Spherotech, Inc.
1840 Industrial Dr., Suite 270, Libertyville, IL 60048

SPHERO™ Technical Note **STN-1 Rev B. 092004**

PARTICLE COATING PROCEDURES

Introduction

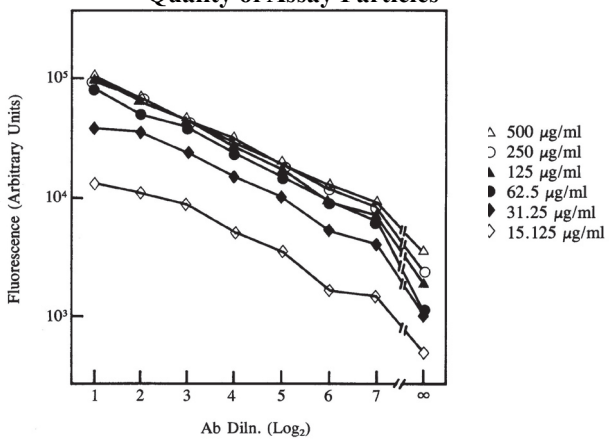
Currently, there are several methods of attaching biological ligands to polystyrene particles. These methods include adsorption to plain polystyrene particles, covalent attachment to surface functionalized particles, and attachment of the ligand of interest to particles that are pre-coated with a binding protein such as Streptavidin, Protein A or Protein G. Presented in this Spherotech Technical Note are protocols such as adsorption, covalent coupling, and other methods used to attach ligands to polystyrene particles .

Procedures and Disscussion

The following information explains generalized protocols for the attachment of ligands to polystyrene particles. These protocols are easily optimized to meet the requirements for specific applications. The following protocols are developed by Spherotech for the convenience of SPHERO™ microparticle users. **They are to be utilized only as initial conditions.** Spherotech encourages the optimization of the coating conditions by changing the buffer, pH or reagents concentration.

In general, polyclonal antibodies are coated to polystyrene particles by adsorption without using any coupling agents. The binding of polyclonal antibodies to polystyrene particles is strong. However, care should be taken not to over load the antibodies to the particles. If over loading occurs, leaching of the coated antibody will happen during storage. This is due to the weak interaction between antibody molecules compared to the interaction of antibody molecules to the surface of polystyrene particles. As shown in Fig. 1., the optimal antibody to particle ratio for passive adsorption is between 62.5 µg to 125 µg/mL of 0.5% w/v (5.0 mg solid / mL) of 0.8 µm polystyrene particles[1].

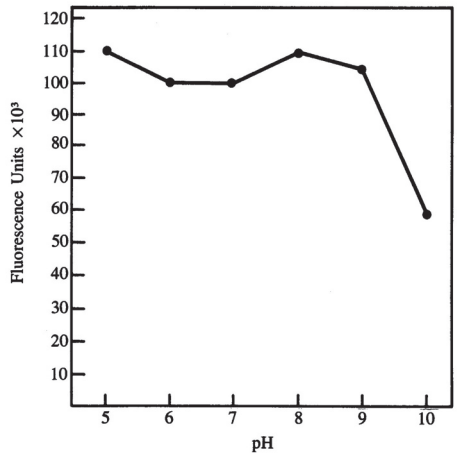
Fig. 1. Effect of Coating Ligand Concentration on Quality of Assay Particles



Performance of polystyrene latex particles coated at pH 7.0 with various concentrations of human IgG.

For 4.0~4.5 µm magnetic particles the optimal antibody to particle ratio is around 250 µg/mL of 2.5% w/v magnetic particles. If different size particles are used, the antibody to particle ratio will need to be adjusted according to the surface area of the particles as compared to the 0.8 µm particles. When the same solid weight of particles are used the total surface area is inversely proportional to the size of the particles. For example, the same weight of 2.0 µm particles will have half as much of the surface area as that of 1.0 µm particles. Likewise, the same weight of 3.0 µm particles will have one third as much of the the surface area as that of 1.0 µm particles. The buffer pH during passive adsorption of antibody to the particles can range from pH 5 to pH 9 as shown in Fig. 2., where pH 5.0 is sodium acetate, pH. 6, 7 and 8 is phosphate and pH 9 and 10 is carbonate[1]. An acidic buffer of pH 5.0 such MES, phosphate or acetate buffer is preferred for covalent coupling of proteins to carboxyl particles. Spherotech has used both acetate or phosphate buffer with EDC without encountering any problems.

