Surface Modification with Protein A for Uniform Binding of Monoclonal Antibodies

Willfried Schramm, Tony Yang, and A. Rees Midgley

We describe optimal conditions for immobilization of two monoclonal antibodies to progesterone for solid-phase assays. Poly styrene surfaces are refined with Protein A to achieve uniform, reproducible, stable, and sterically accessible immobilization of immunoglobulins (lgG). To this end, we optimized the amount of immobilized Protein A, the pH of the medium for immobilization, the concentration of antibody, and the poly styrene surface. We also investigated three carriers for solid-phase assays: 12 × 75 mm polystyrene test tubes, Macrowells™ (Skatron, Inc.; suitable for processing with multiple pipettors), and microwell strips (Immulon II™, Dynatech Inc.). Immunoglobulin does not appreciably dissociate from any of these solid matrices, even if the assay procedure takes several hours. Therefore, we postulate that more than one molecule of immobilized Protein A binds to IgG, or that there is an additional interaction between the antibody and the polymer surface.

Additional Keyphrases: progesterone · polystyrene · solid phase for immunoassays · immobilized antibody · steroids · mechanics of solid-phase binding

Solid-phase assays that involve binding proteins or ligands immobilized on test tube surfaces or similar matrices can substantially improve the simplicity, cost effectiveness, and speed of performance of immunoassays. However, major obstacles keep solid-phase assay from attaining the same quality criteria as liquid-phase assays, including the reproducible and uniform immobilization of ligands, the integrity of adsorbed proteins, and the unpaired interaction between the binding protein and the analyte. For example, enzymes undergo conformational changes in tertiary structure as a result of immobilization on polymer surfaces (7). Also, the physical properties of solid matrices, potentially affected by miniscule variations in the manufacturing process, can result in substantial variations in immobilization of ligands. We have reported variations in immobilized proteins that follow predictable patterns (2) and originate from a different physical microstructure of polystyrene surfaces. Also well known is the "edge effect" in microtiter plates. Several accounts of the variations in directly immobilized proteins (3–7) now make it a common practice in many laboratories not to use the peripheral wells of microtiter plates for immobilization.

In the preceding paper (2) we compared the performance in liquid- and solid-phase assays of two monoclonal antibodies (BQ.1 and BQ.2) to the steroid hormone progesterone. The superior performance of solid-phase assays for quantitative analysis depends on uniform, reproducible, stable, and sterically accessible immobilization of immunoglobulins.

Here we describe optimal conditions for meeting these criteria, which involve immobilization of monoclonal antibodies via Protein A.

Materials and Methods

Immobilization of Protein A. For immobilization on polystyrene surfaces, we dissolved soluble Protein A from Staphylococcus aureus (Miles Labs., Elkhart, IN) either in phosphate buffer (50 mmol/L, pH 7.4) or in carbonate buffer (50 mmol/L, pH 9.6), in various concentrations. We then incubated 1-mL aliquots of these solutions in 12 × 75 mm polystyrene test tubes (VSR Scientific Inc., San Francisco, CA), 500-μL aliquots in Macrowells™ (Skatron Inc., Sterling, VA), or 200-μL aliquots in polystyrene microwell strips (Immulon II™, Dynatech Inc., Alexandria, VA) overnight at room temperature. The areas covered by these incubation volumes are: test tubes 448 mm², Macrowells 368 mm², and microwells 157 mm². After washing the tubes and wells five times with de-ionized water, and letting them air dry, we stored them with desiccant (anhydrous calcium sulfate; Hammond Drierite Co., Xenia, OH) at 4 °C until use. Under these conditions, the binding of Protein A to immunoglobulins was undiminished in the treated tubes and wells for as long as one year.

In experiments to study the stability of Protein A bound to the solid surface, we diluted radiolabeled Protein A (125I-PA) with unlabeled Protein A before immobilization to various concentrations. We prepared the radiolabeled protein with carrier-free 125I (New England Nuclear, Boston, MA) by the Chloramine-T method (9), separating the free iodide from radiolabeled protein by size-exclusion chromatography on polyacrylamide gel (P-30; Bio-Rad, Richmond, CA). The labeled Protein A (spec. acty. 45 Ci/g; 0.9 iodine atom per molecule of Protein A) was used within two weeks.

Binding of Immunoglobulins. Immunoglobulins from peritoneal ascites fluid were precipitated twice with saturated ammonium sulfate solution (pH 7.4), dialyzed, and further purified by ion-exchange chromatography on a matrix of diethylaminoethyl-Sephadex (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) with a gradient of increasing phosphate buffer concentration. Lyophilized antibody was reconstituted in 10 mmol/L phosphate buffer, pH 7.4, containing 9 g of sodium chloride and 1 g of gelatin per liter. The same volume aliquots as above (1 mL, 500 μL, or 200 μL, respectively) were incubated overnight at room temperature in Protein A-coated carriers (tubes, Macrowells, or microtiter strips). We washed the carriers five times with de-ionized water, air dried them, and stored them in the presence of desiccant at 4 °C until use. Tubes and wells retained 95% binding activity of progesterone for more than a year.

