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Probing pore structure of virus filters using scanning electron microscopy with gold nanoparticles

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ABSTRACT

The performance characteristics of virus filtration membranes are strongly influenced by the underlying pore size and morphology of the membrane, but the available characterization techniques provide only limited information on these properties. A new methodology was developed for probing the internal pore size and structure of different virus filtration membranes by direct visualization of captured gold nanoparticles using scanning electron microscopy (SEM). SEM images were obtained using both hollow fiber (Planova™ 20 N and BioEX) and flat sheet (Viresolve® Pro and Ultipor® DV20) membranes after challenging with 20, 40, and / or 100 nm gold nanoparticles. The images were used to identify the location of individual nanoparticles and to calculate the nanoparticle distribution through the depth of the filter (using ImageJ software). The results clearly demonstrate the very different pore structures within these filters and their impact on nanoparticle capture, providing important insights into the performance characteristics of these virus filtration membranes.

1. Introduction

Virus filtration membranes are used to remove viruses in both bioprocessing [1–3] and water treatment [4,5]. In bioprocessing applications, virus filters are designed to remove at least 99.9% of the smallest parvovirus (around 20 nm in size) while providing nearly complete transmission of the therapeutic product, e.g., a monoclonal antibody, which is approximately 8 nm in size. This requires very careful design and control of the membrane pore size and morphology to obtain the desired selectivity while also achieving high capacity (large volumetric throughput before fouling).

A number of different approaches have been used to obtain insights into the pore size / morphology of virus filters. Giglia et al. [6] used liquid-liquid displacement porosimetry with mixtures of polyethylene glycol and ammonium sulfate (referred to as CorrTest wetting and intrusion fluids) to evaluate the pore size distribution of a series of co-cast polyethersulfone virus filtration membranes. The calculated values of the pore size distribution were in good agreement with the measured retention of different size bacteriophages (PR772 and ΦX-174) and model mammalian viruses (MVM - minute virus of mice and PPV – porcine parvovirus). Similar results were obtained by Peinador *et al.* [7] using isobutanol and water for the liquid-liquid displacement tests and PP7 bacteriophage as a model virus. Calvo *et al.* [8] found that data for dextran retention coefficients provided similar results to liquid-liquid porosimetry, although neither approach was able to provide accurate information on the tail end of the pore size distribution, which is likely to be critical for achieving very high levels of virus retention. In addition, liquid-liquid porosimetry and dextran retention measurements provide no information on the location of the most selective pores or of the variation of the pore size with position within the depth of the examined virus filtration membranes.

Bakhshayeshi et al. [9] used confocal laser scanning microscopy with fluorescently-labeled bacteriophages (PP7 and MS2) to identify the location of virus capture within flat-sheet virus filters. The results clearly show the highly asymmetric structure of the Viresolve® Pro membrane, with virus capture occurring very close to the filter exit. In contrast, the selective pores within the Ultipor® DV20 membrane were located several microns into the depth of this membrane, which possesses a more symmetric (homogeneous) structure. This approach was extended by Fallahianbijan et al. [10] using different size nanoparticles, each with different fluorescent dyes. Confocal images of the captured nanoparticles in the Viresolve® Pro membrane provided a clear picture of the asymmetric structure of the membrane, with the pore size decreasing from 100 to 20 nm over approximately a 10–20 μm region near

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the exit of the filter. However, confocal microscopy has a resolution of only ≈500 nm, making it impossible to detect the capture location of individual viruses / nanoparticles.

Marquez-Rocha et al. [11] used Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Spectroscopy to study the pore size of a polyethersulfone ultrafiltration membrane. SEM images of the upper and lower surfaces clearly demonstrated the asymmetric character of these ultrafiltration membranes, but it was not possible to determine the actual pore size of the tight "skin" from the SEM images. Calvo et al. [12] used Field Emission Scanning Electron Microscopy (FESEM) to evaluate the pore size distribution of polysulfone ultrafiltration membranes. Image analysis yielded a much broader pore size distribution than determined from liquid-liquid porosimetry measurements, which the authors attributed to the difficulty in evaluating the very small pores and "the confusion between actual pore entrances and simply darker areas of polymeric surface." In addition, FESEM images of the intact membrane only provide information for the pores on the external surface of the membrane, which may or may not be the critical pores for virus retention.

A number of studies have used gold nanoparticles to evaluate the pore size and retention characteristics of virus filtration membranes. Sekine et al. [13] described the use of gold nanoparticle retention as an integrity test for Planova™ hollow fiber virus filtration membranes. Tsurumi et al. [14,15] were able to image captured gold nanoparticles within frozen fractured sections of a regenerated cellulose hollow fiber (BMM) virus filtration membrane. The nanoparticles were visible throughout the wall of the hollow fiber, with the greatest capture of 30 nm particles occurring approximately 10 μm into the filter as measured from the inner (lumen) surface of the fiber. A subsequent study showed that even gold nanoparticles as large as 100 nm were present through the depth of the pore structure of this filter. However, the resolution of the SEM images in this study was insufficient to identify the location of individual nanoparticles.

