

Recommended Adsorption and Covalent Coupling Procedures

Introduction

Our strength is in offering you a complete microparticle technology. We give you simple, validated protocols for coupling proteins to microparticles (MPs). We take the mystery out of working with MPs by giving you concrete data, backed by years of applications research in our own labs. These recommended coupling procedures are designed for:

- Optimal adsorption of proteins to MPs
- Optimal covalent coupling of proteins to MPs
- Choice of two protocols for covalent coupling
- Simplicity, efficiency, and confidence

A comprehensive laboratory reference manual, *Microparticle Reagent Optimization*, on microparticle sensitization and optimization is available us. This manual is the third in a series of our technical publications. To order, call Customer Service at 1-866-737-2396.

Principles of Protein Binding

Proteins may be bound to polystyrene (PS) or carboxylate-modified (CM) particles by adsorption. Adsorption is mediated by hydrophobic and ionic interactions between the protein and the surface of the microparticles. Proteins may also be covalently attached to the surface of CM-MPs. Carboxyl groups on the MPs, activated by the water-soluble carbodiimide 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC), react with free amino groups of the adsorbed protein to form amide bonds.

Here we give the protocols for both adsorption and covalent coupling which have proven useful. These are written for 1.0 mL “optimization series” reactions. For larger reactions, all volumes may be scaled up proportionally.

Materials

Polystyrene Microparticles (PS-MPs)

PS-MPs for immunoassays are available in standard sizes ranging from 0.05 μM to 2.0 μM . Larger particles are also available. These PS-MPs are manufactured by emulsion polymerization using an anionic surfactant and have surface sulfate groups which arise from the polymerization initiator. PS-MPs are formulated to have low free surfactant, and, generally, the surfactant used does not interfere with protein binding. For this reason, it is recommended that PS-MPs be used without any preliminary clean-up.

Carboxylate Modified Microparticles (CM-MPs)

CM-MPs are available in sizes ranging from 0.05 μM to 1.5 μM . These CM-MPs are manufactured by the copolymerization of styrene and acrylic acid using emulsion polymerization methods. CM-MPs are available in a wide range of carboxyl densities. Titration values in milliequivalents of carboxyl per gram of microparticles (mmoles/g, or $\mu\text{moles/mg}$) are provided with each lot. In addition, the calculated parking area (area per carboxyl group) is provided with each lot (Note 1).

CM-MPs are formulated to have low free detergent. The detergent used does not generally interfere with protein binding. CM-MPs may be rigorously cleaned by ion exchange with mixed bed resin or by tangential flow filtration (Note 2). Such cleaning removes various ionic byproducts, including detergent, soluble polymers, and buffer salts, which may affect coupling chemistry. The need for preliminary clean-up of CM-MPs must be established on a case-by-case basis.

BCA Protein Assay

See Microparticle-bound Protein Assay” bulletin.

MES Buffer

Prepare 10X *stock* buffer at 500 mM, pH 6.1. The pH will not change significantly on dilution. Store at 4°C and discard if yellowed or contaminated.

EDAC 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride

52 $\mu\text{mol/mL}$: Just before use, weigh approximately 10 mg of EDAC on an analytical balance: for each 10.0 mg weighed add 1.0 mL deionized water. Note that EDAC is very sensitive to moisture and undergoes rapid hydrolysis in aqueous solutions. EDAC should be stored in a desiccator at -5 °C and brought to room temperature before weighing.

NHS N-hydroxysuccinimide
50 mg/mL in water (very soluble)

Protein Stocks

The protein to be coated onto MPs should be completely dissolved and not too concentrated. When the protein is added to the MPs, rapid mixing is critical for even coating. Typically, a protein stock in the range of 1-10 mg/mL is recommended. When working at a 1 mL scale, pipet the protein stock directly into the buffered MPs, and using the same pipet tip, “syringe” the solution (mix up and down quickly). When working at a larger scale, have the MPs in a beaker with a stir bar, mixing well, and add the protein stock quickly into the middle of the vortex.

Mixing Wheel or Other Device

Gentle, constant mixing is important for microparticle reactions.

Microcentrifuge and Related Supplies

For optimization scale, it is convenient to run coupling reactions in microcentrifuge tubes. With conventional microcentrifuges such as Eppendorf, coated particles of 0.150 μM or greater diameter are pelleted in 10-30 minutes. Smaller particles may require tangential flow filtration or ultracentrifugation (see Notes 2, 3).

