Journal of Chromatography, 458 (1988) 67–77
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1424

STRATEGY FOR THE IMMOBILIZATION OF MONOCLONAL ANTIBODIES ON SOLID-PHASE SUPPORTS

ROBERT S. MATSON*,* and MICHAEL C. LITTLE
Bio-Rad Laboratories, 1414 Harbour Way South, Richmond, CA 94804 (U.S.A.)

SUMMARY

Using matrices based upon Affi-Gel and Affi-Prep, we have examined conditions during the immobilization of antibodies (immunoglobulin G, IgG) that influence the performance of immunosorbents. Such conditions include: coupling pH, coupling kinetics, antibody density on the immunosorbent and the activation chemistries utilized for the immobilization process. These studies have shown that the capacity for antigen does not increase with increased antibody coupling efficiency. Presumably, increased coupling times or efficiencies lead to multi-site attachment of the antibody to the matrix, thereby causing inactivation. Immunosorbents containing low densities of IgG were found to have greater capacity for antigen on a per mole IgG basis. This suggests steric crowding of antigen at high antibody density. Finally, immunosorbents prepared through IgG carbohydrate linkages (oriented coupling) show dramatic increases in antigen capacity over those prepared by stochastic (random) coupling through IgG primary amino groups. A combination of low IgG density and oriented coupling of the IgG via the carbohydrate moiety may represent the best strategy for the preparation of immunosorbents.

INTRODUCTION

The goal in the construction of an immunoaffinity support (immunosorbent) is to immobilize the antibody to the solid-phase support without adversely affecting the antibody's function to capture antigen. Although sequestering of the antibody through the antigen binding site is not a recommended strategy most activation methods are based upon this approach, since they rely primarily on the reactivity of the immunoglobulin's free lysine residues with an activated ester or other reactive group on the support. To complicate matters further, most commercially available activated supports contain an excess of activation groups such that multi-site attachment of the antibody is assured. The results of this approach is an immunosorbent that is largely inactive, generally on the order of 1–30% of the theoretical antigen binding efficiency.

Of course, the advantage of purification by immunoaffinity chromatography

* Present address: Nuclepore Corporation, 7035 Commerce Circle, Pleasanton, CA 94566-3294, U.S.A.

0021-9673/88/$03.50

generally offsets this shortcoming. However, from an economical viewpoint, especially if one is to consider the scale-up of immunoaffinity chromatography with monoclonal antibodies, it is important to understand the immobilization process.

In these studies, we have examined both cross-linked agarose matrices such as Affi-Gel (Bio-Rad) and the polymer-based activated support, Affi-Prep (Bio-Rad) which is suitable for both analytical high-performance liquid chromatography (HPLC) and preparative chromatography. The Affi-Gel and Affi-Prep supports are N-hydroxysuccinimide (NHS)-activated matrices. The advantage of using monoclonal antibodies (MABs) rather than polyclonals is that all of the immunoglobulin G (IgG) molecules in a monoclonal population have identical primary sequence. Therefore, in altering coupling conditions one does not have to be concerned so much with the selective immobilization of different sub-populations as in the case of polyclonal antibodies. The disadvantage in using purified MAbs is that they may in fact have been isolated by affinity methods that employ harsh elution conditions, thereby reducing their native antigen, binding capacity. We will be contrasting our work on MAb immobilization with the coupling of polyclonal antibodies. They share, of course, some commonality in developing a strategy for antibody immobilization.

We have examined several factors which may influence the outcome of antibody immobilization. Principal among these is the consequence of an increased antibody ligand-matrix density. This is not a new issue since Eveleigh and Levy[1] first demonstrated that a lower density of polyclonal antibody immobilized to CNBr-Sepharose was essential in obtaining the highest antigen-binding capacity. However, this parameter has not generally been considered in the preparation of immunosorbents, especially those that are based upon other matrices using different activation chemistries. We have demonstrated the importance of ligand density; and offer methods by which the immobilization process is controlled to obtain an optimal immunosorbent. We also explored the process of oriented coupling of antibodies via hydrazide chemistry[2] to a high-performance matrix, Affi-Prep 10[3].

