Combining Fluorescence Microscopy and Flux Experiments for Visualizing the Mechanism of BSA Biofouling

Charles S. Widing, Nathan R. Ruffle-Deignan, Elana M.S. Stennett

Department of Chemistry, Hobart and William Smith Colleges, Geneva, NY, USA

For correspondence, please send to stennett@hws.edu, 300 Pulteney St, Geneva, NY 14456, 315-781-3185.

Short Running Title: Visualizing Mechanism of BSA Biofouling

Combining Fluorescence Microscopy and Flux Experiments for Visualizing the Mechanism of BSA Biofouling

Flux experiments and fluorescence microscopy were combined and optimized to visualize the membrane surface during biofouling of two mixed cellulose ester membranes. Using flux measurements, the fouling by bovine serum albumin (BSA) was measured in the presence of 1 to 12% labeled BSA. By fitting the relative flux decays to an exponential decay for statistical analysis, the dye in this range of labeled protein was found to not affect the fouling nature of the protein. A 2.5% or 5% labeled protein sample was determined to be the best percent labeled protein for fluorescence imaging the membrane because the beginning of cake formation was observed within 25 min of experimental time. Finally, by fitting the flux data to four different biofouling mechanism equations, we conclude that both membranes, though at different rates, have BSA depositing inside the membrane pores restricting the flow eventually leading to cake formation. The combination of the two techniques allows for further insight into the biofouling mechanism of BSA, and this method can be applied to other biological molecules.

Keywords: fluorescence; biofouling; cake formation; flux; bovine serum albumin

Introduction

One of the largest issues facing the world is availability of drinking water, voted as one of the top priorities of the UN for the next decade.(Nations, n.d.) Desalination is a potential solution to this issue, but factors like biofouling still limit its efficiency and effectiveness.(Chen, Qian, Zhou, & Yu, 2018; Goh, Lau, Othman, & Ismail, 2018) Biofouling is the reversible and irreversible build-up of biological material on the membrane. Typical solutions for this problem include pre-conditioning and chemical cleaning, though neither can fully eliminate biofouling.(Goh et al., 2018; Jeong, Naidu, Leiknes, & Vigneswaran, 2017) Understanding the mechanism of biofouling would help determine best practices for alleviating biofouling, but as biofouling is believed to be quite complex, there is no easy solution.(Hou, Wang, & Song, 2017) Fluorescence is an excellent technique to better visualize the membrane for understand biofouling.

Fluorescence has become an increasingly popular technique in the past couple of decades due to two facts: 1) few molecules fluoresce causing little background interference and 2) a low concentration of fluorescent molecules are needed for visualization. With the advancement in imaging technologies, fluorescence can be used to visualize biological processes if a fluorophore can be attached to the biomolecule of interest.(Lichtman & Conchello, 2005) In fact, fluorescence has been incorporated into studying fouling of membranes using confocal scanning laser microscopy (CLSM), which uses a pinhole to block out unfocused light allowing for depth measurements of the sample.(Ferrando, Rŏzek, Zator, López, & Güell, 2005) This technique has been used to visualize the interaction of biomolecules inside the membrane pores.(Bacchin, Snisarenko, Stamatialis, Aimar, & Causserand, 2020; Chen et al., 2018; Ferrando et al., 2005; Maqbool, Ly, Asif, Ng, & Zhang, 2020; Marroquin, Vu, Bruce, Powell, et al., 2014; Marroquin, Vu, Bruce, Ranil Wickramasinghe, et al., 2014; Wu, Lin, Chou, Hu,

& Tung, 2019; Zator, Ferrando, López, & Güell, 2007) The challenges with this technique, though, are 1) to differentiate between biomolecules, the samples must be completely fluorescently labeled and 2) depths deeper than micrometers cannot be probed.(Chen et al., 2018; Hao, Liang, Moriya, Matsuyama, & Maruyama, 2012; Meng et al., 2010) In addition, to gather real-time data, modifications in how the membrane is constructed and the fouling experiment conducted must be performed.(Bacchin et al., 2020; Mukherjee, Menon, Liu, Kang, & Cao, 2016) Fluorescence microscopy, though, allows for imaging the surface of the membrane at any time without the drawback of needing a large amount of labeled biomolecules or restructuring of the fouling experiment.

