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Microsphere Specialists

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Tech. Note #13a

ADSORPTION PROTOCOLS

*****This is a collection of recipes or methods for coating polymeric microspheres which have not been tested by us. Feel free to substitute other buffers or make changes according to your judgment.

I. General Considerations

A. Protein

Adsorption of proteins on latex surfaces is due to hydrophobic binding forces (Van der Waals - London type) and thus should be independent of pH. Nevertheless, charge affects the conformation of the protein molecule and determines the amount of protein adsorbed on the latex surface. The formation of the most compact monolayer of proteins is generally maximal at or slightly above the isoelectric point of the protein. (For human IgG, pH $\approx$7.8)

Salt concentrations which will neutralize localized charges on a protein will also promote hydrophobic interactions. In this regard, an ionic strength of at least 0.1M should be used initially.

Raising the temperature during incubation will increase the diffusion rate and increase the probability of the protein molecules coming into contact with the surface of the solid phase within a given period of time. In practice, room temperature is sufficient. Higher temperatures can be used if the molecules being adsorbed are not heat labile.

Incubation time will vary depending on the protein and its concentration. In general, if a saturating level of protein is used, adsorption is rapid. Minutes may be enough to fill most sites, and one or two hours should suffice in most instances. If the protein is stable, overnight incubation can be used. Stirring is advised.

B. Microspheres

Microspheres are usually made and shipped with surfactant present. Although surfactants or detergents may sometimes interfere with binding, it is often not necessary to clean the microspheres prior to use. Ionic detergents (like SDS or carboxylic acid salts) can compete with proteins for binding sites. Nonionic detergents (Tween 20, Triton X-100) can depress protein binding, by hydrating the proteins and hindering hydrophobic binding. The microsphere suspension should ideally contain only the smallest amount of surfactant necessary to maintain its stability. This may mean removing some of the surfactant, e.g., by ion exchange, cross-flow filtration, dialysis, column chromatog-raphy, or centrifugation. For extreme dilution of latexes (less than 1% solid content), surfactant at a 0.01-0.1% concentration may be necessary (before protein coating). Note that in adaptation of the following protocols, centrifuge times and speeds must be varied according

to the size of the particles. Refer to Tech. Note #37 for tips on washing beads.

C. Ratio of Protein to Microspheres to be Used

We suggest a 3-10x excess of protein over that which can be accommodated by the PS surface to ensure crowded, upright adsorption.[1]

1. Achieving Surface Saturation
   - Polystyrene capacity for BSA ≈ 300 ng/cm^2 ≈ 0.30 μg/cm^2 ≈ 3 mg/m^2
     (BSA = bovine serum albumin)
   - Polystyrene capacity for BIgG ≈ 250 ng/cm^2 ≈ 0.25 μg/cm^2 ≈ 2.5 mg/m^2 ref.2

   - Surface Area/Mass for Particles = 6/ρD (m^2/g)
     where ρ = density of a particle (g/cm^3) (ρ = 1.05 g/cm^3 for polystyrene) and D is in μm.
     Then, if D = 1μm, A/M ≈ 6 m^2/g.

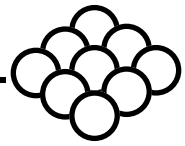
   - Therefore, one gram of 1μm diameter PS particles can adsorb
     ~6 m^2 x 3 mg/m^2 ≈ 18 mg of BSA or
     ~6 m^2 x 2.5 mg/m^2 ≈ 15 mg of BIgG

2. Calculations for Protein Coating of Polystyrene Microspheres

Particle Diameter (μm)	**Surface/Mass Ratio** (m2/g)	**Protein Monolayer Coating** (mg BSA/g beads)
0.1	57.1	171.4
0.2	28.6	85.7
0.3	19.0	57.1
0.4	14.3	42.9
0.5	11.4	34.3
0.6	9.5	28.6
0.7	8.2	24.5
0.8	7.1	21.4
0.9	6.3	19.0
1.0	5.7	17.1
1.5	3.8	11.4
2.0	2.9	8.6
	↓	↓
	6/(1.05 x d)	Assumes monolayer = ~3mg BSA/m^2

Note: For non-agglutination tests, if the coating is antigen, 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 that only enough Ab to cover half the particles' surfaces is ideal for latex agglutination tests.[3]

D. Microsphere Resuspension



To remove surfactant or to remove unbound protein after adsorption we recommend methods of cleaning like ion exchange or ultrafiltration. If you must use centrifugation to wash the particles, then please make certain by microscope or particle size instrument that particles are *completely* resuspended as single particles and redispersed after new buffer is added before proceeding to next steps. This can be done by rolling, sonication, aspiration through a fine pipet tip, vortex mixing, etc.

