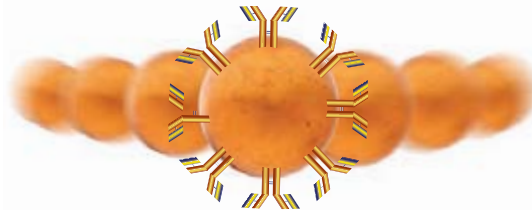


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B E A D S • A B O V E T H E R E S T™



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I. Overview of Adsorption

There are currently several means of attaching biological ligands to the microspheres used as solid phase supports in immunological tests and assays, including adsorption to plain polymeric microspheres, covalent attachment to surface functionalized microspheres (see TechNote 205), and attachment to microspheres that are pre-coated with a generic binding protein, such as streptavidin or protein A (see TechNote 101).

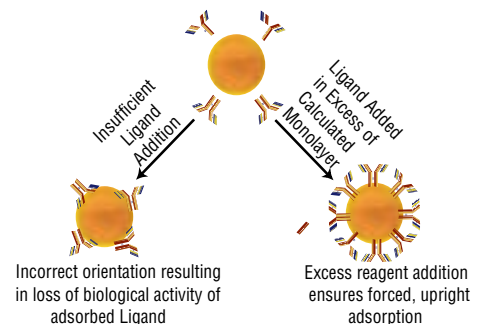
The original method for attachment of proteins to hydrophobic microspheres was passive adsorption. Because of the simplicity and flexibility of this method, it is still widely used today.

The following information explains some of the variables involved in establishing a working adsorption protocol, so that the generalized protocol listed can be easily optimized to your specific

application.

The mechanism for adsorption is based primarily on hydrophobic (Van der Waals, London Type) attractions between the hydrophobic portions of the adsorbed ligands and the polymeric surface of the microspheres. This is the means of attachment for most hydrophobic ligands, including immunoglobulins. In the case of less hydrophobic ligands (or more hydrophilic microspheres, such as -COOH modified), attachment via both ionic interactions and hydrophobic interactions can take place. Human serum albumin and hemoglobin are examples of such ligands. The importance of this is that ligands, whose attachment is due in part to ionic interactions, are affected by the conditions of the environment in which they are suspended, and pH changes are more likely to result in desorption than if the attachment was solely via hydrophobic interactions. For this reason, we generally recommend that the pH of the adsorption buffer be kept at or near the pl of the protein.

In the case of antibodies, the Fc portion of the protein is generally more hydrophobic, and therefore more likely to be adsorbed, than the Fab region (helping to ensure that the proteins are bound in their most biologically active orientation). It is possible, however, that antibodies can be bound in a less than optimal orientation, and this can be prevented by adding a large excess of ligand, to ensure crowded, upright adsorption of the protein.



Adsorption of hydrophobic ligands to polymeric microspheres can be carried out in two ways:

1. *Passive Adsorption*: Adsorption in which the ligand of interest is attached to the microspheres simply by incubating the two together for a fixed amount of time. Because impurities will compete with ligand for space on the particle surface, both through relative affinity and relative concentration effects, maximum ligand adsorption requires the use of ultrapure reagents. If the concentration of an impurity is very high, it could become the principal coating.

2. *Forced Adsorption*: Adsorption in which a precipitating agent is used to "force-precipitate" the ligand onto the microsphere surface, thus obviating the need for highly purified ligand.¹

A. Effect of Different Polymers on Adsorption Efficiency

Adsorption of large ligands to hydrophobic surfaces has been found to be essentially irreversible to dilution in the same buffer used for attachment. However, adsorbed ligands can be displaced by competing molecules, such as proteins or surfactants. The composition of the hydrophobic polymer influences the ability of an adsorbed ligand to be displaced.³ The order of displacement from the polymeric microspheres (in the order of easiest to hardest to displace) is:

PS / PAA > PMMA > PS / PMMA > PS,

where PS / PAA = copolymer of styrene and acrylic acid - the composition of most carboxyl modified microspheres,
 PMMA = polymethyl methacrylate,
 PS / PMMA = copolymer of styrene and methyl methacrylate, and
 PS = pure polystyrene.

