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Antibody immobilization to high-performance liquid chromatography supports Characterization of maximum loading capacity for intact immunoglobulin G and Fab fragments

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Abstract

This study examined various factors that affect the maximum amount of intact immunoglobulin G (IgG) or Fab fragments that can be covalently immobilized to silica and other HPLC-grade supports for use in immunoaffinity chromatography or immunoextractions. Factors that were considered included the amount of surface area available for immobilization, the pore size of the support, the type of immobilization method and the nature of the support matrix. The main factor in determining the extent of immobilization was found to be the relationship between the support's surface area and the ability of the IgG or Fab fragments to reach this surface. Access to the support surface was a function of the size of the protein being immobilized and the support porosity, with maximum immobilization being obtained with supports having pore sizes of approximately 300 Å for intact IgG and 100 Å for Fab fragments. Some differences in the maximum level of immobilization were noted between different coupling methods. Supports like Poros and Emphaze gave similar results to those seen with HPLC-grade silica when a comparison was made between materials with comparable pore sizes. Many of the trends observed in this work for IgG and Fab fragments should apply to other proteins that are to be immobilized to HPLC supports.

Keywords: Immobilized antibodies; Stationary phases, LC; Immunoaffinity chromatography

1. Introduction

High-performance liquid chromatography (HPLC)-based supports containing immobilized antibodies are becoming increasingly important in the development of new separation and analysis methods for compounds of biological, industrial or environmental interest. Examples include methods like chromatographic-based immunoassays, on-line immunoextraction, and multi-dimensional schemes in which antibody affinity columns are coupled with reversed-phase liquid chromatography, gas chromatography or capillary electrophoresis [1–5].

There are several ways that antibodies and other proteins can be attached to HPLC supports for use in affinity columns [1,2,4–8]. The optimization of antibody and protein immobilization to low-perform-

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ance supports has been well-characterized [9–12]. The relationship between analyte size and optimum active ligand density for HPLC supports has also been studied [13]. However, there is little information in the literature regarding the maximum degree of antibody immobilization that can be obtained for HPLC supports. This information would be useful for applications in which high-density affinity supports are needed to develop small columns or to promote fast, quantitative binding of analytes [4,14–19]. Such data should become particularly important as current trends continue in micromachining and in the development of miniaturized separation systems [20–22].

This study will examine the maximum extent of immobilization that can be obtained on several types of HPLC supports for antibodies and antibody-related fragments. Polyclonal rabbit immunoglobulin G (IgG) and its Fab fragments will be used as models for this work. Silica supports with various pore sizes and surface areas will be examined along with perfusion media (Poros) and an azalactone support (Emphaze). Both general and site-specific methods of immobilization will be considered. The overall goal is to identify the main factors that determine the maximum amount of IgG and related ligands that can be coupled to HPLC-grade materials.

2. Experimental

2.1. Reagents

All Nucleosil supports were purchased from Alltech (Deerfield, IL, USA). The Emphaze hydrazide support and Poros AL support were donated by 3M (Minneapolis, MN, USA) and PerSeptive Biosystems (Framingham, MA, USA), respectively. The diameters, nominal pore sizes and surface areas of these supports are summarized in Table 1. Adipic dihydrazide was obtained from Aldrich (Milwaukee, WI, USA). The rabbit, mouse and goat immunoglobulin G (rabbit, goat or mouse IgG, all greater than 95% pure and with no detectable aggregation), *p*-periodic acid reagent, 1,1′-carbonyldiimidazole (CDI) and protein A (recombinant IgG binding fragment) were from Sigma (St. Louis, MO, USA). Reagents for the bicinchoninic acid (BCA) protein assay and immobilized papain were from Pierce (Rockford, IL, USA). Other chemicals were reagent-grade or better. All aqueous solutions were prepared using deionized water from a Nanopure water system (Barnstead, Dubuque, IA, USA).