Probe-type Ultrasonicator

Probe-type ultrasonicator with microtip should be used for resuspending pellets during washing (see Note 4). Sonication is also helpful for redispersing clumped particles in a stabilizing buffer.

**Adsorption
Procedure:
for PS-MPs or
CM-MPs**

1. Set up binding reaction by pipetting into microcentrifuge tubes in the order given:

50 μL of 500 mM stock MES buffer: 25 mM final
Water to make 1.0 mL final volume
100 μL of 10.0% solids stock microparticles: 1.0 % solids final
Protein stock solution (Note 5)

Note that the protein should be added last and mixed very rapidly into the reaction mixture by syringing repeatedly with the pipettor. Improper mixing can yield unevenly coated microparticles.

2. Mix tubes at room temperature on a mixing wheel or other device for one hour.
3. Remove unbound protein: first, pellet microparticles and decant the

supernatant. Next, perform *two* washes with fresh 50 mM MES buffer, pH 6.1. Resuspend pellets between washes by ultrasonication (see Note 4). Alternatively, a tangential flow membrane device may be used for washing (see Note 2).

4. Resuspend final pellet to 1.0% solids by adding 0.97 mL of the same buffer. Adsorbed proteins may elute from the MP surface if the wash/storage buffers are different from the coupling buffer. Many detergents will elute adsorbed proteins and should not be used with the adsorption protocol.
5. BCA Protein Assay: microparticle preparation can be assayed for total bound protein by following our “Microparticle-bound Protein Assay” bulletin.

One-Step Covalent Procedure: for CM-MPs Only

1. First, calculate the amount of EDAC required. It is recommended that approximately a 0.5 to 2.5 fold molar excess over microparticle carboxyl concentration be used (this should be optimized).

Note that (MP acid content) meq/g is equivalent to $\mu\text{mole}/\text{mg}$.
Note that 1 mL of 1% MP contains 10 mg MP.

(Acid content, $\mu\text{mol}/\text{mg}$) (10 mg microparticles) (desired ratio)
= μmol EDAC required
(μmol EDAC required) / (52 $\mu\text{mol}/\text{mL}$) = mL EDAC stock per mL of reaction.

2. Set up binding reaction by pipetting into microcentrifuge tubes in the order given:

50 μL of 500 mM stock buffer: 25 mM final
Water to make 1.0 mL final volume
100 μL of 10.0% solids stock microparticles: 1.0 % solids final
Protein stock solution (Note 5)
Mix the tubes for about 15 minutes on a mixing wheel at room temperature.

3. Prepare the EDAC solution immediately before use and mix the calculated volume rapidly into the reaction by syringing repeatedly with the pipettor.
4. Mix tubes at room temperature on a mixing wheel or other device for one hour. MPs may clump during this time, but this is not unusual or harmful (Note 6).

5. Remove unbound protein: first, pellet microparticles and decant the supernatant. Next, perform *two* washes with fresh 50 mM buffer. Resuspend pellets between washes by ultrasonication.
7. Resuspend final pellet to 1.0% solids by adding 0.97 mL of a buffer that does not contain blocking proteins. This may be the MES buffer or a higher pH buffer of your choice.
8. BCA Protein Assay: microparticle preparation can be assayed for total and covalently bound protein following our “Microparticle-Bound Protein Assay” bulletin.
9. For long term colloidal stability, a stabilizing storage buffer will be needed (Notes 7-9). After protein assay, coated MPs can be centrifuged and resuspended in a variety of storage buffers, and the colloidal stability and reactivity optimized.