EXPERIMENTAL

Antibodies and antigens

Purified (98%), murine anti-tPA IgG_1 and 99+% purity tissue plasminogen activator (tPA) were experimental preparations obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). Horseradish peroxidase (HRP), type IX, was obtained from Sigma (St. Louis, MO, U.S.A.). Murine anti-HRP IgG_1 obtained from Proteins International (Rochester, MI, U.S.A.) was purified from ascites by Affi-Prep protein A affinity chromatography to near homogeneity as determined by sodium dodecyl sulphate-polyacrylamide gel electrophoretic (SDS-PAGE) analysis (data not shown). Affinity purified sheep anti-BSA IgG was obtained from Bethyl Labs. (Montgomery, TX, U.S.A.) and [methyl-^{14}C]BSA (37 μCi/mg) from Amersham (Arlington Heights, IL, U.S.A.).

Activated supports

Affi-Gel 10, Affi-Gel hydrazide (HZ), and Affi-Prep 10 were obtained from Bio-Rad Labs. Affi-Prep hydrazide was prepared from Affi-Prep 10 by quantitative displacement of NHS with hydrazine hydrate[2] by a proprietary process.

Preparation of aldo-IgG for immobilization

The oxidation of immunoglobulins was carried out as previously described[4], using sodium periodate. Essentially, IgG was dialyzed against 0.1 *M* sodium acetate, 0.15 *M* sodium chloride (pH 5.5) overnight at 25°C. To one volume of IgG dialyzate (1–10 mg/ml) was added 0.1 volume of 0.1 *M* sodium periodate, prepared in deionized water. The reactants were mixed for 1 h in the dark at 25°C. The reaction was then stopped by the addition of 20 μl of glycerol per 1.0 ml reaction mixture and the mixing was continued for an additional 30 min. The oxidized or aldo-antibody was then dialyzed against the same buffer used for coupling.

Antibodies were coupled to hydrazide supports by end-over-end mixing for 20 h at 25°C. The resulting immunosorbents were then rinsed in 0.1 *M* sodium phosphate, 0.5 *M* sodium chloride (pH 7.0) and re-equilibrated in this buffer to provide 50% (v/v) slurries. The concentration of immobilized IgG was determined indirectly from estimates of the difference in total protein input for coupling and that remaining in solution (unbound IgG) upon termination of the coupling reaction. The protein content was determined by measuring the absorbance at 280 nm.

Coupling pH studies

Anti-tPA IgG_1 (6.9 mg/ml) was prepared in the following 0.1 *M* Good's Buffers at the indicated pH: 2-(N-morpholino)ethanesulfonic acid (MES, pH 5 and 6), 3-(N-morpholino)propanesulfonic acid (MOPS, pH 6.5, 7.0, 7.5 and 8.0) and 2-(N-cyclohexylamino)ethanesulfonic acid (CHES, pH 8.5, 9.0 and 9.5). Affi-Gel 10 (1.0 ml bed volume) was prepared for coupling as previously described[3]. Equal volumes of protein solution and Affi-Gel were mixed together with constant rotation at 4°C for 12 h in polyprep columns. Each column was then washed with 8 *M* potassium thiocyanate phosphate buffered saline (KSCN-PBS), pH 7.2 and finally reequilibrated in PBS, pH 7.2 containing 0.01% Tween 20. Next, 10 ml of purified tPA (antigen) at 0.47 mg/ml PBS-Tween 20 buffer, pH 7.2 was passed over each column. Bound tPA was eluted in 3.5 *M* KSCN-PBS, pH 7.2. The results are shown in Fig. 1.

Preparative scale coupling of MAb to Affi-Gel 10

A 20.6-g amount of the anti-tPA monoclonal antibody was reacted with 3.4 l of Affi-Gel 10 at 4°C using a turbine-type propeller for efficient batch mixing. Aliquots were removed at specified times and analyzed for coupled protein. Results shown in Fig. 2 are the average of three runs.

Immobilization kinetic studies

The process of fast coupling (15 s to 10 min) of antibody to Affi-Prep 10 was carried out in the following manner. Affi-Prep 10 was rinsed in icc-cold (4°C) 10 m*M* sodium acetate, pH 4.5 and prepared as a 50% (v/v) slurry in the above buffer. Then, 400 μl of slurry was delivered into microfuge tubes to obtain 200 μl packed bed volumes upon centrifugation. The supernatant was drawn off the pelleted resin by suction and 500 μl of antibody solution (0.25–2 mg/ml) was added. Antibodies were prepared in coupling buffer, 0.1 *M* MOPS (pH 7.5) containing varying amounts of salt (0.15–3.0 *M* sodium chloride) depending upon the experiment. Rapid mixing was accomplished with the aid of a vortex mixer. For coupling times greater than 1 min

