the "living" polymer. Thus, methanol was chosen as a good solvent for the termination step. The vacuum-dried "living" polymer was reconstituted with 1 ml or less of methanol using a vortex mixer. This polymer-methanol solution was added to activated HCPC[6-17] every hour at room temperature in aliquots of 20% of the theoretical amount needed for a 1:1 termination of polymer by peptide. A final incubation at 40°C for a minimum of two hours was performed before the reaction mass was vacuum-dried for 3 hours at 0.1 torr and then reconstituted in nanopure, deionized water. Reaction products made from 20 K and 40 K POX "living polymers" were mostly insoluble in aqueous media. The proposed reaction sequence for generation of the live polymer and termination with peptide is given in Figure 2.

Purification of the Reaction Mass

An anion-exchange liquid chromatography method using DEAE-Sephacel was developed for separation of free peptide from polymer-peptide adduct at room temperature. Aliquots of reaction mixtures containing 5 mg of total peptide were dissolved in 1 ml of D.I.H_2O(pH 7.0) and applied to a 2.54 cm diameter x 10 cm bed length column and eluted at a flow rate of 1.0 ml per minute with degassed, D.I.H_2O at pH 7.0, U.V. absorbance of the column effluent was detected at 214 nm. A step change to 1M NaCl was made after a stable baseline was obtained following the elution of peaks using D.I.H_2O. Chromatographic fractions were lyophilized, weighed and reconstituted with 1 ml of deuterium oxide (D_2O) and assayed by 200 MHz pNMR. The products were then recovered from the NMR tubes and lyophilized. While most reaction products initially tended to be wholly aqueous soluble (in excess of 5 mg adduct per ml H2O), sporadic aqueous solubility problems occurred with poly(2-ethyloxazoline) adducts after vacuum-drying. Dissolution of these adducts was facilitated in a TBS-buffer. The parent ethyl-POX and methyl-POX polymers, HCPC[6-17] peptide and poly(2-methyloxazoline)-peptide adducts did not show solubility problems in water or TBS-buffered aqueous media after vacuum-drying.

Competition Studies of Purified Polymer-Peptide Adducts

Immulon II plastic microtiter plates (96-well) were coated with 100 μl of rabbit derived, anti-human Protein C antisera diluted 1:200 with 0.1 M sodium bicarbonate buffer at pH 9.6, for one hour at 37°C. Residual reactive sites were blocked with 0.1 M $NaHCO_3$, pH 9.6, 1% BSA for 1 hour at 37°C. The wells were then washed with 0.05 M Tris, 0.15 M NaCl, pH 7.5 (TBS), containing 0.1 % Tween 20. Solutions of TBS, 0.1% BSA, 25 mM $CaCl_2$, 0.032 μM HPC4 monoclonal antibody, 0.032 μM human Protein C, and polymer-peptide adduct ranging from 0.0064 to 100 μM which had been pre-equilibrated for 1 hour at 25°C were applied (100 μl) to the IgG-coated wells and incubated at 25°C for 1 hour. After washing (TBS, 0.1% Tween 20, 25 mM $CaCl_2$), 100 μl of 1:1000 diluted HRP-conjugated goat anti-mouse IgG was applied to the wells. After a 1 hour incubation and washing with the same buffer, o-phenylenediamine dihydrochloride (OPD) chromogenic substrate was added to each well and the reaction was allowed to proceed at room temperature for 5-10 minutes. Detection of the chromophore was made at 490 nm using a Molecular Devices Vmax kinetic microtiter or Bio-Tek Instruments EL308 plate reader after quenching each well with 100 μl of 3 M H_2SO_4.

RESULTS

The titration of the peptide at pH 10.5 and subsequent reaction with "living" polymer showed little reactivity based upon the amount of free peptide recovered from the reaction mass by anion exchange chromatography and also the absence of pNMR signal attributable to peptide in water-elutable peaks. Anion exchange chromatography and pNMR analysis of the reaction mass consisting of the "living" polymer peptide titrated at pH 12.0-12.4 is shown in Figure 3 and Figure 4, respectively.

An example of a DEAE-Sephacel chromatographic separation of a 1K poly(2-methyloxazoline)-peptide reaction mass detected at 214 nm is shown in Figure 3. Anion-exchange chromatography resulted in three distinguishable elution peaks. The first two peaks (labeled A and B) were eluted with water. Typically at the exprimental conditions stated in the text, the water-elutable peaks appeared approximately at half the column volume. The third peak (labeled C) was eluted with 1 M NaCl. Proton-nuclear magnetic resonance spectroscopy (pNMR) of these peaks are presented in Figure 4. Figure 4a presents the pNMR spectra of a peptide reference. The multiple signal centered at approximately 0.9 ppm is characteristic of the methyl protons associated with leucine and isoleucine. A reference spectra for 1 K methyl-POX in D_2O is provided in Figure 4b. The proton signals with a maximum peak height at

1.9 ppm are characteristic of the 2-methyl protons of the methyl-POX. The pNMR spectra of the first peak (lyophilized and reconstituted in D_2O) of Figure 3 was similar to that of Figure 4b (data not shown). The pNMR spectra of the second peak of Figure 3 is shown in Figure 4c. Proton signals corresponding to both the 2-methyl protons of polymer and the methyl protons of isoleucine and leucine were seen in the second peak. The third peak of Figure 3 possessed a pNMR spectra identical to the reference peptide given in Figure 4a (data not shown). Furthermore, a control chromatography with peptide only produced a single NaCl elution peak which coincided with the third peak of Figure 3 and which possessed a pNMR spectra identical to the reference peptide given in Figure 4a (data not shown). Because the peptide required 1 M NaCl for elution, we conclude that the second water-elutable peak contained peptide covalently linked to the polymer, but no free peptide. Chromatography of the parent ethyl- and methyl-POX polymers gave a water-elutable peak which was very similar to the elution behavior of peak A obtained for the polymer-peptide reaction masses for either of the ethyl- or methyl-POX (data not shown). However, fractionation of water-elutable peaks was performed under similar conditions for ethyl-POX reaction masses because the resolution of peak A and peak B was sometimes poor (data not shown). The chromatographic fractions obtained from ethyl-POX reaction masses which possessed pNMR signals similar to that of Figure 4c were used in the avidity

studies discussed below. In the synthesis of both ethyl- and methyl-POX peptide adducts an average yield of 30% was acheived, based upon yield of polymer-peptide adducts from two or more separate experiments.

The relative avidity of the HPC4-Mab for the HCPC[6-17], poly (2-ethyloxazoline) polymers and polymer-peptide adducts was measured by their ability to compete with hPC for binding to the Mab in solution. The avidity between the HPC4 and purified polymer-peptide adducts was evaluated by competitive enzyme-linked immunosorbent assay (ELISA). HPC4, hPC and polymer-peptide adducts purified by anion exchange chromatography as described above were pre-equilibrated in solution. The resultant amount of hPC-HPC4 complex was then immunosorbed and detected by the chromophore generated from a sandwich of rabbit-derived anti-hPC immunoglobulin (IgG) and horseradish peroxidase conjugated (HRP), anti-mouse IgG derived from goat in the presence of OPD. A decrease in absorbance at 490 nm as a function of increasing concentration of polymer-peptide adducts was indicative of the avidity of the adducts for the HPC4-Mab.

None of the parent ethyl- or methyl-POX polymers showed competition with hPC for HPC4-Mab over the 0.05 to 100 μM range (See Figure 5a and Figure 5b). The half-maximal inhibition of hPC binding siganl to HPC4-Mab in

the presence of HCPC[6-17] peptide occured at about 0.08 μM peptide (Figure 6 and Figure 7). Thus, the HPC4-Mab was observed to possess a similar avidity for the HCPC[6-17] as the parent hPC which was present at a concentration of approximately 0.032 μM. Both titrated (used to maximize reactivity with the living polymer) and untitrated peptide possessed virtually the same avidity as measured using this same assay (data not shown). Polymer-peptide adducts recovered from water-elutable peaks of the ethyl-POX reaction mass possessed half-maximal inhibitions which occured between 0.7-2 μM adduct (See Figure 6). Polymer-peptide adducts from the water-elutable peaks of the methyl-POX reaction mass possessed half-maximal inhibitions which occured between 0.3-2 μM adduct (Figure 7).

DISCUSSION

Polyoxazolines possess the advantage of being synthesized as "living" polymers with no intermediate activation step (16, 10, 19, 23). Termination has been made by reaction with primary amines such as hexylamine but not readily with alcohols. Thus, the reaction with the carboxy-terminal, ϵ-alkyl amino group of the HCPC[6-17] peptide was expected (Velander W.H, ACS abstracts, 1989). In contrast, the reaction with the α-terminal amino residue of HCPC[6-17] was expected to be less predominant due to differences in nucleophilicity as reflected by the lower pKa of the α-amino group. Indeed, a

greater reactivity was seen for the HCPC[6-17] peptide titrated at pH 12.4 relative to that titrated at pH 10.5. Thus, the primary reaction was probably with the carboxy-terminal lysine residue (pKa = 10.53) and much less with that of the amino terminus (pKa = 9.67) of the HCPC(6-17) peptide. While the activation of the peptide occurred only at high pH, the titration was performed rapidly at low temperature in a non-nucleophilic buffer. This procedure was shown not to adversely impact the primary structure of the peptide as indicated by the lack of change in avidity for HPC4 after undergoing titration. Alternatively, coupling chemistry has also been developed using carbodiimide modified POX-polymers which have been terminated with glutaric acid (12).

The anion exchange chromatography of either ethyl- or methyl-POX peptide reaction masses proved to be an efficient method of removing unreacted peptide from the reaction mass. Because the parent ethyl- and methyl-POX polymers tended to elute with water, some free polymer is probably present in pooled fractions of water-elutable peaks obtained from the reaction masses.

The concentration at which any of these adducts inhibited 50% of the maximum hPC binding (signal) by HPC4-Mab was used as a measure of adduct:HPC4-Mab avidity. The differences in avidity between adducts of

different molecular weight and between ethyl-and methyl-POX adducts can be partly attributed to the impurity of these adducts due to the presence of free polymer. Importantly, this effect does not arise from nonspecific interaction by the parent polyoxazolines as the POX polymers show no competitive avidity in ELISA assays. In spite of the presence of free polymer, both ethyl- and methyl-POX adducts retain a significant portion of avidity for HPC4-Mab relative to the underivatized parent peptide or hPC. Furthermore, the avidity of the adducts were not appreciably affected by the size of the polymer employed over the 1K to 10K molecular weight range. As an example of the utility of these adducts, we have subsequently utilized the similariity in avidity of these adducts when compared with hPC to enhance the activity of immunosorbents. These polymer-peptide adducts have been demonstrated to effectively shield the Fab domain of the HPC4-Mab prior to and during immobilization such that a highly active immunosorbent results once the adduct is released from the covalently-anchored antibody (21).

This study should serve as a basis for the further study of POX-protein modification. POX-protein adducts may possess different properties than those reported for PEG-modified proteins due to the differences in chemical structure found between PEG and POX. Furthermore, unterminated POX has the advantage of possessing a single reactive site that is characteristic of the

polymerization chemistry of a "living" polymer(23). For example, the direct coupling of a lysyl-amino group to a single "living" POX-polymer strongly contrasts the multiple reactivity that PEG-hydroxyls could have with lysyl groups activated by cyanuric chloride (1,18).

ACKNOWLEDGEMENTS

This work was partially supported by NSF grant CBT-8803036 AMD2 to W.H. Velander and C.L. Orthner. We are grateful to Dr. Charles T. Esmon of the Oklahoma Medical Research Foundation for supplying the HPC4 monoclonal antibody used in this study.

NOMENCLATURE

D_2O	deuterium oxide
D.I. H_2O	deionized ("nanopure") water
DEAE	diethyl-aminoethane anion exchange moiety
ELISA	enzyme-linked immunosorbent assay
ethyl-POX	poly(2-ethyloxazoline)
HCPC[6-17]	synthetic peptide corresponding to residues 6-17 of the heavy chain of human Protein C
hPC	human Protein C; HPC4 for calcium-dependent murine monoclonal antibody directed against human Protein C
HPC4-Mab	calcium-dependent murine monoclonal antibody directed against human Protein C
IgG	immunoglobulin
HRP	horseradish peroxidase
Mab	monoclonal antibody
Mr	relative molecular weight
methyl-POX	poly(2-methyloxazoline)
OPD	o-phenylenediamine-2HCl chromogenic substrate
PBS	phosphate-buffered saline
PEG	polyethylene glycol
pNMR	proton nuclear magnetic resonance spectroscopy

POX	polyoxazoline polymer
ppm	parts per million corresponding to pNMR signal
TBS	tris-buffered saline

REFERENCES

1. Abuchowski, A., McCoy, J.R., Palczuk, N.C. and Van Es, T., and Davis, F.F. 1977. Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase. The Journal of Biological Chemistry. **252**(11): 3582-3586.

2. Abuchowski, A., Van Es, T., Palczuk, N.C. and Davis, F.F. 1977. Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol. The Journal of Biological Chemistry. **252**(11): 3578-3581.

3. Anderson, W.L. and Tomasi, T.B. 1988. Polymer modification of antibody to eliminate immune complex and Fc binding. Journal of Immunological Methods. **109**: 37-42.

4. Beauchamp, C.O., Gonias, S.L., Menapace, D.P. and Pizzo, S.V. 1983. A new procedure for the synthesis of polyethylene glycol-protein adducts; effects on function, receptor recognition, and clearance of superoxide dismutase, lactoferrin, and α-macroglobulin. Analytical Biochemistry. **131**: 25-33.

5. Berger, Jr., H. and Pizzo, S.V. 1988. Preparation of polyethylene glycol-tissue plasminogen activator adducts that retain functional activity: characteristics and behavior in three animal species. Blood. **71**: 1641-1647.

6. Briand, J.P., Muller S., and M.H.V. Van Regenmortel. 1985. Synthetic Peptides as Antigens: Pitfalls of Conjugation Methods. Journal of Immunological Methods. **78**: 59-60.

7. Chen, R.H.L., Abuchowski, A., Van Es, T., Palczuk, N.C. and Davis, F.F. 1981. Properties of two urate oxidases modified by the covalent attachment of poly(ethylene glycol). Biochimica et Biophysica Acta. **660**: 293-298.

8. Delgado, C., Patel, J.N., Francis, G.E. and Fisher, D 1990. Coupling of poly(ethylene glycol) to albumin under very mild conditions by activation with tresyl chloride: characterization of the conjugate by partitioning in aqueous two-phase systems. Biotechnology and Applied Biochemistry. **12**: 119-128.

9. Keskkula, H. and Paul, D.R. 1986. Thermal Behavior of Polyethyloxazoline. Journal of Applied Polymer Science. **31**: 941-950.

10. Kobayashi, S., Masuda, E., Shoda, S., Shimano, Y. 1989. Synthesis of acryl-N-methacryl-type macro monomers and telechelics by utilizing living polymerization of 2-oxazolines. Macromolecules. **22**: 2878-2884.

11. Koide, A., Suzuki, S. and Kobayashi, S. 1982. Preparation of polyethylene glycol-modified streptokinase with disappearance of binding ability towards anti-serum and retention of activity. FEBS LETTERS. **143**: 73-76.

12. Miyamoto, M.,Naka, K.,Shiozaki. M.,Chujo, M and Saegusa, T. 1990. Preparation and enzymatic activity of poly[(N-acylimino)ethylene]-Modified catalase. Macromolecules. **23**(13): 3201-3205.

13. Noboru, T., Watanabe, K. Awaya, J. Kurono, M. and Fujii, S. 1985. Modification of acyl-plasmin-streptokinase complex with polyethylene glycol. FEBS Letters. **193**: 44-48.

14. Orthner, C. Madurawe, R., Velander, W, Drohan, W.N., Battey, F.D. and Strickland, D.K. 1989. Characterization of monoclonal antibodies to human protein C and their use as probes of metal-induced conformers. Journal of Biological Chemistry. **264**: 18781-18788.

15. Rajagopalan, S. Gonias, S.L. and Pizzo, S.V. 1985. A nonantigenic covalent streptokinase-polyethylene glycol complex with plasminogen activator function. J. Clin. Invest. **75**: 413-419.

16. Saegusa, T., Ikeda, H. 1973. Isomerization polymerization of 2-oxazoline VI. kinetic study on the polymerization of 2-methyl-2-oxazoline initiated by methyl iodide. Macromolecules. **6**: 808-811.

17. Saegusa, T., Kobayashi, S. and Yamada, A. 1976. Kinetics and mechanics of the isomerization polymerization of 2-methyl-2-oxazoline by benzyl chloride and bromide initiators. Effect of halogen counteranions. Makromol. Chem. **177**: 2271-2283.

18. Sakuragawa, N., Shimizu, K., Kondo, K., Kondo, S., Niwa, M. 1986. Studies on the effect of PEG-modified urokinase on coagulation-fibrinolysis using beagles. Thrombosis Research. **41**: 627.

19. Sinai-Zingde, G., Verma, A., Liu, Q., Brink, A., Allison, D., Goforth. A., Patel. N., Marand, H., McGrath, J.E. and Riffle, J.S. 1991. Polyoxazoline containing copolymers useful as emulsifiers for polymer blends. Polymer Preprints. **31**(1). 63-65.

20. Stearns, D.J., Kurosawa, S., Sims, P.J., Esmon, N.L. and Esmon, C.T. 1988. The interaction of a Ca^{2+}-dependent monoclonal antibody with the Protein C activation peptide region. Journal of Biological Chemistry. **263**(2): 826-832.

21. Velander, W., Subramanian, A., Madurawe, R.D. and Orthner, C. 1992. The use of Fab masking antigens to enhance the binding activity of immobilized antibodies. Biotech and Bioengg. 39. 1013-1023.

22. Veronese, F.M., Boccu, E., Schiavon, O., Velo, G.P. Conforti, A., Franco, L. and Milanino, R. 1983. Anti-inflammatory and pharmacokinetic properties of superoxide dismutase derivatized with polyethylene glycol via active esters. J. Pharm. Pharmacol. **35**: 757-758.

23. Webster, W.O. **February 1991**. Living Polymers. Science: 887-893

SEQUENCE OF SYNTHETIC PEPTIDE HCPC[6-17]

GLU - ASP - GLN - VAL - ASP - PRO - ARG - LEU - ILE - ASP - GLY - LYS

Figure 1. Amino Acid Sequence of HCPC(6-17)

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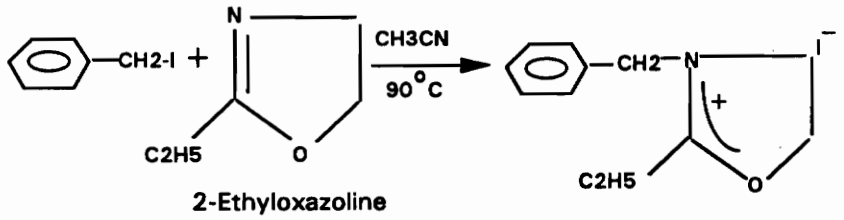

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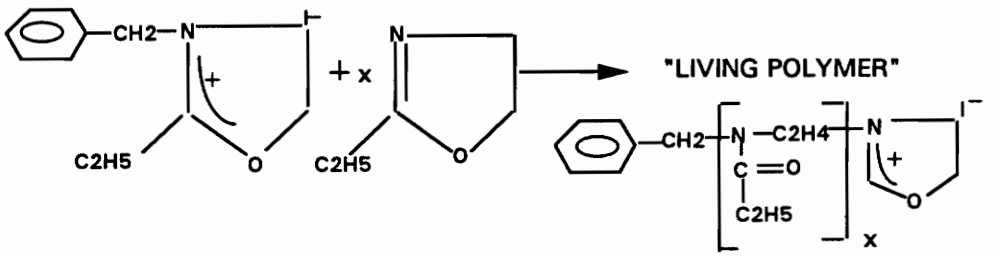

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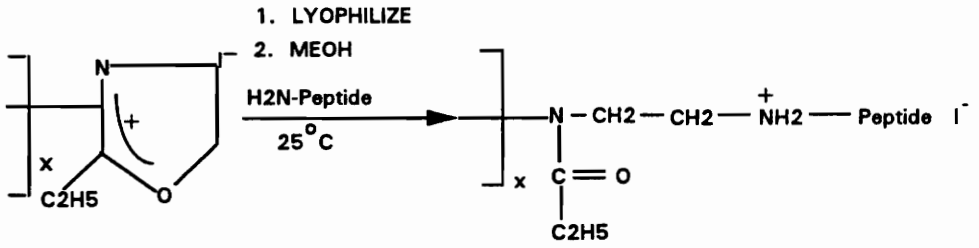
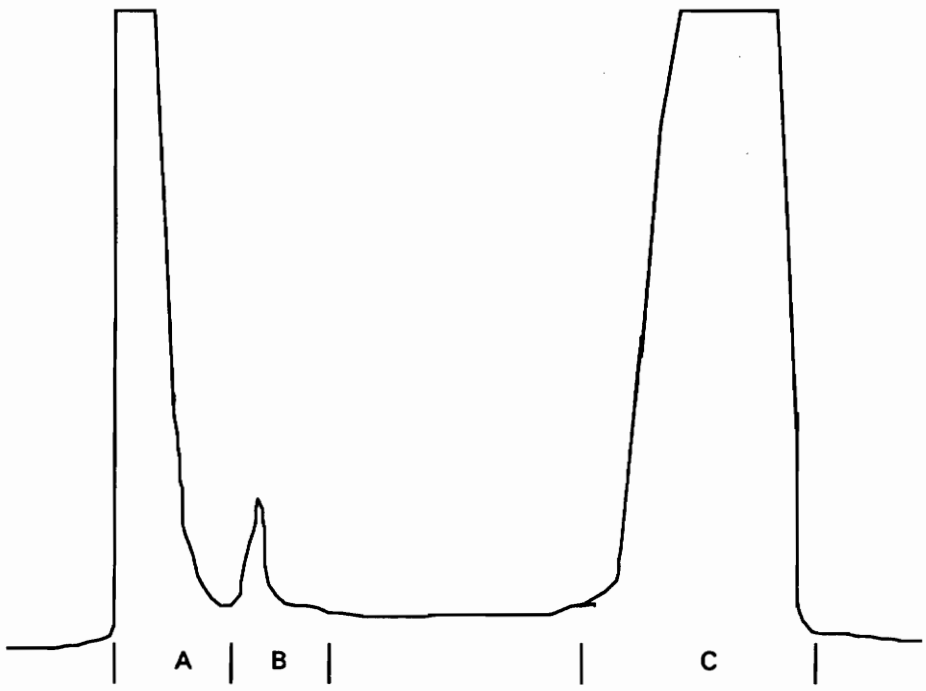


Figure 2. Synthesis of Peptide Terminated Poly(2-ethyloxazoline) polymers



A = First D. I. H2O Elutable Peak
B = Second D. I. H2O Elutable Peak
C = 1 M NaCl Elutable Peak

Figure 3. DEAE-Sephacel chromatography of polymer-peptide reaction mass

An anion-exchange method using DEAE-Sephacel was employed for the separation of unreacted peptide from polymer-peptide adducts. Fractions rich in polymer-peptide adducts were eluted with D.I H2O and the unreacted peptide was eluted with 1M NaCl. The chromatographic products were lyophilized and were analysed by pNMR.

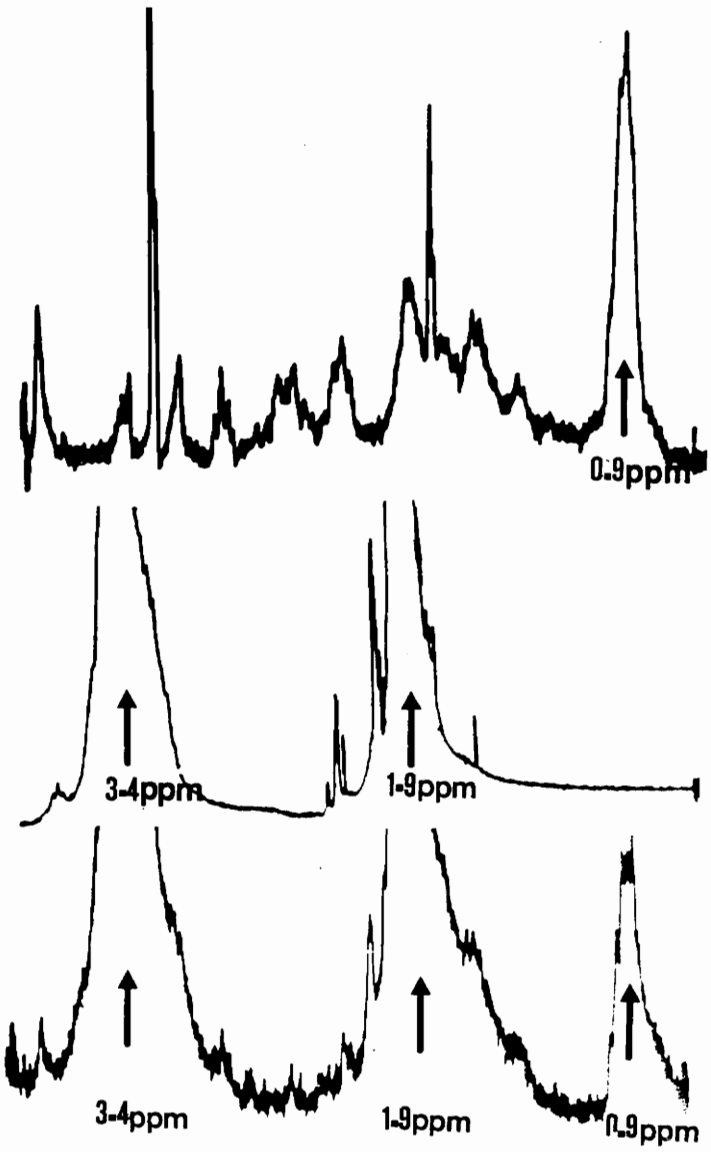


Figure 4. pNMR of fractions obtained from DEAE-Sephacel chromatography

Lyophilized chromatographic fractions were weighed and reconstituted with 1 ml of deuterium oxide (D2O) and assayed by 200 MHz pNMR.