It has also been shown that the ease of displacement of proteins, in the presence of competing proteins in solution, is a function of the composition of the protein. Three representative proteins were tested for their ease of displacement on each of the polymers listed above, and the order of displacement (in the order of easiest to hardest to displace) from each polymer was:

Fibrinogen > Immunoglobulin > Albumin

An adsorbed monoclonal antibody retains more biological activity on the copolymers listed above than on pure polystyrene.³ This illustrates the direct correlation between the ease of displacement of adsorbed proteins and retention of activity. The structural changes in the adsorbed protein leading toward decreased ease of displacement also potentially lead to decreased activity. Stated differently, any opportunity for the protein to interact with a hydrophobic surface is likely to result in some relaxation of its folded structure, with a gain in entropy, as it exchanges intramolecular hydrophobic interactions for similar bonds with the surface, and

the hydrophobicity of the polymer will determine the extent of this structural relaxation. Thus, the degree of displacement of a readily available protein should be a useful tool in determining the ability of a given polymeric surface to cause substantial structural rearrangement of adsorbed proteins.

B. Effect of Different Proteins on Adsorption Efficiency

Studies have demonstrated the adsorption efficiency of various proteins on polystyrene surfaces. The point of these studies is that by knowing the adsorption efficiencies of various proteins, protocols can be developed that allow for adsorption of a mixture of proteins. This comes into play when developing solid-phase immunoassays for the detection of not only pure proteins, but also protein mixtures.

One such study² defines the *region of independence* as an important variable to consider in developing an adsorption protocol using a mixture of proteins. The region of independence can be defined as the range of concentrations of added protein above which surface saturation is reached, and further binding becomes negligible. By staying within this region of independence for the overall concentration of added protein, you help to ensure that the maximum binding efficiency for each protein can be reached. Finding this region of independence is based on both the size and, to a small extent, the charge of the proteins to be adsorbed, as well as the surface area of the microspheres to which they will adsorb. Section D lists calculations for two representative proteins, as well as for a number of sizes of microspheres that are often used in such solid-phase tests and assays.

C. Calculation of Microsphere/Protein Ratio to Achieve Surface Saturation

Most adsorption applications start with a monolayer of protein bound to the microspheres. In order to ensure the correct spatial orientation and decrease the likelihood of nonspecific binding, we recommend adding protein in a 3-10X excess of the calculated monolayer. However, some applications, such as latex agglutination tests, seem to work best with less than a monolayer of coverage. This monolayer amount can be derived from the following equation:

$$S = (6 / \rho D)(C),$$

where S = amount of representative protein needed to achieve surface saturation (mg protein/g of microspheres),
 C = capacity of microsphere surface for given protein, which will vary depending on the size and molecular weight of the protein to be coupled (mg protein/m² of polymer surface),
 6 / ρD = surface area/mass (m²/g) for microspheres of a given diameter (ρ = density of microspheres, which for polystyrene is 1.05 g/cm³), and
 D = diameter of microspheres, in microns.

Data is available for bovine serum albumin (BSA, MW 65kD) and bovine IgG (BlgG, MW 150kD). By comparing the MW of your ligand to that of BSA and IgG, surface saturation of other ligands can be approximated. We base our calculations, as well as the reagent volumes listed in the adsorption protocol, on microspheres with a mean diameter of 1.0 μ m. Therefore, the calculation is carried out as follows:²

For BSA: C ~ 3 mg/m², so:

$$\begin{aligned} S &= (6 / \rho D)(C) \\ &= (6 / 1.05 \text{ g/cm}^3 \cdot 1.0\mu\text{m})(3 \text{ mg/m}^2) \\ &\sim 18 \text{ mg of BSA to saturate 1 gram of } 1\mu\text{m} \\ &\text{ polystyrene-based microspheres.} \end{aligned}$$

For BlgG: C ~ 2.5 mg/m², so:

$$\begin{aligned} S &= (6 / \rho D)(C) \\ &= (6 / 1.05 \text{ g/cm}^3 \cdot 1.0\mu\text{m})(2.5 \text{ mg/m}^2) \\ &\sim 15 \text{ mg of BlgG to saturate 1 gram of } 1\mu\text{m} \\ &\text{ polystyrene-based microspheres.} \end{aligned}$$

Table 1: Surface Saturation Values

Particle Diameter (microns)	BSA Monolayer (mg BSA/g beads)	BlgG Monolayer (mg BlgG/g beads)
0.1	171.4	142.8
0.2	85.7	71.4
0.3	57.1	47.6
0.4	42.9	35.7
0.5	34.3	28.6
0.6	28.6	23.8
0.7	24.5	20.4
0.8	21.4	17.9
0.9	19.0	15.9
1.0	17.1	14.3
1.5	11.4	9.5
2.0	8.6	7.1

Note: For non-agglutination tests, if the coating is antigen, to which the antibody will bind, most often the more antigen on the solid phase, the better. If the coating is IgG antibody (Ab), then the antigen to be subsequently bound must have room to access the binding site of the solid-phase IgG, and you may not need complete coverage. Some experienced users report using only enough Ab to cover about half the particles' surfaces as ideal for latex agglutination tests.