2.2. Apparatus

Samples for the manual BCA protein assay were analyzed using a Shimadzu UV160U absorbance spectrophotometer (Kyoto, Japan). The antibodies and supports were mixed using either an Aliquot 4651 Mixer from Ames (Elkhart, IN, USA) or a Labquake Shaker from Lab Industries (Berkeley, CA, USA).

2.3. Methods

The Emphaze and Poros supports, which had previously been activated with dihydrazide and

Table 1
Summary of support properties[a]

Type of support	Pore size (Å)	Surface area (m^2/g)	Particle diameter (μm)
Nucleosil Si-50	50	350	7
Nucleosil Si-100	100	350	7
Nucleosil Si-300	300	100	7
Nucleosil Si-500	500	35	7
Nucleosil Si-1000	1000	25	7
Nucleosil Si-4000	4000	25	7
Poros AL	10 000/300[b]	75	20
Emphaze	200	340	65

[a] All values were supplied by the manufacturers of these supports.
[b] The first Poros value is the average size of the through-pores, while the second value is for the side-pores.

aldehyde groups, respectively, were used as supplied by the manufacturers. Each Nucleosil silica support was converted into a diol-bonded form before use, as prepared according to a previously-reported method [23]. This diol-bonded support was then activated with aldehyde groups (the Schiff base method) [7,11,12,23], imidazole groups (the CDI method) [24], or hydrazide groups (the hydrazide method) [23], as performed by techniques given in the literature.

IgG that was immobilized by the Schiff base or CDI methods was used without further pretreatment. However, IgG that was to be immobilized onto hydrazide-activated supports had to first be oxidized under mild conditions with periodate in order to generate aldehyde groups in the IgG's carbohydrate regions [23,25,26]. This oxidation was carried out by dissolving 20 mg/ml rabbit IgG into a pH 5.5, 0.02 *M* sodium acetate buffer. A total of 20 mg *p*-periodic acid was added to this solution, and the resulting mixture was reacted for 40 min. It has previously been determined that these conditions yield an average of two hydrazide coupling sites per IgG molecule [26]. These oxidized IgG molecules were then removed from any remaining periodate and placed into a pH 6.5, 0.10 *M* potassium phosphate buffer [26].

Fab fragments were prepared by digesting rabbit IgG with immobilized papain from Pierce, as performed according to the manufacturer's instructions. The Fab fragments that were produced were decanted from the immobilized papain support, and the undigested antibodies or Fc fragments in the supernatant were removed by incubating this solution with a Nucleosil Si-300-7 support that contained immobilized protein A. The Fab fragments that were prepared in this fashion were found by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) to be greater than 99% pure and to contain no detectable amounts of intact IgG. After the Fab fragments had been purified, they were lyophilized and redissolved in a pH 7.0, 0.10 *M* phosphate buffer. All Fab and IgG solutions were immobilized immediately following their preparation and/or isolation.

Most of the IgG immobilization methods were carried out in pH 6.0, 0.10 *M* phosphate buffer; the only exception was the CDI method, which was instead conducted in a pH 7.0, 0.10 *M* phosphate buffer. For the Schiff base method, approximately 8 mg/ml of sodium cyanoborohydride was added to the reaction mixture to promote the formation of stable secondary amine linkages between the immobilized protein and support [11,12,23]. The amount of silica in all the reaction slurries was 13.3 mg/ml and the approximate amount of IgG was 20 mg/ml. Each of these solutions was vortex-mixed, placed onto an inversion shaker, and allowed to react for 7 days at 4°C to allow sufficient time for all immobilization methods to reach completion [27]. For the Schiff base method, the silica was washed with pH 8.0, 0.10 *M* phosphate buffer after immobilization and allowed to react for another 2 h with 4 mg/ml sodium borohydride in the pH 8.0 buffer in order to remove any aldehyde groups that remained on the support [11,12,23].

