Application Of A Novel Ultrathin Nanoporous Silicon Nitride Membrane To The Separation Of Soluble Aggregated Macromolecular Species

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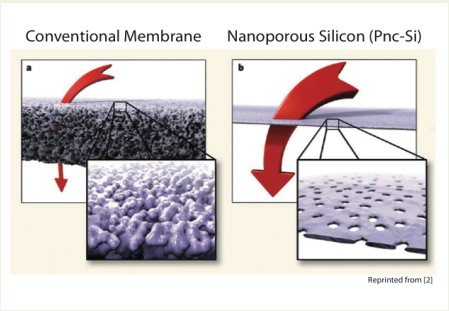
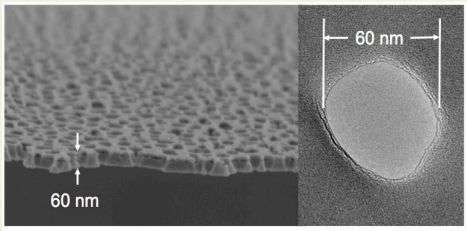
Introduction

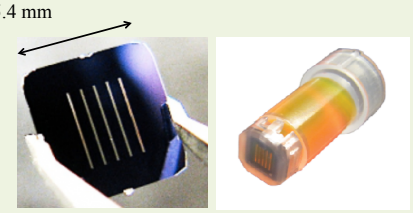
Formation of aggregates in biopharmaceutical products continues to be one of the major quality issues in biotherapeutic development. Protein aggregates can impact the quality, safety and efficacy of the biotherapeutic. While insoluble aggregated species or particles can readily undergo physical separation using traditional membrane-based filtration systems. A more vexing problem is the molecular separation of smaller sized aggregates (e.g. dimer, trimer) from the monomer in the nm size range. Regulatory agencies are expecting soluble aggregated species to be characterized during development, stability testing and commercialization. Typically, size exclusion chromatography is used for this characterization. However, chromatographic conditions can cause disruption of the aggregate and large aggregates can be excluded from the analysis, remaining on the top of the stationary phase. Here we describe use of an ultrathin (15 - 50 nm) free standing silicon nitride nanomembrane to fractionate materials with 5 nm size resolution without dilution or sample loss under intrinsic conditions.

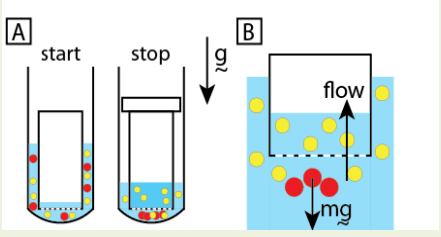


Porous nanocrystalline silicon (pnc-Si) membranes are formed as a result of controlled crystallization of amorphous silicon. The membranes combine a unique combination of nanoscale thickness (LT 50 nm) with robust, millimeter-scale lateral dimensions and tunable pore sizes in the range of 5 to 100 nm. Conventional ultrafiltration membranes made from solvent-cast polymers have tortuous path, sponge-like, pore structures that limit the resolutions of separations. High internal surface area leads to flow resistance, sample loss and clogging. Nanoporous silicon membrane material provides high resolution separation with little sample loss and dilution of the filtrate. In this work we have used the pnc-Si as a template to transfer the pores into a stronger, more chemically robust silicon nitride film that is 50 nm thick. The nanoporous silicon nitride membranes have an average pore diameter of 70 nm and porosity of 25 %.



5.4 mm





(A) `Reverse` centrifugation scheme. (B) Agglomerates of NPs are pulled away from membrane despite flow through membrane.

Materials and Methods

Formation of Antibody Aggregates. FITC labeled Goat anti-Rabbit IgG (affinity purified) was purchased from Life Technologies. All other reagents were obtained from standard sources, high purity water (NANOpure) was used in all solution preparation. FITC-IgG was diluted to ~ 50 µg/mL in PBS + 0.1% Tween 20. An excess of amount of ammonium sulfate was added to purified water to created a saturated solution (100% v/v). Small volume mixtures (200 µL) of IgG and salt were combined, mixed and immediately analyzed by dynamic light scattering or fractionated using SEPCON™ devices.. The total active area of the filter is 1.5 mm^2. Separations were performed in an angled centrifuge for 5 min at 2,000 rpm.

Dynamic Light Scattering Measurements (DLS). Solutions of IgG with increasing salt concentration from 0% to 30% were mixed immediately prior to DLS measurements. Sixty µL of IgG/salt solutions were transferred to a low volume cuvette. DLS was measured using a Malvern Zetasizer Nano ZS. The DLS distribution results were plotted as a function of intensity. The intensity distribution weights species according to the amount of light they scatter, thus responses due to aggregates would predominate.

Fluorescence Measurements. Fluorescence was measured using a Tecan infinite m200 UV/VIS spectrometer. Solutions with volumes of 3 µL were placed on a Nanoquant 16 well plate. Samples were excited at 450 nm and emission was measured at 520 nm.