Through the combination of measuring flux and fluorescence microscopy of membranes after they are fouled, we are able to image the membrane surface to observe biofouling. This method has been optimized by determining the best amount of fluorescent label to ensure good images while not effecting the fouling nature of the protein as well as the best protocol for visualizing the fouled membrane through fluorescence microscope. With this technique, we can now visualize the surfaces at various times throughout the flux measurement to correlate fouling behavior with what is occurring on the membrane providing further evidence for how cake formation occurs.

Materials and Methods

Purified deionized water (Synergy UV, Millipore, 18.0 MΩ) was used throughout this study. Sodium phosphate dibasic (Na_2HPO_4, anhydrous, Baker Chemical) and potassium phosphate monobasic (KH_2PO_4, Sigma-Aldrich) were used to make a 0.150 M buffer at pH = 6.00 ± 0.02, which was vacuum filtered (0.22 μm, PES) before use.

The solutions had a protein concentration of 4.0 g/L and made up of corresponding amounts of unlabeled BSA (called uBSA) or labeled BSA (called F-BSA). Percent of labeled protein varied from 1 – 12% to optimize which was best for this study. Bovine serum albumin (nuclease- and protease-free, VWR, 97061) was used as is as the unlabeled BSA. The labeled protein was albumin-fluorescein isothiocyanate (Sigma-Aldrich, A9771) and used as is. Table 1 shows how the different amounts of uBSA and F-BSA were calculated for each protein solution to maintain a constant mass of protein independent of the mass of the label. Protein concentration was verified using absorbance spectroscopy as described in (Kilmer, Huss, George, & Stennett, n.d.); protein absorbance at 279 nm ranged from 2.45 – 3.45 au depending on the percent of labeled protein present as fluorescein absorbs in this region.

Two different mixed cellulose ester membranes were purchased from Millipore and used as is: 1) MF Millipore (GSWP02500, referred to as MFM) is a white hydrophilic, mixed cellulose ester membrane with 0.22 μm pores and 2) black mixed cellulose ester membrane (HABP02500, referred to as BCE) is a black hydrophilic membrane with 0.45 μm pores. All membranes were conditioned by soaking for 10 min in ultrapure water and then running 400 mL water through the membrane at pressure; the water flux for MFM was between 4,400 – 5,600 kg/(m^2hr) while it was 11,600 – 14,100 kg/(m^2hr) for the BCE at 40 kPa.

The simulator and flux procedure are described in (Kilmer et al., n.d.) with the following modifications: initial protein solution temperature was 21 °C; water flux was measured first to verify J_o, then 400 mL of PBS buffer was run through the membrane at pressure before the protein sample was run. Mass of the permeate was collected every second, and every 11 points were averaged to smooth noise in the data. The protein sample was run until the solution (typically around 125 mL) ran out or after

approximately 25 min, whichever was shorter. Then, the membrane was rinsed by discarding any remaining protein solution and pushing ultrapure water through at pressure for 6 min or a total of 200 mL, whichever came first. As the membrane was removed to dry, the orientation of the membrane in the holder was marked for consistent imaging (Supplemental Figure 1). Drying time varied depending on the membrane: MFM = 48 hrs and BCE = 24 hrs. This time was optimized to get the best pictures with the least amount of membrane curving.

Flux measurements were also conducted where the protein run was interrupted in order to image membranes after a small volume of permeate, called a shortened flux experiment. For the BCE membrane, the permeate was collected for less than 60 sec while for the MFM membrane, the permeate was collected for less than 100 sec. After this experimental time, the remaining protein solution was discarded and the membrane rinsed as described above.