The immobilization of IgG onto the Emphaze and Poros supports was performed by the hydrazide and Schiff base methods, respectively, according to the manufacturer's instructions. These both used pH 6.0, 0.10 *M* phosphate buffer plus 1.5 *M* sodium sulfate as the immobilization buffer. The initial IgG concentrations in this buffer were 20–26 mg/ml and the slurry density was 26 mg/ml, or roughly 65 mg of the support per 2.5 ml of buffer [27–29]. As with the silica supports, each of these reaction slurries was vortex-mixed, placed onto an inversion shaker, and allowed to react for 7 days at 4°C [27].

The Fab fragments were immobilized using both the Schiff base and CDI methods. Both methods were conducted under the same buffer and reaction conditions as described earlier for IgG immobilization, but now using an initial Fab concentration of 36 mg/ml. The suspensions for these immobilization methods were again vortex-mixed, placed onto an inversion shaker, and allowed to react for 7 days (in this case, at room temperature).

At the end of each immobilization reaction, the supports were centrifuged and washed several times with deionized water and pH 7.0, 0.10 *M* phosphate buffer. These supports were then suspended in 1.0 ml portions of pH 7.0, 0.10 *M* phosphate buffer for storage or later use. The protein content of each support was determined in triplicate by a manual BCA assay [30], with rabbit IgG being used as the standard and samples containing only the initial

support (with no IgG or Fab fragments) being used as blanks.

One reason that polyclonal rabbit IgG and its fragments were chosen as the ligands for this work is that these are commonly used in the preparation of antibody-based supports for immunoassays and affinity chromatography. Also, rabbit IgG has been shown in several previous immobilization studies to be a good model in indicating the general behavior that would be expected for alternative types of antibodies (e.g., monoclonal antibodies or antibodies from other species) [26,27].

Each support used in this study had a large excess of activated groups versus the total moles of IgG that was eventually attached to its surface. For example, the hydrazide-activated and Schiff base silica used in this work were each estimated to have almost 70 activated sites in the area covered by only one immobilized IgG molecule [27]. This was desired in these experiments in order to eliminate the degree of support activation as a variable when determining the maximum possible extent of IgG immobilization [27].

3. Results and discussion

3.1. Effect of IgG excess on maximum loading capacity

Since the goal of this work was to determine the maximum amount of IgG or Fab fragments that could be immobilized, it was necessary to see what relative excess of these ligands was needed to achieve this maximum level. This was done by incubating the same activated support with various amounts of IgG under otherwise constant reaction conditions. The results are summarized in Fig. 1. For convenience, the relative amount of IgG that was added to the immobilization slurry is expressed here in terms of the effective number of monolayers that this IgG concentration would have produced if all of it had become attached to the support. The number of effective monolayers was calculated based on the known surface area of the support (see Table 1), the amount of support and IgG added to the reaction slurry, and the approximate surface area that was covered by a single IgG molecule. This latter number

