TechNote 201

Working with Microspheres

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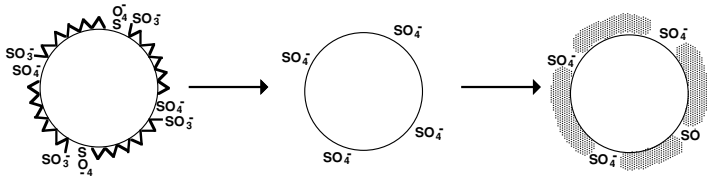

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I. INTRODUCTION

Many tests and assays use uniform latex particles, or microspheres, as substrates or supports for immunologically based reactions - tests and assays. These range from the original "latex" agglutination tests to more recent assays, such as particle capture ELISAs, turbidimetric immunoassays, dyed particle sandwich tests, and solid phase assays using silica or magnetic microspheres. In all cases, the particles must be prepared for binding and coated with a ligand, usually a protein, before they can be used in the chosen test or assay. One must also consider the microspheres' interaction with other test elements, like filters, membranes, and magnets.

A. Analyte

The analyte involved will partly determine the type of format that one chooses. For example, molecules with MW < 6000 may be difficult to detect in a sandwich format, since it's difficult for two antibodies to fit on such a small molecule. Such small analytes require competitive assays. This explains why drug tests are performed via inhibition or competitive binding formats. Large analytes, like proteins, can be measured by either direct or inhibition assays.

Actual clinical samples should be used early in the antibody selection process, to gauge the effect of interferences. "Out of 6-10 good antibodies that have passed all other selection criteria, only one will give these superior results with clinical specimens."[1]

B. Raw Materials

Microspheres, latex particles, or beads are presently available with diameters spanning > 6 orders of magnitude, from ~15nm to 25mm (0.015 - 25,000µm)! They are made from various materials (polymers and minerals), with densities ranging from ~1 to > 2 g/mL, and they offer a wide choice of surface chemistries. Here are some suggestions for microsphere choice (size and type) depending on test/assay format.

C. Microsphere Sizes Suggested for Different Test Formats

Test / Assay Type	Microsphere Size
Slide agglutination (LATs)	0.2-0.9µm
Turbidimetric immunoassays	0.01-0.3µm
Particle capture ELISA	0.3-0.9µm (depends on capture method)
Strip tests (particles migrate)	0.1-0.4µm (depends on strip porosity)
Solid phase immunoassays	>0.8µm

D. Microsphere Types Suggested for Different Applications

Application	Microsphere Type
Protein adsoprtion	Polystyrene (PS)
Nucleic acid adsorption	Silica or COOH-modified polymers
Covalent attachment	Surface-modified polymers or silica
Slide agglutination	PS or surface-modified polymers
Passive agglutination	Polymethyl methacrylate (PMMA) or dense polymers
SP_IA (fill in blank)	PS, PMMA, dense polymers, silica, or magnetic

E. Specialized Microspheres for Special Uses

It is now possible to get the microspheres you need, made to your specifications. Commercial vendors supply particles with special chemistries, higher / lower density than polystyrene, or with refractive indices above/ below that of polystyrene (brighter or dimmer particles for turbidimetric assays).

You can choose "brighter" microspheres with (higher refractive indices) like polyvinylnapthalene (n_D = 1.68 vs. 1.59 for polystyrene). Vinylbenzylchloride also has a higher n_D, so S/VBC copolymers with significant levels of VBC will also yield brighter microspheres, which scatter light better. You can also achieve a higher refractive index by using light of a shorter wavelength: n_{400nm} (PS) = 1.63.[6] ("Don't raise the bridge, lower the river!")

Polybutylmethacrylate (n_D = 1.43) and polymethyl methacrylate (n_D = 1.49) microspheres are "dimmer" and do not scatter as well as polystyrene.[7] Less bright beads may be important in some instruments for lower background scattering (a lower blank value for the calibration curve).

Recently, there has been increased appreciation for non-adsorbing microspheres. It is very difficult to distinguish between adsorption and covalent attachment, and there can be much nonspecific adsorption onto PS-based microspheres. Therefore, some developers want microspheres which will not adsorb any protein. They want to covalently bind their Abs to these microspheres, knowing that there will be no nonspecific binding. There are microspheres made with high surface levels of hydrophilic monomers like acrylic acid and acrylamide which come closest to "non-adsorbing." It is also possible to make very hydrophilic beads with polyethylene glycol extending from the surfaces.

Microspheres with varied surface functional groups are available. See our TechNote 205, *Covalent Coupling*.

Silica microspheres are naturally very hydrophilic, so there should be low nonspecific adsorption of protein onto them.

Silica's higher density (2.0 g/mL vs. 1.05 g/mL for PS) leads to a very important difference in settling velocity. Since settling in water depends on the difference between microsphere density and water density (2.00 - 1.00 = 1.00 for silica vs. 1.05 - 1.00 = 0.05 for PS), the silica microspheres will settle ~20X as fast as the PS! This important difference could lead to some interesting tests and assays, since agglutinated microspheres will settle out that much quicker. Small microspheres might be used for an agglutination test, where the unagglutinated microspheres would remain suspended, but agglutinated ones would drop out of solution very quickly. Then, perhaps settling time could differentiate a positive from a negative. (See TechNote 104, *Silica Microspheres*, for more information.)

Polymeric microspheres may be dyed during or after polymerization in a rainbow of colors. Originally dyed for improved visibility and color discrimination, they are also now dyed with fluorophores (used singly, or several in the same particle) and scintillators (they fluoresce when exposed to γ or β rays). Often only a small amount of these dyes are required to give an intense signal. These dyed particles are available "off-the-shelf" or "custom-tailored." Fluorescent-dyed beads may also improve the sensitivity of agglutination or strip tests. (See TechNote 103, *Fluorescent / Dyed Microspheres*.)

Superparamagnetic microspheres are available in different sizes, size distributions, surface chemistries, and levels of magnetite (for adjustable response to a magnet). The newest ones are core / shell encapsulated in pure polymer to prevent iron from coming into contact with sensitive enzymes. (See TechNote 102, *Magnetic Microspheres*.)

Picking the right microsphere supplier is not a trivial pursuit - it is worth doing well (Table 1). Work with manufacturers who have a good reputation for reliability and the ability to reproducibly deliver the quantities that you need, promptly (ask colleagues about reputations). Also, make sure you can get good, fast technical service, because there will be a few questions or problems. You might consider a microsphere supplier who can guarantee you an uninterrupted supply: two plants, two reactors, or some other arrangement.

Table 1. Proper Selection of Microspheres
(or "Practicing Safe Specs with Latex Products")

- Manufacturer
  - proof of continuity/reproducibity
  - technical service (full service shop vs. discounted store)
  - 2 producers or reactors
- Polymer: Is it polystyrene or "something special"?
- Particle Manufacturing Process
  - Standard or research?
  - Demonstrated lot-to-lot reproducibility
- Particle Size/Acid Content: Broad range - give yourself room
- Pre-Shipment Sample of New Lot: "The only spec you really need!"

We recommend you try standard materials first. If you are using special microspheres, they may take longer to obtain (smaller scale, more involved process) and qualify (are they really reproducible?).

Understand the scale of manufacture of the material you are testing. Did it come from a small research lot (OK for starting out)? Has it been made

in large enough scale for your possible use, or can it be scaled up easily? Prove it. Make your specifications as broad as you can, to give yourself as much flexibility as possible. Obtain beads from a few replicate lots, and test a pre-shipment sample of every new lot. Remember, you are not buying a commodity, and good suppliers will understand this.

F. Protein or Not

Pick your protein carefully. Do you want to use IgA, IgM, or IgG? Monoclonal antibody (mAb) or polyclonal (pAb)? Whole IgG or parts, like F(ab')$_2$, Fab, Fv, or single chain antibody (scAb) (Figure 1)? The Fc portions of IgG can be removed to avoid interferences due to rheumatoid factor (RF), reducing nonspecific binding (NSB) and autoagglutination problems. Or, try "mini-antibodies" (divalent Fv Ab fragments, grown in *E. coli* by recombinant techniques).[8] How about chicken antibodies, which don't react with RF?[9] "Recombinant polymeric IgG" has been designed to have the "best" properties of IgM and IgG for complement response.[10] (But will a different or engineered protein adsorb well on PS or make a better agglutinating protein?)

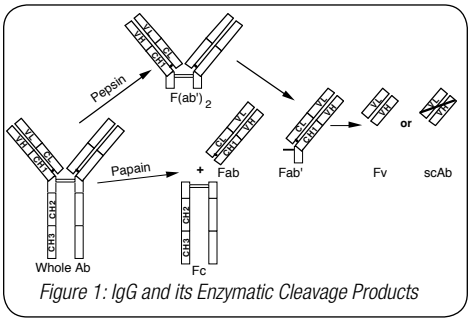


Figure 1: IgG and its Enzymatic Cleavage Products

G. Choosing mAb vs. pAb

1. mAb specificities are usually higher;
2. mAb binding affinities are normally lower;
3. mAbs do not normally cause immunoprecipitation (not recommended for LATs);
4. mAbs may not adsorb well onto PS (IEPs may be acidic);
5. mAb hydrophobicity changes with maturity.

Seaver says it will take less than half the time to find one good pAb for an agglutination test, compared to the time to find *two different* mAbs for a sandwich assay. Also, mAbs are more difficult to work with than originally thought, and careful screening and selection will be necessary.[1] (See also the *Final Words* section of this TechNote.)

