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Working With FluoSpheres® Fluorescent Microspheres

Properties and Modifications

Introduction

Molecular Probes' FluoSpheres® microspheres are manufactured using high-quality, ultraclean polystyrene microspheres. These microspheres are loaded with Molecular Probes' proprietary dyes, making them the brightest fluorescent microspheres available. With our special staining methods, all of the fluorescent dye molecules are contained inside each polystyrene microsphere, instead of merely on the bead's surface. The protective environment within the bead shields the dye from many of the environmental effects that cause quenching or photobleaching of exposed fluorophores. The method we employ also ensures the narrow distribution of fluorescence intensity and size. The stability, uniformity and reproducibility of the FluoSpheres microspheres, as well as the extensive selection of colors available, make them the preferred tools for research and diagnostic assays that use fluorescence. In addition, fluorescent microspheres are potentially more sensitive than colorimetric methods in most, if not all, of the major microsphere-based diagnostic test systems presently in use, including microsphere-agglutination tests, filter-separation tests, particle-capture ELISA methods and two-particle sandwich techniques. Every possible precaution is made throughout the manufacturing process to ensure that the microparticles are kept free of contaminating agents. The final product is sold as a suspension in ultrapure water, in most cases containing 2 mM azide or 0.02% thimerosal as a preservative.

Surface Properties

FluoSpheres are available with four different surface functional groups, making them compatible with a variety of conjugation strategies. Our fluorescent dyes have a negligible effect on the surface properties of the polystyrene beads or on their protein adsorption. Anionic FluoSpheres having aldehyde–sulfate, sulfate or carboxylate-modified surface groups have been used most frequently in biological applications due to their broader general availability and because they are less likely to bind to negatively charged cell surfaces. Cationic microspheres having amine-modified surface groups have been used less frequently, but may have some distinct advantages for certain applications, since they are stable to alkaline pH conditions and to high concentrations of multivalent anions such as calcium and magnesium. We caution, however, that the surface properties have an important role in the functional utility of the microspheres; we cannot guarantee the suitability of a particular bead type for all applications.

Carboxylate-Modified FluoSpheres

The carboxylate-modified microsphere products are made by grafting polymers containing carboxylic acid groups to sulfate microspheres. The result is a microsphere with a highly charged, relatively hydrophilic and somewhat porous surface layer. The external layer produced by this modification process is only a few Å thick, and therefore does not change the size of the seed particles significantly. The surface charges of carboxylate-modified microspheres range between 0.1 and 2.0 milliequivalents/gram, and therefore, they are stable to relatively high concentrations of electrolytes (up to 1 M univalent salt). Carboxylate-modified microspheres will adsorb proteins and other biomolecules, but much less strongly than the hydrophobic microspheres. Carboxylate-modified microperes are often superior for applications in biological systems because they are more highly charged, which reduces their attraction to cells. It is also easier to further reduce nonspecific binding by the introduction of additives such as bovine serum albumin (BSA) or dextrans. A further potential advantage of carboxylate-modified microperes is that they can be covalently coupled to proteins, nucleic acids and other biomolecules. Covalent coupling requires more effort than passive adsorption, but can result in conjugates with greater specific activity and products that remain active longer. Covalent coupling to carboxylate-modified microspheres is the method of choice for conjugating low molecular weight peptides and oligonucleotides. Carbodiimide-mediated coupling of proteins to carboxylate-modified microspheres is discussed in more detail below in *Covalent Coupling of Proteins to Carboxylate-Modified Microspheres*. Their pendent carboxyl groups also make these microspheres suitable for covalent coupling of amine-containing biomolecules using water-soluble carbodiimide reagents such as EDAC.

Amine-Modified FluoSpheres

Amine-modified microsphere products are prepared by further chemical modification of carboxylate-modified microspheres to give hydrophilic particles with positively charged amine groups. The charge density is high, permitting their use in high ionic strength buffers. Amine-modified microspheres contain aliphatic amine surface groups that can be coupled to a wide variety of amine-reactive molecules, including succinimidyl esters and isothiocyanates of haptens and drugs or carboxylic acids of proteins, using a water-soluble carbodiimide. The amine surface groups can also be reacted with SPDP (S1531) to yield (after reduction) microspheres with sulfhydryl groups.