Also, the protein flux ($J_{protein}$) was normalized by dividing by the average of the buffer flux (J_{buffer}) for comparison between trials and between different conditions. Then, all trials were fit to an exponential decay as described in (Kilmer et al., n.d.). As the flux decays for each sample had greater than 10% standard deviation, the τ and y_o from the exponential decay fits were used to statistically analyze if the presence of the dye resulted in differences in the fouling nature. In order to determine which biofouling mechanism is likely occurring, the raw data from a representative run that matched the average for the 2.5% and 5% F-BSA samples was recalculated in terms of the filtrate flow rate (Q, in m^3/s) and the volume of permeate (V, in m^3). To convert the mass data to a volume, the average density of the solutions (1.011 ± 0.003 g/mL) was used. Due to the noise from data collection, the 11 pt average used to smooth the flux data was fit to the four linearized equations of the biofouling mechanisms.(van Reis & Zydney, 2007)

The linearized form of the functions describing the biofouling mechanisms was chosen so that R^2 could report on which model best describes the data.

A Zeiss Axioskop 2 Plus microscope with a 10x objective was used with a Retiga Exi camera, and X-Cite Series 120 PC fluorescence lamp. Excitation at 480 nm and emission at 535 nm were selected by a dichroic fluorescence filter cube. QCapture Pro-7 imaging software (Q Imaging) was used to collect and analyze microscope images. Exposure times ranged from 7 – 15 ms (MFM) and 20 – 50 ms (BCE). The exposure time for each percent label with each membrane type was kept constant for consistent imaging so fluorescence intensity values should only be compared between images of the same percent labeling. The membrane was visually examined under the microscope, and then images recorded in the four quadrants (Supplemental Figure 1) as well as around the center as representative images. Membrane images from the microscope include a calibration bar of 150 μm. All microscope images were contrast adjusted and the dark background subtracted.

Results and Discussion

Many commercial membranes are not suitable for fluorescence microscopy because they auto-fluoresce when excited at 480 nm (Supplemental Table 1). MFM and BCE were chosen as they have low fluorescence background and reasonable water flux at 40 kPa. Fluorescence microscopy images the surface but less than 1% of the membrane area at a time. Therefore, the membrane was surveyed in quadrants (Supplemental Figure 1) so that, using the microscope, the surface of the membrane could be swept and all sections examined before taking representative pictures of the surface. Before imaging, the membrane was rinsed after the protein flux run (see Materials and Methods) because otherwise, the remaining protein solution dried leaving a deposit (Supplemental Figure 2).

The relative flux decay of all protein solutions was measured for both membranes. This was done to ensure that the presence of the dye did not affect the fouling nature of BSA.(Suwal, Doyen, & Bazinet, 2015) All protein samples (no matter the percent label) have a fast decay leading to low relative flux by around 600 sec (Figures 1 and 2). This fouling rate by BSA is faster than seen in earlier experiments but is similar to hemoglobin (Hb).(Kilmer et al., n.d.; Loh et al., 2009) Considering that BSA and Hb have similar molecular weight and Stokes radius, it makes sense that these two proteins could have similar fouling behaviors. What is surprising is that BSA differs from earlier experiments, probably because the source of BSA is different. Since the protein sample causes significant fouling by 1,000 sec, this is a good model system for optimizing the technique of combining flux experiments with fluorescence microscopy within a reasonable experimental time.

Also surprising is that the profile of the flux decays from both membranes are different (Supplemental Figure 3). Both membranes are hydrophilic and made of mixed cellulose ester. The make-up and hydrophilicity of the membrane is usually how differences in biofouling due to the membrane are differentiated in the literature.(Loh et al., 2009; Rabe, Verdes, & Seeger, 2011) However, this distinction is not sufficient as indicated by the different profiles. The differences could be related to how the BCE membranes are dyed.

Also, for all membranes and percent labeling, the fouling decays have larger than 10% deviation which vary with experimental time (Figures 1 and 2). Therefore, all protein samples were run a minimum of four times and up to 10 times to ensure an accurate representation of the fouling nature of each membrane. The deviation in the flux means that statistical analysis on the impact of the percent label cannot be done directly on the flux data. Therefore, all flux data was fit to a single exponential decay to

extract the rate of fouling (τ) and the steady-state relative flux value (y_o) (Supplemental Tables 2 and 3). The two parameters were used to determine if the percent label affected the fouling nature of the protein.

