ADVANCED IMAGING AS A NOVEL APPROACH TO THE CHARACTERIZATION OF MEMBRANES FOR MICROFILTRATION APPLICATIONS

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ADVANCED IMAGING AS A NOVEL APPROACH TO THE CHARACTERIZATION OF MEMBRANES FOR MICROFILTRATION APPLICATIONS

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Chemical Engineering

by
Milagro Marroquin
August 2013

Accepted by:
Dr. Scott M. Husson, Committee Chair
Dr. Douglas E. Hirt
Dr. Charles Gooding
Dr. Terri Bruce
Dr. S. Ranil Wickramasinghe
ABSTRACT

This dissertation focuses on the design, development and implementation of novel, advanced imaging protocols for the characterization of membranes in microfiltration applications. Oftentimes, membrane characterization studies are done with high resolution microscopy techniques like scanning electron microscopy or transmission electron microscopy. The results obtained by these popular imaging techniques are subject to error and their reliability might be, in some instances, compromised because they require drying and metallization of the sample; working under high vacuum and electron beam intensity; and extensive sectioning to retrieve internal information. These factors may disrupt the membrane structure or modify its features. As an alternative to these techniques, confocal microscopy stands out and has gained popularity recently in material studies because its features overcome the aforementioned limitations. Therefore, the primary objectives of my dissertation were to design, develop and implement novel confocal microscopy imaging protocols for the characterization of membranes and highlight opportunities to obtain reliable and cutting-edge information of microfiltration membrane morphology and fouling processes.

My strategy consisted of developing a cross-sectional confocal microscopy imaging protocol that combines minimal mechanical sectioning of the sample with optical sectioning to obtain images from just below the surface of the cross-section and avoid concerns about surface artifacts due to sample preparation. The application of this protocol allowed the visualization of the full thickness of symmetric and asymmetric
membranes, overcoming the limit on depth of penetration inherent in confocal microscopy. Along with image analysis, it is possible to obtain information regarding, but not limited, to membrane morphology and fouling.

After a comprehensive introduction and review of confocal microscopy in membrane applications (Chapter 1), the first part of this dissertation (Chapter 2) details my work on membrane morphology characterization by confocal laser scanning microscopy (CLSM) and the implementation of my newly developed CLSM cross-sectional imaging protocol. Depth-of-penetration limits were identified to be approximately 24 µm and 7-8 µm for mixed cellulose ester and polyethersulfone membranes, respectively, making it impossible to image about 70% of the membrane bulk. The development and implementation of my cross-sectional CLSM method enabled the imaging of the entire membrane cross-section. Porosities of symmetric and asymmetric membranes with nominal pore sizes in the range 0.65–8.0 µm were quantified at different depths and yielded porosity values in the 50-60% range. It is my hope and expectation that the characterization strategy developed in this part of the work will enable future studies of different membrane materials and applications by confocal microscopy.

After demonstrating how cross-sectional CLSM could be used to fully characterize membrane morphologies and porosities, I applied it to the characterization of fouling occurring in polyethersulfone microfiltration membranes during the processing of solutions containing proteins and polysaccharides (Chapter 3). Through CLSM imaging,
it was determined where proteins and polysaccharides deposit throughout polymeric microfiltration membranes when a fluid containing these materials is filtered. CLSM enabled evaluation of the location and extent of fouling by individual components (protein: casein and polysaccharide: dextran) within wet, asymmetric polyethersulfone microfiltration membranes. Information from filtration flux profiles and cross-sectional CLSM images of the membranes that processed single-component solutions and mixtures agreed with each other. Concentration profiles versus depth for each individual component present in the feed solution were developed from the analysis of the CLSM images at different levels of fouling for single-component solutions and mixtures. CLSM provided visual information that helped elucidate the role of each component on membrane fouling and provided a better understanding of how component interactions impact the fouling profiles.

Finally, Chapter 4 extends the application of my cross-sectional CLSM imaging protocol to study the fouling of asymmetric polyethersulfone membranes during the microfiltration of protein, polyphenol, and polysaccharide mixtures to better understand the solute-solute and solute-membrane interactions leading to fouling in beverage clarification processes. Again, cross-sectional CLSM imaging provided information on the location and extent of fouling throughout the entire thickness of the PES membrane. Quantitative analysis of the cross-sectional CLSM images provided a measurement of the masses of foulants deposited throughout the membrane. Moreover, flux decline data collected for different mixtures of casein, tannic acid and β-cyclodextrin were analyzed with standard fouling models to determine the fouling mechanisms at play when
processing different combinations of foulants. Results from model analysis of flux data were compared with the quantitative visual analysis of the correspondent CLSM images. This approach, which couples visual and performance measurements, is expected to provide a better understanding of the causes of fouling that, in turn, is expected to aid in the design of new membranes with tailored structure or surface chemistry that prevents the deposition of the foulants in “prone to foul” regions.

Overall, results from my dissertation demonstrate that CLSM has strong potential for providing reliable and new information that conventional imaging techniques, at present, are not able to provide. Also, CLSM and the cross-sectional imaging protocol developed in this dissertation are worthy tools in, but not limited to, membrane morphology and fouling characterization studies.
DEDICATION

I dedicate this dissertation to my beloved husband, Carlos Prudencio, and to my parents Guillermo and Deisy Marroquin.
ACKNOWLEDGMENTS

First, I am grateful to God because without him I would not be able to be here or finish my PhD. It has been five years full of challenges that I have overcome thanks to Him and my only hope is to put to work all the blessings that He has given me.

I would like to express my deepest gratitude to my advisor Dr. Scott M. Husson for his expert guidance, for letting me work with him and be part of his awesome research group. As advisor, he has always been very patient and respectful. As a professional, he is highly respected, recognized and works really hard to keep that status. For these reasons, I considered myself very fortunate for having him as my advisor and I could not have asked for a better one.

I express my thanks to my parents whom always supported and motivated me to come out of my comfort zone, live out my dreams until my heart gives out and excel in what I do. The word thank you is not enough to show my gratitude to my dear husband, Carlos. He has been very patient all these five years while I was pursuing this dream. His support and love gave me strength to get up and to keep walking during the low points of this journey. Additionally, I would like to thank the Woolbright family for their support and help. Especially, Joey and Debra, whom were like my second parents here in Clemson.

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I would like to thank my committee members Dr. Ranil Wickramasinghe and Dr. Terri Bruce for their valuable input and collaboration with my research and in the development of this dissertation, and to Dr. Charles Gooding and Dr. Douglas Hirt for their valuable time, feedback and suggestions to complete this work.

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CHAPTER 1

INTRODUCTION AND OVERVIEW OF DISSERTATION

This chapter provides an introduction to confocal microscopy as an emerging characterization tool in membrane science. After a brief introduction and background on what confocal microscopy does and what information it provides, a detailed overview is provided on the components of a confocal microscope, the working principle, and operating modes. Thereafter, a more in-depth coverage is given on theory; analysis protocols; case studies and a literature review; and summary of advantages, disadvantages, and limitations of confocal microscopy versus alternative techniques. The level of coverage given in this chapter is meant to provide a starting point for readers who wish to use confocal microscopy for the first time, as well as for experts who wish to keep up with recent developments and trends.

1.1. Brief history

In 1957, Marvin Minsky patented the concept of the confocal microscope [1], but due to technical limitations, the technology became commercially available only three decades later [2]. In 1987, the images of fluorescently stained cells and embryos presented in a publication by White et al. [3] captured the attention of the scientific community by showing clear images that were free from out-of-focus information. Their publication showed the great potential of the confocal microscope for biological research and contributed to the takeoff in its popularity and use [4-6]. Unlike a conventional fluorescent microscope, the confocal microscope used by White et al. was capable of
focusing the incident light on a spot within the sample at a selected depth and detecting the light that was emitted only from this focused spot. This outcome was achieved by using pinhole apertures for the light source and the detector, the latter of which was responsible for blocking any light emitted from planes above and below the selected focal depth. Several factors contributed to the rapid acceptance of the confocal microscope during the 1980s. These included its ability to obtain improved images with higher contrast, better lateral and axial resolutions (i.e., ability to resolve two points from each other), depth imaging without physical sectioning, and its ability to remove out-of-focus light compared to the widely used fluorescence microscope. Furthermore, it provided an alternative to the popular electron microscopes [7].

Although scanning and transmission electron microscopy provide higher resolution than confocal microscopy, the latter has other advantages. It enables noninvasive imaging, depth discrimination and, with appropriate imaging software, three dimensional imaging by stacking a series of 2D images obtained at different focal depths. The confocal microscope performs depth discrimination by changing the position of the focal plane and recording respective images at different depths. Thus, physical cutting or sectioning of the sample is replaced with optical sectioning. Confocal microscopy, therefore, is a nondestructive technique. Eliminating the need for physical sectioning of the sample is advantageous, as sectioning introduces surface artifacts and disturbs the original structure of a sample. Electron microscopes are prone to generating images from samples with surface artifacts due to the sectioning process, leading to questionable analysis from these images.
Thanks to the advancements in lens and laser technology, computers and digital imaging processing, the confocal microscope has become a powerful imaging tool in biological and biomedical studies, with significant growth in use for applications in the material science area. Readers interested in further details on the evolution of confocal microscopy should refer to references [5-8].

1.2. Scanning modes

There are three types of scanning modes on the confocal microscope: specimen (or stage), beam of light, and spinning disk. Stage scanning mode was used in early confocal systems. It scans the specimen by moving the stage and keeping the optical arrangement stationary. This mode can scan larger areas because is not limited by the field of view of the objective lens; rather, it is limited by the range of movement of the stage. A disadvantage of this method is the slow speed for image generation. It also is prone to sample shifting if the sample is not well attached to the slide, resulting in distortion of the final image [6, 9].

Beam light scanning mode is used in most modern commercial confocal microscope systems. It uses a scanning beam of light and a stationary stage. Motorized, adjustable mirrors or acoustic beam deflectors deflect the light beam, and the beam is scanned across the focal plane within the sample in a raster pattern [3, 4, 8]. The advantage of this mode is faster imaging speeds compared to stage scanning mode. There are two types of beam scanning confocal microscopes: slow scan and slit scan. In the slow scan (or point scan) confocal microscope, a focused beam of light scans the
specimen horizontally and vertically. It generates images with high resolution. In the slit scan confocal microscope, a slit of illumination scans the specimen. Imaging rates are faster than those of the point scan confocal microscope because many points on the axis of the illumination slit are scanned in parallel. One disadvantage that the slit scan systems present is lower lateral and axial resolution [5, 10].

Finally, the spinning disk mode uses a stationary stage and light source and a rotating scanning disk (Nipkow wheel) with multiple pinholes (or squares), illustrated in Fig. 1.1 [11].

![Fig.1.1](image)


These microscopes obtain images at a rapid rate. An example of a spinning disk confocal microscope is the tandem scanning confocal microscope (TSCM) developed in 1967 by Petráň et al. [12]. In the TSCM, the disk has thousands of pinholes arranged in a
series of nested clusters of Archimedean spirals. The disk rotates and scans multiple points of the sample simultaneously; the pinholes on one half of the disk illuminate the sample, while the pinholes on the other half collect the light for the detector. The spinning disk illuminates multiple spots in the focal plane and each pinhole images a specific point in the focal plane of the sample in parallel [5, 13, 14]. Another example of a spinning disk microscope is the monoscanning confocal microscope, where the detected light passes through the same illumination aperture in the spinning disk. One disadvantage of disk scanning microscopes is that only a small fraction of incident light is transmitted, and illuminating photons are lost, resulting in dimmer images and lower lateral and axial resolution. To overcome this limitation, the Yokugawa system uses a microlens array to focus the light onto the disk and reduce the loss of light. Another disadvantage is its sensitivity to the vibration of the disk.

The remainder of this chapter will focus on the beam scanning confocal microscope, in point scan and fluorescent mode, since it is the most popular commercial scanning mode in the market and most research laboratories. Fig. 1.2 shows a basic illustration of the light path in most modern confocal systems.
Fig. 1.2. Light path in a confocal microscope and its basic components. Reprinted with permission from Elsevier Science. In “Handbook of Image and Video Processing” (A. Bovik, ed), Elsevier Science, copyright © 2005.

1.3. Optical path in the confocal scanning microscope

According to Fig. 1.2, a source light illuminates a pinhole aperture and the light that emerges from the aperture is reflected by a beam splitter that directs it to an objective lens. The objective lens collects all the incoming light and focuses it onto the specific plane at a selected depth within the sample. The light that returns from the sample (fluorescent emission or reflected) passes through the objective lens and then through the beam splitter. Only the light coming from the in-focus plane can pass through the pinhole aperture shielding the detector. Light coming from planes above and below the in-focus plane is blocked by the pinhole aperture and is rejected. The light coming from the plane
of focus reaches the detector, which transmits the information to a computer for image processing and generation.

### 1.4. Light received by detector

The confocal microscope can work in epi-fluorescence, reflection and transmission mode. The epi-fluorescence mode is used most commonly in commercial confocal microscopes. In this mode, the illuminator and the detector are on the same side of the specimen. Fluorescent dyes are used to label structures and imaging is done by collecting the secondary fluorescence emitted by the dyes on the sample. One advantage of the fluorescent mode is its specificity, which is to say that different structures can be labeled with different dyes that, during imaging, will result in different colors in the final image. On some occasions, the material itself has an intrinsic fluorescence (auto-fluorescence) that can be used for imaging. The disadvantage of this imaging mode is that the dyes are prone to the loss of fluorescence due to light exposure in the presence of oxygen (photobleaching).

Confocal microscopy in reflection mode was used in the early confocal systems [1]. This mode uses backscattered or reflected light to generate an image. In this imaging mode, the illuminator and the detector are on the same side of the specimen. This imaging mode is simpler because it requires only minimum sample preparation (i.e., it does not require staining/labeling of the sample). Beam and stage scanning and spinning disk confocal microscopes can be used in reflection mode [15]. To obtain a good reflectance (ratio of illumination intensity to reflected intensity), the sample has to have
different refractive index than the medium. It is for this reason that reflection mode imaging is used more commonly in material science than biological sciences, due to the low light reflectance of biological samples. Unlike fluorescent mode, the reflection mode does not have photobleaching limitations [16].

In the confocal microscope transmission mode, the image is generated using the transmitted light or light absorbed by the sample. In this system, the illuminator and the detector are located on opposite sides of the sample and there is a second lens that focuses the light onto the detector. This mode was used in the early confocal systems. It has lost favor because of difficulties in aligning the optical system and maintaining the alignment while imaging. Properties like the opacity and turbidity of the sample can affect the light transmission and affect the depth of penetration of the light for imaging. If enough light penetration is not achievable through a thick sample, then microtoming to obtain thinner sections of the sample can overcome this limitation.

1.5. Confocal microscope hardware

This section gives a brief description of the main hardware components of the confocal scanning microscope. For more in-depth coverage, please refer to the references [4, 5, 11].

1.5.1. Light source

Confocal microscopes commonly use lasers (gas or semiconductor) for illumination; alternatively, they can use arc lamps. The use of lasers as light sources in confocal microscopy has coined the name confocal laser scanning microscopy (CLSM).
In fluorescence CLSM, the light source should provide a high flux of photons at a specific wavelength that will excite the fluorochromes [4]. High-intensity excitation light is necessary in the fluorescent mode because it is necessary to compensate the low emission intensity that results from low quantum efficiency of fluorescence dyes and loss of light due to interaction of light with the sample and the media (i.e., scattering, absorption, etc.). Commercial fluorescence systems predominantly use gas lasers, including argon-ion, krypton-argon, helium-neon, helium-cadmium that can be air or water cooled. Lasers can emit multiple wavelengths and can be operated in multiline or single-wavelength mode [11]. Since lasers do not have an unlimited number of wavelength lines to cover the full visible spectrum, CLSM instruments use combinations of lasers (or “laser batteries”) to widen the operating range. Nowadays, there are numerous fluorochromes in the market designed to match the standard laser lines in commercial confocal microscopes.

Although the intensity of the final image should be proportional to the intensity of the light source, that relationship is not always found experimentally. In fluorescence confocal microscopy, photobleaching and saturation might occur when the laser intensity is too high. Photobleaching occurs when the excited state of the fluorochromes reacts with surrounding oxygen producing a non-fluorescent molecule. For fluorescent dyes that are prone to photobleaching, even low intensity illumination light results in loss of image intensity. This loss of intensity will be observed while illuminating a sample over a period of time. The rate that image intensity is lost increases with increasing illumination intensity. Under conditions of saturation, increasing the intensity of the illumination light
does not produce a higher intensity of emitted light by the fluorochrome. Saturation occurs because the rate of absorption of light by the fluorescent molecule exceeds the rate of generation of emitted light [8].

For reflection mode microscopes, low power light is sufficient, and He-Ne or semiconductor diode lasers are used often [17]. Modern spinning disk microscopes can use lasers, mercury (or xenon) arc lamps and light emitting diode arrays [4, 11].

1.5.2. Filters

The function of the emission filters is to make sure that only light emitted by the target fluorochrome reaches the detector by ‘cleaning’ the emitted signal. Also, excitation filters are used in conjunction with non-monochromatic light sources to tune the wavelength of the excitation light to match the excitation light wavelength of the fluorochrome. Filters are particularly important when working with multiple fluorescent dyes, as they are necessary to separate and match the wavelengths of the excitation and emission light to the respective fluorochromes.

**Filters for intensity attenuation:** Filters can be used to attenuate the intensity of the light source and reduce the loss of fluorescence due to photobleaching. Also, attenuation filters can be used when it is desirable to reduce the intensity in the final image. In modern confocal systems, light can be attenuated by using Acousto-optic tunable filters (AOTF) and neutral density filters. AOTFs are devices that work as electronically tunable filters that regulate the intensity of the laser. AOTFs also allow the user to define the shape and size of the region for excitation, as well as the wavelength of
the illumination light. Neutral density filters can be absorptive gray glass filters or reflective metallic filters.

**Filters for wavelength discrimination and separation**: There are four types of filters for wavelength discrimination and separation: (1) Short-pass filters block light with wavelength longer than a specific cut-off value. (2) Long-pass filters block light with wavelength shorter than a specific cut-off value. (3) Bandpass filters only transmit light with wavelengths within a specific range (cut-on and cut-off values). (4) Dichroic mirrors reflect light with short wavelength and transmit light with long wavelength. Excitation filters are usually bandpass or short-pass; whereas, emission filters are usually long-pass to prevent excitation light from reaching the detector. Bandpass filters can be used with excitation and emission light when working with multiple fluorochromes in the same sample.

Commonly used are glass filters containing colored dyes that absorb light at one wavelength and transmit light with a different wavelength. Disadvantages of these filters are that (1) the glass and dyes can show auto fluorescence at short wavelengths (high energy light), (2) the optical properties of the filter change with the thickness of the filter, and (3) they are prone to overheating that can affect their performance. Other commonly used filters are interference filters that are composites comprising multiple layers of reflective materials deposited on glass substrates [11]. These filters are non-fluorescent but can produce stray light.
1.5.3. **Beam splitter**

Epi-fluorescence confocal microscopy commonly uses dichroic mirrors as beam splitters to separate excitation light from emission light before it reaches the detector. Dichroic mirrors usually are long-pass interference filters that are designed to be used at an angle of incidence of 45 degrees with respect to the light path. They reflect light with wavelengths below a fixed threshold value and let light with wavelengths longer than the threshold value pass through [8].

1.5.4. **Objective lens**

The selection of the right objective lens ultimately determines the quality of the confocal images since it is responsible for the image resolution, contrast and magnification. An important characteristic to consider when selecting a lens is its numerical aperture (NA). The NA is a measure of the light gathering and resolving power of the lens and is defined in eq. 1.1:

\[ \text{NA} = n \sin \theta \]  
\[ \text{(eq. 1.1)} \]

\( n \) is the refractive index of the immersion medium between the lens and coverslip glass, and \( \theta \) is illustrated in Fig. 1.3.
The numerical aperture of the objective lens plays an important role in determining the limit of resolution in a confocal microscope. Since the NA is an indication of the light gathering power of the lens, a higher NA will result in brighter images. Resolution (lateral or axial) refers to the smallest feature that can be resolved or the minimum distance between two features that can be differentiated. The Raleigh criterion for resolution in conventional fluorescent microscopes can give an estimation of the lateral and axial resolution, as defined by eqs. 1.2 and 1.3. Muller gives a complete explanation on their derivation [18]:

Lateral resolution (x-y plane) \( r_{\text{Airy}} \approx 0.61 \frac{\lambda}{\text{NA}_{\text{obj}}} \)  

Axial resolution (x-z plane) \( d_z \approx \frac{2n\lambda}{(\text{NA}_{\text{obj}})^2} \)
r_{\text{Airy}} is the radius of the Airy disk, NA is the numerical aperture of the objective lens, \( \lambda \) is the wavelength of the light and \( n \) is the refractive index of the medium between the lens and the coverslip glass. A point of light in the focal plane appears as a bright disk (the Airy disk) surrounded by progressively dimmer rings. The radius of the Airy disk is defined as the distance between the center of the disk (maximum intensity) and the first minimum of the intensity distribution. The Rayleigh criterion says that the images of two Airy disks are resolved, when the maximum intensity of the first disk falls into the first intensity minimum of the second Airy disk, meaning that the distance between the centers of both Airy disks is equal or larger than \( r_{\text{Airy}} \) [5, 18, 19]. Since the confocal microscope has a pinhole collecting the incoming light from the focus plane, the full width half maximum (FWHM) criterion for the limit of resolution is used to account for the efficiency of the detection pinhole. Eqs. 1.4 and 1.5 result from application of this criterion [18]:

\[
\begin{align*}
\text{Lateral resolution} & \quad r_{x-y,\text{conf}} \approx 0.4 \frac{\lambda}{\text{NA}_{\text{obj}}} \\
\text{Axial resolution} & \quad d_{x-z,\text{conf}} \approx \frac{1.4n\lambda}{(\text{NA}_{\text{obj}})^2}
\end{align*}
\]  

(1.4)  

(1.5)

\( r_{x-y,\text{conf}} \) and \( d_{x-z,\text{conf}} \) are the limits of lateral and axial resolution for a confocal microscope based on the FWHM criterion, respectively. The resolving power of the microscope increases as \( r_{x-y,\text{conf}} \) and \( d_{x-z,\text{conf}} \) get smaller. Then, as seen in eqs. 1.4 and 1.5, the resolving power of the confocal microscope is inversely proportional to the

\[
\begin{align*}
\end{align*}
\]
wavelength of the illumination light and directly proportional to the numerical aperture of the objective lens. The axial resolution in the confocal microscope also depends on the refractive index of the immersion medium. The limits of resolution obtained by these equations are approximate only, as assumptions have been made in their derivations [4, 18].

**Working distance of the lens** is the maximum distance between the objective lens and the surface of the coverslip glass. The working distance also defines the maximum depth that the objective can image under the coverslip glass. In general, the working distance is inversely proportional to the lens numerical aperture. Longer working distance helps when imaging thick samples, but the cost is loss of resolution [11].

