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DEVELOPMENT OF IMMUNOAFFINITY RESTRICTED ACCESS MEDIA FOR RAPID EXTRACTIONS OF LOW MASS ANALYTES

Chunling Wa, Rangan Mallik, and David S. Hage*

Department of Chemistry, University of Nebraska-Lincoln, Lincoln, NE 68588-0304 (U.S.A.)

Abstract

Restricted access media using antibodies as immobilized ligands were developed for the rapid and selective capture of small analytes by immunoextraction, giving rise to materials referred to as immunoaffinity restricted access media (IA-RAM). To make such a material, intact antibodies for the desired target were first immobilized onto porous silica, with antibodies at or near the outer surface of the support then being treated with papain (or a related agent) to release and remove their binding domains. The result was a support in which only antibodies deep within the pores remained intact and able to bind to the target. Items evaluated in the development of such media included the immobilization method used for the antibodies, the pore size of the support, and the amount of papain and time that were used for support treatment. A theoretical model was also developed to describe the extent of binding domain removal based on the measured polypeptide content of the IA-RAM support before and after treatment with papain. The final optimized conditions for making the IA-RAM supports were used to prepare columns that contained anti-fluorescein antibodies. Injections of fluorescein and fluorescein-labeled bovine serum albumin onto these IA-RAM columns gave selective and quantitative extraction of fluorescein in 1–2 s. This approach can be used with other antibodies and low mass targets and should be valuable for such applications as the rapid separation of drugs from drug-protein complexes or the isolation of labeled/modified peptides from intact proteins that contain the same modification or label.

INTRODUCTION

Restricted access media (RAM) have been used for many years in work with biological samples to combine the features of size exclusion chromatography with partition or ion-exchange chromatography.¹ The support in RAM is typically porous silica or a porous polymer that has a non-adsorptive and hydrophilic outer surface, while the interior of the support contains a stationary phase that can retain analytes through partitioning or ionic interactions. This provides a medium in which only low mass substances are retained, while larger agents such as proteins elute in the excluded volume. Applications of RAM have included the analysis of drugs, peptides, and endogenous substances in complex samples such as serum, blood, urine and cell cultures, among others.^{2–6}

Despite the various applications that have been reported for RAM columns,^{1–6} there has been no known previous work in the use of affinity ligands within such columns. The use of antibodies within a RAM support was examined in this current study, giving a new class of materials referred to as immunoaffinity restricted access media (IA-RAM). Figure 1 shows the general scheme utilized to prepare IA-RAM supports in this study. Antibodies were first immobilized onto a porous support with a hydrophilic coating. The antigen binding regions on some of these antibodies (as represented by their antigen binding fragments, or F_{ab} regions)

*Author for correspondence. Phone: 402-472-9402; Fax: 402-472-9402; E-mail: dhage@unlserve.unl.edu.

were then removed by treating them with an enzyme such as papain. The pore size of the support was chosen so that this enzyme was excluded from at least some of the antibodies in the support's interior. The goal was to create a support with a non-absorptive, hydrophilic outer surface and an interior that contained intact antibodies with selective binding for the desired low mass target.

One potential application for such a material is its use in separating the free and bound fractions of a drug or hormone in body fluids. For example, many drugs and hormones bind to proteins and other carrier agents in blood and serum. These interactions create both a free and bound form for these small solutes, where the free form is generally thought to represent the biologically-active fraction.⁷⁻¹¹ There have been previous studies examining the use of traditional RAM columns based on reversed-phase supports for the separation of free and bound drugs in drug/protein mixtures.^{2,12-15} However, these reports have noted only a partial resolution of the free and bound fractions,^{2,12-15} and have been limited to drug-protein systems that cover a narrow range of dissociation properties and that have a relatively large free fraction for the solute.¹⁶

It has recently been shown that antibodies immobilized onto HPLC supports can be used for free and bound drug/hormone separations and measurements based on ultrafast immunoextraction (i.e., an antibody-based extraction step taking place on the millisecond-to-second time scale).¹⁷⁻²⁰ This approach requires that the immobilized antibodies be able to recognize and bind the free form of a drug or hormone while having no significant interactions with the protein-bound fraction in a sample. Although meeting this latter requirement has not been a problem in previous work using traditional antibody immobilization methods,¹⁷⁻²⁰ it may be an issue as this technique is extended to new analytes. This report considered an approach to overcome this potential problem by combining the selectivity of antibodies with the use of a RAM support.

This study examined the creation of IA-RAM supports with antibodies that were immobilized through either random or site-selective methods (i.e., the Schiff base method or hydrazide methods, respectively).²¹⁻²³ The conditions required to prepare these supports were optimized and then used to construct columns that contained anti-fluorescein antibodies. These columns were tested by examining their binding to fluorescein (used as a model for a low mass drug or hormone) and a fluorescein-conjugate of the protein bovine serum albumin (used as a model for the protein-bound form of a drug or hormone). The results were then used to provide information on the relative advantages and disadvantages of such media and on their possible applications in the analysis of drugs, hormones and other low mass solutes in samples that contain proteins or other high mass components.

THEORY

An important factor in the development of an IA-RAM support is the amount of active binding sites for the target (i.e., F_{ab} regions) that remain on the support after it has been treated with an agent such as papain. These remaining F_{ab} regions, and corresponding binding sites, form the stationary phase of the support and would be expected to vary with the immobilization and treatment conditions that are employed in making an IA-RAM column.

To compare supports prepared under different conditions, the mole ratio R was used early in this study to describe the relative amount of binding regions (in terms of the moles of F_{ab} regions, n_{Fab}) that remained on an IA-RAM support versus the moles of antibodies that were originally present (n_{Ab}), where $R = n_{Fab}/n_{Ab}$. This ratio was found by measuring the total polypeptide content of the support before treatment with papain (S_0) and after treatment (S_t) by using a BCA protein assay (see Experimental Section).

Site-Selective Immobilization of Antibodies

In the case of the hydrazide immobilization method (i.e., a site-selective coupling technique for antibodies), antibodies are attached to a support through oxidized groups that are generally located in the carbohydrate chains of an antibody's lower stem region (i.e., the F_c region).²⁴ This method helps provide immobilized antibodies that have all their binding sites active and exposed for interacting with the desired target. During treatment with papain, some of the antibodies within the pores of the support will be inaccessible to this enzyme due to steric hindrance. This will leave F_{ab} regions on these antibodies intact, while antibodies on the outside of the support will be cleaved and leave only F_c fragments on the surface, as shown in Figure 2(a). The moles of these two different types of antibody-related species (i.e., intact antibodies versus cleaved antibodies) can be represented by the terms n_I and n_2 . These two terms can, in turn, be related to the measured polypeptide content of the support before and after treatment with papain, as given by S_0 and S_I .