Radiolabeled antibody was prepared as described above for Protein A and purified on polyacrylamide gel (P-100; Bio-Rad). The specific activity was 13 Ci/g, 0.9 iodine atom per molecule of immunoglobulin.

Immunoassays. Test tubes, Macrowells, and microwells were coated with Protein A and incubated with immuno-
globulin as described above. Immunoassays were performed with iodinated progesterone 11α-hemisuccinyl tyrosine methylester as reported elsewhere (2), with simultaneous incubation of radiolabeled derivative (25 000 counts/min; 8.6 pg) and samples. The total volumes used for incubations were 200 μL (microtiter wells), 500 μL (Macrowells), and 1 mL (test tubes).

Samples for these immunoassays were from rat granulosa cells grown in 96- and 24-well plates (Costar, Cambridge, MA). Ovarian follicular cells from 23-day-old rats that had been pretreated with estrogens were harvested and incubated for 48 h in DME-F12 medium (1) with 20 ng of ovine follicitropin (follicle-stimulating hormone) per millilitre, then treated with human chorionic gonadotropin to stimulate progesterone synthesis. The model system for granulosa cell differentiation has been described previously (10, 11). We directly transferred the medium (5–50 μL) either with an eight-channel or a four-channel multiple pipettor (Flow Laboratories, McLean, VA) into the coated microwells and Macrowells, or with single-tip pipettors into the test tubes.

Results

Dissociation of Radiolabeled Protein A and Immunoglobulin

Binding of iodinated Protein A to polystyrene and subsequent "washing off" revealed that some of the protein is not firmly bound to the polymer surface (Figure 1, upper panel). This portion is easily removed during three brief washes with either de-ionized water or with phosphate-buffered isotonic saline. However, the portion remaining does not readily dissociate from the surface, even after several hours of incubation with medium. When a 1 mg/L solution of Protein A was incubated, 30% of the Protein A, expressed in mass per square millimeter, was bound to all carriers after incubation and, of this portion, 40% remained firmly bound on all surfaces.

Immunoglobulins bind to immobilized Protein A rather firmly (Figure 1, lower panel). If incubated with buffer, only 5% of radiolabeled antibody BQ.1 dissociated from the polystyrene surface in 6 h. This is also reflected in slopes of those immunoassay standard curves that change only minimally with different incubation intervals. In the present instance, the slopes of curves obtained after incubation for 90 min are almost identical to those obtained after 18 h (2). The differences in slopes between 15- and 90-min incubation, albeit very similar (Figure 2), might be attributed to incomplete equilibrium between the antibody and the two antigens (progesterone and iodinated progesterone derivative), which do not have the same binding affinity for IgG.

Binding of Protein A

Optimal conditions for binding immunoglobulins via immobilized Protein A depend on four factors: (a) the solid matrix, (b) the concentration of Protein A, (c) the pH of the medium containing Protein A, and (d) the concentration of immunoglobulin bound to immobilized Protein A. Figure 3 shows immunoglobulin BQ.1 bound on Immunon II wells pretreated with different concentrations of Protein A in pH 9.6 carbonate buffer. At concentrations >5 mg/L, Protein A does not increase the binding of IgG. However, we could not determine from this experiment whether the amount of Protein A on the surface had reached saturation and was thus rate limiting for subsequent binding of IgG or whether, instead, the amount of antibody (2.6 fmol per well) was limiting. Therefore, we performed a series of experiments to investigate the interaction between different concentrations of Protein A and IgG. To do this, we prepared polystyrene tubes, Macrowells, and microwells but examined four variables: the solid matrices, the amount of Protein A used for immobilization, the pH for coating, and the concentration of IgG.

Test tubes (12 × 75 mm). Figure 4 (upper panel) shows our results for test tubes coated with Protein A at pH 9.6 in
conditions result from partial denaturation of the protein, because its conformation remains intact over a pH range of 0.99 to 11.8 (12).

We prepared test tubes for immunoassays in two different ways, according to their final purpose. To obtain a lower detection limit in the assay system, we treated the tubes for the immunoassays with 0.5 mg of Protein A per liter in pH 9.6 carbonate buffer. The tubes were subsequently incubated with 2 pmol of antibody BQ.1 per liter. However, when samples contain high concentrations of progesterone, a higher detection limit of the assay is required. In this case, the antibody can be incubated in 10- to 20-fold higher concentrations.