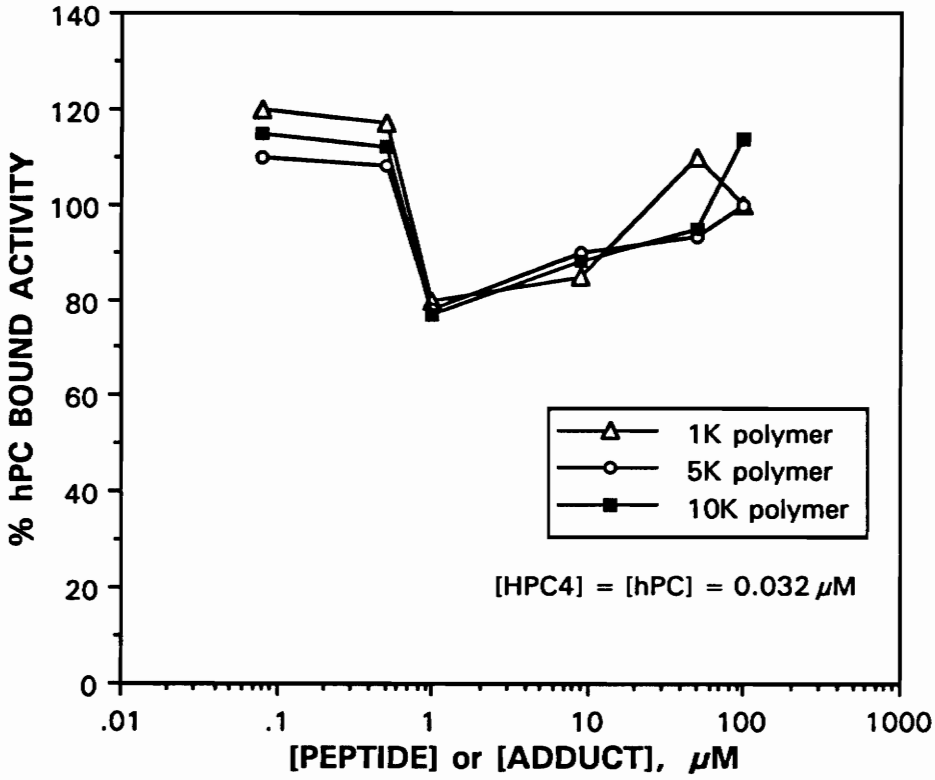


Figure 5a. Antibody avidity for ethyl-POX polymers relative to hPC

Poly(2-ethyloxazoline) polymers of molecular weights 1K, 5K and 10K (competitors) were incubated with 32 nM HPC4 and 32 nM hPC in TBS/CaCl2/0.1% BSA. The resultant hPC-HPC4-Mab complex was captured on Immulon II microtiter wells which had been coated with rabbit anti-hPC antisera and blocked with TBS/0.1% BSA. The captured complex was detected with HRP-goat anti-mouse-IgG and OPD chromophore by absorbance at 490 nm. The data is presented as a percentage of hPC binding signal obtained in the absence of a competitor.

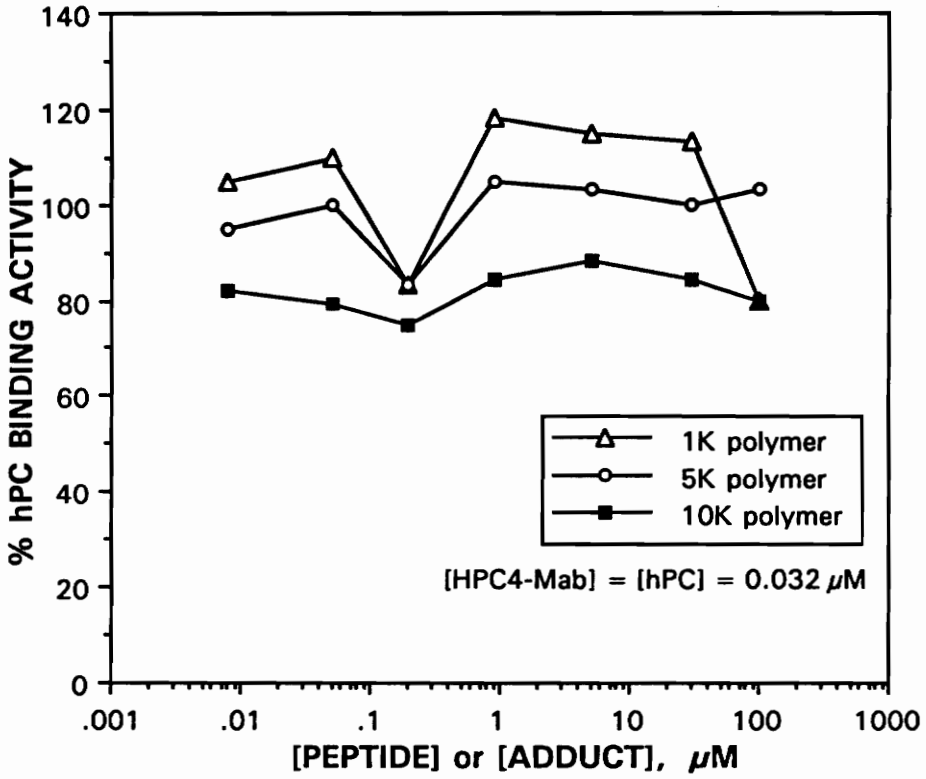


Figure 5b. Antibody avidity for methyl-POX polymers relative to hPC

Poly(2-methyloxazoline) polymers of molecular weights 1K, 5K and 10K (competitors) were incubated with 32 nM HPC4 and 32 nM hPC in TBS/CaCl2/0.1% BSA. The resultant hPC-HPC4-Mab complex was captured and detected as described in Figure 5a. The data is presented as a percentage of hPC binding signal obtained in the absence of a competitor.

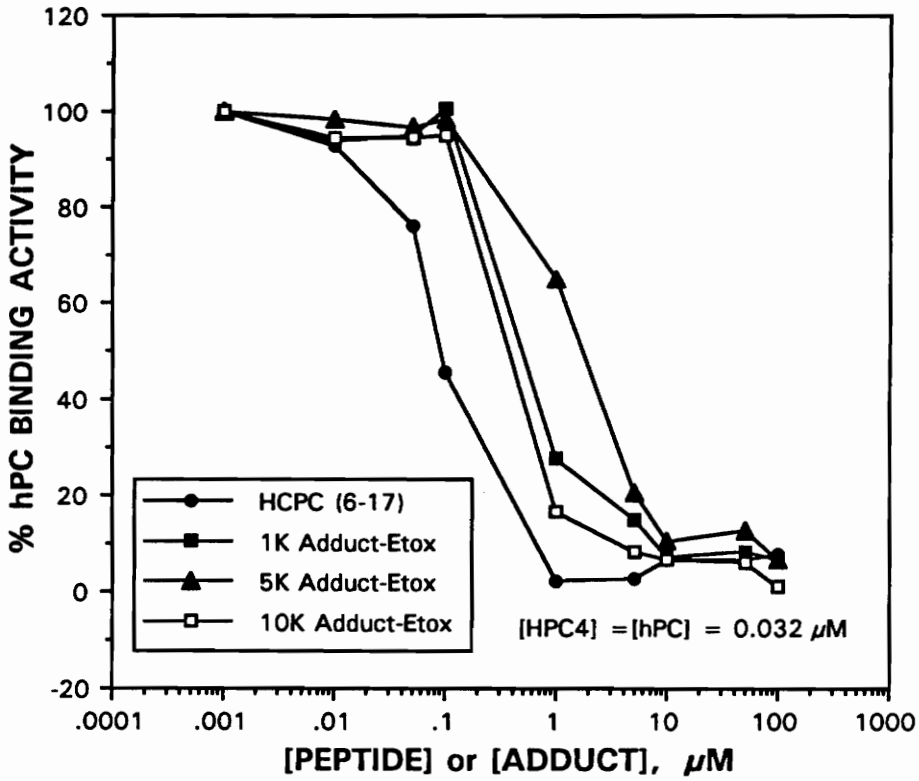


Figure 6. Antibody avidity for peptide or ethyl-POX-peptide adducts relative to hPC

Peptide and polymer-peptide adducts(competitors) were incubated with 32 nM HPC4 and 32 nM hPC in TBS/CaCl2/0.1% BSA. The resultant hPC-HPC4-Mab complex was captured and detected as described in Figure 5a. The data is presented as a percentage of hPC binding signal obtained in the absence of a competitor.

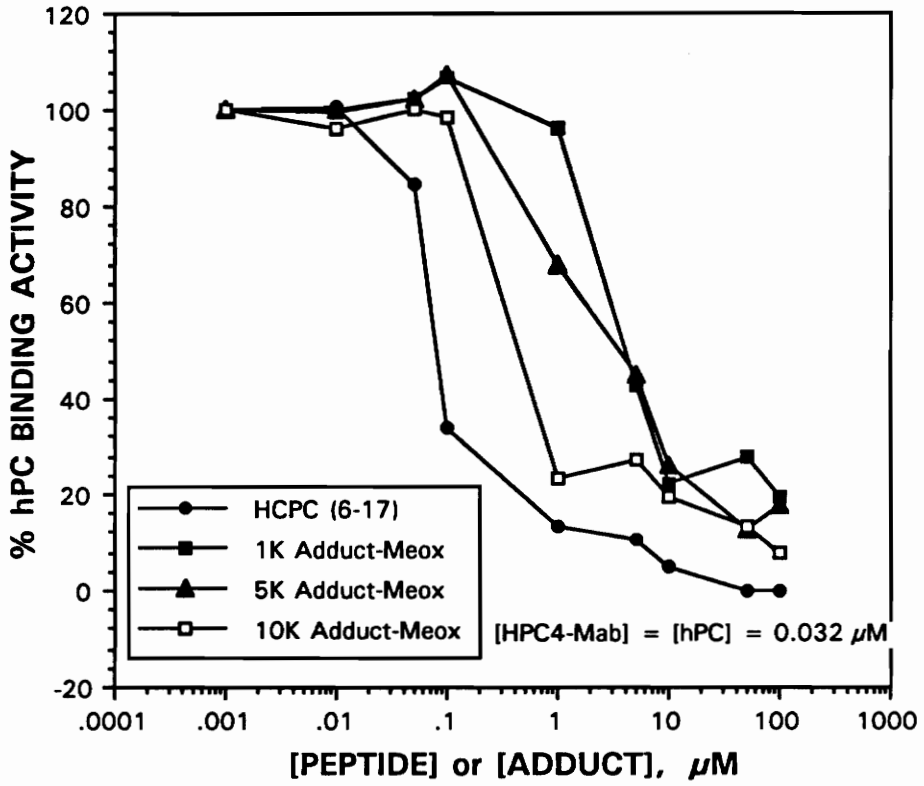


Figure 7. **Antibody avidity for peptide or methyl-POX-peptide adducts relative to hPC**

Peptide and polymer-peptide adducts(competitors) were incubated with 32 nM HPC4 and 32 nM hPC in TBS/CaCl2/0.1% BSA. The resultant hPC-HPC4-Mab complex was captured and detected as described in Figure 5a. The data is presented as a percentage of hPC binding signal obtained in the absence of a competitor.

CHAPTER THREE

THE USE OF Fab-MASKING ANTIGENS TO ENHANCE THE ACTIVITY OF IMMOBILIZED ANTIBODIES

Enhancing the Activity of Immunosorbents

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ABSTRACT

This study demonstrates that masking the Fab regions of a monoclonal antibody (Mab) with synthetic antigens prior to covalent immobilization on a porous membrane results in increased immunosorbent efficiency. Water soluble adducts of poly(2-methyloxazoline) polymers and a synthetic-peptide epitope for the Mab were constructed. These synthetic antigens are referred to as Fab-masking-antigens (FMAs). The antibody used in this study is a Ca^{2+}-dependent murine monoclonal IgG directed against the plasma protein, human protein C (hPC). The FMAs were pre-equilibrated with Mab in the presence of calcium prior to immobilization and were then removed by EDTA which destabilized the FMA:Mab complexes. The antigen binding efficiency and accessibility of the Fab domain of the immobilized antibody was significantly increased for Mab immobilized in the presence of FMA relative to those Mab immobilized without FMA. The increase in binding efficiency was most pronounced for the largest FMA employed. No appreciable differences were detected in the avidity of hPC:Mab complexes formed by immunosorbents produced by either masked or unmasked antibody. These results provide evidence that orientation may play an important role in the binding activity of immobilized antibodies.

INTRODUCTION

Antibodies can possess both high specificity and binding affinity for a given antigen or hapten molecule, and hence immunosorbents have long played an important role in both clinical diagnostics and protein purification (9). The total binding capacity of an immunosorbent is determined by the number of active antigen-binding sites, which is in general a small fraction of the number of immobilized antibodies (3,4). Typical binding capacities for covalently anchored antibodies are usually less than 30% of theoretical capacity based upon a 1:2 stoichiometry of antibody to antigen (3,4,6,8,16). Furthermore, immunosorbents used for protein purification have been shown to exhibit "surface density effects" whereupon concentrations of less than 2 mg of Mab/ml of support matrix result in higher antigen binding efficiencies (21).

While no direct proof has been offered for the deleterious impact of "improper orientation", it has been cited as a probable cause for low bound activity of immobilized antibody (3,4,16). For this reason, much effort has been spent upon the chemistry used to immobilize antibodies. These chemistries are usually based upon covalent coupling of reactive residues of proteins, such as the amino groups of lysine, the carboxyl groups of aspartate and glutamate, and the phenolic groups of tyrosines to a matrix either through prior activation of the matrix or through a coupling reagent (5,9,10,11,16,20,21). Covalent linkage to spacer arms which increase distance

of the antibodies from the matrix surface have been employed to increase binding activity (5,11,20). Being large molecules, antibodies contain many such reactive groups in both the antigen-binding (Fab) and cell receptor (Fc) regions. Hence, these chemistries result in covalent linkages to either region. Thus, the antibody can be coupled through residues which are located in or which allosterically affect the antigen-binding region. Dimethylmaleic anhydride has been used to reversibly but nonspecifically protect reactive amino groups on monoclonal and polyclonal antibodies prior to immobilization on epoxy-activated supports (10). These antibodies were directed against horse-radish peroxidase (HRP). The reduction in the amount of amino groups available for immobilization resulted in a 3-10.7 fold increase in the activity of immunosorbed-HRP.

Another approach has been "Fc-directed" immobilization chemistry which uses Fc-binding proteins such as Staphylococcus aureus-derived protein A (18) or Streptococcus-derived protein G (1). These IgG-binding proteins are covalently-linked to the matrix and then allowed to equilibrate with the antibody of interest. A stable complex of the Fc portion of the antibody with the Fc receptor protein results in a highly active immunosorbent (18). However, protein A does not recognize all subclasses of IgG and has varying avidity for IgG from different species (18). Another Fc-directed chemistry which utilizes the reaction of a hydrazide linker group with an oxidized carbohydrate chain has

been developed to increase bound IgG activity (7,14,17). However, carbohydrate moieties have been found in the Fab domain of most classes of mammalian immunoglobulins, including murine IgG (12,16). This was thought to be the basis for the lack of binding activity for the two immunosorbents prepared from murine monoclonal antibodies (16). Other receptor proteins such as avidin have been employed to couple biotinylated antibodies to matrices (2,9,15,21).

The purpose of the present investigation was to evaluate the binding activity of an antibody whose Fab domain was masked prior to immobilization. A synthetic peptide, HCPC[6-17], which contains the epitope for a Ca^{+2}-dependent murine IgG (HPC4) monoclonal antibody (Mab) directed against human Protein C (19) was employed as a Fab-masking-agent (FMA). The amino acid sequence of the HCPC[6-17] peptide is given in Figure 1. Synthetic adducts of this peptide with several different molecular weight species of water soluble poly(2-methyloxazolines) were also employed as FMAs. Complexes of HPC4-Mab with either peptide (19) or adducts (this work) have been shown to be Ca^{+2}-dependent, and to be destabilized in a chelated environment. The avidity of these adducts for the Mab has been previously reported by us (22). We show here that immunosorbents made from Fab-masked antibody possess considerably higher antigen-binding efficiencies relative to those that are immobilized in an unmasked state.

MATERIALS AND METHODS

MATERIALS

Monomeric 2-methyloxazoline was purchased from Aldrich Chemical Co. (Milwaukee, WI.) A peptide corresponding to residues 6-17 of the heavy chain of human Protein C (HCPC[6-17]) was made by t-boc synthesis. The methyl benzhydrylamine-HCL anchoring resin for peptide synthesis was purchased from Colorado Biotechnology Associates. (Boulder, CO.) Murine anti human Protein C (hPC) monoclonal antibody (HPC4-Mab) was provided by Dr. Charles Esmon of the Oklahoma Medical Research Foundation. (Oklahoma City, OK.) Murine anti human Factor IX monoclonal antibody (1H5-Mab) was provided by American Red Cross. (Washington. D.C.) Rabbit antisera against human Protein C was purchased from American Bioproducts. (Parsippany, NJ.) Affinity purified goat-anti-mouse(whole molecule, Fab-specific, Fc-specific) and anti-rabbit immunoglobulins conjugated to horseradish peroxidase (HRP) and 4-chloronapthol were purchased from Sigma Chemical Co. (St. Louis, MO.) The specificity of HRP-goat anti-mouse (Fc-specific)- and (Fab-specific) antisera were determined by ELISA by the manufacturer (product information on A-0168 and A-9917, Sigma Immunochemicals). The Fc-specific antisera showed no cross-specificity to mouse Fab fragment and was reactive with mouse IgG, IgG-Fc fragment and G1, G2a, G2b and G3 subclasses (Sigma product A-0168). The Fab-specific antisera showed no cross-specificity to mouse Fc fragment and was reactive to mouse IgG-Fab fragment as well as to all mouse IgG

subclasses (Sigma product A-9917). Immobilon AV affinity membranes (IAV) were purchased from Millipore. (Bedford, MA.) All reagents were of the best commercial grade available. Living poly(2-methyloxazoline) polymers of 1K, 5K, and 10K molecular weights were synthesized and covalently linked to HCPC[6-17] peptide (1.5K molecular weight) and the polymer-peptide adducts were purified as described by Velander et al. (22). Human protein C (hPC) was isolated by immunoaffinity methods as described by Orthner et al.(15).

METHODS

Effect of FMA Precoating of IAV Membrane on Mab Immobilization

Stock solutions containing HPC4-Mab, peptide or polymer-peptide adducts were diluted in 0.05 M Imidazole/0.1 M NaCl/25mM $CaCl_2$/pH 7.0 (Buffer A). The IAV membranes were preconditioned by wetting in 0.05 M phosphate buffered saline, pH 7.2 (PBS) and drying at room temperature. A SMI Quick-Set Micropettor was used to apply 3 μL of each sample containing peptide, polymer-peptide adduct or buffer A alone to the preconditioned IAV membranes. Several identical sets of applications on an IAV membrane were made with each set containing 4 replicates of a given sample. The spots were dried at RT and the IAV membranes were then washed with three buffer changes of buffer A over a 3 hour time period and dried at room temerature (RT).

The spots were then overlaid with 1 μl of HPC4-Mab using the SMI Quick-Set Micropettor. The spots were dried at RT and the membrane was rewetted in buffer A and the residual reactive sites on the membrane were then blocked with 10% ethanolamine in 1 M $NaHCO_3$ (pH 9.4) for 2 hours at RT. The blocked membranes were washed with 0.1 M EDTA, 0.05 M Tris HCl, 0.1 M NaCl, pH 7.0 (TBS) over a 3 hour period, and then were divided into identical strips. An anti-murine-IgG enzyme immunoassay (EIA) was performed upon these strips as described below.

Immobilization of Masked and Unmasked HPC4-Mab to IAV membrane

Stock solutions containing HPC4-Mab, peptide, or polymer-peptide adducts were prepared in Buffer A. Samples containing 0.300 μM HPC4-Mab and varying ratios of FMA/HPC4-Mab in the presence of calcium were made from these stock solutions and allowed to incubate for 1 hour at RT. A SMI Quick-Set micropettor was used to apply 1 μL of each sample to an IAV membrane which had been pre-wetted in buffer A and dried at RT. The spots were dried at RT upon which the membranes were re-wetted in buffer A and the residual reactive sites on each membrane were then blocked with 10% ethanolamine in 1 M $NaHCO_3$ (pH 9.4) for 2 hours at RT. The membranes were then washed with 100 mM EDTA-TBS for 3 hours with three buffer changes to remove bound polymer-peptide conjugate or peptide. Each membrane was divided into identical strips containing various replicate applications of masked

and unmasked Mab. These strips were subjected to one of the following EIA or ELISA assays, described below.

As a parallel experiment, chelated stock solutions of HPC4-Mab, peptide, or polymer-peptide adducts were prepared in 0.05 M Imidazole/0.1 M NaCl/25 mM EDTA/pH 7.0. Samples containing 0.300 μM of HPC4-Mab and varying ratios of FMA/HPC4-Mab were prepared from chelated stock solutions and were incubated for 1 hour at RT. Membranes containing replicate applications of these 1H5-Mab samples were subjected to EIA and/or ELISA as described below.

Evaluation of Specificity of the Immunoaffinity Interactions involving FMA

Stock solutions of 1H5-Mab, peptide, or polymer-peptide adducts were prepared in Buffer A. Samples containing 0.300 μM of 1H5-MAb and varying ratios of FMA/1H5-Mab were prepared from these stock solutions and allowed to incubate for 1 hour at RT. Membranes containing replicate applications of these samples were subjected to EIA and/or ELISA as described below.

Calcium Dependent Nature of HPC4-Mab Binding to Peptide and Adducts

Additional membranes with sets of replicate applications identical to that described above were made with the only difference being that Buffer A was used instead of 100 mM EDTA-TBS during the wash step to remove polymer-

peptide or peptide from the immobilized HPC4-Mab.

Assay of Immobilized HPC4-Mab by EIA

Identical strips of membranes prepared in the manner above were washed with PBS and incubated with 1:1000 diluted HRP-goat anti-mouse-IgG in PBS for 3 hours at RT, then washed with PBS. Bound HRP-conjugate was detected with 4-chloronaphthol substrate at 570nm using a Shimadzu CS-9000 diffuse reflectance densitometer. Total area was measured for each 1 μl spot of HPC4-Mab. Identical strips of membranes were incubated with 1:2000 diluted HRP-goat anti-mouse-IgG (Fc-specific) and processed in a manner identical to that described above. Identical strips of membranes were incubated with 1:2000 diluted HRP-goat anti-mouse-IgG (Fab-specific) and processed in a manner identical to that described above.

Effect of FMA upon Immunosorbent Activity as Measured by Sandwich ELISA

Identical strips of membranes prepared in the manner above were incubated in 0.300 μM hPC in 0.05 M Tris-HCl, 0.1 M NaCl and 0.1 M $CaCl_2$, pH 7.0 (buffer B) for 5 hours at RT. The membrane strips were washed with an excess of PBS and incubated with 1:1000 diluted anti-hPC rabbit antisera in PBS for 3 hours at RT, then washed with PBS. These strips were then incubated with 1/1000th diluted HRP-goat anti-rabbit-IgG in PBS for 3 hours at RT and then washed with PBS. Bound HRP-conjugate was detected with

4-chloronaphthol substrate at 570nm using a Shimadzu CS-9000 diffuse reflectance densitometer as described above. An additional strip containing identical set of replicates was treated as above, the only difference being the absence of hPC. These replicates were used to measure the background signal generated by adducts or peptides.

Evaluation of the Avidity of Immobilized HPC4-Mab for hPC Using Competitive Sandwich ELISA

Identical strips of membranes prepared in the manner above were incubated with 0.0012 μM hPC and varying molar ratios of HCPC(6-17) in buffer B for 5 hours at RT. Bound hPC was assayed as described above. Inhibition of the ELISA signal generated by hPC bound to the immobilized HPC4-Mab was measured as a function of increasing HCPC(6-17) concentration.

RESULTS

Characterization of the IAV Membrane System

These studies were performed such that identical sets of replicate applications of masked and unmasked antibody could be subjected to either EIA for detection of immobilized antibody or to an ordinary sandwich-ELISA to measure relative immunosorptive capacity for hPC by HPC4-Mab. In addition, competitive-sandwich-ELISA was used to evaluate the distribution of avidities

for hPC by the immobilized HPC4-Mab. In the case of EIA, a chromophore was generated by an HRP-goat anti-mouse antisera which was used to detect the antibody immobilized upon the membrane. In ELISA experiments, a chromophore was generated by a HRP-goat anti-rabbit antisera which was used to complex with a sandwich of rabbit-anti-hPC-IgG and immunosorbed hPC.

The IAV membrane provides an activated surface for covalent attachment of protein and a high precision detection of EIA and ELISA chromophoric signals by diffuse-reflectance-densitometry. The signal generated by the entire spot area was corrected for background exhibited by the surrounding (ethanolamine-blocked) membrane. The signal to noise ratio was typically greater than 100/1. We have previously documented the use of diffuse-reflectance-densitometry for the sensitive and precise detection of chromophoric substances adsorbed to thin layer chromatographic plates (13). In the present study, the precision of the analysis on IAV membranes was determined from 4 replicate applications of each sample upon a single sheet (4" x 4 7/8" sheet dimension) as well as on different sheets. A minimum of three sheets from the same lot were used to obtain the data for each experiment. The standard error presented in Figure 2-5 for each ratio of FMA/HPC4-Mab was calculated based upon 95% of all values occurring within two standard deviations of the mean. A 2-4% variance in EIA and ELISA signals was found between replicate applications within a sheet. A maximum of 25% sheet to

sheet variance for percent hPC binding activity was found for this membrane with most data points showing less than a 15% variance. Sheet to sheet variance is depicted by the error bars for the experiments presented in Figures 2-5. The EIA signals generated from anti-mouse-IgG, IgG-(Fc-specific), IgG-(Fab-specific) and ELISA signals from anti-hPC-IgG (without FMA present) produced a linear response to 0.1 -1.6 μM reference applications of HPC4-Mab immobilized on the membrane (See Figures 2a,b). It is important to note that all experiments reported here produced signals in the linear range of the reference applications. Thus, in all experiments the signals generated by the anti-murine-IgG antisera used in EIA and the anti-hPC-Ab used in ELISA have been interpreted as being proportional to HPC4-Mab and hPC present on the membrane, respectively.

The IAV membrane was selected for its specificity with respect to covalent attachment of antibody and low nonspecific sorption of protein. Experiments were performed to determine the amount of hPC signal attributable to nonspecific sorption of either hPC itself or the detecting antibody used in the ELISA. When hPC was not included in ELISA procedures, essentially no signal was generated (data not shown). In addition, the substitution of buffer for HPC4-Mab exhibited virtually no background hPC binding signal when incubated with or without hPC and performed with an otherwise identical ELISA protocol (data not shown). Thus, the hPC sandwich ELISA was specific for hPC and

dependent upon immobilized HPC4-Mab.

It has been previously reported (19) that chelation of Ca^{+2} destabilizes hPC:HPC4-Mab complexes as well as the complexes between HPC4-Mab and the synthetic epitope HCPC[6-17] or the poly (2-methyloxazoline)-HCPC(6-17) adducts (22). These studies were performed by solution phase equilibration of the HPC4-Mab with these antigens. In the present studies, membranes were treated with EDTA to disrupt the FMA:HPC4-Mab complex and render the antigen-combining sites of the HPC4-Mab available for subsequent immunosorption of hPC. No hPC binding ELISA signal was obtained for any experiment having EDTA present during the hPC binding step (data not shown). In contrast, all experiments having the EDTA wash step after immobilization generated strong hPC binding signals if calcium was present during the subsequent hPC binding step (data presented in Figure 2b and used to derive Figure 5a). This was the case for both applications of HPC4-Mab in the presence or absence of FMA. In addition, the membrane samples containing FMA and which possessed Ca^{2+} instead of EDTA during the "FMA-removal step" yielded no significant hPC binding signal after a subsequent incubation with hPC and detection by sandwich ELISA (data not shown). This indicates that the calcium-dependent nature of the complex of HPC4-Mab with all antigens employed (hPC, peptide and poly(2-methyloxazoline) adducts) was retained even upon the immobilization of the HPC4-Mab. Furthermore, FMA

removal not only appears to be Ca^{2+}-dependent but is also necessary for hPC immunosorption by HPC4-Mab under the conditions employed here. Because the FMAs are not reactive to the polyclonal antisera used in the hPC detecting ELISA, the experimental design employed here does not easily enable a quantitative assessment of FMA removal. The large increases in relative hPC binding presented below for applications with FMA suggests that the removal of FMA was very efficient.