In work with IgG-class antibodies from rabbits or mice, as were used in this study, the mass of an intact antibody can be taken to be approximately 150 kDa, while the mass of an F_c fragment is roughly 50 kDa (note: these values can be adjusted as needed in the following equations when working with antibodies from other species, subclasses or specific cell lines). Under these conditions, the mole ratio R can be estimated from the measured polypeptide content of the IA-RAM support before and after treatment with papain by using eq 1 (see derivation in the Appendix).

$$R = \frac{n_{Fab}}{n_{Ab}} = \frac{2n_I}{n_I + n_2} = \frac{3S_I}{S_0} - 1 \quad (1)$$

In this equation, the relationship $n_{Fab} = 2 n_I$ that appears in the numerator reflects the fact that there are originally two F_{ab} regions present per antibody for an IgG-class immunoglobulin.

If there is no treatment of an IA-RAM support with papain, the ratio S_I/S_0 will equal one (i.e., there has not yet been any loss of F_{ab} regions), and the ratio R that is obtained from eq 1 will be equal to two because there are two F_{ab} regions for each undigested IgG-class antibody. If there is complete digestion of all immobilized antibodies by papain, S_I/S_0 will equal 1/3 (i.e., only the F_c fragments will remain immobilized) and R will be equal to zero (i.e., no F_{ab} regions will remain on the support). If only some of the antibodies are digested, such as will occur if the remaining antibodies are inaccessible to papain, a value for R between zero and two will be obtained. The result is that the value of R which is calculated from eq 1 can be directly correlated with the relative amount of binding sites that remain on the immobilized antibodies and the IA-RAM support.

Random Immobilization of Antibodies

In an amine-based coupling technique (e.g., the Schiff base method), antibodies can be immobilized through sites throughout their structure. As is illustrated in Figure 2(b), the resulting immobilized antibodies can be divided into three groups according to the location of their immobilization site and their final orientation on the support. The first group (present in an amount of n_I moles) consists of antibodies that are inside the pores and inaccessible to papain, causing these antibodies to be unaffected when the IA-RAM support is treated with papain. Although only one type of orientation is shown for these antibodies in Figure 2(b), immobilization through either the F_c or F_{ab} regions may occur in this group; however, no change will be noted in the activity for either orientation because none of these antibodies will be altered during treatment with papain. The other two groups in Figure 2(b) both consist of

antibodies that are located near the exterior of the support and that are accessible to papain. One of these groups is immobilized through the F_c region (present in an amount of n_2 moles), and the other group is immobilized through the F_{ab} regions (present in an amount of n_3 moles). After treatment with papain, these latter two groups will leave behind single F_c or F_{ab} regions that are immobilized to the support, respectively.

The terms n_1 , n_2 and n_3 in this situation can again be related to the measured values of S_0 and S_t , as demonstrated in the Appendix using a 150 kDa IgG-class antibody as an example. These relationships, in turn, make it possible to use eq 2 to estimate the mole ratio R from the measured values for S_0 and S_t in the case of random immobilization.

$$R = \frac{2n_1 + n_3}{n_1 + n_2} = \frac{2S_t}{S_0} \quad (2)$$

As was noted earlier for site-selective immobilization, the ratio S_t/S_0 will equal one prior to antibody treatment with papain and the ratio R will be equal to two. If there is complete digestion of all the immobilized antibodies, S_t/S_0 will be equal to 1/3 (i.e., only single F_{ab} or F_c fragments will remain immobilized), but R will now equal 2/3 because approximately two-thirds of the antibodies will have been immobilized through their F_{ab} regions. If only some of the antibodies are digested, a value for R between 2/3 and 2 will be obtained for a system with random immobilization.

Effects of Activity Loss Due to Immobilization

It should be noted that the previous equations for calculating R describe the total amount of F_{ab} regions that remain on the support but do not indicate whether these regions are active. It is possible, however, to combine independent information on activity with the estimates of F_{ab} content that are made through the use of eqs 1 and 2. For instance, one previous study indicated that roughly half of the binding sites on some immobilized antibodies are inactive when using the Schiff base method.²⁵ Such information can easily be combined with the results from eqs 1 or 2 to estimate the final binding capacity that would be expected for an IA-RAM support for low mass target solutes. It is assumed in this approach that the fraction of active F_{ab} regions that remain on the support will be the same on the original and papain-treated materials for low mass solutes (e.g., about 50% of all F_{ab} regions will be active when using the Schiff base method). The same assumption is not valid for high mass substances that can bind to the same original antibodies but that are excluded from the final IA-RAM support. In fact, it is this difference in apparent activity that is used in this report to create IA-RAM columns that can separate low and high mass substances with common structural features and that cross-react with a given preparation of antibodies.

EXPERIMENTAL SECTION

Reagents

The Nucleosil Si-50, Si-100, Si-300 and Si-500 silica supports (7 μ m particle diameter; nominal pore size, 50, 100, 300 or 500 Å, respectively) were from Macherey Nagel (Düren, Germany). The following reagents were from Sigma (St. Louis, MO): rabbit immunoglobulin G (IgG, reagent grade), monoclonal anti-fluorescein antibodies (anti-FITC clone FL-D6, produced in mouse ascites fluid), the fluorescein conjugate of bovine serum albumin (fluorescein-BSA; prepared using fluorescein isothiocyanate and giving 12 mol fluorescein/mol protein), papain (85% protein, from papaya latex), fluorescein (sodium salt, $\geq 90\%$ pure), sodium cyanoborohydride ($> 99\%$; a mild reducing agent), sodium borohydride (98%; a strong reducing agent), D/L-dithiothreitol ($> 99\%$; a reducing agent), iodoacetamide ($\sim 99\%$), and

ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA, ~99%). The 3-glycidoxypolytrimethoxysilane (> 94%) was purchased from Aldrich (Milwaukee, WI). Reagents for the bicinchoninic acid (BCA) protein assay²⁶ were from Pierce (Rockford, IL). All aqueous solutions were prepared using water from a Nanopure system (Barnstead, Dubuque, IA) and were filtered using 0.22 μ m nylon filters from Fisher.

Apparatus

Slide-A-Lyzer dialysis cassettes (7 kDa MW cutoff, 0.5-3 mL capacity) were purchased from Pierce. BCA assays were performed using a Shimadzu UV-160A spectrophotometer (Kyoto, Japan). Fluorescence spectra were acquired using a Shimadzu RF-5301PC spectrofluorometer along with the following settings: excitation wavelength, 460 nm; emission wavelengths, 300–700 nm; slit width, 3.0 nm for both excitation and emission; scan speed, medium; sensitivity, high. The buffers employed in preparing samples were used to autozero the spectrofluorometer before each analysis.

The IA-RAM and control columns were packed using a Shimadzu LC-10AT pump and a Rheodyne 710 six-port valve (Cotati, CA). The chromatographic system consisted of a Perkin Elmer Series 200 micropump (Norfolk, CT) and a Shimadzu RF-525 fluorescence detector. Samples were injected using a Rheodyne LabPro valve equipped with a 200 μ L loop. Chromatographic data were collected and processed using in-house programs written in LabView 5.1 (National Instruments, Austin, TX).