The optimal concentration for antibody BQ.2 to bind about 30% of radiolabeled tracer \(B/T\) was 10 pmol/L. The overall pattern for binding this antibody was similar to that described for antibody BQ.1.

**Macrowells.** The profile of bound radiolabeled progesterone, as related to interactions of matrix, Protein A, antibody BQ.1, and antigen interaction, was similar for Macrowells and test tubes. Less antigen was bound at higher concentrations of Protein A, with the decrease starting at 5 mg/L. For immunoassays, we used 1 mg of Protein A per liter, in pH 9.6 carbonate buffer. As with the other matrices, the antibody BQ.2 showed a similar pattern of binding to Macrowells coated with various concentrations of Protein A.

**Microwells.** Unlike the case with polystyrene test tubes, the acidity of the medium for Protein A during immobilization on Immulon II microwells did not change the binding profile of the antibody BQ.1 with its antigen; moreover, this was independent of the antibody, as shown for BQ.2 (Figure 5). The profiles of antigen binding for the two antibodies were similar, although the increase in binding at higher concentrations of Protein A was more pronounced for BQ.2.

**Immonoassays**

The concentration of progesterone in media incubated with granulosa cells correlated well when measured by RIA and by solid-phase assay (Figure 6). If the cells were cultured in 96-well plates, aliquots of the medium could be transferred directly into assay microwells or Macrowells.

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**Fig. 3.** Binding of radiolabeled progesterone derivative to monoclonal antibody BQ.1 (2.6 fmol per well) in the presence of various concentrations of Protein A immobilized at pH 9.6 on Immulon II wells

**Fig. 4.** Ratio of bound to total radiolabeled progesterone derivative \((B/T)\) as a function of different concentrations of immobilized Protein A \((x\text{-axis})\) and different concentrations of monoclonal antibody BQ.1 \((y\text{-axis})\)

- Upper panel: Protein A in pH 9.6 carbonate buffer, was coated on polystyrene test tubes.
- Lower panel: Protein A in phosphate-buffered saline was coated on polystyrene test tubes at pH 7.0

Carbonate buffer. Use of more IgG did not always result in higher binding (cf. 15 and 30 pmol/L). The decrease in binding at higher concentrations of Protein A, regardless of the IgG concentration, is noteworthy.

Immobilization of Protein A at pH 7.4 produces a different profile for bound antibody (Figure 4, lower panel). Binding of IgG at 3 and 15 pmol/L is generally lower at this pH than at pH 9.6. However, higher concentrations of Protein A at pH 7.4 similarly diminish the binding of IgG. It is unlikely that differences in binding of Protein A under alkaline conditions result from partial denaturation of the protein, because its conformation remains intact over a pH range of 0.99 to 11.8 (12).

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**Fig. 5.** Ratio of bound to total radiolabeled progesterone derivative \((B/T)\) as a function of different concentrations of immobilized Protein A \((x\text{-axis})\) and different concentrations of monoclonal antibody \((y\text{-axis})\):

- Upper panels: Protein A was coated in carbonate buffer, pH 9.6, on Immulon II strips.
- Lower panels: Protein A in phosphate-buffered saline was coated on Immulon II strips at pH 7.0
Fig. 6. Correlation between values for progesterone concentrations in luteinized rat granulosa cells, as determined by RIA and solid-phase assays
Dashed lines: 90% confidence interval of the regression line

We typically assayed samples in triplicate. The solid-phase assay was three to five times faster than the RIA. Microwells and Macrowells were broken apart and counted in carrier tubes, which were re-used, except for the wells with total counts.

The average coefficient of variation of results for samples at a concentration that inhibited the binding of radiolabeled progesterone derivative by 40% to 60%, repeatedly measured within one assay (intra-assay variation, n = 12), was 8.9% for the solid-phase assay vs 6.7% for the conventional liquid RIA. The respective interassay CVs were 15.7% and 12.8%. Nonspecific binding of radiolabeled progesterone derivative was between 3% and 5% of the total amount of tracer. We did not find a significant difference in nonspecific binding between carriers, or between untreated carriers and Protein A-coated carriers.

Discussion

To overcome some of the limitations of solid-phase assays, modification of the matrix with specific binding proteins for more controlled binding of immunoglobulins appears to be of great advantage. An alternative to Protein A as a binding protein is the initial coat with a "second antibody" (anti-IgG immunoglobulin) for modification of the solid matrix. Because second antibodies are polyclonal, their composition is neither consistent from animal to animal nor from bleeding to bleeding; therefore, nonspecific binding to the solid matrix and specific binding to the "first antibody" might vary and would need to be investigated from batch to batch for optimal and predictable assay conditions. In contrast, Protein A, a uniform molecule that binds specifically to the Fc region of immunoglobulins, allows systematic studies of interactions on the solid matrix. Nevertheless, we believe the results of this investigation with Protein A as "modifier" for the solid matrix can be extended to the use of second antibodies for refinement of surfaces.

