



Technical Note
TN – 019.02A
August 15, 2003

EVALUATING PORE SIZES OF BIOLOGICAL MEMBRANES WITH FLUORESCENT MICROSPHERES

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Revised 7/15/2003 from a paper published in Particulate Science and Technology 7:217-221, 1989

ABSTRACT

The application of a series of monodisperse fluorescent microspheres to membrane pore-size evaluation is discussed. The microspheres are available in red, green and blue fluorescent colors in a range of diameters from 25 nanometers to 3 micrometers. Particle retention and transmission can be monitored with an epifluorescence microscope or a fluorescence spectrophotometer. Once membranes are challenged with pairs of sizes and colors, the ratio of different colors upstream and downstream can be used as an indication of the membrane's particle retention properties. A case study is presented where fluorescent spheres are used to evaluate changes in fenestral pores of rat liver membranes.

1. INTRODUCTION

Biological membranes are of scientific interest because of their active role in the regulation of biological processes. Membranes can selectively transport or exclude water, ions, hormones, nutrients, foreign matter, or information between internal and external environments. They function by mechanisms such as surface charge, osmosis, hydrophobic interaction, immunological recognition, and size screening (i.e., through pores).

In this paper, we describe a method of using fluorescent microspheres to evaluate the pore sizes of rat liver fenestrae, as an example of their use in biological membrane research. The method is being developed at Tufts University Medical School, where it is part of an extensive research program on liver physiology¹.

2. FLUORESCENT MICROSPHERES

The method utilizes fluorescent polymer microspheres developed at the author's laboratories for membrane filter testing and other scientific applications². The microspheres are available in a range of sizes from 25 nanometers (nm) to 3 micrometers (μm), in red, green, and blue

fluorescent colors. Fluorescent microspheres offer both the sensitivity of fluorescence detection methods and the separation properties of their diameters.

The fluorescent dyes are incorporated into the polystyrene matrix of the spheres so they will not leach out in aqueous suspension media. Fluorescence values are approximately proportional to the volume of the microspheres. The dyes have large differences between the excitation and emission wavelength maxima, which permit flexibility in the selection of fluorescence detection systems. Table 1 summarizes the spectral properties of the dyes.

Fluorescent Color	Excitation Max. (nm)	Emission Max (nm)	25% Max. Emis. Threshold (nm)	Stokes Shift (nm)
Blue	388	447	433	59
Green	468	508	487	40
Red	542	612	589	70

Table 1. Spectral Properties of Fluorescent Particles

In addition to a choice of sizes and fluorescent colors, the microspheres are available with chemically clean surfaces, i.e., Bioclean™⁵ or with surfactant stabilization for the testing of synthetic membranes. The Bioclean surfaces permit the microspheres to be used for hydrophobic phagocytosis studies, or for adsorbing proteins or antibodies, making them suitable for neutral or active immunological response studies.

3. FLUORESCENCE MEASUREMENTS

Fluorescent microspheres can be observed with an epifluorescence microscope equipped with appropriate excitation and barrier filters, or their fluorescence can be quantitatively measured with a fluorescence spectrophotometer. In a typical membrane testing experiment, two sizes of microspheres are used which are two different colors. The sizes are selected so one size is retained by the membrane, the other transmitted by the pores. The fluorescent colors are then used as size indicators, making it unnecessary to measure the size of the particles to see which size was retained and which size transmitted. When two colors are used in this manner, the preferred color pairs are red and green or red and blue, to minimize the effects of spectral overlap.

Microspheres 180 nm or larger in diameter can be seen with a properly equipped fluorescence microscope. Those smaller than 180 nm are easily measured with a spectrophotometer. Larger microspheres may also be measured with a spectrophotometer, subject to light-scattering limitations. When absolute fluorescence values are required for maximum accuracy, a solvent such as methyl pyrrolidone can be used to dissolve the polymer matrix and make a clear solution of the dye for direct fluorescence measurements.

4. RAT LIVER MEMBRANE STUDIES

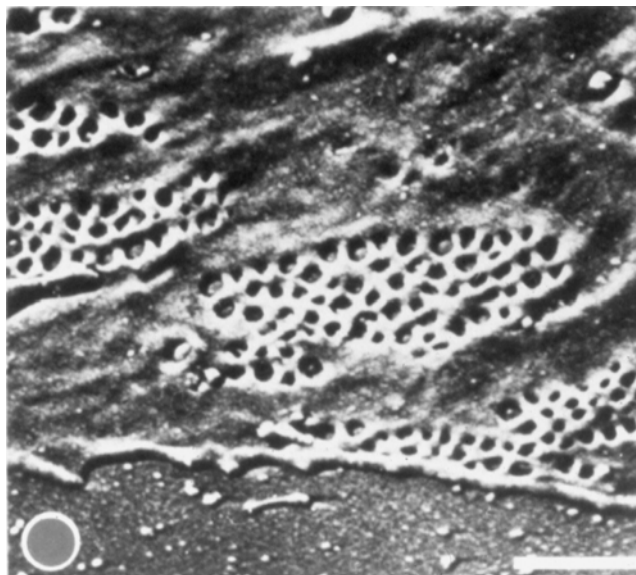


Figure 1. Rat Liver Fenestrae (bar $\approx 1\mu\text{m}$)

Investigations of rat liver fenestrae (openings, or pores) are being conducted at Tufts University Medical School to determine their changes in diameter as a function of stimulating factors in the bloodstream. The fenestrae are located in the endothelial cells of liver capillaries. Illustrated in Figure 1, they have a normal diameter of approximately 100 nm and act as a biological "screen" along the lumen of the capillary³. They are a transport medium between the hepatic cells of the liver and the flow channel of the capillary. The pores are believed to contract or dilate over a range of 80 to 120 nm under certain conditions, which are being studied with this method.