Support Activation and Antibody Immobilization

Each silica support was first converted into a diol form according to a method adapted from the literature.^{21,23} To do this, 500 mg of Nucleosil Si-50, Si-100, Si-300 or Si-500 was placed into 7.5, 6, 4 or 2 mL of pH 5.5, 0.1 M sodium acetate buffer and degassed by sonication under vacuum for 10-15 min. Next, 0.75, 0.60, 0.20 or 0.10 mL of 3-glycidoxypolytrimethoxysilane, respectively, was added to these suspensions, with the resulting mixtures being shaken for 5 h at 90°C. The epoxy silica product was washed several times with water and a pH 3.0 aqueous solution of sulfuric acid. Each type of silica was then suspended in 100-150 mL of a pH 3.0 aqueous sulfuric acid solution and refluxed for one hour to convert the epoxy silica into a diol form. The resulting diol silica was washed with several portions of water, methanol and ether. The silica was then dried overnight under vacuum and stored in a desiccator until use. This diol silica was later activated according to literature methods to produce either aldehyde groups (in the Schiff base method)^{21,27} or hydrazide groups (in the hydrazide method)²³ on its surface for antibody immobilization.

The rabbit IgG (used in part of this study as a general model for IgG-class antibodies) was used without further pretreatment in the Schiff base method.^{21,27} In this method, a 13 mg/mL slurry of aldehyde-activated silica was reacted with 0.69, 0.69, 0.20 or 0.069 mg antibody per mg silica for the Nucleosil Si-50, Si-100, Si-300 or Si-500 silica, respectively. These amounts of rabbit IgG were at least two-fold greater than the maximum amount that could be immobilized to the surface of each support, as determined from previous studies²⁸ and calculations based on the known surface area of these supports and the size of IgG.²⁹ These mixtures were reacted in the presence of pH 6.0, 0.10 M phosphate buffer and 8 mg/mL sodium cyanoborohydride for 3 days at 4°C. The resulting material was washed several times with pH 8.0, 0.10 M potassium phosphate buffer. This support was then suspended in 2 mL of a 4 mg/mL solution of sodium borohydride in pH 8.0, 0.10 M potassium phosphate buffer and shaken for 90 min at room temperature to convert any remaining aldehyde groups into alcohols. The final support was washed several times with water for later use in a BCA assay. The support was then dried overnight under vacuum and stored in a desiccator for later analysis.

Rabbit IgG and anti-fluorescein antibodies that were to be immobilized by the hydrazide method were first oxidized with periodate, according to methods in the literature, to generate aldehyde groups in their carbohydrate regions.^{23,24,30,31} Although some monoclonal antibodies may have insufficient levels of glycosylation for immobilization by this approach, preliminary studies with the FL-D6 monoclonal anti-fluorescein antibodies indicated that these antibodies could successfully be coupled to silica by the hydrazide method. The concentrations of the final oxidized antibody/IgG solutions that were used in this immobilization process were determined to be 0.6-0.7 mg/mL by a BCA assay. The oxidized protein in these solutions was immobilized by combining it with 4-5 mg hydrazide-activated silica/mg protein and reacting this mixture at 4°C in pH 7.0, 0.10 potassium phosphate buffer for 3 days. The supernatant was collected for analysis by a BCA assay. The support was packed within a column or washed several times with water and dried overnight under vacuum for analysis by a BCA assay.

Preparation of IA-RAM Supports

The immunoaffinity supports prepared in the previous section were treated with papain according to solution-phase procedures adapted from the literature.³² In the final optimized method that was developed in this study, a 2 mg/mL solution of papain was prepared in pH 7.5 phosphate-buffered saline (PBS) buffer that contained 50 mM phosphate, 150 mM sodium chloride, and 2.7 mM potassium chloride; the papain solution also contained 2 mM EDTA and 1 mM dithiothreitol, with the latter reagent being used to reduce disulfide bonds within papain for activation. To perform the papain treatment step, the desired immunoaffinity support was washed two times with the PBS buffer, suspended in the same buffer and sonicated for 5 min. The typical concentration of antibodies/IgG in this solution was 0.3-1.2 mg/mL, as determined from the measured protein content of the support and the known slurry density. Next, the papain solution was added to this slurry to give an papain:immunoglobulin ratio of 1:100 (w/w). This mixture was incubated at 37°C for 30 min. The treatment with papain was stopped by adding 30 mM iodoacetamide and incubating this mixture in the dark on ice for 60 min. The treated immunoaffinity support was washed several times with PBS buffer and water to remove the papain and any antibody fragments that were cleaved from the support. Supports that were to be examined by a BCA assay were dried overnight under vacuum and stored in a desiccator at room temperature. The anti-fluorescein support that was to be used in chromatographic studies was washed three times with pH 7.4, 0.10 M potassium phosphate buffer and stored in this buffer at 4°C until use.

The anti-fluorescein IA-RAM support was packed into a sandwich microcolumn according to a previously described method.^{17,33} This column had an inner diameter of 2.1 mm and a total length of 1.0 cm, with a 2 mm long section in the middle of this column containing the anti-fluorescein IA-RAM support while the remainder contained an inert layer of diol silica. The immunoaffinity support was placed into this column by making fifty-three 200- μ L injections of a 0.32 mg/mL slurry of the anti-fluorescein IA-RAM support in pH 7.0, 0.067 M potassium phosphate buffer. Diol silica was placed into the remainder of the column by using a 2 mg/mL slurry of diol Nucleosil Si-100 in pH 7.0, 0.067 M potassium phosphate buffer. A control column was prepared in a similar manner by using diol silica in place of the IA-RAM support.

Evaluation of IA-RAM Supports

The amount of polypeptides on an immunoaffinity support before or after treatment with papain was determined by using a BCA assay. This assay was useful for this work because it gives a response that is correlated with the mass or length of a polypeptide chain,²⁶ as is required to relate the measured values of S_0 and S_t in eqs 1 and 2 to the mole ratio R . All BCA assays were performed in triplicate using rabbit IgG as the standard and the appropriate control support as the blank. The amount of antibodies/IgG placed on a support was determined by comparing the final and initial concentrations of the antibodies/IgG in the supernatant of the reaction slurry

and/or by directly measuring the final content of the protein support. The amount of antibodies/IgG remaining after papain treatment was determined by comparing the protein content of this support before and after treatment with papain.

