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# Nanoporous Membranes with Ultrahigh Selectivity and Flux for the Filtration of Viruses\*\*

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The filtration, separation, and isolation of viruses are critical issues for controlling blood-borne viral infections and for viral research.<sup>[1–3]</sup> Membrane-based technology has been identified as a useful method for the separation of biomaterials including viruses, owing to its efficiency, ease of implementation, and cost effectiveness.<sup>[4–8]</sup> Several types of membranes have been employed for virus filtration.<sup>[9–13]</sup> For example, microfiltration (MF) membranes show a relatively high flux and good retention of viruses on the membrane due to the presence of electrostatic interactions under appropriate conditions.<sup>[9]</sup> However, the pore size of MF membranes is typically much larger than the size of the virus particles, which limits their applicability to biomaterials that are tens of nanometers in size.<sup>[10]</sup> Ultrafiltration membranes with smaller pore sizes have also been employed for the separation of viruses.<sup>[11–13]</sup> However, they have not been very effective, since the virus particles permeate into a small number of abnormally large-sized pores.<sup>[11,12]</sup> Track-etched polycarbonate (PC) and anodized aluminum oxide (AAO) membranes with a uniform pore size have also been studied for the separation of viruses. While the pore size distributions are narrow for these membranes, both types of membranes show a very low flux for virus separation.<sup>[13]</sup>

Thus, a new type of membrane, providing both high selectivity and high flux, is needed to filter viruses. Here, we introduce a new membrane with an asymmetric film geometry,

which shows both high selectivity and flux. This membrane consists of a thin nanoporous layer, prepared from a block-copolymer template, and a support membrane that provides mechanical strength. This asymmetric membrane shows ultrahigh selectivity while still maintaining a high flux for the separation of human rhinovirus type 14 (HRV14), which has a diameter of ~30 nm<sup>[14,15]</sup> and is a major pathogen for the common cold in humans. Since the pore diameter in the top layer can be tuned from 10 to 40 nm,<sup>[16,17]</sup> the cutoff size of the membrane filter can be precisely controlled. With these pore sizes, this membrane allows biomolecules such as proteins present in the unfiltered solution to pass through the membrane, while only the viruses are screened. This unique characteristic of the new membrane filter eliminates the risk of contamination from viruses while processing biotherapeutic proteins such as vaccines and hormones. Therefore, this new membrane can be used to develop new types of blood-filtering systems, such as a haemodialysis membrane that is free of the risk of viral infection. Moreover, this membrane provides an easy means to increase the concentration of the virus, thereby making it possible to investigate virus cultivation and the morphologies of unknown viruses.

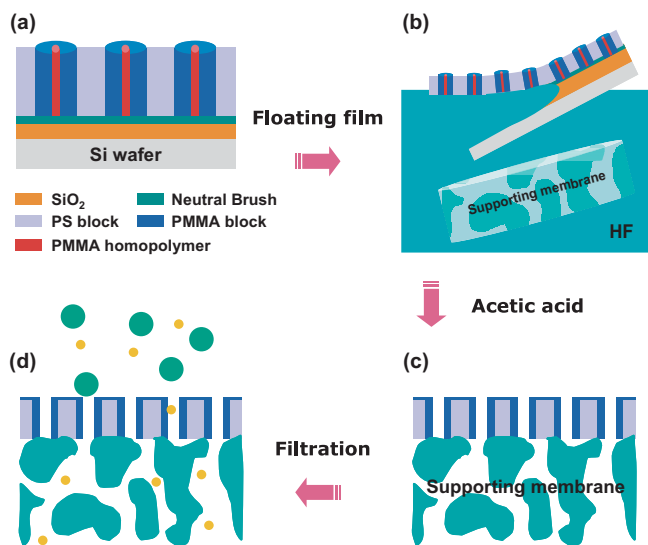
Figure 1 shows a schematic depiction of the fabrication of asymmetric nanoporous membranes. The top separation layer (~80 nm thick) is made from a thin film of a mixture of polystyrene-*block*-poly(methyl methacrylate) copolymer (PS-*b*-PMMA), with cylindrical microdomains of PMMA, on a ~100 nm thick sacrificial silicon oxide layer. As previously reported,<sup>[18]</sup> when the PMMA homopolymer is added to PS-*b*-PMMA, the cylindrical nanodomains orient normal to the surface in films of up to ~300 nm thickness on surfaces where the interfacial interactions have been balanced (Fig. 1a).<sup>[19]</sup> This thin film can be removed from the substrate by using a buffered HF solution to dissolve the oxide layer. The film is then transferred onto the MF polysulfone (PSU) membrane, which acts as a support (Fig. 1b). The adhesion between the block-copolymer-mixture film and the PSU membrane is sufficient to maintain the mechanical integrity of the system during the fabrication and filtration experiments. Porous thin films of the upper layer can be prepared by selectively removing the PMMA homopolymer from the cylindrical PMMA microdomains with acetic acid (Fig. 1c). This produces a well-ordered array consisting of ~15 nm diameter pores with a narrow pore size distribution (see Fig. S1, Supporting Information), which completely prevents the HRV14 virus (colored green) from penetrating into the pores, while proteins, such as bovine serum albumin (BSA) (colored yellow)