If screening pAbs for your assay, first ask for "nephelometry and turbidimetry grade" pAbs. These will have been selected as good precipitating Abs. This is a predictor that they will be good for adsoprtion onto PS and perhaps good for agglutination, too.

There are some very special binding proteins now available, including a "transducing antibody," a bifunctional, or "schizophrenic" Ab, which is apparently designed to recognize both an antigen and an enzyme.[11] This would certainly make for an interesting test or assay - binding together an enzyme to an antigen.

BSA and IgG solutions change with time. Oligomers gradually form in protein solutions, and these oligomers adsorb onto microspheres more quickly and firmly than monomers. If you want oligomers, then wait, or try to accelerate this aging by thermal incubation. There may be ways to create synthetic oligomers, by cross-linking them or binding them to a synthetic polymer.

How about using nucleic acid ligands? "A recent entry in the field of selective binding and molecular recognition is the nucleic acid ligand, an oligonucleotide that exhibits high-affinity specific binding with selected target molecules." These are usually 15-50 base pairs long, the minimal sequence for binding to the target molecule.[12]

H. Water ("Using your microspheres to purify your water?")

The water you are using is probably not good enough! Researchers working with microspheres often assume that their water is clean. It might be clean, but even commercial deionized (DI) water may contain ionic and organic species, which can adsorb onto the particles. Even if you have a conductance meter for your water, the total organic carbon (TOC) is probably unknown. Anatel has an on-line meter to measure TOC to ensure that water is OK,[13] and Millipore is now offering new water systems with an Anatel TOC meter built-in (Milli-Q-A10 systems). (*Warning*: TOC meters may be expensive.)

Washing or cleaning particles with water is only effective if the particles are "dirtier" than the water. The microspheres have a very high specific surface area (~60 m^2/g for 0.1µm microspheres), and they will adsorb organic species from the water, if any are present. If the particles have been made with pure water, they will have only surfactant present. If you clean them with water which is not deionized and organically pure, or water with microbes in it, then you might be adding contaminants to a relatively clean system. Use a good water source and check to be sure it is consistently producing very clean water (low conductivity, zero organics, and no microbes). Otherwise, you might be cleaning your water with the microspheres!

Your "pure" DI water or wash water from microsphere cleaning can be quickly checked for surfactants by the "Seaman Shake Test" (No, it's not a sailor's dance!). Dr. Seaman recommends vigorous shaking of ~5mL of wash water in a 10mL tube and watching the bubbles collapse. In solutions of 0.1ppm Triton™ X-100, the bubbles will collapse within 1-2 seconds after shaking ceases (the same as pure DI) water. At $\geq$1ppm, the bubbles will persist for a significantly longer time, signalling the presence of surfactant.

I. CLEANING MICROSPHERES (BEFORE & AFTER COATING)

Most uniform polystyrene microspheres are made by emulsion polymerization using surfactants. The surfactants, usually negatively-charged alkyl sulfonates, sulfates, or fatty acids, become adsorbed on the microsphere surface, imparting a negative charge and increasing colloidal stability. In addition, surface-modified microspheres (those with COOH, NH_2, and other surface groups) may very well contain water-soluble polymer (WSP, left over from the polymerization process), which might interfere with the coupling of proteins to the surface. WSP, if present, will undoubtedly react first with any coupling chemistry, consuming protein which is intended to be bound onto the microspheres.

Chloromethylated particles, made with vinylbenzyl chloride monomer, come in very acidic aqueous solutions (pH < 3 from HCl lost during polymerization). It may be desirable to remove this acid before coupling. Therefore, before microspheres are coated with protein for use in various diagnostic tests and assays, the surfactant and other solutes may need to be removed (Figure 2).

Uniform silica microspheres are made from pure $Si(OC_2H_5)_4$ reacted with water and ammonia. The resultant microspheres are pure SiO_2 (ammonia and ethanol by-products) with negligible surface-active impurities and therefore may not require clean up before use.

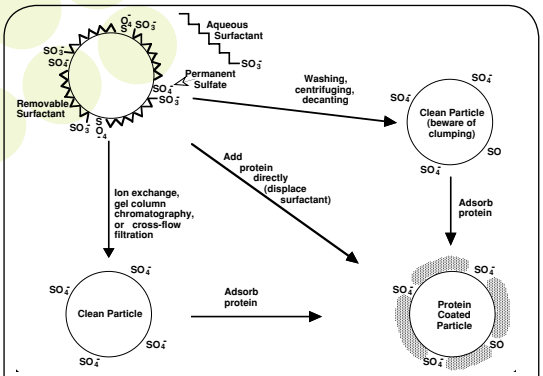


Figure 2: Cleaning Polystyrene Microspheres and Adsorbing Protein to Them

Most polystyrene-based magnetic microspheres are made by copolymerizing styrene with carboxylic acid-containing monomers in the presence of magnetite. Thus, some WSP may be present. Sodium dodecyl sulfate (SDS) is added to ensure long-term colloidal stability. Fatty acid surfactants may also be present.

There are several ways to clean microspheres before protein coating. The method chosen should be easy and effective, without causing problems (Table 2). When choosing any method for particle cleaning to remove surfactant, it is also wise to think about the necessity of removing unbound protein after coating. Most methods will work for the removal of either surfactant or unbound protein. With proper method choice, both cleaning steps can be done in turn, with the same technique.

Table 2. Microsphere Cleaning Methods

- For removing surfactant from "as produced" latex; or
- For removing unbound protein from coated microspheres.

1. Washing (centrifuge, decant, and resuspend in H_2O several times)
   - Labor intensive
   - Easier for larger microspheres (faster settling)
   - Hydrophobic particles might stick together (monitor aggregation)
   - OK for hydrophilic and protein-coated microspheres
2. Dialysis (must be done in very clean tubing)
   - Slow
   - Irreproducible
   - Possible use for the "overcoat-for-sweater" swap (see text)
3. Cross-Flow Filtration (filtration without filter-cake)
   - Easy scale-up
   - Many devices (stirred beaker, tangential-flow, hollow fiber)
   - Clean, coat, and remove unbound protein in same unit
4. Mixed Ion-Exchange Resin
   - Fast
   - Complete
   - Proven for ionic surfactants
5. Column Methods
   - Easy scale-up
   - IX: weak anion or cation
   - Affinity: Protein A or G
   - Size exclusion (example: Sephadex G-25)

J. "Do Nothing" or "NO Washing"

Note that some immunodiagnostic manufacturers successfully use microspheres "as received" - perhaps only diluting the microspheres, surfactant, and other solutes 10X with buffer and coating protein. We DO NOT recommend this practice! The danger in this technique is that, if another lot of latex is used the next time, the binding of protein might be very different. This is especially true if there is more or less surfactant in the new lot, or if another surfactant is used. To be successful with this procedure, you must ensure that you are getting excellent lot-to-lot reproducibility. You should always pre-test any new lots that you use.

K. "Just Enough" Washing

Microspheres are clean enough when "just enough" surfactant has been removed so that coating proceeds well. One wants to remove surfactant until coupling is uninhibited and reproducible, while the microsphere remains singly dispersed. After cleaning, some users actually add-back surfactant (under their own control) to assist single microsphere coupling. And, it is possible to get some (especially < 100nm) too clean and unstable if all the surfactant is removed (see "Dialysis" for a fix).

L. "Washing"

Repetitive centrifuging, decanting, and resuspending in water or buffer is often the first cleaning method considered. This method has caused more grief than joy. The microspheres must be spun down to form a tight "button" to permit the clean separation (decantation) of liquid from solids. The smaller the particles, the more difficult this separation becomes. If the brake is used to stop the centrifuge, the particles may be partially resuspended and some of them lost on decanting.

After decanting, fresh water or buffer is added, and microspheres should be fully resuspended. The resuspending process must not stop after merely stirring all the sediment off the bottom of the tube. Microspheres must be completely redispersed to single entities for effective washing. Resuspension should be monitored with some reliable methods, like microscopic examination or fast instrumental size analysis, to ensure that primarily single particles are observed with only a few doublets. As more surfactant is removed, the microspheres will tend to stick together more tightly, especially if buffer is used. Thus, the redispersal process will become more and more difficult (and beads may begin clinging to tubes and pipette tips, resulting in their "loss").

Larger plain particles (> 0.8µm) are more easily spun down, less likely to stick firmly together, and more easily resuspended. Microspheres < 50nm (< 0.05µm) may require > 300,000 G to sediment them efficiently (i.e., at 10 cm/hr settling rate).

Magnetic microspheres can also be "washed," using a magnet to pull down the solids between repeated DI water rinses. However, as the microspheres go through successive cleaning steps, they will become more hydrophobic and, therefore, more difficult to resuspend and separate from one another. Many wash steps (10-20) may be required to thoroughly clean magnetic microspheres this way. Slightly alkaline (pH 8-10) water may be used to assist colloidal stability and to ensure solubilization of SDS surfactant, WSP, and any fatty acids. Also, for large scale clean-up, it may be difficult to scale up the magnetic separation steps, and other more readily scaled-up processes should be considered. Folks who coat magnetic particles often use other, non-magnetic means of cleaning them.

Resuspension can be greatly assisted with an ultrasonic bath, not a probe. (*Note:* Ultrasonic probes are notorious for introducing contamination. Even metal particles can come off the probe.) Ice may be added to the bath to prevent sample overheating.

So, are you still interested in centrifuging (and magnetic separation) for cleaning? Think of the nightmare that this will create when your production

department must handle kilogram quantities of microspheres which have been diluted to 1% solids. [1000g (dry weight) at 1% solids is 100 liters of latex to be put into 1 liter (?) bottles and spun down, etc.] One of our customers handles > 200kg of microspheres per year, and they do NOT use centrifuges! If you must clean your microspheres this way, investigate a continuous centrifuge (like a cream separator), to concentrate the microspheres without producing a packed cake. If you are determined to spin down your microspheres anyway, see TechNote 206 for equations and estimating times.

