



# Reagent Microspheres-Surface Properties and Conjugation Methods

## 1. GENERAL DESCRIPTION

Duke Scientific Reagent Microspheres are uniform spherical polymer particles composed mainly of polystyrene. Some of the microspheres have other copolymers added in small quantities to produce reactive surfaces to which various molecules can be covalently attached. In general, the particle surface is strongly hydrophobic, although some types of microspheres have limited hydrophilic properties. Colloidal stabilization is achieved by introducing various types of surface charges during the manufacturing process. Reagent Microspheres are prepared without the use of surface-active agents or other contaminants, resulting in clean surfaces that are optimized for attachment of ligands. The microspheres are suspended in deionized water.

## 2. SURFACE PROPERTIES

### 2.1. Hydrophobic Microspheres

Reagent Microspheres with sulfate, carboxyl and aldehyde-modified surfaces are hydrophobic, and are designed for passive adsorption of high molecular weight biomolecules such as proteins. The surface of the sulfate microspheres contains negatively charged sulfate groups that have a pKa of approximately 2, and are therefore stable at acidic pH. The aldehyde-modified microspheres also contain sulfate groups on the surface, but have the ability to form covalent bonds with adsorbed ligands. Carboxyl microspheres have negatively charged surface carboxyl groups, and are stable above pH 5, which is the approximate pKa of the carboxyl group. The hydrophobic microspheres will bind to any molecule that has hydrophobic character, including proteins, peptides and small hydrophobic molecules. The binding affinity tends to increase with increasing molecular weight, and can result in the preferential binding of higher molecular weight proteins in mixtures. Specific adsorption of substances such as antibodies is easily accomplished by mixing the microspheres and the protein together at an optimal pH and then separating the unbound protein from the solid phase.

### 2.2. Hydrophilic Microspheres

Carboxylate-modified (CML) microspheres have a highly charged surface layer of carboxyl groups derived from a copolymerization process. The surface is somewhat porous and relatively hydrophilic, but retains overall hydrophobic properties. The charge density of these particles ranges from about 10-125 Å<sup>2</sup> per carboxyl group, and they are stable to high concentrations of electrolytes (up to 1M univalent salt). The CML latex will adsorb proteins and other biomolecules, but much less strongly than the hydrophobic microspheres. The carboxyl groups on the surface of these microspheres are different from those of the hydrophobic carboxyl microspheres described above in that they are designed to be reactive toward amines after activation with carbodiimide reagents.

### 3. COLLOIDAL STABILITY OF MICROSPHERES

Reagent Microspheres are supplied as a monodisperse suspension in deionized water. The particles are maintained in colloidal suspension by the electrostatic effect of the ionic charges on their surface. The particles will maintain colloidal stability indefinitely, as long as they are not subjected to conditions that cause aggregation. These conditions include: high electrolyte concentration, neutralization of surface charge and/or exposure to unfavorable environmental conditions such as freezing. If the concentration of electrolyte is raised above a certain level, the surface charges are screened and particle-to-particle contact results in aggregation. Therefore, storage or reaction buffers of high ionic strength should be avoided. It is recommended that buffers of no more than 50 mM be used for most types of particles, although some CML particles with very high charge density can tolerate higher ionic strength. Avoid using positively charged buffers such as TRIS in the presence of negatively charged particles, since this may result in neutralization of charge and subsequent aggregation. For long term storage, the pH of the suspending medium should be maintained at least 1-2 pH units above the pKa of the particle surface groups. A high concentration of microspheres tends to promote colloidal instability, therefore particle concentrations should be kept as dilute as possible. Under no circumstances should the microsphere suspension be frozen. This will cause irreversible aggregation.