II. Conversions

Some conversions and physical constants are often helpful when modifying the following protocols for varying sizes, types, and concentrations of microspheres.

Table 2: Conversions

Microsphere % Solids (By weight)

10% solids	~0.1 g/mL = 100 mg/mL
1% solids	~0.01 g/mL = 10 mg/mL
0.1% solids	~0.001 g/mL = 1 mg/mL

Density of Typical Polymers

Polystyrene	1.05 g/cm ³
Polymethyl methacrylate	1.19 g/cm ³

Temperature

Celsius = 5 / 9 (F - 32)
Fahrenheit = 9C / 5 + 32

Mass / Linearity Conversions

10 ⁻⁹ = nano-
10 ⁻⁶ = micro-
10 ⁻³ = milli-

Centrifugation Table (for a standard benchtop centrifuge)

Microsphere Size	Centrifugal Force (G)	Time
300-500nm	9,300	15 minutes
500-800nm	2,200	15 minutes
800nm and up	1,200	15 minutes

III. Buffers

Following are some basic recipes for buffers commonly used in adsorption protocols. Generally, maximal adsorption occurs at or near the pI of the protein, so the choice of buffer should be made accordingly. Additionally, many researchers have reported that the addition of NaCl to the coupling buffer, in physiological concentrations of about 0.15 M, increases adsorption efficiency. This information is intended only as a general guideline. Feel free to substitute buffers and/or adjust concentrations as your application demands.

1. Phosphate Buffered Saline (PBS); pH 7.4

- Potassium Phosphate dibasic: 1.82 g/L (MW 174.2)
- Sodium Phosphate monobasic: 0.22 g/L (MW 120.0)
- Sodium Chloride: 8.76 g/L (MW 58.4)

- Bring to a final volume of 1L using deionized water. Adjust pH to 7.4 using either 1 N HCl or 1 N NaOH.

2. Borate Buffer; pH 8.5

- Boric Acid, H₃BO₃: 12.4 g/L (MW 61.8)
- Sodium Tetraborate: 19.1 g/L (MW 381.4)

- Add 50mL of (a) to 14.5mL of (b). Bring to final volume of 200mL using deionized water. Adjust final pH to 8.5 using 3 M NaOH solution.

3. Acetate Buffer; pH range 3.6 to 5.6

- 0.1 M Acetic acid: (5.8mL made to 1000mL)
- 0.1 M Sodium acetate: 8.2 g/L (anhydrous; MW 82.0)

- Mix acetic acid and sodium acetate solutions in the proportions indicated and adjust the final volume to 100mL with deionized water. Adjust the final pH using 1N HCl or 1 N NaOH.

mL Acetic Acid	46.3	41.0	30.5	20.0	14.8	10.5	4.8
mL of Na Acetate	3.7	9.0	19.5	30.0	35.2	39.5	45.2
pH	3.6	4.0	4.4	4.8	5.0	5.2	5.6

4. Citrate-Phosphate Buffer; pH range 2.6 to 7.0

- 0.1 M Citric acid: 19.2 g/L (MW 192.1)
- 0.2 M Dibasic sodium phosphate: 35.6 g/L (dihydrate; MW 178.0)

- Mix citric acid and sodium phosphate solutions in the proportions indicated and adjust the final volume to 100mL with deionized water. Adjust the final pH using 1N HCl or 1 N NaOH.

mL Citric Acid	44.6	35.9	29.4	24.3	19.7	13.6	6.5
mL of Na Phosphate	5.4	14.1	20.6	25.7	30.3	36.4	43.6
pH	2.6	3.4	4.2	5.0	5.8	6.6	7.0

5. Carbonate-Bicarbonate Buffer; pH range 9.2 to 10.4

- 0.1 M Sodium carbonate: 10.6 g/L (anhydrous; MW 106.0)
- 0.1 M Sodium bicarbonate: 8.4 g/L (MW 84.0)

- Mix sodium carbonate and sodium bicarbonate solutions in the proportions indicated and adjust the final volume to 200mL with deionized water. Adjust the final pH using 1 N HCl or 1 N NaOH.

mL Na Carbonate	4.0	9.5	16.0	22.0	27.5	33.0	38.5
mL of Na Bicarbonate	46.0	40.5	34.0	28.0	22.5	17.0	11.5
pH	9.2	9.4	9.6	9.8	10.0	10.2	10.4

Note: Small concentrations of antimicrobial agents (0.05-0.1% w/v), such as sodium azide or merthiolate, are often added to the storage buffer, especially for long-term storage.