E. Use of "Surface Diluents"

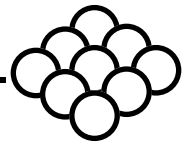
The use of a coadsorbent, surface diluent, blocker or filler is recommended in most protein adsorption or covalent coupling protocols. The following are some examples of the more common items used:

- BSA/ HSA/ ovalbumin
- BSA/Tween 20 in a 20:1 ratio (1% BSA + 0.05% Tween)
- Surfactants, especially non-ionics (Triton X-100, Tween 20, etc.)
- Polypeptides (e.g. Prionex, safer (?) alternative to BSA)
- "Irrelevant" or neutral IgG
- Gelatin / gelatin hydrolysate (enzymatic) or fish gelatin
- “heterophilic (antibody) blocking reagent” (Scantibodies)[4]
- Casein or non-fat dry milk
- KLH (keyhole limpet hemocyanin)
- Blotto (normal goat serum + non-fat dry milk)
- Fish Serum
- Super Block serum-free protein blocker (SLD)[5]
- Megga-block 3: “components of small molecular size” (Bionostics)[6]
- α1-acid glycoprotein
- Polyvinyl alcohol
- Polyethylene glycol

II. Adsorption Protocols

A. PBS/TPG Method— Enhanced adsorption can be achieved by the following treatment:

1. Reagent preparation
   a. PBS Coat/Wash Buffer:
      1.56g $NaH_2PO_4 \cdot H_2O$ (0.01M)
      9.00 g NaCl (0.9%)
      Add approximately 800 ml deionized water. Adjust pH to 7.8 with 1M NaOH. Dilute with deionized water to 1 liter.
   b. TPG Storage Buffer
      7.8 g $NaH_2PO_4 \cdot 2H_2O$ (0.05 M)
      1.0 g NaCl (0.1%)
      Add approximately 800 ml of deionized water. Adjust pH to 6.6 with NaOH. Add 2g Gelatin (dissolved in hot H_2O, while stirring). Dilute with deionized water to 1 liter.
   c. Microspheres: 10% solid content



Centrifuge the microspheres and discard the supernatant.
Wash twice with deionized water using centrifugation.
Re-suspend the particles in PBS Coat/Wash Buffer at a solids concentration of 20 mg/ml (2 wt %) and dialyze overnight against 100 volumes of PBS Coat/Wash Buffer.

d. Protein solution: Dilute purified IgG to 1mg/ml with PBS Coat/Wash buffer at pH 7.8.

2. Adsorption
   a. Place 0.1 ml of 10% solids polystyrene latex (or equivalent wt. of latex solids) in a glass tube.
   b. Add 1 ml protein solution, vortex.
   c. Gently shake for 30 min. at 56°C.
   d. Store overnight at 4°C.
   e. Centrifuge at 4°C
   f. Wash twice with 1 ml PBS Coat/Wash Buffer, centrifuge, discard supernatant.
   g. Resuspend in 1 ml TPG Storage Buffer to block remaining sites not covered by protein.

B. Borate/BSA Method

1. Reagent preparation
   a. Coat/Wash Buffer: 0.1M borate buffer, pH 8.5
   b. Blocking Buffer: 0.1M borate buffer, pH 8.5 + BSA (0.33 mg/ml)
   c. Final Wash Buffer: PBS, pH 7.2 + BSA (5 mg/ml)
   d. Storage Buffer: PBS, pH 7.2 + BSA (5 mg/ml) + 5% glycerol
2. Microsphere Preparation
   a. Start with 50 mg latex solids.
   b. Dilute to 6ml with Coat/Wash Buffer.
   c. Centrifuge/wash 4 times with same buffer.
3. Protein Coating
   a. Re-suspend pellet in 4ml Coat/Wash Buffer with 300µg Ab/ml.
   b. Rotate 16-20 hours at room temperature; centrifuge; discard supernatant. 4.

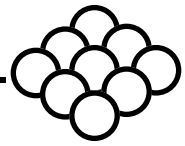
Blocking

   a Re-suspend in 6ml Blocking Buffer.
   b. Rotate 30 minutes at room temperature; centrifuge; discard supernatant.
   c. Centrifuge/wash 3 times in Final Wash Buffer.
5. Storage
   a. Re-suspend in Storage Buffer.
   b. Store at 4°C until used.