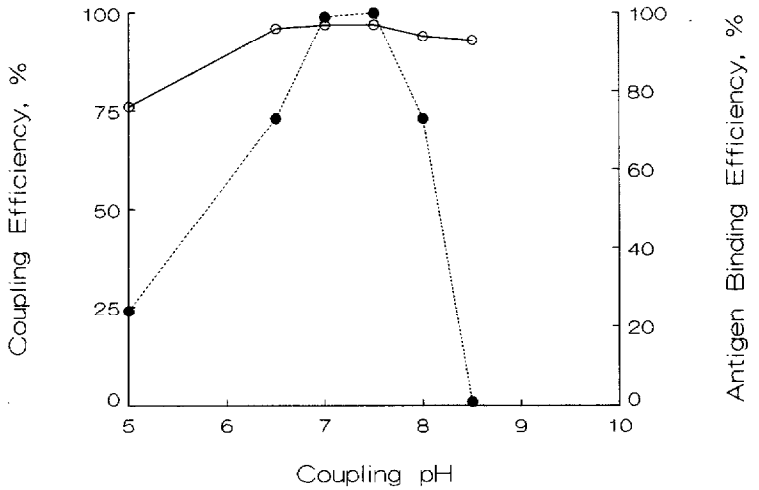

Fig. 1. The effect of coupling pH on the immobilization and activity of a monoclonal antibody. Murine anti-tPA MAb was coupled to Affi-Gel 10 under batch conditions in which the pH for coupling was varied. The tPA antigen binding efficiency for each of the immunosorbents was determined at pH 7.2. Details are provided in Experimental. ⊙ = Relative coupling efficiency (%). ● = Relative antigen binding efficiency (%).

additional mixing was accomplished by end-over-end rotation. After a specified coupling time, 100 μl of 1 *M* ethanolamine (pH 8) was added, the reaction mixture immediately mixed and the immunosorbent pelleted by centrifugation (1 min, 13 000 rpm). The supernatant was removed and placed on ice for analysis of protein content. The pelleted immunosorbent was then extensively washed, first with 0.1 *M* MOPS (pH 7.5), followed by 1 *M* sodium chloride, and finally rinsed and resuspended in antigen binding buffer as a 50% (v/v) slurry. An estimation of non-specific adsorption was

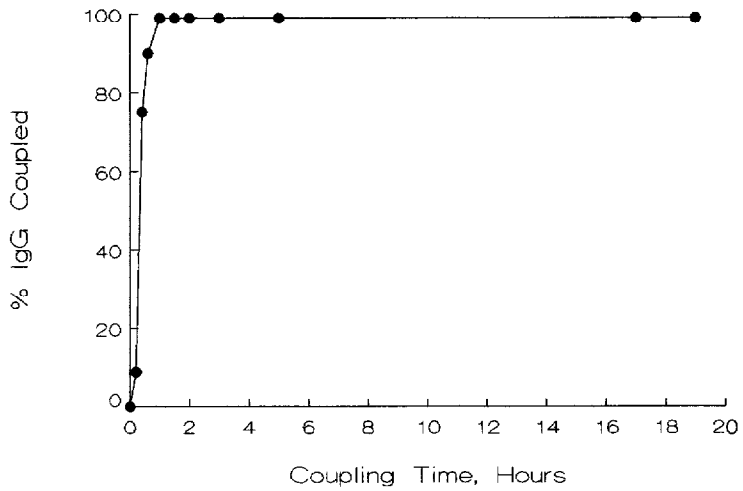

Fig. 2. Time course for preparative scale coupling of anti-tPA MAb to Affi-Gel 10.

attempted from the analysis of protein content of effluents obtained from rinsing the pelleted immunosorbent with 7M urea/1M sodium chloride. Minimal levels of protein ($\leqslant$ 1 μg/ml) were observed indicating that bound IgG was covalently coupled to the matrix.

For example, murine anti-tPA MAb (4 mg/ml) was coupled to Affi-Prep 10 in 0.1 *M* MOPS, pH 7.5 containing 0.15–3.0 *M* sodium chloride. Reactions were terminated after 10 min. Pure tPA was applied to each support and the amount bound determined by the difference between load and unbound (recovered) total protein. The results are shown in Fig. 3.