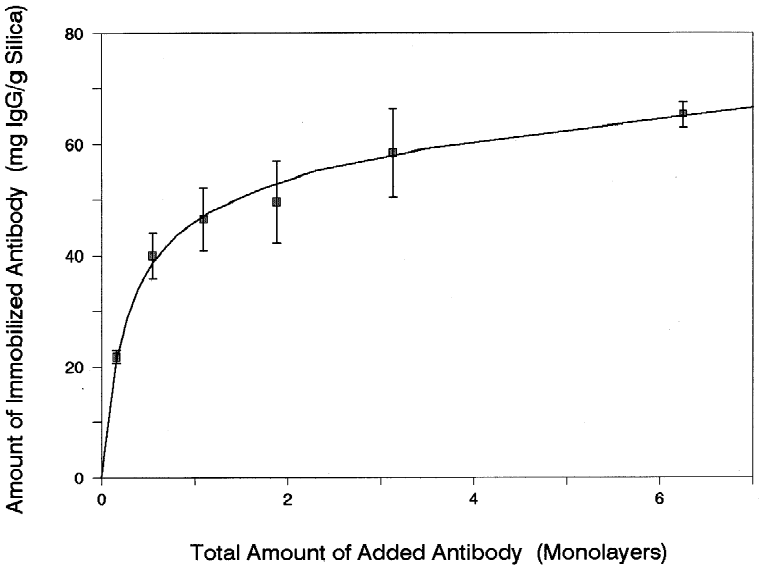

Fig. 1. Effect of increasing the amount of IgG that is used during immobilization on the total amount of immobilized IgG that is actually observed. These results were obtained for rabbit IgG using the Schiff base method and Nucleosil Si-300 silica as the support. The number of equivalent monolayers that are given on the lower axis were calculated by using an average estimated diameter of 80 Å for rabbit IgG (note: using a diameter of 100 Å decreases the values on this axis by roughly 1.5-fold). The error bars represent a range of ±1 standard error of the mean (SEM) for three replicate analyses.

was estimated by describing each IgG molecule as a sphere with an average diameter of 80–100 Å [31].

It can be seen from Fig. 1 that using an IgG excess of less than one effective monolayer gave behavior in which there was a sharp increase in the degree of immobilization as the initial amount of IgG was increased. This was expected since the surface of the support was not yet saturated under these conditions and still had adequate space for the attachment of additional IgG molecules. However, at an IgG excess above one effective monolayer, the final amount of immobilized IgG increased only slightly and appeared to approach a fixed value. There was still some increase in the extent of immobilization when going from an IgG excess of one to three effective monolayers, but this increase did not continue when a higher excess of IgG was used.

Based on the results in Fig. 1, a minimum IgG excess of three effective monolayers was used in all later immobilization studies. Under these conditions, the results that were obtained should have shown little or no variation with slight changes in the amount of IgG that was used in the immobilization study. These same conditions should also have allowed a good approximation to be obtained for the maximum amount of IgG that could be immobilized to any given support.

3.2. Immobilization of intact IgG onto porous silica

After the immobilization conditions had been optimized to provide maximum IgG attachment, a series of studies were performed to examine how this maximum amount varied as the porosity of the support was changed. Fig. 2 shows the results obtained when IgG was immobilized by the hydrazide method onto silica supports with pore sizes that ranged from 50 to 4000 Å. Similar trends were seen with other immobilization methods.

Fig. 2 indicates that the amount of immobilized IgG increased in going from supports with pore sizes of 50 to 300 Å. This can be explained by considering the average size of the IgG molecules and the available surface area on each support. As shown in Table 1, the supports used in this study had a larger total surface area as the pore size was decreased. However, the diameter of rabbit IgG (i.e., approximately 80–100 Å) [31] would have prevented these IgG molecules from reaching the surface located within any pores that were smaller than this size. Thus, as the pore size increased it became easier for the IgG to reach more of the total surface area for immobilization.

Another trend that can be seen in Fig. 2 is a

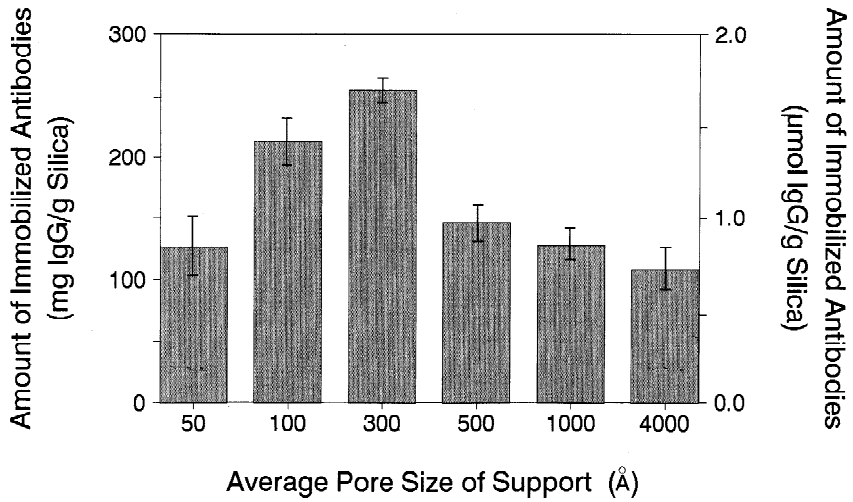

Fig. 2. Total amount of intact rabbit IgG that was immobilized onto dihydrazide-activated silica of various pore sizes. The results shown are the mean values for three replicates. The error bars represent a range of ±1 SD in these results.