**Immersion objective lenses** use water, oil or glycerin as immersion media. They are used because of their higher NA compared to dry objective lenses (where the immersion medium is air) and higher resolution power, and because there is less light scattering between the immersion medium and the coverslip glass compared to the case where air is the medium. The result is that more light can be collected using an immersion objective lens.

Matching the refractive index (RI) of the components within the optical path between the objective lens and sample (glass coverslip, immersion and mounting medium) is important for immersion lenses to achieve their maximum resolving power and avoid aberrations. Incorrect matching among the RI of the objective lens, immersion oil and mounting medium can lead to strong spherical aberrations and loss of contrast and resolution. Oil immersion lenses are designed to work with immersion oil, a mounting
medium with approximately the same refractive index, and a coverslip glass with a specific thickness and a similar refractive index. Water immersion lenses are used with biological samples that require water or aqueous buffer solutions as mounting media; therefore, their NA values are not as high as those for oil immersion lenses. If illumination light moves from regions of higher to lower refractive index, then the focal point will move to a position closer to the coverslip. An example of this situation is when an oil immersion lens is used with immersion oil to image a sample mounted in water. When acquiring images at different depths, spherical aberrations cause a mismatch between the movement of the focal plane and the movement of the objective lens, and this leads to loss of signal since the emitted light (from a plane not selected) will be blocked by the pinhole aperture, as shown in Fig. 1.4 [18].

![Diagram showing the effect on the focal plane caused by mismatched refractive indexes of the elements within the light path of the microscope.](image)

**Fig. 1.4.** Effect on the focal plane caused by mismatched refractive indexes of the elements within the light path of the microscope. Reprinted with permission from SPIE Press. In “Introduction to confocal fluorescence microscopy” (M. Muller), volume TT69, SPIE Press, copyright © 2005.

Using an incorrect immersion medium can damage an objective lens. It is important to pay special attention to use the proper immersion medium according to the lens specification. After using the objective lens, the immersion medium generally can be
removed by blotting with lens paper. For a deep cleaning of the lens, it is imperative to read the manufacturer instructions or call the lens supplier company to request information. Care and cleaning are critical for objective lenses since dirt and scratches to the lens degrade the image formation or render the lens useless.

**Depth of field** is the distance along the z-axis of the optical path that an object can move (while the position of the image plane is maintained) without detectable loss of sharpness in the image [4, 8]. Confocal microscopes have a very shallow depth of field compared to conventional light microscopes. eq. 1.6 can be used to obtain an approximate value for the depth of field [4, 20]:

\[
d_z \approx \frac{n\lambda}{(NA_{obj})^2} + \frac{n}{NA} \cdot d_{\text{pixel}}
\]  

(eq. 1.6)

\(d_{\text{pixel}}\) is the width of a pixel in the object plane. It is the quotient of the physical pixel size by the total magnification of the system [20].

**Other important considerations about objective lenses**

To reduce lens aberrations, it is necessary to work with the specified immersion medium, thickness of coverslip (~0.17 mm) and refractive index for the objective lens to be used. Using non-standard coverslips (with a thickness different from the lens specification) and a mismatch between the refractive index of the immersion and mounting medium (i.e. oil and water, respectively) are the main reasons for **spherical aberrations**. Spherical aberrations occur when the ray paths of the illuminating light do
not converge to a single focus and peripheral rays entering the lens are focused at a different point than the central rays of light. When this happens, a broad region is being illuminated instead of a single point [4, 11]. Blurred images, non-reproducibility of the results, loss of signal and contrast are some of the consequences of this kind of lens aberration. Spherical aberrations increase proportionally with depth. They are especially problematic when imaging thick samples because they reduce the depth of penetration due to the loss of signal.

The absence of **chromatic aberrations** is important in beam scanning confocal microscopy, especially when working with samples labeled with multiple fluorescent dyes. Chromatic aberration occurs when light with different wavelengths (corresponding to different colors) do not focus on the same point in the sample. Lenses with axial chromatic aberrations focus light with shorter wavelength closer to the lens compared to light with longer wavelength. Lenses with lateral chromatic aberrations show differences in the magnification, where light with shorter wavelength is magnified more than light with longer wavelength [4]. Consequences of chromatic aberration are two-fold: (1) Colocalization data may be misinterpreted. (2) Emitted light will not be focused correctly (by the objective lens) at the detection pinhole; thus, it will be lost and the image intensity will be reduced [4, 21].

Problems with chromatic and spherical aberrations were common in the early days of confocal microscopy. Nowadays, there are commercial objective lenses that are corrected for these kinds of aberrations. **Plan** objectives correct curvature of the field that may result in loss of focus and signal from the peripheral regions of the image, making
the field of view look flat from edge to edge [4, 11]. Classic **achromat** lenses are corrected for chromatic aberration for red and blue light and spherical aberration for green light. **Fluar** objectives are corrected for chromatic and spherical aberrations for blue and red wavelengths. **Apochromat** lenses are corrected for chromatic and spherical aberrations for red, blue and green wavelengths of light and possess a complex construction, making them an expensive option [11]. Also, some objective lenses available in the market use correction collars to compensate for incorrect coverslip glass thickness and mounting medium variations. For instance, dry objectives with high numerical aperture are sensitive to spherical aberrations when used with a coverslip glass with a thickness different from the objective specification.

### 1.5.5. Pinhole aperture

The one in front of the light source and the one preceding the detector should be co-focused. The detector pinhole acts as a barrier, blocking incoming light from planes other than the one at the selected depth. As the size of the detector pinhole decreases, the resolution increases, but the intensity decreases. Also, the thickness of the optical section is inversely proportional to the numerical aperture of the objective lens and directly proportional to the size of the pinhole. Thinnest optical sections are generated by high NA lenses and small pinhole apertures. Equations 1.4 and 1.5 for the lateral and axial limit of resolution are developed for the condition where the pinhole diameter is equal to the diameter one Airy disk. If the diameter is less than that of one Airy disk, then the lateral resolution increases with little improvement in the axial resolution [4]. According
to Masters [8], “if the pinhole diameter is set to be smaller (50-75%) than the first minimum of the Airy disk, then a good compromise between the signal strength and the degree of background rejection is achieved.” In cases where the sample has low intensity of fluorescence, it might be necessary to increase the size of the pinhole to collect more signals and increase the intensity. When working with samples containing multiple fluorochromes, some confocal microscopes use multiple detectors and a separate pinhole aperture located in front of each detector. This configuration is beneficial since the size of the Airy disk depends on wavelength, and the optimum pinhole size will be different for each wavelength of light detected.

In the case of confocal microscopes based on the Nipkow disks, the size of the pinholes is fixed and designed to achieve a certain resolution. Also, the spacing between the pinholes is designed to avoid any interference between the images collected by each pinhole. Confocal microscopes based on scanning slits have slits with a fixed length and variable (adjustable) width. The width of the slit can be increased when working with samples with low intensity of fluorescence; although, the resolution will be affected.

1.5.6. Detectors

The purpose of the detector is to collect as many photons coming from the sample as possible for the image formation, since every photon carries information. In modern microscopes, the image is developed from the output of a photomultiplier tube (PMT) or avalanche of photodiodes (APD), or it is captured directly with a charge-coupled device (CCD) camera, and then processed with imaging software [6].
PMTs convert light to electric information and transfer it to a computer for image processing. PMTs have a fast response rate, are stable, sensitive and can be used with low levels of signal detection because they are capable of high amplification and high signal-to-noise ratios. In a PMT, the photocathode converts the received photons to electrons that are passed through a series of amplifying stages called dynodes and then collected by an anode. The photocathode is specific for the wavelengths of light to be detected to ensure high enough sensitivity [8]. Some confocal microscopes have several PMTs to allow the detection of different fluorochromes in samples containing more than one. One downside of PMTs is their low quantum efficiency (~40%), meaning that about 6 out of every 10 photons reaching the photocathode will not produce photoelectrons and will go undetected.

Avalanche of photodiodes is an alternative to PMTs. APD have a high sensitivity, low noise, and higher quantum efficiency (~80%) than PMTs. One downside of APDs is that they easily become saturated by high intensity light.

CCD cameras work by reading out a voltage proportional to the number of photons absorbed by a square area of the sensor over a period of time. CCD cameras offer high sensitivity, low noise, high quantum efficiency and work well with low levels of signal detection. One downside is that they are not as fast or sensitive as PMTs.

1.5.7. Imaging and image analysis

Once a focal plane position has been selected, the laser is scanned to collect information from it. The same plane can be scanned multiple times and the final image
will be the average of them. This process is called ‘averaging’, and it is done to reduce the image noise. The microscopist should use the lowest averaging number that yields a high quality image by achieving a balance between low noise and loss of resolution due to photobleaching. If images from different depths are desired, then the microscope does an ‘optical sectioning’ or ‘sectioning’ that refers to the process of taking images from focal planes at different depths within the sample, resulting in the generation of a z-stack or z-series. The thickness of each optical section relates inversely to the numerical aperture of the lens and directly to the wavelength of the excitation light. Usually, the distance between the optical sections (images) in the z-stack is the same and is called the z-step. The z-step commonly is set to be larger than the optical section thickness.

Computer-controlled confocal microscopes usually come with custom software packages that allow the user to control the microscope, image acquisition, storage, display and processing. For instance, Bio-Rad, Leica and Nikon confocal microscopes come with software programs called LaserSharp, PowerScan and NIS-Elements, respectively. The confocal system offers the option of selecting the desired number of pixels to build the images. Using a high number of pixels (small pixel size) yields high resolution images, where it is easier to distinguish between closely spaced objects in the image. The trade-off when obtaining high resolution images is an increase in the time to scan the sample to generate an image.

The 2D images collected at different depths can be analyzed with freeware and commercial programs such as ImageJ (National Institutes of Health, USA), Photoshop (Adobe Systems Incorporated, USA), Image Structure Analyzer-2 (ISA-2) (Center of
Biofilm Engineering, USA), among others. The z-stacks collected can be processed with freeware like Fiji (National Institutes of Health, USA) or commercial programs like Imaris (Bitplane AG, USA) and Amira (Indeed Visual Concepts GmbH, USA) to produce 3D or 4D reconstructions [11].

1.6. Applications of CLSM for the study of synthetic membranes

This section summarizes the main findings in the literature on the use of CLSM for the characterization of synthetic (i.e., abiotic) membranes.

1.6.1. Morphology characterization

The morphologies of synthetic microfiltration (MF) membranes are quite complex and irregular [22]. [Strictly speaking, “microporous” materials are defined as those with pores that have diameters less than 2 nm; however, in the membrane literature, the word “microfiltration” commonly is used to define membranes with much larger, micron-sized pores.] Observation of morphology is one of the main applications of CLSM in membrane studies. This section provides an overview of work that has been carried out in this area.

Morphology characterization using CLSM in different modes

Some articles have mentioned research with CLSM operated in reflection mode [22-24]. However, most studies use CLSM in fluorescence mode [25-39]. Specific to membrane morphology studies, characterization combining CLSM imaging in reflective and fluorescence modes has been done for comparative studies or to provide complementary information obtained from both imaging modes [23, 40-43]. In a
comparative study, Charcosset et al. [40] reported that the depth of penetration was larger in fluorescence mode than reflective mode; whereas, the image contrast is better in reflective mode (as shown in Fig. 1.5).

(a) image using CLSM in reflective mode   (b) image using CLSM in fluorescence mode (stained with rhodamine)

Fig. 1.5. CSLM images of a Millipore microfiltration membrane surface using CLSM in reflection and fluorescence modes. Reprinted with the permission of Cambridge University Press. In “Characterization of microporous membranes using confocal scanning laser microscopy in fluorescence mode” (Charcosset, C.; Bernengo, J. C.), European Physical Journal-Applied Physics, copyright © 2000.

It should be noted, however, that the selection of the fluorophore affects results during observation using fluorescence mode, e.g., staining with Alexa Fluor 594® provided better images than with 5-(4,6-dichlorotriazinyl) aminofluorescein (5-DTAF) when observing a 0.22 µm nominal pore size mixed cellulose ester membrane (EMD Millipore), as shown in Fig. 1.6 [33].
Fig. 1.6. CLSM images of 0.22 µm nominal pore diameter mixed cellulose ester membrane at z = 4 µm: (A) stained with Alexa Fluor 594® and (B) stained with 5-DTAF. The common scale indicator is 10 µm in diameter. Reprinted with permission from the publisher and the authors. In “Characterization of asymmetry in microporous membranes by cross-sectional confocal laser scanning microscopy” (Marroquin, M.; Bruce, T.; Pellegrino, J.; Wickramasinghe, S. R.; Husson, S. M.), Journal of Membrane Science, copyright © 2011.

In addition, images collected by CLSM in fluorescence mode may be impaired by the presence of residual fluorophore within the membrane pores. Pores that should appear dark are highlighted [44]. Nevertheless, reliable images can be obtained with proper sample preparation that involves covalent attachment of the fluorophore to the membrane material followed by exhaustive rinsing.

**Membrane observations under different states**

Membrane morphology is related directly to the material state, which depends on the sample preparation method [22, 45]. Membranes can be observed in their dry state, hydrated state, or mounted in a medium such as glycerol or immersion oil [24, 28, 37, 41,
Charcosset et al. [45] used CLSM to visualize two microfiltration (MF) membranes, a mixed ester microfiltration membrane (RAWP04700, Millipore) and a reinforced nylon membrane (Immunodyne membrane, Pall), in different states. Good agreement was found between the CLSM membrane images in the dry state and hydrated state (as shown in Fig. 1.7). Their results showed that reliable information on membrane morphology could be obtained from CLSM observation in the hydrated state, which is the general state of MF membranes in operation [45, 46].

In addition, CLSM image quality can be improved by careful selection of a mounting medium. Results indicated that there was a good agreement between CLSM and SEM images of the MF membrane surface when the membrane was mounted for CLSM in glycerol (refractive index \( n = 1.474 \)). Images of the MF membrane cross-section and the Immunodyne membrane surface were best when the membranes were mounted in immersion oil (\( n = 1.583 \)) [45]. Charcosset and Bernengo [22] concluded that immersion oil did not cause any deformation to the structure of cylindrical pores membrane (polycarbonate, Isopore, Millipore), but some polymeric membrane materials may be influenced. Mounting in glycerol can make the MF membranes more transparent, which is beneficial for sub-surface CLSM imaging [40].
Fig. 1.7. CLSM images of a microfiltration membrane under dry and wet conditions. Reprinted with permission from the publisher and the authors. In “Characterization of microporous membrane morphology using confocal scanning laser microscopy” (Charcosset, C.; Cherfi, A.; Bernengo, J. C.), Chemical Engineering Science, copyright © 2000.

Observations at different locations of the membrane

CLSM plays an essential role in the accurate and nondestructive characterization of membrane morphologies. CLSM can be used to observe membrane outer surfaces [29, 41, 47-49] or membrane cross-sections [22, 25-28, 30-34, 40-42, 44-46, 50-56] by setting the optical axis perpendicular or parallel to the membrane surface plane [45]. The agreement was good between CLSM and other microscopy (e.g., SEM, AFM, etc.) images of membrane surfaces for almost all reported research studies [22, 40, 45, 46]. The research on zeolite MFI membranes carried out by Bonilla et al. [25] also revealed that the columnar microstructure of the membrane agreed with SEM cross-section images. Moreover, defects not extending to the membrane surface and information about the degree of intergrowth, which cannot be detected by SEM, were imaged clearly [25]. It also has been illustrated that the surface morphology may be very different from the bulk of most industrial membranes [22]. For example, it was reported in early work that the
pores on a reinforced nylon membrane (Immunodyne, Pall) surface were much larger than those within membrane [45].

**Depth and resolution limitations of CLSM imaging**

CLSM enables researchers to observe morphology within membranes, but light absorption and scattering by the membrane material leads to image degradation as one moves deeper within the membranes [22, 33]. Research showed that for polycarbonate track-etched membrane (TTTP 047 00, Isopore, Millipore), poly(vinylidene fluoride) web supported membrane (SVLP 04700, Durapore, Millipore), and cellulose acetate membrane (SM 123 42-047 N, Sartorius), images were significantly degraded beneath 4 µm, 6 µm and 10 µm from the membrane surface, respectively.

Wang et al. [46] found that clear CLSM images can be observed for a maximum depth of around 23 µm from the active layer surface of a cellulose triacetate membrane (CTA-HW, Hydration Technology Innovations). Marroquin et al. [33] utilized CLSM in fluorescence mode to observe the structures of symmetric mixed cellulose ester (MCE, EMD Millipore) and asymmetric polyethersulfone membranes (PES, Pall Corporation) with nominal pore sizes of 0.22–8 µm. For MCE and PES membranes, the limits of depth penetration were 12–14 µm, and 7–8 µm, respectively. Fig. 1.8 shows CLSM images of MCE membranes at different depths that illustrate the loss of signal intensity with increasing depth [33]. In general, observed depth of penetration limits are roughly 5–60 µm beneath membrane surface [28, 33, 50] depending on membrane material, pore size, sample preparation and observation conditions.
As discussed in an earlier section, the resolution of normal CLSM imaging is limited [28, 33, 40, 42, 48]. The CLSM resolution is roughly 0.1–0.2 µm and 1 µm³ (for 3D) [28, 40, 42, 50]. Marroquin et al. [33] imaged MCE membranes (stained by adsorption of Alexa Fluor® 594 goat anti-rabbit IgG) with different nominal pore diameters at a common depth of 5.6 µm to determine the resolution, as shown in Fig. 1.9. It was found that membrane pores were distinguishable for all of these membranes, and it is clear from the result of the 0.22 µm membrane that imaging was being done near the lateral resolution limit, which was consistent with the theoretical limit of 0.2-0.23 µm based on eq. 1.2.
Fig. 1.9. CLSM images of MCE membranes with different nominal pore diameters. The common scale indicator is 10 µm in diameter. Reprinted with permission from the publisher and the authors. In “Characterization of asymmetry in microporous membranes by cross-sectional confocal laser scanning microscopy” (Marroquin, M.; Bruce, T.; Pellegrino, J.; Wickramasinghe, S. R.; Husson, S. M.), Journal of Membrane Science, copyright © 2011.

To improve the resolution, an indirect method was proposed by Snyder and Vlachos [48] for extending the limit of CLSM imaging resolution, i.e., by establishing a relationship between the results via high-resolution techniques (e.g., SEM) and the fluorescence intensity values from the CLSM image of dye-filled nano-patterned substrate or standards with a pore dimension below the resolution limit of the CLSM measurement. Both SEM and CLSM were used to measure a standard image with hollow
features of a controlled size. By this approach, they were able to calibrate the fluorescence intensity with feature size, and proposed that the lateral resolution limit of CLSM imaging could be improved to be 10 nm, thereby making nanomaterial quantitative characterization possible.

**Cross-sectional imaging**

Normally, CLSM utilizes an optical sectioning method to obtain images inside membranes [33, 42]. The resolution, however, decreases with the increase of depth beneath the membrane surface due to the lowering of signal strength resulting from light absorption and scattering. Marroquin et al. [33] analyzed the relationship between the depth inside a symmetric 5 µm nominal pore diameter MCE membrane and the pixel intensity of the images collected. Fig. 1.10 shows that intensity decreases exponentially with depth, with a slope that depends on the absorption and scattering coefficients for the membrane and the pore filling medium.

Focusing on this obstacle, a mechanical sectioning method was developed to obtain cross-sectional images of the membranes to visualize the membrane through its entire thickness. This innovative method made it possible to obtain the images of asymmetric membranes beneath the observed depth limited by optical sectioning [33]. Fig. 1.11 illustrates the sectioning protocol. An important step was to image the cross section 4 µm beneath the cut surface to avoid the influence of mechanical sectioning artifacts on the observed images. Using image analysis, this cross-sectional CLSM method could provide both qualitative and quantitative information about porosity for symmetric and asymmetric membranes.
**Fig. 1.10.** Normalized mean pixel intensity from CLSM images collected at different depths within a symmetric 5 µm nominal pore diameter MCE membrane. Reprinted with permission from the publisher and the authors. In “Characterization of asymmetry in microporous membranes by cross-sectional confocal laser scanning microscopy” (Marroquin, M.; Bruce, T.; Pellegrino, J.; Wickramasinghe, S. R.; Husson, S. M.), Journal of Membrane Science, copyright © 2011.

**CLSM membrane observations and their applications**

Besides the aforementioned work, the literature contains numerous examples of how CLSM can be used for applications-driven membrane characterization research.

Wakeman et al. [57] compared three methods for producing silicon nitride based microfiltration ceramic membranes with pore sizes smaller than 1 µm and performed microstructural characterization of the membranes using SEM and CLSM. They presented measurements of density, porosity, pore size, water permeability and water flux. Their work suggested that it is necessary to use a thick evaporated silicon coating to produce a continuous, thin silicon nitride layer with ultrafine pores.
**Fig. 1.11.** Illustrations for membrane mechanical sectioning. The left-hand illustration shows that the limited penetration depth (PD) disallows CLSM imaging of the interior membrane if the membrane thickness is >2PD. By sectioning the membrane as shown in the right-hand illustration, it is feasible to image the full membrane cross section. Reprinted with permission from the publisher and the authors. In “Characterization of asymmetry in microporous membranes by cross-sectional confocal laser scanning microscopy” (Marroquin, M.; Bruce, T.; Pellegrino, J.; Wickramasinghe, S. R.; Husson, S. M.), Journal of Membrane Science, copyright © 2011.

Huang et al. [58] used CLSM to observe the morphologies of cellulose acetate membranes to tailor the membrane surface properties using low-pressure plasma processing. CLSM morphology of the plasma-treated cellulose acetate membranes revealed a small change in surface roughness in a qualitative way. It was concluded that low-pressure plasma processing was effective for controlling the surface properties of cellulose acetate membranes.

Bonilla et al. [25] carried out CLSM measurements to observe high silica zeolite MFI membranes prepared by the secondary (seeded) growth method on porous α-alumina disks. The results showed that CLSM in fluorescence mode could provide quantitative information on the intergrowth degree of the membranes. Their research also indicated
clearly that crystal grains were present in the zeolite MFI membranes [52]. Nair et al. [51] observed two types of silicalite (MFI) membranes: Type A (thick) membrane and Type B (thin) membrane. Crystal grain boundaries were able to be observed for Type A membranes, but not for Type B membranes. The results were in accordance with differences in separation performance between the two membranes. This study confirmed the determining role of crystal grain boundaries for the permeation characteristics of MFI membranes. Bonilla et al. [52] simulated the growth and microstructure of zeolite MFI films and membranes made by secondary growth. The simulation results were in good agreement with those obtained by SEM and CLSM.