The Effect of FMA on Immobilization of HPC4-Mab

Experiments were performed to determine whether the presence of FMA during immobilization affected the amount of HPC4-Mab immobilized to the membrane. The blocking of potential sites for HPC4-Mab immobilization by FMA was examined by precoating the membrane with FMA prior to application of the HPC4-Mab. In these experiments, HPC4-Mab was overlaid onto sections of membrane which had been pre-spotted with either buffer, peptide or adducts and then analyzed by EIA for total murine IgG present on the membrane. Figure 3a presents the amount of Mab detected at increasing amounts of precoated FMA and at constant amount of overlaid HPC4-Mab. The total antibody detected on the membrane varied no more than 6% relative to applications spotted with buffer alone. This indicates that neither the peptide nor the adducts strongly interacted with the membrane in such a way that the amount of HPC4-Mab immobilized was significantly affected. Co-applications of FMA

and HPC4-Mab to IAV, under conditions which do not favor the formation of antigen-antibody complexes (in the presence of EDTA), did not yield any appreciable change in the amount of Mab detected on the membrane (Figure 3b).

Fab-masking of the antibody prior to immobilization was performed by pre-equilibrating varying concentrations of FMA with 0.3 μM HPC4-Mab in the presence of calcium prior to application to the IAV membrane. The amount of HPC4-Mab applied to the IAV membrane was held constant while the concentration of FMA was varied from 0-0.03 mM. The FMAs were removed by EDTA-buffer prior to EIA detection as discussed above. The amount of HPC4-Mab immobilized at varying concentrations of FMA as detected by EIA and expressed as a percent of HPC4-Mab immobilized in the absence of FMA is presented in Figure 4a. In contrast to the precoating experiments (Figure 3a), the presence of HCPC[6-17] peptide during the immobilization procedure resulted in a 10-70% increase of the amount of Mab detected on the membrane. Applications of HPC4-Mab with polymer-peptide adducts behaved differently than those with the peptide. A 40% increase in the amount of Mab detected on the membrane was seen at a 1/1 ratio of FMA/HPC4-Mab for the 1 K adduct. The amount of HPC4-Mab detected then steadily decreased to a value of less than 100% at a ratio of 100/1 FMA/HPC4-Mab. The presence of 5 K or 10 K adduct during HPC4-Mab immobilization at FMA/HPC4-Mab ratios

of 1/1 had little effect, while higher ratios resulted in decreasing amounts of Mab detected on the membrane. The 5 K polymer-peptide adduct applications yielded a decrease of about 22% in HPC4-Mab detected, while the 10 K polymer-peptide adduct yielded a decrease of about 55% at a 100/1 ratio of FMA/HPC4-Mab. A precipitous drop in total bound HPC4-Mab was seen at concentrations higher than 0.03 mM FMA in both precoating and FMA-masking experiments (data not shown). This was probably due to nonspecific sorption effects which begin to limit the sites available for immobilization of HPC4-Mab at about 0.03 mM polymer-peptide adduct. Therefore, we have limited this study to a concentration range of 0-0.03 mM for all species of polymer-peptide adduct where decreases in bound HPC4-Mab were minimized (See Figures 3-4a,b).

No change in the amount of 1H5-Mab was detected for co-applications of 1H5-Mab and FMA in the presence of calcium (data not shown).

Effect of FMA on the Accessibility of the Fab Domain of Immobilized HPC4-Mab

Changes in the accessibility of Fab and Fc domains of HPC4-Mab immobilized in the presence or absence of FMA were also evaluated by EIA. The EIA signal generated by a sandwich of HRP-goat anti-mouse antisera [Fc-specific] and goat-anti-mouse antisera [Fab-specific] were used to detect accessible Fc and Fab domains of immobilized HPC4-Mab, respectively. These

Fab and Fc specific EIA are in contrast to the EIA experiments (Figure 4a) which used a polyclonal antisera to form complexes with both Fab and Fc domains. The EIA signals obtained for the detection of Fab and Fc domains were linear with equivalent slopes for increasing HPC4-Mab (no FMA) concentration applied to the membrane (Figure 2a). The ratio of signals (Fab/Fc) at varying concentrations of FMA to HPC4-Mab is presented in Figure 4b. HPC4-Mab immobilized in the presence of a 10/1 ratio of peptide to HPC4-Mab yielded a maximum increase in Fab/Fc ratio of approximately 175% of the signal ratio found for HPC4-Mab immobilized without peptide. This increase in Fab/Fc declined to about 150% as the peptide to HPC4-Mab ratio was increased to 100/1. The 1K and 10K polymer-peptide adducts showed gradual increases to 250% and 180% for applications containing a 100/1 ratio of adduct to HPC4-Mab respectively. The 5K polymer-peptide adduct yielded a maximum increase in Fab/Fc of approximately 225% at a ratio of 50/1 adduct to HPC4-Mab. A 100/1 ratio of 5K polymer-peptide to HPC4-Mab yielded an increase in Fab/Fc of approximately 175% over that of HPC4-Mab immobilized in the absence of adduct.

Evaluation of hPC Binding Activity by Immobilized HPC4-Mab

Identically processed strips of membrane as used for EIA were subjected to ELISA to determine the hPC-binding capacity of HPC4-Mab co-immobilized with FMA in the presence of calcium. The results of these experiments are

presented in Figure 5a. A binding activity of 100% was assigned to the HPC4-Mab applications having had no pre-equilibration with FMA. All of the applications which contained both HPC4-Mab and FMA produced higher hPC signals than those containing only HPC4-Mab. The 1K, 5K and 10K polymer-peptide adducts gave very similar signals corresponding to a maximum increase in hPC binding activity of about 2-fold over the reference. The peptide resulted in a maximum increase in hPC binding activity of approximately 4.5-fold higher than the reference application. No change in hPC ELISA binding signals were observed for HPC4-Mab, co-immobilized with FMA under chelated conditions (Figure 5b).

The hPC binding signal data of Figure 5a does not take into account the differences in the amount of Mab detected on the membrane due to the presence of FMA during the immobilization step (Figure 4a). Hence, we here define the hPC binding efficiency as the total hPC ELISA signal presented in Figure 5a divided by the percent total Mab signal at the same FMA/HPC4-Mab ratio as presented in the EIA data of Figure 4a. The hPC binding efficiency defined here does not account for the stoichiometry of the antigen-antibody complex and is therefore a relative hPC binding efficiency and not a percentage of theoretical antigen binding capacity. The peptide, 1K, and 5K polymer-peptide adducts all yielded a maximum increase in hPC binding efficiency of about 3-fold higher than the applications which contained only HPC4-Mab.

However, the 10K adduct yielded an increase in hPC binding efficiency of approximately 6-fold higher than applications containing HPC4-Mab only. The normalized data of Figure 5c contrasts the unnormalized hPC-binding data of Figure 5a in that the smaller FMAs impart similar increases in hPC-binding-efficiency while the largest FMA yields significantly higher hPC-binding-efficiency. In addition, the increase in the hPC-binding efficiency correlates well with the accessibility of Fab relative to Fc domains of the immobilized HPC4-Mab (Figure 4b). No hPC ELISA binding signals were observed for either 1H5-Mab immobilized alone or with FMA in the presence of calcium.

Effect of FMA on Immobilized HPC4-Mab:hPC Avidity

The conditions placed upon hPC-binding by ELISA favors the retention of strongly immunosorbed hPC. However, order of magnitude effects upon hPC:HPC4-Mab avidity can be discerned for those complexes which are detected by ELISA. In the present studies, changes in antibody avidity for hPC-antigen due to the presence of FMA during immobilization were evaluated by the presence of a competitor species to hPC. The presence of increasing amounts of HCPC[6-17] during the immunosorption of hPC by immobilized HPC4-Mab resulted in the loss of ELISA signal. The concentrations at which half-maximal inhibition of ELISA signal occurred are presented in Table 1 and are reported for immobilizations performed at different molar ratios of FMA/HPC4-Mab. The immunosorbent applications having no FMA present

during immobilization possessed half-maximal inhibition of hPC binding signal by HCPC(6-17) at a 0.050 ± 0.002 molar ratio. The applications having HCPC[6-17] as the FMA possessed half-maximal inhibition of hPC binding at a molar ratio of HCPC[6-17]/hPC ranging from 0.049-0.052. The HPC4-Mab applications which were masked with the 1K adduct produced an avidity for hPC similar to that of peptide with half-maximal inhibition occurring at a molar ratio of HCPC[6-17] / HPC4-Mab ranging from 0.053-0.060. The application masked by 5K and 10K polymer-peptide adducts possessed a slightly higher average avidity with a half-maximal inhibition at molar ratios of HCPC[6-17] / HPC4-Mab ranging from 0.058-0.088 and 0.055-0.073, respectively. For all FMA to HPC4-Mab ratios studied, the standard error places the avidity of the HPC4-Mab immobilized in the presence of FMA similar to that of the immobilizations having HPC4-Mab only. Thus, no significant changes in HPC4-Mab:hPC avidity were seen as a result of the increases in activity due to the presence of FMAs during immobilization.

DISCUSSION

We have here used the IAV membrane as a facile support for studying antigen-binding efficiencies of covalently immobilized Mab. The characteristics of the IAV membrane are comparable to polymeric materials which are typically used for immunodiagnostics or for protein purification. Under the conditions employed here, the manufacturer's specifications for the IAV membrane indicate that greater than 90% of bound antibody will be covalently anchored via reaction of lysyl residues with an activated ester (Technical data on Immobilon AV membrane, Millipore Corporation, MA), albeit that we did not determine the amount of antibody that was strongly physicochemically adsorbed versus covalently-linked. In general, low antigen binding efficiencies are found for covalently anchored antibodies. These effects have been attributed to the concerted actions of surface density of antibody (21), undesirably restrictive conformations imposed by covalent attachment (10,16), and orientation effects (7,10,16). These effects are schematically shown in Figure 6. We have confined these studies to HPC4-Mab concentrations which were low enough to limit "surface density effects" and provide an increase in hPC-binding which was proportional to HPC4-Mab immobilized upon the membrane.

We have previously characterized the avidity of the synthetic antigens used in these studies with HPC4-Mab (22). In these previous studies, the

peptide possessed an avidity for HPC4-Mab which was approximately 2-fold less than hPC. The 10 K-poly(2-methyloxazoline)-peptide adduct was shown to possess an avidity which was approximately 2-4 fold less than that of the peptide. The 1K- and 5K-poly(2-methyloxazoline)-peptide adducts possessed avidities which were approximately 10-20 fold less than that of the peptide. Although the HPC4-Mab avidity for the peptide, 1K and 5K polymer-peptide antigens has a range of 2-40 fold less avidity than for hPC, these smaller FMAs imparted similar increases in hPC binding efficiency to the immobilized HPC4-Mab. In contrast, the 10K polymer-peptide adduct imparted significantly higher hPC binding efficiency to the immobilized HPC4-Mab than did the smaller FMAs. Because the avidity of the HPC4-Mab for the 10K adduct was similar to that of the peptide, the increase in hPC binding efficiency maybe due to its greater size.

The presence of FMAs during immobilization can effect the amount of immobilized Mab by both changing the conformation of the Mab as well as masking the surface of the antibody by FMA. The inability of the FMAs employed to influence the amount of 1H5-Mab detected on the membrane further implicates the specific immunoaffinity interactions of FMAs with HPC4-Mab. The changes in HPC4-Mab conformation as a result of an immunocomplex with HCPC[6-17] has been well-documented by epi-fluorescence studies (19). Conformational changes in the HPC4-Mab may have resulted in an increased

accessibility of lysyl-residues to the reactive ester moieties present on the IAV and a subsequent increase in the amount of immobilized HPC4-Mab. In contrast to the peptide, the increased masking affected by the larger FMAs may have decreased the amount of HPC4-Mab which became immobilized. Congruously, the intermediate masking and conformational effects present for the 1K adduct:HPC4-Mab complex did not appreciably decrease the amount of HPC4-Mab immobilized as occurred for the much larger adducts.

The distribution of hPC:HPC4-Mab complexes recognized by ELISA suggests the presence of a minimum of two populations; one population which has a weak hPC avidity and another with a high avidity for hPC. The multiple washes used in either ELISA or chromatographic immunosorbent protocols do not favor retention of low avidity complexes. Hence, a wide distribution of low avidity complexes may exist that can not be well-studied using our protocol. However, the distribution of high avidity complexes detected by competitive-sandwich-ELISA can be well characterized by the competition for HPC4-Mab between HCPC[6-17] and hPC (19). A similar distribution of avidity was found in the present studies for HPC4-Mab immobilized either in the presence or absence of FMA as for the solution phase ELISA (19,22). The failure of 1H5-Mab, when co-immobilized with FMAs in the presence of EDTA, to generate hPC binding signals further establishes the specific nature of FMA/HPC4-Mab interactions. We interpret the increases in hPC binding efficiency caused by

FMAs as an increase in the population of immobilized Mab which possesses a narrow distribution of high avidity. Furthermore the trends in increased accessibility of Fab domains relative to Fc domains were well-correlated with increases in hPC-binding efficiency at low surface density of Mab.

In summary, Fab masking can be used to increase the percentage of functional and properly oriented antibody which would normally occur to a much lesser extent under conditions where random covalent attachment occurs through accessible amino residues. We are currently evaluating the effect of synthetic FMAs used in this study as well as those produced by less expensive recombinant methods on the enhancement of immunosorbent performance of conventional beaded supports.

LIST OF ABBREVIATIONS

Mab(s)	Monoclonal antibody(ies)
HCPC[6-17]	Synthetic peptide for heavy chain of human Protein
HPC4-Mab	Calcium-dependent murine monoclonal antibody directed against human Protein C
1H5-Mab	Murine monoclonal antibody directed against human Factor IX
Fab	Antigen binding domain of immunoglobulin
Fc	Cell receptor domain of immunoglobulin
FMA	Fab masking antigen
HRP	Horseradish peroxidase
IAV	Immobilon AV membrane
TBS	Tris-buffered saline
PBS	Phosphate-buffered saline
ELISA	Enzyme-linked immunosorbent assay
EIA	Enzyme immunoassay
hPC	Human Protein C
RT	Room temperature
CHO	Carbohydrate
IgG	Immunoglobulin

REFERENCES

1. Åkerström, B., Brodin, T., Reis, K. and Björck. L. 1985 Protein G: a powerful tool for binding and detection of monoclonal and polyclonal antibodies. The Journal of Immunology. **135**: 2589-2592.
2. Babashak, J. V. and Philips, T. M. 1988. J. Chromatogr. **444**: 21-28.
3. Comoglio, S., Massaglia, A., Rolleri, E. and Rosa, A. 1976. Factors affecting the properties of insolubilized antibodies. Biochimica et Biophysica Acta. **420**: 246-257.
4. Cress, M. C. and Ngo, T. T. 1989. Site specific immobilization of immunoglobulins. American Biotech. Lab. **21**: 16-19.
5. Cuatrecasas, P. 1970. The Journal of Biological Chemistry. **245**: 3059-3065.
6. Eveleigh, J. W. and Levy, D. E. 1977. Immunochemical characteristics and preparative application of agarose-based immunosorbents. Journal of Solid-Phase Biochemistry. **2**(1): 45-77.
7. Fleminger, G., Hadas, E., Wolf, T. and Solomon, B. 1990. Oriented immobilization of periodate-oxidized monoclonal antibodies on amino and hydrazide derivatives of eupergit C. Applied Biochemistry and Biotechnology. **23**: 123-137.
8. Gersten, D. M. and Marchalonis, J. J. 1978. A rapid, novel method for the solid-phase derivatization of IgG antibodies for immuno-affinity chromatography.

9. Goding, J.W. 1983. Monoclonal Antibodies: Principles and Practice. Academic Press. New York.Journal of Immunological Methods. **24**: 305-309.

10. Hadas, E., Koppel, R., Schwartz, F., Raviv, O. and Fleminger, G. 1990. Enhanced activity of immobilized dimethylmaleic anhydride-protected poly- and monoclonal antibodies. Journal of Chromatography. **510**: 303-309.

11. Lowe, C.R., Harvey, M.J., Craven, D.B. and Dean, P.D.G. 1973. Biochem. J. **133**: 499-506.

12. Melchers, F. 1969. The attachment site of carbohydrate in a mouse immunoglobulin light chain. Biochemistry. **8**(3): 938-947.

13. Morcol, T. and Velander, W. H. 1991. An o-Toludine method for detection of carbohydrates in protein hydrolysates. Anal. Biochem. **195**: 153-159.

14. Nose, M. and Wigzell, H. 1983. Biological significance of carbohydrate chains on monoclonal antibodies. Proc. Natl. Acad. Sci. **80**: 6632-6636.

15. Orthner, C. L., Madurawe, R. D., Velander, W. H., Drohan, W. N., Battey, F. D., Strickland, D. K. 1989. Conformational changes in an Epitope Localized to the NH_2-terminal Region of Protein C. J. Biol. Chem. **264**(31): 18781-18788

16. Orthner, C.L., Tharakan, J., Highsmith, F. A., Madurawe, R. D., Morcol, T. and Velander, W.H. 1991. Comparison of the Performance of Immunosorbents Prepared by Site-Directed or Random Coupling of Monoclonal Antibodies. Journal of Chromatography. **558**: 55-7018.

17. O'Shannesy, D. J. and Quarles, R. H. 1985. J. of Applied Biochemistry. **7**: 347-355.

18. Schneider, C., Newman, R. A., Sutherland, D. R., Asser, U. and Greaves, M. F. 1982. A one-step purification of membrane proteins using a high efficiency immunomatrix. The Journal of Biological Chemistry. **257**(18): 10766-10769.

19. Stearns, D. J., Kurosawa, S., Sims, P. J., Esmon, N.L. and Esmon, C.T. 1988. The interaction of a Ca^{2+}-dependent monoclonal antibody with the Protein C activation peptide region. Journal of Biological Chemistry. **63**(2): 826-832.

20 Steers, E.Jr., Cuatrecasas, P and Pollard, H.B. 1971. The Journal of Biological Chemistry. **246**: 196-203.

21. Tharakan, J.P., Clark, D.B. and Drohan, W.N. 1990. Effect of feed flow-rate, antigen concentration and antibody density on immunoaffinity purification of coagulation factor IX. Journal of Chromatography. **522**: 153-162.

22. Velander W. H., Madurawe, R. D., Subramanian, A., Sinai-Zingde, G., Riffle, J. S., Drohan, W. N. and Orthner, C. L. 1991. Polyoxazoline-Peptide Adducts which retain antibody avidity. (submitted, 1991).

Table 1

AVIDITY of MASKED and UNMASKED IMMOBILIZED HPC4-Mab for hPC

FMA	RATIO OF [FMA] / [HPC4-Mab][a]	[HCPC (6-17)] / [hPC][b] to YIELD HALF-MAXIMAL INHIBITION OF hPC BINDING SIGNAL
HCPC (6-17)	50	0.052 ±0.002
	10	0.049 ±0.003
	1	0.051 ±0.001
1K POX-Adduct[c]	50	0.060 ±0.002
	10	0.055 ±0.003
	1	0.053 ±0.004
5K POX-Adduct[c]	50	0.070 ±0.005
	10	0.088 ±0.006
	1	0.058 ±0.003
10K POX-Adduct[c]	50	0.055 ±0.005
	10	0.073 ±0.004
	1	0.071 ±0.003
HPC4-Mab (no FMA)	0	0.050 ±0.002

a [HPC4-Mab] = 0.300 μM, applied to the membrane.

b [hPC] = 0.00120 μM, incubated with peptide [HCPC(6-17)]

c Poly(2-methyloxazoline)-HCPC(6-17) adduct

SEQUENCE OF SYNTHETIC PEPTIDE HCPC[6-17]

GLU - ASP - GLN - VAL - ASP - PRO - ARG - LEU - ILE - ASP - GLY - LYS

Figure 1. Amino acid sequence of HCPC(6-17) (19).

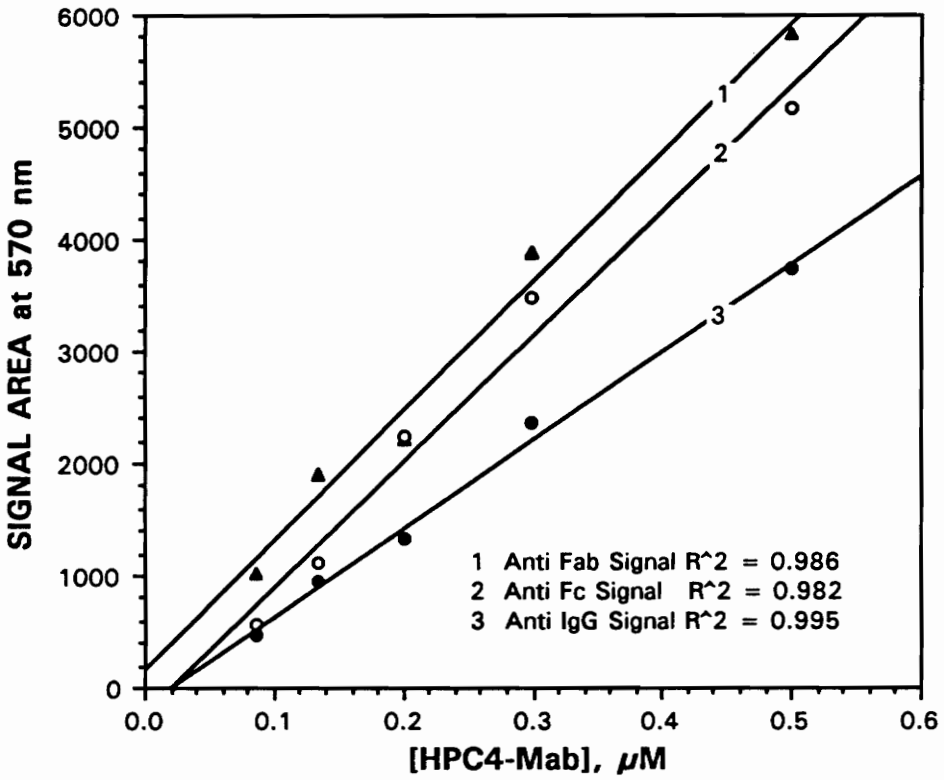

Figure 2a. EIA of Varying Concentrations of Immobilized HPC4-Mab

Varying concentrations of HPC4-Mab (0.08 μM to 1.0 μM) were spotted onto a pre-conditioned IAV membrane. The spots were dried at RT and the membranes were rewetted, blocked and washed with 100 mM EDTA/TBS, to simulate identical conditions. The amount of HPC4-Mab immobilized to the membrane was detected by EIA using HRP-conjugated-anti-mouse-antisera. The amount of accessible Fab and Fc domains at various concentrations of HPC4-Mab was detected by EIA using HRP-conjugated-anti-mouse-Fab(specific)- and Fc(specific) antisera respectively with 4-chloro-1-napthol as substrate. Bound chromophore was detected at 570 nm using a Shimadzu CS-9000 densitometer. The precision of the assay is such that the error bars are imperceptible as presented.

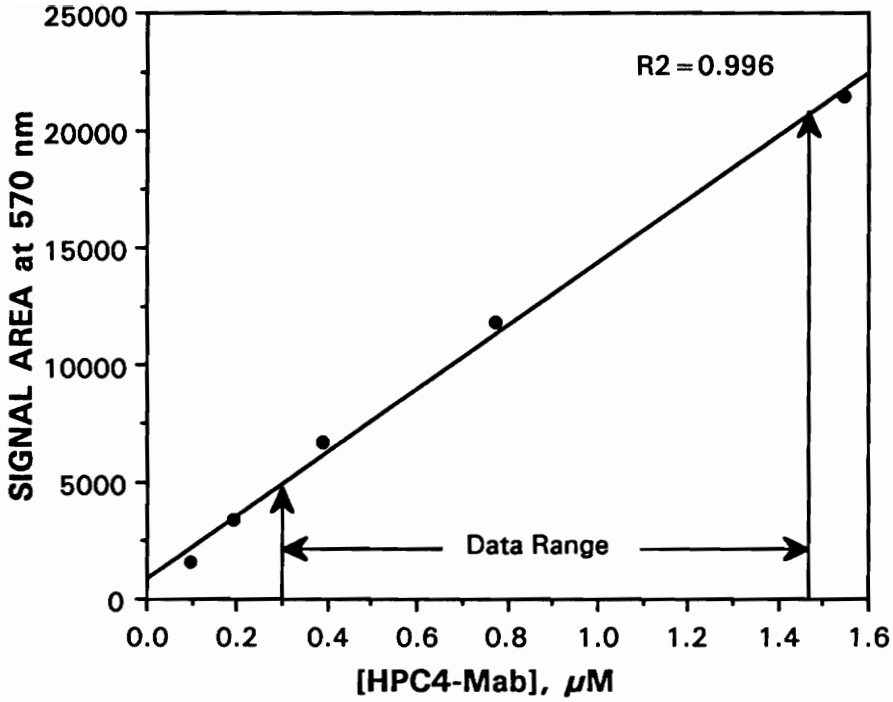

Figure 2b. ELISA of hPC Binding Activity at Varying Concentrations of Immobilized HPC4-Mab

Varying concentrations of HPC4-Mab (0.08 μM to 1.0 μM) were spotted onto a pre-conditioned IAV membrane. The spots were dried at RT and the membranes were rewetted, blocked and washed with 100 mM EDTA/TBS, to simulate identical conditions. The membranes were incubated with hPC in TBS/CaCl2. The hPC binding activity of the various concentrations of immobilized HPC4-Mab was detected by a sandwich of rabbit anti-hPC antisera and goat-HRP-conjugated-anti-rabbit-antisera with 4-chloro-1-napthol as substrate. Bound chromophore was detected at 570 nm using a Shimadzu CS-9000 densitometer. The precision of the assay is such that the error bars are imperceptible as presented.