C. GBS/ HSA Method: Adsorption of IgG on Polystyrene

•This method provides a large excess of protein over that necessary for complete monolayer coverage.

1. Reagent Preparation
   a. Microspheres: 10% w/v (100ml), 0.3-0.5µm. Adjust protocol appropriately for other sizes. *Note that in this protocol, microspheres



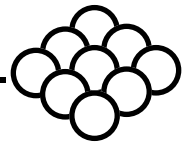
are used without prior cleaning.

b. Coat/Wash Buffer (4.5 liters): 0.15% w/v glycine in deionized water; adjust pH to ~9.2; add 0.2% w/v NaCl.
c. IgG Solution (30ml): 2% w/v in deionized H_2O; heat to 63°C for 30 minutes; cool to room temperature.
d. Blocking Buffer: HSA 2% w/v in Coat/Wash Buffer (300ml)
e. Storage Buffer (Glycine buffered saline (3 liters)): Deionized water + glycine, 0.75% w/v (adjust pH to 8.2) + NaCl, 1% w/v + BSA, 1%w/v + thimerosal 0.01%v/v (adjust pH to 8.2)

2. Protein Coating/Blocking
   a. Mix 800ml Coat/Wash Buffer + 30ml IgG solution (600mg lgG) + 300ml Blocking Buffer (6g HSA) + 100ml,10% solids microspheres (10g latex solids). *****Microspheres are the final ingredient to be added to the mixture.
   b. Stir 15 - 30 minutes to mix thoroughly. Allow to coat for 2 hours at room temperature.
3. Storage
   a. Centrifuge particles, decant supernatant, resuspend particles in 2 liters of Coat/Wash Buffer.
   b. Centrifuge particles, decant supernatant, resuspend particles in 2 liters of Storage Buffer (0.5% solids).

D. Sodium Phosphate/PBS/BSA Method[7]

1. Reagent Preparation
   a. Coat Buffer: 0.1M sodium phosphate buffer, pH 6.0 + 0.1 g/L monoclonal antibody.
   b. Blocking Buffer: PBS + 10 g/L BSA
   c. First Wash/Storage Buffer: PBS
   d. Second Wash Buffer: PBS + 1M glycine
   e. Microspheres: 0.8µm Polystyrene, 10% solids
      *****Particles are not washed prior to use.
2. Protein Adsorption
   a. Mix 2.5 ml of the microsphere suspension and 50 ml of Coat Buffer.
   b. Incubate overnight at 4°C.
   c. Pellet microspheres by centrifugation at 12,000 x g for 2 minutes.
   d. Collect supernatant and measure to estimate the amount of immobilized antibody.
3. Blocking
   a. Resuspend particles in Blocking Buffer.
   b. Incubate for 3-4 hours at room temperature.
   c. Centrifuge, decant supernatant and resuspend in First Wash/Storage Buffer. Repeat.
   d. Centrifuge, decant supernatant and resuspend pellet in 8.25 ml of First Wash/Storage Buffer.
4. Removal of Loosely Bound Protein
   a. Incubate the bead suspension in a water bath at 55°C.
   b. Allow the water bath to cool to room temperature over 2-3 hours.



c. Centrifuge, decant the supernatant and resuspend in Second Wash Buffer. Repeat.

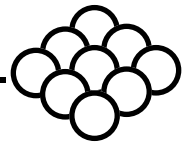
5. Storage
   a. Centrifuge and decant the supernatant.
   b. Resuspend in 8.25 ml of First Wash/Storage Buffer.

E. Forced Adsorption Method[8]

1. Reagent Preparation
   a. Microspheres: Dilute particles to 1%(w/v) with deionized water.
   b. Coat Buffer: 0.1M glycine buffered saline (GBS), pH 8.2
   c. Incubation Buffer: 95% ethanol containing 0.5% Na acetate.
2. Particle Preparation
   a. Centrifuge dilute particle suspension at 16,000 x g for 30 minutes (4°C).
   b. Save the supernatant for resuspending the particles later in the procedure.
3. Antigen Coating
   a. Resuspend the pellet in 1/20th the volume with Coat Buffer (15 minutes with a magnetic stirrer).
   b. Checkerboard titrate antigen to microspheres (usually 15-50 µl/ml).
   c. Stir for 15 minutes at room temperature.
4. Forced Coating
   a. Add 10 volumes of Incubation Buffer.
   b. Stir mixture for 30 minutes at room temperature.
   c. Incubate overnight at 4°C.
5. Blocking/Storage
   a. Centrifuge at 16,000 x g for 30 minutes at 4°C. Discard the ethanolic supernatant.
   b. Slowly resuspend the pellet with the original supernatant (See 2.b above).
   c. Add 0.1-1% Bovine Serum Albumin (BSA)
   d. Sonicate with a probe for 15 seconds to get a smooth suspension.