With large-scale magnetic particle usage imaging having to clean the beads with some large-scale magnetic separation. Does such equipment exist or will you need to invent it?

The good news is: hydrophilic microspheres (with COOH, OH, etc. surfaces) and protein-coated microspheres will be much less likely to stick together after centrifuging.

M. Dialysis

A slow and unreliable method, it may be used in research to begin. It is difficult to remove all impurities this way because of the time it takes for the surfactant to completely desorb and diffuse through the dialysis tubing.

The old "overcoat-for-sweater" swap: There is one possible situation where dialysis can be very effective. In some cases, hydrophobic particles with low inherent surface charge are not comfortable without their surfactant "sweaters." They might "freeze" (i.e., they may become colloidally unstable and clump), especially in certain higher ionic strength buffer solutions, before they can put on their protein "overcoats." In such as case, the idea is to mix protein with surfactant-containing particles and dialyze the mixture in a membrane chosen to let the surfactant diffuse out while holding the particles and protein in. The protein will gradually coat the particles as the surfactant leaves the particle surface and diffuses out through the dialysis tubing. (It's like avoiding freezing by putting on your overcoat outdoors, while simultaneously taking off your sweater.) Then, after protein coating, you could use larger pore dialysis tubing to remove the unbound protein.

Dialysis might also be the most practical method for cleaning extremely small nanoparticles. And, if the particles become unstable when you get them too clean by dialyzing with water, then you can dialyze against a very dilute surfactant solution with just enough soap to stabilize them, but not enough to interfere with coating.

N. "Dead-End" or Bed Filtration

Standard filtration is generally unacceptable, because the small microspheres can easily plug a filter designed to catch them. Also, flow through a packed bed of submicron particles is extremely slow. Then, after filtration, one still has the problem of redispersal of a filtercake, especially if the cleaned beads are clumped.

O. Cross-Flow Filtration

It could also be called "dialysis under pressure" or "filtration without a filtercake." Various mechanical means are employed to permit liquid to permeate the filter medium, while preventing a particle layer build-up. Several filter manufacturers carry equipment to handle volumes from a few milliliters to many liters. The best methods permit development of a lab method which can be easily scaled up to production quantities. Best of all, you never need to handle clumped microspheres.

There are stirred breakers with a filter in the bottom and a stirrer just above the filter to keep a filtercake from forming while the liquid is filtered under pressure. Convenient for small scale lab work, they are available in several sizes with different filters.

Some devices have flat membranes sandwiched between monofilament screens. The latex is pumped horizontally through the screen, over and under the filaments of the screen, so that net flow is parallel to the plane of the screen. This tortuous path causes turbulent mixing of particles and liquid, while a small amount of the total flow passes perpendicularly through the membranes, located on either side of the screen.

Disposable hollow-fiber filter cartridges, with areas from a few cm^2 to several m^2 and pore sizes as small as 0.05µm, allow clean-up of all but the very smallest particles. Small and large scale units use the same fibers, so the process is very "scalable."

The same device can often be used for initial cleaning of microspheres, as a reaction chamber for protein-coating, and for removing unbound protein. This eliminates a great deal of (your) labor-intensive particle handling.

"Ultrafiltration membranes" are cross-flow membranes "tight" enough to retain proteins. These might be useful for the "sweater-to-overcoat" method of coating microspheres with protein (see above under the *Dialysis* section). After protein coating, one would still need a separate cross-flow filtration step to remove the unbound protein from the microspheres.

Some cross-flow filters are reusable. However, they must certainly be thoroughly cleaned and perhaps sterilized before reuse.[14] If disposable filters are used, clean-up is minimized.

After cross-flow filtration, the microspheres' surface ionic groups will be only half neutralized: $[-SO_4^-] \approx [-SO_4H]$ and $[-COO^-] \approx [-COOH]$. Microspheres cleaned this way should thus be more stable colloidally than those cleaned by ion exchange (below), where all ionic groups are neutralized (all in $-SO_4H$ and -COOH forms) -- an important advantage.

Cross-flow filtration systems easily clean magnetic microspheres of various magnetite contents and with different size distributions. Centrifugation or magnetic separation may result in losses of the smaller, slower moving microspheres, with a significant shift in the size distribution.

Hollow-fiber cross-flow filtration is fast, efficient, and automatable, with much less clumping, minimizing particle instability and material loss. Ron Pong describes a typical use of hollow fiber for microsphere processing.15

We sometimes refer to "unstable" microsphere suspensions. In the spirit of political correctness, perhaps we should instead call them "colloidally challenged."

P. Mixed Ion-Exchange (IX) Resins

Used successfully for > 35 years, ion-exchange resins actively remove all ionic species from latex particles. One mixes equal volumes of rigorously cleaned strong acid and strong base resins in the H+ and OH- forms, respectively, then combines the resins with the latex to be cleaned. This method removes all ionic surfactant and inorganic buffers from the aqueous phase and quantitatively strips them off the particles' surfaces. One then separates clean microspheres from the much larger IX beads by decantation and coarse filtering.

Mixed ion-exchange is the only cleaning process that rapidly and actively removes adsorbed surfactant. (All other cleaning methods are passive -- the microspheres become cleaner as surfactant spontaneously desorbs from their surface and is subsequently removed from the aqueous phase.)

Commercial IX resins (i.e., from Dow or Rohm & Haas) must be carefully and rigorously cleaned before they are used. Otherwise, the resins will add contamination to the microspheres. The resins do not need to be put into a "bed" or column; they can be mixed with the latex in a slurry and then coarse-filtered to remove the IX resins. BioRad supplies pre-purified IX resins.

These strong acid and base resins are not designed to remove proteins. In fact, they might denature some proteins. And, of course, IX resins will not remove nonionic surfactants.

Q. Column Methods

Any bed packing should be as large as possible for good fluid flow and to allow microspheres to percolate freely through the bed. Any hang-ups will result in loss of microspheres and plugging of the bed. The packing bead porosity should be large enough to easily let the unbound solute enter, while excluding any microspheres. Only the unbound protein and other water-solubles should be caught within the pores.

Various affinity columns will probably work well (if expensively) to remove unbound protein from microspheres. Several column makers claim binding of a wide variety of proteins. Some columns contain genetically engineered affinity agents, which bind various immunoglobulins selectively or comprehensively - your choice.

Weak anion and/or cation columns can remove proteins. DEAE cellulose is recommended.

Gel phase chromatography, or size exclusion chromatography, can be used to separate free surfactant or unbound protein from microspheres.[16] As the microsphere suspension is poured or pumped through the bed, microspheres move quickly through the void volume between the beads, while dissolved surfactant (or unbound protein) diffuses into the pores of the beads, where it remains briefly and exits the column after the microspheres. Sephadex G-25 columns have been used for this job; pre-packed, disposable PD-10 columns (G-25 M) are available with bed volumes as small as 1.7mL. Various vendors sell a wide variety of other columns with different porosities and gel bead sizes, in columns large enough to satisfy any contemplated scale of operations.

R. Ultrasonics

Ultrasonics may help in single particle dispersion during cleaning and processing.[17, 18]

S. Microsphere Characterization

We advise that you check microspheres at various stages in their processing- before and after cleaning, protein coating, blocking, and final formulation (buffer adjustment). One can monitor the microspheres' monodispersity (single microspheres), colloidal stability, surface charge, and changes in electrokinetic behavior, which relate to protein coverage. Table 3 outlines various methods for characterizing microspheres.

Table 3. Microsphere Characterization

- Size Distribution
  - Light microscopy
  - Electron microscopy
  - Instruments: Coulter-type, light scattering, disc centrifuge, others
- Surface Titration: 'Surface Charge Density' Parking Area
  - Potentiometric
  - Conductometric
  - Soap (adsorption area)
- Critical Coagulation Concentration (stability against flocculation)
- Electrokinetics/Particle Electrophoresis
  - Brookhaven
  - Coulter (DELSA 440)
  - Malvern Zetasizer (size and zeta potential)
  - Matec Acoustosizer (size and zeta potential)
  - Micromeritics Zeta Potential Analyzer 1202
  - Pen Kem (Lazer Zee Meter and automated electrokinetics analyzer)
  - Zetameter
  - Rank Bros. (UK)
- Field Flow Fractionation (FFFractionation, Inc., Salt Lake City) (can measure size distribution, density, and other parameters)

T. Size / Monodispersity

It is important to make sure that the microspheres are singly dispersed. (Or do you want them to be partly flocculated?) If they are clumped, was it due to centrifugation or instability in the buffer, and at what point did clumping occur?

By using light microscopy, you can ensure that the particles are well dispersed after each washing or coating step. You can monitor progress and identify where/when problems are arising. Clumped beads are easily seen with an ordinary microscope at 400X. We have used light microscopy to monitor our progress while coating magnetic and other particles. We could easily see when the microspheres stayed well dispersed, and could quickly pinpoint where a potential problem arose.

Many sizing instruments measure particle size distribution. Any size distribution change between processing steps indicates possible clumping of the particles and should be investigated. An article by Nicoli, et al., describes typical modern methods and equipment, especially dynamic light scattering (DLS) and single particle optical sensing (SPOS).[19] Wide dynamic range, measuring 0.02-30.00µm particles with good resolution, is claimed for a disc centrifuge sizing system.[20]

U. Surface Titration

The capacity of certain lots of COOH-modified microspheres for covalent coupling can be determined. Potentiometric or conductometric titrations of active surface COOH groups are performed on clean or coated microspheres to determine lot-to-lot reproducibility. Titration might also be used to help explain the inability to adsorb as much protein, after a particular treatment. (Was sufficient surfactant removed?)