The binding properties of the anti-fluorescein IA-RAM support before and after digestion were initially determined by reacting this support with a known excess of fluorescein (sodium salt, MW = 376 Da) or the fluorescein-BSA conjugate (average MW = 69.5 kDa). These studies were performed using anti-fluorescein antibodies that were immobilized by the hydrazide method onto Nucleosil Si-100. A 2 mg portion of the desired anti-fluorescein support was incubated with 1 mL of a 0.040 $\mu\text{g/mL}$ fluorescein solution or 1 mL of 20 $\mu\text{g/mL}$ fluorescein-BSA solution in pH 7.4, 0.1 M potassium phosphate buffer at 4°C for 30 min on a shaker. These mixtures were then centrifuged and the supernatant was analyzed for its content of fluorescein or fluorescein-BSA based on the fluorescence of these solutions. Standard solutions were also prepared and analyzed that contained 0.002-2.0 $\mu\text{g/mL}$ fluorescein or 1-30 $\mu\text{g/mL}$ of the fluorescein-BSA conjugate in pH 7.4, 0.10 M potassium phosphate buffer. The amount of fluorescein or fluorescein-BSA that had bound to the anti-fluorescein supports was determined by comparing the final and initial concentrations of these agents that remained in solution.

In the chromatographic studies, 200 μL injections of samples containing 0.00019 $\mu\text{g/mL}$ (0.5 nM) fluorescein or 0.21 $\mu\text{g/mL}$ (3 nM) fluorescein-BSA were made at flow rates ranging from 0.1-1.0 mL/min. These injections were performed at room temperature using pH 7.4, 0.067 M potassium phosphate buffer as the application buffer. Two or three replicate injections were made at each flow rate onto columns containing the anti-fluorescein IA-RAM support or the corresponding control support. The non-retained fluorescein or fluorescein-BSA was monitored using an on-line fluorescence detector set at excitation and emission wavelengths of 488 nm and 520 nm, respectively. The fraction of extracted fluorescein was calculated by comparing the areas of the non-retained peaks to those obtained for the same samples when the control column was instead present in the system. Measurements made at 0.01 mL/min (i.e., an incubation time 10-fold longer than the longest sample contact time used in the extraction study) were used to correct the peak areas for any small amount of non-binding, fluorescent contaminants that were present in the samples. The retained species were eluted between studies by passing a pH 2.5, 0.067 M potassium phosphate buffer through the column for 20 min. This was followed by a column regeneration step for 10 min by applying the pH 7.4, 0.067 M phosphate application buffer before each new sample injection. Although much shorter elution and regeneration times were probably possible for this system, these particular conditions were chosen to avoid any detectable carryover and to provide essentially complete antibody regeneration, as has been noted in previous work with the same anti-fluorescein antibodies in ultrafast extraction studies.³³

RESULTS AND DISCUSSION

General Considerations in Preparing IA-RAM Supports

One factor considered in the design of an IA-RAM support was the choice of an enzyme for antibody digestion. There were several criteria when selecting an enzyme for this purpose. First, the enzyme had to be the same size as or smaller than any proteins which were to be excluded from the IA-RAM support. Second, the enzyme had to be able to digest antibodies at only a few well-defined sites and leave the binding regions largely intact, as needed to provide a material with relatively homogeneous binding sites. Third, it was desirable to have enzymes that cleaved antibodies into only a few large fragments. The presence of these large fragments on the support's surface was expected to work with the pores of the support to help exclude large substances such as proteins that might otherwise be able to access antibodies in the pores

of the support. It was also desired for the selected enzyme to be readily available, inexpensive and easy to use for the routine preparation of IA-RAM supports.

Based on these criteria, several enzymes were initially considered for the digestion of immobilized antibodies. These enzymes included papain, pepsin, and ficin (see Figure 3). Papain (MW, 23 kDa) generally cleaves an antibody into two F_{ab} fragments and one F_c fragment, while pepsin (MW, 35 kDa) cleaves an antibody into one $F_{(ab')_2}$ fragment and a F_c fragment.³⁴ The cleavage of antibodies by ficin (MW, 25 kDa) is strongly dependent on the concentration of cysteine, generating $F_{(ab')_2}$ fragments in the presence of 1 mM cysteine and F_{ab} fragments in the presence of 10 mM cysteine.³⁵

In theory, all three of these enzymes were good candidates for the digestion of immobilized antibodies on porous supports. However, other considerations led to papain being chosen as the enzyme that was employed in this current study. The chief reason for this selection was that the anti-fluorescein antibodies that were used to evaluate the IA-RAM supports were mouse IgG₁-type antibodies. Pepsin was not suitable for this particular type of antibody because this antibody is resistant to the pepsin digestion due to a lack of the cleavage site Leu 234, which is conserved in most species except mouse IgG₁ and contributes to the flexible hinge region for pepsin cleavage.³⁶ Disadvantages of using ficin for this work included its relatively high cost compared to papain or pepsin and the need for its digestion conditions to be carefully controlled to obtain reproducible cleavage patterns.

Another factor that needed to be considered when preparing an IA-RAM support was the extent of digestion that could occur for the immobilized antibodies. The general goal in this particular study was to have binding sites cleaved from antibodies that were on the outside of the support while keeping at least some of the antibodies within the pores intact. This was achieved by choosing the pore size of the support so that both the pores and the remaining antibody fragments near the pore's entrance could limit access by papain or a similar enzyme to the support's interior. It was also necessary to control conditions such as the amount of added enzyme and digestion time during this treatment process. All of these factors were examined and optimized later in this study.

Effect of Pore Size in the Preparation of an IA-RAM Support

The amount of antibodies that can be immobilized onto porous silica with different pore sizes is known to be a function of both the total surface area of the support and the accessibility of antibodies to the pores. Silica with pore sizes of 100-300 Å have been found to give maximum antibody content when using either the Schiff base or hydrazide methods.²⁸ When preparing an IA-RAM support, the pore size will also determine the extent to which an enzyme like papain or a protein from a sample will be able to access antibodies inside these pores.

This effect was examined by looking at the polypeptide content and estimated amount of F_{ab} regions that were present on IA-RAM supports with various pore sizes. Figure 4 shows the results that were obtained when rabbit IgG was immobilized by the Schiff base method onto silica with pore sizes that ranged from 50 Å to 500 Å. All of these supports were digested by using papain for 3 h at an initial enzyme:antibody ratio of 1:100 (w/w).

Figure 4 shows that the total amount of immobilized rabbit IgG increased when going from pore sizes of 50 Å to 300 Å and decreased between 300 Å and 500 Å. This trend agrees with a previous report examining the immobilization of antibodies to porous silica,²⁸ and was noted in this current study for silica supports both before and after their treatment with papain. The relative amount of F_{ab} regions that remained after treatment was highest for the 100 Å pore size support. After treatment with papain, the estimated value of R was 0.99-1.01 for the 50 Å, 300 Å and 500 Å pore size silica when calculated according to eq 1; these values corresponded

with 50 to 51% (± 3 to 6%) of the original F_{ab} regions being present on these supports after papain treatment. An estimated R value of 1.15 was obtained for the 100 Å pore size silica, which corresponded to 58 (± 5)% of the original F_{ab} regions being present after treatment of this support with papain.