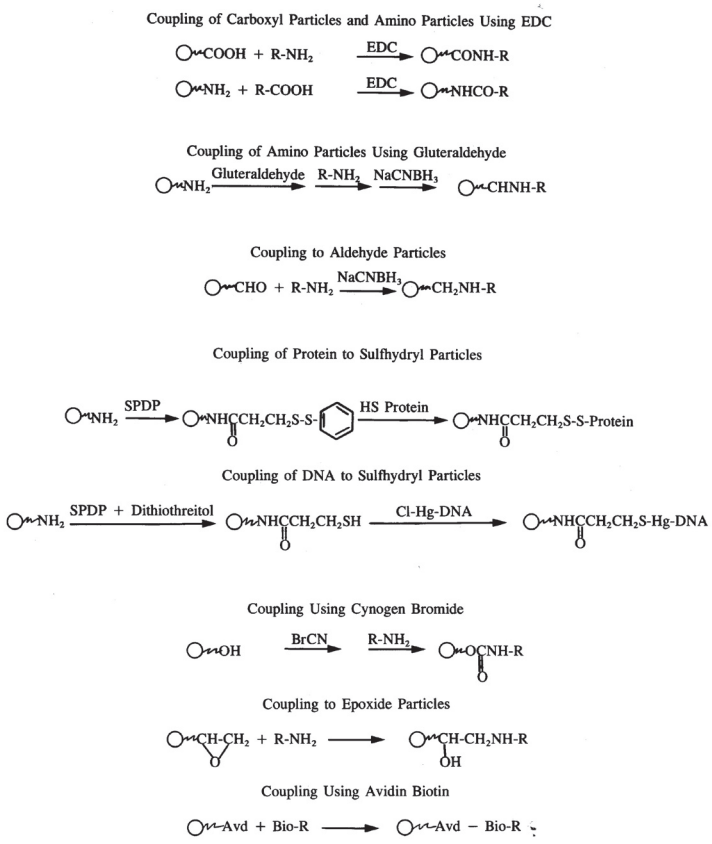
Fig. 2. Effect of Buffer pH on the Quality of ParticleCoating



Human IgG was incubated with plain polystyrene particles for 2 hours at ambient temperature. 0.1M buffers of varying pH (sodium acetate pH 5, sodium phosphate pH 6-8, sodium carbonate pH 9,10). Quality of coating was determined through PCFIA using labeled anti-human IgG.

Typically, centrifugation is used for removing unbound proteins after coating for polystyrene particles larger than 0.4 µm. On the contrary, gel filtration, dialysis or diafiltration is used for particles smaller that 0.4 µm. The magnetic particles are processed easily during the coating with a commercially available magnetic separator. The FlexiMag Separator or FlexiMag Separator, Jr., which are available from Spherotech, will provide a very convinient and cost effective way of cleaning magnetic particles after coating. A schematic presentation of covalent coupling of ligands to functionalized particles is shown in Fig. 3.

Fig. 3. Covalent Coupling of Ligands to Particles



General Procedures For Particle Coating

Passive adsorption:

1. Add the following to a 15 mL glass centrifuge tube:
   a. 1.8 mL of phosphate buffer, 0.1 M, pH 7.4
   b. 0.2 mL of 1 mg/mL protein solution
   c. 0.2 mL of 5% w/v 0.8 µm polystyrene particles
2. Vortex and incubate for at least one hour at ambient temperature.
3. Centrifuge at 3000x g for 15 minutes
4. Remove the supernatant carefully.
5. Add 4 mL of Isotonic Buffered Saline (IBS).
6. Mix well using a vortex mixer.
7. Centrifuge at 3000x g for 15 minutes.
8. Remove the supernatant carefully.
9. Add 4 mL of IBS and mix well to obtain 0.25% w/v suspension.