A "soap" or surfactant titration is a standard colloid chemist's technique for determining the amount of open surface area on latex microspheres. One titrates a known amount of clean or as-received microspheres with a standard "soap" solution, and measures the surface tension. Soap adsorbs onto the PS microspheres and the aqueous surface tension remains steady, until the particle surface is full of a monolayer of soap molecules oriented perpendicular to the surface. Then, the extra surfactant goes to the water/air interface, and the surface tension starts to drop. The amount of soap

added, up to the surface tension break point, is the surface capacity of the microspheres.

Other specialized titrations can be done, depending on the microsphere type and binding chemistry. For example, chloromethylated microspheres [made of styrene/vinylbenzylchrloride (S/VBC)] may become dehydrohalogenated, losing HCl, on long-term storage. One can measure Cl^- ion (perhaps with a simple Cl^- electrode) to monitor the reactivity and colloidal stability of the microspheres at various stages - after polymerization, after storage, or before/after cleaning. To titrate the reactive chloromethyl surface groups, one can measure the Cl^- concentration before and after reaction of S/VBC microspheres with some small molecule, like dihydroxyl amine. Cl^- release should be proportional to available CH_2Cl surface groups. And, Cl^- released by protein binding should precisely measure the number of covalent bonds formed - proving covalent coupling.

V. Critical Coagulation Concentration

Rather like the opposite of soap titration, one can pre-determine stability against coagulation, by titration with a standard salt solution. Gradually increase the salt concentration until microspheres coagulate to determine if the microspheres will flocculate in the buffer you intended to use to coat, couple, or store the particles.

W. Electrokinetics

There are several methods and appropriate instruments to monitor the progress of cleaning and coating of the microspheres. The most commonly used method measures the direction and speed-of-motion of individual microspheres in a standard set-up. With these methods, one can determine whether the microspheres are clean, or uniformly coated with protein. Field-flow fractionation is only one of the more interesting newer methods for characterization.21 Without some method like these, you are "flying blind" in your coupling process.

X. Measuring Bound Protein

To determine the protein bound to your microspheres, you can use BCA reagent from Sigma, Pierce, or Bio-Rad. Measure protein in your solutions before and after adsorption onto microspheres, and calculate adsorption per gram of particles used. You can also measure protein directly on the microspheres using this reagent. Molecular Probes also provides a protein quantification assay named NanoOrange®.

Some say you can measure protein covalently bound vs. adsorbed by stripping adsorbed material from the microsphere surface with hot, alkaline SDS. Beware of denaturing and tearing protein apart to get it off the surface. If you want to determine what comes off, wait to see what naturally desorbs. If it doesn't come off naturally, it is tightly bound, whether adsorbed or covalently coupled.

II. COATING MICROSPHERES

A. Simple Adsorption

Protein adsorbs onto polystyrene (PS) readily and permanently. Simply adsorbing protein, especially polyclonal immunoglobulin G (IgG), on the surface of polystyrene microspheres is successful > 95% of the time.

The microspheres' surface capacity, for the protein being used, should be known. For example, the surface saturation capacity of polystyrene for bovine serum albumin and bovine IgG is calculated22 at ~3 mg/m^2 and ~2.5 mg/m^2, respectively. These numbers are consistent with their Stokes diameters of ~7nm (BSA) and ~10nm (IgG). Molecular packing onto a sphere of given diameter can be calculated and the numbers come out similar to these.

The specific surface area to mass ratio for a sphere is calculated: A/M (m^2/g) = $6/\rho D$, where ρ = density in g/mL and D = diameter in µm. For PS, where ρ = 1.05 g/mL, A/M = 5.7/D. Thus, if D = 1µm, then A/M = 5.7 m^2/g ≈ 6 m2/g. Therefore, 1µm PS microspheres may adsorb ~ 3 mg/m^2 x 6 m^2/g ≈ 18mg BSA/g of microspheres. This represents a monolayer of protein, and will probably be the maximum amount that can be put on the microspheres reproducibly and stably, whether by adsorption or covalent attachment.

For maximum surface coverage up to a monolayer, buffer pH should be at, or slightly more basic than, the IgG's isoelectric point, IEP (i.e., pH ~8.0). Protein is in its most relaxed, compact form (**Y**, not **T**) at its IEP. Because the pH at the particle surface will be more acidic than the solution pH, it is better to operate with a bulk pH that is more basic than the IEP for the protein of interest. Costar says, "Binding is favored when pH is close, but not equal to, the pI of the protein being immobilized," and "IgG binds best at a slightly basic pH which exposes hydrophobic groups due to partial denaturation."[23] Tris buffer (pH = 8.0) and phosphate buffer (pH = 7.4) work well for IgG adsorption. The Fc and Fab portions of IgG adsorb differently in response to pH changes. Thus, one can arrange for the optimal adsorption of the Fc portion, and relative suppression of Fab adsorption, by choosing slightly alkaline pH.[24] Note that some mAbs have low (acidic) IEPs.

Use a dilute microsphere suspension (≤ 1% solids) to ensure that you are coating particles singly, so clumping during coating will be less likely. While a final protein concentration of ~0.1 mg/mL may be enough to achieve a monolayer, we recommend that you add a 3-10X excess of protein. This ensures favorable stoichiometry, a good driving force for adsorption and crowded, upright positioning (**YYY**, not ≺ ≺ ≺). Stir the suspension to increase the probability that the protein will encounter the PS surface. Allow to incubate, with stirring for 24 hours at 4˚C, 16 hours at 20˚C, 4-8 hours at 37˚C, or 1-2 hours at 56˚C. Remove excess protein by one of the methods described above for removing surfactant.

We know that many practical, experienced microsphere users do not add a large excess of their Ab. This might be because of the extra expense of using (and losing) precious Ab. One recipe uses 1mg IgG/m^2, a fraction of the amount of Ab that could be bound.

The recipe does not call for removal of any unbound protein in solution. We feel that this may be dangerous practice, because of the unknown orientation of the adsorbed Ab and the unadsorbed Ab left in solution. We know of other experienced coaters who add only enough protein so it will be adsorbed; then they believe they have none to remove. This is fine if you have your coating process well documented and know what you are doing, but we cannot recommend this. ("Don't try this at home, kids! We're trained professionals!")

Adjusting protein coverage will also be discussed later in the section, *Achieving Optimal Coating*.

Researchers in Uruguay found a unique method for coating large 250µm PS beads with gliadin, a very hydrophobic protein from wheat gluten. Adsorption of the protein was done from 70% ethanol solution, followed by ethanol washing and BSA blocking.[25]

For more theory, mechanisms, and good data for protein adsorption, we recommend Olal's thesis[26] and the book by Horbett, et al.[27]

Ultrasonics, Vortexing, and Adsorption Don't Mix: Gentle mixing during adsorption is recommended - to overcome slow diffusion and help Abs find their places on the microsphere surface. But, experienced particle coaters report that violent agitation (as with ultrasonic baths and vortexers) can tear much of the adsorbed Ab off the microsphere surface!

Adsorption onto Silica Microspheres: In 1990, it was shown that DNA adsorbs to silica in the presence of chaotropic agents like 3 M KI, NaI, or NaSCN, with 8 M urea. HIV RNA is isolated from serum using $NaClO_4$ and guanidinium thiocyanate (or guanidinium hydrochloride).[28] Now, "Chaotropic-salt-induced adsorption of DNA to silica is one of the most common methods for purifying both chromosomal and plasmid DNA from cell homogenates." Apparently, the DNA adsorbs lying down at ~500 mg/m^2.[29] A newer, simplified, automatable, high throughput protocol uses much milder conditions for reversible binding of bacterial DNA onto silica in 96-well plates.[30] The DNA or RNA can then be desorbed and use for sequencing, PCR, library construction, or restriction digestion.

B. Complex Adsorption

IgG and albumin (BSA or HSA) can be mixed and adsorbed together. One commercial protocol calls for a weight ratio of 1 IgG/ 10 albumin in a co-adsorption mixture. Other methods of coating particles should be considered, if simple adsorption is inadequate (Table 4).

We have even heard of coating with BSA before adsorption of IgG. Perhaps this was done at a BSA concentration that was low enough that it didn't prevent enough IgG from adsorbing.

Table 4. Coating Microspheres

- Simple Adsorption
  - Ab or Ag onto PS
  - DNA onto silica
- Complex Adsorption
  - Ab + BSA coadsorption
  - Adsorption + Cross-linking (e.g., glutaraldehyde)
  - Adsorption of hapten/polymer conjugate
  - Adsorption of polymer, then coupling hapten
  - Peptide adsorption, then linking more peptide
  - Adsorb one ligand which captures another
    - Ab1 captures Ab2 (pAb α-mouse captures mAb)
    - Protein A, G, or L (they bind various Igs)
    - Avidin captures biotinylated Ag or Ab
    - Concanavalin A and other haemagglutinins (bind carbohydrates of MW >2000)
    - Jacalin binds IgA
- Covalent Chemical Coupling

Adsorption of protein can be followed by glutaraldehyde cross-linking of the mixed proteins on the surface to preclude any protein desorption. (Like wearing a belt and suspenders?)