decrease in IgG immobilization when going from 300 Å to 500–4000 Å pore supports. This is related to the total surface area of these materials.

All of these supports had nominal pore sizes that were at least three times bigger than the size of rabbit IgG, so most of their surface area should have been available for IgG immobilization. This is illustrated in Fig. 3, where the results in Fig. 2 have now been normalized according to the total surface area that was present on each support. Fig. 3 shows that supports with pore sizes of 50, 100 or even 300 Å had a large fraction of their total surface area which was not available for IgG immobilization. For instance, these gave maximum IgG coverages of only 0.07, 0.12, or 0.51 monolayers, respectively, when using 80 Å as the average diameter of an IgG molecule. But for the larger pore supports approximately the same degree of IgG coverage was obtained per unit surface area. Furthermore, the estimated extent of this coverage was 0.85–1.03 monolayers (average, 0.92±0.10 monolayers) for the 500–4000 Å pore supports, which confirmed that essentially all of their surface area was available for IgG immobilization. This also confirmed that the net decrease in immobilized IgG in going from the 300 Å to the 500–4000 Å pore supports (as shown in Fig. 2) was directly related to the smaller surface areas of the larger pore materials.

3.3. Changes in IgG coverage with immobilization method

Studies were next performed to determine the maximum amount of IgG immobilization that could be obtained on porous silica by other coupling methods. The other techniques that were examined were the Schiff base and CDI methods. Both of these approaches couple through free amine groups on IgG but have been shown to have differences in their reactivity and specificity for these groups [32,33]. In contrast to this, the hydrazide method used in Figs. 2 and 3 is thought to be a more site-selective approach that immobilizes IgG through aldehyde groups generated within the carbohydrate residues of IgG's Fc region.

Both the Schiff base and CDI methods gave the same trends as shown in Figs. 2 and 3 for the hydrazide method. There were some differences, though, in the total degree of IgG immobilization that was obtained by these techniques. For example, the Schiff base method gave an average of only 61±4% (1 standard error of the mean, SEM) of the maximum IgG coverages that are shown in Fig. 2 for the hydrazide method. On other hand, the CDI method produced values that were statistically indistinguishable from the hydrazide data, with an average ratio of 110±20% being observed between

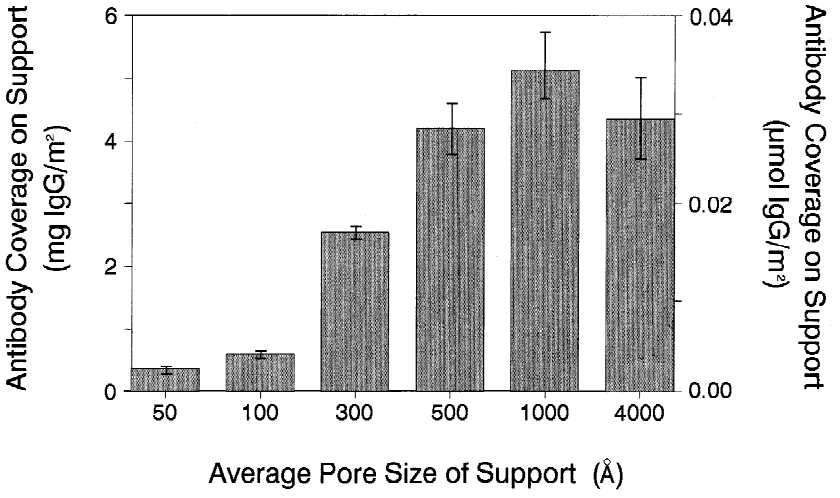

Fig. 3. Change in the degree of protein coverage for intact rabbit IgG as a function of pore size for hydrazide-activated silica. Each bar represents the mean of three results; the error bars represent a range of ±1 SD.