F. CAPS/PBS Method of Peptide Adsorption to Paramagnetic Microspheres[9]

1. Reagent Preparation
   a. Peptide: Synthetic peptide 42 amino acids in length
   b. Microspheres: Superparamagnetic carboxylate-modified surface, 4µm diameter, 2.5% w/v solution.
   c. Coat/Wash Buffer: 0.1M 3-cyclohexylamino-1-propane-sulfonic acid (CAPS) buffer.
   d. Blocking Buffer: Phosphate buffered saline (PBS), pH 7.4 with 0.05% Tween 20.
   e. Storage Buffer: PBS, pH 7.4.
2. Microsphere Preparation
   a. Centrifuge wash 1ml of magnetic particles with 70% ethanol at 5000 rpm for 5 minutes.



b. Resuspend pellet in 1 ml Coat/Wash Buffer.
c. Centrifuge at 5000 rpm for 5 minutes.

3. Peptide Adsorption
   a. Dissolve peptide (125 μg/ml) in Coat/Wash Buffer.
   b. Resuspend pellet in 1 ml of peptide solution.
   c. Tumble mixture overnight at room temperature.
4. Blocking
   a. Centrifuge as above, discard supernatant, resuspend in 1 ml Blocking Buffer.
   b. Repeat twice.
5. Storage
   a. Centrifuge as above, discard supernatant, resuspend in 1 ml Storage Buffer
   b. Centrifuge as above, discard supernatant, resuspend in 1 ml Storage Buffer. Final microsphere concentration is 2.5% w/v.

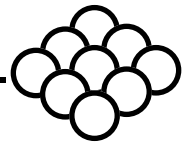
*****At this point, microspheres may be either diluted for use in an immunoassay or used in concentrated form for further peptide attachment via glutaraldehyde cross-linking of peptides.

G. Sodium Phosphate/PBS Method[10]

•One Cell Systems reports that this method has worked well with a variety of antibodies.

1. Reagent Preparation
   a. Microspheres: Polystyrene, 0.6-0.9μm diameter, 5% w/v.
      *****Particles are used without any prior cleaning.
   b. Coat/Wash Buffer: 0.1M Sodium Phosphate Buffer, pH 5.5
      Soln. A: 27.6 g Sodium Phosphate, monobasic ($NaH_2PO_4 \cdot H_2O$) in 1 L of deionized water.
      Soln. B: 28.4 g of Sodium Phosphate, dibasic (Na_2HPO_4, anhydrous) in 1 L of deionized water.
      Add 470 ml of Soln. A to 30 ml of Soln. B.
      Adjust pH to 5.5 with 0.5M HCl or NaOH if necessary.
      Adjust volume to 1 L with deionized water.
   c. Protein Solution: 200 μg IgG in 4 ml of Coat/Wash Buffer
   d. Wash/Storage Buffer: PBS
2. Protein Adsorption
   a. Thoroughly suspend microspheres in solution before sampling.
   b. Mix 0.4 ml of microsphere solution, 4 ml Protein Solution.
   c. Incubate 1 hour at room temperature with periodic mixing.

3. Washing
   a. Centrifuge at 10,000 x g for 30 minutes, discard supernatant, resuspend in 4 ml Coat/Wash Buffer.
   b. Centrifuge as above, discard supernatant, resuspend pellet in 1 ml



Wash/Storage Buffer. Transfer to microcentrifuge tube.

4. Storage
   a. Centrifuge on maximum speed for 3 minutes, discard supernatant, resuspend pellet in 0.4 ml Wash/Storage Buffer.
   b. ***If desired,** 0.1% sodium azide may be added for storage.

III. Frequently-Asked Questions

Q 1: To detect a certain virus by latex agglutination we tried to adsorb polyclonal and monoclonal antibodies onto 0.8 and 2.5µm polystyrene particles. First, we washed 50mg of particles several times with buffer. Then we resuspended them in 4ml of 0.3mg of Ab/ml buffer solution and incubated (overnight at room temp.). Next, we measured the amount of Ab in solution after adsorption and found 0mg/ml for 0.8µm particles and ~0.3mg/ml for the 2.5µm particles that we tried. What happened here?