Small molecules, like haptens, will not adsorb well or remain attached for a long time. Covalent binding of these small molecules to proteins like BSA, dextran, polylysine, or other polymers which will adsorb well, enhances and stabilizes the attachment to the microspheres. These "polyhaptens" are used commercially. Alternatively, one could couple the hapten or other label after adsorbing the polymer or protein on the microspheres.[31] Others favor a hybrid method: adsorption of phenylalanine/lysine copolymer onto polystyrene with glutaraldehyde binding of protein to the amino surface groups from the polymer.[32] One novel idea is to adsorb peptide onto the microspheres, then covalently link more peptide onto the surface.[33]

If you adsorb one Ab and use it bind another Ab, the outer Ab will certainly be more accessible, sticking further out into the aqueous phase. Paul Hemmes compared activities of primary Ab adsorbed directly, with primary Ab which was bound by an adsorbed secondary Ab. He reports better activity, or recognition of Ag with the Ab which was further away from the microsphere surface. Even pAbs bound this way perform better.[34] This is perhaps true because the antibodies are away from the surface and are more free to move around. They may also be oriented more favorably and therefore be significantly more active (Figure 3).

Sometimes the only way to bind monoclonal antibodies (mAbs) to microspheres is by first adsorbing a polyclonal antibody (pAb) directed against your mAb - something like goat anti-mouse (GAM). Please make sure that the goat serum responds to the particular sub-type of mAb that you have.

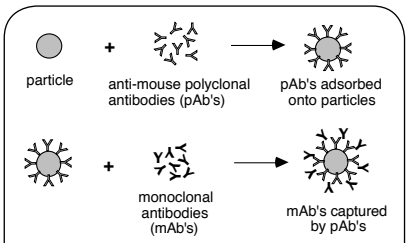


Figure 3: Second Antibody Binding to Microspheres

Beckman-Coulter cites this technique frequently in publications about their magnetic microspheres assays. These assays, such as one for ferritin,[35] are run on their Access® instrument. One could possibly make a whole series of tests or assays from a single pAb preparation used to bind mAbs specific for various analytes.

One can also use Proteins A or G to attach various Abs to particles. Some claim superior orientation this way. Since Protein A binds specifically to the Fc portion of IgG, the Fab portions of the Ab are supposed to be pointed away from the surface. You have a choice of native Proteins A or G, recombinant forms of Proteins A or G (these have deleted sequences for reduced non-specific binding potential), or even with recombinant fusion Protein A / G. The latter is supposed to bind IgGs better than either A or G alone.[36] After the IgG is bound, some claim that dimethyl pimelimidate (DMP), or dimethyl suberimidate (DMS), should be used to crosslink the IgG to the Protein A. (Belt and suspenders again?)

Protein L is supposed to be a more universal monoclonal antibody-binding protein. Since it apparently binds to the Fab portion of the Ab, we wonder if "L" can provide the same directed binding as claimed for "A"? We have not evaluated any yet, but it might be easier/better than anti-mouse secondary antibodies (worth consideration?).[37]

Avidin adsorbs onto PS and captures biotinylated Ag or Ab (Figure 4). Often avidin or streptavidin are covalently coupled to get more secure binding. One vendor offers *improved*, more tightly adsorbing streptavidin which may make covalent attachment unnecessary.[36] In molecular biology applications, the avidin-biotin system is used with paramagnetic particles for mRNA isolation.[37] Note that, in this case, the streptavidin is covalently linked to the particles.

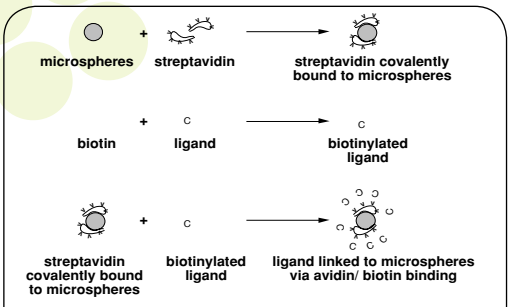


Figure 4: Avidin / Biotin Binding of Ligand to Particles

After producing streptavidin-coated microspheres, one can produce many different tests or assays merely by adding any variety of biotinylated ligands. Peptides or other ligands can be bound to biotin, usually through a spacer, for more secure attachment and better activity. Using the avidin-biotin linkage scheme should save on precious ligand to be bound, because it is easier to estimate and control the quantities needed for the final biotin/avidin reaction.

Other options: Monomeric avidin has been mentioned for coating microspheres. It supposedly permits easy binding and release. Due to its lower affinity, it will let go of biotinylated ligands more easily. Free biotin is added to make avidin-coated beads release the ligand. A special bifunctional protein binder, a streptavidin•protein A conjugate, is also available, which could enable special binding combinations with a long spacer between them. Lectins, like Concanavalin A and other haemagglutinins, bind carbohydrates of MW > 2000. Thus, they can be used to bind immunoglobulins via the carbohydrate moieties linked to their Fc portions. Similarly, another lectin, Jacalin, binds IgA specifically.

You can also purchase many different sizes and types (dyed, magnetic, etc.) of protein-activated microspheres with various generic coatings -- like streptavidin, Protein A, and anti-Mouse IgG.

C. Reasons for Covalent Attachment (Don't Get in a Bind Unless You Need To)

Adsorption seems to be more than adequate to put IgG onto polystyrene microspheres, at least for most test / assay systems. Why then would anyone want to consider using covalent attachment?

1. It might be possible to put more protein on the surface by covalent binding. There is some evidence that you can get 10-40% more on the surface this way.[40]
2. When the desired coverage is low, it may be easier to control coating level and uniformity by covalent coupling.
3. Some tests or assays might be so sensitive that they will be influenced by minute quantities of adsorbed IgG which might leach off the particles over time. Covalent coupling can be used whenever more secure binding is needed.
4. Covalently bound protein should be more thermally stable. After 1 hour at 56°C, 70% of the IgG remained adsorbed, but 99.7% of covalently linked IgG remained.[23] This could be very important if the particles are to be used in PCR work, or other applications requiring thermocycling.
5. Lot-to-lot reproducibility should be improved.
6. If reagents are precious, then covalent coupling will be much more economical, since crowded adsorption requires an excess of protein.
7. Small molecules must be covalently linked to the particle surface. Unless chemically bound to the surface, they will surely desorb when the equilibrium is disturbed by removing unbound soluble molecules from solution.
8. Covalent coupling also permits the inclusion of spacer arms for small molecules. There is always the concern that molecules will be bound "face-down" (with the recognition site hidden) or too close to the surface. The introduction of a spacer arm or "tether" should permit secure, but flexible, attachment of many different molecules.
9. Some claim much better binding capacity using spacers.
10. With a spacer, one can change the coupling chemistry. For example, by binding H_2NCH_2-Ø-CH_2NH_2 or H_2N-C_6H_{12}-NH_2 onto COOH-modified microspheres, one can change the surface chemistry to amino groups. On amino-modified microspheres, use glutaraldehyde, "polyglutaraldehyde," or amino reactive spacers or cross-linkers to add a spacer and change surface binding groups. (Put on more ligand!)
11. Directional binding (e.g., periodate oxidation of vicinal hydroxyls on the carbohydrate portion of IgG Fc, and binding to hydrazide microspheres) ensures that recognition sites are pointing outward and accessible.
12. Covalent attachment at relatively few sites may overcome the "Gulliver effect." There is some evidence that large, well-adsorbing protein molecules may become so tightly adsorbed over a wide area or at many contact points that they become distorted or denatured - rather like Gulliver was immobilized on the beach by the Lilliputians.[41] This is a frequent problem with gold.
13. One might need to covalently bind the smaller, specialized antibody pieces, such as the $F(ab')_2$, Fab, or Fv portions or the new "miniantibodies." These smaller molecules won't adsorb as well as IgG. (See also the *Protein or Not* section above).
14. Some mAbs have isoelectric points ~4, and, at this pH, some microsphere may be "colloidally challenged." In these cases, it may be easier to covalently couple mAbs to microspheres than to adsorb them.
15. Working with very small (< 100nm) hydrophobic polystyrene microspheres can be troublesome. Obtaining them surfactant-free is difficult-to-impossible because of colloidal instability, and cleaning them is not easy. They may flocculate when contacted with the protein coating buffer. There are ways to minimize the problems, but it may be easier to covalently bind to surface-modified particles. Microspheres with hydrophilic surface groups (-COOH, -OH, etc.) tend to be more colloidally stable.
16. After covalent attachment, the protein will not come off the surface. It is possible to add as much surfactant as necessary (possibly > 1% Tween®) to eliminate nonspecific binding.[42, 43] Adding that much surfactant to adsorbed protein / microspheres could displace unbound protein, but not adding the surfactant could result in significant interference from serum effects in samples. This may be the most important reason for covalent coupling.
17. If DNA is adsorbed to microspheres by multiple site attachment, the hybridization of DNA will likely be hindered (analogous to the "Gulliver effect" with proteins).
18. The best way to put DNA on any microsphere is probably via covalent coupling, using one point of attachment, preferably at the 5' or 3' end. (The best method of attachment of DNA or protein to hydrophilic silica particles is probably via covalent linking to any of several coupling groups. Chloromethyl or amine are only two examples of the many possible functional groups possible, using silane coupling agents.)

D. Carboxylate Covalent Coupling Chemistries

Surface-modified microspheres are often made by copolymerizing styrene with a small amount (< 5%) of a functional monomer, like acrylic acid. Emulsion polymerization (with potassium persulfate as the initiator) yields particles with COOH groups covering the surface (Figure 5). Other acids and

monomers can be used to yield particles with different surface chemistries.