In another experiment, murine anti-HRP MAb (1 mg/ml) was coupled to Affi-Prep 10 in 0.1 *M* MOPS, pH 7.5 containing either 0.15 *M* or 2.0 *M* sodium chloride. Reactions were terminated at the specified times, beginning at 15 s. HRP was applied to each support and the extent of binding determined by the Bio-Rad ELISA peroxidase assay. The results of this study are provided in Fig. 4a and b.

RESULTS AND DISCUSSION

The effect of coupling pH

Immunoglobulins can be covalently coupled to activated, solid supports over a broad pH range without difficulty. For example, an anti-tPA IgG_1 was immobilized to Affi-Gel 10 over the pH range of 5–9.5 in an overnight (18 h) reaction at 4°C. As shown in Fig. 1, near quantitative coupling of the antibody was obtained between pH 6 and 9. However, these immunosorbents varied dramatically in their ability to effectively bind tPA antigen. Optimal antigen binding was observed only for the immunosorbents constructed from the immobilization of the MAb at coupling pH 7–7.5.

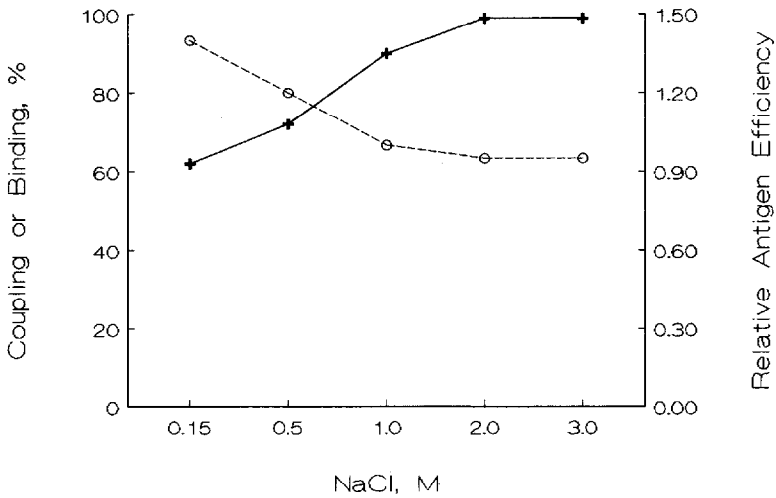

Fig. 3. Coupling efficiency *vs.* antigen binding to Affi-Prep 10. Anti-tPA MAb was coupled to Affi-Prep 10 as described in Experimental. In this case, reactions were terminated after 10 min. Pure tPA was applied to each immunosorbent and the amount bound determined by the difference between load and unbound (recovered) total protein. ○ = Antigen binding (%) or relative antigen binding efficiency (0.00–1.50). + = Antibody coupling efficiency (%).