The different flux profiles of the two membranes means that the goodness of the fit varied depending on the membrane: the fast decay with the BCE membrane resulted in good exponential fits while the more gradual decay with the MFM membrane were not single exponential (Supplemental Figure 4.a and 4.b). While the rate of fouling does vary depending on the membrane (Supplemental Figure 3), we focused only on the $y_o \times 100$ parameter from the MFM membrane fits because these were in line with the data (Supplemental Figure 4.c). The fitting parameters were analyzed in three ways: 1. a two-way ANOVA test was performed on the fit parameters for both membranes. All p values were greater than 0.15 showing no correlation between the percent label and either fitting parameter (Supplemental Table 4). 2. The average ± 95% confidence interval of the fit parameters overlap (Table 2). In fact, $y_o \times 100$ values are similar no matter the membrane type. 3. The fitting parameters were plotted versus the percent label (Figures 1.e and 2.d) and found to have no linear correlation. In addition, flux experiments where the dye (with no protein) was measured show that at concentrations similar to the amount of dye present in the 2.5% F-BSA solution, less than 14% fouling occurs with both membranes while higher dye concentrations can result in significantly more fouling (Supplemental Figure 5). Therefore, we conclude that the percent labeling studied (amount of F-BSA) does not significantly affect the fouling nature of the protein and are appropriate for study by fluorescence microscopy.

After J_o dropped to 1 – 3% of the initial value, the fouled membranes from all percent label solutions were imaged. In general, a splotchy, dark/light motif was observed (Figure 3.a and 3.b, Supplemental Figures 6 and 7). While the flux profile

differs for both membranes, the relative J_o and the y_o values are similar (Table 2). Therefore, the similarities in the images are not surprising no matter the percent label present or the membrane type. This kind of motif could be related to the membrane pore structure and how the fibers form the pores and the protein interacts with the fibers (as indicated by SEM images from the manufacturer website(Sigma, 2020)).

While scanning the membrane surface for the 2.5% and 5% F-BSA samples, though, structures of increased height were observed (Figure 3.c and Figure 3.d, Supplemental Figure 8). With the BCE membrane, long, thin structures are apparent (Figure 3.c and Supplemental Figure 8). With the MFM membrane, the protein is seen building on top of each other (Figure 3.d, vertical relief was needed for in-focus images). These different structures appear to be the beginning of cake formation occurring on the membrane. These structures were not seen with the 1% or 12% F-BSA samples, which could be due to different reasons including not enough dye to visualize the structures (1%), fluorescence quenching due to higher amounts of dye (12%), or the presence of the dye affecting the formation of these structures (12%). Either way, we conclude that the 2.5% and 5% F-BSA solutions are best for these types of measurements. Marroquin, et al. used a 5% labeled protein solution in their CSLM experiments and similarly reported no dye effects on the fouling nature though they do not report testing different labeled protein concentrations.(Marroquin, Vu, Bruce, Powell, et al., 2014)

Membrane fouling depends on a complex series of interactions including protein-membrane interactions and protein adsorption.(Belfort, Davis, & Zydney, 1994) To study the effect of the protein with the membranes in the absence of pressure, a “sit-test” was performed where the membrane was conditioned, both water and buffer flux were collected, then the protein solution sat in the stirred cell for a set amount of time

before the membrane was rinsed and imaged. For the BCE membrane, a 5% F-BSA solution sat in the stirred cell for 5 min (by allowing the permeate to flow out due to the large pore size) and for 30 min (by feeding the permeate back into the stirred cell). The J_o decreased 13%, even after sitting for 5 min. Images of the membranes after both tests show some deposit of protein on the membrane (Supplemental Figures 9.a and 9.b). The average intensity of the images compared to the images after a full flux run (Supplemental Figure 7.b) is 75% lower. This suggests that there is some strong initial interaction between the protein and the membrane occurring and likely why this membrane shows fast fouling.