the CDI and hydrazide results. The exact reason for the lower degree of immobilization by the Schiff base method is not clear at the present. However, this is probably related to the different specificities of these methods, which in turn may lead to different orientations or spacing of the IgG molecules that are immobilized by each of these techniques.

Studies were also performed to compare the use of IgG from other animal sources, such as mice or goats. Using the Schiff base method of immobilization, it was found that both mouse and goat IgG gave identical trends to those seen for rabbit IgG in Figs. 2 and 3. However, the mouse and goat IgG did have slightly smaller values for the amount of coverage that was obtained on each support. The mouse IgG gave an average of 78±8% of the coverage that was noted for rabbit IgG under identical immobilization conditions, while the goat IgG gave 70±4% of the coverage that was seen for rabbit IgG. The exact reason for these differences is not known, but it is probably due to the slightly different sizes and structures of these proteins.

3.4. IgG immobilization onto other support materials

A comparison was also made between the results obtained with porous silica and those seen with other HPLC-grade media that can be used for immunoaffinity supports. Two alternative materials that were considered were Emphaze and Poros AL. The Emphaze support was hydrazide-activated, so its immobilization was performed using the same oxidized IgG as utilized with the hydrazide-activated silica [23,26,27]. Poros AL is an aldehyde-activated material, so the Schiff base method was used for IgG immobilization with this medium [11,12]. Table 2 gives a summary of the immobilization results that were obtained with these supports.

A comparison of Table 2 with Fig. 2 shows that Emphaze produced an IgG coverage that was in between the values observed for the Nucleosil Si-100 and Si-300. This is not surprising since Emphaze has an average pore size of 200 Å, which is also intermediate between the average pore sizes of the Si-100 and Si-300 silica. The maximum coverage of IgG on Emphaze was estimated to be 0.09–0.10 monolayers. This was in the same range as seen for the Nucleosil Si-100 and Si-300. It was determined from this that only a small amount of the total surface area on Emphaze was actually available for IgG coupling. In addition, the similarity of the Emphaze and Nucleosil Si-100/Si-300 results suggests that pore size played a dominant role in determining the overall IgG coupling capacity of these supports.

The maximum antibody coverage of 2.33 mg/m^2 that was measured for Poros AL indicated that only about half of its total surface area was available for IgG immobilization. This coverage was close to that seen earlier with the Nucleosil Si-300 silica. This is in agreement with the fact that the side-pores of the Poros medium (where most of the surface area is located) were approximately the same size as the pores on Nucleosil Si-300. These results again indicate that support porosity is an important factor in determining the maximum coverage that can be obtained for IgG on such media.

3.5. Immobilization of Fab fragments

The final group of studies considered the immobilization of Fab fragments (derived from rabbit IgG) onto HPLC-grade supports. This was performed using Nucleosil silica with a variety of pore sizes.

Table 2
Immobilization results for non-silica supports[a]

Support/immobilization method	Maximum amount of immobilized IgG (mg IgG/g support)	IgG coverage (mg IgG/m^2 support)
Poros/Schiff base method	140 (±30)	0.41 (±0.09)
Emphaze/hydrazide method	175 (±10)	2.33 (±0.14)

[a] The numbers in parentheses represent a range of ±1 standard error of the mean for three replicate analyses.

The amount of immobilization that could be obtained with respect to pore size was examined in a similar manner to the previous study with intact IgG. The amount of Fab fragments originally placed in each immobilization mixture was 40 mg/ml; this provided at least a 10-fold excess versus that needed to provide a theoretical coverage of one monolayer on the support. Other conditions, such as the slurry density and incubation time for the Fab immobilization, were the same as used in the earlier experiments with intact IgG.