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**Figure 1.** Schematic depiction of the procedure for the fabrication of asymmetric nanoporous membranes.

low) with a size of  $\sim 7.2$  nm, can pass freely through the pores in the membrane (Fig. 1d).

Figure 2 shows the flux of deionized (DI) water and phosphate buffered saline (PBS) solution containing  $2.5 \times 10^5$  plaque forming units (PFU) mL<sup>-1</sup> of HRV14 through two different membranes: a) the nanoporous membrane developed in this study, and b) a track-etched PC membrane. The flux through the membrane prepared in this study is two orders of magnitude greater than that through the PC membrane. This increase can be attributed to two causes. The first cause is that the nanoporous copolymer film is much thinner (80 nm) than the PC membrane (6.5  $\mu$ m). The reduced thickness of the top layer results in an increased flux since the resistance against mass transfer is mainly determined by the top layer, which has a smaller pore size. The second cause is the higher areal density of pores (20 % in the copolymer film versus 2 % in the PC film). It is not possible to achieve such a high areal density of pores in the track-etched PC membrane.

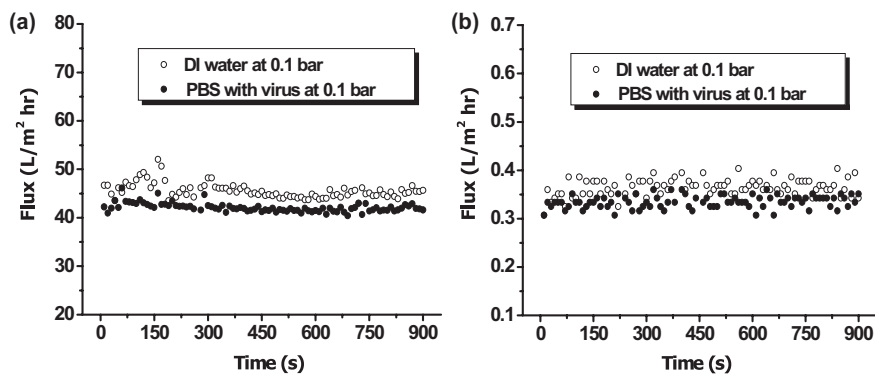
Figure 3 shows scanning electron microscopy (SEM) (Fig. 3a) and scanning force microscopy (SFM) (Fig. 3b) images of HRV14 virions that have been blocked by the nanoporous membrane. In Figure 3a, many spherically shaped

particles with structures characteristic of picornaviruses<sup>[20]</sup> are seen to be deposited on the membrane. From the SFM image, the diameter and height of the HRV14 particles are measured to be 34 and 30 nm, respectively, consistent with their icosahedral structure.<sup>[15]</sup> However, these results do not imply that all the virus particles have been screened by the membrane.

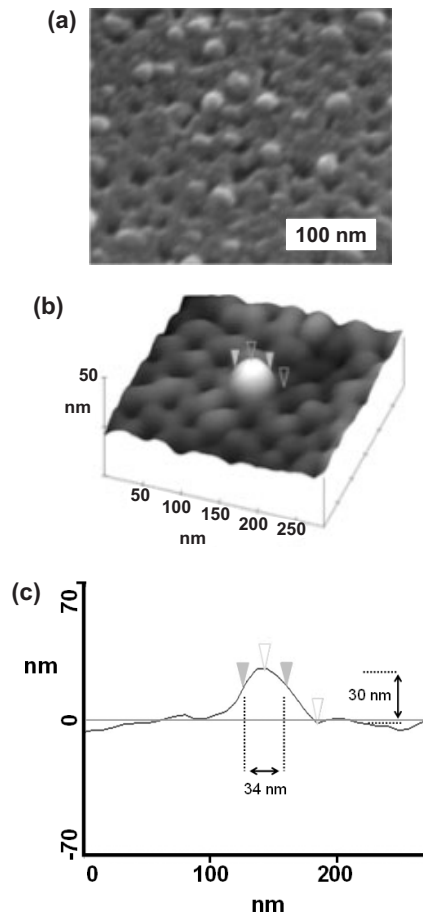
To answer this question, we have performed a plaque assay for HRV14, and the results are shown in Figure 4. The plaques, shown as white circles, are areas covered by dead HeLa cells on a culture plate, and are surrounded by live HeLa cells shown in black (Fig. 4a). A single viral plaque in the monolayer of HeLa cells represents one infection of a virion particle, which infects and proliferates in the cell, the progenies of which infect neighboring cells. Therefore, the titer of the virus in solution can be measured very precisely and with high sensitivity by this plaque assay. About 2 % of the virion particles penetrate through an AAO membrane, even though the viral titer is reduced from  $5 \times 10^6$  to  $9.5 \times 10^4$  PFU mL<sup>-1</sup>, as shown in Figures 4a,b. Although the AAO membrane shows a high flux ( $90$  L m<sup>-2</sup> h<sup>-1</sup>) owing to its highly porous structure and hydrophilic properties, some virion particles are able to pass through the membrane because of the presence of larger pores with diameters of  $\sim 50$  nm; these pores are much larger than the regular pores with a nominal diameter of  $\sim 20$  nm (see Fig. S2, Supporting Information). However, no plaque formation is observed for solutions filtered through either the bilayer membrane (Fig. 4c) or the PC membrane (Fig. 4d) developed in this study. These results indicate that both the PC and nanoporous membranes completely block the penetration of virion particles through the filters.