For the MFM membrane, a 5% F-BSA solution sat in the stirred cell for 10 min and for 30 min. The J_o decreased less than 8% after 10 min and less than 12% after 30 min while the membrane images show a little protein presence (Supplemental Figures 9.c and 9.d). Examining the images of the membrane, less protein appears than from the BCE experiment, though the average intensity from the MFM membranes sit-test is 60% lower than the average intensity from a full flux experiment (Supplemental Figure 6.b). Therefore, we draw two conclusions: 1. When using these two techniques, average intensity alone cannot be the matrix for comparison and 2. The protein-membrane interaction varies greatly not just depending on the membrane type.

As *in situ* imaging is not possible to conduct in these experiments, to gain further insight into the interactions leading to biofouling, the flux experiments were stopped at earlier times to visualize the surface. The flux graphs are shown due to the variation in the fouling experiment to demonstrate that the interrupted trials are typical of what is occurring during the flux experiment (Figure 4). A 5% F-BSA solution was stopped 50 sec into a flux experiment with the BCE membrane and resulted in 40% lower J_o. Images of this membrane (Figure 4.b) compared to the full flux runs

(Supplemental Figure 7.b) have a 70% lower average intensity. With the MFM membrane, a 2.5% F-BSA flux experiment was stopped after 60 sec and the J_o was 16% lower than the full run. The average intensity of the membrane image (Figure 5.d) is around 50% lower than the full run (Figure 4.b). The images from the shortened flux run show increased areas of complete blackness (as indicated by the lower average intensity). Protein depositing on the membrane surface is apparent but not to the same extreme as the images from the full flux experiments. For both membranes, the results from the shorter flux experiment are similar to those seen from the sit-tests which makes sense as the addition of pressure allows for faster mass transport to the membrane surface.

As the results illustrate, combining flux experiments with fluorescent membrane images means we are able to visualize how BSA is interacting with the membrane in real-time. To further classify the biofouling mechanism, the flux data was fit to the linearized equations describing the four different biofouling mechanisms using the assumption that the pores are uniform cylinders (Table 3).(van Reis & Zydney, 2007) As seen in Table 3, the complete blockage and cake filtration models do not fit the data, suggesting that these mechanisms are not at play. For all fits, the standard blockage mechanism (described as lowering the diameter of membrane pores by consistent protein deposition (Vu, Darvishmanesh, Marroquin, Husson, & Wickramasinghe, 2016)) has $R^2 > 0.99$, suggesting good linear correlation. The intermediate blockage mechanism (which is similar to the standard pore mechanism except protein deposits more randomly in the pores (Vu et al., 2016)) also shows R^2 values > 0.9, meaning that this mechanism could also potentially explain the data. This makes sense as both models rely on the filtrate flow rate. We note that these models are not without their limitations. Previous research suggests that complete, standard, and intermediate

blockage occur first leading to cake filtration.(van Reis & Zydney, 2007) In fact, researchers have begun combining models to fully describe fouling observed over long timescales(Hou et al., 2017) or to account for more realistic pore structure(van Reis & Zydney, 2007). While cake formation has started (as evidenced by Figures 3.c and 3.d and Supplemental Figure 8), these structures are not seen consistently over the membrane, likely why the cake filtration mechanism does not result in higher R^2 values. This further supports previous work into the order of how biofouling progresses. Not being able to see into the membrane pores with this technique, but comparing the images from the sit-tests, shortened flux experiments, and full flux experiments, we believe that BSA is depositing in the membrane pores leading to coverage of the membrane before cake formation begins.