Turner and Cheng [50] used CLSM to visualize polydimethyl siloxane (PDMS)/polymethacrylic acid (PMAA) Interpenetrating Polymer Network (IPN) membranes prepared by using the monomer immersion method. They concluded that the layer near the membrane surface with dispersed hydrogel domains made such IPN membranes impermeable to water-soluble compounds. They also imaged the membrane at various depths [37]. These observations were helpful for the understanding of morphology development in IPNs. In addition, the pH responsiveness of IPNs was probed [38]. CLSM characterization indicated that the permeation through IPN was pH-dependent due to the pH-dependent morphology.

Wang et al. [46] characterized the internal pore structure of forward osmosis (FO)/pressure retarded osmosis (PRO) membranes using CLSM for the first time. Comparison of CLSM results with those obtained using SEM and TEM showed
reasonably good agreement. The study provided some information on the pore structure of commercial FO/PRO membranes.

Lu et al. [43] used CLSM to characterize an organic-inorganic microfiltration membrane comprising poly(vinylidene fluoride) (PVDF) (FR904) and alumina nanoparticles. The membrane surface morphology, porosity and inorganic particle distribution in the modified membrane was compared with those in the unmodified one. It was found that the addition of nanometer-sized alumina particles altered the membrane surface morphology by increasing the surface roughness, which in turn increased the permeation-flux of the membrane.

Cannon et al. [31] observed nanofluidic channels inside focused-ion beam milled poly(methylmethacrylate) (PMMA) films. It was revealed that pores spanned the thickness of the PMMA film. The study on single pores is expected to provide a better understanding of nanoscale transport phenomena, and allowing more efficient applications of nanoscale fluidic components in many areas.

Suryanarayan et al. [32] used CLSM to obtain the morphology of gel-filled microporous poly(propylene) hollow fiber membranes. The membranes were characterized by factors including the amount of the gel polymer volume fraction, ion exchange capacity and wall thickness. The performance characteristics of the membranes were quantitatively examined by mixed-salt feed solutions with varying salt concentrations. Applications of these membranes in water softening were also discussed.

Wickramasinghe et al. [27] observed the morphology of anion- and cation-exchange membranes by characterizing solute binding within the membranes. Results
showed that CLSM could be a useful tool for the optimized design of membrane adsorbers. Wang [28] also investigated commercial Sartobind® cation-exchange membranes, based on stabilized regenerated cellulose with sulfonic acid (S) or carboxylic acid groups (C). The dynamic protein binding in these macroporous membrane adsorbers was monitored in situ by CLSM.

Risbud and Bhonde studied the morphology of polyamide 6 composite membranes blended with gelatin and chondroitin sulfate, analyzed the properties, and evaluated the in vitro biocompatibility [59]. Presented data indicated that polyamide 6 composite membranes were biocompatible and had potential applications in the field of tissue engineering.

Observation of membrane morphology using CLSM is a fundamental tool for further analysis of how membrane structure impacts performance. Vandenberg et al. [44] observed the microstructures of two types of porous polypropylene membranes, CELGARD and ACCUREL. Images were collected for membrane surfaces and at a depth of around 20 µm beneath each membrane surface. The side views of membranes also were collected (as shown in Fig. 1.12). The authors concluded that the ACCUREL membrane had a “random” structure, while the CELGARD membrane had a relatively straight and ordered pore structure. Differences in structure well explained the experimental results that CELGARD membranes have a much higher permeability than ACCUREL membranes.
**Fig. 1.12.** Side views of CLSM in fluorescence mode images for two types of membranes. Reprinted with permission from the publisher and the authors. In “Influence of Membrane Microstructure on the Diffusion Barrier of Supported Liquid-Crystalline Membranes” (Vandenberg, R.; Schulze, D.; Boltwesterhoff, J. A.; Dejong, F.; Reinhoudt, D. N.; Velinova, D.; Buitenhuis, L.), Journal of Physical Chemistry, copyright © 1995, American Chemical Society.

Wandera et al. [30] used CLSM in their work to develop advanced membranes for produced water treatment through the modification of low-molecular weight cut-off regenerated cellulose ultrafiltration membranes with grafted poly(N-isopropylacrylamide)-block-poly(oligoethylene glycol methacrylate) nanolayers. The research aimed to understand how grafting density and polymerization time influenced membrane performance. By using the CLSM results of one unmodified membrane and two modified membranes at different depths, the correlation between membrane depth and image pixel intensity was obtained. Visualization by CLSM showed that the modification occurs at the membrane external surface and internally within the porous cellulose layer. The membrane surface modification protocol could be used to produce membranes with high instantaneous permeate flux and low rate of flux decline, so as to
design highly advanced membranes for separating emulsified oils from oilfield produced water.

Green et al. [23] used CLSM in fluorescent and reflective mode to observe and quantify the micromechanical deformations caused by stretching. Additionally, the interconnecting pores that resulted from the stretching of composite membranes were visualized (as shown in Fig. 1.13).

Li et al. [36] characterized biotin incorporated polylactic acid (PLA) nanofiber membranes with SEM, electron probe microanalysis (EPMA), and CLSM. The CLSM in fluorescence mode was used to track the biotin–streptavidin specific binding from the top layer of each membrane towards the deepest layer where no fluorescence emissions could be detected.

![CLSM images of low-stretched region for the stearic acid calcite/polypropylene composite membrane.](image)

**Fig. 1.13.** CLSM images of low-stretched region for the stearic acid calcite/polypropylene composite membrane. Reprinted with permission from the publisher and the authors. In “Three-dimensional pore connectivity in bi-axially stretched microporous composite membranes” (Green, D. L.; McAmish, L.; McCormick, A. V.), Journal of Membrane Science, copyright © 2006.
Tan et al. [56] characterized the phase-separated microstructure of polymeric blended membranes by using combined multiphoton and reflective confocal imaging techniques. It was found that this combined method could be used to define the interfacial boundaries between two materials with different refractive indices and to reveal interfacial morphological features.

Synder et al. [55] established a link between the secondary growth of NaX zeolite membrane at different temperatures and the subtle differences in its separation performance. They concluded that the protocols they developed had pushed the limit of CLSM as a quantitative characterization tool for polycrystalline membranes. The authors concluded that the CLSM could be used to analyze currently unexplored zeolite films.

Charcosset and Bernengo [40] measured porosity and pore size from the analysis of images obtained using CLSM in fluorescent mode. The images were binarized (turned into black-white images) using Adobe Photoshop software (Adobe Systems Incorporated, USA) followed by an appropriate threshold. In their study, it was noted that the selection of a threshold point on the gray level scale affects the size of the analyzed features. Similar work also was presented by using ImageJ software (as shown in Fig. 1.14) [33]. The gray scale images could be utilized to calculate the porosity of membranes.

Ease of 3D reconstruction is another important advantage of CLSM over other imaging techniques, e.g., SEM [59]. Using Imaris software, Ferrando et al. [41] obtained information about the fouling pattern of proteins inside a membrane by 3-D reconstruction. Zhang et al. [26] characterized the nanostructure of stimuli-responsive polymeric composite membranes, and a 3D reconstruction of a membrane was presented.
The 3D reconstruction technique also was applied to the study of micromechanical deformation caused by stretching [23]. Tan et al. [56] reported the first application of multiphoton and reflected confocal imaging for analyzing the 3D phase-separation phenomena in immiscible nylon-chitosan blends. 3D imaging provides a more complete visual understanding and information about the microstructure of membranes and presence of defects or fouling within membranes.

![Image](image_url)

(a) image in fluorescence mode  
(b) binary image (black and white)

**Fig. 1.14.** CLSM image in fluorescence mode and the converted binary image by ImageJ software. Reprinted with permission from the publisher and the authors. In “Characterization of asymmetry in microporous membranes by cross-sectional confocal laser scanning microscopy” (Marroquin, M.; Bruce, T.; Pellegrino, J.; Wickramasinghe, S. R.; Husson, S. M.), Journal of Membrane Science, copyright © 2011.

### 1.6.2. Using CLSM to evaluate membrane performance

CLSM has been used by a number of groups to study membrane performance, including analysis of chemical separations; adsorption; and the influence of membrane properties on permeability, virus retention, fouling, among others [30, 44, 51, 57, 60-64]. Nearly all the observations reported were carried out by CLSM in fluorescence mode. In
many cases, such studies analyzed performance changes and their association with membrane morphology observations. Results from such work can be used to inform new membrane design.

Protein adsorption to a cation-exchange membrane was investigated by Reichert et al. [39]. They introduced CLSM as a method to evaluate protein purification. The membranes with adsorbed, labeled protein were observed by CLSM in fluorescence mode. The membrane structure itself and proteins were visualized by using multiple dyes, which enabled one to simultaneously observe the membrane and the protein bound to its pore structure.

Vandenberg et al. [44] presented CLSM results of two types of porous polypropylene membranes, CELGARD and ACCUREL. Observations of the membrane microstructures helped to explain the experimental finding that the CELGARD membrane achieved a much higher permeability than the ACCUREL membrane.

Wandera et al. [30] introduced the modification of low molecular weight cut-off regenerated cellulose ultrafiltration membranes with uniquely structured block copolymer nanolayers. An unmodified membrane and two modified membranes were contrasted. CLSM results provided visual evidence that the chain spacing within the nanolayer coating could be varied utilizing the membrane surface modification protocol to produce membranes with a good combination of high enough instantaneous permeate flux and a low rate of flux decline.

Local water content inside a membrane can affect the fluorophore concentration, which influences the fluorescence emission intensity. Dai and Barbari used CLSM to
study the water content of hydrogel membranes based on this understanding that fluorescence intensity was correlated to water content [64]. The ability to predict permeability and selectivity for asymmetric membranes may enable a priori design of gradient modification conditions that will lead to membranes with desired transport properties.

Nair et al. [51] presented results on the separation of close-boiling hydrocarbon mixtures by using zeolite membranes MFI (silicalite). The differences between observed membrane microstructures were found to be consistent with the differences in separation performance between two types of MFI membranes.

Fujimori et al. [63] described CLSM as a highly specific and sensitive method to demonstrate adsorption of biological substances to dialysis membranes. Finally, Bakhshayeshi et al. [61] and Hayama et al. [60, 62] studied the retention of endotoxins and virus particles inside membranes to understand how retention occurs and inform the design of optimized membrane materials.

1.6.3. Fouling characterization

Wide applicability of membranes in liquid separation processes is highly limited due to service life and change-out costs due to fouling [65]. Membrane fouling negatively affects performance and increases the operation cost by requiring frequent membrane cleaning/replacement and higher energy consumption. Also, membrane fouling can compromise the properties of the final product. For instance, in the dairy product industry,
fouling can influence the rejection of caseins and whey proteins, altering the quality of the final product.

Membrane fouling can occur due to pore constriction, pore blocking or cake formation. At constant pressure operation, fouling causes decay in the flow rate and at constant flux operations, fouling increases the transmembrane pressure [66]. Understanding how fouling occurs is the first step toward developing fouling mitigation strategies for membranes. In the literature, researchers have reported characterization and monitoring the fouling of microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), reverse osmosis (RO) membranes through techniques like electron microscopy, permeate flux tests, infrared spectroscopy, contact angle measurements, atomic force microscopy, rejection/transmission measurements, fluorescent microscopy, direct observation with standard light microscopes, energy dispersed spectroscopy, among other techniques [41,67-71]. Also, these techniques (and others) have been explored to evaluate the efficiency of membrane cleaning processes [72-74]. The advantage that CLSM presents over the aforementioned techniques is that CLSM can differentiate between foulants provided that they can be labeled with fluorescent dyes that have different emission wavelengths. Also, CLSM allows the visualization of the fouling within the sample (up to certain depth for thick samples, see depth of penetration limit) at different depths without the need of cutting or section the sample. By stacking the images collected at different depths, it is possible to re-construct a three dimensional representation of the sample. For thick samples, it was discussed earlier the approach taken by Marroquin et al. [33, 70] to obtain
cross-sectional CLSM images and visualize the full thickness of membranes for morphology and fouling studies.

Next, we will talk about the reports found in the literature centered on the application of CLSM to characterize membrane fouling.

**Microfiltration**

Membranes for MF applications are used in the food and pharmaceutical industries, among others. During the MF of biological streams, large particles like bacteria, yeast, cell debris and small molecules like proteins, polysaccharides, lipids and/or polyphenols commonly are present and responsible for membrane fouling.

Park et al. [75] developed a method to measure cake porosity with CLSM and image analysis in their study of the coagulation-microfiltration of fluorescent polystyrene latex beads with MF cellulose ester membranes. Their estimated porosity values from image analysis were in agreement with those calculated from specific cake resistances using the Carman–Kozeny equation. Also, they studied the effects (at different pressures) of size and fractal dimension of aggregates on cake porosity and compressibility.

By using CLSM in fluorescent mode, Ferrando et al. [41] characterized the fouling of polycarbonate track-etched MF membranes when filtering single and binary protein solutions. Fluorescently labeled bovine serum albumin (BSA) and ovalbumin were used to visualize fouling with CLSM in fluorescent mode. Image analysis was done to quantify, from the CLSM images, the fraction of the pore surface in which protein was detected. Information on protein deposition within the membrane was collected up to 3
μm within the membrane and 3 μm within the cake deposited on the membrane surface. Imaris software was used to construct 3D images of the fouled polycarbonate membranes. From their analysis, they concluded that ovalbumin caused more fouling in single and binary solutions compared to BSA. Kanani et al. [42] used CLSM to locate BSA protein (labeled with Alexa Fluor 488) on and within MF membrane. Also, CLSM was used to study the effectiveness of front washing in removing deposited BSA from the membrane.

Zator et al. [69] studied the fouling of mixed cellulose ester membranes and polycarbonate track-etched membranes during the cross-flow MF of BSA and dextran mixtures to get a better understanding of fouling that occurs in membrane bioreactors (MBRs), where the main foulants are extracellular polymeric substances like proteins, polysaccharides and nucleic acids. CLSM and image analysis were done to calculate the fraction of pore surface where fluorescently labeled protein (BSA-FITC) and/or polysaccharide (dextran-TRITC) were detected. Information for the fraction of the pore occupied by protein or polysaccharide was obtained up to a depth of 3 μm inside the membrane. In a separate publication, Zator et al. [72] assessed the efficiency of water rinsing and chemical cleaning of MF polycarbonate membranes post cross-flow MF of protein and polysaccharide (BSA/dextran) mixtures. Using CLSM and image analysis, they quantified the amount of protein/polysaccharide detected inside the pores and performed 3D reconstructions of CSLM images to compare the effects and efficiencies of the cleaning protocols.

In a similar study, Zator et al. [54], performed cross-flow MF of protein, polyphenol and polysaccharide mixtures with polycarbonate membranes to gain deeper
insight regarding the fouling that occurs in beverage clarification, where these components commonly are found and foul the membrane despite their small size. CLSM and image analysis were done to estimate the fraction of pore where protein and/or polysaccharide were visualized. Also, 3D reconstruction of the CLSM images was done, as shown in Fig. 1.15. Although, they were not able to visualize the internal fouling of the membrane below the first few microns, they discovered that the addition of a polyphenol (tannic acid) in protein/polysaccharide solutions appears to result in a much more severe fouling than is observed for binary BSA/dextran solutions. They also assessed the efficiency of chemical cleaning and water rinsing with CSLM along with permeate flux evolution measurements. An interesting finding in their research was that water rinsing does not improve membrane permeability if protein is involved in membrane fouling, and that, during water rinsing, some of the proteins appear to be driven deeper inside the membrane, causing further blockage and reducing water flux.

In a similar investigation, Marroquin et al. [70] investigated the fouling occurring in polyethersulfone MF membranes in the dead end filtration of component, binary and ternary mixtures of proteins, polyphenols and polysaccharides. They observed drastic flux decay when the casein/tannic acid binary mixture was filtered. They also investigated the influence of the polysaccharide β-cyclodextrin on the protein-polyphenol aggregation. It was found an appropriate concentration of polysaccharide that prevented the protein/polyphenol aggregation and concentrations above and below the ‘sweet spot’ concentration fouled the membrane even more or had no significant effect, respectively. Cross-sectional CLSM images of the fouled membranes allowed the location of the
foulants throughout the entire thickness of the membrane (see Fig. 1.16) and were in agreement with their results obtained from the flux measurements. Also, the amount of foulant within the membrane was quantified based on calibration curves (intensity versus amount of foulant) produced in their study.

Fig. 1.15. A volumetric 3D reconstruction of a 0.8 µm polycarbonate membrane fouled by ternary solutions of BSA-FITC/dextran-RITC/tannic acid. Concentration of protein (BSA: 0.25 g/L) and polysaccharide (dextran: 0.25 g/L) in the feed was kept the same for (a), (b) and (c). Concentration of the polyphenol tannic acid (TA) in feed was (a) TA025: 0.25g/L; (b) TA05: 0.5g/L; (c) TA1: 1 g/L. Grey and black colors represent membrane and membrane pores, respectively. Green color represents signal coming from BSA-FITC, red color represents signal coming from dextran-RITC. The white/yellow square indicates the membrane surface (scale bar = 10 µm). Reprinted with permission from the publisher and the authors. In “Microfiltration of protein/dextran/polyphenolic solutions: Characterization of fouling and chemical cleaning efficiency using confocal microscopy” (Zator, M.; Ferrando, M.; Lopez, F.; Guell, C.), Journal of Membrane Science, copyright © 2009.
Yang et al. [29] also used CLSM to gain a better understanding of the fouling occurring in MBRs. They followed a 6-step staining protocol to label nucleic acids, proteins, β-D-glucopyranose polysaccharides, α-D-glucopyranose polysaccharides and lipids in the biofouling layer after the direct-flow filtration of waste-activated sludge obtained from a wastewater treatment plant, using mixed cellulose ester MF membranes. Fig. 1.17 shows the CLSM image of the deposited layer on the mixed cellulose ester membrane surface.

It is observed that the extracellular polymeric substances (EPS) are distributed in clusters within the fouling layer and that proteins, β-D-glucopyranose, and lipids correspond closely to cells leading to the believe that these EPS might be bound to the cell membrane. Based on the three-dimensional volumetric grid model of the fouling layer structure observed from the series of CLSM images, the intra-layer flow field during filtration was simulated using commercial software and effective permeability of the fouling layer was estimated.

Le-Clech et al. [76] compared the observed formation of a polysaccharide fouling layer on polyvinylidene fluoride (PVDF) MF membranes by environmental SEM (ESEM), CLSM and direct observation. Using CLSM, they were able to visualize alginate (polysaccharide) labeled with DTAF on PVDF membranes after direct-flow filtration experiments. The cake was visualized on the surface and thickness was measured.

Brans et al. [77] studied with CSLM the transmission process (in-line) of monodispersed latex particles through cellulose acetate membranes and polyethersulfone
(PES) micro-sieves in cross flow mode. They visualized with SEM and CLSM where the particles were deposited on and within the membrane. Also, they commented that morphology of the membrane and the transmembrane pressure influenced the behavior of deposition of the latex particles.

**Fig. 1.16.** Cross-sectional CLSM images of casein/casein-FITC and β-cyclodextrin/β-cyclodextrin-RITC binding within an asymmetric 0.65 μm PES membrane at a depth of 4 μm. Feed comprised 25 mg/L casein (1:20 fluorescently labeled to non-labeled protein), 150 mg/L tannic acid, and 25 mg/L β-cyclodextrin (1:20 fluorescently labeled to non-labeled polysaccharide). The dense surface is on the LEFT of all images. TOP row images are for samples taken after processing 125 mL permeate volume: casein (LEFT), β-cyclodextrin (MIDDLE), superimposed image of casein and β-cyclodextrin (RIGHT). SECOND row images are for samples taken after processing 250 mL permeate volume. BOTTOM row images are for samples taken after processing 500 mL permeate volume. The scale indicator is 10 μm in diameter.

Hwang et al. [78] used polyethylene hollow fiber MF membranes in a submerged MBR set-up for the treatment of synthetic wastewater. They used CLSM to gain a better understanding of the structure of the bio-cake formed on the membrane surface. By incubating the biofilm with a mixture of dyes, they stained the nucleic acids to locate bacterial cells with a commercial fluorescent dye, SYTO 9 (Molecular Probes, USA), and stained polysaccharides with concanavalin A (ConA) lectin-TRITC (Molecular Probes, USA). The excess of dye was washed out with buffer solutions. From CLSM image analysis, they obtained thickness and roughness of the bio-cake layer. Additionally,
CLSM images showed the distribution of cells and polysaccharides within the cake. They performed studies at three permeate fluxes, and collected samples from each run when the same, specific transmembrane pressure had been reached. Interestingly, the membrane corresponding to a lower flux (and longer operation time) had a higher accumulation of polysaccharide near the surface of the membrane. The authors indirectly quantified the amounts of bacteria cells and polysaccharides along the cake depth by estimations of their respective volume fractions. From the analysis of the CLSM images, they concluded that the bio-cake formed at the highest flux consisted mostly of bacterial cells, rather than polysaccharide; whereas, at the lowest operating flux, the bio-cake was largely polysaccharide (see Fig. 1.18). The authors also found differences in the spatial distribution (volume fraction versus depth within cake) of bacterial cells and polysaccharides under the different fluxes: the spatial distribution of the volume fraction of bacterial cells was relatively higher at the highest flux; whereas, spatial distribution of the volume fraction of polysaccharide shows the converse. In a similar investigation, Hwang et al. [79] determined the live-to-dead cell ratio along the thickness of the cake by using a BacLight Live-Dead staining kit (Invitrogen, USA) and CLSM imaging. They related the differences in the spatial distribution of extracellular polymeric substances (EPS) to the differences in the live-to-dead cell ratio at different depths.

Hwang et al. [80] applied CLSM to assess the flux enhancement that occurs when a membrane fouling reducer (MFR) was added to the MBR. By incubating the cake with a mixture of fluorescent dyes, they were able to label proteins (benzoaxanthene yellow, Sigma Aldrich, USA), polysaccharides (Concanavalin A lectin-TRITC, Molecular Probes,
USA) and nucleic acids within cells (SYBR Green I, Molecular Probes, USA). They presented 3D reconstructed images of the cake, and were able to visualize the spatial distribution of cellular and polymeric constituents. From CLSM image analysis, they were able to obtain information about the porosity, bio-volume and average run length of the cake. It was noted that even though the cake formed during operation of the MFR/MBR reactor was thicker than that of the control reactor (with no MFR), loss of performance due to membrane fouling was decreased primarily as the result of increased cake porosity.

Fig. 1.18. CLSM images of volumetric 3D reconstructed images of bacterial cells and polysaccharides in the bio-cake corresponding to 27 L/m²h (a, b), 20 L/m²h (c, d) and 13 L/m²h (e, f) operating flux. Green color: bacterial cell; red color: polysaccharide. Area of each image: 512 × 512 µm². Reprinted with permission from the publisher and the authors. In “Membrane bioreactor: TMP rise and characterization of bio-cake structure using CLSM-image analysis” (Hwang, B.K; Lee, C. H.; Chang, I.S.; Drews, A.; Field, R.) Journal of Membrane Science, copyright © 2012.
Yun et al. [81] compared the fouling occurring in two MBRs using polyethylene MF membrane in a hollow fiber membrane module and treating synthetic dye wastewater at aerobic and anoxic conditions, respectively. By incubating the cake, they labeled nucleic acid within the cell with SYBR Green I (Molecular Probes, USA), and polysaccharides with Concanavalin A lectin-TRITC (Molecular Probes, USA) and Wheat Germ Agglutinin-TRITC (Molecular Probes, USA). They were able to visualize the spatial distribution of cells and polysaccharides within the cake and characterize the structure of the biofilm with CLSM. They concluded that fouling was faster for the MBR at anoxic conditions and that it was related to the structural parameters, obtained from CLSM image analysis. Their analysis showed that the anoxic biofilm was thinner than the aerobic biofilm; however, the anoxic biofilm was spread out on the membrane surface more uniformly and densely, resulting in a more severe loss in performance due to membrane fouling.