Direct immobilization of antibodies to surfaces limits the usefulness of solid-phase assays for at least three reasons:

* Variations in the physical structure of surfaces are extended to the analytical system. This requires, for example, careful selection of batches of microtiter plates in immunosorbent assays (13) and can cause concern regarding quality control and continuity of analytical procedures when a particular batch has all been used.

* Lateral surface interaction of the adsorbed immunoglobulin distorts the molecule (14), which might affect the idiotypic site of the antibody.

* Interaction of lipophilic domains of the immunoglobulin with the solid matrix might either include the antigen binding site directly or contribute to steric hindrance for optimal antigen access and (or) binding.

By comparing the antigen binding to monoclonal antibodies directly immobilized on polystyrene vs those immobilized via Protein A, we have shown substantially higher consistency in reproducible binding with the latter method (2). That the immunoglobulin binds better to its antigen when immobilized via another binding protein is not unexpected. Studies by x-ray crystallography indicate that Protein A binds to the Cg-region of IgG—i.e., distal to the antigen binding site (15). With the Protein A system, "ends-on" immobilization (16) seems to be the preferred configuration. Thus, the idiotypic region of IgG appears to be more favorably exposed at the solid/liquid interface. This is also reflected in the amount of antibody required to obtain binding. For direct immobilization of the monoclonal antibodies described here, 20- to 50-fold higher concentrations of IgG were necessary to bind the same amount of progesterone derivative. These results, together with the high variation in binding and the poor performance in Scatchard analysis, suggests that many idiotypic sites of directly immobilized IgGs are not available for antigen binding.

Protein A contains five distinct regions, four of them highly homologous in amino acid structure and capable of binding IgGs (17, 18), although the molecule is considered functionally bivalent (19). The fifth region is anchored in the phospholipid bilayer of Staphylococcus aureus. The composition of this region is about 27% lipophilic amino acids; most probably the molecule strongly adheres with this region to the hydrophobic surface of polystyrene, leaving free the binding regions that would interact with IgG.

There is an optimum concentration of Protein A for maximum binding of antibody. That is, greater concentrations of Protein A do not necessarily result in higher immunoglobulin concentrations on the surface. Protein A does not appear to be immobilized onto the solid matrix at the greatest possible density of a monomolecular layer. Because Protein A is a relatively elongated molecule (20), it is difficult to predict the exact surface area it would occupy on a solid matrix. Assuming that the Stokes' radius, 5 nm (21), would represent the midsection of a sphere, we can calculate the surface area occupied by Protein A (A = π × r²) as 78.5 mm². In a dense packing, the protein would occupy the surface at 21.2 fmol/mm² (about 1 ng/mm²). Our coating experiments with radiolabeled Protein A indicated that no more than a third of this amount was firmly bound to the surface, whether the binding of IgG had started to reach asymptotically maximal values (Figure 5) or had just started to decrease (Figure 4). These calculations probably overestimate the surface area that Protein A occupies, because the molecule is not spherical. Thus, there apparently are "empty spaces" between the Protein A molecules.

We postulate that the spacing of Protein A contributes to a thermodynamically favored complex involving the surface, Protein A, and IgG. In this complex, each IgG molecule does not interact with only one Protein A molecule; rather, it binds simultaneously either to the surface or to another molecule of Protein A (Figure 7). This hypothesis is supported by the stability of the protein A–IgG complex, which does not dissociate appreciably within several hours (Figure 1). We cannot conclude from these experiments, however, whether IgG binds simultaneously to Protein A and the surface, to more than one Protein A molecule, or to more
than one Protein A molecule and the surface. In support of the model of cooperative interaction between the two binding proteins and the solid matrix are our previous experiments, in which titration curves indicated an optimum ratio of immobilized Protein A and firmly bound antibody (2). Titrations curves with increasing amounts of antibody showed a "hook effect" in the solid phase at higher concentrations of IgG, contrary to liquid-phase assays.

Currently, an attempt to explain the different binding of antigen (B/T) with increasing amount of Protein A for test tubes and Macrowells vs microtiter strips (Figures 4 and 5) must remain speculative. Although all materials consisted of polystyrene, surface binding varied substantially. Immuno II microtiter strips are treated, according to the manufacturer, by a proprietary method of a physical nature to enhance binding of proteins. However, maximum adsorption seems to depend on the protein structure and can be greater on nontreated polystyrene surfaces (13).

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References