Based on the results in Figure 4 and the calculated amount of F_{ab} regions that remained after treatment, the 100 Å pore size support was selected as the primary material to be examined in all further studies. This material offered the best compromise between its final relative F_{ab} content and its potential to exclude sample proteins such as BSA (the model protein used in this study; MW, 66.5 kDa) or human serum albumin (HSA, the human counterpart of BSA; MW, 66.5 kDa), which have diameters of roughly 80-85 Å.³⁷ It should also be noted, though, that Figure 4 indicates that the 50 Å, 300 Å or even 500 Å pore size materials might be useful in some cases for preparing IA-RAM columns when exclusion is needed of either smaller or larger sample components.

The similarity of the fractions of F_{ab} fragments that remained on the treated 50-500 Å pore size supports suggested that pore size was not the only factor that led to the exclusion of papain from the immobilized antibodies. There are two other exclusion effects that could also have occurred in this system. First, an exclusion effect was present during immobilization which prevented antibodies from accessing any surface area that was within sufficiently narrow pores. This was the effect responsible for the decrease in the total initial amount of immobilized IgG that is seen in Figure 4 when going from the 300 Å to 100 Å and 50 Å pore size supports.²⁸ Second, the presence of immobilized antibodies near the upper regions of the pores could have acted to exclude papain from antibodies deeper within the pore's interior, as illustrated earlier in Figure 1(b). The combination of these various exclusion effects would have resulted in a zone of intermediate cross-sectional diameters within the support pores in which antibodies could be immobilized but were later protected from cleavage by papain. The relative size of this zone would be expected to vary much less between support materials than would be expected from only their nominal pore sizes. This is consistent with the relatively narrow range of 50-58% F_{ab} regions that were found to be present in this study for the 50-500 Å pore supports after treatment with papain.

Effect of Time of Treatment with Papain

Studies were next performed to determine if sufficient treatment times were being used to allow papain to cleave all accessible F_{ab} regions from the immobilized antibody supports. Figure 5 shows the results that were obtained when rabbit IgG was immobilized by the Schiff base method onto 100 Å pore size silica that was then treated with papain (Note: the same trends were seen when using silica with a pore size of 300 Å). The antibodies immobilized on this support were again treated using an initial enzyme:antibody ratio of 1:100 (w/w), as used in the last section, but the treatment time was now allowed to range from 0 to 6 h.

Figure 5 indicates that there was a sharp decrease in the amount of polypeptides that were left on the 100 Å pore size silica as the treatment time with papain was increased from 0 to 30 min. At 30 min, the total polypeptide content had decreased to 36 (± 2) mg/g silica, which was 62 (± 6)% of the polypeptide content before treatment. However, as the treatment time was further increased, the amount of polypeptides left on the support decreased only slightly and approached a fixed value. This result indicated that essentially all of the accessible antibodies on the support had now been cleaved by papain. Based on these data, it was determined that a treatment time of at least 30 min should be used for digesting immobilized antibodies by papain when working with 100 Å pore size silica. The same conclusion was reached in identical experiments performed using 300 Å pore size silica.

The apparent reaction order and rate constant for the treatment of immobilized antibodies by papain were examined by measuring the change in the overall polypeptide content for the support over time. The amount of remaining antibodies that were accessible to papain was determined by comparing the polypeptide content seen at treatment times greater than 30 min (representing inaccessible antibodies) to the polypeptide content that was measured for treatment times of 0-30 min. These results were then analyzed according to several kinetic models, including those for pseudo zero-order, first-order, and second-order reactions. The first-order reaction model gave the best fit to the experimental data, with a correlation coefficient of 0.9990 ($n = 5$). This type of model is consistent with an enzyme-catalyzed reaction described by Michaelis-Menton kinetics that involves the use of a limited amount of substrate. It was determined from the slope of this plot that the rate constant for the papain treatment process was $0.114 (\pm 0.007) \text{ min}/(\text{mg polypeptide/g silica})$. From this result, it was predicted that 95% of the accessible antibodies were digested in 30 min when using an enzyme:antibody ratio of 1:100. In all later studies, a digestion time of 3 h was actually used to still provide 95% or greater cleavage of accessible antibodies when using smaller amounts of papain for the treatment of an IA-RAM support (see next section).

Effect of Enzyme Amount on Support Treatment

The relative amount of papain added to a support for treatment (as reflected by the enzyme:antibody ratio) was another factor considered in controlling the amount of F_{ab} regions that remained on the IA-RAM support after digestion. A small amount of enzyme would lead to slow or incomplete digestion of antibodies while a large excessive might lead to aggregation or adsorption onto the support. In this study, the relative amount of papain that was used for digestion was varied from an enzyme:antibody ratio of 1:400 to 1:10 (w/w). The effect of varying this ratio was examined using rabbit IgG that was immobilized by the hydrazide method onto 100 Å pore size silica and that was later treated with papain for 3 h.

The results in Figure 6 show that there was a sharp decrease in the amount of polypeptides that were left on immunoaffinity supports after their treatment with papain using enzyme:antibody ratios of 1:400 to 1:100, or 0.0025 to 0.010 papain:IgG (w/w). However, similar polypeptide contents were observed after the treatment of supports using larger enzyme:antibody ratios of 1:50-1:10, or 0.020-0.10 papain:IgG (w/w). This information indicated that most of the accessible antibodies had been cleaved by papain within 3 h when using an enzyme:antibody ratio of 1:100 or greater. Based on this information, an enzyme:antibody ratio of 1:100 was used in all further work in this study.

Effect of Immobilization Method

Once the digestion conditions and support pore size had been selected, the effect of immobilization method was also considered in the preparation of IA-RAM supports. The hydrazide method was used as an example of a site-selective coupling technique, in which antibodies were coupled to a support through carbohydrate chains that are mainly located in their F_c region. This type of immobilization method is represented by the situation in Figure 2 (a). The Schiff base method was used as an example of a more random amine-based coupling technique for antibodies. Such a method, as illustrated in Figure 2(b), would be expected to produce a population of immobilized antibodies with a variety of orientations and sites of attachment to the support.

The effect of the immobilization method was examined by immobilizing rabbit IgG by the Schiff base method or hydrazide method onto silica with a pore size of 100 Å. These supports were then treated with papain for 3 h using an enzyme:antibody ratio of 1:100. In the Schiff base method, the polypeptide content of the immunoaffinity support before and after treatment with papain was $58 (\pm 4)$ or $33 (\pm 2) \text{ mg/g silica}$, respectively. According to eq 2, this meant

that around 57 (± 6)% of the original F_{ab} regions remained on the support after treatment with papain, or $R = 1.14 (\pm 0.11)$, as was observed earlier in this report.