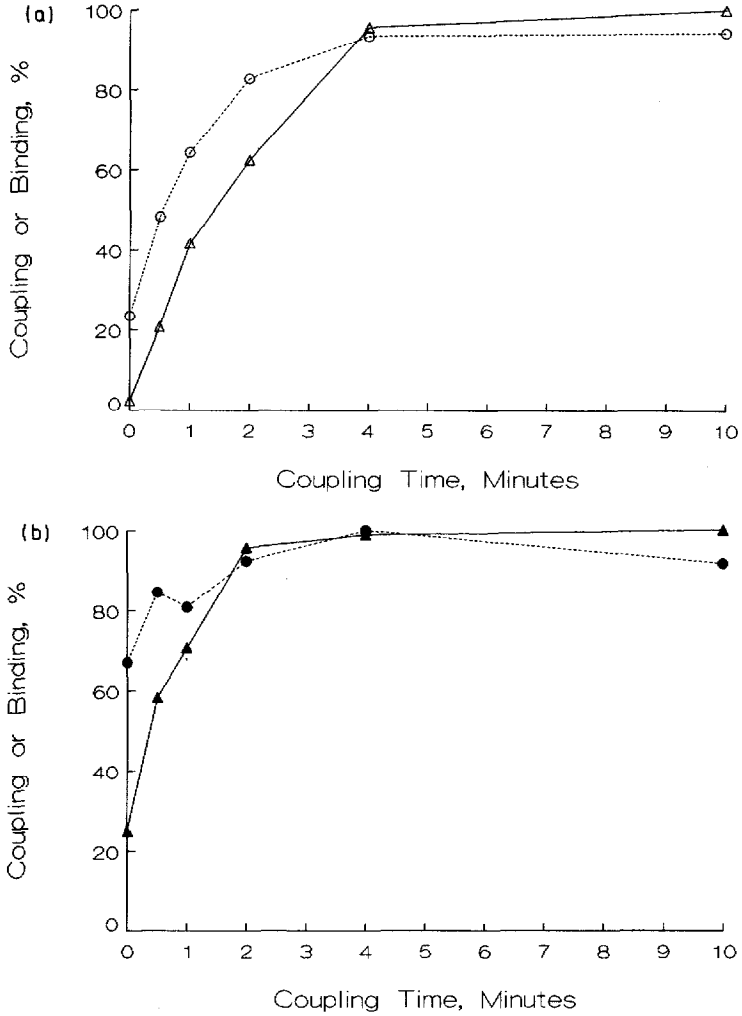

Fig. 4. The effect of coupling rate on the antigen binding capacity. (a) Anti-HRP MAb was coupled to Affi-Prep 10 in buffer containing either 0.15 *M* sodium chloride or (b) 2 *M* sodium chloride. Reactions were terminated at the specified times, beginning at 15 s. HRP was applied and the extent of binding determined by the Bio-Rad ELISA peroxidase assay. (a) △ = Antibody coupling efficiency (%); ○ = HRP binding efficiency (%). (b) ▲ = antibody coupling efficiency (%); ● = HRP binding efficiency (%).

The effect of monoclonal antibody-matrix density

Eveleigh and Levy[1] studied the effect of increased polyclonal antibody density on CNBr-Sepharose 4B and porous glass beads. They concluded from these studies that lower protein densities (*ca.* 1 mg/ml) improved the performance of the immunosorbent in terms of specific antigen binding activity (moles antigen bound/moles IgG coupled). Unfortunately, these studies cannot be regarded as definitive since the amount of CNBr was also varied over a 20-fold range in order to achieve these results. And as they report, other variables such as the activation procedure that is

operational during the immobilization process may influence the immunoreactivity of the sequestered antibody.

We have studied the antibody ligand-matrix density problem on several matrices using different activation chemistries. Three approaches have been used and will be briefly outlined here.

The first method involved using a fixed volume ratio of MAb to matrix such that the total available number of activation groups remained constant but the concentration of antibody was varied to achieve a desired ligand density. The coupling reaction was allowed to proceed to completion (Fig. 2). Any remaining reactive groups were displaced by the addition of ethanolamine. For example, the anti-tPA monoclonal antibody was immobilized onto Affi-Gel 10 (NHS) matrix at ligand densities of *ca.* 5–7 g MAb/l gel in this manner (Table I). In this particular experiment there was nearly a 2-fold increase in antigen binding efficiency when the MAb ligand–matrix density is decreased by 25%.

In the second approach, MAb was rapidly (within 10 min) coupled to the matrix, Affi-Prep 10 (NHS) by the addition of salt (Fig. 3). The result of this coupling procedure was that increased salt concentration led to an increased immobilization of MAb. At a fixed time interval such as 10 min, a series of different MAb ligand densities could be achieved by varying the input salt concentration. When the antigen binding efficiency was determined over this density range, it was found that substantially greater binding of antigen could be accomplished at the lowest MAb ligand density. This was further verified in the rapid coupling of an anti-HRP monoclonal antibody (also an IgG_1) in which enzymatic activity of the bound HRP antigen was monitored rather than mass as in the previous case. Interestingly, with the anti-HRP system little difference was found either in the amount of MAb coupled nor in the antigen binding efficiency between low salt (0.15 *M* sodium chloride) and high salt (2 *M* sodium chloride) buffers at 10 min (Fig. 4). The very early coupling times did indicate that higher salt was more effective in bringing the antibody to the solid phase for coupling. So at these earlier time points (below 2 min) the antigen capacity appeared to be better under high salt coupling conditions (Fig. 4b). The benefit gained by lower density couplings was substantially reduced with coupling time for either situation. Therefore, a similar trend in MAb ligand density *vs.* antigen binding efficiency was achieved by this approach using two different monoclonal antibodies. However, it is clear that antibodies that are even of the same isotype may interact with the matrix under different optimal coupling conditions.

TABLE I

AFFI-GEL 10 (NHS) COUPLING: EFFECT OF ANTIBODY LIGAND DENSITY ON CAPACITY FOR ANTIGEN

MAb coupled refers to the preparative scale coupling of anti-tPA MAb to Affi-Gel 10.