CLSM has been used to compare the fouling behaviors of bulking sludge, deflocculated sludge, and normal sludge in MBRs [82]. CLSM was used to see the differences in the cake layers formed for each case. In this case study, nucleic acids and polysaccharides within the cake were stained, and the authors visualized the spatial distribution of cells and polysaccharides. From the 3D reconstructed CLSM images, cake thickness was obtained for each case, and it was observed that the bulking sludge and deflocculated sludge could form denser cake layers compared with normal sludge.
Ultrafiltration

Wei et al. [83] used CLSM to assess if the surface modification of polyacrylonitrile (PAN) ultrafiltration membranes with D-gluconamidoethyl methacrylate improved their fouling resistance towards the adsorption of protein. In this study, CLSM was used to evaluate the resistance to protein adsorption of the modified membrane by static adsorption experiments. Fluorescein isothiocyanate (FITC)-labeled BSA adsorption on membranes was visualized with CLSM. For different degrees of grafting of D-gluconamidoethyl methacrylate, the authors measured the fluorescence emission intensity of the membranes with adsorbed FITC-BSA. A relative intensity (or relative adsorption) was calculated for each sample, adjusted for the intensity of the unmodified UF membrane. (PAN membranes showed some autofluorescence). Results were compared to determine the role of surface glycosylation on protein fouling resistance. CLSM results showed that the relative intensity of the samples decreased as grafting degree increased. From the CLSM image analysis, protein resistance of the PAN UF membrane was improved, and the adsorption of protein was inhibited significantly.

Gao et al. [84] used a flat sheet PVDF ultrafiltration membrane module submerged in an anaerobic MBR for treating thermomechanical pulping whitewater. They identified that cake formation was the main fouling mechanism, and they implemented CLSM to visualize, locate and quantify the spatial distribution of EPS (proteins and polysaccharides) within the cake layer. By incubation of the cake with a mixture of fluorescent dyes, they stained protein with a commercial fluorescent dye, Sypro Orange (Invitrogen, USA), and polysaccharides were stained with concanavalin A
conjugated with Alexa Flour 633. Microtoming was used to isolate sections of the cake layer corresponding to different depths, as a way to overcome depth of limitation of the CLSM microscope. Three-dimensional reconstruction of the cake was done. From the analysis of the CLSM images, they concluded that the coverage of proteins and polysaccharides on the membrane substrate increased along the depth of the cake, creating a loose outer surface compared with the bottom cake layer.

_Nanofiltration (NF)_

Marconnet et al. [85] used CLSM to evaluate the fouling of NF membranes (composed of an ultrathin top layer made of polypiperazine and a polysulfone and polyester microporous support) by two different pre-treated feed waters with equivalent microbial cells content but different total and biodegradable organic carbon (BDOC) concentrations. By incubation of the fouling layer with fluorescent dyes, they labeled the cells within the fouling layer with DNA binding stain DAPI (4′, 6-diamidino-2-phenylindole) and polysaccharides were labeled with lectins conjugated with fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC). The biofilm on the membrane fed by the water with higher BDOC was thicker, denser and contained more microorganisms and polysaccharides than the biofilm obtained with the water with lower BDOC, as seen in Fig. 1.19. They concluded that the BDOC in the feed water is a good indicator to predict membrane biofouling, and its reduction through raw water pretreatments is critical to prevent biofouling and maintain the performances of NF membranes.
Following a similar labeling of the fouling layer, Di Martino et al. [86] used CLSM to assess the efficiency of cleaning procedures to remove the fouling deposit from the surface of NF membranes. From their observations, they concluded that the cleaning of the NF fouled membranes only partially removed the organic fouling deposit characteristic of a microbial biofilm.

**Fig. 1.19.** CLSM visualization of membrane biofouling after filtration of water higher BDOC (top) and lower BDOC (bottom). Blue color: cells labeled with DAPI; red and green color: polysaccharides labeled with lectins conjugated with FITC or TRITC. Reprinted with permission from the publisher and the authors. In “Biodegradable dissolved organic carbon concentration of feed water and NF membrane biofouling: a pilot train study” (Marconnet, C; Houari, A.; Galas, L.; Vaudry, H.; Heim, V.; Di Martino, P.), Water Science and Technology, copyright © 2008.
Reverse Osmosis (RO)

To understand the mechanisms governing the decline in RO membrane performance caused by cell deposition and biofilm growth, Herzberg and Elimelech [87] studied the biofouling of a thin-film composite reverse osmosis membrane (aromatic polyamide composition, neutral surface charge) during the cross-flow filtration of a synthetic wastewater containing *Pseudomonas aeruginosa*. Post filtration, the biofilm was incubated with a mixture of concanavalin A conjugated to tetramethylrhodamine isothiocyanate (TRITC) and propidium iodide (PI) to label EPS and dead cells, respectively. The *P. aeruginosa* bacteria strain contained green fluorescent protein, and there was no need to label it. Three-dimensional images were constructed, and the biofilm growth dynamics were studied by monitoring active cells, dead cells, and EPS with CLSM. The specific biovolumes of the biofilm components were estimated from the image analysis for membranes collected at different operational times.

Kent et al. [88] evaluated tertiary membrane filtration and MBR as pre-treatments to prevent RO membrane fouling. They used polyamide thin-film composite membranes in cross-flow units to process municipal wastewater from a treatment plant. The biofilm was stained with concanavalin A-Alexa Fluor® 633 (polysaccharides) (Invitrogen, USA) and SYPRO orange (proteins) (Sigma Aldrich, USA). Confocal microscopy was used to visualize the accumulation of organic matter (proteins and polysaccharides) on the surface of the membrane. Differences in the intensity coming from polysaccharides and proteins at different permeate volumes were analyzed for RO membranes processing feed following the two different pretreatments. The CLSM results suggested that proteins
made up a higher proportion of fouling layers initially (in the first 2 weeks), after which the polysaccharide deposition densities increased dramatically and became much higher than the protein deposition densities for both pretreatment methods. The RO with tertiary membrane filtration pretreatment had higher organics deposition and more rapid biofilm development compared with the RO membrane with MBR pretreatment.

Surface modification of cellulose acetate and polyamide RO membranes by attaching selenium to the membrane surface was tested by Low et al. [89] as an approach to prevent biofouling. Selenium acts as a catalyst for oxygen reduction and the production of superoxide radicals that kill cells. The surfaces of RO membranes were modified by attaching Se-acetoacetoxy ethyl methacrylate (Se-AAEMA) or selenocystamine. CLSM was used to visualize the presence of *Staphylococcus aureus* bacterial growth. The *S. aureus* strain contains a green fluorescent protein that was used to locate the bacteria with CLSM. CLSM was used to monitor the efficacy of selenium on inhibiting biofouling. Fig. 1.20 shows that both selenium attachment protocols were successful in inhibiting the fouling of the membrane by *S. aureus*. The authors concluded that selenium incorporation by the addition of the monomer selenocystamine is more suitable since it does not affect significantly the membrane flux, unlike addition of Se-AAEMA to the surface of the membrane.
Fig. 1.20. Confocal laser scanning microscope (30× magnification) of RO membrane surface after 24 h exposure to *S. aureus*. RO membranes were coated with selenium using either Se-AAEMA or selenocystamine (SCA). Reprinted with permission from the publisher and the authors. In “Attachment of selenium to a reverse osmosis membrane to inhibit biofilm formation of *S. aureus*” (Low, D.; Hamood, A.; Reid, T.; Mosley, T.; Tran, P.; Song, L.; Morse, A.) Journal of Membrane Science, copyright © 2011.

1.7. Conclusions

This chapter has examined in detail confocal microscopy as an advanced imaging technique and its application in synthetic membrane studies. Great interest in confocal microscopy has developed in recent years and it has become popular in material science. Moreover, its advantages over conventional microscopy techniques and versatility in applications make confocal microscopy an attractive characterization tool. In this chapter, I described the basics of confocal microscopy and highlighted its features (sections 1.1 – 1.5). Attention was drawn to the importance of ‘knowing’ the instrument to ensure proper operation and to obtain reliable, accurate and reproducible results. The aim was to teach
readers about how the ‘black box’ called the confocal microscope works and how its settings and variations affect performance and final results. I finish with a compilation of studies that have implemented confocal microscopy as a main or complementary tool in research on the characterization of membranes. Section 1.6 shows that morphology, performance and/or fouling observation and analysis by CLSM, when combined with mechanisms analysis, 3D reconstruction, simulation and prediction, etc., is emerging as an important tool for membrane science and engineering studies. It also demonstrates the versatility, wide applicability of CLSM and the valuable information provided by this technique that, in most cases, has not been provided by any other. Finally, this chapter is meant to provide a better understanding of confocal microscopy to the membraneologist so that he/she can effectively use it in membrane research. My hope is that the ideas presented here help the researcher in implementing CLSM in more creative ways.

1.8. Dissertation outline

This dissertation comprises three main chapters that compile the investigations done through my doctoral research work. Chapter 2 presents my work on developing the protocols for sample preparation and cross-sectional CLSM imaging. The objective of this part of the study was to characterize asymmetry in microporous membranes by confocal laser scanning microscopy in fluorescent mode. Initially, I determined the limits of resolution and depth of penetration of the state-of-the-art confocal microscope. Two MF membranes (symmetric mixed cellulose ester and asymmetric polyethersulfone) were the subjects of study. Defect-free cross-sectional images were obtained and porosities
were estimated throughout the entire thickness of the membranes from the image analysis. Additionally, I determined the effect of the fluorescent dye chosen on the porosity results.

Chapter 3 describes the application of the cross-sectional CLSM imaging protocol developed in Chapter 2 to the characterization of membrane fouling in the microfiltration of proteins and polysaccharides mixtures. The objective of this part of the study was to locate and quantify foulants within MF membranes and gain a better understanding of the effect of solute-solute and solute-membrane interactions on fouling. Flux data were collected and membranes were autopsied to obtain the cross-sections and CLSM images. Foulants were located at different depths within the polyethersulfone asymmetric membrane used in the experiments at different levels of fouling or flux decline. Fouling behaviors were analyzed in the frame of solute-solute and solute-membrane interactions. Finally, accordance between flux data, visualization of fouling through CLSM images, and fouling mechanisms occurring was discussed.

In Chapter 4, I describe the application of the cross-sectional CLSM imaging protocol in the characterization of membrane fouling in the microfiltration of protein, polysaccharide, and polyphenol mixtures, emulating a beverage clarification process where these components commonly are found and foul the membrane despite their small size. The objective of this part of the study was to analyze the fouling behavior of the feed components when they were alone and mixed in solution. I evaluated the effect of polysaccharides in the disruption of protein and polyphenol aggregates and determined where these foulants had the tendency to accumulate within the membrane. The fouling
behaviors observed in the flux data and CLSM images were discussed in the frame of the component interactions happening in solution and within the membrane.

Chapter 5 presents the conclusions of my studies and recommendations for future studies.
1.9. References


CHAPTER 2

CHARACTERIZATION OF ASYMMETRY IN MICROPOROUS MEMBRANES
BY CROSS-SECTIONAL CONFOCAL LASER SCANNING MICROSCOPY

[As published in Journal of Membrane Science 379 (2011) 504–515, with minor revisions]

2.1. Introduction

Methods of manufacturing filtration membranes such as air casting, immersion casting, track etching, etc. lead to a wide array of membrane structures, and the structural characteristics of these membranes affect their performance during a separation process. The performance factors considered in the design of filtration membranes are selectivity, productivity and capacity. Selectivity is the ability of the membrane to reject non-product components while maximizing passage of the product. Productivity refers to the average volumetric filtrate flux, and capacity is the total volume of feed that can be processed per unit membrane area before the membrane must be replaced [1]. Filtration membrane design aims to maximize selectivity, productivity and capacity.

For polymeric membranes, four structure levels can be defined that affect the membrane performance regardless of its application [2]: (1) chemical composition of the selective polymer separation layer, (2) steric relationships in repeat units of the selective polymer, (3) morphology of the separation layer, and (4) macroscopic membrane structure. In the case of sterile filtration membranes, structure levels 3 and 4 arguably play the most important roles in determining filtration performance and selection of a
suitable membrane for a specific process. For example, isotropic membrane capacities are limited since retention occurs at the surface. Anisotropic membranes enhance capacity and maintain productivity for feeds with a particle size distribution because different membrane ‘layers’ retain different size particles. Composite and multilayer membranes provide many degrees of freedom; for example, the top layer may serve as a built-in prefilter [3]. Given the large number of possible membrane structures, it is essential to characterize average membrane pore diameter, pore-size distribution, porosity and asymmetry (parameters describing structure levels 3 and 4) to design membranes with structures that maximize performance. It is not enough to compare membranes based on nominal pore size alone because this property only refers to the size of the particles retained by the membrane; it does not provide direct information about the membrane morphology [4].

No membrane asymmetry characterization method is completely reliable. Bubble-point porosimetry, mercury intrusion and liquid/gas permeability methods depend on model assumptions [5]. Microscopic techniques like scanning and transmission electron microscopy (SEM and TEM) are invasive and require time-consuming sample preparation (embedding and microtome-sectioning) to obtain information beyond superficial images. Furthermore, this sample preparation may generate defects that distort the actual morphology. Despite these disadvantages, SEM and TEM continue to be used because they are high-resolution methods (around 10 nm) and provide direct morphology information [4].
Confocal laser scanning microscopy (CLSM) is a more recent technology (commercially available since 1983) that provides an alternative to SEM and TEM. It has been utilized widely in biological sciences; however, it is now being utilized with increasing frequency in the study of materials. The paramount advantages of CLSM over SEM and TEM are that CLSM is a less invasive technique (performs an optical sectioning and provides images from the bulk of the sample without sample destruction), and generates images that provide reliable information about the internal morphology without artifacts introduced by sample sectioning. CLSM requires sample staining with a dye. However, staining must be done only once for each membrane sample to enable 3-dimensional imaging. In contrast, 3-dimensional imaging by electron microscopy requires numerous sample preparation steps (i.e., mechanical sectioning and metallization for each section).

Ferrando et al. [6] provide a comprehensive overview of CLSM fundamentals. Briefly, CLSM works by focusing on a single plane at a selected depth. Images are recorded at different depths by moving the objective lens, thus changing the position of the focal plane [5]. As a result, a 3-dimensional image can be created by stacking the images collected from adjacent planes. When using CLSM in fluorescent mode, the excitation light delivers energy in the form of photons to fluorophores in the sample causing electrons in the outer shell of the fluorophore to move to a higher energy state. To return to their original energy state, these electrons release energy in the form of photons with a slightly lower energy level resulting in emission light of a longer wavelength than the original excitation light. The emission light released from the
fluorophores follows the same path as the excitation light, passing through the objective lens and a dichroic mirror. A pinhole aperture placed in front of the photomultiplier tube detector allows the light from the focal plane to pass through to the detector, while blocking light scattered from other planes, resulting in a completely focused image.

Relatively few studies are found in the literature where CLSM is applied to characterize the morphology of membranes because of its lower resolution compared to SEM and TEM methods and due to its limited depth of penetration. Table 2.1 presents several studies that have implemented CLSM to characterize membranes. Snyder et al. [7] and Bonilla et al. [8] were able to collect structural information throughout the full thickness of thin zeolite materials. Green and coworkers used CLSM and three-dimensional image reconstruction to visualize the interconnecting pores that result from the bi-axial stretching of thin (8–10 μm) CaCO₃/polyolefin composite membranes [9]. For thicker materials, Charcosset and Bernengo [5] identified a depth limit, beyond which the images became degraded because the intensity of light reaching the detector from the focal plane decreased with increasing depth. Reichert et al. [10] and Ulbricht and coworkers [11] used CLSM to observe protein binding to fluorescently labeled ion-exchange membranes. By using different fluorescent labels for the proteins and membrane, they were able to visualize membrane structure and the location of protein binding. These studies recognized that the detection sensitivity and resolution both decrease with increasing depth, as less emitted light reaches the detector. They reported depths of penetration of 20 and 50 μm, respectively. Wickramasinghe et al. [12] also used CLSM to study protein binding patterns in ion-exchange membranes using fluorescently
labeled proteins. While direct characterization of membrane morphology was not the objective of their work, they did provide information on depth of penetration, which was reported to be 60 μm. These previous studies highlight a limitation of CLSM, which is the inability to characterize the internal morphology of thick membranes fully. This limitation provided the driving force for developing a cross-sectional imaging methodology.

The goals of this research were to develop cross-sectional CLSM methods to characterize asymmetry in thick microporous membranes, to quantify sub-surface porosity as a function of depth using CLSM, and to identify limits of resolution and penetration depth. Membranes with different nominal pore diameters were imaged using CLSM to determine experimental limits of resolution and depths of penetration. ImageJ software [13] was used to analyze CLSM images to quantify porosity at different depths. Our development of a cross-sectional CLSM imaging method will enable researchers to perform full morphological characterization of thick, asymmetric membranes, leading to better understanding of the effects of membrane structure on performance.
### Table 2.1. Summary of studies that used CLSM to characterize membrane structure.

<table>
<thead>
<tr>
<th>Authors</th>
<th>CLSM application</th>
<th>Material thickness</th>
<th>Pore size</th>
<th>Depth of penetration limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Snyder et al. [7]</td>
<td>Characterization of the morphology, microstructure and polycrystallinity of NaX zeolite membranes</td>
<td>10–16 μm</td>
<td>24–97 nm</td>
<td>N/A&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bonilla et al. [8]&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Study of grain boundary of zeolite MFI membranes</td>
<td>&gt;20 μm</td>
<td>Not reported</td>
<td>N/A&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Green and co-workers [9]</td>
<td>Visualize pore connectivity in bi-axial stretched CaCO&lt;sub&gt;3&lt;/sub&gt;/polyolefin composite membranes</td>
<td>8–10 μm</td>
<td>0.1–1 μm</td>
<td>N/A&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Charcosset et al. [5]&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Characterization of the morphology of fluorescently labeled cellulose acetate (CA), poly(vinylidene fluoride) (PVDF) and polycarbonate (PC) membranes</td>
<td>CA: 145 μm</td>
<td>1.2 μm</td>
<td>10 μm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PVDF: 125 μm</td>
<td>5 μm</td>
<td>6 μm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PC: 10 μm</td>
<td>2 μm</td>
<td>4 μm</td>
</tr>
<tr>
<td>Reichert et al. [10]&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Visualization of protein adsorption to Sartobind S® and enzyme adsorption to Sartobind Q® fluorescently labeled ion-exchange membranes</td>
<td>200 μm</td>
<td>≥3 μm</td>
<td>50 μm</td>
</tr>
<tr>
<td>Wickramasinghe et al. [12]&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Visualization of protein adsorption to non-labeled Sartobind S® ion-exchange membrane</td>
<td>275 μm</td>
<td>≥3 μm</td>
<td>60 μm</td>
</tr>
</tbody>
</table>

<sup>a</sup>Corresponds to CLSM in fluorescence mode. <sup>b</sup>Corresponds to CLSM in reflective mode. <sup>c</sup>Full membrane thickness could be imaged.
2.2. Experimental materials and methods

2.2.1. Materials

Isotropic mixed cellulose ester (cellulose nitrate/cellulose acetate, MCE) membranes (EMD Millipore) and asymmetric polyethersulfone (PES) membranes (Pall Corporation) were used. The MCE membranes have average effective pore diameters of 0.22 μm (SCWP04700), 0.45 μm (SMWP04700), 0.65 μm (SSWP04700), 0.80 μm (RAWP04700), 1.2 μm (AAWP04700), 3.0 μm (DAWP04700), 5.0 μm (HAWP04700) and 8.0 μm (GSWP04700). MCE membrane thickness was 160–180 μm, according to the manufacturer and supported by measurements in this study. The PES membranes have effective pore diameter of 0.65 μm (Supor®) and thickness of 114–175 μm, according to the manufacturer and supported by measurements in this study.

Membranes were stained with 5-DTAF [5-(4, 6-dichlorotriazinyl) aminofluorescein] (Invitrogen, D-16) or Alexa Fluor® 594-conjugated goat anti-rabbit IgG (Invitrogen, A-11012). The 5-DTAF dye has excitation/emission wavelengths of 492/516 nm and Alexa Fluor® 594 dye has excitation/emission wavelengths of 590/617 nm. Phosphate buffered saline (PBS) solution was prepared using bioreagent 1× powder concentrate (Fisher Scientific, BP661-10) and deionized Milli-Q system (EMD Millipore) water. Sodium carbonate buffer (100 mM, pH 9.6) was prepared using sodium carbonate (Sigma Aldrich, S2127), sodium hydrogen carbonate (Sigma Aldrich, S6014) and deionized Milli-Q system water. Sodium chloride (Sigma Aldrich, S9888) was used with sodium carbonate buffer to prepare the staining solution. The rinse solution for
membranes stained with 5-DTAF was prepared using absolute ethanol (Sigma Aldrich, E7023) and deionized Milli-Q system water.

All samples were mounted using VECTASHIELD® (Vector Labs, H-1000) aqueous mounting medium (glycerol-based aqueous sample mounting medium and anti-fading agent for the fluorescent dye) and covered with micro cover glasses (VWR, 48393 092) before imaging. The Type A immersion oil (Nikon) specified for the objective lens was used with the optical system.

2.2.2. Sample preparation

MCE membranes were stained by adsorption of Alexa Fluor® 594 goat anti-rabbit IgG or by covalent coupling of 5-DTAF. PES membranes were stained by covalent coupling of 5-DTAF. Alexa Fluor® 594-conjugated goat anti-rabbit IgG binds to the membrane structure through hydrophobic interactions and electrostatic interactions between the membrane and IgG protein. 5-DTAF attaches covalently to the membrane by reaction with hydroxyl groups. Alexa Fluor® 594-conjugated goat anti-rabbit IgG was added to PBS buffer solution to achieve a 0.03 mg/mL concentration. 5-DTAF dye solution (15 μg/mL in sodium carbonate buffer, with 100 mM NaCl) was prepared as described by Ulbricht and co-workers [11].