In the hydrazide method, the polypeptide content of the immunoaffinity support before and after treatment with papain was 98 (± 4) or 47 (± 2) mg/g silica, respectively. The original amount of immobilized antibodies in this case was 1.6-fold larger than that obtained by the Schiff base method, while the amount of remaining polypeptides after treatment with papain was 1.4-fold larger. The larger amount of immobilized antibodies in the hydrazide method versus Schiff base method has been noted in previous studies²⁸ and is probably related to the more uniform orientation of antibodies in a site-selective coupling method. According to eq 1, R was 0.44 (± 0.03) in this case, meaning that roughly 22 (± 2)% of the original F_{ab} regions were present on the hydrazide support after treatment with papain. This difference versus the result for the Schiff base method may also reflect the more ordered orientation of antibodies in the hydrazide method, which could have made more F_{ab} regions accessible to papain for cleavage. It should be noted that larger differences between these two immobilization methods may be observed for some types of antibodies and, in particular, monoclonal antibodies that have a relatively low degree glycosylation compared to typical polyclonal antibodies.^{38,39} However, this issue was not found to be a problem for the monoclonal anti-fluorescein antibodies that were used later in this current study.

These results indicated that the use of a random versus site-selective method may affect the preparation of an IA-RAM support. However, the overall binding capacity of this support will depend on the activity of the immobilized antibodies along with the total antibody content and fraction of F_{ab} regions that are present on the final material. As an example, if there is approximately a 50% retention of activity for F_{ab} regions in the Schiff base method,²⁵ the resulting binding capacity for an IA-RAM support like the one made in this study would be roughly 110 nmol/g silica for a low mass target. If 85-100% retention of activity occurs in the hydrazide method,⁴⁰ this would give a binding capacity of 104-122 nmol/g silica for a low mass target on an IA-RAM support like the one made in this section, even though fewer F_{ab} regions are present in the final material. Slightly different results might again be obtained for other types of antibodies or when using alternative immobilization methods.^{34,41}

Use of IA-RAM Supports in Static Binding Studies

After conditions had been selected for the creation of IA-RAM supports, the binding and selectivity of these supports for high mass and low mass targets were evaluated. These properties were examined by using fluorescein and a fluorescein-BSA conjugate as the model targets and anti-fluorescein antibodies as the ligands for preparation of the IA-RAM support. Fluorescein was useful as a model for drugs or other low mass solutes in this work because of its size (MW, 376 Da), its ease of detection, and the availability of monoclonal antibodies for this agent. Fluorescein-BSA (MW, 69.5 kDa) was valuable as a model for a high mass target because both BSA and its human counterpart HSA (MW, 66.5 kDa) are the main proteins present in bovine or human serum and each binds to a variety of drugs, hormones and other small solutes.³⁷ This made it important to test this type of protein when evaluating an IA-RAM support for use in the separation of free and protein-bound drug fractions and for future use with biological fluids such as serum or blood. Also, the fluorescein-BSA conjugate was prepared using the same reagent (i.e., fluorescein isothiocyanate, or FITC) that was utilized to prepare the fluorescein-carrier protein conjugate and resulting anti-fluorescein monoclonal antibodies that were obtained for this study. This meant that these anti-fluorescein monoclonal antibodies should have able to recognize and bind both free fluorescein and the fluorescein groups that were attached to the surface of the BSA conjugate.

In many ways the use of the fluorescein-BSA conjugate as a high mass target represented a worse-case scenario in this study because it maximized the probability of interaction for such

a target with an IA-RAM support. This is the case because the group that was being recognized by the antibodies (i.e., fluorescein) was fully exposed on the surface of the protein and had the same orientation as the carrier protein conjugate that was used to raise these antibodies. In addition, there were many copies of this group present per protein, with an average of 12 fluorescein groups being linked to BSA for the conjugate used in this report. These properties are in contrast to those for a typical system involving a drug that is non-covalently bound to BSA or HSA, in which the chance of cross-reaction with an IA-RAM support would be expected to much be lower. For instance, many drugs and small solutes interact at only one or a few sites on BSA or HSA, limiting the number of copies of this solute per protein. In addition, this solute-protein interaction often involves the solute at least partially entering a binding pocket, which would limit exposure of this protein-bound form to an antibody.^{27,37,42-44}

The use of an IA-RAM support under such conditions was evaluated by first immobilizing anti-fluorescein antibodies by the hydrazide method onto silica with a pore size of 100 Å. A portion of this support was set aside for use as a reference while the remainder was treated with papain for 90 min at 37°C using an enzyme:antibody ratio of 1:100 (w/w). The resulting IA-RAM support was thoroughly washed with PBS buffer to remove the enzyme plus any F_{ab} fragments that had been cleaved from the antibodies. Known amounts of the treated and original anti-fluorescein immunoaffinity supports were then incubated for 30 min with an excess of fluorescein or fluorescein-BSA conjugate to saturate any accessible binding regions for these targets on the support. The fluorescein or fluorescein-BSA that was bound by anti-fluorescein antibodies was then determined by looking at the amount of these analytes that remained in solution after the incubation step.

The original binding capacity of the immobilized antibodies was estimated by this approach to be 0.97 ($\pm$ 0.02) µg/g silica for fluorescein or 0.69 ($\pm$ 0.01) mg/g silica for the fluorescein-BSA conjugate. It was also found that the binding capacity for fluorescein-BSA decreased by 92 ($\pm$ 3)% on the anti-fluorescein immunoaffinity support after this support had been treated with papain. However, the binding capacity for fluorescein decreased by only 47 ($\pm$ 2)%. These results clearly indicated that the treated immunoaffinity support was now more selective in binding to a low mass target such as fluorescein and had gained some size selectivity, as would be predicted for an IA-RAM material.

There was a small amount of residual activity that remained on the treated support for the fluorescein-BSA conjugate, but this was not surprising given the large number of fluorescein labels that were present per protein and the fact that an excess of this conjugate was incubated for a relatively long period of time with the IA-RAM support. Under these conditions it is possible that some fluorescein groups on the BSA conjugate were able to reach and react with F_{ab} regions that were near the areas on the support that could not be reached by papain. The presence of any unfolding of the BSA during the incubation step would have also helped to make this type of binding possible. However, it will be shown in the next section how even this small amount of residual binding was not a problem when much shorter times for contact were allowed between the fluorescein-BSA conjugate and IA-RAM support.

Use of IA-RAM Supports in Ultrafast Extraction

The IA-RAM support created in the previous section for fluorescein was next tested for use in a flow-based system for the selective, ultrafast extraction of fluorescein versus a fluorescein-BSA conjugate. This was accomplished by comparing the results that were obtained with the IA-RAM column to those that were seen for a control column containing only diol silica. The amount of injected fluorescein or fluorescein-BSA in these studies was 1.5% or 20% of the total binding capacity for these two analytes on the IA-RAM support, as determined from data in the previous section.