Fig. 4 shows results obtained for the immobilization of Fab fragments onto silica by the CDI method. This gave a similar profile to that seen in Fig. 2 for intact IgG in that the largest amount of immobilized protein was obtained at intermediate pore sizes. Again, this can be viewed as representing a compromise between the ability of the Fab fragments to reach the support's surface and total amount of surface area that was available on the support for immobilization (see Fig. 5).

The main difference in Figs. 2 and 4 was that the maximum immobilization of the Fab fragments occurred at a smaller average pore size than was noted for the intact IgG molecules (i.e., 100 Å in Fig. 4 versus 300 Å in Fig. 2). It is believed that this is due to the smaller size of the Fab fragments, which have a diameter approximately half that of intact IgG molecules. It is interesting to note that both intact IgG and Fab fragments had optimum pore sizes for maximum coverage which were roughly two- to three-times the estimated diameter of the protein (i.e., a diameter of 40–50 Å for Fab fragments and 80–100 Å for intact IgG molecules). This may represent a general guideline that could be used for maximizing the immobilization of other proteins to porous supports.

Another difference between Figs. 2 and 4 was that the overall level of coverage, in terms of the mass of immobilized protein per gram of support, was lower for the Fab fragments than it was for the intact IgG. This occurred because of the smaller molecular mass of the Fab fragments (i.e., 50 000 versus 150 000 for intact rabbit IgG). However, the extent of immobilization in terms of moles of immobilized protein was about the same, with both the Fab fragments and intact IgG having a maximum coverage between 1 and 2 μmol per gram of support. Since Fab fragments are smaller than intact IgG molecules, this might be expected to result in a larger coverage for the Fab fragments in terms of the moles of immobilized protein. But Fab fragments also have a more elongated shape than intact IgG, which would probably make them more difficult to pack together on a surface's surface. The branched ''Y''-shaped structure of intact IgG may also play a role in making this

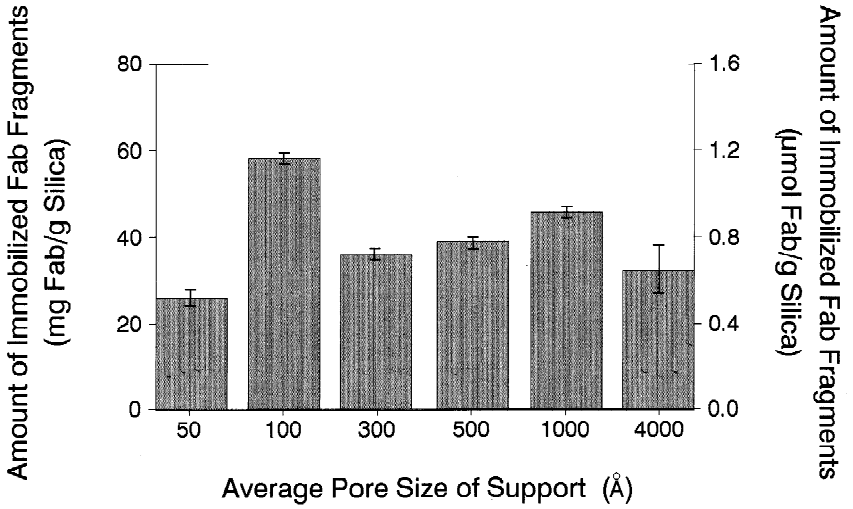

Fig. 4. Total amount of rabbit IgG Fab fragments that were immobilized onto CDI-activated silica of various pore sizes. The results shown are the mean values for three to four replicates. The error bars represent a range of ±1 SD in these results.