The results shown in Figures 2–4 indicate that the nanoporous membrane developed here exhibits excellent selectivity for the HRV14 virus and yields a high solution flux. In another permeation experiment, using a PBS solution containing 1 mg mL<sup>-1</sup> of BSA, almost all of the BSA proteins permeate through the nanoporous membrane, as verified by the Bradford method (see Fig. S3, Supporting Information).<sup>[21]</sup>

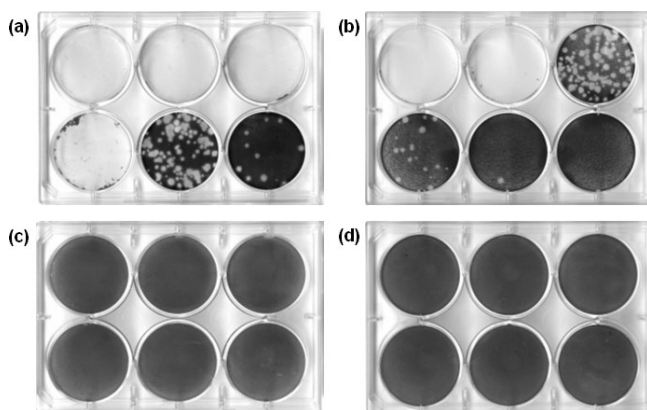
Since the pore size in the top layer can be easily tuned between 10 and 40 nm,<sup>[16,17]</sup> this technique can be used for the filtration, purification, and concentration of various viruses. The purification and concentration of viruses represent criti-



**Figure 2.** Flux data of DI water and PBS solution containing  $2.5 \times 10^5$  PFU mL<sup>-1</sup> of HRV14 using a) the nanoporous membrane prepared in this study and b) a PC membrane.



**Figure 3.** a) Film surface image of the nanoporous membrane after filtration of a PBS solution containing  $2.5 \times 10^5$  PFU mL<sup>-1</sup> of HRV14 investigated by SEM; b) SFM. b) The height SFM image of HRV14 virions deposited on the nanoporous membrane. c) Height profile of a HRV14 virion determined from (b).



**Figure 4.** Plaque assays of the HRV14 solution a) before filtration and b–d) after filtration through various filters: b) AAO membrane with a nominal diameter of  $\sim 20$  nm, c) asymmetric membrane prepared in this study, and d) PC membrane with a diameter of  $\sim 15$  nm. Serially diluted solutions are applied to monolayers of HeLa cells. The well at the upper-left corner contains the virus from 0.2 mL of the original solution permeating through a filter, and each well is consecutively diluted by a factor of ten up to a dilution of  $10^5$  for the well at the lower-right corner.

cal steps in the identification of pathogenic viruses from patient samples, the titration of viruses with low titer in patient sera, the in-vitro cultivation of viruses, and the observation of viruses after purification. The development of a device to concentrate virus particles from dilute solutions enables the measurement of viral titers in drinking water, which will improve public hygiene by preventing the spread of water-borne viruses. The filtration of viruses can be used in the cultivation of human cells for therapeutic purposes using animal sera, without risk of infection from zoonotic viruses. Moreover, this enables the development of a virus-proof filter for the haemodialysis of patients with renal failure who are at a greater risk of viral infection.<sup>[22]</sup>

In summary, a new double-layered nanoporous membrane suitable for virus filtration has been introduced in this work. This membrane is an 80 nm thick film with cylindrical pores of diameter 15 nm with a narrow pore size distribution. This layer is prepared by using a thin film of a mixture of a block copolymer and a homopolymer, and mainly acts as a virus separator. The support layer (ca. 150  $\mu$ m thick) is a conventional microfiltration membrane with a broad pore size distribution. This asymmetric membrane showed very high sensitivity and flux for the separation of the human pathogen of the common cold in humans.