Membrane coins with 25 mm diameter were cut out and placed in a plastic filter holder (Whatman Ltd, 420200). A plastic syringe was used to flow 20 mL of dye solution through each membrane, and the time for manual delivery of the solution was held constant to ensure a constant rate of approximately 10 mL/min in each experiment.
Membranes were stored at 4 °C for 12 h in the same 20 mL of dye solution that had passed through the membrane. Each was stored in a separate Petri dish and protected from light exposure. After staining, the membrane samples were rinsed. MCE membranes dyed with Alexa Fluor 594® were rinsed by flowing 10 mL of 1× PBS buffer solution through the membrane with a syringe and then immersing the membrane in 1× PBS buffer solution three times for 10 min to remove unbound dye. PES and MCE membranes dyed with 5-DTAF were rinsed by flowing 10 mL of 20% (v/v) ethanol:water and 10 mL of 1× PBS buffer solution through the membrane with a syringe, immersing the membrane in 20% (v/v) ethanol:water for 10 min, and then immersing in 1× PBS buffer solution twice for 10 min to remove unbound dye.

### 2.2.3. Sample mounting for lateral CLSM imaging

From each membrane coin, a 5 mm square sample was cut out. The sample was mounted on a microscope slide (Fisherbrand, 12-550-A3), followed by addition of 2 drops (approximately 50 μL) of VECTASHIELD® as mounting medium to (1) completely fill the void between the cover glass slides containing the membrane sample, (2) maintain uniform thickness between the slide and cover glass during the scanning, (3) prevent photobleaching (fading) of the dye, and (4) match the sample refractive index to that of the immersion oil for the microscope lens. VECTASHIELD® was applied using a special pipette supplied with the product. A micro cover glass was applied to disperse the mounting medium over the entire membrane sample, and the four corners were fixed and sealed with clear nail polish.
2.2.4. Sectioning and mounting samples for cross-sectional CLSM imaging

A cryostat (Leica model CM3050S) was used for sample cryosectioning. The dimensions of the cutting blade (Fisher Scientific, 12-634-4) were 76(L) × 14(W) × 0.3(H) mm. The embedding medium was Tissue-Tek® O.C.T. Compound 4583 (VWR, 25608-930). From each membrane coin, a 5 mm square sample was cut out. Embedding media was poured into a Tissue-Tek® Intermediate Cryomold 4566 (15 mm × 15 mm × 5 mm, Fisher Scientific, NC9542860) to fill it halfway. The membrane sample was placed flat on top of the embedding medium and more embedding medium was added to fill the mold completely. Samples were frozen in liquid nitrogen. The solid block of embedding medium containing the membrane sample was removed from the cryomold and mounted on the cryostat vertically. With this orientation, the blade cut parallel to the z axis (see Fig. 2.1) of the membrane to yield flat cross sections. The cryostat chamber temperature was set to −20 °C. The cryostat was set to cut 30 μm thick sections which were transferred onto adhesive, electrostatically charged Superfrost® Plus Micro Slides (VWR, 48311-703). From each 5 mm square sample, multiple cross-sections were obtained and three cross-sections were selected for the image analysis described in Section 2.3.4. Embedding medium was removed by immersing the slide for 20 min in 1× PBS at 35 °C. Fifty microliters of VECTASHIELD® mounting medium were added, a micro cover glass was applied to disperse the mounting medium over the entire membrane sample, and the four corners were fixed and sealed with clear nail polish.
Fig. 2.1. Overcoming limited penetration depth (PD) by cross-sectional CLSM imaging. The left-hand illustration shows that the limited PD disallows CLSM imaging of the membrane interior, since membrane thickness is >2PD. By sectioning the membrane as shown in the right-hand illustration, we are able to image the full membrane cross section.

2.2.5. Optical system and imaging

For all measurements, a Nikon Ti Eclipse C1si confocal laser scanning microscope system (Nikon Instruments Inc, Lewisville, TX) was used in fluorescent mode with a Nikon CFI Plan Apochromat 60× TIRF oil immersion objective with Nikon's highest numerical aperture of 1.49 [14]. This CLSM system was used to visualize fluorescently stained membranes and to store images as 12-bit scans with a resolution of 512 × 512 pixels, which represented an area of 212 μm × 212 μm. No digital zoom was used while taking pictures. The excitation light sources were a helium-neon laser (561 nm wavelength for Alexa Fluor® 594) and an argon laser (488 nm wavelength for 5-DTAF). Once the surface of the membrane was identified (depth z = 0), lateral x–y scans were performed. More than 40 images of the x–y planes and sets of 10 cross-sectional images from the surface of each cross-section were collected for each membrane at depth
increments of 0.4 μm. Each image is the result of averaging the signal/information collected from four scans to reduce signal noise.

2.2.6. Image analysis

Image analysis was performed using ImageJ version 1.42q (National Institutes of Health, USA) [13]. The images were converted to 8-bit (gray scale images) with pixel intensity values from 0 (black) to 255 (white). Next, they were converted to black and white images. ImageJ converts 8-bit images to binary (black and white) images following an iterative algorithm to calculate a segmentation threshold level for the intensity of the gray pixels that form the image. All the pixels with intensity values higher than the threshold were converted to white, corresponding to the membrane material. All the pixels with intensity values lower than the threshold were converted to black, corresponding to the pore volume. Porosity was estimated as the ratio of void area (black pixels) to total area. Section 2.3.4 gives further details about this process.

2.3. Results and discussion

Characterizing membrane asymmetry is important for developing structure-performance relationships of composite and multilayer filtration membranes. The objective of this study was to develop a CLSM method to quantify the porosity in the thickness direction of microporous membranes. Ultimately, we were interested in employing this method to characterize asymmetric membranes; however, work also was done with symmetric membranes for two reasons: (1) By knowing the nominal average pore diameter of each membrane, this characteristic dimension can be employed as an
imaging standard to determine the experimental limits of image resolution. (2) By using symmetric membranes, the imaging standard will apply at different focal depths and can be used to determine the depth of penetration of the optical system for each type of membrane.

2.3.1. Limits of lateral resolution

The limit of lateral resolution of CLSM can be estimated using the point objects method [15]. The in-focus image of a luminous object point is a diffraction image with a central bright spot and weaker concentric dark and bright rings. The radius of the first dark ring depends on the wavelength of the excitation source (λ) and the numerical aperture (NA\textsubscript{obj}) of the objective lens:

\[
  r = 0.61 \frac{\lambda}{\text{NA}_{\text{obj}}}
\]  

(eq. 2.1)

Images of two equally bright spots are resolved if the distance between them is equal to or larger than the value of r [15]. Analysis of membranes was carried out using the Nikon Ti Eclipse C1si CLSM system with a 60\times TIRF oil immersion objective with NA\textsubscript{obj} = 1.49. Alexa Fluor 594® and 5-DTAF were used as fluorescent labels for the structure of MCE and PES membranes, respectively. According to the Rayleigh criterion (eq. 2.1), the helium-neon laser source (561 nm) used to excite Alexa Fluor 594® gives a theoretical lateral resolution limit of 0.23 μm, and the argon laser source (488 nm) used to excite 5-DTAF gives a theoretical resolution limit of 0.20 μm for our system. Membrane structural features larger than these values and/or separated by distances larger than these
resolution limits should be resolved [16]. These theoretical limits of resolution highlight one limitation of CLSM, which is that it cannot be used for porosity characterization of ultrafiltration membranes that normally have pore sizes in the range of 0.001–0.1 μm [17].

Fig. 2.2 shows CLSM images of six symmetric MCE membranes with nominal average pore diameters from 8 to 0.22 μm. Images represent single x–y planes at a common depth of z = 5.6 μm. Pores are distinguishable for all membranes; although, it is clear from inspection of the 0.22 μm membrane that we are near the limit of lateral resolution, consistent with the theoretical limit.

Fig. 2.2. CLSM images of mixed cellulose ester membranes with different nominal pore diameters. Membranes were stained by adsorption of Alexa Fluor® 594 goat anti-rabbit IgG. Images are shown at a common depth of 5.6 μm. The common scale indicator is 10 μm in diameter. Top row, left to right, are 8, 1.2, 0.8 μm membranes. Bottom row, left to right, are 0.6, 0.45, 0.22 μm membranes.
2.3.2. Penetration/sampling depth

Fig. 2.3 and Fig. 2.4 present the x–y lateral scans at different depths for MCE (8 μm average pore size) and PES (0.65 μm average pore size) membranes, respectively. Since the dye is present only on the membrane structure, the fluorescent signal (green for 5-DTAF or red for Alexa Fluor 594®) corresponds only to the membrane structure and the dark areas correspond only to the pores. In Fig. 2.3 and Fig. 2.4, signal loss is observed as the depth increases. Morphological features are observable to a depth of approximately 12–14 μm for MCE membranes and 7–8 μm for PES membranes.

Fig. 2.3. CLSM images of 8 μm nominal pore diameter mixed cellulose ester membranes at different depth (z) values. Lateral x–y scans were performed at z-increments of 0.4 μm. Every third image is shown. The common scale indicator is 10 μm in diameter.
Fig. 2.4. CLSM images of 0.65 μm nominal pore diameter polyethersulfone membranes at different depth (z) values. Lateral x–y scans were performed at z-increments of 0.4 μm. Every third image is shown. The common scale indicator is 10 μm in diameter.

From Fig. 2.3 and Fig. 2.4, the decrease of intensity per depth increment is larger for PES membranes stained by 5-DTAF compared to MCE membranes stained by Alexa Fluor 594®. The likely cause of this behavior is greater loss of lower wavelength excitation and emission light caused by scattering of the light. According to the Rayleigh scattering criterion, light scattering is inversely proportional to the fourth power of wavelength [18]. Light scattering occurs in the excitation and emission light paths.
Scattering decreases the amount of excitation light that reaches the focal plane. Since scattered emission light does not come from the focal plane, it does not pass through the pinhole aperture and is lost, decreasing the amount of emitted light (signal) that reaches the detector [18]. Close to the surface of the sample, where the effect of scattering is low, shorter wavelength light enables higher resolution imaging according to eq. 2.1. However, at increased depth, the shorter wavelength light yields lower signal recovery efficiency, and, therefore, smaller features, close to the experimental limit of resolution, are more difficult to resolve.

Another factor contributing to the loss of intensity for membranes stained by 5-DTAF is that this dye has lower resistance to photobleaching (i.e., fading of the fluorophore because of light exposure). Photobleaching affects the intensity of the emitted light that arrives at the detector. Alexa Fluor 594® is a sulfonated rhodamine derivative and a registered trademark of Molecular Probes (Carlsbad, CA). According to the manufacturer, Alexa Fluor 594® presents higher quantum yields and enhanced photostability compared to similar dyes, since the sulfonation of the rhodamine inhibits the fluorescence quenching [19] and [20].

Despite 5-DTAF having lower excitation/emission wavelengths than the Alexa Fluor® 594, which generates a lower theoretical limit of resolution according to the Rayleigh criterion eq. 2.1, this dye did not enable higher resolution because of more significant photobleaching and scattering of light. Fig. 2.5 compares CLSM images at common depth for the 0.22 μm nominal pore diameter MCE membrane stained with Alexa Fluor 594® and 5-DTAF. Although the pore size is close to the theoretical limit of
resolution, it was possible to observe membrane pore features on the membrane stained with Alexa Fluor 594®. No distinct pore features were discernable on the membrane stained by 5-DTAF.

Fig. 2.5. CLSM images of 0.22 μm nominal pore diameter mixed cellulose ester membrane at same depth (z = 4 μm). (A) x–y scan of membrane stained with Alexa Fluor 594®. (B) x–y scan of membrane stained with 5-DTAF. The common scale indicator is 10 μm in diameter.

Penetration depth (PD) depends on factors that include the characteristics of the optical system used; how closely the refractive index of the mounting medium, sample and immersion oil match with the lens refractive index specifications (to reduce spherical lens aberrations) [21]; the excitation and emission light wavelengths; and the sample material properties (described by its scattering and absorption coefficients, as shown in eq. 2.2) [18, 22].

\[
I(z) = I_0 e^{-\left[\left((1-g)\mu_s + \mu_a\right)(1-\varepsilon) + \left((1-g)\mu_s + \mu_a\right)(\varepsilon)\right]z} \quad \text{(eq. 2.2)}
\]
Eq. 2.2 shows the dependence of light intensity on depth (z) and the sample material properties. \( I_0 \) is the intensity of the excitation light at the surface (\( z = 0 \)); \( g \) is a factor that quantifies the directional change of the photon after scattering and, specifically, measures the cosine of the average angular change of the photon direction after scattering; \( \mu_s \) is the scattering coefficient and measures the propensity of membrane (component 1) or mounting medium (component 2) to deflect photons from their path; \( \mu_a \) is the absorption coefficient and measures the propensity of the membrane material or mounting medium to absorb photons; and \( \varepsilon \) is the void fraction, representing the porosity of the membrane. Thus, intensity of the excitation light (and consequently the emission light) decreases with scanned depth, and eventually reaches a limit of detection at the PD.

Fig. 2.6 presents representative data from image analysis of x–y scans of 5 μm MCE membranes. Values of \( I(z) \) represent average pixel intensities determined using ImageJ software. These data show that intensity decreases with increasing depth according to eq. 2.2. Using the slope value determined from linear regression in eq. 2.2), a PD of 24 micron was determined for this system at \( I/I_0 = 0.16 \), which corresponds to the background intensity within the pores. This value is well below the membrane thickness.

As PD is dependent upon many characteristics of the specimen itself, even when using the same optical system, PD will not necessarily be the same for all materials. Nevertheless, it is useful to compare findings to previous work. Ulbricht and coworkers [11] reported for regenerated cellulose based ion-exchange membranes (nominal pore size = 3–5 μm; thickness = 200–250 μm) that the differences in refractive index between cellulose (1.45) and aqueous buffer (1.33) caused a steep decay of excitation intensity
over thickness, thereby limiting PD to 20 μm. According to Millipore, the refractive index of MCE is 1.5 and the literature reports a refractive index of 1.64 for PES [23]. The VECTASHIELD® mounting medium that we used has a refractive index of 1.44. By using a mounting medium with a similar refractive index to the membrane materials, we reduced the spherical aberrations of the lens and decreased the deviation of the experimental resolution from the theoretical resolution, as demonstrated in Fig. 2.5 by our ability to resolve pore structure in 0.22 μm membranes.

![Graph showing image mean pixel intensity at different depths for symmetric 5 μm nominal pore diameter MCE membrane from x–y lateral scan images.](Image)

**Fig. 2.6.** Image mean pixel intensity at different depths for symmetric 5 μm nominal pore diameter MCE membrane from x–y lateral scan images. Data are plotted to evaluate goodness of fit to eq. 2.2. Error bars represent the standard deviation of the measurements.

In the case of MCE membranes, we expected to increase the PD beyond 20 μm by achieving a smaller difference between the refractive index of the mounting medium (VECTASHIELD®) and the sample material and by using a mounting medium with a refractive index close to 1.5, which is the refractive index required for the lens to work at
its designed numerical aperture. Our results showed that the PD is about 24 μm for MCE membranes and 7–8 for PES membranes using the Nikon CLSM system. The only marginally higher PD obtained for MCE compared to the PD reported by Ulbricht and coworkers for cellulose membranes could be attributed to differences between the scattering and absorption coefficients of cellulose and mixed cellulose esters and differences in membrane porosity, which affect the light intensity profile throughout the membranes according to eq. 2.2).

Based on the measured depth of penetration limits for MCE and PES membranes, following this standard procedure to study their internal morphology would allow only about 20% of the sample to be observed, as exemplified in Fig. 2.1. Since a goal of this work is to quantify the asymmetry of the morphology (in the thickness direction) of membranes with thicknesses >2PD, we developed a new method for imaging the entire membrane cross-section. While cross-sectional imaging is possible by SEM, the advantages of CLSM for cross-sectional imaging are two-fold: (1) Concerns about surface artifacts due to sample preparation are avoided, since CLSM can be used to image just below the exposed surface. (2) CLSM does not require metallization of the sample; although, it does require sample staining with a dye.

2.3.3. Cross-sectional imaging

To study the membrane morphology throughout the full thickness, we developed a protocol that uses cryo-sectioning to prepare samples for cross-sectional CLSM imaging. Studies that combine cryo-sectioning and CLSM imaging have been reported
recently to study fouling layers at membrane surfaces [24] and [25]. Our protocol is similar to the one reported by Gao et al. [25] in their study of membrane fouling; however, instead of sectioning parallel to the x–y plane at different depths, we cross section the membrane perpendicular to the x–y plane. Membrane sectioning and sample mounting were described in Section 2.2.4. Samples were cryo-sectioned as is common practice when preparing samples for visualization (e.g., SEM). While there may be concern about changes in membrane morphology due to cryo-sectioning, any changes would be the same as occur when preparing samples for analyses by SEM. Furthermore, comparison of top surface and cross-sectional x–y images at a common depth show that cryo-sectioning does not alter the morphology or estimated porosity (vide infra) of MCE membranes. Fig. 2.1 illustrates the method that we developed in this study. Using this method, the cross-section becomes the x–y plane for the confocal scanning. Surface artifacts due to sectioning are avoided by imaging just below the surface, as illustrated in Fig. 2.7, which compares the surface image of a cross-sectioned membrane (Fig. 2.7A) to an image collected 4 μm below the surface of the cross-section (Fig. 2.7B).

Fig. 2.8 and Fig. 2.9 present cross-sectional CLSM images for MCE and PES membranes. With this method, we were successful at imaging the MCE and PES membranes throughout their entire thicknesses (z-dimension). As expected and quantified (vide infra), the porosity of the MCE membranes was constant throughout their thickness, and the porosity of PES membranes showed asymmetry, having one dense surface layer (top side in Fig. 2.9) and one open pore surface layer (bottom side in Fig. 2.9).
Fig. 2.7. Cross-sectional CLSM images of 3 μm nominal pore diameter MCE membrane stained with Alexa Fluor® 594. (A) Surface image of cross section (z = 0) showing artifacts from sectioning. (B) Image of cross-section at 4 μm depth (z = 4 μm). Common scale bar is 10 μm.

Fig. 2.8. Cross-sectional CLSM image of symmetric 5 μm MCE membrane at a depth of 4 μm (left). Membrane was stained with Alexa Fluor® 594. Image scale is 210 μm × 210 μm. A cross-sectional SEM image of this membrane is provided for comparison (right). Scale bar for CLSM image is 10 μm.
Fig. 2.9. Cross-sectional CLSM image of asymmetric 0.65 μm PES membrane at a depth of 4 μm (left). Membrane was stained with 5-DTAF. The dense surface is at the top. Image scale is 210 μm × 210 μm. A cross-sectional SEM image of this membrane is provided for comparison (right). Scale bar for CLSM image is 10 μm.

Fig. 2.8 and Fig. 2.9 provide SEM images of MCE and PES membranes for comparison to the CLSM images. There is good agreement between the morphological features shown by SEM and CLSM images. For the PES asymmetric membrane, a region of high dye concentration in the CLSM image coincides with the dense surface layer (top side in Fig. 2.9).

2.3.4. Porosity versus depth

Porosity of MCE and PES membranes was quantified as a function of depth. ImageJ software was used to convert CLSM images to 8-bit (gray scale) images, where the software assigns to each pixel an intensity value between 0 (black) and 255 (white). Next, the images were converted to black and white by setting a segmentation threshold level for the pixels on each image based on their intensity. The program uses an iterative procedure to calculate the threshold level. It sets an initial threshold and separates pixels into two groups: those in the background (pixels with intensity below the threshold) and
those in objects (pixels with intensity above the threshold). It computes the average intensity of pixels in the background and pixels in objects, and computes a new threshold using eq. 2.3.

\[
\text{threshold} = \frac{\text{average background} + \text{average objects}}{2} \quad \text{(eq. 2.3)}
\]

This process is repeated, based upon the new threshold calculated by eq. 2.3, until the threshold value becomes constant [26]. The program displays all the pixels below the threshold level in black (pores) and all the pixels above the threshold level in white (membrane structure).

Fig. 2.10 shows representative CLSM images for MCE and PES membranes and the corresponding black and white images. Porosity was estimated as the ratio of void area (black regions) to total area in the binary image. For both MCE and PES membranes, porosity at different depths was determined from cross-sectional images with z increments of 20 μm by calculating the average porosity of each 20 μm × 210 μm section from top to bottom of one cross-sectional image, as depicted in Fig. 2.11 for an MCE membrane. Reported porosities are an average of the values from the respective depths of three separate cross-sectional samples.
Fig. 2.10. Processed CLSM images at common scale. CLSM images were converted to binary (black and white) images using ImageJ software. (Top) Initial and binary images of 5 μm nominal average pore diameter MCE membrane. (Bottom) Initial and binary images of 0.65 μm asymmetric PES membrane. Common scale bar is 10 μm.

Fig. 2.12 gives porosity estimates for MCE symmetric membranes. Comparison is made between estimates from x–y images at various depths and cross-sectional images at a constant depth of 4 μm. From Fig. 2.12A we see the disadvantage of using x–y images at various depths for quantification of porosity (i.e., the porosity appears to increase as a function of depth as a result of the inability to see structure beyond PD). Even if the threshold value is recalculated at each new depth, there eventually reaches a point where
no emission light is observed, and the apparent porosity approaches 100% as a result of loss of light intensity with depth. Using cross-sectional images for analysis, we see in Fig. 2.12B that, as expected, porosity is essentially constant throughout the membrane thickness for these symmetric membranes. Cross-sectional imaging enables this analysis of porosity versus depth throughout the entire membrane thickness. To further support the reliability of the cross-sectional porosity estimates for MCE symmetric membranes, CLSM images were collected for MCE membranes stained with a second dye, 5-DTAF. Fig. 2.13 shows that the porosity remains constant and the porosity values are similar to those presented in Fig. 2.12B. This result also should dispel any concerns that the large IgG protein-Alexa Fluor 594® dye conjugate may lead to pore filling and underestimation of porosities.

![CLSM image sectioning procedure](image)

**Fig. 2.11.** Illustration of CLSM image sectioning procedure used to calculate the porosity as a function of depth. Each section is approximately 20 μm × 210 μm. Scale bar is 10 μm.
Fig. 2. Porosity estimates for symmetric 3, 5 and 8 μm nominal average pore diameter MCE membranes stained with Alexa Fluor 594®. Image analysis was used to measure the fraction of void area as a function of depth. Comparison is made between estimates from (A) x–y images at various depths from the membrane surface and (B) cross-sectional images at a constant imaging depth of 4 μm from the surface of the cross-sectioned sample. The apparent increase in estimated porosity seen in (A) results from decreasing fluorescence signal. Error bars represent the standard deviation of the measurements.

Fig. 2.12 also shows that the cryo-sectioning procedure used to prepare samples for cross-sectional imaging does not impact the estimates for membrane porosity. The porosity values measured from near-surface images of the membranes in Fig. 2.12A are in good agreement to those measured from cross-sectional images in Fig. 2.12B.