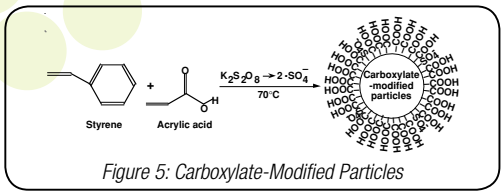


Figure 5: Carboxylate-Modified Particles

1. *One Step Process:* Probably the best knows coupling chemistry uses COOH groups on some carboxylate-modified latex (CML) microspheres to couple amino groups on protein, via water- soluble carbodiimide (WSC) chemistry, in a one step process at pH 6.0-8.0[42] (Figure 6). One typical water-soluble carbodiimide is EDAC [1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride], which is available from Sigma and Pierce. The original Hager patent has now expired, so everyone can use it freely. The chief advantage is that it is only one step - "only one pot gets dirty."

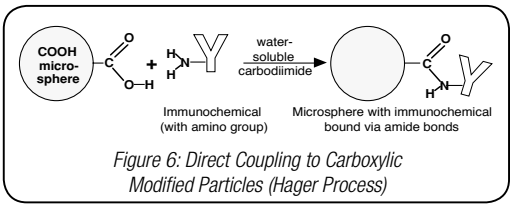


Figure 6: Direct Coupling to Carboxylic Modified Particles (Hager Process)

One disadvantage to "one step" coupling is that the pH normally used is a compromise between the ideal pH for both halves of the coupling reaction. The BIG disadvantage is that the WSC is indiscriminate; it can cross-link protein as well as bind it to the particles, and it is possible to bind everything together - protein, cross-linked protein, and microspheres. You can easily form clumps of particles, which are covalently bound together by cross-linked protein. The clump sizes are uncontrollable, and a wide distribution of clump sizes can be formed. It may be possible to perform this reaction successfully in one pot, but we mostly hear about problems with clumping.

2. *Active Ester Process*: In an improvement on the Hager work, Dorman used an active ester intermediate.[45] (Dorman's patent also got around the Hager patent!) Its chief advantage is that the WSC is used to create the active ester, then excess WSC is removed by cleaning. This two-step process keeps the protein away from the WSC to avoid any possible cross-linking of the protein. Dorman's patent expired in 1994.

First, form the active ester (Figure 7a) and clean the particles to remove unreacted chemicals. Then, react the active ester with the immunochemical (Ag or Ab) (Figure 7b).

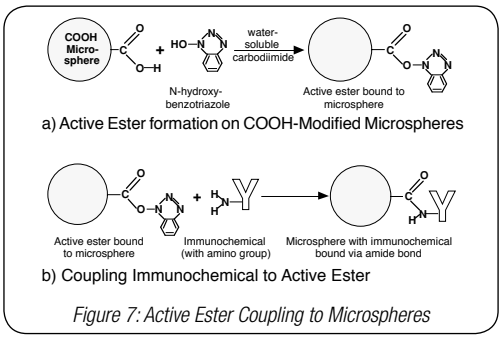


Figure 7: Active Ester Coupling to Microspheres

3. *Two Step Process:* Recent practice seems to borrow from the above two concepts by doing direct coupling in two steps:

1. React CML microspheres with WSC at acidic pH, where the carboxylic acid surface groups are in the COOH form. An active intermediate is formed by the WSC. Clean the latex to remove free WSC. (Note that the first reaction goes rapidly [~15 minutes is enough time], but the active intermediate is unstable and immediately begins to hydrolyze. Hermanson says the rate constant of hydrolysis is 2-3 seconds^{-1} at pH 4.7.46 Thus, cleaning should be rapid to minimize hydrolysis and maximize the number of active sites that will bind protein.)
2. Quickly react activated microspheres with protein at basic pH (where the amino groups on the protein are in the NH_2 form). Clean to remove unbound protein.

While the "two-step" process indeed takes more steps, we think you have better control of each step in the process and can easily avoid forming microsphere clumps and cross-linking of protein to itself.

One idea to simplify things is to use cross-flow systems, especially hollow-fiber membranes, to do all coupling and cleaning steps in the same reactor. (Ask membrane suppliers for assistance.)

4. *One and a Half Step Process:* If you need a simplified "one pot, one pH" process, then I would suggest the following "1.5 step" method:

1. Carefully calculate how many COOH groups you need to activate (dry weight of particles x millequivalents of COOH/gram).
2. Add only enough WSC (at acid pH) to activate all surface groups. This may require some excess.
3. Let this first part of the reaction proceed for ~15 minutes at room temperature.
4. Calculate how much protein you intend to bind to the microspheres.
5. Add only enough protein (in basic pH buffer) to saturate the surface. Such a process should allow covalent binding of protein to microspheres without any extra WSC or protein to permit cross-linking of protein or microspheres.
6. Clean to remove unbound protein.
7. Check for clumps.

E. Beyond WSC

The wide array of microsphere surface chemical groups now available (Table 5) make many coupling chemistries beyond WSC possible.[47] We have two favorite reference works for the wide range of possibilities.[48, 49]

Table 5. Microsphere Surface Chemistries for Covalent Coupling

• -COOH	Carboxylic acid
• -RNH2	Primary or aliphatic amine
• -ArNH2	Aromatic amine or aniline
• -ArCH2Cl	Chloromethyl (vinyl benzyl chloride)
• -CONH2	Amide
• -CONH NH2	Hydrazide
• -CHO	Aldehyde
• -OH	Hydroxyl
• -SH	Thiol
• 	Epoxy

F. GA Binding

Other classical binding techniques include using glutaraldehyde (GA) to bind amino groups on protein to amino-modified microspheres. We recommend using pure glutaraldehyde. It works better if you remove the oligomers /

polymers, which form with age. You can easily remove polymeric GA with activated charcoal; monitor GA for purity, and store monomeric GA at -20˚C.[50]

After reacting an amino group with an aldehyde group, a relatively unstable aldimine or Schiff base is formed. This can be converted to a stable alkylamine by saturation of the double bond by treatment with $NaBH_4CN$.

G. Easiest (?) Binding

Chloromethyl-activated beads have been described by a user as "the simplest to bind to and for non-organic chemists, and the gentlest chemistry for proteins." These sytrene / vinylbenzylchloride copolymers, available in a wide range of monomer ratios, permit easy coupling. One simply adds protein in a pH 7.5 buffer solution to the microspheres, and the reaction of protein amino groups is spontaneous (Figure 8).

Similar easy reactivity is obtained with epoxy-modified microspheres at a pH > 9.

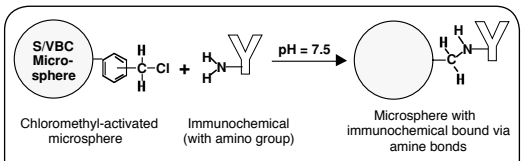


Figure 8: Direct Coupling to Chloromethyl-Activated Microspheres

H. Special Applications

The chemistries in Table 5 will be sufficient in most cases, but other situations deserve special mention.

Covalent Binding to Silica: Silica microspheres have natural hydroxyl surface groups, which can be used for covalent coupling. These silanol surface groups are readily reacted with a wide variety of aqueous or solvent-based silane coupling agents. You can do this yourself, or select functionalized silica microspheres offered with surface functional groups, such as COOH groups. If there is a silane coupling agent available, these chemistries can be put on the silica microspheres. The chemistries are stable and no hydrolysis should be expected at pH < 10.0. Ligand binding is then straightforward.

Lipids: Lipids can be bound via the ω-carboxyl group on the fatty acid chain of the lipid using propylamine surface groups on the silica microspheres.[52] Avanti has special lipid linkers.

Binding Oligonucleotides to Polymeric and Magnetic Microspheres: We suggest WSC coupling of 3'-aminoalkyl-derivatized oligonucleotides to COOH-modified particles.[52] Or react your oligonucleotide with biotin. Biotinylated DNA binds with high affinity to streptavidin-coated microspheres.

I. Quenching Active Coupling Reactions

After your covalent reaction is complete, you may want to ensure that the coupling chemistry is stopped, by adding a reactant which will quench or sop up all remaining active groups. In the case of polymeric COOH groups activated by WSC, for example, one can add ethanolamine, glycine, or other similar chemicals to convert the activated species to hydrophilic groups, which are no longer active, leave no ionic charge, and which are not hydrophobic (and therefore will not cause non-specific adsorption). Choline will also possibly work here. For other groups, pick molecules which are analogous in their reactivity and effect on the system.

III. ACHIEVING OPTIMAL COATING FOR YOUR TEST OR ASSAY

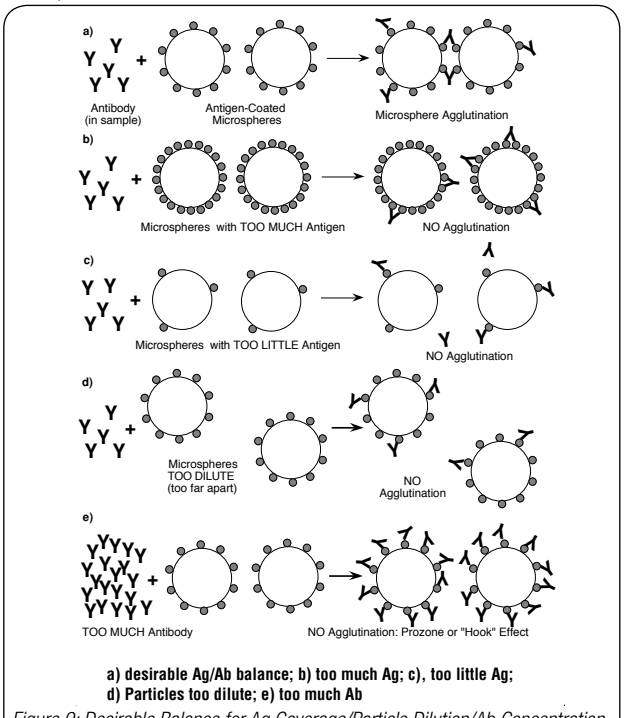
Whether to coat microspheres with protein completely or incompletely depends upon the type of assay. Decide how much protein is needed or wanted on the surface of the microspheres. How far apart should the molecules be?

