TechNote 203

Washing Microspheres

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Bangs Laboratories, Inc.

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CONTENTS

I. INTRODUCTION

Bangs' Laboratories microspheres are presented to the customer in several different ways; dry (large particles and some silica), in methanol (spacers), and, most frequently, in an aqueous suspension. The aqueous suspension contains surfactant, unless otherwise specified, and, in some cases, there may be an antimicrobial agent.

There is a common view that microspheres must be washed before they can be used by the customer. This is not always the case. If the aqueous solution will not harm the system in which you are working, the microspheres may be used directly out of the bottle. The surfactant is in the solution to help keep the microspheres monodisperse, and removing it may not be necessary. We suggest that you first try using microspheres without washing them.

Please also note that our ProActive® protein-coated microspheres come in a buffer with specified salts and surfactants that may not be suitable for your system. This may make it necessary to replace the initial buffer.

If the surfactant in the aqueous solution needs to be decreased or eliminated, or water should not enter your system, then the microspheres will have to be washed. There are many different techniques that can be used; however, the microsphere size dictates which method is preferred. The standard methods of washing microspheres use centrifugation, dialysis, mixed-bed ion exchange, cross-flow filtration (tangential flow filtration, ultrafiltration), and serum replacement.

Centrifugation is the most commonly used, and perhaps easiest, cleaning method, but is difficult to use with microspheres smaller than 300nm in diameter. The speeds required to centrifuge microspheres this small would be very high, and the pellet would be very hard to resuspend.

The other four cleaning methods that were mentioned can be used on any type of microsphere, with some reservations. In this technical note, procedures for these methods are outlined. General considerations for each of the procedures are also covered. The procedures presented in the body of this technical note are for dyed and undyed, nonmagnetic microspheres. The appendix will outline the washing of magnetic and silica microspheres.

II. GENERAL CONSIDERATIONS

Although it is often not necessary that microspheres be completely cleaned of surfactant prior to use, there are applications that call for this condition. One can measure surfactant coming off the microspheres with instrumental methods, including surface tension analysis, to determine when the wash liquid is free of surfactant. An alternative and more practical method comes from Dr. Geoffrey Seaman of Emerald Diagnostics. The wash liquid can be considered to be free of surfactant when the foam or bubbles on top of 5mL of H_2O, shaken in a clean 10mL test tube, collapse in 2-3 seconds. We call this the "Seaman Shake Test." This method can be used with many of the methods outlined in this TechNote.

Use the cleanest water possible when mixing buffers or washing

microspheres. This is water with low conductivity, zero organics, and no microbes. Any organic or inorganic contaminants, present in even commercial deionized water, will tend to stick to the microspheres, effectively cleaning your water, but making matters worse for the microspheres.

Many cleaning methods result in a microsphere pellet or filter cake at some point. It is important that the microspheres be completely resuspended between washes, or before continuing with ligand coating. Resuspension can be made easier by forming the loosest pellet or filter cake that is possible or practical to work with, without losing microspheres in the supernatant. After addition of clean wash liquid or buffer, any microsphere aggregates can normally be broken up using brief (1-2 minute) bath sonication. If a bath sonicator is not available, vortexing or repeated pipetting and dispensing can often do the trick. We do not recommend the use of sonic probes, as they are a potential source of microbial contamination, and have been known to shed metal into the solution.

Complete microsphere resuspension can be verified by microscopic evaluation. Even for particles smaller than ~0.8µm in diameter (the practical single particle limit for 1000X magnification with a light microscope), aggregates can usually be seen quite easily. A uniform suspension of small microspheres will appear as a hazy vibrating background.

III. CENTRIFUGATION

A. Considerations

- Microspheres less than 300nm in diameter should not be cleaned by centrifugation (see dialysis, serum replacement, or ion exchange).
- Times are estimates based on settling 10cm through water. Settling times will, of course, vary for large volumes and viscous or high specific gravity solutions.