In conclusion, the combination of flux experiments with fluorescent images of the membrane surface was optimized to learn more about how BSA interacts with the membrane and leads to membrane fouling. Using two different colored mixed cellulose ester, hydrophilic membranes and testing different percentages of F-BSA, a 2.5% or 5% F-BSA solution was found to provide the best images while also not affecting the fouling nature of the BSA protein. Examining the membrane images shows that even within 25 min run time, cake formation has begun on the membrane facilitated by BSA depositing inside the membrane pores and restricting the flow through the membrane. Optimization of this technique now opens the door for understanding and visualizing how other biological molecules interact and facilitate fouling, a crucial need for increased implementation of water purification technologies.

Acknowledgements, Brad Cosentino for the coding of the two-way ANOVA statistical test; Meg Quint for initial photo-physical studies; Patricia Mowery and Brianna Hurysz for helpful conversations on operating the fluorescence microscope; Walter Bowyer for fruitful conversations on different data analysis. Funding for this research was provided by HWS start-up funds and HWS Faculty Research Grants.

References:

Bacchin, P., Snisarenko, D., Stamatialis, D., Aimar, P., & Causserand, C. (2020). Combining fluorescence and permeability measurements in a membrane microfluidic device to study protein sorption mechanisms. *Journal of Membrane Science*, 118485. https://doi.org/10.1016/j.memsci.2020.118485

Belfort, G., Davis, R., & Zydney, A. (1994). The Behavior of Suspensions and Macromolecular Solutions in Crossflow Microfiltration. *Journal of Membrane Science1*, *96*(1–2), 1–58. https://doi.org/10.1016/0376-7388(94)00119-7

Chen, W., Qian, C., Zhou, K., & Yu, H. (2018). Molecular Spectroscopic Characterization of Membrane Fouling : A Critical Review. *Chem*, *4*, 1492–1509.

Ferrando, M., Rŏzek, A., Zator, M., López, F., & Güell, C. (2005). An approach to membrane fouling characterization by confocal scanning laser microscopy. *Journal of Membrane Science*, *250*(1–2), 283–293. https://doi.org/10.1016/j.memsci.2004.10.043

Goh, P. ., Lau, W. ., Othman, M. H. ., & Ismail, A. F. (2018). Membrane fouling in desalination and its mitigation strategies. *Desalination*, *425*, 130–155.

Hao, Y., Liang, C., Moriya, A., Matsuyama, H., & Maruyama, T. (2012). Visualization of protein fouling inside a hollow fiber ultrafiltration membrane by fluorescent microscopy. *Industrial and Engineering Chemistry Research*, *51*(45), 14850–14858. https://doi.org/10.1021/ie302111w

Hou, L., Wang, Z., & Song, P. (2017). A precise combined complete blocking and cake filtration model for describing the flux variation in membrane filtration process with BSA solution. *Journal of Membrane Science*, *542*, 186–194.

Jeong, S., Naidu, G., Leiknes, T., & Vigneswaran, S. (2017). 4.3 Membrane Biofouling: Biofouling Assessment and Reduction Strategies in Seawater Reverse Osmosis Desalination. In E. Drioli, L. Giorno, & E. Fontananova (Eds.), *Comprehensive Membrane Science and Engineering II, Volume 4.* (2nd ed., pp. 48–71). https://doi.org/10.1016/B978-0-12-409547-2.12261-9

Kilmer, N. T., Huss, R. L., George, C. C., & Stennett, E. M. S. (Submitted June 2020). The Influence of Ion Identity and Ionic Strength on Membrane Biofouling of a Binary Protein Solution. *Separation and Purification Technology*.

Lichtman, J. W., & Conchello, J. A. (2005). Fluorescence microscopy. *Nature Methods*, *2*(12), 910–919. https://doi.org/10.1038/nmeth817

Loh, S. T., Beuscher, U., Poddar, T. K., Porter, A. G., Wingard, J. M., Husson, S. M., & Wickramasinghe, S. R. (2009). Interplay among membrane properties, protein properties and operating conditions on protein fouling during normal-flow microfiltration. *Journal of Membrane Science*, *332*(1–2), 93–103. https://doi.org/10.1016/j.memsci.2009.01.031