### 4. CONJUGATION OF PROTEINS TO REAGENT MICROSPHERES

#### 4.1. Hydrophobic Adsorption

Adsorption of proteins and other ligands that have hydrophobic domains is the simplest and most straightforward method of attaching these substances to particle surfaces. In this method, microspheres are mixed with a solution of the desired protein and then the excess unbound protein is removed by a washing procedure to obtain protein bound microspheres that are ready for use. Hydrophobic adsorption methods should only be used with the hydrophobic microspheres of Section 2.1. (sulfate, carboxyl and aldehyde-modified surfaces). Aldehyde-modified microspheres are a special case, since hydrophobic adsorption results in subsequent covalent attachment. This will be discussed further in Section 4.2. Although hydrophobic adsorption is not pH dependent, the pH of the reaction buffer can have a strong effect on the conformation of the protein being conjugated, and thus affect the efficiency of protein adsorption. In general, proteins have been shown to bind most efficiently at or near their isoelectric point (1). The following general procedure for physical adsorption of proteins is presented as a guideline for preparing conjugates with hydrophobic microspheres.

1. Prepare a 10 mg/mL solution of the protein in the reaction buffer (Note 1)
2. Prepare a 1% w/v suspension of microspheres in the reaction buffer.
3. Add one volume protein solution to ten volumes microsphere suspension. Incubate at room temperature for 2 hours (Note 2).
4. Remove unbound protein (Note 3).
5. Store the microsphere conjugate in the appropriate storage buffer (Note 4).

#### 4.2. Covalent Attachment

Carboxylate-modified (CML) Reagent Microspheres and aldehyde-modified Reagent Microspheres are designed for covalent attachment of proteins and other biomolecules (2). The following general procedures are presented as a guideline for preparing conjugates with carboxylate-modified or aldehyde-modified microspheres and can be modified to fit requirements.

##### 4.2.1 Reaction of proteins with CML Reagent Microspheres

The following procedures are presented as guidelines for preparing conjugates with CML Reagent Microspheres. In these reactions, the carboxylate-modified microspheres are activated using a water

soluble carbodiimide reagent that makes the carboxyl groups reactive with primary amines on the proteins to be coupled.

### A. One Step Procedure

1. Prepare a 50 mM reaction buffer at pH 6.0 (Note 1) Sodium acetate or 2-[N-morpholino]ethanesulfonic acid (MES) are suitable buffers.
2. Dissolve the protein in the reaction buffer at a concentration of 10 mg/mL.
3. Prepare a 1% (w/v) suspension of microspheres in the reaction buffer.
4. Add one volume protein solution to ten volumes microsphere suspension (Note 2). Allow the mixture to incubate at room temperature for 20 minutes.
5. Prepare a solution of 10 mg/mL (52  $\mu$ Mol/mL) of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) in deionized water. Use this reagent immediately. (Note 5).
6. Add a calculated amount of EDAC solution to the microsphere suspension (Note 6).
7. Adjust the pH of the reaction mixture to  $6.5 \pm 0.2$  with 0.1N NaOH. Incubate the mixture on a rocker or mixing wheel for 2 hours at room temperature (Note 7).
8. Remove unbound protein and store the product in storage buffer (Notes 3 and 4).

### B. Two Step Procedures

To avoid bridging of the particles by covalently coupled protein and cross-linking of the protein to itself, a two-step procedure is often preferred, in which the excess EDAC is washed away prior to addition of the protein to be coupled. This two-step approach also allows the protein to be dissolved in a buffer of a higher pH. This is often advantageous from the standpoint of protein stability, and speeds up the reaction of the protein with the activated particles. Two approaches are commonly used.

#### B.1 Simple Two Step Procedure

1. Prepare a 1% w/v suspension of the microspheres in reaction buffer as described in Section A, steps 1 and 3.
2. Add 20 mg EDAC per mL of microsphere suspension and incubate for 40 minutes at room temperature, with a second addition of 20 mg/mL EDAC after 20 minutes (Note 7).
3. Wash the microspheres by adding an equal volume of the same buffer, centrifuge and repeat with one volume of buffer (Note 3).
4. Meanwhile, dissolve the protein to be coupled in a buffer. This coupling buffer is preferably at pH 7 to 9, and preferably at a buffer molarity of 50 mM to 100 mM (Note 1). A typical protein concentration is 1 mg/mL.
5. Resuspend the microspheres in either deionized water or coupling buffer, and quickly add dissolved protein. Incubate for 2 to 5 hours with intermittent mixing (Note 2).
6. Add 2.5  $\mu$ L ethanolamine per mL of reaction mixture and incubate for 10 minutes (Note 9).
7. Remove unbound protein and ethanolamine and store the product in storage buffer (Notes 3 and 4).