In addition to the endothelial cells containing the fenestrae, the capillary has macrophages known as Kupffer cells located at intervals inside the membrane. The Kupffer cells play an immunological role in the membrane; they phagocytize large foreign particles. The two kinds of cells combine their functions to capture or delay the passage of particulate matter through the capillaries. Using fluorescent microspheres in various sizes, colors, and surface treatments, both the size changes and the screening mechanisms of the fenestral pores can be studied.

5. PERFUSION SYSTEM

A pump and temperature controlled reservoir are used to perfuse Krebs-Ringer buffer through the rat liver in-vivo. The stream enters the portal vein and exits from the vena cava, where it is collected as a series of fractions for further analysis. A precision syringe is used to inject a bolus of microspheres into the perfusion stream for the test. The rat liver is maintained by continuously adjusting the perfusion medium to a pH of 7.4 with NaOH or HCl, and monitoring its oxygen uptake as an indication of its viability.

Fluorescence spectra for the microspheres are measured on a Perkin-Elmer Model LS-5 Spectrometer⁴. They are typically measured at fixed wavelengths of both excitation and emission, which provides an index of microsphere perfusion through the system. The

fluorescence spectra are used to distinguish between particle sizes in the eluted fractions. Since the two sizes of particles take different amounts of time to perfuse through the liver, the fluorescence spectra of each fraction permits an elution profile to be plotted for each microsphere size.

6. EXPERIMENTAL PROCEDURE

The perfusion buffer and solutions consist of 2 liters of Krebs-Ringer (bicarbonate) at pH 7.4; 100 mL of physiologically buffered saline (PBS) at pH 7.2; and a solution of bovine serum albumin (BSA) for coating the microspheres (4% in PBS). After sonicating the microspheres for 30 to 60 seconds to assure their dispersion, a 1.6 mL suspension of coated microspheres is prepared by combining 0.800 mL of BSA solution, 0.560 mL of PBS solution, and 0.240 mL of microspheres (1% solids). This 1.6 mL combination is allowed to incubate for 2 hours at room temperature so the BSA molecules can adsorb onto the microsphere surfaces. The 0.240 mL of microsphere suspension should include the two different colors and sizes of the microspheres at the desired ratios.

The baseline fluorescence values are obtained by taking a 0.2 mL aliquot of the 1.6 mL microsphere suspension, diluting it in 6ml of perfusion buffer, measuring the fluorescence of the two dyes, and multiplying the fluorescence values by 30 and by 0.2 mL (or the exact weight of the sample in grams). These are the total uninjected fluorescence values per unit volume.

The perfusion system is set up with the rat liver in place and the perfusion flow rate set at 30 mL per minute. After the viability of the rat liver is verified by observing the oxygen uptake rate, another 0.2 mL aliquot of the microsphere suspension is injected into the perfusion stream. The fraction collector is adjusted to collect 50 fractions in one minute, approximately 0.64 mL each. The fluorescence values for each color are measured for each fraction and compared to the standard fluorescence curve to obtain the ratio of recovered spheres to injected spheres at each time interval. In most cases, the first 25 fractions contain sufficient fluorescence for one set of data. The 1.6 mL of microsphere suspension is usually enough for four or five experiments including the fluorescence baseline calibration.

7. RESULTS AND CONCLUSIONS

As expected, the larger particles were generally eluted before the smaller particles, which were delayed by their tortuous path through the fenestrae. However, higher percentages of the smaller particles were typically recovered, probably because many of the larger particles were retained by the phagocytic action of the Kupffer cells. The BSA coated microspheres were recovered at a much higher rate than the uncoated microspheres, due to the interaction between the various protein surfaces along the membrane and the hydrophobic surface of the uncoated polystyrene microspheres. No significant difference was found between microspheres coated with rat serum albumin and BSA. Table 2 summarizes the recovery values for a series of coated and uncoated microspheres, in various sizes and fluorescent colors.

Microsphere		Bovine Serum Albumin	
Diam. (nm)	Color	Uncoated	Coated
47	Red		69.7
76	Green	13.7	
86	Green		75.5
105	Red	13.2	
111	Green		57.6
150	Blue	15.6	
185	Green	18.6	55.3
204	Red		46.8
210	Green	18.8	
312	Red	22.5	51.4
532	Red	21.3	38.5

Table 2. Recovery Rates of Fluorescent Microspheres

Additional procedures are being studied to refine the method, but it has been proven to be effective and useful by the Tufts University group. Changes in fenestral pore diameters as a function of stimulating agents in the bloodstream can now be studied in detail, meeting the original goals of the program.

8. SUMMARY

Evaluation of rat liver fenestrae is only one example of many potential applications of fluorescent microspheres to the field of membrane studies. The microspheres can be used to study a variety of screening and capture mechanisms, and can be adapted to the study of membrane immunological properties.

9. REFERENCES

1. Method being developed by I. M. Arias, Z. Gatnaitan, and K. Akamatsu, Dept. of Physiology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111.
2. S. D. Duke, "Particle Retention Testing of 0.05 to 0.5 Micrometer Membrane Filters", Proceedings, *International Technical Conference on Filtration and Separation*, p. 523-532, (1988), American Filtration Society, P.O. Box 6269, Kingswood, TX 77325.
3. Kirn, A., Ed., D.L. Knook and E. Wisse, *Cells of the Hepatic Sinusoid*, Vol. 1 (1986).
4. The Perkin-Elmer Corporation, Norwalk, CT 06856
5. The BIOCLEAR™ product line is a trademark of Duke Scientific Corporation and is no longer available for retail sale. Duke Scientific Corporation offers many alternative products for this type of testing.