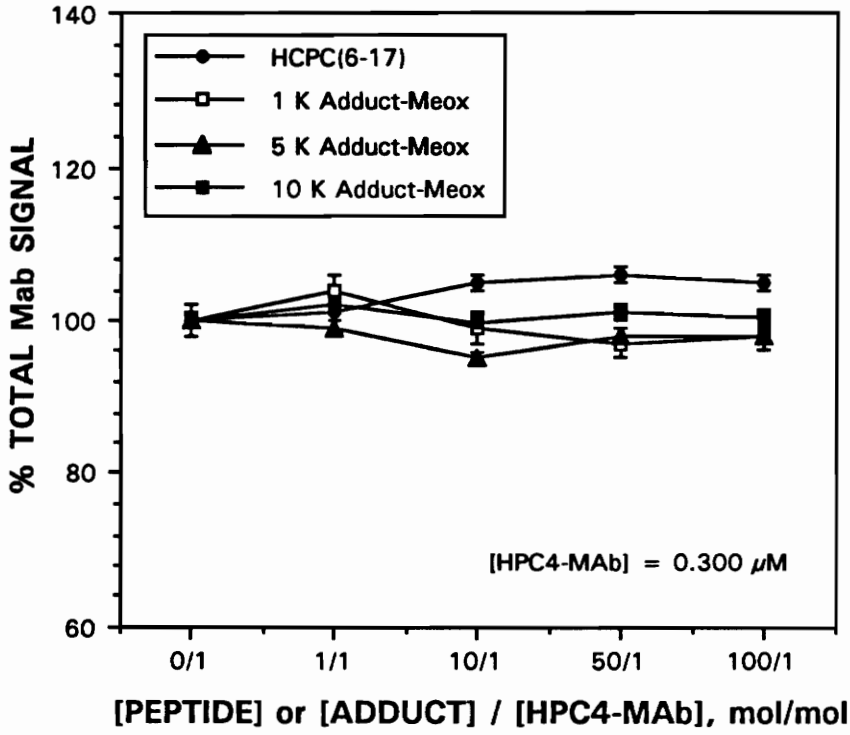

Figure 3a. Effect of Precoating the Membrane with Peptide or Poly(2-methyloxazoline)-Peptide Adducts on HPC4-Mab Immobilization

Varying concentrations of FMA (0-0.3 mM) were spotted on to a pre-conditioned IAV membrane. The spots were dried at RT and the membranes were thoroughly washed with buffer A upon which the membranes were dried again at RT. The membranes were overlayered with 1 μl applications of 0.3 μM HPC4-Mab, dried at RT, rewetted, blocked and washed with 100 mM EDTA/TBS. The amount of immobilized HPC4-Mab was detected by EIA using HRP-conjugated-anti-mouse-antisera with 4-chloro-1-napthol as substrate.Bound chromophore was detected at 570 nm using a Shimadzu CS-9000 densitometer.

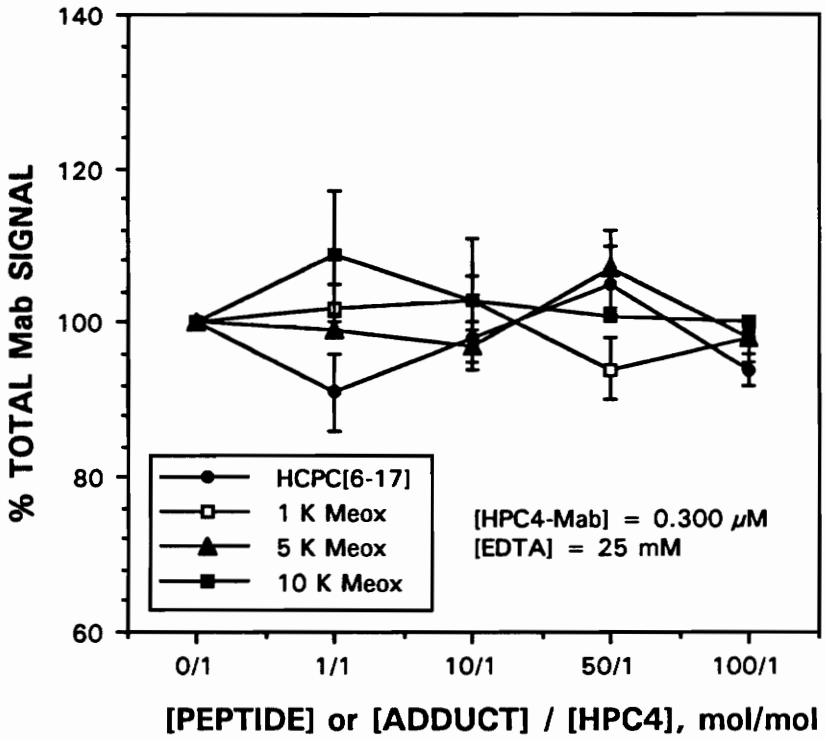

Figure 3b. Effect of Peptide or Poly(2-Methyloxazoline)-Peptide Adducts on HPC4-Mab Immobilized to the Membrane when incubated under chelated conditions.

Peptide and adducts at varying concentrations were incubated with 0.3 μM HPC4-Mab in the presence of 25 mM EDTA for 1 hour ar RT prior to spotting on to pre-conditioned Immobilon membranes. The spots were dried at RT and the membranes rewetted, blocked and washed with 100 mM EDTA/TBS to destabilize the FMA/HPC4-Mab complexes. Immobilized HPC4-Mab on the membrane was detected by HRP-conjugated-anti-mouse-antisera with 4-chloro-1-napthol as substrate. Bound chromophore was detected at 570 nm using a Shimadzu CS-9000 densitometer. The data is presented as a percentage of the EIA signal obtained in the absence of FMA.

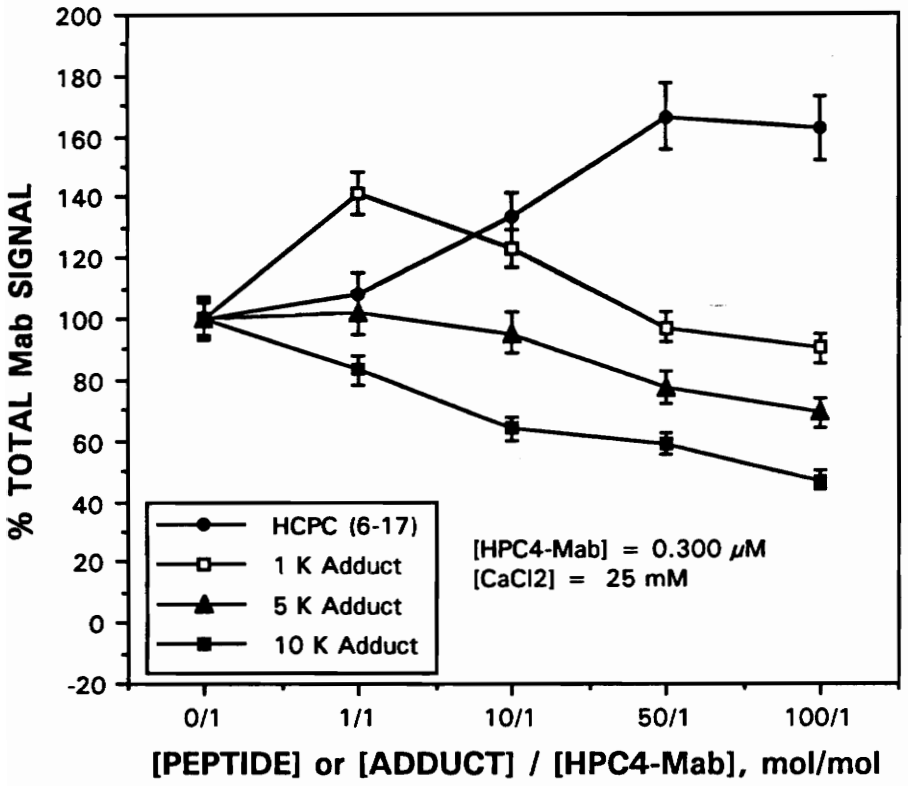

Figure 4a. Effect of Peptide or Poly(2-Methyloxazoline)-Peptide Adducts on HPC4-Mab Immobilized to the Membrane

Peptide and adducts at varying concentrations were incubated with 0.3 μM HPC4-Mab in the presence of calcium for 1 hour ar RT prior to spotting on to pre-conditioned Immobilon membranes. The spots were dried at RT and the membranes rewetted, blocked and washed with 100 mM EDTA/TBS to destabilize the FMA/HPC4-Mab complexes. Immobilized HPC4-Mab on the membrane was detected by HRP-conjugated-anti-mouse-antisera with 4-chloro-1-napthol as substrate. Bound chromophore was detected at 570 nm using a Shimadzu CS-9000 densitometer. The data is presented as a percentage of the EIA signal obtained in the absence of FMA.

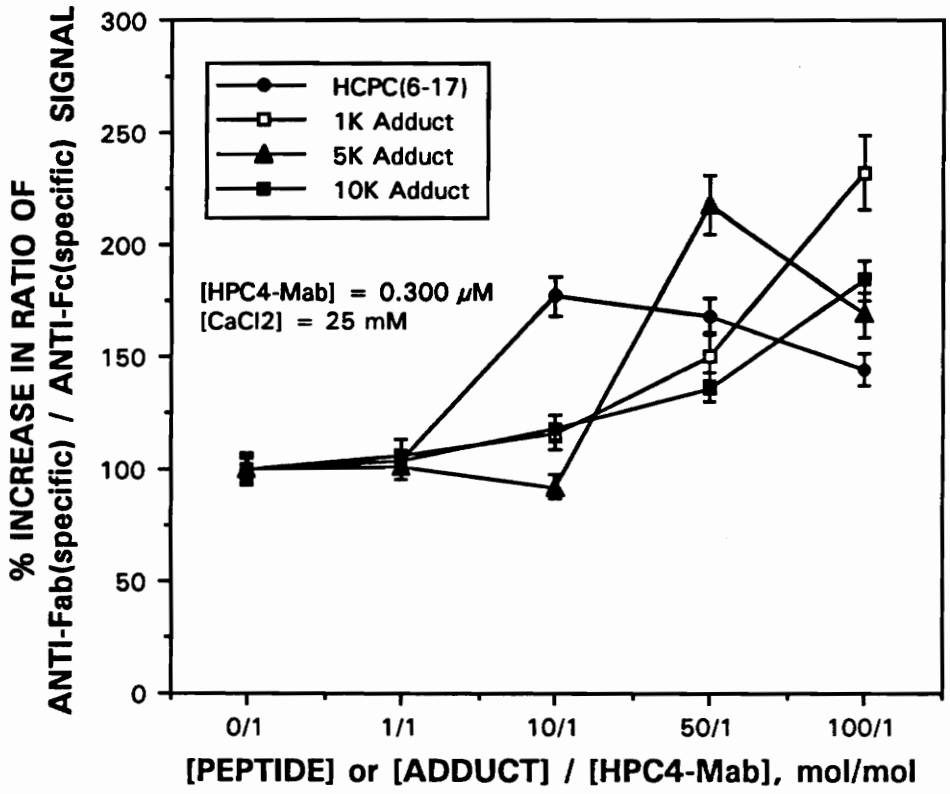

Figure 4b. Effect of Peptide or Poly(2-Methyloxazoline)-Peptide Adducts on the Ratio of the Anti-Fab(specific) to Anti-Fc (specific) EIA Signals

Peptide and adducts at varying concentrations were incubated with 0.3 μM HPC4-Mab for 1 hour ar RT prior to spotting on to pre-conditioned Immobilon membranes. The spots were dried at RT and the membranes rewetted, blocked and washed with 100 mM EDTA/TBS to destabilize the FMA/HPC4-Mab complexes. Immobilized HPC4-Mab on the membrane was analyzed for accessibility of both Fab and Fc domains by HRP-conjugated-anti-mouse-Fab specific- and Fc specific antisera with 4-chloro-1-napthol as substrate. Bound chromophore was detected at 570 nm using a Shimadzu CS-9000 densitometer. The data is presented as a percentage of the EIA signal obtained in the absence of FMA using standard curves similar to that presented in Figure 2a.

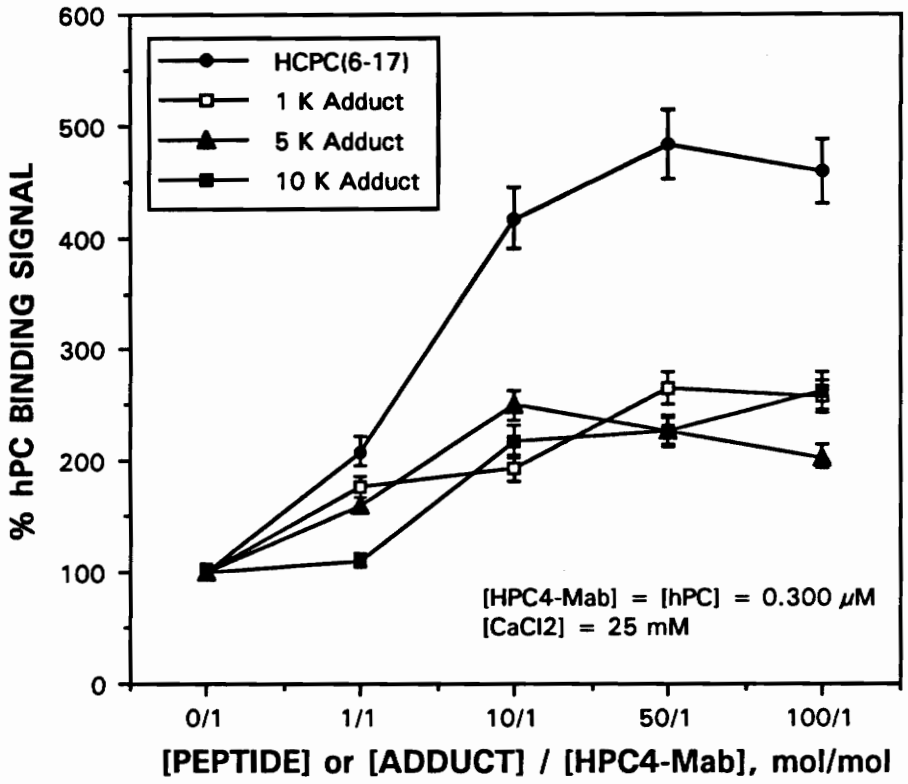

Figure 5a. Effect of Peptide or Poly(2-Methyloxazoline)-Peptide Adducts on hPC Binding Activity of Immobilized HPC4-Mab

Peptide and adducts at varying concentrations were incubated with 0.3 μM HPC4-Mab for 1 hour ar RT prior to spotting on to pre-conditioned Immobilon membranes. The spots were dried at RT and the membranes were rewetted, blocked and washed with 100 mM EDTA/TBS to destabilize the FMA/HPC4-Mab complexes. The membranes were then incubated with 0.3 μM hPC in TBS/CaCl2 for 5 hrs. The hPC binding activity of the immobilized HPC4-Mab on the membrane was detected by a sandwich of rabbit anti-hPc antisera and goat-HRP-conjugated-anti-rabbit-antisera with 4-chloro-1-napthol as substrate. Bound chromophore was detected at 570 nm using a Shimadzu CS-9000 densitometer. The data is presented as a percentage of the ELISA signal obtained in the absence of FMA.

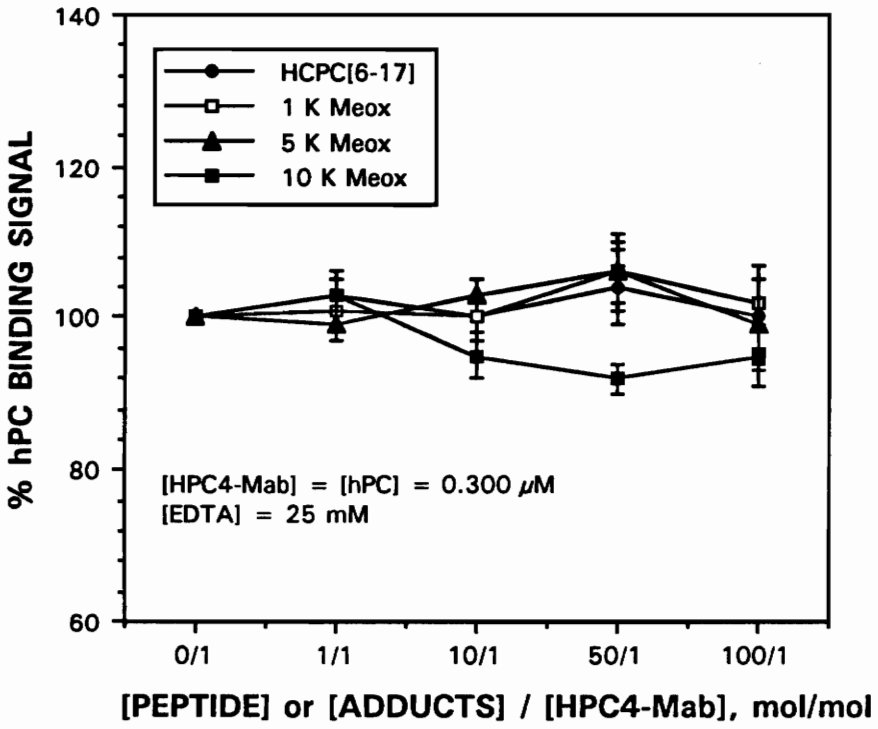

Figure 5b. Effect of Peptide or Poly(2-Methyloxazoline)-Peptide Adducts on hPC Binding Activity of Immobilized HPC4-Mab when incubated under chelated conditions.

Peptide and adducts at varying concentrations were incubated with 0.3 μM HPC4-Mab in the presence of EDTA for 1 hour ar RT prior to spotting on to pre-conditioned Immobilon membranes. The spots were dried at RT and the membranes were rewetted, blocked and washed with 100 mM EDTA/TBS to destabilize the FMA/HPC4-Mab complexes. The membranes were then incubated with 0.3 μM hPC in TBS/CaCl2 for 5 hrs. The hPC binding activity of the immobilized HPC4-Mab on the membrane was detected by a sandwich of rabbit anti-hPC antisera and goat-HRP-conjugated-anti-rabbit-antisera with 4-chloro-1-napthol as substrate. Bound chromophore was detected at 570 nm using a Shimadzu CS-9000 densitometer. The data is presented as a percentage of the ELISA signal obtained in the absence of FMA.

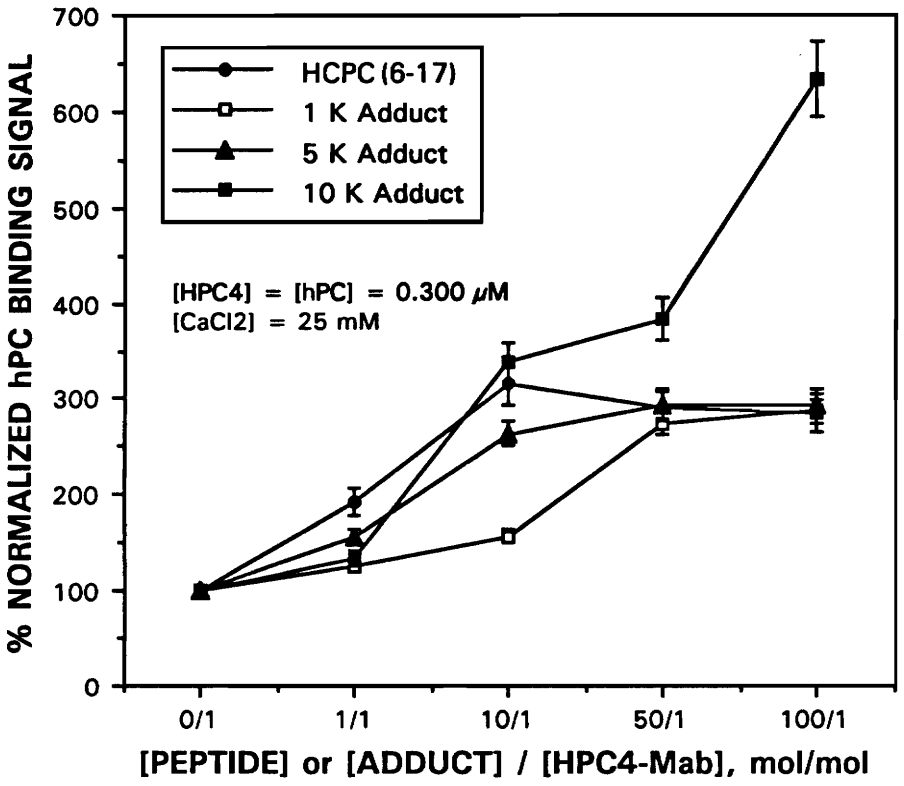

Figure 5c. Effect of Peptide or Poly(2-Methyloxazoline)-Peptide Adducts on hPC Binding Efficiency of Immobilized HPC4-Mab

The hPC binding activity of the immobilized HPC4-Mab presented in Figure 5a has been normalized with respect to the amount of HPC4-Mab immobilized on the membrane as detected by anti-mouse EIA at the respective FMA:HPC4-MAb ratio and concentration (Figure 4a).

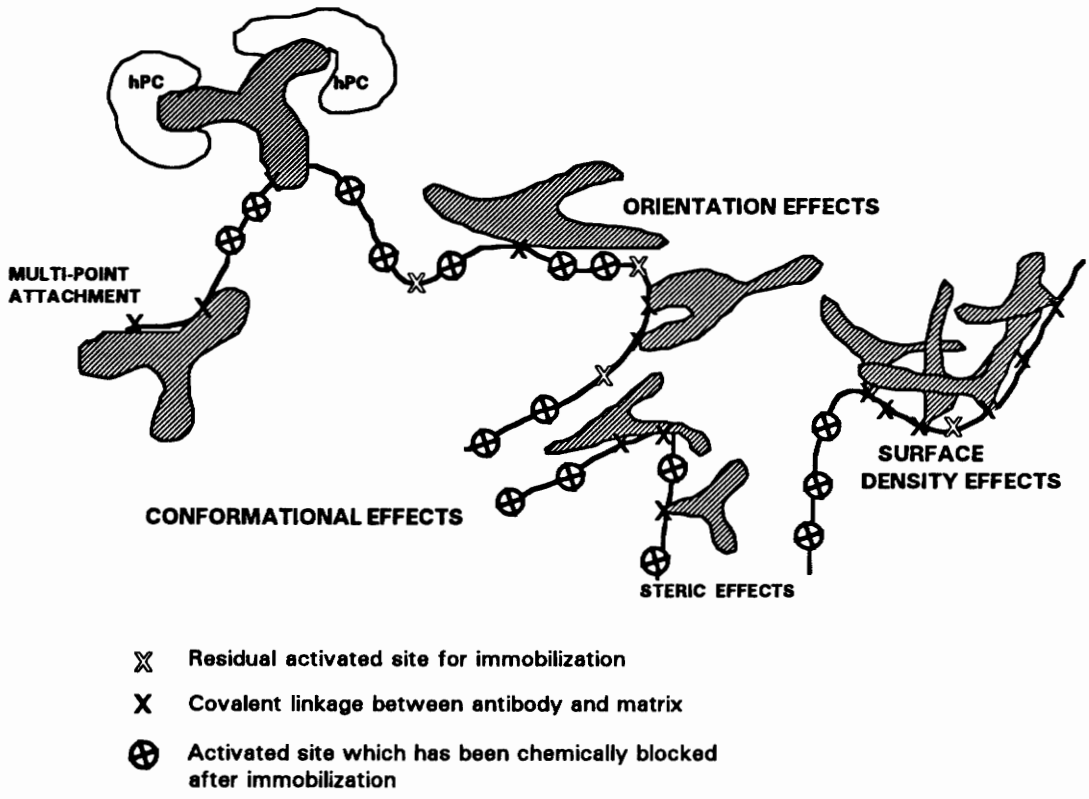

Figure 6. Model for Immunosorption

Effect of conformational restrictions, orientation and surface densities of immobilized antibody on the antigen binding activity of porous immunosorbents.

CHAPTER FOUR

The Role of Localized Antibody Density Effects on Immunosorbent Efficiency

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ABSTRACT

This study evaluates the effect of immobilized antibody density on the performance of an immunosorbent. In contrast to previous studies that emphasize the correlation of high volume averaged antibody density with immunosorbent performance, we have studied the effects of locally high antibody density and spatial distribution on the antigen binding efficiency under conditions of dynamic loading and elution. The distribution of an anti-human Protein C monoclonal antibody immobilized on Emphaze™ AB1 Biosupport Medium was evaluated. The amino groups of the antibody readily react with Emphaze AB1 azlactone functionalities at pH 7 or greater to yield stable covalent linkages. The distribution of immobilized antibody was controlled by a two-step sequence of permeation and reaction. Labeled antibody was visualized by immunofluorescence. Conditions of low pH, low temperature, and the presence of a competitor nucleophile sufficiently depressed the Thiele modulus for coupling to enable permeation of the antibody. The adsorption of the permeated antibody was enhanced by the presence of 0.75 M Na_2SO_4, and then the pH was raised to achieve rapid covalent coupling. Bead-averaged antibody densities of 1-11 mg/ml of hydrogel support were studied. Immunosorbents containing more evenly distributed antibody gave a three-fold greater antigen binding efficiency than those with locally high antibody densities. No appreciable changes in mass transfer characteristics were

observed using breakthrough analysis for immunosorbents with distributed versus locally high antibody density.

INTRODUCTION

Immunoaffinity chromatography is a process in which the binding affinity of an antigen (Ag) to a parent antibody is used as the basis for separation. Antibodies recognize specific regions of their antigens and bind them by a web of low energy forces including ionic interactions, hydrogen bonds, van der Waals forces, and hydrophobic interactions. After washing away unbound material, the interactions between the antigen and immobilized antibody can be disrupted to yield a highly purified product. Monoclonal antibodies (Mab) can be selected for a high degree of avidity for an antigen and gentle elution conditions (1-3). While being a powerful tool for protein purification, the high cost of Mab production and low volume averaged antigen binding efficiency ($<\eta_{Ag}>$) of immobilized Mab makes immunoaffinity chromatography an expensive purification technique. The development of methods that increase the antigen binding efficiency can make immunosorbents more cost effective both at laboratory and industrial scales.

Antigen binding efficiency can be affected by the orientation and localized density of the immobilized antibodies (1,3). At high localized densities the antigen binding sites of the antibodies may be in sufficient proximity to cause steric hindrance and restricted access of antigen to the binding site on the antibody (3). It also has been postulated that restricted access to the

support interior can be caused by high densities of immobilized protein near the surface (4,5). However, immunosorbent performance is usually correlated with volume averaged antibody densities ($<\rho_{Mab}>$, mg antibody/ml of resin or hydrogel), not local immobilized densities (ρ_{Mab}).

Many researchers have observed lower immunosorbent activity at high volume averaged antibody densities (Table I). Eveleigh and Levy observed a reduction in the immunosorbent efficiency of a polyclonal anti-Human Albumin antibody immobilized on Sepharose 4B with increased antibody density (6). For a Sepharose 4B/anti-β-galactosidase Mab system, Fowell and Chase reported that the lumped kinetic parameter that includes mass transfer and intrinsic adsorption kinetics decreased with increasing Mab density (7). Tharakan et al. studied the effects of Mab density, feed flow rate, and antigen concentration using CNBr Sepharose CL2B and immobilized anti-FIX (8). The antigen-binding efficiency of immobilized anti-Factor IX was relatively independent of feed flow rate and antigen concentration in the ranges studied; however, the immunosorbent efficiency decreased with increased immobilized Mab density. Studies reported by Katoh (9), Strauss et al. (10), and Weston and Scorer (11) also report similar effects of antibody density on immunosorbent performance. Antibody density effects similar to those that occur in agarose-based supports have also been reported in silica supports (12-14). In all the immunoaffinity

systems reported, the efficiency of the immobilized antibody decreased or remained constant as immobilized antibody density increased. None of the above studies distinguished between $<\rho_{Mab}>$ and ρ_{Mab}.

Most protein immobilization on particulate supports is done in a batch mode where intraparticle diffusion is the rate-limiting mass transfer step. Modelling studies have shown that it is primarily the ratio of the reaction rate to the intraparticle diffusion rate governs the distribution of immobilized antibody (15-18). In these studies, the Thiele modulus [Φ^2] was used to scale the relative rates of the protein coupling reaction to diffusion, and was correlated with more even antibody distribution for values ≤ 1.0, and a shell-like distribution with values of 10 or more. However, low coupling yields were achieved for Φ^2 less than 1.0.