MAb coupled (g/l gel)	*Antigen bound/released (g/l gel)*
7.3	2.0
5.6	3.7
5.2	3.9

So in these first examples, we have attempted to optimize on the basis of coupling pH and protein-ligand density. However, as we have observed there are other factors that may influence the success of the immobilization process. In particular, one area that is not generally addressed is that of coupling kinetics. There are a number of good reasons to study kinetics in the preparation of an immunosorbent. First, longer reaction times may lead to increased inactivation of the immobilized antibody by allowing for multi-site attachments and an increased ligand density creating a steric effect that effectively reduce capacity. Furthermore, Wilchek and Miron[5] have pointed out the potential for leakage created by the immobilization of antibodies to unstable activated ester intermediates. These interactions are favorable at longer reaction times. Finally, non-specific adsorption can dramatically increase with time.

We have found that the NHS-mediated immobilization of MAbs using Affi-Gel 10 or Affi-Prep 10 is essentially complete within 1 h with proper mixing. Using more efficient methods of delivery of the antibody to the matrix, the coupling time can be reduced to approximately 10 min. For example, in earlier studies concerned with the *in situ* or on column immobilization of a rat polyclonal antibody to Affi-Prep 10 for HPLC, we could control the level of coupling by varying the flow-rate, duration and protein load. In these studies, coupling efficiency is nearly the same at 7.5 min and 85 min for similar protein loads[3].

However, the real question is what effect does this have on the antigen binding efficiency? We have examined the benefit of very rapid coupling times using high salt to enhance delivery of the immunoglobulin to the surface and found that such methods can be useful in obtaining optimal ligand densities. The action of salt upon delivery of the MAb to the matrix is presumably due to increased hydrophobic interaction of the antibody with the support. However, charge interaction phenomena with the matrix cannot be ruled out. Further studies will be necessary to delineate this effect.

Therefore it is possible to control the immobilization process to some extent from a careful study of ligand-matrix interaction. Nevertheless, the interaction remains largely a random event since the reactive lysine residues of antibodies may be distributed throughout the molecule. This approach fails particularly if these lysine residues are located in close proximity to the antigen binding site.

Oriented vs. random coupling

Recently a different strategy has been utilized. This involves sequestering of the antibody via its Fc-region. We call this oriented coupling. There are actually several ways to accomplish this event. One method involves binding the antibody to a Protein A support and then cross-linking the antibody to Protein A. For example, Philips *et al.*[6] successfully cross-linked an IgG_1 to Protein A Sepharose using dimethyl suberimidate to purify human adenosine deaminase to near homogeneity in one step.

There are some potential problems with this approach. First, not all antibodies will bind to Protein A. Second, once the antibody is bound by Protein A, the cross-linking process must be accomplished with care so as not to cause premature elution of the antibody; or inactivation by excessive cross-linking. Third, Protein A is itself expensive.

A more straightforward approach has recently been suggested by O'Shannessy and Hoffman[2], and successfully demonstrated by Little *et al.*[4]. This involves the coupling of aldo-IgG to solid-phase supports which have been activated with

a terminal hydrazide group. The aldo-IgG is generated by the sodium periodate oxidation of vicinal hydroxyl groups of the antibodies' carbohydrate moieties to aldehydes. These aldehydes are quite reactive with the matrix hydrazide. Since the reactive carbohydrates are usually located outside the antigen binding domain and no other groups within the protein will react with the matrix hydrazide, an oriented coupling is favored. The chemical bond that is formed is a hydrazone rather than a Schiff's base and does not have to be reduced for example with cyanoborohydride[7].

We have converted both Affi-Gel and Affi-Prep NHS matrices to the corresponding hydrizide (Hz) supports. Our initial studies were conducted on the cross-linked agarose matrix hydrazide; and we examined the consequences of oriented coupling for both polyclonal and monoclonal antibody-antigen pairs relative to activated supports that favor a random coupling of immunoglobulin[4]. In the case of polyclonal antibodies, dramatic increases in the antigen binding capacities were observed relative to CNBr-Sepharose. Similar results were obtained using Affi-Gel 10 (NHS). Therefore, we believe that this is a clear demonstration of the superiority afforded by the oriented coupling mechanism. In contrast, the monoclonal antibodies that we examined showed only slightly better or equivalent antigen capacity relative to those obtained on Affi-Gel 10 or CNBr-Sepharose. However, our studies have been limited to only a few examples using affinity purified species. It is possible that both the antigen binding site and the carbohydrate moiety could have been damaged during affinity purification using strongly acidic (pH 2–3) or basic (pH 9–11) conditions. Still another possibility is that excessive oxidation of the MAbs' occurred and that milder conditions for the conversion to aldo-IgG should be examined. Nevertheless, Rodwell *et al.*[8] failed to detect any significant alteration in the antigen binding affinity of periodate oxidized monoclonal antibodies.