Finally, Fig. 2.14 shows porosity estimates for the PES asymmetric membrane measured using cross-sectional images. Porosity increases from the surface (z = 0) to about 80 μm. Thereafter, the porosity remains constant. Again, cross-sectional imaging enables this analysis, which provides reliable information on the membrane porosity throughout the entire thickness of the membrane.
Fig. 2.13. Porosity estimates for symmetric 3, 5 and 8 μm nominal average pore diameter MCE membranes stained with 5-DTAF. Image analysis was used to measure the fraction of void area as a function of depth for cross-sectional images at a constant imaging depth of 4 μm from the surface of the cross-sectioned sample. Error bars represent the standard deviation of the measurements.

Fig. 2.14. Porosity estimates for asymmetric 0.65 μm effective pore size PES membrane as a function of depth for cross-sectional image at a constant imaging depth of 4 μm from the surface of the cross-sectioned sample. Error bars represent the standard deviation of the measurements.
2.4. Conclusions

Cross-sectional confocal laser scanning microscopy has been developed to provide qualitative and quantitative information about the morphology of symmetric and asymmetric microporous membranes. Our innovative technique combines minimally invasive imaging with software analysis of the images to obtain quantifiable data. CLSM is a frequently utilized technique in biological sciences, but there are relatively fewer studies applying this technique to materials or membranes, mainly due to resolution and depth of penetration limitations and difficulty of data analysis. This study overcomes limited sampling depths of CLSM by imaging the membrane cross section, providing information about porosity throughout the full thickness of the membranes in a way that, to our knowledge, has not been done previously. Our method to extract morphology information from CLSM images is advantageous over electron microscopy techniques as it avoids concerns about introduction of surface defects during sample preparation by imaging below the surface. The new characterization strategy developed in this work will enable future studies of membrane materials by confocal microscopy.

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2.5. References


CHAPTER 3
LOCATION AND QUANTIFICATION OF BIOLOGICAL FOULANTS IN A WET MEMBRANE STRUCTURE BY CROSS-SECTIONAL CONFOCAL LASER SCANNING MICROSCOPY

3.1. Introduction

Microfiltration (MF) is used in the food and beverage industry on a large scale for clarification, sterilization (bacteria/microorganism removal), stabilization and pretreatment prior to unit operations such as ultrafiltration, reverse osmosis or crystallization (to ensure high quality of crystals) [1]. Processing of milk, beer, soft drinks, whisky, fruit juices, edible oils and vinegar are a few examples where MF is applied. In beer production, MF is used for sterilization and for removal of any remaining yeast cells, chill and permanent haze flocs (protein-polyphenol aggregates), and other components that prevent its final crisp clarity. In the dairy product industry, MF is used to separate fats, remove bacteria and maintain protein levels in the milk year round for automated cheese making [2]. However, filtration of these streams suffers from permeate flux decline caused by membrane fouling.

Fouling negatively affects the performance of the membrane and increases the operating cost by requiring frequent membrane cleaning/replacement and consequently higher energy consumption. Further, membrane fouling can compromise the properties of the final product. For example, in the dairy product industry, fouling can influence the rejection of caseins and whey proteins, altering the quality of the final product. Fouling
can be caused by particulate matter with a size equal to or larger than the nominal pore size of the MF membrane (e.g., cellular components, microorganisms, fat globules, etc.) that completely or partially block the pores. In addition, components like proteins, polysaccharides and polyphenols that are present in the feed solutions can foul microfiltration membranes despite having a much smaller size relative to the nominal pore diameter of the membranes [3]. These components tend to adsorb to the pore walls constricting it over time. During MF, the extent of fouling depends on a number of factors that include operating conditions, feed and membrane properties [4]. In addition to these factors, solute-solute interactions among components commonly found in the feed (i.e. proteins, polysaccharides and polyphenols) have been shown to affect filtration performance significantly. Polyphenols are thought to behave like physical crosslinkers among protein molecules, forming insoluble aggregates due to hydrophobic and/or hydrogen bonding interactions [5]. These interactions are altered by the presence of polysaccharides, which can disrupt the binding of polyphenols to proteins by molecular association between the polysaccharides and polyphenols or by forming complexes among protein, polyphenol and polysaccharide molecules [6, 7, 8]. Also, protein and carbohydrates may interact to form plugging agents [9]. Membrane-solute interactions may also affect the fouling of the membrane. For example, Ulbricht and co-workers [10] reported that dextran and myoglobin significantly fouled porous membranes and non-porous films of polyethersulfone (PES) simply by contacting the PES with the polysaccharide or protein solutions under static conditions. They found that the degree of
fouling was less on cellulose membranes by the same components using the same conditions.

Understanding how fouling occurs is the first step toward developing fouling mitigation strategies for microfiltration of biological streams. Previous researchers have characterized membrane fouling by proteins, polysaccharides and/or polyphenols [5, 10, 11]. In these studies, relative flux reductions and flux profiles of the fouled membranes were reported to characterize how the fouling occurs and quantify its impact on performance. Infrared (IR) spectroscopy has been used for indirect evaluation of the ‘degree’ of fouling by quantifying the increase in IR band area of distinct peaks corresponding to the foulants deposited on the membrane surface [10] or the decrease (or disappearance) in IR band area of the peaks that correspond to the clean base membrane [12]. Changes in contact angle and zeta potential have been quantified to characterize how the foulants affect the surface properties of the polymeric membrane material. Imaging techniques like environmental scanning electron microscopy (ESEM) and scanning electron microscopy (SEM) have been used to visualize the a fouled membrane surface [11]. Although ESEM and SEM allow the observation of surface fouling of membranes by proteins, polysaccharides and/or polyphenols, it is not possible to conclude from the images how (i.e., by what mechanism) the fouling occurs when there is more than one component involved, since it is not possible to distinguish individual components/foulants. An additional disadvantage is that these techniques only provide only superficial information of the sample. Although, it is possible to use ESEM or SEM to visualize the internal structure of the membrane post filtration, doing so requires
sectioning of the sample. This step introduces surface artifacts that compromise the reliability of the information obtained from the imaging. Another requirement to study samples with conventional SEM is that the sample must be dehydrated, preventing the study of samples in the wet state, a disadvantage for samples prone to alteration of morphology due to drying. Even though ESEM allows imaging of a sample in a wet state and under a moderate vacuum, obtaining clear images is difficult due to the low electron density of the components in the fouling layer (i.e. proteins, polysaccharides and polyphenols). Finally, while collecting images, beam damage due to local heating and structural collapse due to vacuum can occur.

There is much interest to explore alternative characterization methods that may overcome the limitations that have been mentioned above. Particularly, confocal laser scanning microscopy (CLSM) is a technology that has recently become an important tool for studying membranes. Briefly, CLSM works by focusing light into a small spot on a single plane at a selected depth within the membrane structure. Images are recorded at different depths by changing the position of the focal plane [13]. Thus, CLSM performs an ‘optical sectioning’ to collect images from the membrane interior. Stacking the images from adjacent planes can create three-dimensional volume elements. Recent studies by Zator and coworkers reported the use of CLSM in the fluorescent mode for studying fouling of microfiltration of mixed protein-polysaccharide [14] and protein-polysaccharide-polyphenol solutions [5]. These studies were limited to polycarbonate and polyester membranes using BSA as model protein. Confocal images were collected after processing a fixed volume of solution, and depth of imaging was 3 microns. By using
CLSM, they were able to locate the foulants individually within the cake and within the first 3 microns of the membrane by using foulants labeled with fluorescent dyes. Zator et al. [5] worked with foulants labeled with different dyes, and they collected images showing the location of each foulant, represented by a different color, at different depths within the membrane. In their conclusions, they suggest that, even though they did not find significant pore blockage by protein, dextran and polyphenols within a depth of 3 microns from the surface of the membranes after enzymatic cleaning, aggregates of these components may have been blocking the pores at depths not reachable by their confocal analysis. They hypothesize that such aggregates were the reason why the enzymatic cleaning process did not restore the water fluxes of the membranes after cleaning. This hypothesis could not be tested due to the CLSM depth of penetration limit, beyond which images become degraded as the emitted light (photons) originating at the focal plane is lost due to scattering or absorption by the membrane material. In a previous publication [13], we explained the causes and consequences of the depth of penetration limit for membrane imaging, and we developed a cross-sectional CLSM imaging method that overcomes this limitation and provides images throughout the entire thickness of the membrane. This method was used in this study to locate biological foulants in a wet membrane structure post-filtration.

The objectives of this research were to determine where proteins and polysaccharides deposit inside a polymeric microfiltration membrane when a fluid containing these materials is being filtered and to better understand the role of each component on membrane fouling. Using mixed-component feeds, we sought to
determine whether proteins and polysaccharides deposit inside the membrane in the same manner or location within the wet membrane structure when they are present together in a mixture as they do when present individually, and to investigate how they affect one another in fouling a membrane. Our ultimate goals were (1) to use the intensity information provided by the cross-sectional CLSM images of the fouled membranes to quantify the amount of foulant at different depths within the membrane as a function of volume processed; (2) to compare flux decline measurements of single-component and mixed solutions against CLSM images of membranes at different degrees of fouling to gain insights on the reasons for observed loss of performance; and (3) to search for evidence of fluorescence resonance energy transfer (FRET) between fluorescently labeled protein and polysaccharide in membranes that processed mixed solutions of these components, as a marker for protein-polysaccharide interactions. In this contribution, we used casein and dextran as the model protein and polysaccharide, respectively. We used our newly developed CLSM protocol to conduct cross-sectional imaging of membranes following filtration, overcoming limitations of depth of penetration observed in previous CLSM work [15-18]. The extension of our cross-sectional CLSM imaging method offered in this work should be useful to researchers who wish to use CLSM to study internal fouling within wet membrane structures. Also, the knowledge acquired in this study will contribute to better understanding of the mechanisms that lead to fouling, as is needed to develop more effective fouling mitigation strategies for microfiltration of biological streams.
3.2. Experimental materials and methods

3.2.1. Materials

Asymmetric polyethersulfone (PES) membranes (Pall Corporation) were used for filtration experiments. The Supor® PES membranes that were used have effective pore diameter of 0.65 µm and thickness of 114–175 µm.

Fluorescently labeled probes used in these experiments were fluorescein isothiocyanate (FITC) labeled casein from bovine milk (Sigma-Aldrich, C-0403) and Alexa Fluor® 594 labeled dextran, 10 kDa molecular weight, anionic-fixable (Life Technologies, D-22913). Non-labeled compounds used in the filtration experiments were casein from bovine milk (Sigma Aldrich, C6554) and dextran from Leuconostoc mesenteroides, 9 -11 kDa molecular weight (Sigma Aldrich, D9260).

For filtration experiments, sodium phosphate buffered solutions were prepared using sodium phosphate monobasic (anhydrous, ≥99%, Sigma Aldrich, S0751), sodium phosphate dibasic (anhydrous, ≥99%, Sigma Aldrich, S9763), and deionized (DI) Milli-Q system (EMD Millipore) water.

Dow Filmtec™ NF90 membranes were used to prepare the calibration plots of intensity versus areal protein or polysaccharide mass. The NF90 membranes were pre-treated to enhance the permeability of the membranes while maintaining their rejection properties [19]. Pre-treatment was done by soaking the membranes for 2 days in a 1:10:9 (by volume) mixture of absolute ethanol (≥99.5%, Sigma Aldrich, 459836), sulfuric acid (ACS reagent, 95-98%, Sigma Aldrich, 258105), and DI water.
For membrane cryosectioning, the embedding medium was Tissue-Tek® O.C.T. Compound 4583 (VWR, 25608-930). Tissue-Tek® 15×15×5 mm Intermediate Cryomolds 4566 were used (Fisher Scientific, NC9542860). The high-profile cutting blades were 76.2×1.4×0.03 mm (Fisher Scientific, 12-634-4). Superfrost® Plus Micro Slides (VWR, 48311-703) were used to collect and mount cryosections. Samples for XY lateral CLSM scans were mounted on microscope slides (Fisherbrand, 12-550-A3). All samples were mounted using VECTASHIELD® aqueous mounting medium (glycerol-based aqueous sample mounting medium and anti-fading agent for the fluorescent dyes) and covered with micro cover glasses (VWR, 48393 092) before imaging. The immersion oil Type A (Nikon) specified for the objective lens was used with the optical system.

3.2.2. Filtration experiments

A direct-flow filtration cell, Amicon 8050 from EMD Millipore, was used at a constant pressure of 14.5 kPa. The Amicon 8050 cell has an effective filtration area of 13.4 cm².

Sodium phosphate buffer solutions were prepared with an ionic strength of 0.125 M and pH of 6.8. A stock solution of casein (25 g/L) was prepared by mixing the casein powder from bovine milk in a 0.04 M sodium hydroxide solution. Stirring at 250 rpm for 4 hours was applied to facilitate the casein dissolution. A stock solution of dextran (9-11 kDa, 25 g/L) was prepared by mixing the dextran powder in DI water.

Single component protein and polysaccharide solutions were prepared with a final concentration of 25 mg/L or 12.5 mg/L in phosphate buffer solution. Binary component
solutions were prepared with a final mixture concentration of 25 mg/L comprising 50:50 (w/w) protein-polysaccharide in phosphate buffer solution. These solutions were prepared by adding the appropriate volume of stock solution(s) to a volumetric flask and adding phosphate buffer to achieve the desired volume. To allow confocal visualization of the protein (casein) and polysaccharide (dextran), fluorescently labeled casein and dextran were added to the solutions in a ratio of 1:20 fluorescently labeled to non-labeled component.

PES membranes were pre-wetted before each filtration experiment by soaking them in a 25% (by volume) aqueous ethanol solution for 10 minutes. Then the membranes were soaked in DI water for 10 minutes. Membranes were kept in DI water until use. Each filtration experiment was done at constant pressure until 1 L of permeate was collected. The membrane was placed with the more open surface facing the feed, and filtration was done in direct flow mode with constant stirring speed of 250 rpm. Flux versus permeate volume data were recorded during each experiment.

After filtration, 5 mL of a solution of non-labeled component(s) at the concentration(s) used in the filtration run was filtered with the purpose of emptying out the pores of unbound fluorescently labeled probes. This step was taken as a precaution to ensure that the confocal microscope visualized only fluorescently labeled probes that were physisorbed to the membrane. Membranes were used just once and sacrificed to collect samples for confocal visualization.
3.2.3. Calibration curve preparation

A pressure of 380 kPa was used to filter solutions containing different masses of fluorescently labeled probes (1:20 ratio of fluorescently labeled to non-labeled protein or polysaccharide) through pre-treated Dow Filmtec™ NF90 membranes. Post filtration, the samples were collected for confocal microscopy visualization. Confocal lateral XY scans were performed on the surface of NF90 membranes to determine the average intensity and relate it to the mass per area of fluorescently labeled probe retained on the surface. This information was used to generate calibration plots as average intensity versus mass per area of fluorescently labeled probe retained on the surface. We followed a procedure described by Marroquin et al. [13] to mount the NF90 calibration curve membranes for lateral XY CLSM imaging.

3.2.4. Sectioning and mounting of samples for cross-sectional CLSM imaging

Cross-sections of the membranes used in the filtration experiments were obtained and prepared for confocal imaging following the procedure described by Marroquin et al. [13]. Fig. 3.1 is a schematic for the sectioning process to access the sample cross-sections.

3.2.5. Optical system and imaging

A Nikon Eclipse Ti confocal laser scanning microscope system (Nikon Instruments Inc, Lewisville, TX) was used in fluorescence mode with a Nikon 60X oil immersion objective with a numerical aperture of 1.49. This CLSM system was used to visualize fluorescently labeled probes bound throughout the entire thickness of the PES
membranes or on the surface of the NF90 membranes. Images were stored as 12-bit scans with a resolution of $512 \times 512$ pixels, which represented an area of $212 \times 212$ micron. The excitation light source was a helium-neon laser (594 nm excitation wavelength for Alexa Fluor® 594 and 488 nm wavelength for FITC). Each image is the result of averaging the signal/information collected from four scans, which reduces noise, producing better resolved images.

**Fig. 3.1.** Schematic for sample sectioning and cross-sectional CLSM images.
3.2.6. **Image analysis**

Images collected by the confocal microscope were processed using NIS-Elements 3.2 Software Package (Nikon Instruments Inc, Lewisville, TX).

3.2.7. **Dynamic Light Scattering (DLS) measurements**

DLS measurements were conducted using a Malvern Zetasizer Nano ZS instrument (Malvern Instruments Ltd., Malvern, UK) at a wavelength of 633 nm from a 4.0 mW, solid-state He-Ne laser at a scattering angle of 173°. Number average diameters were calculated from the autocorrelation function using Malvern Zetasizer Nano 7.01 software utilizing a version of the CONTIN algorithm.

3.3. **Results and discussion**

3.3.1. **Flux measurements**

Flux experiments were done with casein, dextran, and casein-dextran mixtures and the asymmetric PES membrane. Fig. 3.2 shows direct-flow flux data for solutions of the individual components, as well as data for the casein-dextran mixture.
It was observed that the mixed feed (casein and dextran) showed a less severe flux decline than dextran alone. One difference between these two solutions is that the concentration of dextran in the mixed solution was lower than the concentration in the single component solution (12.5 mg/L versus 25 mg/L). To determine if the difference in fouling behavior was due to a difference in dextran concentration, we prepared a dextran solution at 12.5 mg/L and measured the flux versus permeate volume for this single component solution. As shown in Fig. 3.2, even at this lower concentration, the dextran solution yielded a more severe flux decline than the mixture. To understand what might
be the cause of this difference, we carried out DLS measurements of the solutions. Fig. 3.3 shows the DLS data for casein and casein-dextran feed solutions.

![DLS data](image)

**Fig. 3.3.** DLS data for 12.5 mg/L casein single component solution (TOP) and a 50:50 (w/w) mixture of casein and dextran at 25 mg/L (BOTTOM).

The apparent size of 215 nm and broad peak size for the casein feed are consistent with the findings of Gebhardt et al. [20] and indicate the presence of casein micelles. Fig. 3.3 also shows the size distribution of the micelles after addition of dextran to the solution. The apparent size of the micelles increases to 290 nm after addition of dextran. We hypothesized that the increase in size after dextran addition may be due to some form of association between the casein micelles and dextran. Association of the dextran with casein may be the reason that the casein-dextran mixture is less fouling than dextran alone.
3.3.2. Sample preparation for CLSM imaging

Samples were collected at 5, 10, and 15% flux decline and they were prepared for cross-sectional CLSM imaging according to the protocol followed by Marroquin et al. [13]. During sample preparation, it was necessary to flush the pores to remove unbound fluorescent probe molecules, and also it was necessary to remove the embedding medium used for cross-sectioning by immersing the sample in phosphate buffered saline for 20 minutes at 35 °C. Control experiments were done to verify that sample preparation removed unbound probe and did not lead to foulant migration or leaching and a change of the bound probe intensity profile. In the first experiment, a membrane was challenged with 500 mL of 25 mg/L casein-FITC/casein (1:20) in buffer solution and then rinsed twice to remove fluid in the pores. The second rinse solution was analyzed, and there was no detected fluorescence. In a second experiment, membranes were loaded with 12.5 mg/L of dextran (10kDa)-dextran (9-11 kDa)-Alexa Fluor® 594 (1:20) until the flux declined by 15%. Three samples were prepared for cross-sectional CLSM using our standard protocol. Sample 1 was washed once, sample 2 was washed twice, and sample 3 was washed three times with PBS buffer for 20 minutes at 35 °C. Fig. 3.4 gives the CLSM images of the three samples. Intensity measurements showed that, within the standard error, the degree of washing has no measureable effect on CLSM image intensity (Fig. 3.5), consistent with the first control experiment.
**Fig. 3.4.** Cross-sectional CLSM images of dextran-Alexa Fluor® 594 binding within an asymmetric 0.65 μm PES membrane at a depth of 4 μm. Images are for samples taken after 15% flux decline. The dense surface of the membrane is on the left of all images. Images are for samples washed once (LEFT), twice (MIDDLE), and thrice (RIGHT). The scale indicator is 10 μm in diameter.

### 3.3.3. Determining the location of foulants by CLSM: single and binary component solutions

Using cross-sectional CLSM imaging, we were able to image foulant at all depths within the membrane structure, overcoming previous depth of penetration limitations for such studies [13]. Figs. 3.6–3.8 show the cross-sectional CLSM images (at a depth of 4 μm) of the asymmetric 0.65 μm PES membranes that processed single and binary component solutions of casein and dextran. We imaged just below the surface to avoid concerns about surface defects caused by sample preparation.
Fig. 3.5. Intensity profile for CLSM images presented in Fig. 3.4. Profiles are for samples washed once (●), twice (○), and thrice (▼). Error bars represent the standard deviation of the intensity measurements.

Fig. 3.6 shows the fouling profile within membranes after filtering a casein solution. Protein accumulates throughout the membrane structure and is not concentrated at the feed surface (right side of each image). The intensity of the green color (emission by FITC) increases towards the dense surface, demonstrating that the membrane acts as a depth filter. Fouling of the PES membranes by casein is due to hydrophobic interactions between the protein and the membrane material as other authors have stated [10, 21, 22].

Fig. 3.7 shows the fouling profile of dextran at different degrees of fouling. In this case, we observed some accumulation of dextran at the feed surface, consistent with the more severe flux decline in this system (see Fig. 3.2).
Fig. 3.6. Cross-sectional CLSM images of casein and casein-FITC binding within an asymmetric 0.65 μm PES membrane at a depth of 4 μm. Feed was 25 mg/L casein (1:20 fluorescent probe to non-tagged compound). The dense surface is on the LEFT of all images. Images are for samples taken after 5% flux decline (LEFT), 10% flux decline (MIDDLE), and 15% flux decline (RIGHT). The scale indicator is 10 μm in diameter.

Fig. 3.7. Cross-sectional CLSM images of dextran and dextran-Alexa Fluor® 594 binding within an asymmetric 0.65 μm PES membrane at a depth of 4 μm. Feed was 25 mg/L dextran (1:20 fluorescent probe to non-tagged compound). The dense surface is on the LEFT of all images. Images are for samples taken after 5% flux decline (LEFT), 10% flux decline (MIDDLE), and 15% flux decline (RIGHT). The scale indicator is 10 μm in diameter.
PES fouling by dextran at static and dynamic conditions has been reported by Susanto et al. [10, 23]. They propose that attractive forces between dextran and PES are due to van der Waals interactions and hydrogen bonding between hydroxyl groups of dextran (donor) and the oxygen atoms in the SO$_2$ group of PES (acceptor). It is also proposed that the displacement of water molecules off of the hydrophobic surface by the adsorbed polysaccharide results in an increase in entropy, indicating a spontaneous process. According to Mochizuki et al. [24], under some conditions, dextran can deposit on the surface of the membrane and form a ‘gel layer’. In this study, it is believed that interactions between dextran and the PES membrane might be causing pore narrowing on the surface facing the feed. The significant flux decline shown in Fig. 3.2 is counter-intuitive since the hydrodynamic radius of dextran (10 kDa) is approximately 2.2-3.6 nm [25]. Another explanation for this unexpected flux decline behavior may be related to the findings that neutral polysaccharides (like dextran) have a low solubility due to the presence of a large number of hydrogen bonds that stabilize intra- and inter-chain interactions [26, 27]. Thus, the severe flux decline observed for the dextran solution might be due to dextran aggregates that are not well solubilized in solution. Indeed, when high intensity sonication was applied to the dextran stock solution, the solution was less fouling (data presented in Appendix A) compared to the results presented in Fig. 3.2.