Note: 1. For 4.0~4.5 µm magnetic particles, use 250 µg of proteins per mL of 2.5% w/v magnetic particles and FlexiMag Separator orFlexiMag Separator, Jr. for the separation of particles in Steps 3 and 7.

2. This procedure is used for the passive adsorption of immunoglobulis, antigens or other ligands to polystyrene particles or polystyrene magnetic particles.

Coating of Amino Particles with Ligands or Proteins Using EDC

Covalent Coupling (one step EDC coupling):

1. Add the following to a 12x75 mL glass centrifuge tube:
   a. 2 mL of phosphate buffer, 0.1 M, pH 7.4
   b. 2 mL of ligands or proteins
   c. 2 mL of 5% w/v 0.8 µm Amino particles
   d. 20 mg of EDC
2. Vortex and incubate for two hour at ambient temperature on a rotary mixer or with occasional vortexing or shaking.
3. Centrifuge at 3000x g for 15 minutes
4. Remove the supernatant carefully.
5. Resuspend the pellet in 4 mL of Isotonic Buffered Saline.
6. Repeat Steps 3 and 4 and resuspend the pellet in 2 mL of IBS to obtain 2 mL of 5% w/v suspension.

Note: 1. For 4.0~4.5 µm magnetic particles use 0.5 mg of ligands or proteins per 2 mL of 2.5% w/v magnetic particles and 5 mg of EDC. Use the FlexiMag Separator orFlexiMag Separator, Jr. for the separation of particles in Step 3.

2. For 1.0~2.0 µm magnetic particles, use 1.0 mg of ligands or proteins per mL of 2.5% w/v magnetic particles and 10 mg of EDC.

3. EDC(1-ethyl-3(-3-dimethylaminopropyl) carbomiimide hydrochloride), Sigma Chemical Cat. No. E7750.

Coating of Carboxyl Particles with Avidin Using EDC

Covalent Coupling (one step EDC coupling):

1. Add the following to a 15 mL glass centrifuge tube:
   a. 2 mL of sodium acetate buffer, 0.01 M, pH 5.0
   b. 2 mg of Avidin or Streptavidin
   c. 2 mL of 5% w/v 0.8 µm Carboxyl particles
   d. 20 mg of EDC
2. Vortex and incubate for two hour at ambient temperature on a rotary mixer or with occasional vortexing or shaking.
3. Centrifuge at 3000x g for 15 minutes
4. Remove the supernatant carefully.
5. Resuspend the pellet in 4 mL of Isotonic Buffered Saline.
6. Repeat Steps 3 and 4 once and resuspend the pellet in 2 mL of IBS to obtain 2 mL of 5% w/v suspension.

Note: 1. For 4.0~4.5 µm magnetic particles, use 0.5 mg of Avidin per 2 mL of 2.5% w/v magnetic particles and 5 mg of EDC. Use the FlexiMag Separator or FlexiMag Separator, Jr. for the particle separation in Step 3.

2. This procedure is also for covalent coupling of other proteins such as monoclonal or polyclonal antibodies, antigens or other ligands. Acidic buffers such as phosphate, 0.1M or MES, 0.05 M can be used instead of acetate buffer.