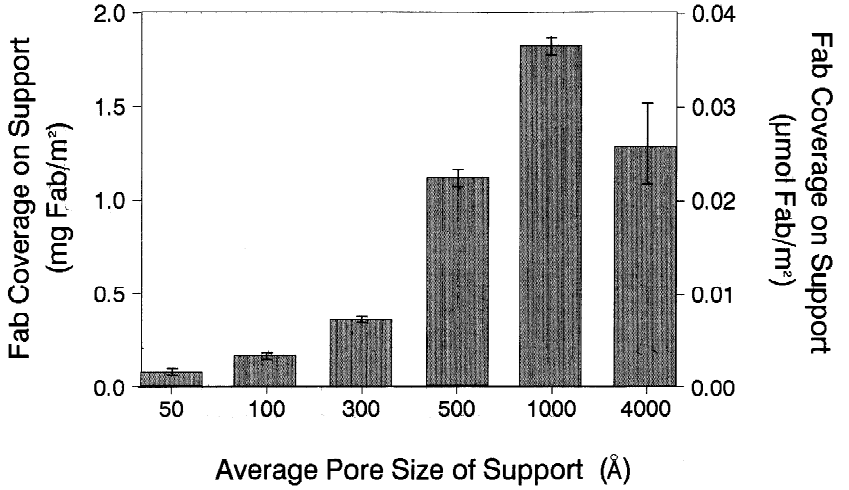

Fig. 5. Change in the degree of Fab coverage for rabbit IgG fragments as a function of pore size for CDI-activated silica. Each bar represents the mean of three to four results, and the error bars represent a range of ±1 SD.

easier to pack on a surface than an Fab fragment. The similarity of the results in Figs. 2 and 4 (in terms of the moles of immobilized protein) indicates that such packing and shape effects are important when comparing the degree of immobilization that can be obtained for Fab fragments and intact IgG.

Another immobilization technique that was used with the Fab fragments was the Schiff base method. This gave similar trends to those seen with the CDI method in Figs. 4 and 5. However, as with the intact IgG, it was again observed that the Schiff base method gave a maximum level of immobilization that was only 50–60% (50±17%) of that obtained with the CDI method. This difference between the two coupling techniques was statistically identical to the difference seen for the intact IgG. As stated previously, this may be a result of the known differences in specificity of the CDI and Schiff base methods [32,33].

4. Conclusion

This work examined various factors that affect the maximum amount of intact IgG or Fab fragments that can be immobilized to HPLC supports. It was found that the maximum extent of immobilization to a porous support was mainly determined by the relative size of the protein, the total surface area of the support and the size of pores within the support. The largest degree of immobilization for both intact IgG molecules and Fab fragments was obtained at pore sizes which were roughly two-to-three times the diameter of these proteins. These conditions allowed good access of the proteins to the interior of the support while also providing an optimum surface area for attachment. Similar results were obtained regardless of whether the support was based on silica or other porous media, such as Emphaze or Poros. This indicated that the physical structure of the support, rather than its chemical nature, was more important in determining the maximum possible extent of protein immobilization.

Another factor found to affect the maximum coverage of intact IgG molecules or Fab fragments was the type of immobilization method that was used. For instance, with intact IgG the Schiff base method gave results that were 50–60% lower than those seen with the CDI method, while the hydrazide method gave comparable results. The reasons for these differences are not yet clear, but they are probably related to the different reaction specificities of these methods. The shape of the protein also appeared to play a role in determining the extent of maximum immobilization. This was suggested by the fact that Fab fragments gave similar coverages (in terms of moles of immobilized protein) to that

measured for the larger, but less elongated, structures of intact IgG molecules.

Some of the effects examined in this study for IgG, such as the role of pore size and surface area in immobilization, are general phenomena that should apply to other proteins. But the effects related to immobilization method and protein shape are more specific and should be considered on a case-by-case basis. Future work will examine additional factors, like the influence of analyte size, support porosity, and the extent of IgG coverage on the apparent binding activities that are observed for immunoaffinity supports. Both this present study and such future work should provide valuable information that can be used in the more rapid development and optimization of new affinity chromatographic methods.

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