B. Procedure

1. Place aliquot of microspheres in appropriate centrifuge tube.
2. Centrifuge the microspheres at the appropriate G forces for 15 minutes to clear the supernatant (see Table below).
3. Remove and discard supernatant.
4. Resuspend the microspheres in water or buffer of choice.
   - Use proper amount of liquid to arrive at desired percent solids.
   - The microspheres may need to be vortexed or sonicated to redisperse.
   - Use a sonic bath. Probe sonicators introduce the possibility of contamination in the sample, so care should be taken that the probe tip is completely clean before use.
5. Repeat these steps the number of times needed for your application.
   - For coupling of protein, at least 3 washes are recommended, with the final wash being done in the buffer to be used for protein attachment.

Table 1: Centrifugation Table
(for a standard benchtop centrifuge)

Microsphere Size	Centrifugal Force (G)	Time
300-500nm	9,300	15 minutes
500-800nm	2,200	15 minutes
800nm and up	1,200	15 minutes

IV. DIALYSIS

This method can be used with any size microsphere, but as particle diameter and membrane pore size change, dialysis time for complete cleaning might also vary.

A. Considerations

1. Concentration Differential

The driving force in dialysis is the concentration differential between the two solutions on the opposite sides of the membrane. Maximum efficiency occurs when the membrane is thin and the concentration differential is large.

2. Molecular Weight Cut Off (MWCO)

Dialysis membrane performance is characterized by the molecular weight at which 90% of the solute will be retained by the membrane. In addition to the MW, the exact permeability of a solute is dependent on the shape of the molecule, its degree of hydration, and its charge. Each of these may be influenced by the nature of the solvent. Extreme pH, ionic strength, or non-aqueous solvents may cause a deviation in the MWCO. Because of this, the MWCO should be used as a guide, and not an absolute prediction of performance. In the case of microspheres, a pore size close to the mean microsphere diameter will ensure a rapid exchange, but also a potential loss of 10% of the material.

3. Pore Size

A narrow pore size distribution is important. The pores should be very uniform and very close to reported size.

Table 2: Pore Sizes

MWCO	Diameter	
100,000	0.0100µm	100Å
50,000	0.0040µm	40Å
10,000	0.0025µm	25Å
5,000	0.0015µm	15Å

4. Flow Direction

Sample flow perpendicular to the membrane may cause blockage. Blockage can be reduced by sample mixing during dialysis. Mixing can be achieved by stirring the dialysis buffer, or by passing the sample parallel to the membrane. A good way to keep both the dialysate and buffer passing over the membrane at the same time, when dialyzing small volumes, is to place the microsphere suspension in a dialysis sack, inside a 50mL conical tube containing wash buffer. The tube is then placed on a rocker. Alternatively, a slow stirring rate will inhibit dialysis by creating a concentration polarization barrier at the membrane. Increased stirring will improve dialysis to the maximum rate allowed by the intrinsic properties of the membrane.

5. Hydrodynamic Flow

Flow is not a molecular process, but includes bulk movement of the fluid through a porous medium. The flow rate of the fluid through the medium is influenced by pressure, porosity, and the viscosity of the fluid.

Table 3: Hydrodynamic Flow

	Increased Flow	Decreased Flow
Membrane Porosity	high	low
Pressure Difference	large	small
Fluid Viscosity	low	high

6. The Donnan Effect

The Donnan Effect arises when one of the solutes (i.e., protein or polymer) is charged, but non-diffusible. As a result, an unequal distribution of ions impedes diffusion of the ionic species of the opposite charge from the non-diffusible species. This results in a membrane potential.

7. Concentration Polarization

This is a phenomenon that affects solute transfer across a semi-permeable membrane. Although this effect is more prevalent in ultrafiltration, an effort should be made to reduce concentration polarization in dialysis. Stirring or circulating the buffer is perhaps the easiest and most effective way to minimize this effect, caused by the accumulation of retained solutes, or particles, on the membrane or in the boundary layer adjacent to the membrane surface. This layer of solute is known as a gel layer, and may have a higher retention rate than the membrane itself. Diluting the solution will also help to minimize this effect.