Maqbool, T., Ly, Q. V., Asif, M. B., Ng, H. Y., & Zhang, Z. (2020). Fate and role of fluorescence moieties in extracellular polymeric substances during biological wastewater treatment: A review. *Science of the Total Environment*, *718*, 137291. https://doi.org/10.1016/j.scitotenv.2020.137291

Marroquin, M., Vu, A., Bruce, T., Powell, R., Wickramasinghe, S. R., & Husson, S. M. (2014). Location and quantification of biological foulants in a wet membrane structure by cross-sectional confocal laser scanning microscopy. *Journal of Membrane Science*, *453*, 282–291. https://doi.org/10.1016/j.memsci.2013.11.011

Marroquin, M., Vu, A., Bruce, T., Ranil Wickramasinghe, S., Zhao, L., & Husson, S. M. (2014). Evaluation of fouling mechanisms in asymmetric microfiltration membranes using advanced imaging. *Journal of Membrane Science*, *465*, 1–13. https://doi.org/10.1016/j.memsci.2014.03.077

Meng, F., Liao, B., Liang, S., Yang, F., Zhang, H., & Song, L. (2010). Morphological visualization, componential characterization and microbiological identification of membrane fouling in membrane bioreactors (MBRs). *Journal of Membrane Science*, *361*(1–2), 1–14. https://doi.org/10.1016/j.memsci.2010.06.006

Mukherjee, M., Menon, N. V., Liu, X., Kang, Y., & Cao, B. (2016). Confocal Laser Scanning Microscopy-Compatible Microfluidic Membrane Flow Cell as a Nondestructive Tool for Studying Biofouling Dynamics on Forward Osmosis Membranes. *Environmental Science and Technology Letters*, *3*(8), 303–309. https://doi.org/10.1021/acs.estlett.6b00218

Nations, U. (n.d.). Sustainable Development: Decade of Action. Retrieved June 3, 2020, from https://www.un.org/sustainabledevelopment/decade-of-action/

Rabe, M., Verdes, D., & Seeger, S. (2011). Understanding protein adsorption

phenomena at solid surfaces. *Advances in Colloid and Interface Science*, *162*(1–2), 87–106. https://doi.org/10.1016/j.cis.2010.12.007

Sigma, M. (2020). MF-Millipore Membrane Filter, 0.45 um pore size. Retrieved July 24, 2020, from https://www.emdmillipore.com/US/en/product/MF-Millipore-Membrane-Filter-0.45m-pore-size,MM_NF-HABP02500?ReferrerURL=https%3A%2F%2Fwww.google.com%2F

Suwal, S., Doyen, A., & Bazinet, L. (2015). Characterization of protein, peptide and amino acid fouling on ion-exchange and filtration membranes: Review of current and recently developed methods. *Journal of Membrane Science*, *496*, 267–283.

van Reis, R., & Zydney, A. (2007). Bioprocess membrane technology. *Journal of Membrane Science*, *297*(1–2), 16–50. https://doi.org/10.1016/j.memsci.2007.02.045

Vu, A., Darvishmanesh, S., Marroquin, M., Husson, S. M., & Wickramasinghe, S. R. (2016). Fouling of microfiltration membranes by biopolymers. *Separation Science and Technology (Philadelphia)*, *51*(8), 1370–1379. https://doi.org/10.1080/01496395.2016.1150295

Wu, S. E., Lin, N. J., Chou, C. Y., Hu, C. C., & Tung, K. L. (2019). Biofouling mechanism of polysaccharide–protein–humic acid mixtures on polyvinylidene fluoride microfiltration membranes. *Journal of the Taiwan Institute of Chemical Engineers*, *94*, 2–9. https://doi.org/10.1016/j.jtice.2017.08.013

Zator, M., Ferrando, M., López, F., & Güell, C. (2007). Membrane fouling characterization by confocal microscopy during filtration of BSA/dextran mixtures. *Journal of Membrane Science*, *301*(1–2), 57–66. https://doi.org/10.1016/j.memsci.2007.05.038

Table 1. Equations used to determine the mass (in grams) of uBSA and F-BSA for different percent label (L) where V_t is the total volume (in liters) and 0.063 is the experimentally determined mass difference of the dye.