Fig. 3.8 presents the CLSM cross-sectional images that show where casein and dextran deposit within the PES membranes post-filtration of the casein-dextran mixture. Shown are the fouling profiles for casein and dextran individually, as well as the overlaid profiles. Interestingly, there appears to be no accumulation of dextran at the feed surface.
when casein also is present in the feed. Additionally, the fouling profiles follow the same trend; the color intensity increases toward the dense layer. The CLSM images agree with the flux decline data, which show that the mixed feed has a less severe flux decline than dextran alone. It is apparent that the presence of casein in solution is changing the fouling behavior of dextran. Interactions between dextran (molecular weight 10 kDa) and a protein (myoglobin) were observed by Susanto et al. [28]. They observed higher degrees of fouling in dextran-myoglobin mixtures compared to the single solute feeds and explained this behavior is a possible interpenetration of both biopolymers to form a network structure stabilized by multiple hydrogen bonds.

According to the work by Dickinson [29], polysaccharides and proteins are capable of interacting favorably through hydrogen bonding or electrostatic interactions. Also, weak complexation between proteins and neutral polysaccharides can occur due to secondary, non-Coulombic interactions at low ionic strength conditions [30]. To gain a better understanding of the effect of pH and ionic strength on the interactions occurring between casein and dextran, additional flux measurements were done for the binary mixture at two ionic strength conditions (0.125 and 0.25) and three pH values (7, 6.25 and 5.5). The flux profiles showed no change over the range of pH and ionic strength that was studied, suggesting that electrostatic interactions between casein and dextran are not predominant at the conditions tested (data presented in Appendix B).
Fig. 3.8. Cross-sectional CLSM images of a 50:50 (w/w) casein-dextran mixture binding within an asymmetric 0.65 μm PES membrane at a depth of 4 μm. Feed was 25 mg/L (1:20 fluorescent probe to non-tagged compound). The dense surface is on the LEFT of all images. TOP row images are for samples taken after 5% flux decline: casein (LEFT), dextran (MIDDLE), superimposed image of casein and dextran (RIGHT). SECOND row images are for samples taken after 10% flux decline. BOTTOM row images are for samples taken after 15% flux decline. The scale indicator is 10 μm in diameter.
Motivated by the observations that dextran (used in some medical applications to lower blood viscosity and to prevent platelet aggregation) is capable of coating platelets, red and white cells, Ponder et al. [31] found, through electrophoretic methods, that a complex between the protein albumin and dextran was formed and that this complex comprises one molecule of albumin for every four dextran molecules. Based on the results of that study, together with the flux and DLS data and CLSM imaging, we hypothesized that the co-localization of casein and dextran shown in Fig. 3.8 is due to association between casein and dextran, and that this association helps to solubilize/disperse dextran and prevent its accumulation at the membrane feed surface. The observed increase in the apparent casein micelle size given by the DLS measurements supports our hypothesis about the association between casein micelles and dextran, likely due to van der Waals and hydrogen bonding interactions.

Fluorescence resonance energy transfer (FRET) and immunoprecipitation assays were done to further support our hypothesis that a complex forms between casein and dextran. Details on these experiments are available in Appendix C. For the FRET experiment, casein-fluorescein isothiocyanate (FITC) was used as a donor and dextran-tetramethyl isocyanate (TRITC) was used as an acceptor. Briefly, the FRET experiment consisted in exciting the donor (FITC) with the 488 nm laser. If the distance between the fluorescent labels is between 2-7 nm (possible in a complex), then the light emitted from FITC is able to excite the acceptor (TRITC). The CLSM microscope was configured to detect only the light emitted by TRITC. When illuminating the sample with the 488 nm laser, if TRITC is visualized then it is indication that FRET is happening between FITC
and TRITC (attributed to the close distance between casein and dextran in a complex). Additionally, in order to assess interaction between casein and dextran, we performed a modified immunoprecipitation assay. The experiment consisted in adsorbing casein on the surface of magnetic beads coated with anti-rabbit antibodies, immersing the beads in a solution containing dextran-Alexa Fluor® 594, measuring the fluorescence intensity of the beads with a plate reader, and comparing results against the control samples to determine if dextran had an affinity for binding to casein. Results for both experiments were inconclusive. FRET was not observed, likely due to low extent of labeling by FITC and TRITC on casein and dextran, respectively. To observe FRET, the distance between the donor (FITC) and the acceptor (TRITC) should be 2-7 nm [32]. Even if there is a complex formed between casein and dextran, FRET will not be observed if the distance between their fluorescent tags is too long. With a low extent of labeling on these macromolecules, the distance between FITC and TRITC may be greater than 7 nm within the complex. The immunoprecipitation assays showed that dextran tends to adsorb to immunoprecipitation assay beads that have casein bound on their surface. However, binding was not specific to casein. Dextran also bound to the base immunoprecipitation assay beads containing sheep anti-rabbit IgG and to the immunoprecipitation assay beads with anti-casein on their surface (controls). Since the manufacturer of the beads does not supply plain beads (with no sheep anti-rabbit IgG covalently bound to the surface), we cannot conclude that the adsorption of dextran to the beads depends on the presence of a protein such as casein.
3.3.4. Image analysis and quantification of foulants

Cross-sectional CLSM images presented in Figs. 3.6–3.8 give qualitative information about the fouling occurring within PES membranes by casein and dextran. The quantitative intensity data for fluorescent probes covalently bound to casein and dextran (FITC and Alexa Fluor® 594, respectively) within the PES membranes at different degrees of fouling was obtained from Figs. 3.6–3.8 by measuring the average color intensity at locations throughout the entire thickness of the cross-sections. Figs. 3.9–3.12 show average color intensity versus depth plots for the cross-sectional CLSM images of the PES that processed solutions of casein/casein-FITC (Fig. 3.9), dextran/dextran-Alexa Fluor® 594 (Fig. 3.10), and casein/casein-FITC/dextran/dextran-Alexa Fluor® 594 (Figs. 3.11 and 3.12, where data reported at the same flux decline correspond to the same run and sample) at three levels of fouling (5, 10, and 15% flux decline). Fig. 3.9 for the fouling profile of casein single-component solution shows that the average intensity increases with depth and also with degree of fouling. Since the FITC is bound covalently to casein, the intensity of the green color is proportional to the mass of casein. The intensity profile shows that the membrane behaves like a depth filter, where the maximum adsorption occurs near the dense surface.
Fig. 3.9. Intensity profiles for the cross-sectional CLSM images of casein-FITC binding (Fig. 3.6) within an asymmetric 0.65 μm PES membrane at a depth of 4 μm, after filtering a single-component solution. Profiles are for samples taken after 5% flux decline (●), 10% flux decline (○), and 15% flux decline (▼). Error bars represent the standard deviation of the intensity measurements.

Fig. 3.10 for the fouling profile of dextran single-component solution shows that, at 5% and 10% flux decline, the intensity is higher near the surface facing the feed and then it plateaus. At 15% flux decline, the intensity profile presents a “U” shape where the intensity near the surface facing the feed and the dense surface (permeate side) have the highest values. It was explained in section 3.3.3 that dextran aggregates may have accumulated on the membrane surface leading to high intensity values close to the surface facing the feed.
Fig. 3.10. Intensity profiles for the cross-sectional CLSM images of dextran-Alexa Fluor® 594 binding (Fig. 3.7) within an asymmetric 0.65 μm PES membrane at a depth of 4 μm, after filtering a single-component solution. Profiles are for samples taken after 5% flux decline (●), 10% flux decline (○), and 15% flux decline (▼). Error bars represent the standard deviation of the intensity measurements.

The intensity profiles for samples that filtered the casein-dextran mixture, Figs. 3.11 and 3.12, show that mass of protein and polysaccharide both increase with depth and with degree of fouling. The CLSM images and intensity profiles show that dextran no longer accumulates near the top surface. It was explained in section 3.3.3 that the presence of casein and its interactions with dextran might be the reasons why dextran no longer accumulates at the feed surface.
Fig. 3.11. Intensity profiles for the cross-sectional CLSM images of casein-FITC binding (Fig. 3.8) within an asymmetric 0.65 μm PES membrane at a depth of 4 μm, after filtering a binary component (casein-dextran) solution. Profiles are for samples taken after 5% flux decline (●), 10% flux decline (○), and 15% flux decline (▼). Error bars represent the standard deviation of the intensity measurements.

The intensity values for casein within membranes that filtered casein-dextran mixtures are roughly 2-3 times higher than the respective intensity profiles for membranes that filtered single-component casein solutions. In contrast, the intensity values of dextran show about a 30% decrease for membranes that processed the binary mixture compared to those that processed the single-component dextran solution. This behavior can be explained by the association between casein and dextran, which increases the casein micelle size (Fig. 3.2) and improves the solubility of dextran. The increase in micelle size leads to a higher sieving coefficient for casein within the
membrane structure. The improved solubility of dextran lessens the degree to which it adsorbs to the membrane surface.

![Intensity profiles for the cross-sectional CLSM images of dextran-Alexa Fluor® 594 binding (Fig. 3.8) within an asymmetric 0.65 μm PES membrane at a depth of 4 μm, after filtering a binary component (casein-dextran) solution. Profiles are for samples taken after 5% flux decline (●), 10% flux decline (○), and 15% flux decline (▼). Error bars represent the standard deviation of the intensity measurements.](image)

**Fig. 3.12.** Intensity profiles for the cross-sectional CLSM images of dextran-Alexa Fluor® 594 binding (Fig. 3.8) within an asymmetric 0.65 μm PES membrane at a depth of 4 μm, after filtering a binary component (casein-dextran) solution. Profiles are for samples taken after 5% flux decline (●), 10% flux decline (○), and 15% flux decline (▼). Error bars represent the standard deviation of the intensity measurements.

Intensity is a measure of the mass of protein or polysaccharide deposited at different depths within the membrane. It is directly proportional to the mass of labeled protein or polysaccharide. We developed calibration curves that relate intensity to the mass of fluorescent probe per membrane area for casein-FITC and dextran-Alexa Fluor® 594 by filtering known masses of fluorescently labeled casein (and separately dextran) through Dow NF90 ultrafiltration membranes that reject the protein and polysaccharide
completely. The small pore size of the membrane compared to the hydrodynamic size of the protein (or dextran) allowed us to retain it all on the membrane surface. CLSM images of the NF90 membrane surfaces were obtained at the same optical conditions used to obtain the CLSM images of the PES membranes at different degrees of fouling. The calibration curves (included in Appendix D) for casein and dextran were prepared by measuring the mean intensity of the fluorescently labeled protein or polysaccharide deposited on the surface of the NF90 membrane and plotting it versus the quotient of the known mass of fluorescent labeled protein or polysaccharide and the effective filtration area of the membrane. By knowing the ratio of fluorescently labeled to non-labeled protein or polysaccharide (1:20), we estimated the mass/area of casein and/or dextran at different depths from the intensity profiles presented in Figs. 3.9–3.12. Results are presented on secondary y-axes of Figs. 3.9–3.12 for the areal mass of casein and dextran found at different depth for PES membranes that filtered single component casein and dextran solutions and binary component casein-dextran solutions. Values also are tabulated in Appendix D.

3.3.5. Fouling mechanisms

Filtration data were analyzed with the Hermia model for constant pressure filtrations (eq. 3.1) to obtain more information about the fouling mechanisms. A more detailed discussion of the underlying assumptions and mathematical development of eq. 3.1 was provided by Hermia [33].
In eq. 3.1, \( t \) and \( V \) are the filtration time (s) and cumulative permeate volume (m\(^3\)), respectively. \( \frac{\partial t}{\partial V} \) is the reciprocal of the permeate flow rate; \( \frac{\partial^2 t}{\partial V^2} \) is defined as the resistance coefficient or the rate of change of the instantaneous resistance to filtration with respect to permeate volume; and \( k \) and \( n \) are two model parameters, where \( n \) depends on the fouling model or mechanism (\( n = 0 \) for cake filtration, \( n = 1 \) for intermediate blocking, \( n = 2 \) for complete blocking, and \( n = 1.5 \) for standard blocking). Plotting on a logarithmic scale \( \frac{\partial^2 t}{\partial V^2} \) versus \( \frac{\partial t}{\partial V} \) should give a straight line with slope equal to the \( n \) parameter [34].

Filtration data presented in Fig. 3.2 were analyzed with the Hermia model (eq. 3.1) and the \( n \) parameter from eq. 3.1 was obtained for each case. For single-component solutions of casein and dextran, steep slopes (\( n > 2 \)) were observed in early stages of filtration, and the slope values decreased throughout the filtration. This phenomenon has been observed by other researchers, and it has been attributed to the fact that particle deposition is unable to block or seal pores since fluid can flow under and around any blocked surface when there is a highly interconnected membrane pore structure [35, 36]. Thus, the Hermia model, which does not account for interconnected pores, fails to describe fouling behavior during the early stages of filtration of casein and dextran single-component solutions. Half way through the filtration of single-component solutions of dextran, the slope value converged to \( n = 1 \), indicating an intermediate pore blocking fouling mechanism, where dextran aggregates partially block the pores on the
surface of the PES membrane. Fig. 3.7 supports this result, where we can visualize the deposition of dextran on the surface of the membrane partially blocking the pores. According to Starbard [9], soft (deformable) particles like carbohydrates typically plug a filter through a pore blockage model. For single-component solutions of casein, the slope values were greater than 2 for the whole filtration data set, making it impossible to conclude by flux measurements alone what fouling mechanism dominates throughout the filtration experiment. In this case, CLSM images provide a visual description of how fouling occurs by depth filtration.

For the binary mixture of casein and dextran, we obtained a slope value of \( n = 0.4 \) for the entire filtration data set. As mentioned earlier, a zero slope indicates that the main fouling mechanism is cake filtration. We might be tempted to submit that a slope value of \( n = 0.4 \) is close to zero and that we might be having a cake filtration fouling mechanism is this system. This finding statement is counter-intuitive since cake filtration generally is interpreted as a cake formed at the surface of the membrane facing the feed. Yet, CLSM images in Fig. 3.8 clearly show that the accumulation of foulants occurs on the dense side of the membrane. Then, based on the CLSM images presented in Fig. 3.8, we can interpret the results from analysis of flux decline data using the Hermia model as ‘cake’ formation on the dense surface of the PES membrane opposite to the feed side of the membrane. According to Bhattacharyya and Butterfield [35], governing filtration equations have been derived for different values of the \( n \) parameter ranging between 0 and 2 (i.e. \( n=1/4, 1/3, 1/2, 2/3, 3/4, 5/4 \) and \( 4/3 \)) but no physical interpretation has been provided for these model equations. It is important to note that fouling visualization by
cross-sectional CLSM imaging can provide information that can be used to give the physical interpretation that these derived filtration equations are missing.

3.4. Conclusions

This research provides a methodology for direct visual observation of membrane fouling within a wet, asymmetric membrane structure. The application of a protocol developed previously by our research group for cross-sectional CLSM imaging allowed the location and quantification of protein and polysaccharide foulants within the full thickness of a PES asymmetric microfiltration membrane, something, to our knowledge, no one has reported previously. Flux experiments provided information on the fouling behavior of casein and dextran when they were alone in solution and when they were mixed. Cross-sectional CLSM images and calibration images provided quantitative information about the location and mass/area of the fluorescently labeled foulants throughout the wet membrane structure. Comparing quantitative analysis of the CLSM images with flux decline data analysis using the Hermia model allowed a better understanding about how fouling occurs. Also, it was demonstrated that information provided by CLSM imaging can be used to infer the fouling mechanism(s) when fouling models that are based on assumed membrane structure, such as the Hermia model, do not apply, fail to provide physically meaningful information or do not lead to the right conclusion.

Hydrophobic and hydrogen bonding interactions are believed to be responsible for casein and dextran adsorption onto the PES membrane material. The presence of casein
in solution prevented surface fouling by dextran likely due to association between these components that facilitates dissolution and prevents aggregation of dextran in solution. This association between casein and dextran in the binary solution explains why the fouling profiles of both components are similar. The knowledge generated in this study is relevant to industry and membrane manufacturers since proteins and polysaccharides are present in beverages and play a role in the fouling of membranes during microfiltration processes. The results from this investigation will enable future investigations on membrane fouling by multicomponent solutions and the elucidation of the roles that membrane structure and material of construction play on foulant deposition/accumulation on and within the membrane. Such knowledge may aid in the design of new membranes with tailored structure or surface chemistry that prevents the deposition of the foulants in “prone to foul” regions.

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3.5. References


CHAPTER 4

EVALUATION OF FOULING MECHANISMS IN ASYMMETRIC MICROFILTRATION MEMBRANES USING ADVANCED IMAGING

4.1. Introduction

Microfiltration (MF) is a key process in the beverage industry that is used to remove bacteria, yeast, colloidal particles and even other filtration media such as diatomaceous earth, to ensure final product quality and/or consumer safety. For instance, in beer production, microfiltration is used to remove chill haze flocs and microorganisms that can spoil the final product [1]. A major problem for MF membranes is fouling, which decreases service life and increases change-out costs compared to other filtration materials [2]. In beverage processing, fouling of MF membranes occurs not only due to the presence of microorganisms or suspended particles, but also by the presence of low molecular weight components in solution. Furthermore, interactions among these components can exacerbate fouling of the membrane.

Common constituents found in beer, wine, juices and tea are polyphenols, proteins and polysaccharides. Polyphenols are responsible for the astringency sensation when drinking these beverages, which is believed to be caused by the precipitation of salivary proteins by polyphenols on oral surfaces, preventing palate lubrication and inducing the drying, puckering and roughing sensation in the buccal cavity [3]. To some degree, astringency is perceived as a positive quality factor in certain beverages; for instance, it is one of the most important organoleptic sensations perceived when drinking
wine or tea [3]. In addition, polyphenols have antioxidant, antimutagenic and anticarcinogenic properties, among other health benefits [4, 5].

Polyphenols behave like cross-linkers between protein molecules, and the complexation between proteins and polyphenols in solution has been well documented [e.g., 4, 6-9]. It has been reported that polyphenols bind to proteins (especially to proline-rich proteins) and form soluble or insoluble complexes through hydrogen bonding and hydrophobic interactions [10, 11]. The protein-polyphenol interaction is affected by parameters that include ionic strength, pH, concentration ratio of polyphenol to protein, solvent composition, and the presence of certain components in solution like polysaccharides [12, 13].

Polysaccharides are capable of disrupting the protein-polyphenol interaction possibly due to hydrophobic interactions and/or hydrogen bonding between oxygen atoms of the polysaccharide and the phenolic hydroxyl group of the polyphenols [3, 6, 10]. An everyday example of the disruption of protein-polyphenol interactions by polysaccharides is the loss of astringency during the ripening process of many edible fruits because of the increase of soluble pectins during maturation [3, 14]. There are two possible mechanisms by which polysaccharides disrupt interactions between proteins and polyphenols: (1) Polysaccharides form a ternary complex with proteins and polyphenols that enhances the solubility in solution. (2) There is a molecular association between polysaccharides and polyphenols that disrupts protein-polyphenol aggregation. It has been proposed that some polysaccharides, like xanthan gum and cyclodextrins, develop
structures in solution that provide hydrophobic pockets to encapsulate polyphenols preventing further interaction with proteins [3, 6, 12, 14-16].

The understanding of solute-solute and solute-membrane interactions that lead to fouling during the microfiltration of beverages is important to develop fouling mitigation strategies and decrease costs of processing. To characterize membrane fouling, techniques like electron microscopy (EM), atomic force microscopy (AFM) and confocal microscopy have been implemented to visualize foulant accumulation [12, 17-21]. Confocal Laser Scanning Microscopy (CLSM) is a light microscopy technique that gradually has gained popularity in membrane studies, and it has been used by our group and others to characterize fouling (internal and external) [12, 22-24], morphology [25-27], performance [28, 29] and surface chemistry [30]. Price et al. [31] provide a comprehensive overview of CLSM fundamentals. Briefly, CLSM in fluorescent mode focuses a laser on a plane at a selected depth within the sample. The laser excites the fluorescent molecules in the sample (present in the sample originally or added deliberately), and the emitted light is collected by the microscope detector to produce an image. By changing the position of the focal plane, it is possible to collect images of different depths within the sample (i.e., optical sectioning).

Advantages that CLSM offers over EM and AFM are non-invasive depth imaging (optical sectioning), wet state imaging capability, and, by using fluorescently labeled probes, ability to locate and identify foulants within the sample. Several authors have identified a limit of depth of penetration (LDP) for CLSM [12, 26, 28, 32]. Beyond the LDP, excitation and emitted light is lost significantly, which prevents the construction of
The LDP is not the same for every sample or microscope; rather, it depends on parameters that include sample material, light wavelength, optical instrument, immersion-mounting media refractive index match, among others [26]. To overcome LDP, we have developed a cross-sectional CLSM imaging protocol that produces defect-free images throughout the full thickness of membranes [26].

The main goal of this study was to gain a better understanding of the solute-solute and solute-membrane interactions and their impact on fouling of asymmetric microfiltration membranes. Flux data were collected for single-component and binary and ternary component mixtures of protein, polyphenol, and polysaccharide. Cross-sectional CLSM imaging was used for direct visual observation of the fouling profiles of fluorescently labeled protein (casein) and polysaccharide (β-cyclodextrin) within the membranes, as well as visualization of how these fouling profiles changed when a polyphenol (tannic acid) was present in solution. The mass of foulants accumulated within the membrane was estimated based on the light emission intensity captured in the CLSM images using calibration curves developed in this study. Finally, flux data obtained in this study were analyzed using standard fouling models to determine the apparent mechanisms of fouling occurring within the PES membrane when processing different combinations of foulants. The consistency of these results was discussed based on the results of the quantitative and visual analysis of their correspondent CLSM images.

The knowledge generated in this research is relevant to industry users of MF and membrane manufacturers. Our hope is that it will aid in the design of new membranes.
with tailored structure or surface chemistry that prevents the deposition of the foulants in “prone to foul” regions, as well as the development of improved cleaning procedures.

4.2. Experimental materials and methods

4.2.1. Materials

Asymmetric polyethersulfone (PES) membranes (Pall Corporation) were used for filtration experiments. The Supor® PES membranes that were used have manufacturer reported effective pore diameter of 0.65 μm and thickness of 114–175 μm.

Non-labeled compounds used in the filtration experiments were casein from bovine milk (Sigma Aldrich, C6554), β-cyclodextrin (Sigma Aldrich, C4767), tannic acid (Sigma Aldrich, 403040), (+)-catechin hydrate (Sigma Aldrich, C1251), xanthan gum (Sigma Aldrich, G1253) and pectin (Sigma Aldrich, P9135).