A. Microsphere Capture ELISAs and Tests, Dyed Particle Sandwich Tests, and Solid Phase Assays

For these uses, one probably wants to coat the microspheres as heavily as possible; a monolayer of protein. Then the Ab will be able to bind the maximum amount of Ag and second Ab (with whatever tag it carries), resulting in a maximum signal.

B. Latex Agglutination Tests (LATs) & Immunoassays; Filter Separation Agglutination Tests & Assays

Proper coating for these tests and assays is critical and requires careful calculation or experimentation to achieve the desired coverage. In the classic view of agglutination, when Ag is bound to the microsphere surface, the desired agglutination begins when the two Ab recognition sites on an IgG molecule react with Ags on separate microspheres, linking the microspheres together (Figure 9a). If Ag is packed too closely on the surface, Ab can bridge between adjacent Ags on a single microsphere rather than linking groups on separate microspheres (Figure 9b). If Ag is too sparsely distributed on the surface (Figure 9c), or the microspheres are too dilute (Figure 9d), agglutination will also be less likely to occur. If too much Ab is present in the sample, it can overwhelm the microsphere coating and prevent agglutination, too (Figure 9e). If there were one Ab for each Ag site on the microspheres, no bridging would occur, and this would prevent agglutination. This is called the "hook" or "prozone" effect.



a) desirable Ag/Ab balance; b) too much Ag; c), too little Ag; d) Particles too dilute; e) too much Ab

Figure 9: Desirable Balance for Ag Coverage/Particle Dilution/Ab Concentration

To determine the proper coating level for LATs, we recommend that you use the "box titration" or checkerboard approach, [53, 54] adapted for slide

agglutination format. This technique is designed to optimize the coverage level of the microspheres and the proper dilution, or ratio, of microspheres to sample for best agglutination sensitivity.

See TechNote 205, *Covalent Coupling*, for information on nucleic acid probe density.

C. Use of Coadsorbant, Surface Diluent, Filler, or Blocker

These materials (Table 6) can be used to promote the adsorption of hard-to-adsorb proteins, like mAbs, by coadsorbing with them. They can also help to space out the Ags or Abs bound to the microsphere surface, to achieve optimum coverage while still permitting outward orientation of the Ab. They also fill in any unoccupied sites on the PS surface, to prevent, or block, unwanted proteins from interfering with, or causing agglutination of, the particles. Depending on the ratio of blocker molecules to desired protein and the strategy for their use, blocker molecules may be put on the microspheres before, during, or after coating with the primary protein (i.e., IgG).

BSA, casein (or non-fat dry milk), gelatin, and Tween® seem to be the most popular blockers. BSA may only be used once, after IgG coating; its effect seems to be permanent, but many users keep BSA in all rinses and storage buffers. Tween® is certainly more labile and will desorb if the equilibrium solution concentration is removed. Therefore, it must be added to every rinse and buffer, in order to keep it on the surface. Effective concentrations are ~1% for BSA, ~0.1 % for casein and 0.01-0.05% for Tween®. Note that, if Ag or Ab have been covalently coupled to microspheres, some people recommend concentrations >1% of Tween® 20 or Triton™ X-100 to prevent nonspecific reactions. [42, 43]

Table 6. Coadsorbants, Surface Diluents, Blockers, or Fillers

- BSA, HSA or ovalbumin - especially fatty acid free grades
- "irrelevant" or neutral IgG or serum
- gelatin / gelatin hydrolysate (enzymatic)
- fish gelatin, fish skin gelatin or fish serum
- casein or non-fat dry milk
- Blotto (normal goat serum + non-fat dry milk)
- surfactants, especially non-ionics (Triton™ X-100, Tween® 20, etc.)
- protein / surfactant combination (especially 1% BSA + 0.05% Tween®)
- miscellaneous specialized commercial blockers55, 56, 57 and polypeptides [e.g., Prionex®, non-animal alternative to BSA]
- synthetic polymers (e.g., PVA, PEG and PVP)
- KLH (keyhole limpet hemocyanin)
- α1-acid glycoprotein

IV. FINAL FORMULATION OF WET AND DRY REAGENTS

A. Microsphere Agglutination Tests

Optimization of the pH, electrolyte, and stabilizer content is important to obtain sensitive, yet stable reagents, which will agglutinate only in the presence of Ag or Ab. One approach to the principles involved is as follows:

1. Coat microspheres with Ag or Ab.
2. Make coating as highly charged as possible (adjust pH), or make coated microspheres highly charged, without reducing the binding of the coating.
3. Adjust electrolyte content until microsphere flocculation almost occurs.[58]

Recipes or protocols for coating microspheres often contain surprises, like the use of DMF as a stabilizer of some sort.[59] Dr. Jacques Singer recently suggested the idea of using aspartame in reagent formulation to neutralize RF interference in patients' serum samples. The idea came from a paper he found about rheumatoid arthritis patients who ingested the artificial sweetener and had fewer and milder symptoms.[60] BLI's TechNotes offer suggested protocols to start from, but each unique combination of assay format, microspheres, protein, and analyte may require a special protocol - not one right out of any book. We urge people to develop their own recipes.

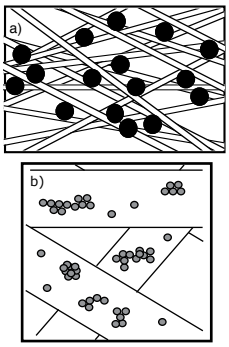
B. Nephelometric/Turbidimetric Assays

Additional consideration must be given to the timing of the agglutination reaction. In an automated instrument, reagent and sample must be mixed and turbidity measured at "zero time." If it takes even 5 seconds to get this first reading, the agglutination of very small (~100nm), fast moving microspheres may already be very far along. The reaction may need to be slowed down, so that after mixing the reagents and sample, a good blank reading can be taken before agglutination actually starts. Dilution of the microspheres will keep the particles apart, and sucrose, polysaccharides, or polyethylene glycol (PEG) will add viscosity, to literally slow them down and perhaps otherwise influence agglutination. PEG content has also been found to influence aggregate size.[61] Salt, at 100 mM, and 3% PEG 6000 are both mentioned as important for formulation of turbidimetric assays. PEG effect may be partially due to exclusion of analyte from hydrated PEG domains which effectively increases analyte concentration.[62]

C. Choosing Filter Media and Membranes

Choose your material depending on the intended use, and whether the microspheres are to be caught on a filter to stay, or migrate through a membrane like a chromatographic strip. Gibbs recommends cellulose acetate (a non-binding membrane) for general filtration, concentrating microspheres, membrane capture assays, or for bead washing;[23] if you want to bind protein-coated microspheres to a membrane, then a more hydrophobic membrane, like nitrocellulose or nylon, would be good.

Physical Entrapment of Particles on Filter (PCELISAs): For the first particle capture ELIST, Hybritech chose an inert fiber to capture ~1µm coated microspheres. Scanning electron micrographs (SEMs) from their early work showed a physical entrapment of the microspheres in the intersections of the filter fibers (like Figure 10a).



a. "Balls and Sticks" or physical entrapment: Microspheres too large to pass through fiber filter (like Hybritech ICON®)

b. Beads sticking to fibers (like Abbott Test Pack® and IMx®)

Figure 10: Particles Caught on Filters or Membranes

Xenith Biomed (Ireland) offers a device (like the Hybritech ICON®) which combines a membrane, designed to physically capture microspheres, with an absorbent base which wicks reagents and wash solutions away from the membrane and microspheres. To catch the microspheres on the surface of the membrane, they recommend that the microsphere diameter/membrane porosity ratio be significantly > 1 (i.e., with a 1µm membrane, you might need microspheres with diameter > 1.3µm). Similarly, Costar recommends

capturing 0.3µm microspheres on a 0.2µm membrane (a bead/porosity ratio of 1.5).

If a filter is used to physically entrap the microspheres, as Hybritech did, then we recommend microspheres ~50% larger than the filter porosity to ensure catching them at the top of the filter.

Chemical Interaction of Particles Sticking to Filters (PCELISAs): For their particle capture ELISTs and ELISAs (Test Pack®, IMx®, and AxSym®), Abbott chose a glass fiber filter, whereon small coated microspheres seemed to stick to the fibers of the filter - a very different capture mechanism. SEMs from Abbott IMx® product literature clearly show microspheres adhering to the fibers of the filter (like Figure 10b), not physically entrapped.

An anonymous recipe, from a university researcher, for binding microspheres to glass fiber filters is as follows:

1. Saturate glass fiber filter, like Whatman GF/D (2.7µm porosity), with protein-coated ~0.5µm PS or CML microspheres.
2. Incubate for 30 minutes in 100% humidity chamber (to prevent drying).
3. Wash 3 times with phosphate-buffered saline.
4. Allow to dry.

D. Membrane or Chromatographic "Strip Tests"

Ordinarily, cellulose nitrate (nitrocellulose) or nylon membranes are recommended to immobilize capture proteins (refer to TechNote 203, *Lateral Flow Tests*, for more information regarding this type of test) in the test and control windows. The idea is to immobilize anti-hCG Ab_2 in a line placed in the first, or larger, test result window. This line must capture and hold the microspheres coated with Ab_1 and hCG. In the second, smaller, control window polyclonal anti-Mouse Ab_3 could be placed in a line to capture m-Ab coated microspheres which get past the test window. To be successful, both of these protein lines must survive drying and rehydration to remain in place.