This work evaluates the effect of local Mab density on the performance of immunosorbents prepared from Emphaze AB1. The Thiele modulus for antibody immobilization was engineered to yield immunosorbents with lower local but higher bead-averaged Mab densities at high coupling yields.

METHODS

Materials and reagents

Murine pH and EDTA dependent anti-human Protein C (hPC) monoclonal antibodies (8861-Mab and 7D7B10-Mab) were provided by the American Red Cross (Rockville, MD). Rabbit antisera against human Protein C, affinity purified goat anti-mouse (whole molecule), and sheep anti-goat, goat anti-mouse immunoglobulins conjugated to horseradish peroxidase (HRP) were purchased from Sigma Chemical Co. (St.Louis, MO.) Goat anti human Protein C antisera was purchased from American Diagnostics Inc. (Greenwich, CT.). Emphaze AB1 Biosupport Medium was provided by 3M. (3M Bioapplications, 3M Center, St. Paul, MN). Immulon II microtiter plates were purchased from Fisher Scientific. Human protein C (hPC) was provided by the American Red Cross. (Washington. D.C.). Recombinant hPC (rhPC) was isolated from transgenic porcine whey using ion exchange and immunoaffinity chromatography (19). JB4 histological embedding kit was purchased from Polysciences, Inc. (Warrington, PA). Texas Red labeled anti-mouse antisera was purchased from Vector Laboratories (Burlingame, CA.). Texas Red (powder) was purchased from Sigma Chemical Co. (St.Louis, MO). O-phenylenediamine-2HCl tablets were purchased from Abbott Laboratories (Chicago, IL.). Immunoaffinity chromatography was performed with C10 columns from Pharmacia. (Piscataway, NJ). A Rainin data acquisition system

along with a Knauer spectrophotometer was used to monitor column-mode separations. All other reagents were purchased from Sigma at the best grade available.

Emphaze AB1 (D_p = 60 μm) is a polymer based affinity support, synthesized by a copolymerization reaction of 2-vinyl-4,4-dimethyl-1,3-oxazolin-5-one and methylene-bis-acrylamide at various ratios to yield a rigid and macroporous support for bioseparations (20). The azlactone functionality readily undergoes a ring-opening reaction with nucleophiles (ex: -NH_2 moieties on the antibody/proteins) to yield stable covalent linkages. The stability of the reactive azlactone functionality under a wide range of pH and temperature makes Emphaze AB1 Biosupport Medium a suitable support for this study.

Emphaze AB1 Immunosorbent Preparation

Reference Coupling Procedure [R] (20):

8861 Mab was incubated with dry azlactone beads in coupling buffer (0.05 M sodium phosphate, 0.75 M Na_2SO_4, pH 7.0) containing 1 -10 mg of antibody, to yield 1-3 ml of immunosorbent. For target densities of 1-2 mg Mab/ml of gel a final Mab concentration of 0.8-1.0 mg Mab/ml was used, and for target densities of 3-10 mg Mab/ml of gel, a final Mab concentration of 3 mg/ml was used. The coupling reaction was carried out for 1 hour at room

temperature (RT) on a rotator shaker. Residual reactive sites were blocked with 1 ml of 1.0 M ethanolamine in 0.05 M sodium pyrophosphate, pH 9.3 for 30 minutes at RT. Beads were allowed to settle and the supernatant was pipetted off. An additional 4 ml of blocking solution was combined with the beads and incubated for 60 minutes at RT. Upon completion of the second blocking step, beads were washed with four column volumes of 0.5 M NaCl and equilibrated with loading buffer for protein immunosorption. Supernatant from all blocking and wash steps were saved for Mab determination by ELISA.

Alternate Immobilization Strategy [A] (two-step method)

8861-Mab (at the same final Mab concentrations as the reference method) was incubated with dry azlactone beads at pH 4.0 for 10 minutes at 4°C in the presence of 0.5 M Tris and in the absence of Na_2SO_4 in a binary buffer system (50 mM Acetate, 0.5 M Tris). At the end of the permeation step, the salt concentration was raised to 0.75 M Na_2SO_4 and the reaction was allowed to proceed for an additional 10 minutes. The pH was increased to 9.0 with 1 N NaOH and the reaction was allowed to proceed for another 40 minutes at 4°C at pH 9.0 (a total of 60 minutes at 4°C). The supernatant was pipetted off and saved, and the reference blocking protocol was followed. The supernatant from all blocking and wash steps were assayed for Mab by ELISA.

Effect of pH/competitor on Coupling Efficiency

Small scale experiments (final immunosorbent volume of 40-50 μl) were conducted to determine the pH dependent Mab coupling efficiency of Emphaze AB1 biosupport medium at 4°C. A ternary buffer system of acetate, phosphate and pyrophosphate (50 mM each, 0.75 M Na_2SO_4) was adjusted to a final pH of 4-9 with either HCl or NaOH. 7D7B10-Mab (200 μg) was suspended in the ternary buffer at the indicated pH, and dry azlactone beads were added to the reaction tubes. A target $<\rho_{Mab}>$ of 5 mg/ml gel was used in these experiments, and the reactions were run in duplicate. The reaction tubes were kept on ice and all coupling experiments were carried out at 4°C on a rotator shaker. To monitor the extent of reaction, the reaction was terminated at 5, 15, 30 minutes. Supernatant was drawn off for Mab determination by ELISA. The effect of Tris (0.1 M, 0.5 M) and salt (Na_2SO_4) on the Mab coupling efficiency at pH 4.0 was determined by similar methods. The condition leading to the slowest rate of immobilization was selected for our alternate immobilization strategy.

Chromatography

Columns (D = 1 cm) were packed with approximately 1 ml of 8861/Emphaze AB1 immunosorbent, cleaned with at least 10 column volumes (CV) of 2 M NaSCN, and then equilibrated with at least 20 CV of loading buffer

(20 mM sodium citrate, 80 mM sodium chloride, pH 6.5). After equilibration, pure recombinant hPC (from transgenic pig whey) at a concentration of 1-2 mg/ml was loaded at about 1 ml/min (linear velocity (u_o) = 1.3 cm/min) until the breakthrough front leveled off. Immunosorbents at similar bead-averaged densities ($<\rho_{Mab}>$) (e.g., 9.3 and 11.8 mg/ml), columns were challenged with the same amount of identical feed solutions. The columns were then washed with the loading buffer until baseline was reached. Bound rhPC was eluted with either a two step sequence of a pH 10 buffer (0.1 M sodium bicarbonate, 0.15 M sodium chloride, pH 10) and then 2 M NaSCN, or by a single 2 M NaSCN elution. The columns were re-equilibrated in the loading buffer. All chromatographic fractions were saved for rhPC determination by ELISA.

Assays

Determination of Mab bound to the resin

Immulon II microtiter plates were coated with 100 μl/well of 1:200 diluted anti-mouse whole molecule in 0.1 M $NaHCO_3$ (pH 9.3) for 24 hrs at 4° C. Wells were washed with 0.05 M Tris/0.1 M NaCl/0.05 % Tween (TBS-Tween) and the residual reactive sites were blocked with TBS/0.1% BSA for 20 minutes at RT. Various dilutions of standard and samples in TBS/0.1% BSA were added to the wells, 100 μl in each well and incubated for 20 minutes at 37°C. Upon incubation wells were washed four times and 1:1000 diluted HRP

conjugated goat anti-mouse IgG was added to the wells and incubated for 20 minutes at 37°C. Wells were washed four times and 100 μl of OPD substrate was added to each well. The colorimetric reaction was stopped after 3 minutes by the addition of 100 μl of 3 N H_2SO_4 to each well. Bound chromophore was detected at 490 nm using an EL308 Bio-Tek Microplate reader. The amount of antibody immobilized on the support matrix was evaluated by subtracting the amount of Mab in the coupling step supernatant,
blocking step supernatant, and 0.5 M NaCl wash step from the total Mab input.

Determination of protein C by Polyclonal ELISA

Immulon II plates were coated overnight with 100 μl/well of 5 μg/ml of rabbit anti-human Protein C in 0.1 M $NaHCO_3$, pH 9.3 at 4° C. Wells were washed with TBS/Tween. 100 μl of standard and samples in the dilution buffer (TBS /0.1% BSA, pH 7.0) was added to all the wells and incubated at 37°C for 20 minutes. Wells were washed four times and the bound PC was detected by a sandwich of goat anti-human protein C and HRP conjugated sheep anti-goat IgG. Bound chromophore was detected at 490 nm using an EL308 Bio-Tek Microplate reader.

Avidity of $(Fab)_2$ domains

Immulon II plates were coated overnight with 100 μl/well of 5 μg/ml of

rabbit anti-human Protein C in 0.1 M $NaHCO_3$, pH 9.3 at 4° C. The wells were then washed with TBS-Tween and residual reactive sites were blocked with TBS, 0.1% BSA for 15 minutes at RT. Solutions of 0.032 μM 8861-Mab (final concentration) with increasing amounts of human Protein C and/or rhPC ranging from 0.04 to 0.32 μM (final concentration) were pre-equilibrated for 1 hour at 25°C, and 100 μl of the Mab/hPC or rhPC mixture were added to the IgG-coated wells and incubated at 37°C for 1 hour. After washing with TBS-Tween 20, 100 μl of 1:1000 diluted HRP-conjugated goat anti-mouse IgG was applied to the wells. After a 1 hour incubation and washing with the same buffer, o-phenylenediamine dihydrochloride (OPD) chromogenic substrate was added to each well, and the reaction was allowed to proceed at room temperature for 3 minutes, and then quenched with 3 N H_2SO_4. Bound chromophore was detected at 490 nm using an EL308 Bio-Tek Microplate reader.

Texas Red Labeling and Immunofluorescent Staining of Emphaze AB1 Beads

The distribution of Mab in azlactone beads at various densities was determined immunofluorescent microscopy. 7D7B10-Mab was labeled with Texas Red by the procedure described in Titus *et al.* (21). Texas Red labeled anti-murine IgG (whole molecule)/ 7D7B10-Mab was bound to azlactone beads by the reference as well as two-step procedure mentioned above at targeted densities of 1, 3 and 8 mg Mab/ml of hydrogel. Texas Red labeled beads were

stored at 0 - 4°C in the dark. Fluorescent detection of bound 7D7B10/IgG was accomplished by allowing Texas Red labeled anti-mouse IgG/7D7B10 to immobilize on azlactone beads via the reference/two-step methods. The beads were prepared for embedding by dehydrating with successive ethanol solutions of 30, 50, 70, 80, and 95%. Beads were allowed to equilibrate for 20 minutes between steps. Dehydrated beads were infiltrated and embedded in JB-4 embedding resin according to manufacturer's instructions. Blocks were allowed to cure overnight before sectioning. Cross-sections, 10 μm thick, were cut from the block using an LKB automatic microtome. These sections were placed on glass slides and fixed in place. Bead slices were viewed under fluorescent light in a microscope equipped with a 35 mm camera. Fluorescent photomicrographs were taken with a shutter speed ranging from 20 seconds to 3 minutes using Kodak Ektar 1000 film.

RESULTS

Avidity Experiments

The avidity of divalent Fab domains on 8861-Mab for hPC (from human plasma) or rhPC (from transgenic porcine milk) was evaluated by ELISA. Figure 1 presents the ELISA signal resulting from the immunocapture of the (r)hPC:8861-Mab complex formed in solution. Error bars are imperceptible for some data. A low and relatively constant response resulted at Ag:Mab ratios

up to 0.1:1 for both hPC and rhPC. The signal then gradually increased for both rhPC and hPC for Ag:Mab ratios from 1:1 to 4:1. A sharp maximum in absorbance was observed at an Ag:Mab ratio of 6:1. Recombinant Protein C was used for our studies because of the availability of rhPC and the similar avidity of 8861-Mab to hPC and rhPC.

Effect of pH/Competitor on Coupling Kinetics

To gain a better understanding of the impact of surface density of immobilized Mab on the functional efficiency, we have developed an immobilization strategy (Figure 2) to yield a uniform distribution of Mab throughout the bead cross-section. The distribution of Mab within the porous support was achieved by controlling both the Thiele modulus for Mab immobilization and by simultaneous titration of reactive sites with a nucleophilic competitor (0.5 M Tris).

The effect of pH on Mab coupling efficiency (total Mab immobilized/total Mab input) for small scale columns (40-50 μl) is shown in Figure 3A. Immobilization of 7D7B10-Mab at pH 4.0 yielded a Mab coupling efficiency of 65% with a $<\rho_{Mab}>$ of 3.2 mg/ml. Mab coupling efficiency increased with pH, with an efficiency of 95% obtained at pH 9.0 to yield a $<\rho_{Mab}>$ of 4.8 mg/ml of gel.

The effect of salts, nucleophilic competitor species (i.e., Tris), and temperature on Mab coupling efficiency is shown in Figure 3B. A target Mab density of 5 mg/ml was used in these experiments. Mab immobilized at pH 7.0 (0.75 M Na_2SO_4, no Tris) at RT for 15 minutes resulted in as average Mab coupling efficiency of 95% (Case 5). Immobilization of Mab at pH 4 in the presence of 0.75 M Na_2SO_4 with Tris at 0.1 M and 0.5 M gave average Mab coupling efficiencies of 64% (Case 1) and 37% (Case 2), respectively. Upon elimination of Na_2SO_4, coupling at pH 4 and in the presence of 0.1 M Tris and 0.5 M Tris yielded average Mab coupling efficiencies of 54% (Case 3) and 26% (Case 4), respectively. Mab incubated at pH 4.0, 0.5 M Tris, 4°C for 10 minutes in the absence of Na_2SO_4, followed by incubation in 0.75 M Na_2SO_4 for 10 minutes and a step change to pH 9.0 at 4 °C gave a coupling efficiency of 90% with a $<\rho_{Mab}>$ of 4.3 mg/ml.

Analysis of Coupling Efficiencies

Table II summarizes the Mab coupling efficiencies attained for larger columns (1-3 ml) with the alternate immobilization (A) and the reference protocol (R) recommended by the manufacturer (see Methods). The immunosorbents prepared with both methods at similar target $<\rho_{Mab}>$ were challenged with the same solution concentration of Mab. Incubation with lower concentrations of Mab (< 0.1 mg/ml) at the same total Mab challenge gave

lower coupling efficiencies (data not shown).

Using the reference protocol (R), 8861-Mab was immobilized at Mab densities (mg Mab/ml of hydrogel) ranging from 1-12 mg Mab /ml of gel. A coupling efficiency of 100% was achieved at a targeted Mab density of 1.1 mg 8861-Mab/ml of hydrogel. Coupling efficiencies of 96% and 98% were attained for 8861-Mab immobilized at 2.2 and 11.8 mg/ml of hydrogel, respectively (Table II).

Immunosorbents were prepared using the two-step procedure (A) with targeted Mab densities of 1-20 mg/ml. A coupling efficiency of 90% was achieved at Mab density of 1.0 mg/ml of hydrogel. However, at higher targeted Mab densities lower coupling efficiencies were obtained. Coupling efficiencies of 71%, 57% and 47% were obtained for targeted Mab densities of 4.5, 10, 20 mg Mab/ ml of hydrogel respectively (Table II).

Immunofluorescent Staining Results

Texas Red labeled antibodies were immobilized according to reference (R) and alternate immobilization procedures (A) at targeted $<\rho_{Mab}>$ of 1-8 mg/ml respectively. Photomicrographs of 10 μm thick bead cross-sections with Mab density of 5-6 mg/ml using fluorescent microscopy are shown in Figure 4A,B. Beads made with the reference procedure (R) gave a shell-like profile of

fluorescent signal in the outer 10 μm of the bead radii (Figure 4A). Beads made with the alternate procedure (A) gave a more even distribution of the fluorescent signal throughout the cross section (Figure 4B).

Breakthrough Analysis

Figure 5 shows typical breakthrough and elution profiles at 280 nm for rhPC loaded on 8861-Mab immunosorbent columns made by either reference (R) or two-step (A) methods. The shapes of the breakthrough profiles and respective 2 M NaSCN eluate profiles were similar for immunosorbents having comparable $<\rho_{Mab}>$. The NaSCN product eluates spanned about 2 column volumes for the 0.9 (A) and 1.1 (R) mg/ml columns. The NaSCN product eluates spanned about 4 column volumes for the 9.3 (A) and 11.8 (R) mg/ml columns. Table III gives operating conditions and antigen binding efficiencies obtained from similar breakthrough experiments. The antigen-binding efficiencies were calculated from the total amount of rhPC eluted from the immunosorbents as detected by ELISA. Since the 8861-Mab is an IgG-2A class immunoglobulin, antigen binding efficiency ($<\eta_{Ag}>$) of the immobilized Mab was calculated by assuming a binding stoichiometry of two Ag per immobilized Mab. Average antigen-binding efficiencies of 23%, 30% and 25% were obtained for 1.1(R), 2.2(R), and 11.8(R) mg/ml columns, respectively. Average antigen-binding efficiencies of 23%, 59% and 62% were obtained for 0.9(A),

3.2(A), and 9.3(A) mg Mab/ml of support, respectively.

DISCUSSION

The distribution of immobilized Mab within a support matrix can be controlled by changing the Thiele modulus [Φ^2] for the coupling reaction. The Thiele modulus is derived from dimensional analysis of governing equations that model the simultaneous processes of intraparticle diffusion and reaction (15-18). These modeling solutions are scaled by parameters that pertain to the relative rate of Mab coupling to the rate of intraparticle diffusion. Φ^2 is of the form:

$$\Phi^2 = \frac{R^2 k c}{D_{eff}} \qquad (1)$$

where k is a rate coefficient, c is the ligand concentration, R is the radius of the bead support, and D_{eff} is the effective diffusivity of the protein in the matrix. The rate of diffusional transport of protein into the matrix is primarily determined by the characteristic length over which diffusion will occur (R^2) and by D_{eff}. For low solids content hydrogels, D_{eff} of proteins may be on order of magnitude of that in a purely aqueous environment, essentially not affected by pH 4-9, and a weak function of temperature. Tyn and Gusek (22) have compiled experimental values of protein diffusivities and predictive correlations

of the form

$D_{eff} = f\,[T,\ 1/Mr]$(2)

Once Mab is delivered to reactive sites within the support matrix, the rate of the Mab coupling can be divided into a sequence of adsorption and then covalent reaction. Mab concentration may be seen as the major driving force for the adsorption step. Previous studies have shown that increasing the adsorption of proteins onto matrices also increases the yield of immobilized protein (20,23,24). We have used Na_2SO_4 to enhance adsorption of Mab onto Emphaze AB1 and increase coupling efficiencies (20). Once adsorbed, the rate of coupling of ligand (i.e., Mab) to the reactive matrix is a strong function of pH and temperature. For example, the lysyl residues of a protein (pK_a about 10) will become markedly better nucleophiles at pH greater than about 9. Conversely, the activated (electrophilic) sites on the matrices will have an optimal pH range where competition between acid catalyzed hydrolysis at low pH and base-catalyzed hydrolysis at high pH will compete with the coupling reaction with lysyl residues. The functional form of the rate of the combined adsorption and coupling reaction (R_c) for protein near the reaction site can be expressed as the following:

$$R_c = \frac{k \cdot f1 \left[C_{Mab} \cdot N_{sites} \right]}{C_{Mab} + f2 \left[N_{sites} \right]} \qquad (3)$$

$$k = k_o \, e^{-\frac{E_{act}}{RT}}$$

where we view k as a strong function of pH. The number of reactive sites N_{sites} will strongly affect R_c. The radial profile of N_{sites} has not been well studied, but we note that the nature of many derivatization procedures may well result in a higher concentration of reactive sites near the edge of beaded supports relative to that found in the bead interior. The complex functions f1 and f2 of equation 3 contain the first order effects of N_{sites} described by Michaelis-Menten or Langmuir adsorption kinetics (23). As seen in equation 3, R_c can be decreased by lowering the N_{sites} available for Mab immobilization. We have achieved this by simultaneous reaction with a nucleophilic competitor such as a primary amine like Tris.

In general, Φ^2 is much greater than 1 for most protein immobilization procedures (25), and thus the rate of coupling is much faster than the rate at which protein is delivered into and transported throughout the bead. For example, in the absence of a competitor nucleophile, a shell of high density, fluorescently-labeled Mab results at immobilization conditions of $\Phi^2 >> 1$ (Figure 4A). This is in part due to the high N_{sites} that occurs on Emphaze AB1

(1.12 meq/g of Emphaze AB1(20)) as well as in many other activated supports. Thus, the Mab cannot achieve an evenly distributed density at conditions where the extent of permeation is limited by fast reaction. For conditions of $\Phi^2 < 1$, a majority of Mab will permeate the support interior before being coupled. In the case of Emphaze AB1 as well as several other activated supports (data for other supports not shown), we have found that the simultaneous conditions of low pH, low temperature and the presence of a competitor nucleophile to the Mab greatly lower the coupling efficiency and enable permeation. The increased permeation is seen in the more evenly distributed fluorescently-labeled Mab that was immobilized and visualized in bead cross-sections (Figure 4B). A subsequent adsorption of the permeated Mab induced by Na_2SO_4, and then a shift to conditions of $\Phi^2 >> 1$ enabled coupling efficiencies of 60-80% for $<\rho_{Mab}>$ equal to about 6 mg/ml or less (Table II), leading to the conclusion that the combined levels of Mab and Tris were still appreciably less than N_{sites}. However, less than 50% yields were obtained at $<\rho_{Mab}>$ of about 9 mg/ml, and therefore the combined levels of Mab and Tris were on the order of N_{sites}. While the increased Mab loadings were performed at a constant level of 0.5 M Tris, the Tris concentration may need further optimization to achieve a balance between ρ_{Mab} and higher coupling efficiencies at high $<\rho_{Mab}>$.

The process of immunosorption onto a support matrix can be

mechanistically viewed as diffusional transport of antigen to the immobilized Mab followed by adsorption and complexation steps. A rate limitation due to mass transfer was not evidenced by the Ag breakthrough and wash profiles (Figure 5a-d). Thus, we here emphasize the effects of ρ_{Mab} on the kinetics of Ag binding and its relationship to the pointwise antigen binding efficiency ($\eta_{Ag}(r)$) and then to the bead-average functional efficiency ($<\eta_{Ag}>$). For clarity, we first discuss the elementary reaction steps for antigen capture by Mab in solution, and then we will extend the concept to the more complex situation of immobilized Mab. The elementary reactions steps for antigen capture by Mab in solution can be described by

$$Mab + Ag \underset{k_{r1}}{\overset{k_{f1}}{\rightleftharpoons}} [Mab{\cdot}Ag_1] \qquad (4a)$$

$$[Mab{\cdot}Ag_1] + Ag \underset{k_{r2}}{\overset{k_{f2}}{\rightleftharpoons}} [Mab{\cdot}Ag_2] \qquad (4b)$$

The elementary reactions for filling the first antigen binding site are given by equation 4a. The elementary reactions for filling the second antigen binding

$$R_{ic} + R_{id} \sim 0 \qquad (4c)$$

$$K_{eq1} \equiv \frac{C_{Mab \cdot Ag_1}}{C_{Mab}\, C_{Ag}} = \left(\frac{k_{f1}}{k_{r1}}\right)_{solution} \qquad (4d)$$

$$K_{eq2} \equiv \frac{C_{Mab \cdot Ag_2}}{C_{Mab \cdot Ag_1}\, C_{Ag}} = \left(\frac{k_{f2}}{k_{r2}}\right)_{solution} < K_{eq1} \qquad (4e)$$

site are given by equation 4b. At conditions near to that of equilibrium binding, the sum of the forward (R_{ic}) and backward (R_{id}) reactions rates for each site is approximately zero (equation 4c). Based upon protein structure (26), the two Ag binding sites on an empty Mab molecule are assumed to be kinetically indistinguishable. It is only when a large Ag is bound to the first site that a kinetic distinction between the first and second Ag binding site of an individual Mab molecule becomes necessary. From the solution phase experiments presented here (Figure 1), we conclude that K_{eq2}, which describes the equilibrium filling of the second antigen binding site, is less than K_{eq1}. An increase in % ELISA signal as a function of increasing molar concentration of either hPC or rhPC is indicative of the relative avidity of the two antigen binding domains for hPC or rhPC. Figure 1 suggests that a large concentration driving force (corresponding to a stoichiometric ratio of Ag:Mab = 6:1) is required to overcome the decreased avidity for filling of the second Ag binding site in

solution. This behavior is typical of the Mab we have studied for binding large Ag such as hPC (M_r = 62 kDa).

The rate expressions for R_{ic} and R_{id} are functions of C_{Mab}, C_{Ag}, $C_{Mab \cdot Ag1}$, $C_{Mab \cdot Ag2}$, and the elementary forward (k_{f1}, k_{f2}) and backward (k_{r1}, k_{r2}) rate coefficients. For solution phase Mab:Ag reactions the equilibrium constants defined for K_{eq1} and K_{eq2} can be related to k_{f1}/k_{f2} (equation 4d), and k_{r1}/k_{r2} (equation 4e). In an analogous way, the combined forward (R_{ic}) and backward (R_{id}) reactions rates can be related to the respective K_{eq1} and equation K_{eq2} for Ag in equilibrium with immobilized Mab. Under conditions where Ag diffusional transport is not rate limiting, the forward rate of immunocapture (R_{ic}) of Ag by a Mab immobilized with proper orientation can be given by

$$R_{ic} = k_{f1}\, \rho_{Mab}\, C_{Ag} + k_{f2}\, \rho_{Mab \cdot Ag_1}\, C_{Ag} \qquad (5a)$$

$$k_{f1} = k_{f1,0} \exp\left[- \frac{E_{act,0} \cdot f1(\rho_{Mab}, \rho_{Mab \cdot Ag_1}, \rho_{Mab \cdot Ag_2})}{R\,T} \right] \qquad (5b)$$

$$k_{f2} = k_{f2,0} \exp\left[- \frac{E_{act,0} \cdot f2(\rho_{Mab}, \rho_{Mab \cdot Ag_1}, \rho_{Mab \cdot Ag_2})}{R\,T} \right] \qquad (5c)$$

where a kinetic expression of the local solution phase concentration of antigen (C_{Ag}) and the local solid phase concentrations of immobilized Mab (ρ_{Mab}) and Mab-Ag complexes ($\rho_{Mab \cdot Ag1}$, $\rho_{Mab \cdot Ag2}$) is proposed. The expressions for the two rate constants k_{f1} and k_{f2} govern the high avidity of the first Ag binding site and the lesser relative avidity of the proximal Ag binding sites due to intra/inter-molecular steric hindrance by neighboring Mab or a sufficiently large Ag occupying that first site. Conceptually, steric hindrances of the Ag binding site result in a higher activation energy in R_{ic} that is needed to "push" the antigen into a combined state with the Mab. Hence, f1 (equation 5b) and f2 (equation 5c) serve as scaling factors to increase the unhindered activation energy $E_{act,0}$ for Ag binding as ρ_{Mab}, $\rho_{Mab \cdot Ag1}$, and $\rho_{Mab \cdot Ag2}$ are increased. We assume the $E_{act,0}$ is the same for solution phase or immobilized Mab having proper orientation. Note that R_{ic} becomes highly dependent upon intraparticle position if ρ_{Mab} varies with position.