Species	**Mass Determination**
F-BSA	$\left(4\frac{g}{L}\right)(V_T)(L)$
uBSA	$\left(\left(4\frac{g}{L}\right)(V_T) - \text{F-BSA}\right) + (0.063 \times \text{F-BSA})$

Table 2. Fit parameters (average ± 95% confidence interval) for both membranes and percent labeled F-BSA. The first row is $y_o \times 100$ and the second row is τ (sec, shown only for BCE membrane since the fits from the MFM membrane were not good for τ).

Membrane	**% F-BSA**				
	0% (uBSA)	*1%*	*2.5%*	*5%*	*12%*
BCE	1.3 ± 0.5	1.2 ± 0.5	1.0 ± 0.4	2.0 ± 1.9	1.1 ± 0.2
	40 ± 11	59 ± 38	38 ± 5	55 ± 14	64 ± 26
MFM	1.9 ± 0.3	1.3 ± 0.3	1.5 ± 0.3	0.8 ± 0.9	1.14 ± 0.05

Table 3. R^2 values from fitting the four biofouling mechanisms to representative trials from the flux experiments that correlate with the average of the run. Values where $R^2 >$ 0.99 are bolded. Two different BCE runs at 5% F-BSA are shown as they were collected for different experimental times.

Membrane	**% F-BSA**	**Approx. Run Time (s)**	**Biofouling Mechanism**			
			Complete Blockage	*Standard Blockage*	*Intermediate Blockage*	*Cake Filtration*
BCE	2.5%	1,850	0.604	**0.998**	0.929	0.687
	5%	1,700	0.649	**0.996**	0.952	0.812
		350	0.878	**0.998**	**0.992**	0.829
MFM	2.5%	1,350	0.800	**1.000**	0.969	0.631
	5%	1,300	0.809	**0.999**	0.980	0.560

Figure 1. Flux experiments of uBSA (black) compared to varying amounts of F-BSA with the MFM membrane: (a) 1% F-BSA, (b) 2.5% F-BSA, (c) 5% F-BSA, and (d) 12% F-BSA. Inserts zoom in on the largest deviation in the relative flux. (e) Comparison of $y_o \times 100$ versus percent labeled (% F-BSA, best-fit line: $y = 0.048x + 1.57$, $R^2 = 0.1737$) to analyze the influence of the dye on the protein fouling nature.

Figure 2. Flux experiments of uBSA (black) compared to varying amounts of F-BSA with the BCE membrane: (a) 1% F-BSA, (b) 2.5% F-BSA (red) and 5% F-BSA (blue), (c) 12% F-BSA. Inserts zoom in on the largest deviation in the relative flux. (d) Comparison of the percent labeled (% F-BSA) to the exponential fit parameters $y_o \times 100$ (left axis, red squares, best-fit line: $y = -0.0046x + 1.31$, $R^2 = 0.0007$) and τ (right axis, black circle, best-fit line: $y = 1.42x + 46.9$, $R^2 = 0.051$) to analyze the influence of the dye on the protein fouling nature.

Figure 3. Fluorescent images of membrane surface after flux experiment with 2.5% F-BSA solution with the (a) BCE membrane showing the splotchy, dark/light motif and (b) MFM membrane showing the splotchy, dark/light motif versus (c) BCE membrane showing structures and (d) MFM membrane showing structures. Scale bar is 150 μm.

Figure 4. BCE membrane with 5% F-BSA (a) shortened flux run (black, 50 sec) versus the full averaged flux experiment (red, with standard deviation) and (b) representative microscope image after 50 sec, which is 30% as intense as the full run. Scale bar is 150 μm. MFM membrane with 2.5% F-BSA (c) shortened flux run (black, 60 sec) versus the full averaged flux experiment (red, with standard deviation) and (d) representative microscope image after 60 sec which is 66% as intense as the full run. Scale bar is 150 μm.