8. Sample Volume

Tubing size should be selected to ensure that the sample can be contained within 5-20cm of tubing. The flat width of the tubing should provide the greatest surface area to volume ratio to enhance the rate of dialysis. There should also be an allowance for increased volume to retentate (2X).

9. Membrane Cleaning

Dialysis membranes must be washed thoroughly before being used for microsphere cleaning, since they are made with various water soluble surfactants, polymers, and other organics.

10. Solvent Concentration

The solvent volume should be maintained at least 10X the sample volume. The solvent should be changed frequently to ensure that diffusion takes place across the membrane against essentially zero concentration.

B. Procedure

1. Choose dialysis tubing according to the considerations listed above. A good guide is to choose the largest pore size that will still retain the microspheres.
2. Cut the proper length of tubing for your sample. Remember that the sample volume will increase from osmotic pressure during the dialysis.
3. Thoroughly wash the tubing free of any water soluble impurities, with the best available water, making sure that no air bubbles remain. At this point, you should also check your tubing with water to make sure that there are no leaks.
4. Place your sample inside the dialysis tubing. Again, remember to allow for increased volume inside the tubing.
5. Place the tubing containing the sample into the solvent you are dialyzing against. Make sure that you have securely clamped or tied off the ends of the tubing.
6. Allow to dialyze for ~24 hours at room temperature. Mixing will help increase the rate of diffusion. Changing the external solvent frequently will increase the exchange rate.
7. Remove the cleaned microsphere suspension for use in your experiment.

C. Dialysis Equipment Suppliers

These are only some companies that supply this equipment.

Fisher Scientific	Phone: 800-766-7000	Fax: 800-926-1166
Pierce Chemical	Phone: 800-874-3723	Fax: 800-842-5007

** Try Pierce's Slide-A-Lyzer® dialysis cassettes.*

Spectrum/Microgon	Phone: 800-634-3300	Fax: 800-445-7330

** Try Spectrum's 300K MWCO DispoDialyzers® for removing unbound IgG.*

V. CROSS-FLOW FILTRATION

A. General Information

Cross-flow filtration, also known as ultrafiltration or tangential-flow filtration, is another method that can be used to clean microspheres. In this method, the sample passes across the membrane tangentially, allowing solutes that are small enough to pass through the membrane, but retaining the microspheres. Making the flow run tangential to the membrane reduces the chance of clogging the pores and developing a filter cake. This method is effective for small microspheres as well as large ones, and may be a nice alternative to centrifugation, especially with large volumes.

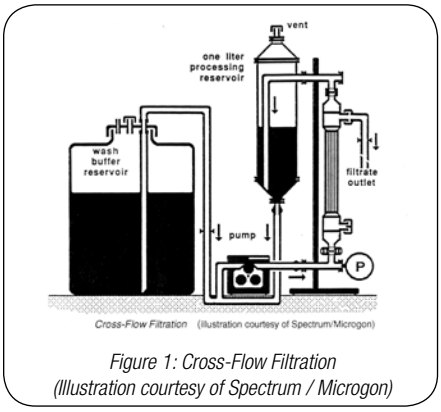

Figure 1: Cross-Flow Filtration
(Illustration courtesy of Spectrum / Microgon)

Cross-flow filtration is an effective method for cleaning microspheres, but there are several things that must be taken into consideration before choosing to use it. There is some loss of sample with this method, due to material being incompletely removed from the filtration unit. Although advances have allowed for the running of smaller sample volumes, there is always loss involved, so the volume you are filtering should be evaluated. This method will have a tendency to concentrate the microspheres.

A detailed protocol for this method is not included, as it will vary according to your application and equipment. We have listed several suppliers of this equipment below. If you decide that this is the proper method for your experiment, you will get technical help and protocols from the supplier, as well as advice on equipment choice.