At equilibrium, the pointwise antigen binding efficiency $\eta_{Ag}(r)$ can be related to K_{eq1} and K_{eq2} through $\rho_{Mab \cdot Ag1}$, $\rho_{Mab \cdot Ag2}$. Antigen binding efficiency $\eta_{Ag}(r)$ is defined by

$$\eta_{Ag}(r) \equiv \left[\frac{\rho_{Mab \cdot Ag_1} + \rho_{Mab \cdot Ag_2}}{\rho_{Mab}} \right]_{r=r} \cdot \frac{1 \ mole \ Mab}{2 \ mole \ Ag} \cdot 100\% \qquad (6)$$

At conditions of equilibrium, equation 6 can be rewritten in terms of the defined equilibrium constants that are analogous to those given by equations 4d-e, but that are now written in terms of the solid phase concentrations of immobilized Mab and Mab-Ag complexes (ρ_{Mab}, $\rho_{Mab \cdot Ag1}$, $\rho_{Mab \cdot Ag2}$).

$$\eta_{Ag}(r) = \left[K_{eq1} C_{Ag} \{ 1 + K_{eq2} C_{Ag} \} \right]_{r=r} \cdot \frac{1 \ mole \ Mab}{2 \ mole \ Ag} \cdot 100\% \qquad (7)$$

Note that if the functional forms of both R_{ic} and R_{id} are known, they are relatable to $\eta_{Ag}(r)$ by K_{eq1} and K_{eq2}. Finally, $\eta_{Ag}(r)$ is integrated over the whole volume of the bead (V) to give the bead-average functional efficiency ($<\eta_{Ag}>$).

$$<\eta_{Ag}> = \frac{\int_V \eta_{Ag}(r) \, dV}{\int_V dV} \qquad (8)$$

$<\eta_{Ag}>$ is the most commonly used measure of immunosorbent efficiency (6-

10). From equation 4c and equations 6-8, a local increase in R_{ic} relative to R_{id} will result in an increase in $\eta_{Ag}(r)$; those Mab with very slow R_{ic} due to locally high ρ_{Mab} will have lower $\eta_{Ag}(r)$.

Because $<\eta_{Ag}>$ can be related to kinetic phenomena described by R_{ic} and R_{id}, both the reference and two-step immunosorbents were exposed to the same Ag concentration driving forces in each set of Ag breakthrough experiments. The steep return to baseline absorbance during the wash step of these breakthrough experiments supports our assumption that the Ag binding reactions are essentially irreversible. We have purposefully chosen 2 M NaSCN elution conditions for the chromatograms in Figure 5 to elicit fast desorption kinetics and make R_{id} very large relative to R_{ic}. Past studies with 8861 immunosorbents used a pH 10 elution step (19,27). These previous studies at pH 10 resulted in broad and tailing peaks, which is indicative of conditions where R_{id} is a rate-limiting process relative to mass transfer. It is noted that shell-type and distributed forms of immobilization at similar $<\rho_{Mab}>$ yield similar elution profiles at given loading and elution conditions (Figure 5), although the characteristic length needed to permeate all Mab sites with Ag is much different (Figure 4A and 4B). Furthermore, the narrow peak widths obtained using NaSCN as an eluate indicate that any increased penetration needed to access interior Mab sites does not affect the Ag mass transfer rate within a

high $<\rho_{Mab}>$ immunosorbent.

Our method of immobilization has enabled study of the relationship between $<\rho_{Mab}>$ and ρ_{Mab} to $<\eta_{Ag}>$. High $<\rho_{Mab}>$ immunosorbents prepared by the reference method have threefold lower values of $<\eta_{Ag}>$ than the two-step method (Table III). This behavior was found over a range of $<\rho_{Mab}>$ from about 3 to 11 mg Mab/ml support, but not for $<\rho_{Mab}>$ less than 3 mg Mab/ml. We note that the highest combined levels of C_{Mab} and N_{sites} occur near the edge for both the reference and two-step methods during the initial contacting of the Mab with the matrix. Thus, we expect that significant reaction may occur even at bead average conditions of $\Phi^2 < 1$ to form locally high ρ_{Mab} at the outer edge of the beads ($r = R$). Coupling yields of about 20%-30% after the first step of the two-step method (at bead average conditions of $\Phi^2 < 1$) probably represent Mab that has coupled near the edge at high ρ_{Mab}. The similar appearance of fluorescent-photomicrographs of bead cross sections of the two-step or reference immunosorbents at a labeled $<\rho_{Mab}>$ of less than 3 mg Mab/ml supports this conclusion for most Mab coupled at about $r = R$. These analyses showed a narrow band of similar fluorescent signal near the edge of the bead cross-sections for two-step and reference methods (data not shown). In terms of equations 4c and 5a-c, the ρ_{Mab} at about $r = R$ is sufficiently high to reduce the ratio of R_{ic} to R_{id} at that radial position.

Congruently, the threefold higher $<\eta_{Ag}>$ found at about 3 mg/ml or greater in two-step relative to reference immunosorbents is consistent with the locally lower ρ_{Mab} at r< R, as evidenced by the more evenly distributed fluorescent signal seen in Figure 4B. Thus, the lower $<\eta_{Ag}>$ seen for reference relative to two-step immunosorbents at ρ_{Mab} greater than about 3 mg/ml is kinetically consistent with a lower ratio of R_{ic} to R_{id} for a large percentage of the immobilized Mab. In summary, it is primarily the Mab immobilized away from the surface by the two-step method at $<\rho_{Mab}>$ greater than about 3 mg/ml that benefit from higher $<\eta_{Ag}>$ due to locally lower ρ_{Mab}.

This present work establishes the highly nonuniform distribution of Mab and $\eta_{Ag}(r)$ in immunosorbents, and thus the oversimplifications in previous modelling studies presenting the effects of mass transfer and sorption kinetics on intraparticle concentration profiles of bound Ag (28). Due to the high values of $<\eta_{Ag}>$ we have achieved with our two-step method (>50%), we have proposed a bivalent kinetic model for Mab-Ag interactions (equation 5a), rather than the traditional univalent Langmuir model presented in most modelling studies (28). A more detailed evaluation of kinetic and mass transport effects using similar but more specific functional forms for R_{ic} (equations 5a-c) and R_{id} (not given) is beyond the scope of this work, but is currently being studied to gain further insight into the role of Mab coupling on immunosorbent

performance.

CONCLUSIONS

In the above discussion, we have hypothesized that intermolecular steric hindrance of Ag-combining sites of immobilized Mab by other proximally immobilized Mab and associated Ag:Mab complexes exists and is superimposed upon the inherent intramolecular steric hindrance by bound Ag. The local Mab density in an immunosorbent is a more critical parameter than bead average Mab density in immunoaffinity chromatography. Mab coupling methods with a large Thiele modulus during permeation will likely result in a shell-type profile with high localized densities. Using a two-step strategy, where the Thiele modulus is low during the permeation step, a more even distribution of immobilized Mab can be achieved at high $<\rho_{Mab}>$. This modified coupling method results in increased binding efficiency at high $<\rho_{Mab}>$, increasing the overall usefulness of the resulting immunosorbent. The improvement is not strongly related to nor results in mass transfer restrictions in the 60 μm diameter beads studied, and thus is related to changes in the kinetics of Ag adsorption.

Acknowledgements

This work was supported by NSF grant BCS-9011098 AMD-2. K. E. Van Cott was supported in part by the DuPont PhD Fellowship. The technical assistance of K. Hess and T. Moore is greatly appreciated. The gift of 8861-Mab and 8861-Mab immunopurified hPC from the ARC is greatly appreciated.

References

1 Chase, H. A., Chem. Engr. Sci., 39 (1984) 1099-1125.

2 Orthner, C. L, Madurawe, R. D, Velander, W. H, Drohan, W. N, Battey, F. D, and Strickland, D. K., J. Biol. Chem., 264 (1989) 18781-.

3 Velander, W. H., Subramanian, A, Madurawe, R. D, and Orthner, C. L., Biotechnol. Bioengr., 39 (1992) 1013-1023.

4 Dennis, K. E, Clark, D. S, Bailey, J. E, Cho, Park, Y. H., Biotechnol. Bioengr., 26 (1984) 892-900.

5 Hossain, Md. M, and Do, D. D., Chem. Eng. J., 34 (1987) B35-B47.

6 Eveleigh, J. W, and Levy, D. E., J. Solid-Phase Biochem., 2 (1977) 45-77.

7 Fowell, S. L, and Chase, H. A., J. Biotechnol., 4 (1986) 1-13.

8 Tharakan, J. P, Clark, D. B, and Drohan, W. N., J. Chromatogr., 522 (1990) 153-162.

9 Katoh, S., TIBTECH, 5 (1987) 328-331.

10 Strauss, W. M, Broze, G. J, Miletich, J. P, and Null, H. R., Biotechnol. Appl. Biochem., 9 (1987) 462-477.

11 Weston, P. D, and Scorer, R., Affinity Chromatography, (1978) 207-210.

12 Narayanan, S. R, and Crane, L. J., TIBTECH, 8 (1990) 12-16.

13 Sportsman, J. R., and G. S. Wilson, Anal. Chem., 52 (1980) 2013-2018.

14 Wu, D, and Walters, R. R., J. Chromatogr., 458 (1988) 169-174.

15 Do, D. D, and Bailey, J. E., Chem. Eng. Comm., 12 (1981) 221.

16 Scharer, R, Hossain, Md. M, and Do, D. D, Biotechnol. Bioengr., 39 (1992) 679-687.

17 Hossain, Md. M, Do, D. D, and Bailey, J. E., AIChE J. 32 (1986) 1088-1098.

18 Do, D. D, Clark, D. S, and Bailey, J. E., Biotechnol. Bioengr., 24 (1982) 1527-1546.

19 Morcol, T, Akers, R. M, Johnson, J. L, Gwazdauskas, F. C, Knight, J, Lubon, H, Drohan, W. N, and Velander, W. H., A.N.Y.A.S. (in press).

20 Coleman, P. L, Walker, M. M, Milbrath, D. M, Stauffer, D. M, Rasmussen, J. K, Krepski, L. R, and Heilmann, S. M., J. Chromatogr., 512 (1990) 345-363.

21 Titus, J. A, Haugland, R, Sharrow, S. O, and Segal, D. M., J. Immunol. Meth., 50 (1982) 193-204.

22 Tyn, M. T, and Gusek, T. W., Biotechnol. and Bioengr. 35 (1990) 327-338.

23 Wheatley, J. B, and Schmidt. Jr, D. E., J. Chromatogr., 644 (1993)

11-16.

24 Coleman, P. L, Walker, M. W, and Milbrath, D. S., US Patent 5,200,471.

25 Borchert, A and Buchholz, K., Biotechnol. Bioengr., 26 (1984) 727-736.

26 Kang, K, Ryu, D, Drohan, W. N, and Orthner, C. L., Biotechnol. Bioengr., 39 (1992) 1086-1096.

27 McCoy, M. A, and Liapis, A. I., J. Chromatogr., 548 (1991) 25-34.

TABLE I

Reference	Support	Mab/Antigen	Density [mg/ml]	Efficiency
Eveleigh and Levy (6)	Sepharose 4B	Anti-HSA/HSA	1.5	27%
			22	10.5%
Fowell and Chase (7)	Sepharose 4B	Anti-β-galactosidase/β-galactosidase	0.3	5.5%
			13.8	5.5%
Tharakan *et al.* (8)	Sepharose CL2B	Anti-FIX/FIX	1.6	61%
			9.67	17%
Katoh (9)	Sepharose 4B	Anti-BSA/BSA	1	22%
			30	15%
Katoh (9)	Activated CH Sepharose 4B	Anti-BSA/BSA	2	18%
			25	10%
Katoh (9)	Formyl Cellulofine	Anti-BSA/BSA	2	30%
			20	10%
Strauss *et al.* (10)	CM BioGel A	Anti-FIX/FIX	0.71	20%
			15.83	8%
Strauss *et al.* (10)	Affi-Gel 202	Anti-FIX/FIX	0.58	28%
			19.52	8%

TABLE II

Target Density [mg/ml gel]	Actual Density [mg/ml gel]	Coupling Efficiency [%]
Two Step Coupling		
1.1	0.9	82%
4.5	3.2	71%
10	5.7	57%
20	9.3	47%
Reference Coupling		
1.1	1.1	100%
2.3	2.2	96%
12	11.8	98%

TABLE III

Mab Density [mg/ml]	Run No.	CV [ml]	F [ml/min]	C_{feed} [mg/ml]	PC Eluted [μg]	Efficiency [η_{Ag}]
Two Step Coupling						
0.9	1	1	1.0	1.1	156	22%
	2	0.86	0.86	0.8	141	23%
3.2	1	1.0	1.0	0.8	1407	55%
	2	1.0	1.0	2.0	1595	62%
9.3	1	1.0	1.0	1.1	4891	65%
	2	1.0	1.0	2.0	4402	59%
Reference Coupling						
1.1	1	1.0	1.0	2.1	180	20%
	2	1.0	1.0	0.8	234	26%
2.2	1	0.86	0.86	2.3	471	31%
	2	0.86	0.86	0.8	429	28%
11.8	1	1.0	1.0	1.1	2092	22%
	2	1.0	1.0	2.0	2700	28%

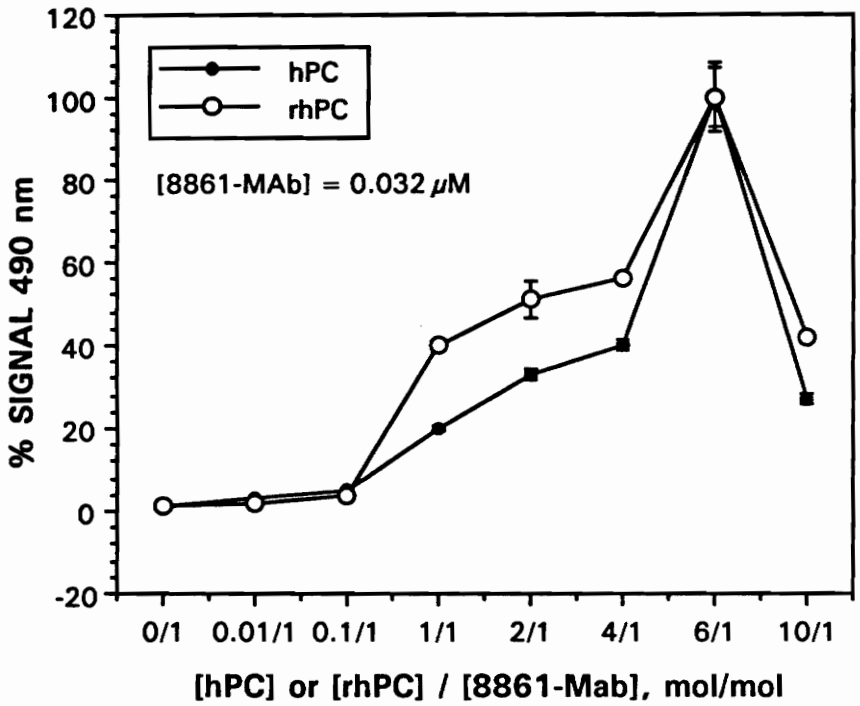


Figure 1

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

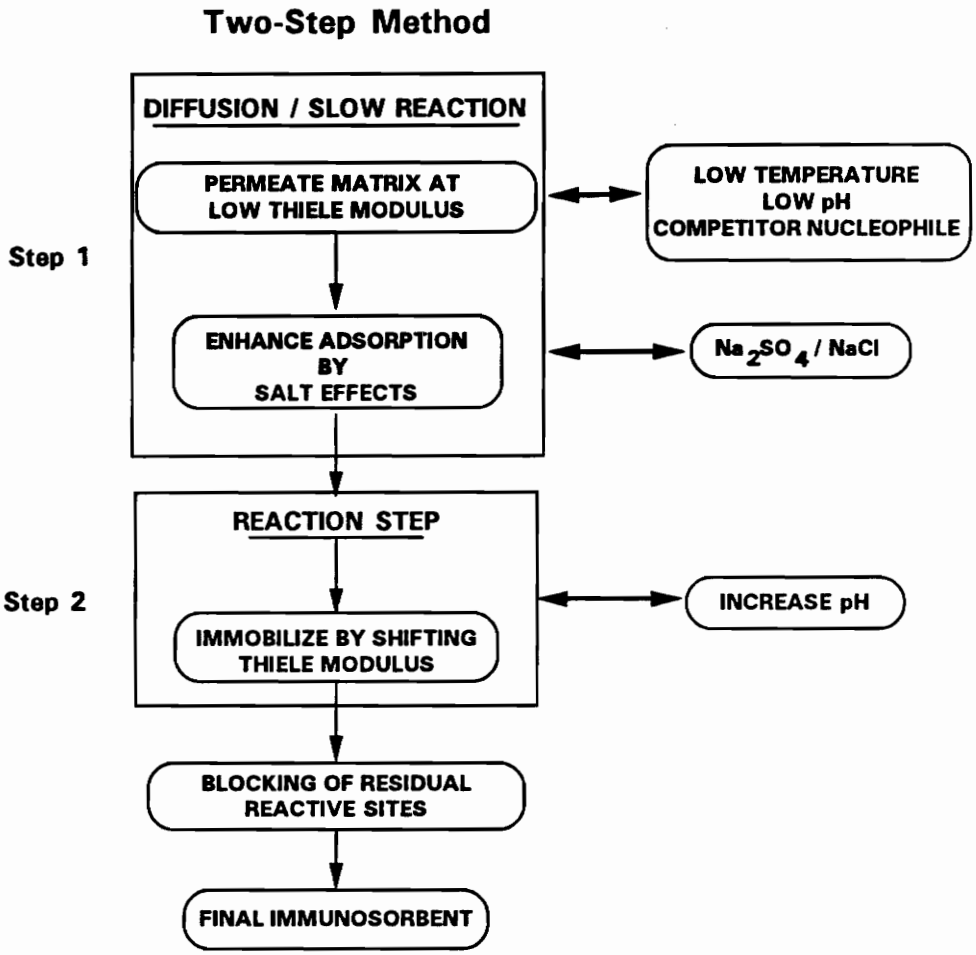


Figure 2

Alternate two-step immobilization procedure
A flowsheet representation of proposed alternate immobilization strategy (two-step method). The first step involves permeation of Mab into the support by selecting a low pH (4.0) and temperature (< 4°C) to yield a low rate of Mab immobilization. Simultaneous diffusion coupled with slow coupling kinetics and the presence of a competitor (0.5M Tris) will result in a more even solution-phase concentration profile in the bead. Once complete penetration of the bead has been reached, the pH is increased (pH 8-9), resulting in an increase in the Thiele modulus [Φ^2] of Mab immobilization.

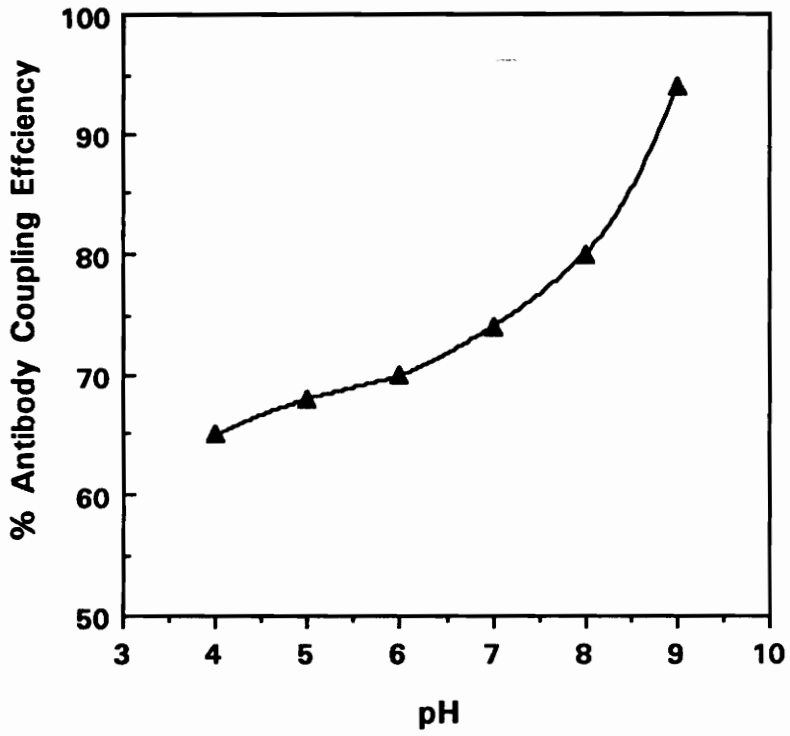


Figure 3a

Effect of coupling pH on Mab immobilization efficiency.
Murine EDTA dependent anti-hPC Mab (7D7B10-Mab) was incubated in a ternary buffer (50 mM acetate, phosphate, and pyrophosphate) with 0.75 M Na_2SO_4 for 30 minutes at predetermined pH values. Immobilizations were performed as outlined in the Methods section. Mab in feed and coupling step supernatants was assayed by ELISA described in Methods. Mab coupling efficiency is defined as the ratio of total Mab bound to the total Mab input.

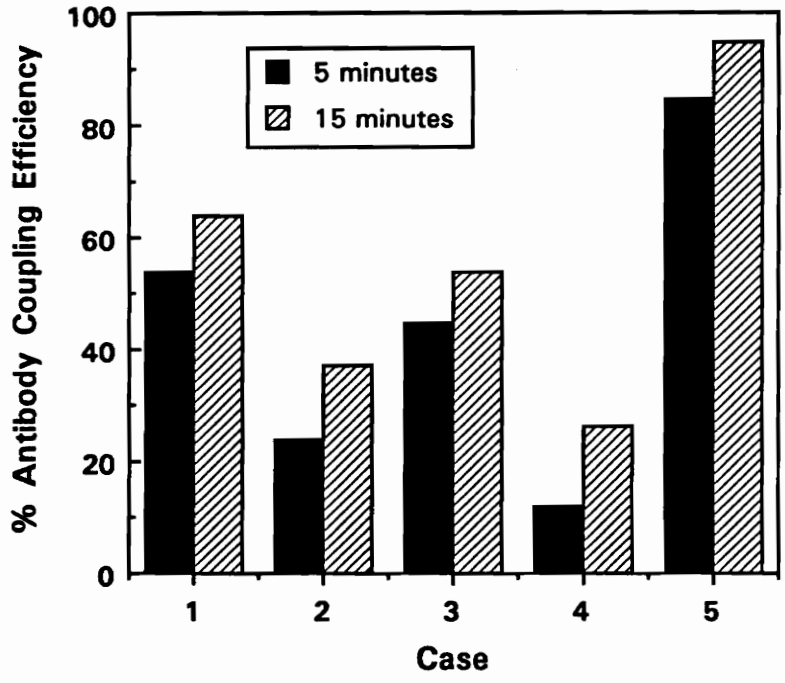
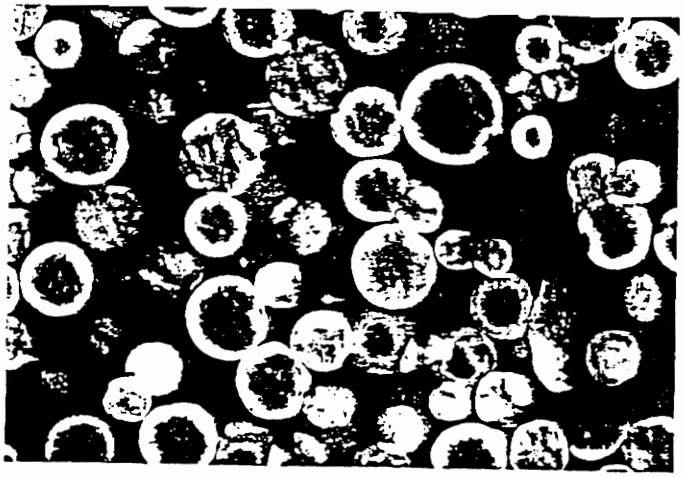


Figure 3b

Effect of salts/Tris on Mab immobilization efficiency.
Murine EDTA dependent anti-hPC Mab (7D7B10-Mab) was incubated in 50 mM acetate buffer (pH 4.0) both in the presence and absence of 0.75 M Na_2SO_4. Immobilizations were performed in the presence of 0.1 or 0.5 M Tris as outlined in the Methods section. Mab coupling efficiency is calculated as in Figure 3a. **Case 1**: 0.1 M Tris/0.75 M Na_2SO_4, 4°C. **Case 2**: 0.5 M Tris/0.75 M Na_2SO_4, 4°C. **Case 3**: 0.1 M Tris/0.0 M Na_2SO_4, 4°C. **Case 4**: 0.5 M Tris/0.0 M Na_2SO_4, 4°C. **Case 5**: 0.05 M sodium phosphate/0.75 M Na_2SO_4/pH 7.0, RT.

A



B

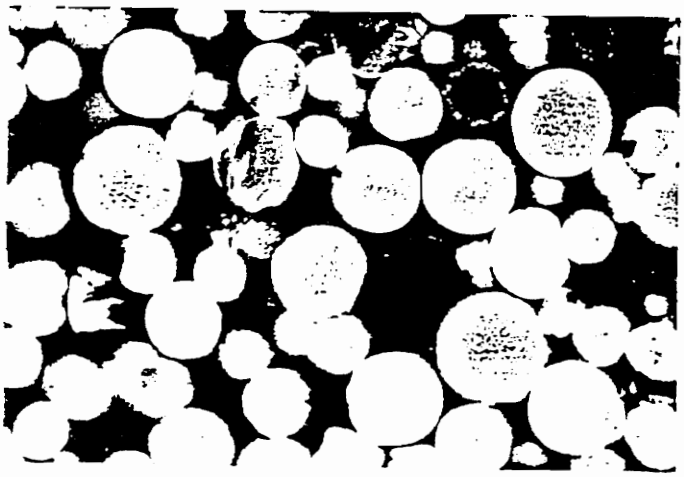


Figure 4

Emphaze AB1 immunosorbents were challenged with Texas Red labeled 7D7B10-Mab or anti-mouse IgG as in the legend to Table II. Texas Red labeled beads were sectioned as detailed in Methods section and viewed under fluorescent microscope. Panel A presents fluorescent micrograph of immunosorbents prepared via the reference method at a $<\rho_{Mab}>$ of 6.0 mg/ml. Panel B presents fluorescent micrograph of immunosorbents prepared via the alternate immobilization strategy (two-step method) at a $<\rho_{Mab}>$ of 5.5 mg/ml.