Sulfate and Aldehyde-Sulfate FluoSpheres

The FluoSpheres having sulfate surface groups are relatively hydrophobic particles that will passively adsorb almost any protein, including BSA, IgG and avidin or streptavidin. The aldehyde-sulfate microspheres are sulfate microspheres modified by the addition of surface aldehyde groups. These microspheres are designed to react with proteins and other amines under very mild conditions. The microsphere suspensions are stable at up to about 0.2 M univalent electrolyte concentrations, but will readily agglomerate in the presence of low concentrations of divalent cations unless stabilized by a hydrophilic coating. Sulfate microspheres (pK_a <2) are stable at acidic pH above their approximate pK_a. Even though they have charged surfaces, the hydrophobic microspheres will bind strongly to any molecule that has hydrophobic character, including proteins, nucleic acids and many small biomolecules such as drugs and hormones. The hydrophobic microsphere products are usually suitable for applications in systems that are free of biologicals and need no further modifications. In biological systems, including immunoassay applications, the microspheres can be easily coated with various proteins or polysaccharides that will greatly reduce their capacity to adsorb biomolecules nonspecifically. Specific, stable adsorption of proteins such as avidin, streptavidin and antibodies is accomplished simply by mixing the microspheres and the protein together and then separating the microsphere-bound protein from the unbound protein. Refer below to *Passive Adsorption of Proteins to Hydrophobic Microspheres* for details.

Optimization of Buffers

The type and density of the surface charges on the microspheres will dictate the best choice of buffer systems for their use in experiments. As a general rule, cationic buffers such as Tris should be avoided when using anionic microspheres (aldehyde–sulfate, sulfate, carboxylate-modified); conversely, borate, citrate or phosphate buffers should be avoided when using cationic microspheres (amine-modified). The ionic strength of the buffer should be kept as low as possible, especially when the microspheres are very small or have a low charge density. Since anionic microspheres are very sensitive to low concentrations of multivalent cations, calcium and magnesium salts should be avoided if at all possible. Cationic microspheres are not sensitive to these ions and may be best for applications in which high concentrations of these ions are anticipated. Because of their hydrophobic character, microsphere particles are great scavengers, therefore, the water used for preparation of buffers should be as pure as possible. Either doubly distilled water or high-purity ion-exchanged water is strongly recommended. In general, the smaller the particle size, the more critical are these requirements, since very small microspheres have fewer charge groups for stabilization. The pH of the buffer can be important when using carboxylate-modified or amine-modified microspheres. The carboxylate-modified microspheres should be used at a pH greater than about 5.0, while the amine-modified microspheres require a pH of less than about 9.0. If these conditions are not followed, the charge groups on these particles may be neutralized, leading to agglomeration. If agglomeration does occur as a result of incorrect pH, the particles can usually be redispersed by adjusting the pH to the correct range followed by gentle sonication.

Controlling Nonspecific Binding of Microspheres

Nonspecific binding is probably the most common problem that is encountered in working with microspheres and is often the major reason for abandoning an otherwise well-conceived experiment with microsphere particles. As mentioned above, the particles are generally hydrophobic, and although various modifications tend to make them less hydrophobic, it must be realized that the particles are polystyrene-based and therefore always retain some hydrophobic characteristics. In biological systems, most of the nonspecific binding problems are a result of hydrophobic interactions; however, some of the problems may also be caused by charge-based interactions (for example, a positively charged molecule attracted to a negatively charged microsphere surface). The best way to minimize these nonspecific binding events is to coat the microsphere with a large macromolecule such as a protein or a polysaccharide, which reduces nonspecific binding by blocking the hydrophobic or charged binding sites on the microsphere surface. Although many types of coating agents may be used, the most frequently employed are bovine serum albumin (BSA), egg albumin and whole serum. Egg albumin should be avoided in systems that employ biotin–avidin binding. When using the hydrophobic microspheres, all that is usually necessary is to suspend the particles in a 1% solution of the protein-based coating agent, since at this concentration, the particles will be completely and stably coated.