Separation of Nanospheres from IgG

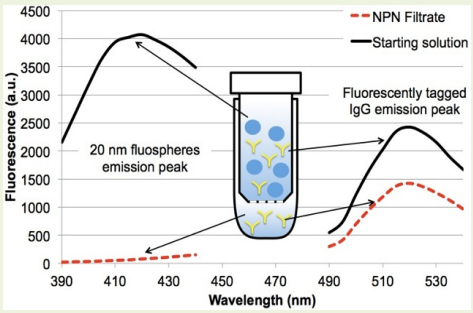


Figure 1. The starting solution was a mixture of 25 µg/mL fluorescent IgG and 20 nm Fluospheres at a concentration of ~5x10^{14} particles/mL in 0.1% Tween 20 and 1xPBS. The solution was centrifuged for 30 min at 5,000 rpm. The peak at 420 nm in the stock solution corresponds to the the fluorescence of the Fluospheres. There is almost no signal in the filtrate, indicating no Fluospheres passed through the filter. The peak at 520 nm is caused by the fluorescent IgG and the peak from the filtrate shows that IgG is passing through the filter.

Dynamic Light Scattering Measurements

IgG Peak Intensity Shifts to Larger Apparent Diameter in 25% (v/v) Ammonium Sulfate

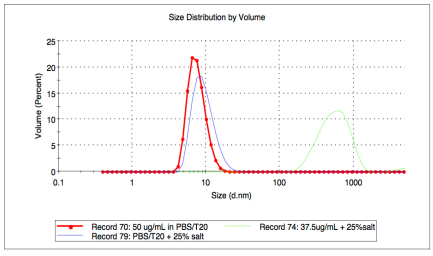


Figure 2. Size distributions for (red) 50 µg/mL IgG PBS/Tween20, (green) 38 µg/mL IgG + 25%(v/v) $(NH_4)_2SO_4$ and (blue) 25% (v/v) $(NH_4)_2SO_4$ in PBS/Tween20 only are shown above. The intensity size distribution of IgG shows a shift to increasing apparent diameter in the presence of ammonium sulfate from ~ 9 nm in PBS/0.1%Tween20 to ~ 700 nm in 25% $(NH_4)_2SO_4$ in PBS/Tween20. The peak observed ~ 9 nm in 25% PBS/Tween20 is likely due to the presence of micelles since Tween 20 at 0.1% is in excess of the critical micelle concentration of 0.01% .

IgG Size (nm) with Increasing Salt (%v/v)

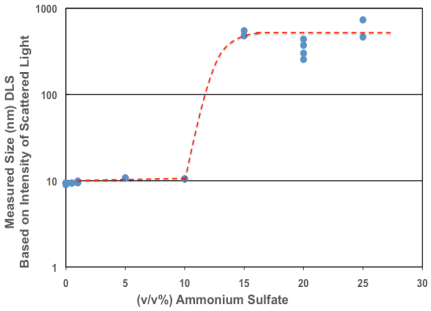


Figure 3. The primary scatter intensity peak was monitored in mixtures of 0.5, 1, 5, 15, 20, 25 and 30% $(NH_4)_2SO_4$ /IgG. A sharp increase in apparent diameter was observed at ~ 15% ammonium sulfate from ~ 10 nm to a plateau value ~ 700 nm at 25% salt. Based on a hydrodynamic diameter of ~ 10 nm for monomeric IgG, assuming a spherical shape, an aggregate with an apparent diameter of ~ 700 nm corresponds to a 70mer.

Separation of IgG Aggregates

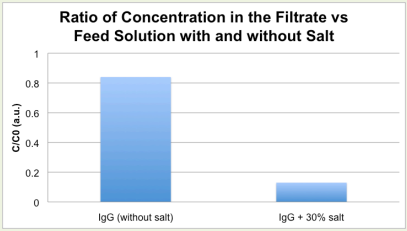


Figure 4. The ratio of filtrate to feed solution fluorescence response at ~ 50 µg/mL FITC shows little change after ~ 5 mins of centrifugation indicating minimal separation. In contrast a ~ 35 µg/mL + 30% salt mixture showed a 70% reduction demonstrating a significant fractionation of aggregates from the starting solution.

Conclusion

Ultrathin pnc-Si membranes exhibit sharp size discrimination, low sample loss and little dilution of the filtrate. Separations occur in minutes using standard laboratory equipment. The centrifugation devices provide an easy and rapid way to perform separations of biotherapeutics. The devices could be used to assess aggregate formation during formulation development, stressed stability testing and bioprocess scale up to rapidly screen conditions and rank order product development pathways

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About SiMPore

SiMPore was founded by the inventors of pnc-Si in 2007. Today SiMPore develops and commercializes novel ultrathin membranes for applications ranging from electron microscopy to separations and cell culture. SiMPore's membrane products are available directly and through international distributors . Please see www. Simpore.com for more information.