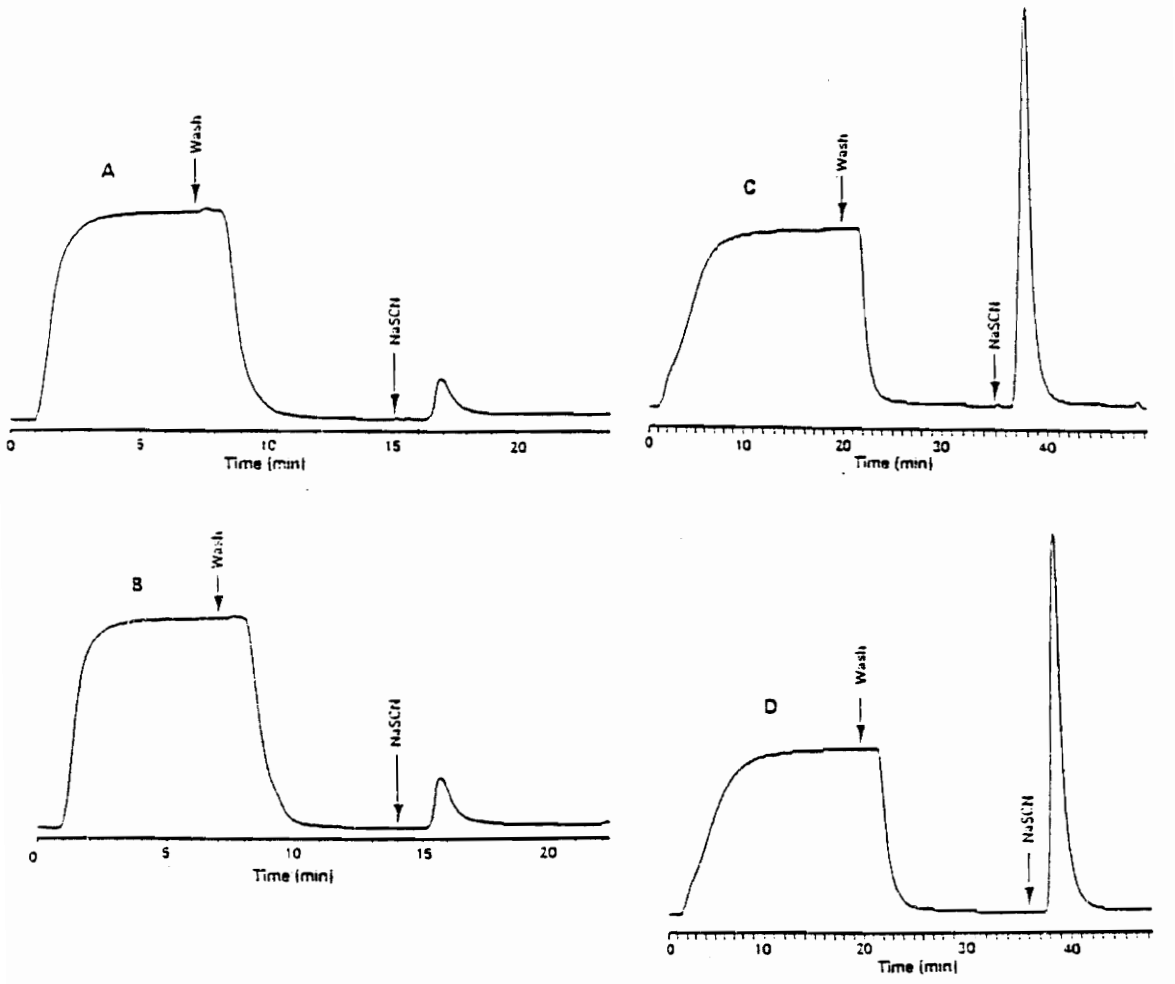


Figure 5

Breakthrough loading of pure rhPC on Emphaze AB1 immunosorbents. Columns (D = 1 cm) were packed with immunosorbents referred to in Table II and loaded with pure rhPC at 1 ml/min. After washing, rhPC was eluted with 2 M NaSCN. **(A)** Two-step procedure, $<\rho_{Mab}>$ = 0.9 mg/ml, F = 1 ml/min, CV = 0.86 ml, C_{feed} = 1.1 mg/ml. **(B)** Reference procedure, $<\rho_{Mab}>$ = 1.1 mg/ml, F = 1 ml/min, CV = 0.79 ml, C_{feed} = 1.1 mg/ml. **(C)** Two step procedure, $<\rho_{Mab}>$ = 9.3 mg/ml, F = 1 ml/min, CV = 1 ml, C_{feed} = 1.0 mg/ml. **(D)** Reference procedure, $<\rho_{Mab}>$ = 11.8 mg/ml, F = 1 ml/min, CV = 1.2 ml, C_{feed} = 1.0 mg/ml.

CHAPTER FIVE

RANKING THE EFFECTS OF ORIENTATION, LOCAL DENSITY AND MULTIPOINT ATTACHMENT IMPACTING IMMUNOSORBENT PERFORMANCE

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ABSTRACT

This work seeks to address the relative importance of the phenomena of orientation, multipoint attachment, and local density upon immunosorbent efficiency. The masking of the antigen binding domains of monoclonal antibodies (Mab) by Fab Masking Antigens (FMAs) is used as a tool to determine the impact of orientation on the performance of Emphaze AB1 and Affiprep™ based immunosorbents. Murine monoclonal antibodies against human Protein C were immobilized in the presence of either synthetic or recombinant FMAs. Immobilizations performed with "FMA-masked" antibody prior to covalent coupling on beaded supports resulted in two- to three-fold higher antigen binding efficiencies as compared to immobilizations with "unmasked" antibody via random coupling. The increase in antigen binding efficiencies produced by "FMA-masked" immobilization correlated well with increased amounts of (Fab)2 released from pepsin digestion of "FMA-masked" immunosorbents relative to "unmasked" immunosorbents. Thus "improper orientation" was correlated with low immunosorbent activity. The results of this study can serve as a basis for a rational design of immobilization procedures which yield highly active immunosorbents.

INTRODUCTION

The performance of an immunosorbent is dependent on physical characteristics of the matrix, localized density of the immobilized antibody and "orientation" of antibody. The chemical structure of the antibody such as lysyl-residue and carbohydrate content will also impact the above phenomena. Although immunosorbent performance is characterized on a volume or bead averaged basis, each of these phenomena may be a function of antibody position within the bead. From a localized view of the bead or support interior, it is necessary for the immobilized antibody to be in the right "orientation" to specifically interact with the antigen in solution. Furthermore, antibody density which is a strong function of position, can adversely impact immunosorbent efficiency. Multipoint attachment to the support matrix will also negatively impact immunosorbent efficiency (1).

Lower immunosorbent activity has been observed at very high levels of antibody density (mg Mab/ml of resin or hydrogel). The effect of surface density (Mab/ml of resin) on immunosorbent performance has been investigated and reviewed in detail (2). Antibodies were immobilized onto a variety of matrices using spacer arms or molecular extenders (3) to reduce the deleterious effects of surface density by minimizing the ligand concentration per unit surface area of the matrix. Mabs against Protein S and Protein Z when

immobilized on beaded supports using molecular extenders failed to show any appreciable improvement in functional efficiency.

Oriented coupling can be achieved by anchoring the Mab such that the antigen binding sites (Fab domains) are directed away from the matrix backbone. One method of accomplishing of "oriented" coupling is to utilize the affinity of protein A to bind Mabs. Mabs anchored in this fashion were used to isolate transferrin receptor and leukemia-associated antigens from cell lysates (4). The potential of protein A leakage in the product coupled with varying affinity of protein A for Mabs from different species makes this technique very tedious. Recently protein G has been employed to co-anchor Mab to the matrix.

Recent efforts have been focused on the immobilization of Mabs via their Fc region by using "Hydrazide Chemistry". Murine monoclonal Mabs commonly used in affinity chromatography are reported to contain CHO moieties in the Fc domains. Oxidation of Mabs by sodium metaperiodate to reactive aldehyde groups and reaction with activated support yields stable hydrazone linkages (5). Immobilized avidin via the hydrazide chemistry demonstrated increased "bound activity" when compared to random immobilization via CNBr chemistry. A 30-400% enhancement in immunosorbent activity per mole of IgG relative to CNBr method was observed for both avidin and anti-b-galactosidase system (6). A

two to three times enhancement in the bound activity of Mabs against carboxypeptidase A and horse radish peroxidase was observed when immobilized on Eupergit C using hydrazide chemistry (7). Near quantitative immobilized activity was observed for anti Human Serum Albumin immobilized on Carbo Link Gel (8). A 70% higher functional activity of anti-Soy Bean Trypsin Inhibitor on Sephacryl immobilized via hydrazide chemistry was observed as compared to activity via CNBr immobilization (9). However, Mabs against human Protein C and Factor IX when immobilized on solid matrices using hydrazide chemistry failed to exhibit an enhancement in functional efficiency as compared to random immobilization via CNBr chemistry (10). Carbohydrate analysis of papain digested Fab fragments of anti Factor IX and anti human Protein C Mabs revealed the presence of sugar moieties. Upon reduction with sodium borohydride, its postulated that coupling to the solid matrix occurred via the CHO moieties found both in Fab as well as Fc domains, thus resulting in the loss of antigen binding Fab sites.

A more recent effort to enhance the bound activity of both polyclonal and monoclonal antibody is to reversibly but non-specifically protect the reactive NH2 groups on the antibody molecule by dimethyl maleic anhydride (DMA) prior to covalent immobilization (11). It is postulated that reactive NH2 groups are found in the Fab domain thus shielding with DMA restricts the

interaction of Fab domain with the activated matrix backbone. Reactive NH2 moieties on monoclonal antibodies when protected by DMA resulted in 3-10.7-fold increase in immunosorbent activity whereas polyclonals under similar conditions showed a moderate 1.6-1.8 fold enhancement.

From previous work, we have demonstrated that shielding of the Fab domains during the immobilization process results in greater immunosorbent efficiency for Immobilon membranes and Affiprep™ affinity beaded support (a synthetic polymer); both of these matrices were activated to react with lysyl residues using ester chemistry (1). A variety of antigens of different size containing the same binding epitope were used. We refer to these Fab-masking antigens as FMAs. This study seeks to elucidate the effect of antibody orientation upon covalent immobilization on the antigen binding efficiency of beaded immunosorbents.

Materials

Murine pH dependant anti human Protein C (hPC) monoclonal antibody (8861-Mab) was provided by American Red Cross (Rockville, MD). Murine metal dependant anti human Protein C (hPC) monoclonal antibody (HPC4-Mab) was purchased from Oklahoma Research Foundation (Oklahoma, OK). Rabbit antisera against human Protein C, affinity purified goat-anti-mouse (whole

molecule) and anti-goat/anti-mouse immunoglobulins conjugated to horseradish peroxidase (HRP) were purchased from Sigma Chemical Co. (St.Louis, MO.) Goat anti human Protein C antisera was purchased from American Diagnostics Inc. (Greenwich, CT.) Emphaze™ biosupport medium was provided by 3M. (Minneapolis , MN) and Affiprep™ polymeric support was purchased from Bio-Rad Laboratories (Anaheim, CA). Immulon II microtiter plates were purchased from Fisher Scientific. Human protein C (hPC) was provided American Red Cross. (Washington. D.C.). Recombinant hPC (rhPC) was isolated from transgenic porcine whey using immunoaffinity chromatography. O-phenylenediamine-2HCl tablets were purchased from Abbott Laboratories (Chicago, IL.). All other reagents were purchased from Sigma at the best grade available. Immunoaffinity separations were performed with Pharmacia C-10 columns (15 cm x 1 cm), a Masterflex peristaltic pump, a Knauer spectrophotometer, and a Rainin data acquisition system was used to monitor chromatography. Columns were kept at 4° C with a Lauda Super RMT water cooler.

Use of Synthetic polymer-peptide adducts as FMAs

A synthetic peptide, equivalent to the binding epitope, as recognized by the monoclonal antibody HPC4-Mab was constructed by t-boc synthesis. Peptide was conjugated to a family of methyl polyoxazoline polymers and

adducts of different molecular weight were synthesized (12). Peptide and the 10K adduct were used to shield HPC4-Mab prior to immobilization.

Use of recombinant hPC as FMA

A cDNA of human protein C was expressed in the mammary gland of pigs (13). The recombinant hPC (rhPC) product expressed in the milk of transgenic pigs was isolated and purified using affinity chromatography and was employed to shield 8861-Mab prior to immobilization.

Characterization of Supports studied

These studies were performed to evaluate the effect of FMAs on the activity of immobilized Mab on two different kind of supports. Three types of FMAs were used; A 12 amino acid long peptide (HCPC[6-17]), a 10K methyl-polyoxazoline-HCPC[6-17] adduct, and modified recombinant protein C. The peptide and 10K adduct contained the binding epitope recognized by HPC4-Mab. The supports used in this study were subjected to different activation chemistries. Affi-prep™ a commercially available support via Bio-Rad laboratories has a reactive ester moiety as an end group, which in turn reacts with the -NH2 group on the proteins to yield a stable amide linkage.

Emphaze AB1 (D_p = 60 μm) is a polymer based affinity support,

synthesize by a copolymerization reaction of 2-vinyl-4,4-dimethyl-1,3-oxazolin-5-one and methylene-bis-acrylamide at various ratios to yield a rigid and macroporous support for bioseparations (14). The azlactone functionality readily undergoes a ring-opening reaction with nucleophiles (ex: -NH_2 moieties on the antibody/proteins) to yield stable covalent linkages.

METHODS

Preparation of "unmasked" and "FMA-masked" Immunosorbent

Affi-prep™ polymeric support was washed with 10 mM sodium acetate buffer, pH 4.5 according to manufacturers instructions and was suspended as a 50% (vol/vol) slurry in the coupling buffer, 0.1 M MOPS, 0.15 M NaCl at pH 7.2. In order to obtain a 1 ml column, 2 ml of the slurry was pipetted in to 10 ml polypropylene tubes. In order to minimize surface density effects a working Mab density of 1-3 mg of Mab/ ml of gel was chosen. For the preparation of "masked" immunosorbent, solution containing 1 mg of Mab with varying ratios of either the FMAs and/or rhPC was preequilibrated for 2 hours at RT. In the case of "unmasked" immunosorbent Mab was incubated with buffer for the same period of time. The supernatant was pipetted drawn off 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 drawn off and saved for ELISA

assays. In order to block the remaining active sites on the resin, 0.1 ml/ml gel of 1.0 M ethanolamine was added and rotated for 1 hour at 4° C, upon which the supernatant was drawn and saved for ELISA assays. The gel was washed in a column mode with 0.05 M Tris, 0.1 M NaCl, pH 7.0 till the absorbance of the column effluent dropped to zero. At this step by passing the appropriate buffer system the FMA:Mab complex was disrupted, to yield FMA in the column eluate, which was saved for ELISA assays. 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 column were stored in the protein coupling buffer at 4° C.

Mabs were immobilized on azlactone beads as per manufacturer's instructions. In brief, 125 mgs of azlactone beads were suspended in 1.0 ml coupling buffer containing 0.5 mgs of 8861-Mab and varying ratios of FMA to yield 1.0 ml of affinity sorbent. Both binding and elution of the antigen was carried out in a batch mode.

Determination of Mab bound to the resin

(a) Liquid Phase EIA

Immulon II microtiter plates were coated with 100 μl/well of 1:200 diluted anti-mouse whole molecule in 0.1 M $NaHCO_3$ (pH 9.3) for 24 hrs at 4°

C. Wells were washed with 0.05 M Tris/ 0.1 M NaCl/0.05 % Tween and the residual reactive sites were blocked with 0.1% BSA/TBS for 15 mins at RT. Various dilutions of standard and samples in TBS/1 mg/ml PEG were added to the wells, 100 μl in each well and incubated for 30 mins at 37°C. Upon incubation wells were washed four times and 1:1000th diluted HRP conjugated goat anti-mouse IgG was added to the wells and incubated for 30 mins at 37°C. Wells were washed four times and 100 μl of OPD substrate was added to each well. The colorimetric reaction was stopped ater 3-5 mins by the addition of 100 μl of 3N H_2SO_4 to each well. Bound chromophore was detected at 490 nm using a EL308 Bio-Tek Microplate reader. Samples from the starting solution and supernatant from coupling steps were subjected to the above described ELISA.

b) Bicinchoninic Acid Assay

The amount of Mab immobilized on the support was determined by the modified BCA assay (15). 50 μl of gel in 10 μl water was incubated with 200 μl of BCA reagent for 2 hours at 60° C. 100 μl of the reaction mixture was transferred to a microtiter plate and the absorbance was measured at 560 nm. As a parallel experiment a control sample was subjected to the assay to subtract the background signal and a standard with either HPC4-Mab or 7D7B10-Mab was generated.

Immunoaffinity Separation

Antibody columns prepared were equilibrated in a batch mode with either plasma-derived or recombinant (transgenic pig milk) hPC for 6 hours or more at 4°C. All immunoaffinity separations were performed at a flow rate of 1.0 ml/min, in a column mode using C20 column systems from Pharmacia. The affinity columns were washed with ligand coupling buffer till the absorbance at 280 nm dropped to zero and the bound hPC antigen was eluted using TBS/25 mM EDTA, pH 7.0. Following elution all columns were successively washed with 2 M NaSCN and then re-equilibrated with ligand coupling buffer. The fractions from each chromatographic step were saved for hPC detection by ELISA assays.

Breakthrough Experiments

Columns were packed with approximately 1 ml of 8861-Mab/Emphaze AB1 or Affi-prep immunosorbent (D = 1 cm), cleaned with at least 10 column volumes (CV) of 2 M NaSCN, and then equilibrated with at least 20 CV of loading buffer (20 mM sodium citrate, 80 mM sodium chloride, pH 6.5). After equilibration, pure recombinant hPC (from transgenic pig whey) at a concentration of 1-2 mg/ml was loaded at about 1 ml/min (linear velocity (u_o) = 1.3 cm/min) until the breakthrough front leveled off. For each immunosorbent column was challenged with the same concentration of rhPC

feed solutions. The columns were then washed with the loading buffer until baseline was reached. Bound rhPC was eluted by a single 2 M NaSCN elution. The columns were re-equilibrated in the loading buffer. All chromatographic fractions were saved for rhPC determination by ELISA.

Determination of protein C in feed and column eluates

Immulon II plates were coated overnight with 100 μl/well of 5 μg/ml of rabbit anti-human Protein C in 0.1 M $NaHCO_3$, pH 9.3 at 4° C. Wells were washed with TBS/0.5% Tween. 100 μl of standard and samples in the dilution buffer (TBS / 0.5% BSA, pH 7.0) was added to all the wells and incubated at 37°C for 20 mins. Wells were washed four times and the bound hPC was detected by a sandwich of goat anti-human protein C and HRP conjugated sheep anti-goat IgG. Bound chromophore was detected at 490 nm using EL308 Bio-Tek Microplate reader.

Pepsin Digestion of Immobilized Mab

100 μl of a 50% (vol/vol) slurry of both "masked" and "unmasked" immunosorbent was pipetted into screw cap tubes to yield a final immunosorbent volume of 50 μl. Immunosorbents were washed four times with 0.1 M sodium citrate, pH 4.2 and suspended in 30 μl of citrate buffer. 5 μl of a 10 mg/ml solution of pepsin in acetate buffer was added to the reaction tubes

and digestion was carried out at 37°C for 5 hours. Enzymatic digestion was terminated with an addition of 100 µl of 2M Tris. The reaction mixture was centrifuged for 10 mins at 2000 x g and the supernatant was subjected to (Fab)2 determination by EIA.

Determination of (Fab)2 in pepsin digest

Liquid Phase ELISA

Immulon II microtiter plates were coated with 100 µl/well of 5 µg/ml diluted Fab specific murine antisera in 0.1 M NaHCO3 (pH 9.3) for 24 hrs at 4° C. Wells were washed with 0.05 M Tris/ 0.1 M NaCl/0.05 % Tween and the residual reactive sites were blocked with 0.1% BSA/TBS for 10 minutes at RT. Various dilutions of standard and samples in TBS/0.1% BSA were added to the wells, 100 µl in each well and incubated for 20 mins at 37°C. Upon incubation wells were washed four times and 1:1000th diluted HRP conjugated goat anti-mouse IgG was added to the wells and incubated for 20 mins at 37°C. Wells were washed four times and 100 µl of OPD substrate was added to each well. The colorimetric reaction was stopped ater 3-5 mins by the addition of 100 µl of 3N H_2SO_4 to each well. Bound chromophore was detected at 490 nm using a EL308 Bio-Tek Microplate reader.

DMA Modification of the Reactive Amino Residues

The reactive amino moities on either 8861-Mab or rhPC were modified using DMA (16). A 3 mg/ml solution of 8861-Mab or rhPC in 0.05 M borate buffer/0.14 M NaCl, pH 8.6 was slowly mixed with 200 molar excess of ester in DMF (10 μl). The pH of the reaction was maintained at 8.5 by the addition of 1 N NaOH and the reaction was allowed to proceed for 24 hours at 4° C. To quench the unreacted ester 0.4 M glycine solution, pH 8.5 was added by maintaining the glycine/ester ratio at 40. After 4 hours of stirring, the modified protein solution was extensively dialysed against PBS for 48 hours to yield a protein-ester conjugate.

TNBS Assay to determine the degree of modification

The degree of modification of the amino residues were determined by the modified TNBS assay (18). Samples and standard were diluted to a concentration range of 1 mg/ml -0.0156 mg/ml in 0.1 M borate buffer. One ml of the sample or standard solution was mixed with 25 μl of 0.03 M aqueous TNBS solution and the mixture was incubated at RT for 30 mins. The blank consisted of 1 ml of buffer with 25 μl of TNBS solution. The absorbance of the samples and standards were read against the blank at 420 nm.

RESULTS

Modification of the Reactive Primary Amines

The number of available reactive primary amines on both 8861-Mab and rhPC were modified by covalent and irreversible reaction with an ester moiety. Using the procedure outlined in the methods section, 55% of the primary amines on 8861-Mab and 60% of the primary amines of rhPC were modified. The degree of modification was estimated by TNBS assay.

The avidity of immunocomplexes formed from either modified Mab and rhPC was determined by solution phase assay and was compared to that of native or unmodified 8861-Mab. Figure 1 presents the ELISA signal resulting from the immunocapture of the (r)hPC-modified:8861-Mab (native or modified) complex formed in solution. The modification of the primary amines on rhPC had no significant effect on the ELISA profile of native or modified Mab; both ELISA signals show similar increases with increasing antigen concentration. In contrast, assay samples containing modified 8861-Mab and modified rhPC showed reduced signals.

Effect of FMA on Immobilization of Mab

The presence of HCPC[6-17] or 10K methoxy polymer peptide adduct during HPC4-Mab immobilization on Affi-prep™ resulted in decrease in the

amount of Mab immobilized (Table 1). A coupling efficiency of 63% was observed for the immobilization of "FMA-masked" HPC4-Mab in the presence of 10 molar excess HCPC[6-17]. When immobilized in the presence of 10K adduct, (at a ratio of 10:1 FMA/Mab) a Mab coupling efficiency of 54 ± 4% was obtained. Mab coupling efficiencies of 40-50% were achieved for HPC4-Mab immobilized in the presence of HCPC[6-17] and/or 10K adduct at a FMA/Mab ratio of 25: 1. Immobilization of "unmasked" HPC4-Mab gave coupling efficiencies of 73 ± 3%. Table 1 lists the normalized coupling efficiency of HPC4-Mab in the presence of FMAs. Coupling efficiency obtained with "unmasked" antibody was assigned a maximum value of 100 and coupling efficiencies obtained in the presence of FMA were reported as a fraction of the maximum value. Immobilization of HPC4-Mab in the presence of FMAs (HCPC[6-17] and 10K adduct) at a ratio of 10/1 gave 14% and 26 % reduction in Mab coupling efficiencies. When masked at a ratio of 25/1 (HCPC[6-17] or 10K adduct) a 25% and 38% reduction in coupling efficiency were observed.

The use of modified-rhPC as an FMA resulted in the reduction of 8861-Mab which was immobilized on both Affi-prep™ or Emphaze AB1 (Table 2). A 40-50 % reduction in the amount of Mab immobilized upon Emphaze AB1 was observed for 8861-Mab complexed to modified rhPC at a ratio of 6:1 (FMA/8861-Mab). A 50% reduction in the amount of immobilized Mab on

Affiprep™ was observed for 8861 complexed with modified rhPC at a ratio of 6:1 (FMA/8861-Mab). Immobilization of modified 8861-Mab in the presence of modified rhPC at a ratio of 6/1 (FMA/ modified 8861-Mab) resulted in Mab coupling efficiency of 51%. Immobilization of "unmasked" 8861-Mab gave a Mab coupling efficiency of 96%.

Prior to testing the immunosorbent capacity, the columns with (r)hPC for immunoaffinity separation analysis, buffers to be used in the course of chromatography were passed columnwise and all the column washes were assayed for either leached Mab or rhPC by EIA/ELISA. No detectable leakage of covalently bound rhPC (when used as FMA) or Mab, was observed in the column washes of the blank operation for azlactone (sensitivity of Mab ELISA assay: 0.1 ng/ml).

Using the modification procedure outlined in the methods section, a 55% reduction in the number of primary amines was obtained. Modified 8861-Mab was coupled to Emphaze AB1 via the manufacturer's protocol (reference method) and alternate immobilization (2-step) method (2) and the Mab coupling efficiencies obtained are listed in Table 3.

Using the reference protocol (R), modified 8861-Mab was immobilized

at Mab densities (mg Mab/ml of hydrogel) of 1 and 10.0 mg Mab /ml of gel. A coupling efficiency of 99% was achieved at a targeted Mab density of 1.1 mg 8861-Mab/ml of hydrogel. Coupling efficiencies of 97% was obtained for modified 8861-Mab immobilized at a targeted Mab density of 9.5 mg/ml of hydrogel.

Immobilizations of modified 8861-Mab using the 2-Step method were performed to yield targeted Mab densities of 1.0 and 9.5 mg/ml respectively. Coupling efficiencies of 61% and 55% were obtained which resulted in immobilized Mab densities of 0.6 and 5.2 mg 8861 /ml of hydrogel.

Effect of FMA on the Antigen Binding Efficiency of Immobilized Mabs

Both "unmasked" and "FMA-masked" HPC4-Mab:Affiprep columns were equilibrated (batch loading) with either plasma derived or recombinant hPC and the bound hPC or rhPC was eluted with TBS/25 mM EDTA, pH 7.0 in a column mode. HPC4-Mab immunosorbents were challenged with pure hPC or rhPC at 30-40% of its maximum theoretical antigen binding capacity. All efficiencies are computed assuming a 2:1 stoichiometry of the antigen:antibody complex. The effect of HCPC[6-17] and 10K methoxy adduct on the efficiency of immobilized HPC4-Mab on Affi-prep™ is presented in Table 4. HPC4-Mab immobilized in the absence of FMA on Affi-prepTM has an efficiency of 19%

(assuming a 2:1 Ag:Mab stoichiometry). Efficiencies of 39 ± 3% were observed for HPC4-Mab immobilized on Affi-prepTM in the presence of HCPC[6-17] at FMA:HPC4-Mab ratios of 10:1 and 25:1 respectively. Efficiencies of 37 ± 2% and 56% were observed for HPC4-Mab immobilized on Affi-PrepTM in the presence of 10K adduct at FMA:Mab ratios of 10:1 and 25:1 respectively.