Figure 7(a) shows some typical chromatograms that were obtained when fluorescein was injected at 0.1 mL/min onto the IA-RAM column or a control column with no antibodies present. In this experiment, the fluorescein was quantitatively extracted by the IA-RAM column but gave no signs of retention on the control column under the same conditions. When the same experiment was performed with fluorescein-BSA, no significant degree of binding was seen for this conjugate on either the IA-RAM column or control column.

Figure 7(b) illustrates how the extent of this extraction varied when using different flow rates and residence times for contact between fluorescein or the fluorescein-BSA with the anti-fluorescein antibodies in the IA-RAM column. It was found that 90% of the fluorescein was extracted when using a contact time of 1.2 s and 80% fluorescein was extracted in 0.9 s. Although this rate of extraction was quite fast, it was slower than what has been noted for the same type of antibodies on more traditional silica-based immunoaffinity supports that have not been treated with papain;³³ this difference was expected due to the decreased amount of F_{ab} regions that were present in the IA-RAM support versus these other materials. Under the same conditions that were tested in Figure 7(b), less than 1% of the fluorescein-BSA conjugate was extracted by the IA-RAM support. This latter result indicated that an IA-RAM support such as this could be quite selective for a low mass target versus a high mass substance when used under these conditions. This data further demonstrated that the IA-RAM supports developed in this report could be used in a flow-based system to prevent cross-reaction of immobilized antibodies with by solute-protein complexes that had a size comparable to that of BSA or HSA.

Although these studies were conducted with standard solutions, the results indicate that IA-RAM supports should be useful with many types of real clinical samples that contain large amounts of proteins. For instance, the extremely low binding that was noted for the fluorescein-BSA conjugate indicates that non-specific binding by HSA, the main serum protein in humans (typical concentration, 35-50 g/L), will not be an issue with columns and supports like those used in this study. This low non-specific binding is aided by 1) the selective nature of antibody-analyte interactions in the IA-RAM column, 2) the low surface area that is present for non-specific binding in columns like that used here for ultrafast immunoextraction, and 3) the small residence times for these columns, which makes it possible to quickly wash off any weakly bound sample components. It is possible that the F_c regions/antibody fragments that remain on the exterior of the IA-RAM support may create non-specific binding for some proteins or solutes, which will have to be evaluated on a case-by-case basis. However, it is expected that these non-specific interactions will also be minimized by the low surface area and small size of IA-RAM columns that were used for immunoextraction in this study.

The extraction results in Figure 7 can be explained by considering both the binding capacity of the IA-RAM support for low versus high mass solutes and the effect that small incubation times will have on the extraction of these agents. It has been shown in several previous studies with immunoaffinity columns made from HPLC supports like those used in this study that the overall rate of binding of an analyte to the immobilized antibody can be described by an adsorption-limited process.⁴⁵⁻⁴⁷ Under these conditions, the following equations can be used to estimate the fraction (b) of the analyte that will be bound by the immunoaffinity column at a particular application flow rate (F) and using a given amount of injected analyte (load A) for an analyte-antibody interaction with an association rate constant of k_a .⁴²⁻⁴⁴

linear conditions (load $A \approx 0$):

$$b=1 e^{(k_a m_L)/F}=1 e^{1/S_0} \quad (3)$$

nonlinear conditions (load $A > 0$):

$$b=1\{S_o/\text{load A}\} \ln[1+\{e^{(\text{load A})/S_o}-1\}e^{1/S_o}] \quad (4)$$

In these equations, m_L is the total moles of active binding sites in the column, and load A represents the relative moles of injected analyte versus these binding sites, where load A = (moles of A)/ m_L . The term S_o is a dimensionless factor that is often used to represent the combination of terms $F/(k_a m_L)$ that appear in both eqs 3 and 4. The relationship in eq 3 shows that the relative amount of analyte that will be extracted by an IA-RAM column will decrease as the apparent value of m_L decreases. Such a decrease occurs when comparing the binding capacity for a small target like fluorescein to the binding capacity for a large target like the fluorescein-BSA conjugate. This effect is further compounded in eq 4 by the larger relative load of the high mass target that will be present as m_L is decreased for this species after treatment of the IA-RAM support with papain.

Another factor in eqs 3 and 4 that favors extraction in an IA-RAM column of a small target over a large species is the association rate constant k_a . Many antibody-analyte interactions approach a diffusion-limited rate, which tends to result in lower k_a values for high versus low mass analytes.⁴⁸ This, in turn, would further result in a less efficient extraction of high mass targets by an immunoaffinity support. However, this factor was counter-acted in this particular study by the large number of fluorescein labels that were present on BSA, which would have increased the apparent association rate constant of this conjugate for the immobilized antibodies. Differences in k_a for high versus low mass targets would also not be a factor when an IA-RAM support is used under static conditions or with long extraction times, such as were employed in the previous section of this study.

CONCLUSIONS

This report examined the development of immunoaffinity restricted access media for the selective extraction of low mass solutes (e.g., drugs) from high mass solutes that bind to the same initial preparation of antibodies. This approach made use of the immobilization of antibodies onto porous silica, followed by treatment of these antibodies with papain to remove any binding regions that were exposed at the support's exterior surface. The effects of varying the support pore size, immobilization method, and treatment conditions were all considered in making these materials. The final optimized conditions were used to prepare a model IA-RAM column, which was able to selectively and quantitatively extract fluorescein (but not a fluorescein-BSA conjugate) in as little as 1-2 s. The selectivity of this IA-RAM column was found to be a result of both its decreased number of accessible binding regions for a high versus low mass analyte and the slower rate of extraction that this situation created for high mass species. These effects also explained why the rate of extraction observed in this study for an IA-RAM column was slightly slower than that noted previously when using intact antibodies on porous HPLC-grade silica.³³

The technique described in this report for generating IA-RAM columns is a general one which could be used for a variety of other targets and applications. The advantages of this approach compared to current RAM columns that are based on partitioning or ion-exchange interactions include the greater selectivity and retention factors that are possible when using immunoaffinity columns.^{1,34} This advantage should make the IA-RAM columns more amenable for work in the analysis or pretreatment of complex samples. The advantages of IA-RAM columns compared to more standard immunoaffinity media are the capability of these new columns to distinguish between analytes based on both their size and reactivity with antibodies.³⁴ One potential application for these IA-RAM columns would be their future use in separating the free versus protein-bound fractions of small drugs or hormones in biological samples, as

directly indicated by the results of this report.⁷⁻¹¹ Another expected application would be the use of IA-RAM columns for the separation of labeled/modified peptides from digests or samples containing intact proteins with the same modification or label.^{49,50}

APPENDIX

Derivation of Eq 1

When an IA-RAM support is prepared using a site-selective coupling technique that attaches through F_c regions on an antibody, the moles of intact antibodies versus cleaved antibodies that remain after treatment with papain can be represented by the terms n_1 and n_2 , as illustrated in Figure 2(a). These two terms can be related to the measured polypeptide content of the support before and after treatment with papain (S_0 and S_t) by using the following expressions.