B. Ultrafiltration Suppliers

Millipore	Phone: 800-645-5476	Fax: 800-645-5439
Pall / Gelman	Phone: 800-521-1520	Fax: 516-484-5228
Spectrum	Phone: 800-634-3300	Fax: 800-445-7330

VI. MIXED ION-EXCHANGE

A. General Information

This method will remove all electrolytes from the suspension, including charged surfactants. A mix of positive (cation) and negative (anion) exchange resins are used in this process. The sample containing the microspheres is added to the mixed resins, and any charged material on the particle or in the solution is sequestered by the exchange resins.

There are several considerations when using this method. One must carefully choose which type of resin to use. Although some precleaned and ready to use mixed resins are commercially available (e.g. through Bio-Rad), other resins may have to be cleaned or activated prior to use. They may have material on them that will adhere to the microspheres, and end up making them dirty instead of cleaning them. (We can offer additional guidance and protocols for cleaning commercial ion-exchange resins, upon request.) It is also true that uncharged material will not be removed from the particle suspension. This includes nonionic surfactants and some water-soluble polymers, which may be present in the microsphere suspension.

B. Ion - Exchange Resin Suppliers

Dow Chemical	Phone: 800-447-4369	Fax: 517-638-9331
Bio-Rad	Phone: 800-424-6723	Fax: 510-741-5800

VII. SERUM REPLACEMENT

A. General Information

Serum replacement is a cleaning method that is very similar to dialysis or cross-flow filtration. The considerations for dialysis can be carried over to this method. In the case of serum replacement, the particle suspension is placed in a "cell." The same osmotic process takes place, as in dialysis.

B. Stirred Cells for Serum Replacement

Millipore	Phone: 800-645-5476	Fax: 800-645-5439

VIII. CLEANING COATED DRY PARTICLES

Some dry particles may have a coating (i.e., calcium phosphate or colloidal silica) or contaminants that may need to be removed. Following is a simple procedure for doing so.

1. Place particles in a Büchner funnel that is attached to a vacuum source.
2. Ensure that the funnel has the appropriate frit or membrane to catch particles.
3. Disperse phosphate-coated particles in 2N Hydrochloric acid.
4. Disperse silica-coated particles in 10% NaOH (sodium hydroxide).
5. Stir for 1 hour.
6. Remove liquid by vacuum filtration.
7. Wash with DI water by filling funnel with water, stir for 5 minutes, then vacuum off water. Repeat water washes until pH of water in = pH of water out.

IX. APPENDIX

A. Washing of (Superpara)Magnetic Microspheres

Magnetic microspheres were made and are utilized for their ease-of-use. They are easy to separate from solution and, therefore, easy to wash. The same method of washing can be used for all magnetic particles, regardless of their size. The microsphere suspension is placed adjacent to a magnet, and the microspheres will pull over to the magnet. As soon as the supernatant is clear and has been aspirated or decanted, the tube can be removed from the magnet and the microspheres resuspended in fresh water or buffer. It should be noted that our magnetic microspheres contain a large amount of surfactant and may require many (10-15) washes at pH 8-10 to remove it. There are many suppliers of magnetic separation racks specialized for test tubes and centrifuge/microfuge tubes.

B. Washing of Silica Microspheres

Silica microspheres are supplied as either dry powders or as aqueous suspensions without surfactant. Because they might contain trace impurities as a result of the precipitation process, users often wash them prior to use. They may be washed using any of the methods outlined above. Their density (1.96 g/cm^3 versus 1.05 g/cm^3 for polystyrene) allows for easier handling. They will pellet more quickly and at lower speed than polystyrene microspheres will, and will resuspend more readily due to their hydrophilic surface. Please note, though, that silica microspheres will settle more quickly than polymeric microspheres, and so will need to be mixed or agitated when being processed by dialysis.

X. REFERENCES

1. **Ahmed, S.M., M.S. El-Aasser, G.H. Pauli, G.W. Poehlein, J.W. Vanderhoff.** 1980. Cleaning latexes for surface characterization by serum replacement. *J Coll Interface Sci,* 73(2): 338-406.

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