Antigen binding efficiencies of 8861-Mab immobilized on either Emphaze AB1 or Affiprep were evaluated by breakthrough analysis. Figure 2 shows typical breakthrough and elution profiles at 280 nm for rhPC loaded on both "FMA-masked" and "unmasked" 8861-Mab immunosorbent column on either Emphaze AB1 or Affiprep. The shapes of the breakthrough profiles and 2M NaSCN elution peaks were similar for "FMA-masked" and "unmasked" 8861 immunosorbents. The peak area under 2M NaSCN eluate fractions for "FMA-masked" immunosorbents were 2-3 fold higher than immunosorbents made with "unmasked" 8861 at comparable Mab densities.

The effect of modified rhPC on the efficiency of immobilized 8861-Mab on Emphaze AB1 and Affiprep is presented in Table 5. 8861 immobilized at 0.5 and 0.9 mg Mab/ ml of hydrogel in the absence of FMA on Emphaze AB1 has an efficiency of 22 ± 1%. Efficiencies of 44% was observed for 8861-Mab immobilized on Emphaze AB1 (at 0.7 mg Mab/ml of hydrogel) in the presence

of modified rhPC at FMA:HPC4-Mab ratios of 6:1. Antigen binding efficiency of 42 % was observed for 8861-Mab immobilized at 1.0 mg/ml of gel in the presence of modified rhPC at a ratio of 6:1 (FMA:Mab).

Similar effects of modified rhPC on the efficiency of immobilized 8861-Mab on Affiprep was observed. 8861-Mab immobilized in the absence of FMA gave an efficiency of 18%. Efficiencies of 45% were observed for 8861-Mab immobilized on Affiprep at 0.42 mg Mab /ml of gel in the presence of modified rhPC at FMA:HPC4-Mab ratios of 6:1.

Effect of Multipoint Attachment on Immunosorbent Efficiency

Using the modification "chemistry" (reaction of primary amines with N-Hydroxtsuccinimide ester of acetic acid) proposed, 55% modification of the amino groups on 8861-Mab was achieved. Modified 12A8-Mab when immobilized using the reference protocol gave a functional efficiency of 14 ± 3 % when compared to an efficiency of 7 ± 2 % attained with unmodified Mab.

(Fab)2 Determination

Both "unmasked" and "masked" immunosorbents were subjected to pepsin digestion for 5 hours at 37°C. The total amount of Fab [Fab' + (Fab)2]

present was determined by EIA. The amount of Fab detected in supernantant was normalized with respect to total Mab input. As pepsin digestion partially or completely degrades the Fc portion, no attempt was made to quantify the amount of Fc in the supernatant. Tables 4 and 5 lists the total amount of (Fab) detected per mg of immobilized Mab.

Immobilizations performed, on either Affi-prepTM or Emphaze AB1, by masking the Mab with FMA prior to covalent coupling gave higher total amounts of (Fab) in the pepsin digest when compared to immobilizations performed with unmasked Mab. 8861-Mab immobilized on Emphaze AB1 in the presence of FMA (modified rhPC) gave 0.04 μg of $(Fab)_2$ / mg of immobilized 8861-Mab whereas "ummasked" 8861 on Emphaze AB1 gave 0.01 μg of $(Fab)_2$ / mg of immobilized 8861-Mab. 8861 immobilized on Affi-prepTM in the presence of FMA (modified rhPC) gave 0.1 μg of $(Fab)_2$ / mg of immobilized 8861-Mab whereas "ummasked" 8861 on Emphaze AB1 gave 0.03 μg of $(Fab)_2$ / mg of immobilized 8861-Mab.

HPC4 immobilized on AffiprepTM in the presence of HCPC[6-17] and 10K methoxy adduct at FMA to Mab ratio of 25:1 gave 0.01 and 0.020 μg of $(Fab)_2$ / mg of immobilized HPC4-Mab respectively whereas "ummasked" HPC4 on AffiprepTM gave 0.01 μg of $(Fab)_2$ / mg of immobilized HPC4-Mab. Tables 4 and 5 gives the percent incrase in antigen binding efficiencies and total

(Fab)2 signal for both "masked" and "unmasked" immunosorbents.

DISCUSSION

A loss in the functional activity of antibodies upon covalent immobilization can be attributed to local Mab density, multipoint attachment and improper orientation. These deleterious effects may be excascerbated by the molecular topography of the support. However at low surface density of immobilized Mab, our results show that improper orientation and multipoint attachment of immobilized Mab can account for 40-80% and 5-10% loss in functional activity respectively. At higher local densities of Mab effects may lead to a 10-30% loss in functional activity.

To gain a better understanding of the effects of supports and support activation chemistries, we have the evaluated the performance of HPC4-Mab and 8861-Mab immobilized on Affi-prepTM and Emphaze AB1 in the presence of both synthetic and recombinant FMAs. Immobilizations on these supports occur via the primary amines on the protein and/or Mab. These commercially available supports offer minimum non-specific and/or other interactions like hydrophobic/hydrogen bonding.

At low immobilized Mab densities, we postulate that orientation and

multipoint attachment will have a more dominant impact on functional activity of the immunosorbent. We have used FMAs to alleviate the effect of orientation and irreversible modification of primary amines on Mab to counteract the effect of multipoint attachment. The lower coupling efficiencies seen with immobilizations in the presence of FMAs and modified Mab are consistent with the physics of shielding and modification of the Mab itself. In each case the reactvity of the Mab is reduced. FMAs which are comparable in size to the Mab (ie modified rhPC) may offer better Mab shielding Mab as compared to smaller FMAs like HCPC[6-17] or 10K adduct.

The lower avidity of the synthetic polymer-peptide masks required higher concentration than would be used for the native antigen. From our previous studies, we estimate HCPC[6-17] and the 10K methoxy adduct to possess avidities which are 4-fold and 16-fold less than hPC (12). In order to attain effective shielding of the divalent Fab domains on HPC4-Mab, incubations of HPC4-Mab with HCPC[6-17] at ratios greater than 4/1 and ratios greater than 16/1 with 10K adduct were necessary. An increase in the ratio of masked antibodies to unmasked antibodies which were immobilized maybe indicated by the increase in the antigen binding efficiency from 36% to 58% for 10K adduct-FMA treatments of 10/1 and 25/1 respectively. Furthermore, no significant improvement in the functional activity was observed at incubations

with HCPC[6-17] at ratios greater than 10/1. Thus, the orientation phenomena may be impacting about 60% of the immobilized antibodies for these immunosorbents.

The impact of FMA larger than 10K adduct is discussed below. Increases in antigen binding efficiencies of "FMA-masked" immunosorbents as compared to "unmasked" immunosorbents correlated well with the percent change in the total amount of (Fab)2 detected in the pepsin digests. Pepsin digest of HPC4-Mab immunosorbent masked with 10K adduct at a ratio of 25/1 gave 1.54 times higher total (Fab)2 as compared to "unmasked" HPC4-Mab, whereas pepsin digest of HPC4-Mab immunosorbent masked with HCPC[6-17] at a ratio of 25/1 gave similar amount of total (Fab)2 as "unmasked" HPC4-Mab. This finding suggests that immobilizations of HPC4-Mab carried out in the presence of smallest mask (HCPC[6-17] peptide) did not cause the Mab to be oriented but caused a conformational change in the 3-D structure of the antibody resulting in a higher functional activity. These results correlate well with Fab/Fc accessibility detected by ELISA format (1) for each of the masks studied on membrane immunosorbents.

The modified rhPC possessed an avidity for 8861-Mab which was similar to that of plasma derived hPC or immunopurified rhPC. A sharp increase in

ELISA signal at ratio of 6:1 (rhPC/8861) suggests that the secondary Ag combining site of the Mab possess lower avidity than the first site (2). Therefore shielding of the secondary site requires higher concentration of FMA (modified rhPC). Furthermore, higher efficiencies were obtained after equilibration of 8861-Mab with rhPC at ratios greater than 2:1 (data not shown).

While 10K masks could enable 60% "oriented" antibody, we sought to evaluate the effects of immobilizations of 8861-Mab on Emphaze AB1 or AffiprepTM in the presence of a larger FMA such as modified rhPC. Thus we used rhPC as a larger mask relative to 10K adduct. The use of modified rhPC as a FMA did not produce further increase in immunosorbent activity indicating that the 10K adduct provided sufficient shielding. Immobilizations of 8861-Mab in the presence of modified rhPC gave higher total (Fab)2 in the pepsin digest as compared to immobilizations via random coupling. The increase in percent total (Fab)2 for "FMA-masked" immunosorbents correlated well with the increases in functional efficiencies. Thus any increase in the antigen binding efficiency of the "FMA-masked" immunosorbents can be attributed to oriented immobilization as opposed to conformational or allosteric effects.

It is clear that "orientation' has an impact upon immunosorbent efficiency

of Emphaze AB1 and AffiprepTM. Local density phenomena and multipoint attachment may still exert a deleterious effect on immunosorbent performance even with the use of FMAs. In summary, FMAs can be employed to enhance the percentage of functional antibody relative to that obtained using random covalent attachment. The degree of enhancement appears to be dependent on the surface activation chemistry as well as on the distribution of reactive moieties on the matrix surface. We are currently evaluating the effects of masking at high local Mab density.

REFERENCES

1 Velander, W. H., Subramanian, A., Madurawe, R. D., and Orthner, C. L., (1992). Biotechnology and Bioengineering, 39, 1013-1023.

2 Subramanian, A., Van Cott, K. E., Milbrath, D. S., and Velander, W. H., submitted to J. Chromatography.

3 Strauss, W. M., Broze, G. J., Milteich, J. P, and Null, H. R., (1987), Applied Biochemistry and Biotechnology, 9, 462.

4 Schneider, C., Newman., R. A., Sutherland, R., Asser, U., and Greaves, M. F., (1982). Journal of Biological Chemistry, 257 (18), 10766-10769.

5 O'Shanessey, D. J., and Hoffman, W. L., (1987). Biotechnology and Applied Biochemistry, 9, 488-496.

6 Little, M. C., Siebert, C. J., and Matson, R. S., (1988). BioChromatography, 3 (4), 156-160.

7 Fleminger, G., Hadas, E., Wolf, T., and Solomon, B., (1990). Applied Biochemistry and Biotechnology, 23, 123-137.

8 Domen, P. L., Nevens, J. R., Mallia, A. K., Hermanson, G. T., and Klenk, D. C., (1990). Journal of Chromatography, 510, 293-302.

9 Bonde, M., Pontoppidan, H., Pepper, D. S., (1991). Preparative Chromatography, 1 (3), 269-277.

10 Orthner, C. L., Highsmith, F. A., Tharakan, J., Madurawe, R. D.,

Morcol, T. M., and Velander, W. H, (1991). Journal of Chromatography, 558, 55-70.

11 Hadas, E., Koppel, R., Schwartz, F., Raviv, R., and Fleminger, G., (1990). Journal of Chomatography, 510, 303-309.

12 Velander, W. H., Madurawe, R. D., Subramanian, A., and Orthner, C. L., (1992). Biotechnology and Bioengineering, 39, 1024-1030.

13 Velander, W. H. et al., (1992). Procedings of the National Academy of Sciences, 89, 12003-12007

14 Coleman, P. L., Walker., M. M., Milbrath., D. S., Stauffer, D. M., Rasmussen, J. K., Krepski, L. R., and Heilmann, S. M., (1990). Journal of Chromatography, 512, 345-363.

15 Plant, A. L., Brown, L. L., Haller, W., and Durst, R. A., (1991). Applied Biochemistry and Biotechnology, 30, 83-92.

16 Endo, N., Umemoto, N., Kato, Y., Takeda, Y., and Hara, T., (1987). Journal of Immunological Methods, 104, 253-259.

17 Snyder, S. L., and Sobocinski, P. Z., (1975). Analytical Biochemistry, 64, 284-286.

ACKNOWLEDGEMENTS

This work was supported by NSF grant BCS-9011098. The technical assistance of K. Hess greatly appreciated. The gift of 8861-Mab and 8861-Mab immunopurified hPC from the ARC is greatly appreciated.

Table 1 Effect of Synthetic Antigens on HPC4-Mab Coupling Efficiency

Support Matrix AffiprepTM

Mab Immobilized HPC4-Mab

FMA Employed HCPC[6-17] and 10K methoxy adduct

FMA	Ratio of FMA/Mab	% Mab Coupling Efficiency	Normalised Coupling Efficiency	Mab density (mg Mab/ml of gel)
None	0/1	73 + 3	100	0.73
HCPC[6-17]	10/1	63 + 3	86	0.63
10 K adduct	10/1	54 + 4	74	0.54
HCPC[6-17]	25/1	55 + 3	75	0.55
10 K adduct	25/1	45 + 5	62	0.45

Table 2 Effect of Recombinant Antigens on Mab Coupling Efficiency

Support Matrix AffiprepTM and EMPHAZE AB1

Mab Immobilized 8861-Mab

FMA Employed modified rhPC

Support	Ratio of FMA/Mab	% Mab Coupling Efficiency	Normalized Mab Coupling Efficiency	Mab Density (mg Mab/ml of gel)
native 8861-Mab				
Affiprep™	0/1	60	100	0.4
	6/1	51	85	0.4
native 8861-Mab				
EMPHAZE AB1	0/1	97	100	0.5
	0/1	94	100	1.0
	6/1	66	69	0.7
	6/1	52	55	1.1
modified 8861-Mab				
EMPHAZE AB1	6/1	51	55	1.6

Table 3 Coupling Efficiencies of modified 8861-Mab on Emphaze AB1

Case No	Coupling Method	Mab Coupling Efficiency	Mab density (mg /ml of gel)	Total (Fab)2 mg (Fab)2 / mg of immobilized Mab
Modified 8861-Mab				
1a	Reference	99%	1.0	0.02062
1b	Reference	97%	9.2	0.0116
2a	2-Step	61%	0.6	0.01032
2b	2-Step	55%	5.2	0.0093
Native 8861-Mab				
	Reference	94%	11.8	0.01388

Table 4 Effect of Synthetic Antigens on Immunosorbent Efficiency

Support Matrix AffiprepTM

Mab Immobilized HPC4-Mab

FMA Employed HCPC[6-17] and 10K methoxy adduct

FMA	Ratio of FMA/Mab	% Antigen Binding Efficiency	% Increase in Antigen Binding Efficiency	Total (Fab)2 [μg (Fab)2/mg immobilized Mab]	% increase in total (Fab)2
None	0/1	22 ± 2	100	0.00862	100
HCPC[6-17]	10/1	39 ± 3	176	n.d	
10 K adduct	10/1	37 ± 2	171	n.d	
HCPC[6-17]	25/1	38	181	0.0082	95
10 K adduct	25/1	56	267	0.020	232

n.d not determined

Table 5 Effect of Recombinant Antigens on Immunosorbent Efficiency

Support Matrix AffiprepTM and EMPHAZE AB1

Mab Immobilized 8861-Mab

FMA Employed modified rhPC

Support	Ratio of FMA/Mab	% Antigen Binding Efficiency	%Increase in Antigen Binding Efficiency	Total (Fab)2 μg (Fab)2/mg of immobilized Mab	% change in total (Fab)2
native 8861-Mab					
Affiprep™	0/1	18	100	0.0259	100
	6/1	48	267	0.0939	362
native 8861-Mab					
EMPHAZE AB1	0/1	22	100	0.012	100
	0/1	19	100	0.0103	100
	6/1	44	220	0.037	308
	6/1	42	210	0.032	301
modified 8861-Mab					
EMPHAZE AB1	6/1	45	225	0.035	305

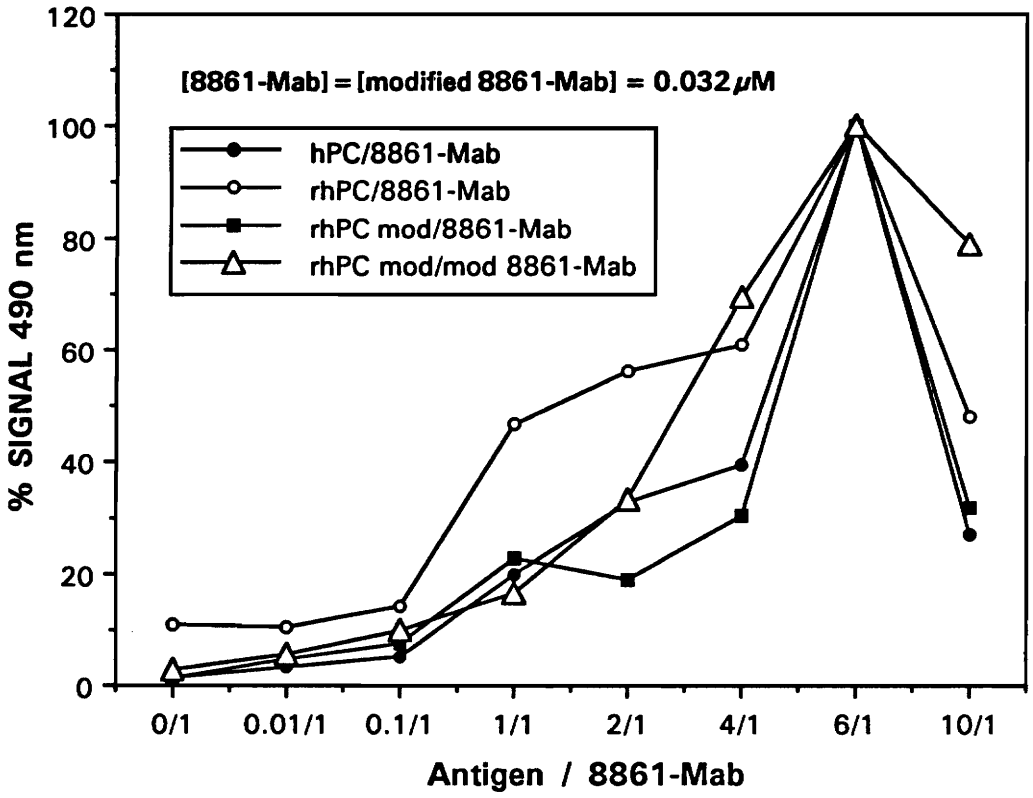

Figure 1

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

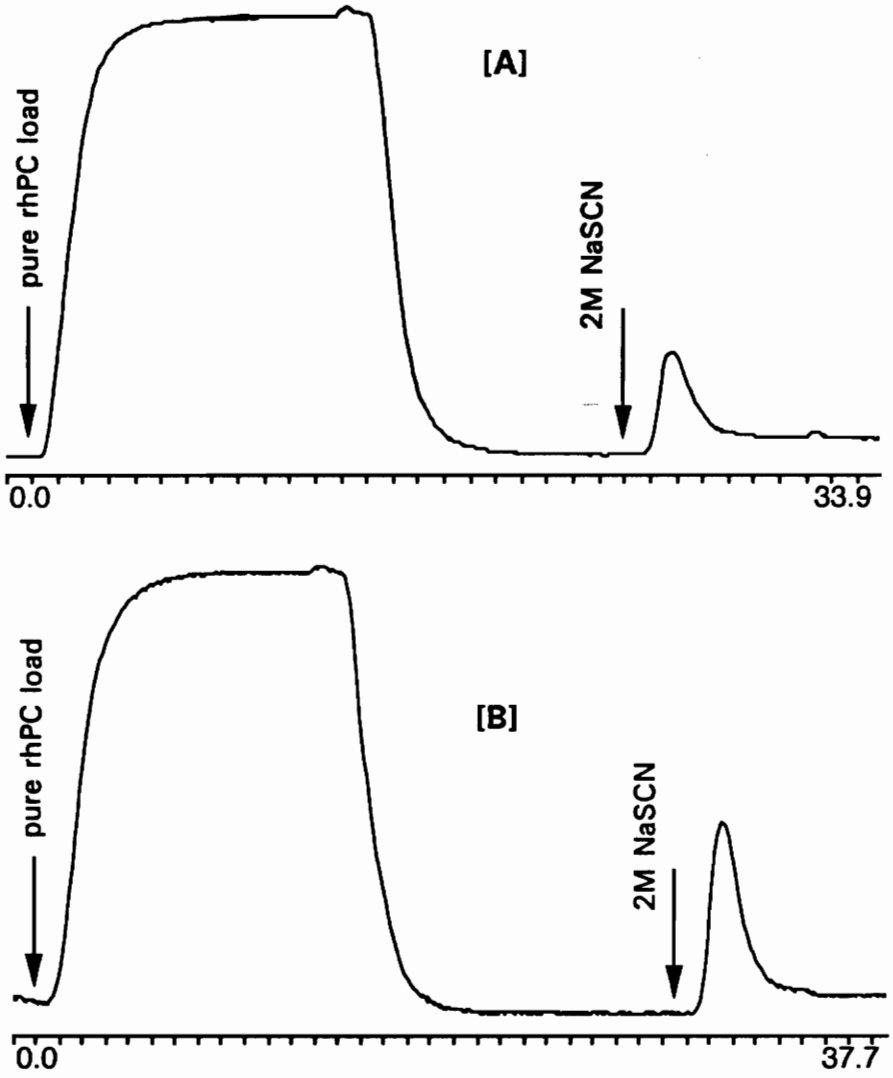

Figure 2

Breakthrough loading of pure rhPC on Emphaze AB1 immunosorbents. Columns (D = 1 cm) were packed with immunosorbents referred to in Table 2 and loaded with pure rhPC at 1 ml/min. After washing, rhPC was eluted with 2 M NaSCN. **(A)** "unmasked 8861, $<\rho_{Mab}>$ = 0.5 mg/ml **(B)** "FMA-masked" $<\rho_{Mab}>$ = 0.7 mg/ml

APPENDIX 1

Determination of the size of synthetic adducts (polymer-peptide) using worm-like chain model

The size of the adducts in an aqueous system were estimated by the theoretical prediction of the radius of gyration (R_g). The following assumptions were made; (i) adducts have a linear structure, (ii) R_g can be estimated using the "worm-like chain model" which predicts the lower range of R_g in the Gaussian coil limit for the theta state of the macromolecule (1).

Where $(R_g^2)_\theta$ is given by $(L_c \cdot L_k) / 6$; L_c is defined as the contour length given by $(DP.L_{mon})$ and L_k is defined as the Kuhn length. For the polyoxazoline family of polymers, the predicted values of L_{mon} and L_k are 0.46 and 0.77 nm respectively (2). Using the parameters defined above, the calculated values of R_g for 1K and 10K adducts are listed in Table 1.

The average dimensions of the active site in the Fab domain and the Fab/Fc fragments of an immunoglobulin (IgG) molecule were estimated by low angle x-ray scattering and /or electron density mapping of intact IgG crystals (3,4). The predicted dimensions are also listed in Table 1. The proposed three dimensional structure of an IgG molecule is schematically depicted in Figure 1.

DISCUSSION

We have used recombinant human Protein C (rhPC) from transgenic pig whey as a Fab masking antigen (FMA). The predicted molecular weight of rhPC is about 62000 daltons which is comparable to the monovalent (Fab)' fragment, which has a molecular weight of 45000 daltons. Assuming similar globular behavior, the size of the Fab domain on an IgG molecule and rhPC-FMA can be expected to be similar on a molecular level. From Table 1, the predicted size of 1K adduct is about one fifth the Fab fragment and the 10K adduct is similar in size to the Fab fragment.

Our studies show that both the 10K adduct and rhPC when used as FMAs were effective and led to a 3-4 fold increase in immunosorbent efficiency while the 1K adduct when used as a FMA did not impact the efficiency. Estimation of the sizes of these FMAs suggest that effective masks should approach the size of the antigen combining site on the Fab fragment which is about 2-3 nm.

REFERENCES

1. Ferdinand and Rodrigues, Principles of Polymerization
2. Personal Communication with Dr. R. M. Davis.
3. Sarma, V. R., Silverton, E. W., Davies, D. R., and Terry, W. D., (1971). Journal of Biological Chemistry, 246(11), 3753-3759.
4. Novotny, J., Bruccoleri, R., Newell, J., Murphy, D., Haber, E., and Karplus, M., (1983). Journal of Biological Chemistry, 258(23), 14433-14437.

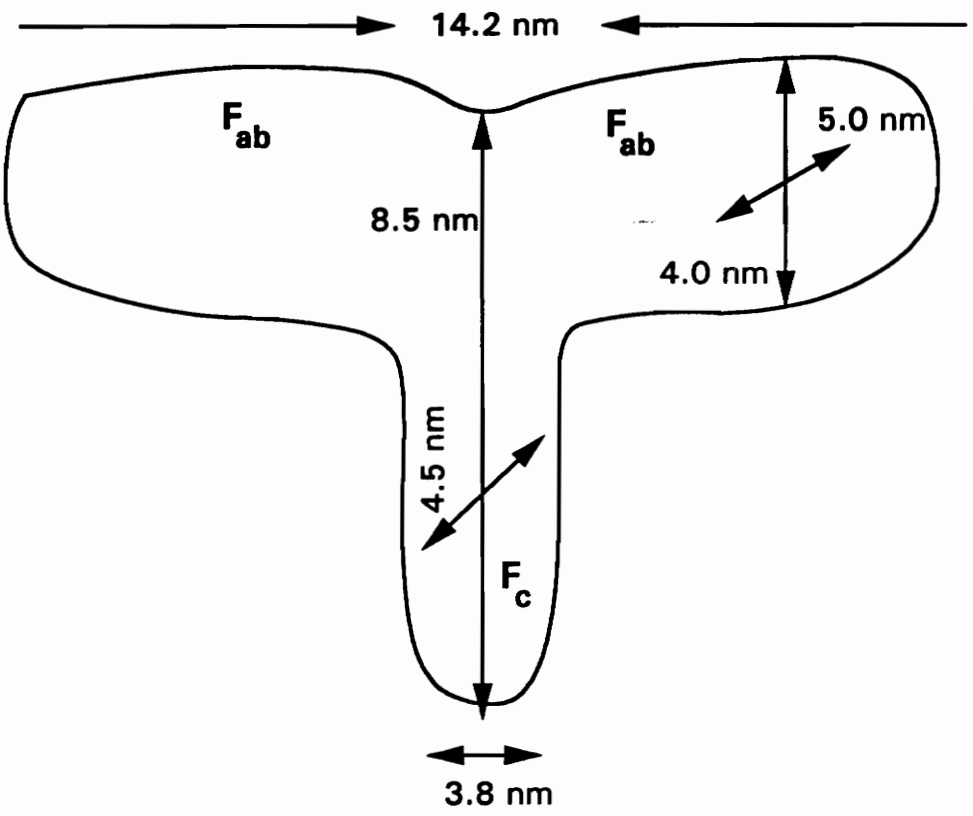

Figure 1 Dimensions of an IgG molecule

Table 1

	Estimated size, nm
1K adduct	1.0
10K adduct	3.0
(Fab)'	8.0 x 5.0 x 4.0
Fc	8.5 x 4.5 x 3.8
Active site	2.5 x 2.0

VITA

Anuradha Subramanian was born in Bangalore, India. Upon completion of her Bachelors Degree in Chemical Engineering from India she joined a Masters degree program in Chemical Engineering at the University of Iowa, Iowa City in August 1987. In the course of her work at the University of Iowa she developed an interest in the field of protein separations. She joined the doctoral program at Virginia Polytechnic Institute and State University, Blacksburg, Virginia in the fall of 1989. She was involved both in the downstream processing of therapeutic proteins from complex mixtures and in improving the performance of immunoaffinity sorbents. She plans to be in academia after gaining industrial experience in the field of biotechnology.