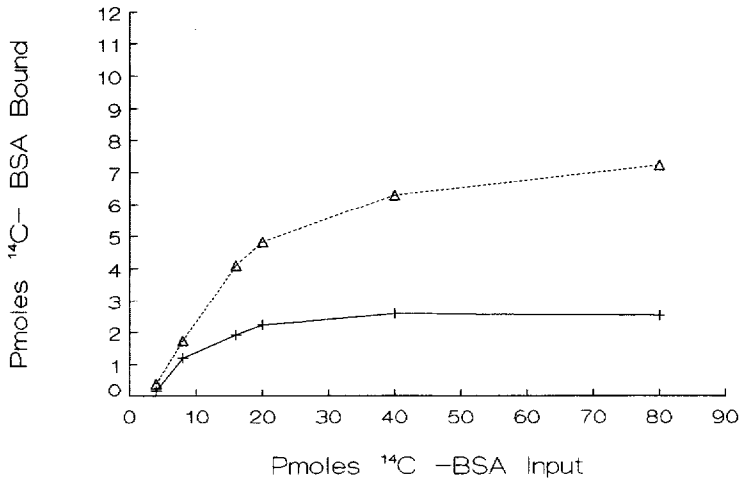

Fig. 5. Comparison of antigen binding capacities for oriented (Affi-Prep 10 HZ) *vs.* random (Affi-Prep 10) immunosorbents. Sodium periodate oxidized polyclonal anti-BSA antibody was immobilized to either Affi-Prep 10 or Affi-Prep 10 HZ and the antigen binding capacity measured using 14C-BSA. Details are described in the text. + = BSA binding to Affi-Prep 10 immunosorbent. △ = BSA binding to Affi-Prep 10 HZ immunosorbent.

There are other plausiable explanations. By definition of monoclonality, primary sequence homology is preserved. Therefore, coupling is much less random than for a polyclonal population, and large differences in antigen binding capacity from oriented *vs.* random coupling mechanisms may not be seen. On the other hand, when the immunoglobulin tends to couple through free amino residues located in close proximity to the antigen binding domain, then random coupling should lead to a substantial inactivation, while oriented coupling will allow for the successful preparation of an immunaffinity support (Fig. 5).

As previously mentioned, it is possible to convert the polymer-based Affi-Prep 10 (NHS) to the hydrazide form, Affi-Prep 10 Hz. We have found little difference in the characteristics of an immunosorbent based upon the agarose gel[4] or this polymeric Affi-Prep resin. For example, we have observed the enhanced binding capacity for immobilized polyclonal antibody that has been reported for Affi-Gel Hz (Fig. 5). In fact, this is very much the same capacity that was obtained using Affi-Gel Hz under similar antibody–ligand density and antigen load. Furthermore, the optimization