### Experimental

Asymmetric PS-*b*-PMMA with an average molecular weight ( $M_w$ ) of 89 000 g mol<sup>-1</sup> and a polydispersity of 1.06 was prepared by atom transfer radical polymerization [18]. The volume fraction of PMMA was 0.3. Atactic PMMA homopolymer with  $M_w = 31\,800$  g mol<sup>-1</sup> and a polydispersity of 1.08 was purchased from Polymer Source. A hydroxy end-functionalized random copolymer of styrene and methyl methacrylate, denoted PS-*r*-PMMA, with a styrene fraction of 0.6, was synthesized in bulk by means of a 2,2,6,6-tetramethylpiperidin-1-yl oxide (TEMPO) “living” free-radical polymerization [19]. The  $M_w$  was 9 600 g mol<sup>-1</sup> with a polydispersity of 1.80.

PS-*r*-PMMA was spin-coated onto a silicon oxide sacrificial layer with a thickness of 100 nm, which had been evaporated onto a silicon wafer, and then annealed at 170 °C under vacuum for 2 days. This procedure permitted the coupling reaction between the hydroxyl group of PS-*r*-PMMA and the oxygen group in the silicon oxide layer [19]. A thin layer ( $\sim 6$  nm) of PS-*r*-PMMA remained on the surface after rinsing with toluene.

Thin films ( $\sim 80$  nm) of the mixture of PS-*b*-PMMA and 10 wt.-% homopolymer PMMA relative to the PMMA block were prepared by spin-coating 2% (w/v) toluene solutions onto the modified silicon wafers and then annealing at 170 °C under vacuum for 2 days, followed by quenching to room temperature. The films were floated onto the surface of a 5 wt.-% HF solution, transferred to a water bath, and then transferred to porous membrane supports (HT Tuffryn, Pall Life Science), which had an average diameter of 0.2  $\mu$ m and thickness of 150  $\mu$ m. The PMMA homopolymer was removed by washing with acetic acid for 60 min at room temperature. The nanopores in the thin film were observed by field-emission scanning electron microscopy (FESEM, Hitachi S-4200) [18]. We also used a track-etched PC membrane (Nucleopore, Whatman Co.), with pores of average diameter 15 nm and height of 7  $\mu$ m, and AAO membranes (Anodisc, Whatman Co.) with pores of nominal diameter 20 nm and thickness of 60  $\mu$ m.

Permeation experiments were performed at a stirring speed of 200 rpm and a pressure of 0.1 bar at room temperature in a stirred cell module (Amicon 8010, Millipore Co.) (1 bar = 100 000 Pa). The

stirred cell had a working volume of 10 mL and an effective membrane area of 4.1 cm<sup>2</sup>. It was necessary to immerse the nanoporous copolymer membrane in ethanol for 1 h for the aqueous solution to wet the nanopores [23] prior to loading into the preautoclaved stirred cell. Excess ethanol was completely removed by flushing with DI water for at least 30 min. The morphology of the deposited virions on the membranes was investigated by SFM (Digital Instruments D3000) using silicon-nitride tips on the cantilever (Nanoprobe) in the tapping mode and by FESEM. To prepare samples for FESEM, virions were first fixed by 1 % glutaraldehyde in a PBS solution and then dehydrated by ethanol [20]. Subsequently, the membrane was dried under vacuum for 24 h at 25 °C, and finally sputter-coated with platinum.

The cultivation and purification of HRV14 was performed as described by Erickson et al. [14]. A virus solution (5 mL, 5 × 10<sup>6</sup> PFU mL<sup>-1</sup> in PBS) was forced to pass through various filters, and then plaque assays were performed using solutions penetrating through the filters [24]. HeLa/E cells were grown in a 35 mm petri dish with Dulbecco's Modified Eagles Medium, 1 % penicillin/streptomycin, and 10 % fetal bovine serum. The HeLa cells were washed once with PBS and once with a serum-free medium. The solutions permeating through the filters were serially diluted (tenfold) and then applied to the cells. The first well in each dish contained viruses from 0.2 mL of the permeated solutions. An overlaying medium, composed of Dulbecco's Modified Eagles Medium, 1 % penicillin/streptomycin, 5 % fetal bovine serum, and 50 % gum tragacanth, was added to the virus-infected cells, which were then incubated for 3 days. After the virus cultivation, the overlaying medium was removed and the plaques were visualized with 0.5 % crystal violet dissolved in 70 % ethanol.

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