IV. Blockers

Blockers can be added to the storage buffer in varying amounts, a standard concentration being 0.05% (w/v). Using a substance dissolved in the storage buffer that will block the exposed hydrophobic surfaces of the polymeric microspheres will reduce

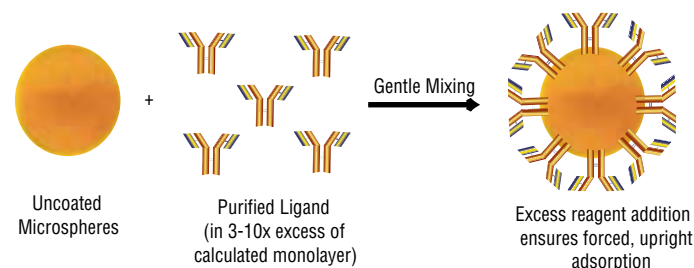
nonspecific binding and self-aggregation of the microspheres. A separate incubation in a higher concentration of blocker (up to 0.1%) is also recommended before storage, in order to saturate the exposed hydrophobic surfaces of the microspheres. Some commonly used blockers are as follows:

- BSA (Bovine Serum Albumin):* Often used alone, but can be combined with other blockers, most commonly surfactants.
- Casein:* A milk-based protein, containing indigenous biotin, which should be avoided when working with systems involving biotin to prevent interference.
- Pepticase (Casein Enzymatic Hydrolysate):* An enzymatic derivative of casein, which should also be avoided when working with systems involving biotin.
- Non-Ionic Surfactants:* Tween® 20 and Triton X-100 are typical. When used in combination with another blocker, a common ratio is 1% Blocker; 0.05% Surfactant.
- “Irrelevant” IgG:* Often used when conjugating a specific IgG to microspheres. For example, if coupling mouse IgG, rabbit (or any non-cross reacting IgG) can be adsorbed as a blocker.
- FSG (Fish Skin Gelatin):* Pure gelatin or gelatin hydrolysate can also be used.
- Polyethylene Glycol:* A very versatile blocker, available in a number of sizes, configurations, and charges.
- Sera:* Non-cross-reacting serums, such as horse or fish serum, are very inert in terms of cross-reactivity with various types of antibodies.
- Commercial Blockers:* Many companies offer preparations which are a composite of 2 or more single blocking substances of various molecular weights, and which can be used effectively over a wide range of conditions. These go under various trade names, and most chemical vendors will offer a variety of these.

There are many others, and we suggest experimenting with various blocker concentrations and combinations when optimizing your application.

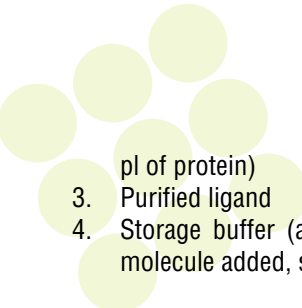
V. Procedures

A. Passive Adsorption



Reagents:

- Polymeric microspheres (often supplied at 10% solids)
- Adsorption buffer (low ionic strength buffer of pH at or near

- 
3. Purified ligand
 4. Storage buffer (adsorption buffer with 0.01-0.1% blocking molecule added, see Section III)

Procedure:

1. Dilute the microspheres to 1% solids (10 mg/mL) with adsorption buffer. *Note:* Although surfactants or detergents, in which microspheres are normally shipped, may sometimes interfere with binding, it is often not necessary to clean the microspheres prior to use. If cleaning is desired, this can be done by techniques like centrifugation, dialysis, or ion exchange as described in our TechNote 203.
2. Dissolve appropriate amount of purified ligand (as determined by calculations is Section 1.C) in adsorption buffer.
3. Add the microsphere suspension to the appropriate volume of dissolved protein, and mix gently for 1-2 hours. *Note:* By adding microspheres to protein, rather than protein to microspheres, efficiency is maximized and even distribution of adsorption is more likely.
4. Incubate suspension overnight at 4°C, with constant mixing. *Note:* Although the vast majority of ligand adsorption occurs very rapidly, the extended incubation seems to aid in achieving correct orientation by allowing an equilibrium to be reached. Other options are to incubate at room temperature for 1-2 hours, or at 37°C for 15-30 minutes (in cases where the ligand will not be adversely affected by the elevated temperatures).
5. Centrifuge, remove supernatant, and resuspend microsphere pellet in storage buffer to desired storage concentration (often 10 mg/mL). A separate blocking step may be added here, if necessary. *Note:* Supernatant can be saved to determine the amount of free protein, from which the amount of adsorbed protein can be indirectly quantified. A common assay to determine the amount of free protein in solution is the BCA assay (Pierce Chemical Company). A more crude measurement can be made by measuring the A_{280} of the supernatant on a spectrophotometer (Section V.B).