#### B.2. Two Step Procedure with Intermediate Formation of NHS-Ester

(In this procedure, an intermediate active ester is formed by reacting the carboxyl groups on the particles with N-hydroxysuccinimide (NHS) in the presence of EDAC. This ester is more stable than the active intermediate formed with EDAC alone, and so is less prone to hydrolysis during the wash step prior to protein addition.)

1. Per mL of reaction mixture, add the following:
  - Deionized water to make 1.0 mL final volume
  - 0.1 mL of a 10x stock buffer at a pH of 6.0 to 6.5 (0.5M MES buffer is typically used)
  - Microparticles to a final concentration of 1% (w/v)
  - 0.23 mL of a 50 mg/mL NHS solution in deionized water (Note 5)
  - a chosen volume of a 19.2 mg/mL (100 mM) solution of EDAC in deionized water (Notes 5 and 6)
2. React this mixture for 15 to 30 minutes at room temperature with stirring (Note 7).

3. Wash the particle suspension to remove unreacted NHS and EDAC (Note 3). Washing may be done with MES buffer or with deionized water.
4. Resuspend the particles at a final concentration of 1% (w/v) in deionized water.
5. Meanwhile, dissolve the protein to be coupled in a buffer. This buffer is preferably at pH 7 to 9, and preferably at a buffer molarity of 50 mM to 100 mM (Note 1). A typical protein concentration is 1 mg/mL.
6. Immediately after washing the activated particles, add one volume of the protein solution to each volume of activated particles (Note 2). (Note that in the example given here, the concentrations of particles, protein, and buffer in the coupling mixture will be 0.5% (w/v), 0.5 mg/mL, and 25 to 50 mM, respectively.)
7. Incubate the mixture with gentle mixing for at least 2 hours.
8. Add 2.5  $\mu$ L of ethanolamine per mL of reaction mixture, and incubate for 10 to 30 minutes with gentle stirring.
9. Remove unbound protein and ethanolamine and store the product in the appropriate storage buffer (Notes 3 and 4).

#### 4.2.2 Reaction of proteins with aldehyde-modified Reagent Microspheres

Aldehyde-modified Reagent Microspheres are hydrophobic particles that adsorb and react with proteins at neutral pH through the formation of a Schiff base between the aliphatic aldehyde surface groups and the amines on the protein (primarily lysine amines). These microspheres do not require any chemical pre-activation steps for covalent binding to occur. Formation of a Schiff base is a reversible reaction; however, since several different amine groups on a protein are bound simultaneously to the latex surface aldehydes, thermodynamics are very unfavorable for dissociation of the protein. The Schiff base can be reduced, if desired, to a stable alkylamine bond by addition of sodium cyanoborohydride after addition of the protein to the particles. Stabilization by reduction is necessary when peptides or other molecules with only a single reactive amine group are conjugated to aldehyde-modified microspheres. The following general procedure outlines the conjugation of proteins to aldehyde-modified microspheres.

1. Prepare a 50 mM reaction buffer at pH 7.0-7.5 (Note 1).
2. Prepare a 10 mg/mL solution of the protein in reaction buffer.
3. Prepare a 1% w/v suspension of aldehyde-modified microspheres in reaction buffer.
4. Add one volume protein solution to ten volumes microsphere suspension. Incubate at room temperature for 2 hours (Notes 2 & 8).
5. Remove unbound protein (Note 3).
6. Store the microsphere conjugate in the appropriate storage buffer (Note 4).