**Preactivation
Covalent
Procedure:
for CM-MPs only**

1. Preactivation step: pipet into microcentrifuge tubes in the order given:
 - 100 μ L of 500 mM MES buffer: 50mM final
 - Water to make 1.0 mL final volume
 - 100 μ L of 10.0% solids stock microparticles: 1.0 % solids final
 - 230 μ L NHS solution: 100 mM final
 - EDAC solution, calculated amount
2. Mix tubes at room temperature on a mixing wheel or other device for 30 minutes.
4. Centrifuge and discard supernatant. Resuspend MPs with 1 mL 50 mM MES buffer, pH 6.1. Centrifuge again, discard supernatant.
5. Resuspend pellet by adding the following and sonicating:
 - 100 μ L 500 mM MES buffer: 50mM final
 - Water to make 1.0 mL final volume
 - Protein stock solution (Note 5)
6. Mix tubes at room temperature on a mixing wheel or other device for 1 hour.
7. Remove unbound protein: First, pellet microparticles and decant the supernatant. Next, perform two washes with fresh 50 mM buffer. Resuspend pellets between washes by ultrasonication.
8. Resuspend final pellet to 1.0% solids by adding 0.97 mL of a

buffer that does not contain blocking proteins. This may be the MES buffer or a higher pH buffer of your choice.

9. BCA Protein Assay: microparticle preparation can be assayed for total and covalently bound protein using our Microparticle-Bound Protein Assay bulletin.
10. For long term colloidal stability, a stabilizing storage buffer will be needed (Notes 7-9). After protein assay, coated MPs can be centrifuged and resuspended in a variety of storage buffers, and the colloidal stability and reactivity optimized.

Procedure Notes

1. Parking area (PA) is a parameter that allows comparison of CM MPs of different diameters and titration values (meq/gm). It is an area-normalized density of carboxyl groups, given in $\text{\AA}^2/\text{COOH}$. If two particles have the same PA, a particular protein molecule will “park on” the same number of carboxyl groups on the surface of either particle, and have an equivalent opportunity for covalent coupling (provided all the carboxyls are activated).
2. Tangential flow membrane devices are available from Microgon Corporation in sizes suitable for processing microparticles in mL to liter quantities. Particles as small as $0.05\ \mu\text{M}$ may be reliably processed with Microgon membranes. For more detailed information contact us at 1-866-737-2396.
3. Small particles ($<0.150\ \mu\text{M}$ diameter): longer centrifugation times are needed for smaller particles, and the pellets are more difficult to resuspend. Colloidal stability problems increase with decreasing MP diameter. Lowering the percent solids in the coupling step to 0.5 % instead of 1 % will help prevent clumping during coupling. Washing by tangential flow filtration rather than centrifugation will also help.
4. An immersible ultrasonic probe is the ideal tool for efficient resuspension of MP pellets. For 1.0 mL reactions a few seconds of sonication is sufficient. Alternatively, pellets may be stirred or resuspended by repeated aspiration with a fine pipet tip. Vortex mixing and bath-type sonicators are not effective for resuspending most pellets.
5. The optimum amount of protein to use depends on several factors:
 - (1) Surface area available: surface area per mg of MP increases linearly with decreasing MP diameter.
 - (2) Colloidal stability: proteins can have stabilizing or destabilizing effects on Mps.

(3) Immunoreactivity: the optimum amount of bound sensitizing protein must ultimately be determined by functional assay.

Performing a protein titration or binding isotherm is a good first experiment. For a 0.3 μM diameter MP, a reasonable starting range would be 10-200 μg protein/mg MP.

6. The MPs may clump during coupling due to the electrostatic effect of the positively charged EDAC molecules, the effect of the protein itself, or consumption of negative charge by amide bond formation. Washing into fresh buffer to remove EDAC and unbound protein, followed by sonication, generally reverses the clumping. Long term colloidal stability of coated MPs requires development of the right storage buffer (see below).
7. The selection of storage buffer and pH is one of the single most critical variables in achieving optimum microparticle performance. Zwitterionic buffers such as MOPSO, blocking proteins such as bovine serum albumin (BSA) and fish skin gelatin (FSG), higher pH, detergents and sodium salicylate have all proven to be useful for stabilizing microparticle preparations while permitting specific agglutination reactions to occur.
8. Blocking proteins with high negative charge, such as BSA and FSG, may be used to add colloidal stability as well as block the surface against nonspecific sample adsorption. FSG works especially well with antibody-coated microparticles.
9. Covalently bound protein will not elute when subjected to detergent washes or buffer changes; thus, covalently coupled reagents are compatible with a wider variety of buffer additives than reagents where the proteins are merely adsorbed to the particles.

Other Products

You are cordially invited to visit our facilities any time you are in the Indianapolis area. We are just minutes from the Indianapolis International Airport. Please inquire about our other exceptional microparticle products. We have a complete range of microparticles to suit your individual requirements.