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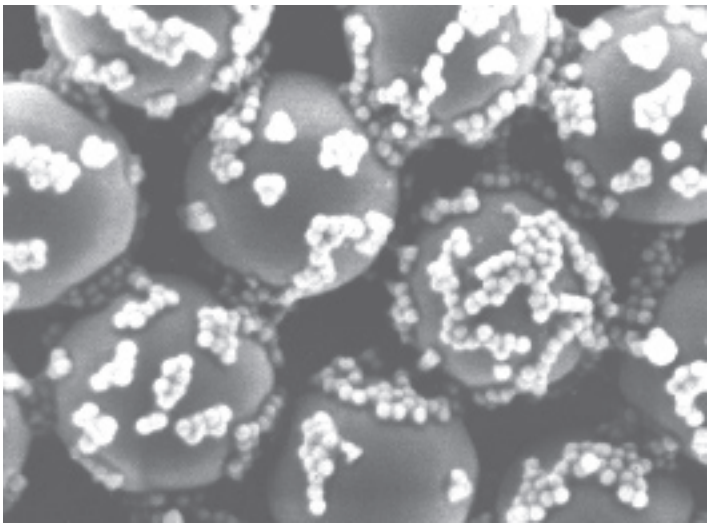
Covalent Coupling (two step EDC coupling):
For two step EDC coupling, wash the particles with coupling buffer, centrifuge and remove ~80% of the supernatant. Add EDC to the pellet, mix, and incubate for 1 hour. Wash the particles with coupling buffer and resuspend with protein solution. Continue with Steps 2 to 6 of the Covalent Coupling (one step) procedure.

Coating of Avidin Particles with Biotinylated Proteins

Affinity Coupling:
1. Add the following to a 15 mL glass centrifuge tube:
   a. 2.0 mL of biotinylated protein (100 µg/mL protein) in sodium phosphate buffer (PB), 0.1 M, pH 5.5
   b. 0.2 mL of Avidin coated polystyrene particles, 5% w/v
2. Vortex and incubate for at least one hour at ambient temperature.
3. Centrifuge at 3000x g for 10 minutes
4. Remove the supernatant carefully.
5. Resuspend the pellet in 4 mL of 0.1M PB.
6. Repeat Steps 3 and 4 once and resuspend the pellet in 4 mL of PB to obtain 4 mL of 0.25% w/v suspension.

Note: 1. This procedure is also used for coating biotin-polystyrene or magnetic particleswith various avidin-protein conjugates or other avidin-ligand conjugates.

Scanning Electron Microscope (SEM) photo of 0.4 µm Avidin fluorescent particles binding to the surface of 6.0 µm Biotin coated polystyrne particles.



Coating of Protein to Hydroxyl Particles

Covalent Coupling using Cyanogen Bromide (CNBr):
1. Add 2 mL of 1.25% w/v 0.8 µm hydroxyl polystyrene particles to a centrifuge tube:
2. Adjust the pH to 10.5 with 1N NaOH.
3. Add 10 mg of CNBr in a fume hood.
4. Readjust the pH to 10.5 with 1N NaOH.
5. Incubate for 15 minutes.
6. Add 2 mL of cold borate buffer (0.1M, pH 8.5).
7. Cool to 4^{o}C and add 1 mL of protein at a concentration of 1 mg/mL.
8. Incubate at 4^{o}C for at least four hours.
9. Add 5 mL of glycine buffer (0.1M, pH 8.5).
10. Centrifuge for 30 minutes at 2000x G.
11. Remove the supernatant and resuspend the pellet in 10 mL of 0.1M phosphate buffer, pH 7.2.
12. Repeat Steps 10 and 11 twice to give 10 mL of particles at 0.25%.

Coating of Dimethylamino Particles with DNA

Ionic Interaction Coupling:
1. Add the following to a 15 mL glass centrifuge tube:
   a. 100 µL of 0.25% w/v 0.8 µm dimethylamino particles in carbonate buffer, 0.1 M, pH 9.0
   b. 5.0 µL of DNA (200 ng/mL) in 0.1M carbonate buffer, pH 9.0
2. Vortex and incubate for three hours at ambient temperature.
3. Centrifuge at 3000x g for 15 minutes
4. Remove the supernatant and resuspend the pellet in 150.0 µL of 0.1M carbonate buffer, pH 9.0.
5. Centrifuge at 3000x g for 15 minutes
6. Remove the supernatant and resuspend the pellet in 100.0 µL of 0.1M tris buffer, pH 7.5. Final particle concentration is 0.25% w/v. Store rerigerated.