Dextrans can be used as coating agents in place of or in addition to proteins. Unlike proteins, the hydrophilic dextrans bind reversibly to microspheres. They form a layer at the surface of the particles and make them more hydrophilic, thus reducing nonspecific interactions. If dextrans are used as coating agents, 40,000 MW dextran at a 2% weight/volume ratio is recommended. If the particle is a hydrophilic carboxylate-modified microsphere, the coating agent may not bind strongly enough to the particles and may fail to prevent nonspecific binding. In this case, covalent coupling of a coating agent such as BSA may solve the problem. In this method, specific binding proteins, such as immunoglobulin or avidin, can be mixed with BSA and simultaneously coupled covalently, resulting in a specifically active microsphere with a covalently bound BSA coating. As a last resort, or in situations where the use of detergents is acceptable, nonionic surfactants such as polyoxyethylenes (Triton® X-100 or Tween® 80) can be coated onto the microsphere at concentrations ranging from 0.01–0.1% (the exact amount to be determined by experimentation).

Our BlockAid™ blocking solution (B10710), a protein-based blocking solution, is designed for use with our streptavidin-, NeutrAvidin-, biotin- and protein A–labeled FluoSpheres microspheres. In flow cytometry applications, we find BlockAid blocking solution to be superior to other commercially available blocking solutions and to a number of “home-made” blocking solutions described in the scientific literature. We expect BlockAid blocking solution to be useful for preventing the nonspecific binding of protein-coated or other macromolecule-coated microspheres in a variety of flow cytometry and microscopy applications. BlockAid blocking solution is available in a 50-mL unit size.

Keeping FluoSpheres in a Monodisperse State

Microsphere particles, which are hydrophobic by nature, will always tend to agglomerate. In aqueous suspensions, the only thing preventing this is the surface charge on the particles. Surfactant-free microspheres do not have detergents to aid in dispersion, so these preparations are slightly more sensitive to conditions that can lead to agglomeration. These conditions include: 1) high concentration of particles; 2) high electrolyte concentration; and 3) neutralization of surface charge groups. To minimize these adverse conditions, it is always wise to keep the microsphere suspensions dilute. Recommended particle concentrations to be used when coating microsphere particles with proteins (both passive adsorption and covalent coupling) is 0.5–1.0% solids. Reaction buffers and storage buffers of relatively low ionic strength (100 mM or less) are best. The use of multivalent cations should be especially avoided with anionic microsphere particles. Finally the pH should be maintained so that all of the surface charges on the microsphere particles are fully ionized. If agglomeration does occur, the particles can frequently be rescued by either diluting the microsphere suspension, adjusting the pH or reducing the ionic strength and then redispersing the suspension by means of a bath sonicator. The use of a bath sonicator greatly aids in working with microsphere suspensions, and it is strongly recommended that this device be utilized if possible. Routine sonication of microsphere preparations is advised before each use, especially in critical applications where a high degree of monodispersity is required. In the case of very small particles (less than 0.1 µm), the sonicated suspension can be briefly centrifuged at high speed (12,000 rpm) to further remove agglomerates from the suspension (the monodisperse particles will remain in suspension under these conditions).

Passive Adsorption of Proteins to Hydrophobic Microspheres

Passive adsorption of proteins and other molecules having hydrophobic domains to microspheres is the simplest method of coating, since no chemical reactions are necessary. The coated microsphere product can often be purified from unbound ligand by a simple centrifugation and washing procedure. Passive adsorption should be used only with the hydrophobic microspheres (sulfate surface groups). The hydrophobic interactions that bind macromolecules to the microsphere particles are essentially independent of pH; however, pH and charge can influence the conformation of protein molecules and thus facilitate their binding. Virtually every protein studied to date has been shown to bind to hydrophobic microspheres, and, in general, proteins have been shown to bind most efficiently at a pH that is near their isoelectric point.