More recently, Kosiol et al. [16] examined the filtration of different size gold nanoparticles through a series of virus filtration membranes from different manufacturers. Optical micrographs of the tested membrane showed that 20 nm particles were captured primarily close to the feed-facing side of the membrane in the absence of sodium dodecyl sulfate (SDS), but the particles were observed deeper in the membrane upon the addition of SDS to the feed. This difference in behavior was attributed to the reduction in nanoparticle adsorption in the presence of SDS. Data for the retention of different size nanoparticles in the presence of SDS were used to evaluate the membrane pore size distribution, but the results provide no information on the nature of the membrane asymmetry, and the resolution of the optical microscopy was on the order of a micron (50 times the size of the nanoparticles). In their most recent work, Kosiol et al. [17] examined the capture of gold nanoparticles using SEM, with the membranes prepared by freeze-fracture with liquid nitrogen following procedures that were similar to those used in the current study. However, SEM images were shown for only a single membrane (manufacturer not identified) using very small 7.5 nm gold particles (well below the typical pore size of the virus filter), and the high nanoparticle loading used in these experiments made it difficult to identify individual particles. In addition, the use of gold nanoparticles in the presence of SDS could significantly alter the surface properties of both the nanoparticles and the membranes, potentially leading to artifacts in the observed capture behavior.

The objective of this study was to use scanning electron microscopy to evaluate the internal pore structure of different virus filtration membranes by direct visualization of the capture of different size gold nanoparticles (captured within the membrane during filtration). SEM images were obtained using both hollow fiber (Planova™ 20 N and BioEX) and flat sheet (Viresolve® Pro and Ultipor® DV20) membranes. The results provide important insights into the pore size / morphology and virus capture properties of different commercial virus filters.

Table 1
Properties of gold nanoparticles used in this study.

Product number	Size (nm)	Stock concentration (particles/mL)
741965	25 ± 8	6.5×10^{11}
741981	42 ± 12	7.2×10^{10}
742031	100 ± 30	3.8×10^{9}

2. Experimental

2.1. Gold nanoparticles

Gold nanoparticles were purchased from Sigma-Aldrich (St. Louis, MO) as stabilized suspensions in citrate buffer (Table 1). The nanoparticle size was determined by Dynamic Light Scattering (DLS) measurements performed using a Zetasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, UK). Nanoparticle suspensions were prepared by diluting the stock solutions with DI water obtained from a Direct-Q® 3 UV Water Purification System (MilliporeSigma, Billerica, MA) with resistivity greater than 18 MΩ cm.

2.2. Virus filters

Nanoparticle capture was examined using four different virus filters (Table 2): Planova™ 20 N and Planova™ BioEX hollow-fiber virus filters from Asahi Kasei Medical Co., Ltd (Tokyo, Japan), highly asymmetric flat sheet Viresolve® Pro filters from MilliporeSigma (Bedford, MA), and relatively homogeneous flat sheet Ultipor® DV20 filters from Pall Corp. (Port Washington, NY). These membranes were cast from different polymers (cellulose, polyethersulfone, and polyvinylidene difluoride) and with different morphologies as summarized in Table 2. The flat sheet membranes were cut into 47 mm disks and used in single layer format to facilitate observation by electron microscopy; the commercial flat sheet filters employ multi-layer membranes to obtain the high levels of virus removal needed in bioprocessing applications. The hollow fiber membranes were provided in cartridges containing 10 or 12 parallel fibers.

2.3. Gold nanoparticle filtration

Filtration experiments were performed at constant pressures of 70 and 210 kPa (10 and 30 psig) maintained by nitrogen pressurization of a feed reservoir filled with the nanoparticle suspension. Hollow fiber membranes were used in 0.001 m^2 modules oriented vertically as per the manufacturer's recommendations. Flat sheet membranes were used as 47 mm disks placed in a stainless steel filter holder (MilliporeSigma, Bedford, MA) with the skin-side away from feed (unless noted otherwise). Filters were initially flushed with at least 50 L/m^2 of DI water to ensure proper wetting and to eliminate any air bubbles. At the end of the filtration, the hollow fiber cartridge was cut open using a BrassCraft rotary tube cutter. Individual fibers were removed using a tweezer for examination by SEM.

2.4. Membrane preparation for SEM

Membranes were prepared using either a Leica EM UC6 Ultramicrotome or a Leica CM1950 Cryostat (Leica Biosystems Inc., Buffalo Grove, IL). For ultra-microtomy, small lengths of individual hollow fibers (~5 mm) or rectangles of the flat sheet membranes (10 × 3 mm) were cut using a scissor and dehydrated using progressively more concentrated ethanol solutions, beginning with 50% ethanol in water followed by 70%, 90%, and then 100% ethanol. The samples were then infiltrated with a 1:1 ratio (v/v) of 100% ethanol and LR white medium grade embedding resin (Electron Microscopy Sciences, Hartfield, PA) for a minimum of one hour (when working with