Coating of Epoxy Particles with Proteins

1. Add 1 mL of 5 µm, 5%w/v epoxy polystyrene particles to a microfuge tube.
2. Microfuge at 15000 rpm for 20 seconds.
3. Aspirate the supernatant and resuspend the pellet in 1 mL of 0.1M carbonate buffer, pH 9.0.
4. Add 0.25 mL of proteins in 0.25 mL of carbonate buffer.
5. Rotate at 60^{o}C for at least 20 hours.
6. Microfuge at 15000 rpm fir 20 seconds and wash two times with PBS (0.1M, pH 7.4) and resuspend to 5 mL with PBS to give 5 mL with PBS to give 5 mL of 1% particles..

Note: 1. For 3.0 µm epoxy polystyrene particles use 0.5 mL of proteins in 0.5 mL of carbonate buffer.

Tel.: 800-368 0822 or 847-680 8922; Fax: 847-680 8927; E-Mail: service@spherotech.com
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1840 Industrial Dr., Suite 270, Libertyville, IL 60048

Coating of Ligands to Modified Amino Proteins

Covalent Coupling Using SPDP[2]:

2-Pyridyldisulfide particles:

1. Add the following to a 15 mL glass centrifuge tube:
   a. 5.0 mL of sodium phosphate buffer (PB), 0.1 M, pH 7.0
   b. 5.0 mL of amino polystyrene particles, 0.8 μm, 5% w/v
   c. 0.5 mL of DMSO containing 12.5 mg of SPDP [3-(2-pyridyldithio) propionic acid N-hydroxy succinimide ester)]
2. Incubate for at least one hour at ambient temperature on a rotary mixer.
3. Centrifuge at 3000x g for 30 minutes.
4. Remove the supernatant carefully.
5. Resuspend the pellet in 5 mL of 0.1M PB, pH 7.0.
6. Centrifuge at 3000x g for 30 minutes.
7. Repeat Steps 3 and 4 and resuspend the pellet in 5 mL of deionized water. Store at 4°C.

Thio Ester Particles:

1. Repeat Steps 1-5 from the above procedure, or use the suspension obtained in Step 6 and centrifuge at 3000x g for 30 minutes.
2. Remove the supernatant carefully.
3. Resuspend the pellet in 5 mL of 0.1M acetate buffer, pH 5.0.
4. Add 40 mg of DTT (1,4-dithiothreitol).
5. Incubate for 30 minutes at ambient temperature on a rotary mixer.
6. Centrifuge at 3000x g for 30 minutes.
7. Aspirate and save the supernatant. If cloudy, filter through a 0.22 μm Acrodisk. Save the filtrate for optical density (OD) measurment at 343 nm. Multiply the abosorbance by 8.08×10^3 to obtain the molar concentration of the thio ester on the particles.
8. Resuspend the pellet in deionized water.
9. Centrifuge at 3000x g for 30 minutes.
10. Remove the supernatant and resuspend the particles in 5 mL of deionized water to give a 5% w/v suspension of thio particles. Use the thio particles as soon as possible for coupling to thiolated ligands. The thio groups can be oxidized to disulfide groups upon prolonged storage.

Modification of Ligands with SPDP and Coupling to Thio Particles:

1. Add a solution containing 1 mg of SPDP in 0.5 mL of methanol to a solution containing 4 mg of ligand in 2 mL of phosphate buffer (PB), 0.1M, pH 7.0.
2. Incubate for at least one hour at ambient temperature on a rotary mixer.
3. Dialyze the mixture using a dialysis tubing of appropriate molecular weight cut off against three changes of PB in 24 hours.
4. Add the resulting thio ligand to 2 mL of 5% w/v thio particles.
5. Incubate overnight at ambient temperature on a rotary mixer.
6. Centrifuge at 3000x g for 30 minutes.
7. Remove the supernatant and resuspend the particles in 10 mL of PB.
8. Repeat Steps 6 or 7 and centrifuge at 3000x g for 30 minutes.
9. Remove the supernatant and resuspend the particles in 40 mL of PB to give a 0.25% w/v suspension.