The microsphere particle concentration most suitable for adsorption is in the range of 0.5–1% solids. At this relatively low concentration of particles, the aggregation caused by protein bridging is minimized. The ligand to be attached should be added to the dilute suspension of microsphere particles at a concentration of 20–50 µg/mL of final suspension in a buffer with an ionic strength of less than 100 mM. The suspension is stirred, shaken or rocked gently for a period of a few minutes to a day or more at room temperature. While the physical adsorption is very fast and is complete in just a few seconds, protein-dependent conformational changes can take an hour or two for completion. It should be noted that partial coverage of the microsphere particles with a ligand usually produces a system with greater binding specificity than one where full surface coverage is achieved.

In order to avoid nonspecific adsorption of further proteins when the coated microsphere is used in applications such as a diagnostic test, the microsphere can be back-coated with albumin, gelatin or other macromolecules (0.5–1 mg/mL). These substances will fill in any remaining hydrophobic areas on the particles. If the application of the coated microsphere particles is such that detergents can be tolerated, a nonionic surfactant such as Tween 20 or Triton X-100 can be added to increase hydrophilicity of the particles.

The coated microsphere particles can be separated from unbound ligand by centrifugation and washing if the particle diameter is greater than about 0.2 µm. The smaller the particles, the greater the centrifugation force and time that will be required to cause them to sediment. Some care should be taken to avoid excessive centrifugation force, however; otherwise, the particles may be packed together too tightly and will overcome the repulsive forces between the particles. After the supernatant is carefully removed, the particles are resuspended in washing buffer by vortexing or sonication. In the case of microsphere particles with diameters of less than about 0.2 µm, some type of filtration process will be necessary for separation of unbound ligand. If the ligand is small, ordinary dialysis tubing (12,000–14,000 MW cut-off) can be used; otherwise, cellulose ester dialysis tubing with a MW cut-off of 300,000 daltons can be used for most proteins, including IgG. When dialyzing the particles, the buffer should be the same as that used for the adsorption process, and at least five changes of buffer should be made. Other separation processes such as gel filtration can also be used, although in our experience, the microsphere particles tend to stick nonspecifically to some types of gels.

Covalent Coupling of Proteins to Carboxylate-Modified Microspheres

There are many procedures published in the literature that describe covalent coupling of proteins and other macromolecules to carboxylate-modified microspheres. Almost all of these use a water-soluble carbodiimide (EDAC) to activate the surface carboxyl groups on the microsphere particles. The following procedure is a simple one-step method we have used with excellent results in our laboratory for coupling avidin, streptavidin, BSA and goat anti-mouse to our carboxylate-modified FluoSphere microspheres. The reaction can be easily scaled up or down to fit individual needs.

1.1 Prepare 100 mL of 50 mM phosphate buffer, pH 7.4, containing 0.9% NaCl (50 mM PBS).

1.2 Prepare 100 mL of 50 mM MES buffer, pH 6.0.

1.3 Dissolve 10–25 mg of protein (avidin, streptavidin, IgG, BSA, etc.) at 2–5 mg/mL in MES buffer in a glass centrifuge tube.

1.4 Add 5 mL of a 2% aqueous suspension of carboxylate-modified microsphere (note **A**). Incubate at room temperature for 15 minutes.

1.5 Add 40 mg of EDAC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; Molecular Probes' E2247). Mix by vortexing (note **A**).

1.6 Adjust the pH to 6.5 ± 0.2 with dilute NaOH. Incubate the reaction mixture on a rocker or orbital shaker for 2 hours at room temperature (or overnight, if desired) (note **A**).