$$150,000 \cdot (n_1 + n_2) = S_0 \quad (\text{A1})$$

$$150,000 \cdot n_1 + 50,000 \cdot n_2 = S_t \quad (\text{A2})$$

In these equations and related expressions in this paper, the mass of an intact IgG-class antibody (such as polyclonal rabbit IgG or the monoclonal anti-FITC antibodies that were used in this study) is taken to be approximately 150 kDa, while the mass of an F_c fragment is given as roughly 50 kDa.⁵¹ Eqs A1 and A2 can then be combined and rearranged to solve for n_1 and n_2 , as shown below.

$$n_1 = \frac{S_0}{150,000} - \frac{S_0 - S_t}{100,000} \quad (\text{A3})$$

$$n_2 = \frac{S_0 - S_t}{100,000} \quad (\text{A4})$$

It is also known that the following mass balance equations will apply to this situation.

$$n_{Fab} = 2 n_1 \quad (\text{A5})$$

$$n_{Ab} = n_1 + n_2 \quad (\text{A6})$$

Eqs A5 and A6 can then be substituted into the equation that provides the definition of R , as stated in the Theory. Finally, using eqs A3 and A4 to replace n_1 and n_2 with equivalent expressions in terms of S_0 and S_t provides the final expression in eq 1.

The masses of an intact antibody and F_{ab} fragment that were used in these equations are typical of those expected for many types of IgG-class antibodies but can be adjusted as needed for working with antibodies from different species, subclasses, or specific cell lines. In this study,

only approximate masses were required for these calculations (i.e., values with two significant figures) because the measured values of S_0 and S_t provided a typical precision of only ± 10 -12% for estimates of R and the remaining fraction of F_{ab} fragments.

Derivation of Eq 2

If an antibody is immobilized by a random coupling method, the moles of the three types of immobilized antibodies that will be present, as shown in Figure 2(b), will be described by n_1 (antibodies that are inside the pores and inaccessible to papain), n_2 (antibodies accessible to papain and immobilized through the F_c region) or n_3 (antibodies accessible to papain and immobilized through an F_{ab} region). These terms can be related to the measured values of S_0 and S_t through the following equations (note: these relationships again use an approximate mass of 150 kDa for an intact IgG-class antibody and 50 kDa for an F_c fragment, but these values can be varied as needed).

$$150,000 \cdot (n_1 + n_2 + n_3) = S_0 \quad (\text{A7})$$

$$150,000 \cdot n_1 + 50,000 \cdot (n_2 + n_3) = S_t \quad (\text{A8})$$

In an intact IgG-class antibody, the mole ratio of the F_{ab} to F_c regions is 2:1. If the possible immobilization sites are distributed in a truly uniform manner throughout the antibody's structure, the following mass balance equation will then be valid.

$$n_3 = 2n_2 \quad (\text{A9})$$

By substituting eq A9 into eqs A7 and A8, the modified expressions in eqs A10-A12 can be used to relate n_1 , n_2 and n_3 to S_0 and S_t .

$$n_1 = \frac{S_0}{150,000} - \frac{S_0 - S_t}{100,000} \quad (\text{A10})$$

$$n_2 = \frac{S_0 - S_t}{300,000} \quad (\text{A11})$$

$$n_3 = \frac{S_0 - S_t}{150,000} \quad (\text{A12})$$

The following mass balance equations will apply to this situation.

$$n_{Fab} = 2n_1 + n_3 \quad (\text{A13})$$

$$n_{Ab}=n_1+n_2 \quad (A14)$$

Eqs A13 and A14 can be substituted directly into the definition of R , as given in the Theory, while eqs A10-A12 can be used to further substitute the values of n_1 through n_3 with expressions for S_0 and S_t . The resulting, combined equation then reduces to the final expression shown in eq 2.

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Figure 1. (a) General scheme for preparing immunoaffinity restricted-access media (IA-RAM) and (b) the use of this media for binding low mass targets in a sample that also contains proteins or other high mass substances. The enzyme shown in (a) is either pepsin or papain in this particular example.

(a) Site-Selective Immobilization (Hydrazide Method) **(b) Random Immobilization (Schiff Base Method)** **Figure 2.**

Nomenclature used to describe the general types of antibodies or antibody fragments that are immobilized to a solid support before and after digestion with an enzyme such as pepsin or papain. The structures in (a) are for a site-selective immobilization method that couples antibodies through their F_C regions (e.g., the hydrazide method). The structures in (b) are for a random immobilization technique (e.g., an amine-based coupling technique like the Schiff base method). For the sake of simplicity, only one type of antibody orientation is shown in (b) for antibodies that are immobilized inside the pores of a support. If other types of orientations are present in the pores, eq 2 can still be used because these other orientations will still result in intact antibodies after digestion and will cause no apparent change in the overall

activity of the support (note: this is true even if some of these antibodies are immobilized in a way that makes them inactive).

Figure 3.

The products that result from the digestion of an intact antibody (IgG in this case) by papain or pepsin. Although the fragments shown to the far right for pepsin and papain have slightly different lengths, both are referred to as “F_c” fragments in this report.

Figure 4. Total amount of immobilized polypeptides (measured using rabbit IgG as a reference) that were present before and after the treatment of immunoaffinity supports made using silica with various nominal pore sizes. Rabbit IgG was immobilized to each of these supports by using the Schiff base method. The error bars represent a range of ± 1 S.D.

Figure 5. Total amount of immobilized polypeptides (measured using rabbit IgG as a reference) remaining on an immunoaffinity support after treatment with papain for various lengths of time. These results were obtained for rabbit IgG that was immobilized to Nucleosil Si-100 silica by the Schiff base method. Other conditions are given in the text. The error bars represent a range of ± 1 S.D.

Figure 6. Total amount of immobilized polypeptides (measured using rabbit IgG as a reference) remaining on an immunoaffinity support after treatment with different amounts of papain. These results were obtained for rabbit IgG that was immobilized to Nucleosil Si-100 silica by the hydrazide method. Other conditions are given in the text. The error bars represent a range of ± 1 S.D.

(a) Extraction of Fluorescein

(b) Extraction of Fluorescein and Fluorescein-BSA by IA-RAM Column

Figure 7.

(a) Typical chromatograms for the injection of fluorescein at 0.1 mL/min onto a control column or an IA-RAM column containing anti-fluorescein antibodies, and **(b)** extraction curves for the injection of fluorescein and a fluorescein-BSA conjugate onto these columns at various flow rates. These results were obtained using columns with dimensions of 2.1 mm i.d. $\times$ 10 mm and an effective length of 2 mm for the immunoaffinity layer containing the anti-fluorescein antibodies and IA-RAM support. The data in (a) were smoothed using a 5-point box car average. Other conditions are given in the text.