Periodate Oxidation of Polysaccharide and Coupling to Amino Particles

1. Add a solution containing 1 mg of sodium m-periodate (Sigma S-1878) in 1 mL of deionized water dropwise with stiring to a solution containing 10 mg of polysaccharide in 2 ml of deionized water.
2. Stir the mixture at room temperature for 30 minutes and add 10 μL of 1 M ethylene glycol to the mixture. After five minutes, add the mixture to the packed 0.8 μm amino polystyrene particles obtained from 5 mL of 5% w/v suspension by centrifugation at 4000x g for for 10 minutes.
3. Adjust the pH of the mixture to 9.0 to 9.5 with 10% K_2CO_3 and stir the mixture at room temperature for at least 45 minutes.
4. Add 6 mg of sodium cyanoborohydride (Sigma S-8628) to the mixture and stir the mixture at room temperature overnight.
5. Wash the particles twice with 5 mL of deionized water and resuspend the particles in 5 mL of 0.1 M PBS containing 100 mg of BSA.
6. Stir the mixture at room temperature for two hours and wash the particles twice as before.
7. Resuspend the particles in 5 mL of 0.1M PBS to give 5 mL of 5% w/v suspension.

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Coating of Carboxyl Polystyrene Particles with Amino Modified Oligonucleotides

1. Add $2.5x10^6$ carboxyl polystyrene particles to 62 µL of 0.1M MES (2-[N-morpholino]ethanesulfonic acid)
2. Add 5nmoles of amino modified oligonucleotide in 25 µL of 0.1M MES
3. Add 0.3 mg of EDC(1-ethyl-3(-3-dimethylaminopropyl) carbomiimide hydrochloride)
4. Vortex and incubate for 20 minutes at ambient temperature.
5. Add 0.3 mg of EDC.
6. Repeat Steps 4 and 5.
7. Incubate for another 80 minutes on a rotary mixer.
8. Centrifuge and remove the supernatant carefully.
9. Resuspend the pellet in 1 mL of 0.1M PBS containing 0.02% Tween-20.
10. Repeat Step 8 and resuspend the pellet in 150 µL of 10mM Tris [hydroxymethyl]aminomethane hydrochloride / 1mL EDTA (ethylenediamine-tetraacetic acid) pH 8.0 (TE)
15. Centrifuge and remove the supernatant carefully.
16. Resuspend the pellet in 200 µL of TE or IBS. Store at 4^oC.

Note: This method was provided by Fuja & Hou, Developmental Biology Center, University of California, Irvine.

Conclusion

Proteins and ligands absorbs onto polystyrene readily and permanently. Coating the surface of polystyrene particles with proteins is sucecessful most of the time using adsorption techniques. Adsorption is adequate for the coating of most polyclonal IgG for assay systems. Other methods of coating particles can be considered if simple adsorption is inadequate. A wide array of particle coating mechanisms with different surface chemistries of particles are now available. For instance, monoclonal antibodies with low isoelectric points will require covalent coupling. Figure 3 will show the mechanism for other methods of coating particles. In this technical note, Spherotech has recommended different initial procedures for the production of tests and assays that provide good sensitivity and stability using polystyrene particles.

Important Notes:

1. Since the quality of the coated particles depends on the quality of reagents and on the coating procedures, high quality reagents should be used while optimizing the coating conditions. As a result of Spherotech's lack of control over the reagents and coating condition, Spherotech can not guarantee the quality or performance of the coated particles even if the provided procedures are followed.

2. Isotonic Buffered Saline (IBS) is prepared using the following formula:

NaCl	8.0g
KCl	0.28g
$NaHPO^4$	0.275g
Na^2HPO^4	2.021g
Sodium Azide	0.2g
Deionized Water	1000mL

References

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2. J. Carlson et al, Biochem. J. Vol. 173, 723-737 (1878)