1.7 Add glycine to give a final concentration of 100 mM to quench the reaction. Incubate 30 minutes at room temperature.

1.8 Centrifuge to separate the protein-labeled microsphere particles from unreacted protein. The time and speed of the centrifugation will vary with the diameter of the microsphere particles. As a guideline, 0.5 µm particles and smaller should be centrifuged at 25,000 × g for 30–60 minutes. Particles 1.0 µm and greater can usually be sedimented at 3000–5000 × g for 20 minutes. It may not be possible to centrifuge 20 nm and 40 nm particles without extended spin times. We therefore recommend that dialysis be used with beads of this size (note **B**).

1.9 Resuspend the pellet in 50 mM PBS by gentle vortexing or by use of a bath sonicator. Centrifuge as described in step 1.8.

1.10 Repeat step 1.9 twice more (a total of 3 washes).

1.11 Resuspend the protein-conjugated microspheres in 5 mL of 50 mM PBS. Other buffers compatible with the microspheres (see above) are also suitable. If desired, the microspheres can be resuspended in a final buffer containing 1% BSA. The BSA will adsorb to the remaining hydrophobic sites on the microspheres and help to provide a more stable suspension that may be less prone to nonspecific interactions with other proteins.

1.12 Add 2 mM sodium azide and store the microspheres at 4°C. DO NOT FREEZE.

Covalent Coupling of Proteins to Aldehyde–Sulfate Microspheres

Aldehyde–sulfate FluoSpheres microspheres can be conjugated to proteins via the formation of a Schiff base between the aliphatic aldehyde surface groups and the lysine ϵ-amines of proteins. Coupling of proteins to aldehyde–sulfate microspheres is simpler than the method described above for carboxylate-modified microspheres, in that there is no need to use an activating reagent. The procedure given below is a one-step mix-and-wash protocol, resulting in a coated microsphere with covalently bound protein on the surface. As with the procedure for coupling proteins to carboxylate-modified microspheres (previous section), the reaction can be scaled to meet individual needs.

2.1 Prepare 100 mL of 50 mM phosphate buffer, pH 7.4, containing 0.9% NaCl (50 mM PBS).

2.2 Prepare 100 mL of 50 mM phosphate buffer, pH 6.5.

2.3 Dissolve 4 mg protein (avidin, streptavidin, IgG, BSA, etc.) in 2 mL phosphate buffer, pH 6.5, in a 15 mL glass centrifuge tube.

2.4 Add 5 mL of a 2% aqueous suspension of aldehyde–sulfate microspheres. Incubate at room temperature overnight.

2.5 *(optional)* The reaction of aldehyde groups with amines to form a Schiff base is a reversible reaction; however, since several different amine groups on a given protein molecule are coupled simultaneously to the microsphere particle, thermodynamics are unfavorable for dissociation of the protein from the microsphere surface. The Schiff base adduct can be reduced, if desired, to a stable alkylamine bond by addition of 15 mg of sodium cyanoborohydride immediately after addition of the microsphere suspension in step 2.4. Reduction of the Schiff base with cyanoborohydride is advised when small peptides or other molecules with only one reactive amine group are conjugated to aldehyde-sulfate microspheres.

2.6 Centrifuge to separate the protein-labeled microsphere particles from unreacted protein. See steps 1.8–1.11 for details for purification of the protein-coated microspheres.

Notes

[A] Agglomeration of the microsphere particles may be observed at this point in the procedure. This agglomeration can be caused by bridging of the particles by protein, neutralization of the charged carboxyl groups or both. Adjusting the pH to 6.5 and sonication of the mixture in a bath sonicator usually will redisperse the particles. If the particles do not redisperse with these treatments, try a lower concentration of particles and reagents (begin with a 50% reduction in concentration).

[B] Particles with a diameter <0.2 µm can be separated from unbound ligand by dialysis using 100,000–300,000 MW cut-off cellulose ester dialysis tubing (Spectrapor, Spectrum Medical Industries, Los Angeles). We have confirmed that this method effectively removes BSA, avidin and IgG from microsphere particles with average diameters ranging from 0.03 to 1.0 µm.

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