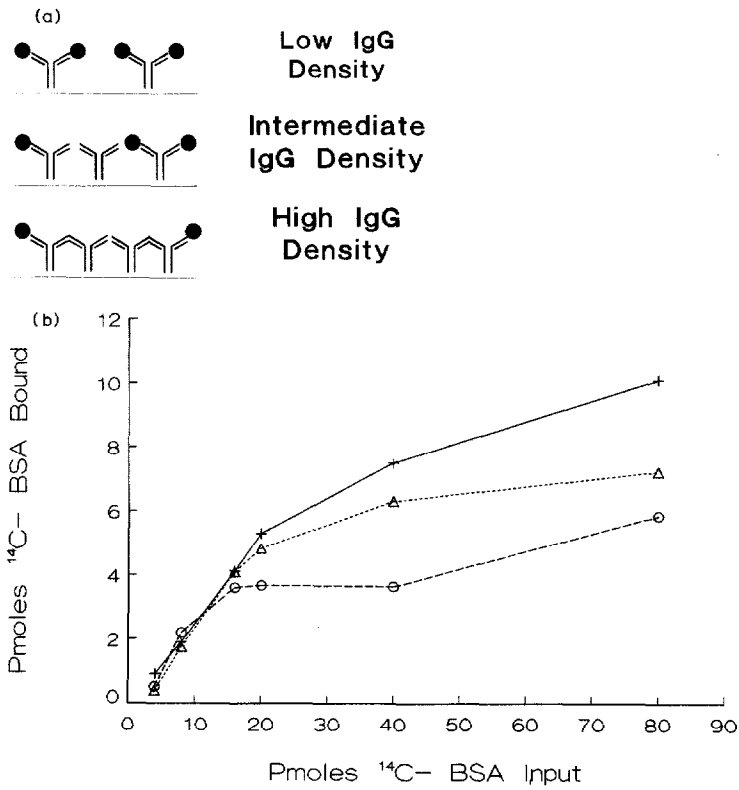

Fig. 6. (a) Artist conception of antibody ligand-matrix density relationships. Antigen molecules (closed circles) can bind freely to the antibody binding sites at low IgG density but experience steric hindrance at increased antibody density. (b) Effect of antibody ligand-matrix density on Affi-Prep 10 HZ antigen binding capacity. Anti-BSA immunosorbent was prepared at various IgG densities using Affi-Prep 10 HZ and the BSA binding capacity determined as described in the text. IgG density: ○ = 5.57 mg/ml; △ = 1.17 mg/ml; + = 0.34 mg/ml.

process should again include a regard for the antibody–ligand density because, as we have seen for CNBr or NHS-mediated coupling, lower densities lead to higher antigen capacities. In this case, antibody immobilized at 0.34 mg/ml had a 2-fold higher capacity for antigen than antibody coupled at 5 mg/ml (Fig. 6B), while the level of IgG required was reduced by 16-fold.

CONCLUSIONS

In our experiments we have attempted to focus upon those factors that are important for the preparation of an efficient immunosorbent. Presentation of the antibody to the solid-phase is critical. In particular, we have observed that monoclonal antibodies are more sensitive to coupling conditions than polyclonal antibodies. For this reason, a careful consideration of the coupling pH is especially important to obtain optimal antigen binding. Cuatrecasas and Parikh[9] in their earlier work suggested that pH control and short reaction times could be used to selectively couple one type of amino acid group over another. Indeed, this is one way in which to reduce the tendency for random coupling that results in a sterically hindered immunoligand.

An alternative to this approach is to abandon amino–ligand coupling altogether. This can be accomplished by condensation of aldehyde groups (generated by periodate oxidation of the carbohydrate moiety) with a solid-phase linked hydrazide group as suggested by O'Shannessy and Quarles[7] and subsequently demonstrated by Little *et al.*[4] for polyclonal antibodies but not for monoclonals. Nevertheless, unless there are significant differences between the kind and location of the carbohydrate moiety between monoclonal and polyclonal antibody molecules, theoretical grounds imply that this technique of oriented coupling via the Fc localized carbohydrate should work equally as well in both cases. Finally, regardless of the technique utilized for immobilization of monoclonal antibodies it is clear that the density of the antibody on the matrix is of principal importance. Steric hindrance created by crowding of adjacent antibody molecules immobilized via oriented coupling or by random coupling mechanisms leads to the same net effect, a decrease in antigen binding efficiency.

ACKNOWLEDGEMENTS

We would like to extend our appreciation to Chris Siebert and Dr. Ken Talmadge for their helpful discussions during the course of these studies.

REFERENCES

1 J. W. Eveleigh and D. E. Levy, *J. Solid-Phase Biochem.*, 2 (1977) 45.
2 D. J. O'Shannessy and W. L. Hoffman, *Biotech. Appl. Biochem.*, 9 (1987) 488.
3 R. S. Matson and C. J. Siebert, *Preparative Chromatogr.*, 1 (1988) 67.
4 M. C. Little, C. J. Siebert and R. S. Matson, *BioChromatogr.*, 3 (1988) 156.
5 M. Wilchek and T. Miron, *Biochemistry*, 26 (1987) 2155.
6 A. V. Philips, D. J. Robbins, M. S. Coleman and M. D. Barkley, *Biochemistry*, 26 (1987) 2893.
7 D. J. O'Shannessy and R. H. Quarles, *J. Immunol. Methods*, 99 (1987) 153.
8 J. D. Rodwell, V. L. Alvarez, C. Lee, A. D. Lopes, J. W. F. Goers, H. D. King, H. J. Powsner and T. J. McKearn, *Proc. Natl. Acad. Sci. U.S.A.*, 83 (1986) 2632.
9 P. Cuatrecasas and I. Parikh, *Biochemistry*, 11 (1972) 2291.