Meanwhile, the dyed Ab_1-coated microspheres are sometimes dried directly upon the nitrocellulose or nylon strip. They must remain in place until the strip is wet with sample (urine). Then, they must rehydrate readily, redisperse as single beads (no clumps, please!) and move freely along the membrane, stopping only when they encounter the Ab_2 or Ab_3 lines in the windows downstream. But, one might expect that the Ab_1-coated microspheres would also naturally stick to the nitrocellulose (just as the Ab_2 or Ab_3 lines stick). How does one get the Ab_2 or Ab_3 lines to stick, while preventing the Ab_1-coated microspheres from sticking, after rehydration with sample?

One way to protect the Ab_1-coated microspheres from sticking permanently is to pretreat the microspheres and / or the membrane where they will be dried with something very hydrophilic. Then, when the sample is added, it will readily rehydrate the microspheres and they will be released from the surface and move freely along the strip with the liquid flow. This release agent could be protein (like blockers), surfactant, and/or saccharides like sucrose or trehalose applied before the beads are laid down (Figure 11).

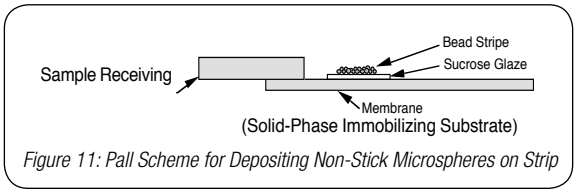


Figure 11: Pall Scheme for Depositing Non-Stick Microspheres on Strip

Triton™ X-100 and Tween® 20, among others, are known to be effective at blocking microspheres from nonspecific binding (adsorption) of protein. Therefore, surfactants like these could be applied carefully and precisely on the right part of the membrane so they do not interfere with placement of Ab lines. Kits of 16 different polymers and 25 different surfactants for use on strip tests are available for testing especially for strip tests.[63]

Consult with your membrane supplier for specific protocols and for membrane selection for use with strip tests (or see TechNote 303, *Lateral Flow Tests*, for more information).

Another way to keep Ab_1 microspheres from sticking is to deposit the dyed Ab_1-coated microspheres in or on the hydrophilic sample receiving pad. When the sample is added to the pad, the microspheres will be released from the pad, flow onto the membrane and along the membrane to the immobilized Ab strips.

Roche has modified this format for strip tests with more sensitivity as follows: Ab_1-coated, dyed microspheres are placed in a second, conjugate support pad above the strip, downstream from the sample receiving pad (Figure 12).[64, 65] Thus, sample flow down the strip is split, with some liquid staying in the main strip and some liquid going through the second pad. This split sample flow causes dyed microspheres to be metered into the primary strip and migrate gradually along the strip to the immobilized Ab_2 line. This spreading out and slowing down of the flow of dyed microspheres makes for a more sensitive test. Each microsphere has a better chance of finding some Ag (to cause it to bind) and a binding spot on the Ab_2 line. Compare this to the earlier situation, where all dyed microspheres may arrive at once, and before the Ag gets there, and many will flow past the Ab_2 line without ever finding a spot to bind to or enough Ag to cause binding.

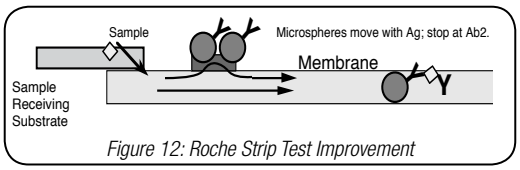


Figure 12: Roche Strip Test Improvement

All strip tests mentioned above are unidirectional. Other companies, like IDEXX (Portland, ME) and SmithKline Diagnostics (San Jose, CA), have made flow tests and devices which are bidirectional for better test performance. In one case, the Ab-containing sample flows one way and reacts with the Ag bound on the strip. Then, after an operator manipulation, the reagent with Ab-dyed microsphere conjugate flows in the other direction.[66]

Strip Test Microsphere Diameter/Membrane Porosity Ratio: For chromatographic strip tests, one needs dyed microspheres which are small enough to move freely through the membrane. The microsphere diameter/membrane porosity ratio should therefore be < 1, perhaps < 0.1, for good flow properties (i.e., with a 5µm membrane, you might need microspheres with a diameter < 0.5µm, maybe 0.3µm). But, small microspheres will not give as intense a color signal as will larger, darkly dyed microspheres. So, choose the best compromise between small size for mobility and large internally colored microspheres for good sensitivity.

Filter, device, and equipment companies who know microspheres are shown in Table 7.

Table 7. Suppliers with "Particular" Interests

- Filter Membrane Suppliers
  - Corning-Costar (suggestions for beads/membrane selection)
  - Millipore (membranes with suggestions for chromatographic strip assays[67])
  - Pall BioSupport[68] (variety of membranes and supports)
  - Sartorius (variety of membranes and supports)
  - Schleicher & Schuell[69] (variety of membranes and supports)
  - Whatman[70] (variety of membranes and supports)

- Devices and Equipment Manufacturers
  - Bio•Dot, Irvine, CA & Huntingdon, UK (striping and slitting machines)
  - Dexter Magnetics Corporation, Freemont, CA (magnets and systems for magnetic particle separation)
  - Ismeca USA, Carlsbad, CA (striping dispensers for particles and Ab lines)
  - IVEK, North Springfield, VT (striping machines for particles and Ab lines)
  - Kinematic Automation, Twain Harte, CA (dispensing, striping, and slitting)
  - Xenith Biomed, Galway, Ireland (LAT track slides and ICON-like devices for your tests)

E. More Ideas for Preventing Microspheres from Sticking to the Membrane, and Other Ideas

1. *Wet Reagent Scheme:* Do not allow the small, dyed Ab_1-microspheres to dry on the strip and become bound there. Use Ab_1-microspheres as a liquid or reconstituted / lyophilized reagent. Mix sample with Ab_1-coated microspheres; add mixture to the strip, beads immediately start to migrate to Ab_2 (bound to the strip). (This might not be practical for an OTC test, but might be useful to determine if beads will move properly on the strip.)

2. *"Boulders in a Stream" Scheme:* Use a membrane like cellulose acetate, which does not bind protein or protein/microspheres well. Then, particles won't get stuck after drying. The membrane is only the conduit for moving sample and dyed marker microspheres. The capture proteins (Ab_2 and Ab_3) are immobilized in their respective windows by binding onto large, colorless Ab-coated microspheres (beads). These beads, which are too big to move through the membrane, would be sprayed or printed on the membrane and would never move. The ratio of microsphere diameter / membrane porosity should be > 1 and perhaps > 2, so that, like boulders in a stream, they don't have a chance of moving with the sample flow. Small dyed microspheres with Ab1, which were dried in place, are easily rehydrated by the sample and move downstream toward the large colorless microspheres with Ab_2 (and Ab3). This is essentially the same as Carter-Wallace's concept for their First Response® 1-step test (See Figure 11 in TechNote 301, *Immunologic Applications*.)

1 + 2 = 3. "Wet Boulders?": Some Japanese researchers used both of the above ideas for plant disease tests for cucumber mosaic virus and tobacco mosaic virus. [71] They put large Ab_2-coated beads onto a strip, where they were dried. Then they dipped the strip into a mixture of Ab_1-coated, dyed microspheres and sample. The dyed particles migrated up the strip and formed a colored line if Ag was present in the sample.

4. Color Coded Tests: For several tests run together, like the Biosite 8 drug test panel, different colors of dyed particles could be coated with Abs to different analytes for color coded tests. (ANI Biotech Finland has done this already.)

5. Creative Flow Patterns for Strip Tests: Study the Roche improvement (Figure 12) and the bidirectional tests. Think about other ways to direct the flow in a strip to achieve better results. Consider printing liquidic circuits by the folks at Wolfson Applied Technology (University of Birmingham, UK).[72]

F. Strip Test References

Since the success of the Unipath ClearBlue One Step™ immunochromatographic strip test, many folks are working to develop similar tests. We have found a good number of patents and patent applications covering this field.[73-90] Undoubtedly there are more. You must decide applicability to your case.

Millipore and Whatman have good materials for better understanding of membranes and their interactions with proteins, surfactants, and microspheres.[91, 92]

V. FINAL WORDS

A. About Development Strategy

Immunoassay research seems to progress as follows: 1. screen Abs for an analyte; 2. find coupling method for Ab to microsphere; 3. develop the assay; 4. if something does not work right in Step 3, return to Step 2. Repeat for the next assay.

The folks at Biotrol (now BioMerieux) adopted an alternative strategy adopted at Biotrol during the development of assays for the MAGIA 120 immunoassay system, using carboxylate-modified magnetic microspheres. Early in the process, looking ahead to production problems which they could solve in advance, they decided to select only one binding method for all assays to simplify the manufacturing process, even though it might make the research phase longer and more difficult. Then they screened mAbs to fit the binding and assay methods. This did make extra research work. For example, for their TSH assay, they screened > 400 clones to get the right one for their binding and assay methods.[93] But now their production people are smiling or at least they have fewer headaches!

B. "Take the Time!"

I have a sign with the above message to remind me to do things the right way the first time. You do not need to "reinvent the wheel" or check everything, but we recommend that you monitor your progress to know where you are after every step of your process.

An older, experienced lab technician I worked with at Dow had this sign over his desk:

"Why is there never enough time to do it right the first time, but always enough time to do it again?"

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