A 1: The surface capacity of polystyrene for adsorption of protein is ~3mg IgG/m^2 particle surface area. The specific surface area of particles (m^2/g) is ~6/d, where d is in µm. Therefore, 0.8µm particles will have a surface area of 6/0.8 or 7.5m^2/g. Thus, they will adsorb 7.5 x 3 = 22.5mg IgG/g of particles or 22.5 x 0.05g = 1.1mg IgG/0.05g particles. You added ~1.2mg IgG (4ml x 0.3mgIgG/ml) to 0.05g of particles, or about what we would expect to saturate the surface, if it all were to adsorb onto the particles. You found 0 mg of protein/ml in the supernatant, and this result is consistent with the amount you added and the capacity of the particles. (If the IgG adsorbed to capacity on the polystyrene surface, we would expect to find none left in solution.)

Likewise, the 2.5µm particles will have a surface area of 6/2.5 = 2.4m^2/g, and will adsorb 2.4 x 3 = 7.2mg IgG/g of particles or 7.2 x 0.05g = 0.36mg IgG/0.05g particles. You added ~1.2mg of IgG/0.05g of particles (an amount which is about 3.33 times what we would expect to saturate the surface). The excess 1.2-0.36 or 0.84mg will be found in the supernatant at a concentration of 0.84/4 = 0.21mg/ml which is quite close to the 0.3 mg/ml concentration which you found. Thus, your adsorption results are about what we would expect.

Q 2: After adsorption of Ab, we washed and resuspended with BSA/ buffer. We next tried agglutination of particles using a virus culture, but no agglutination appeared.

A 2: In this case agglutination should be caused by a virus attaching itself to two or more antibody-coated particles and binding the particles together. You might need to adjust the amount of virus added to cause agglutination. If your sample contained a very high concentration of virus, they could completely cover the

antibody-coated particles and no agglutination could occur, because no bridging between particles could happen. Try diluting your virus concentrations by 10x and 100x to see if you can get agglutination this way. In some cases, but not in this case, perhaps, you may also need to adjust the amount of antibody on the particle surfaces to less than a monomolecular layer. (See our reprint #41 - especially about adjusting the coating density of Ab or Ag on the particles and the ratio of agglutinating agent to particles.)

Footnotes

[1] Spitznagel, T.M., D.S. Clark, "Surface-Density and Orientation Effects on Immobilized Antibodies and Antibody Fragments", *BioTechnology,* **11**/7, 825-829 (1993). *Gives a better understanding of adsorption and binding of whole proteins and pieces onto solids.

[2]Cantarero, L.A., J.E. Butler, J.W. Osborne, "The Adsorptive Characteristics of Proteins for Polystyrene and Their Significance in Solid-Phase Immunoassays", *Anal. Biochem.,* **105,** 375-382 (1980).

[3] Maehara, T., Y. Eda, K. Mitani, S. Matsuzawa, "Glycidyl Methacrylate-Styrene Copolymer Latex Particles for Immunologic Agglutination Tests", *Biomaterials,* **11**/March, 112-126 (1990). *See especially for test optimization using box titration technique.

[4]Anon., "Heterophilic Blocking Reagent Minimizes Antibody Interference", *Biomedical Products*, **19**/6, 58 (1994). (Press Release, Scantibodies Laboratory, 9336 Abraham Way, Santee, CA 92071)

[5]Press Release, SLD, 80 Rue des Romains, L-8041 Strasse n, Luxembourg.

[6]Press Release, Bionostics Ltd., The Business Centre, Tythe Farm, Wyboston, Beds MK44 3AT, UK.

[7]Vaidya, H., S.E. Porter, Y. Landt, D. Silva, D. Dietzler, J. Ladenson, “Quantification of Lactate Dehydrogenase-1 in Serum with Use of an M-Subunit-Specific Monoclonal Antibody”, *Clin.Chem.,* **34**/12, 2410-2414 (1988).

[8]Krambovitis, E., “Latex Technology with Special Reference to Applications in Diagnostic Microbiology”, *The Latex Course*, Strasbourg, France, November, 1990.

[9]Leahy, D.C., D.O. Shah, J.A. Todd, “A Method for Attachment of Peptides to a Solid Surface with Enhanced Immunoreactivity”, *BioTechniques*, **13**/5 (1992).

10One Cell Systems, Cambridge, MA, “Microdrop Technology: Experimental Procedures”, 10-11.

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