B. Absorbance at 280nm (A_{280})⁴

Equipment:

1. Spectrophotometer (equipped for UV reading)
2. Matched quartz cuvettes
3. Pasteur pipettes and pipette bulbs for solution transfer

Reagents:

1. Adsorption buffer (for blank)
2. Supernatant (from Procedure A)

Procedure:

Single Beam Spectrophotometer

1. With no cuvette present in instrument, set A_{280} to zero.
2. With adsorption buffer in cuvette, read A_{280} , then reset to zero. (This step determines whether the adsorption buffer has a significant absorbance.)

3. Remove buffer and add supernatant to cuvette, then record absorbance.

Dual Beam Spectrophotometer

1. With matched, empty cuvettes in machine, set instrument to zero.
2. Add adsorption buffer to sample cuvette, leave reference cuvette empty. Record absorbance. (This step determines whether the adsorption buffer has a significant absorbance.)
3. Remove buffer from sample cuvette. Add supernatant and adsorption buffer to sample and reference cuvettes, respectively, then record absorbance.

Comments:

1. It is a common laboratory shortcut (although a very imprecise one) to assume that an absorbance of 1.0 in a 1cm cuvette roughly approximates 1 mg/mL of protein. For comparison, measured A_{280} values of a sampling of proteins at 1 mg/mL follow:

<u>Protein</u>	<u>A_{280} (1 mg/mL)</u>
Bovine Serum Albumin	0.70
Ovalbumin	0.79
γ -Globulin	1.38
Trypsin	1.60
Chymotrypsin	2.02
α -Amylase	2.42

2. If absorbance is off scale, the sample can be diluted with buffer and the assay repeated. Alternatively, a cuvette with a shorter path length may be used.
3. Glass or plastic cuvettes absorb light in the UV range and should not be used for this assay.

VI. Covalent Coupling to Non-Functionalized Polymeric Microspheres

Although adsorption of hydrophobic ligands to polymeric microspheres is advantageous in many situations, there are times when the hydrophobic attractive forces may not be strong enough to resist the incubation and wash steps included in many assay procedures. In other cases, the antibody in question might not be able to be adsorbed and still retain its immunoreactivity. One answer to such situations is to modify the surface of the microspheres so that covalent coupling becomes an option. Following are approaches that can be taken for such modification.

1. A practical and reproducible coating method for plain polystyrene involves the adsorption of poly-phenylalanine-lysine to polystyrene microspheres, followed by activation with pentane 1, 5-dial and coupling of the required ligand.⁵ The poly phe-lys fulfills two important requisites. First, the strong hydrophobic interactions between the phenylalanine residues and the polystyrene surface create an essentially

irreversible adsorption. Secondly, the introduction of 'reactive' amino groups offers a means of covalent attachment via standardized chemistries (see TechNote 205).

2. It is possible to directly derivatize, and covalently couple ligands to, non-functionalized polystyrene microspheres. References and a brief explanation for derivatizing both polystyrene and polymethyl methacrylate microspheres can also be found in our TechNote 205. Many other surface functionalized microspheres are available for easy covalent coupling.

VII. Troubleshooting

Problem: I can't get my protein to adsorb.

- Solution:
- a. Add more ligand (a more concentrated solution).
 - b. Remove some surfactant to make room on the surface for ligand adsorption.
 - c. Pre-coat the microspheres with an intermediate material, which will stick well to the particles and to which the ligand can adsorb.
 - d. Use a different buffer (different pH, ionic strength, etc.).

Problem: There is plenty of ligand bound, but it is not active (incorrect orientation or packed too closely).

- Solution:
- a. Try more or less ligand. (Orientation of adsorbed molecules is often strongly influenced by steric factors.)
 - b. Use a 'surface diluent' - another biochemical which will co-adsorb onto the surface to prevent ligand molecules from getting too close together.

Problem: After cleaning to remove unbound ligand, the microspheres clump.

- Solution:
- a. Try to put more ligand on the surface.
 - b. Add a blocker, such as BSA or a non-ionic surfactant, to stabilize the suspension.

Problem: Ligand adsorption is initially optimal, but after extended storage, desorption has occurred.

- Solution:
- a. Reduce storage temperature (if stored at room temperature) to 2-8°C.
 - b. Lower the concentration of blocker in the storage buffer.
 - c. Verify that the storage buffer does not contain impurities that could compete with bound protein and cause desorption over time.

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