#### NOTES:

Note 1 The composition of the reaction buffer will depend on the type of protein that is being adsorbed to the microspheres. Some useful buffers are acetate, phosphate, borate and MES (2-[N-morpholino]ethanesulfonic acid). As mentioned above, proteins tend to adsorb more efficiently at their isoelectric point (pI), because the molecule usually has more hydrophobic sites exposed under this condition. In the case of antibodies, the molecules are also more compact at the pI (1), thus allowing more antibodies per unit surface area to bind. However, the final determinant in the choice of reaction buffer is usually the biological activity of the final conjugate and therefore, several different protein concentrations and reaction buffers at different pH should be tried in a series of optimization experiments. For initial experiments, the ionic strength of the reaction buffer should be in the range of 25-50 mM.

Note 2 The protein solution should be added to the microsphere suspension rapidly and with very efficient mixing. In small volumes, this can be carried out using a vortex mixer. When the reaction is carried out in larger volumes, the microsphere suspension should be vigorously stirred using a magnetic stirrer (do not allow foaming to occur) in a flask or beaker and the protein solution added rapidly to the center of the vortex.

Note 3 Removal of unbound chemicals or protein can be accomplished by several methods. If the particles are greater than about 0.2 micrometers in diameter, washing by centrifugation is possible. The pellet can be

re-suspended between washes by vortexing followed by gentle ultrasonication using a bath sonicator. Excessive centrifugation forces should be avoided, since the particles will be difficult to re-suspend. Purification can also be carried out using cross-flow filtration or dialysis. When using filtration methods, it is essential that the pore size of the filter or dialysis material be large enough to allow the dissolved protein molecules to pass through efficiently. The buffer used for washing the microspheres should be the same as the storage buffer. Any change in buffer composition or pH after the particles are washed could result in additional desorption of protein.

**Note 4** Addition of detergents or other surface active agents to the microspheres after they are coated may desorb the protein from the surface. In the case of covalent conjugates, desorption of covalently bound protein will not occur. Blocking proteins such as BSA, casein or gelatin can be used to coat any remaining unbound hydrophobic sites on the microspheres and stabilize them against non-specific aggregation. However, these are surface active materials, and will cause some desorption of primary protein that is not covalently attached.

**Note 5** This reagent reacts with water and therefore should be used within a few minutes after preparation.

**Note 6** The amount of EDAC required will depend on the microsphere carboxyl concentration. An excess of 2-3 moles EDAC for every mole of carboxyl groups is recommended as a starting point. Further optimization may be necessary, depending on the results of functional testing.

**Note 7** Aggregation of the particles is commonly observed at this point in the process. Depending on the procedure being followed, this can be caused by bridging of the particles by protein, neutralization of the carboxyl groups, or both. Adjusting the pH to 6.5 or above optimizes the coupling reaction and usually helps to disperse the particles. If the particles are still aggregated after the reaction is over, washing with fresh buffer to remove excess EDAC and unbound protein will generally reverse aggregation. If aggregation persists in the final product, try a lower concentration of particles and reagents (begin with a 50% reduction). If aggregation still persists, a nonionic detergent such as Tween-20 or Tergitol NP9 at 0.01% to 0.05% can be included in all reaction buffers. This does not interfere with particle activation or coupling, but care must be taken that the protein to be coupled is stable in the presence of this detergent.

**Note 8** If reduction of the Schiff base with sodium cyanoborohydride is desired, the following procedure is used: Add a ten-fold molar excess of borohydride reagent over the total moles of available aldehyde to the protein/microsphere reaction mixture. If the preparation is carried out on a small scale, the borohydride reagent is best dissolved in deionized water at a concentration of 10 mg/mL before addition.

**Note 9** The addition of ethanolamine is carried out to react with (“cap”) any remaining activated carboxyl groups that remain after protein addition.

1. Bagchi P. and Birnbaum S.M.(1981): “Effect of pH on the adsorption of immunoglobulin G on anionic poly(vinyltoluene) model latex particles”. *J. Colloid and Interface Sci.*, **83**(2), 460-478.

2. Brinkley M. (1992): “A brief survey of methods for preparing protein conjugates with dyes, haptens, and cross-linking reagents”. *Bioconjugate Chemistry* **3**(1) 2-14.

**CAUTION: Reagent Microspheres are raw materials. They require processing and validation before diagnostic use.**