Fluorescently labeled probes used in filtration experiments were fluorescein isothiocyanate (FITC) labeled casein from bovine milk (Sigma-Aldrich, C-0403) and rhodamine B isothiocyanate (RITC) labeled β-cyclodextrin. RITC (Sigma Aldrich, 283924) was bound covalently to β-cyclodextrin by a slight modification of the method of Belder and Granath [33] in which β-cyclodextrin was substituted for dextran and RITC for FITC. Briefly, pyridine (0.3 mL) (Sigma Aldrich, 360570), dibutyltin dilaurate (20 μL) (Sigma Aldrich, 291234) and β-cyclodextrin (1 g) were added to anhydrous dimethyl sulfoxide (10 mL) (Sigma Aldrich, 276855) in a screw-top scintillation vial, and the mixture was placed in a water bath at 95°C until β-cyclodextrin was dissolved completely. Then, RITC (100 mg) was added to the mixture and the vial was incubated at 95°C for 2
h with continuous magnetic stirring (250 rpm). At the end of the reaction, the mixture was divided evenly into three screw-top tubes, and 25 mL of ethanol (Sigma Aldrich, 459836) were added to each one. Precipitation of labeled β-cyclodextrin was observed after vortexing for 2 min, and the tubes were centrifuged at 8000 g for 10 min to separate precipitate from the supernatant containing free dye. The supernatant was collected and divided evenly into three tubes. Ethanol (25 mL) was added to each tube to precipitate remaining labeled β-cyclodextrin from solution, and then the tubes were centrifuged at the same conditions specified earlier. After discarding supernatant, the precipitated β-cyclodextrin was washed by resuspending in 10 mL of ethanol and centrifuging the tubes. Resuspension-centrifugation cycles were repeated (5-6 times) until no dye was visible in the supernatant. Finally, RITC-labeled β-cyclodextrin was dried overnight at 45 °C.

For filtration experiments, sodium phosphate buffered solutions were prepared using sodium phosphate monobasic (anhydrous, ≥99%, Sigma Aldrich, S0751), sodium phosphate dibasic (anhydrous, ≥99%, Sigma Aldrich, S9763), and deionized (DI) Milli-Q system (EMD Millipore) water.

Dow Filmtec™ NF90 membranes were used to prepare the calibration plots of intensity versus areal protein or polysaccharide mass. Before using the NF90 membranes, they were pre-treated according to the procedure explained in Marroquin et al. [24] to enhance the permeability of the membranes while maintaining their rejection properties.
4.2.2. Filtration experiments

A direct-flow filtration cell, Amicon 8050 from EMD Millipore, was used at a constant pressure of 14.5 kPa. The Amicon 8050 cell has an effective filtration area of 13.4 cm$^2$.

Sodium phosphate buffer solutions were prepared with an ionic strength of 0.125 M and pH of 6.8. A stock solution of casein (25 g/L) was prepared by mixing the casein powder from bovine milk in a 0.04 M sodium hydroxide solution. Stirring was applied at 250 rpm for 4 h to facilitate the casein dissolution.

Single, binary and ternary solutions containing protein, polyphenol and/or polysaccharide were prepared. The concentrations of the protein (casein) and polyphenol (tannic acid or catechin) were 25 mg/L and 150 mg/L, respectively, in phosphate buffer solution. Solutions containing polysaccharide were prepared with a final concentration of 200, 50 or 25 mg/L in phosphate buffer solution. These solutions were prepared by adding the appropriate volume of casein stock solution, mass of polysaccharide and/or mass of polyphenol to a volumetric flask and adding phosphate buffer to achieve the desired volume. The polysaccharide and polyphenol were sonicated in 20 mL of DI water before addition to the volumetric flask. To allow confocal visualization of the protein (casein) and polysaccharide, fluorescently labeled casein and polysaccharide were added to the solutions in a ratio of 1:20 fluorescently labeled to non-labeled component.

PES membranes were pre-wetted before each filtration experiment by soaking them in a 25% (v/v) aqueous ethanol solution for 10 min. Then the membranes were soaked in DI water for 10 min. Membranes were kept in DI water until use. Each
filtration experiment was done at constant pressure until 1 L of permeate was collected. The membrane was positioned with the more open surface facing the feed, and filtration was done in direct-flow mode with a constant stirring speed of 250 rpm. Flux versus permeate volume data were recorded during each experiment.

After filtration, 5 mL of a solution of non-labeled component(s) at the concentration(s) used in the filtration experiment was filtered to displace solution containing unbound fluorescently labeled compounds from the membrane pores. Using solutions of non-labeled component(s) at the same concentration(s) was done to minimize desorption of physisorbed components from the membrane. Membranes were used once and sacrificed to collect samples for confocal visualization.

4.2.3. Calibration curve preparation

We follow the procedure described in Chapter 3 to develop the calibration curve relating fluorescence emission intensity to mass of fluorescently labeled polysaccharide per area. The corresponding calibration plot for casein-FITC was developed in Chapter 3.

4.2.4. Sectioning and mounting of samples for cross-sectional CLSM imaging

Cross-sections of the membranes used in the filtration experiments were obtained and prepared for confocal imaging following the procedure described by Marroquin et al. [26].
4.2.5. Optical system and imaging

A Nikon Eclipse Ti confocal laser scanning microscope system (Nikon Instruments Inc, Lewisville, TX) was used in fluorescence mode with a Nikon 60X oil immersion objective with a numerical aperture of 1.49. This CLSM system was used to visualize fluorescently labeled probes bound throughout the entire thickness of the PES membranes or on the surface of the NF90 membranes. Images were stored as 12-bit scans with a resolution of 512 × 512 pixels, which represented an area of 212 × 212 micron. The excitation light source was a helium-neon laser (594 nm excitation wavelength for RITC and 488 nm wavelength for FITC). Each image is the result of averaging the signal/information collected from four scans, which reduces noise, producing better resolved images.

4.2.6. Image analysis

To compare information from CLSM images, special care was taken to keep the confocal microscope settings the same for each sample (laser intensity, gain, pinhole size, pixel dwell time, resolution, field zoom, averaging number) while imaging. Images collected by the confocal microscope were processed using NIS-Elements 3.2 Software Package (Nikon Instruments Inc, Lewisville, TX).

4.2.7. Dynamic Light Scattering (DLS) measurements

DLS measurements were conducted using a Malvern Zetasizer Nano ZS instrument (Malvern Instruments Ltd., Malvern, UK) at a wavelength of 633 nm from a 4.0 mW, solid-state He-Ne laser at a scattering angle of 173°. Number average diameters
were calculated from the autocorrelation function using Malvern Zetasizer Nano 7.01 software utilizing a version of the CONTIN algorithm.

4.3. Results and discussion

4.3.1. Selection of study system

The first polyphenol tested in this study was (+)-catechin. We observed no significant fouling when filtering casein-catechin mixtures (flux data included in Appendix E). This observed result was counterintuitive based on the well-documented complexation (physical crosslinking) that occurs between polyphenols and proline-rich proteins such as casein [7, 8, 11, 13]. Since complexation between proteins and polyphenols commonly results in larger aggregates in solution, we expected to observe a decline in the flux due to fouling by the newly formed aggregates. According to Mateus et al. [16], the size of the polyphenol plays an important role in the crosslinking of proteins. Small polyphenols are not capable of crosslinking several proteins since the number of sites able to associate or interact with the proteins is proportional to the molecular weight of the polyphenol [34]. Shukla et al. [8] described in their studies that small polyphenols, like epigallocatechin gallate (main polyphenol in green tea), incorporate within casein micelles, increasing the density of the micelle without modifying its apparent size. Since casein and epigallocatechin gallate both have low molecular weights (290 g/mol and 458 g/mol, respectively), we submit that catechin behaves similarly to epigallocatechin gallate and is incorporated within the casein micelles. Due to its small size, it is not capable of associating with several proteins and
forming aggregates large enough to significantly foul the MF membranes at the conditions and permeate volume collected during this study. Therefore, we adopted a polyphenol with higher molecular weight for our study system. We selected tannic acid (1,700 g/mol), which can cross-link casein proteins and form aggregates without significantly fouling the membrane on its own.

Anionic polysaccharides, like xanthan gum and pectin, have been reported to be effective in preventing the formation of insoluble aggregates between proteins and polyphenols [6]. Initially, we tested xanthan gum and pectin as model polysaccharides for this study; however, the fouling was more drastic when these polysaccharides were in solution along with the protein and polyphenol and even when they were alone in solution (flux data included in Appendix F). Interestingly, the flux declined faster when filtering the pectin ternary mixture (mixed with casein and tannic acid) compared to when filtering the pectin single-component solution. This phenomenon is attributed to the tendency of pectin to form ternary complexes with proteins and polyphenols, as other researchers have reported it [3]. Single-component xanthan gum solution and its ternary mixture showed similar flux profiles. According to Freitas et al. [6], xanthan gum is believed to form gel-like networks in solution (by lateral association of ordered chain sequences) that might be able to encapsulate the polyphenols. While this gel-like network prevents polyphenols from interacting with proteins, it also increases the fouling of the filtration membrane by plugging the pores at the membrane surface and allegedly due to the xanthan gum gel-like network, that it is being retained at the surface of the membrane, single-component and ternary mixtures of xanthan gum present similar flux
profiles [16]. Thus, we decided to test the lower molecular weight polysaccharide β-cyclodextrin, which, according to the literature, associates strongly with polyphenols [14]. β-cyclodextrin is a cyclic (doughnut-shape) oligosaccharide with hydrophilic residues on the exterior and a structural interior that is more hydrophobic than the exterior. Consequently, the mechanism that β-cyclodextrin follows to prevent the association of proteins and polyphenols is believed to be the encapsulation of the polyphenol in the hydrophobic pocket [3].

4.3.2. Flux measurements

Flux versus volume data were collected as a function of pH and ionic strength for casein, casein/tannic acid binary mixtures, and casein/tannic acid/β-cyclodextrin ternary mixtures. Fig. 4.1 presents one set of data at constant pH and ionic strength. It is observed that the mixture of casein (25 mg/L) and tannic acid (150 mg/L) shows a severe flux decline compared to the single component solutions of casein, tannic acid, and β-cyclodextrin, which do not foul the membrane significantly. This result suggests that protein aggregation is caused by the presence of tannic acid, and that these aggregates cause significant pore blockage.

To test for protein-polyphenol association, DLS data were collected for single component solutions of casein (25 mg/L) and tannic acid (150 mg/L) and for the casein-tannic acid binary mixture (25 mg/L and 150 mg/L, respectively). DLS results presented in Fig. 4.2a show that the apparent diameter of the aggregates present in the casein solution are approximately 220 nm, which is an indication of the presence of casein
micelles [35]. Fig. 4.2b shows that the majority of aggregates present in the tannic acid solution are approximately 5–6 nm in diameter.

Fig. 4.1. Permeate flux evolution for casein (25 mg/L) single component (□), tannic acid (150 mg/L) single component (●), β-cyclodextrin (200 mg/L) single component (◊), casein-tannic acid binary component (25 mg/L and 150 mg/L, respectively) (▲), casein-tannic acid- β-cyclodextrin (25 mg/L, 150 mg/L, 200 mg/L, respectively) ternary component (♦), casein-tannic acid- β-cyclodextrin (25 mg/L, 150 mg/L, 50 mg/L, respectively) ternary component (○), casein-tannic acid- β-cyclodextrin (25 mg/L, 150 mg/L, 25 mg/L, respectively) ternary component (Δ).

Unexpectedly, Fig. 4.2c shows that the majority of aggregates in the casein-tannic acid mixture are 26 nm in diameter. Casein micelles are still present in solution, as seen in Fig. 4.2d (size distribution based on intensity) and their size has increased from 220 nm to 241 nm. Fig. 4.2d also shows a small amount of aggregates with a 5.4 μm size that probably are aggregated casein micelles by tannic acid. The 241 nm and 5.4 μm
aggregates have been outnumbered by the new 26 nm aggregates and that is the reason why these peaks do not show up in the DLS size distribution based on number. We submit that the abundant 26 nm aggregates in solution are responsible for the fouling of the MF membrane observed when filtering the casein-tannic acid mixture. Model-based analysis of the flux data (vide infra) suggest fouling by pore blocking. These nanosized clusters probably derive from the association of free casein in solution (in equilibrium with the casein micelle) and tannic acid [4]. From these results we conclude that tannic acid is not large enough to effectively cause micelle-micelle aggregation (as there were very few 5.4 μm aggregates seen in Figs. 4.2c and 4.2d), but it is capable of binding multiple free casein proteins in solution and form the observed 26 nm aggregates. Also, the lack of a significant number of micelles at 220 nm in Fig. 4.2c suggests that the tannic acid breaks up many of the casein micelles by forming more stable 26 nm clusters. As tannic acid scavenges free casein in solution, a thermodynamic driving force exists for dissolution of the micelles into free protein.

Next, we tested the effect of the polysaccharide on the fouling behavior of a solution containing the protein and the polyphenol. The concentration of protein and polyphenol were kept constant, and the concentration of polysaccharide was varied. Three different molar ratios of polyphenol to polysaccharide were tested (1:2, 2:1, 4:1). Adding 50 mg/L β-cyclodextrin (ratio 2:1 polyphenol to polysaccharide) to the mixture yielded some improvement to the flux compared to the protein-polyphenol system (Fig. 4.1).
Fig. 4.2. DLS data for (a) casein (25 mg/L) single component, size distribution by Number (b) tannic acid (150 mg/L) single-component size distribution by Number, (c) casein-tannic acid binary component (25 mg/L and 150 mg/L, respectively) size distribution by Number and (d) casein-tannic acid binary component (25 mg/L and 150 mg/L, respectively) size distribution by Intensity.

Unexpectedly, a higher concentration of 200 mg/L β-cyclodextrin (ratio 1:2 polyphenol to polysaccharide) yielded a more severe flux decline than for the system protein-polyphenol (i.e., there was no benefit to adding more polysaccharide). From these results, it is apparent that higher concentrations of β-cyclodextrin (>50 mg/L) hinder the beneficial effect of the polysaccharide. Our explanation for this observed phenomenon is that at 200 mg/L ternary complexes might be formed between casein/tannic acid/β-cyclodextrin. It was mentioned in Section 4.1 that there are two possible mechanisms by which the polysaccharide disrupts protein/polyphenol interactions: (1) by the association of polyphenols and polysaccharides (i.e. physical encapsulation) and (2) by the formation of a ternary complex (protein/polyphenol/polysaccharide). We believe that at the 50 mg/L of β-cyclodextrin, mechanism (1) is observed and at 200 mg/L of β-cyclodextrin,
mechanism (2) is observed. A concentration of 25 mg/L of β-cyclodextrin (ratio 4:1 polyphenol to polysaccharide) showed a fouling behavior that was similar to the initial mixture with no polysaccharide present in solution. This result may be attributed to the low concentration of polysaccharide that is insufficient to effectively prevent the protein-polyphenol association.

Based on the fouling behavior of the solutions tested in Fig. 4.1, we found that the ‘sweet spot’ ratio (2:1) proved to be an appropriate concentration of polysaccharide to limit the protein-polyphenol association in our system. These findings suggest that, when feasible, addition of aggregate disrupting sugars at low concentrations may be an effective approach to limit fouling during the microfiltration of beverages. DLS data obtained from the ternary mixtures was not conclusive to support our different theories regarding to the aggregates happening at the three polysaccharide concentrations tested in this study. Resolution of our DLS instrument was not good enough to determine the difference between the peaks corresponding to the aggregates occurring at the three polysaccharide concentrations (DLS data presented in Appendix G).

### 4.3.3. Cross-sectional CLSM imaging

Flux measurements presented in section 4.3.2 provided indirect evidence for the cause of fouling. Thus, CLSM was used to visualize protein and polysaccharide binding within the wet membrane structures for the samples corresponding to the flux experiments presented in Fig. 4.1. As mentioned earlier, to allow confocal visualization of the protein (casein) and polysaccharide (β-cyclodextrin), fluorescently labeled casein-
FITC and β-cyclodextrin-RITC were added to the solutions in a ratio of 1:20 fluorescently labeled to non-labeled component. In this section, the cross-sectional CLSM images are shown for three degrees of fouling (corresponding to membranes collected after processing 125, 250 and 500 mL permeate volume). All images correspond to the asymmetric 0.65 μm PES membranes used for the flux experiments in Fig. 4.1. Using cross-sectional imaging [26], we were able to image all depths and overcome previous depth of penetration limitations for such studies. Images were taken at a depth of 4 μm below the cross-section surface to avoid concerns about surface defects caused by sample preparation.

![Cross-sectional CLSM images](image)

**Fig. 4.3.** Cross-sectional CLSM images of casein/casein-FITC binding within an asymmetric 0.65 μm PES membrane at a depth of 4 μm. Feed was 25 mg/L casein (1:20 fluorescently labeled to non-labeled protein). The dense surface is on the LEFT of all images. Images are for samples taken after processing 125 mL permeate volume (LEFT), 250 mL permeate volume (MIDDLE), and 500 mL permeate volume (RIGHT). The scale indicator is 10 μm in diameter.

For membranes that processed casein single component solution, Fig. 4.3 shows that protein accumulates throughout the membrane structure and is not concentrated at the feed surface. It appears that the membrane functions as a depth filter. The bright spots...
on Fig. 4.3 are attributed to accumulation of possible protein aggregates in ‘blind pores’ (dead-end pores that do not permit flow). Fouling of the PES membranes by casein is attributed to hydrophobic interactions between the protein and the membrane material, as other authors have stated [17]. To quantify the mass of casein observed in the CLSM images, we used a calibration curve developed in a previous publication that relates intensity to the mass of fluorescent dye per membrane area for casein-FITC [24] (refer to Appendix D for casein-FITC calibration curve).

Fig. 4.4. Intensity profiles for the cross-sectional CLSM images of casein/casein-FITC binding (Fig. 4.3) within an asymmetric 0.65 μm PES membrane at a depth of 4 μm, after filtering a single-component solution. Profiles are for samples taken after processing 125 mL permeate volume (●), 250 mL permeate volume (○), and 500 mL permeate volume (▼). Please note that the y-axis range in this figure is different from those used for mixed-component systems in Figs. 4.8 and 4.12. Error bars represent the standard deviation of the intensity measurements.
The left-hand ordinate in Fig. 4.4 presents the average intensity versus depth corresponding to images in Fig. 4.3, while the right-hand ordinate presents the mass of casein foulant per area obtained based on the calibration curve. Casein tends to accumulate more towards the dense side of the membrane, and the mass of casein within the membrane increases with increasing volume of permeate that is collected. However, the apparent increases are not statistically significant based on measurement uncertainties at these low intensity values.

Fig. 4.5 shows the CLSM images of membranes post filtration of single component β-cyclodextrin solutions. The adsorption of β-cyclodextrin to the PES membrane occurs through van der Waals interactions and hydrogen bonding between hydroxyl groups of dextran (donor) and the oxygen atoms in the SO_2 group of PES (acceptor) [17, 36]. To quantify the mass of polysaccharide within the membrane, we developed a calibration curve for β-cyclodextrin-RITC to relate intensity captured in CLSM images to the mass of β-cyclodextrin-RITC per area (refer to Appendix H for β-cyclodextrin-RITC calibration curve). Consequently, by knowing the ratio of fluorescently labeled to non-labeled component, we can determine the mass of foulant within the membrane. The development of the β-cyclodextrin-RITC calibration curve was done following the procedure explained by Marroquin et al. [24].
Fig. 4.5. Cross-sectional CLSM images of β-cyclodextrin/β-cyclodextrin-RITC binding within an asymmetric 0.65 μm PES membrane at a depth of 4 μm. Feed was (a) 50 mg/L β-cyclodextrin (1:20 fluorescently labeled to non-labeled polysaccharide), and (b) 200 mg/L β-cyclodextrin (1:20). The dense surface is on the LEFT of all images. Images are for samples taken after processing 125 mL permeate volume (LEFT), 250 mL permeate volume (MIDDLE), and 500 mL permeate volume (RIGHT). The scale indicator is 10 μm in diameter.

Fig. 4.6 presents the average intensity versus depth and the mass of β-cyclodextrin per area at different depths within the membranes corresponding to images in Fig. 4.5. A slight accumulation of the polysaccharide is observed on the surface of the membrane facing the feed and also on the dense surface. Accumulation of β-cyclodextrin on the surface facing the feed is attributed to the presence of aggregates in solution. Interchain and intrachain interactions in neutral polysaccharides like β-cyclodextrin are stabilized by a large number of hydrogen bonds, causing a relatively low solubility for
this kind of polysaccharide [37]. Accumulation of a neutral polysaccharide also was observed during the microfiltration of dextran solutions with PES asymmetric membranes (same membrane orientation) in a previous study [24].

![Graph](image)

**Fig. 4.6.** Intensity profiles for the cross-sectional CLSM images of β-cyclodextrin/β-cyclodextrin-RITC binding (Fig. 4.5) within an asymmetric 0.65 μm PES membrane at a depth of 4 μm, after filtering (a) 50 mg/L and (b) 200 mg/L single-component solutions. Profiles are for samples taken after processing 125 mL permeate volume (●), 250 mL permeate volume (○), and 500 mL permeate volume (▼). Error bars represent the standard deviation of the intensity measurements.

Fig. 4.7 shows the CLSM images of the PES membranes that processed the mixture of casein and tannic acid (25 and 150 mg/L respectively). The casein and tannic acid mixture significantly fouls the PES membrane compared to the minimal fouling observed during filtration of the casein and tannic acid single component solutions. In Fig. 4.7, we did not observe the bright spots seen in Fig. 4.3. It appears that casein aggregates now are bound together by tannic acid and are retained on the membrane surface facing the feed.
Fig. 4.7. Cross-sectional CLSM images of casein/casein-FITC binding within an asymmetric 0.65 μm PES membrane at a depth of 4 μm. Feed comprised 25 mg/L casein (1: 20 fluorescently labeled to non-labeled protein) and 150 mg/L tannic acid. The dense surface is on the LEFT of all images. Images are for samples taken after processing 125 mL permeate volume (LEFT), 250 mL permeate volume (MIDDLE), and 500 mL permeate volume (RIGHT). The scale indicator is 10 μm in diameter.

We can explain this result based on the combination of solute-membrane interactions (i.e., hydrophobic adsorption of casein to PES membrane) and perhaps more significantly solute-solute interactions (i.e., casein and tannic acid association by hydrophobic interactions and hydrogen bonding) contributing to severe flux decline.

After collecting 125 mL of permeate, protein accumulated mostly on the dense side of the membrane and the top surface facing the feed. After collecting 250 mL of permeate, protein fouling on the dense side of the membrane and the surface facing the feed has increased (perceived by a more intense green color in Fig. 4.7). After collecting 500 mL of permeate, protein fouling within the membrane has increased, and the highly fouled region close to the dense side of the membrane is thicker. Fig. 4.8 shows the corresponding average intensity versus depth and the mass of casein per area at different depths within the membranes corresponding to Fig. 4.7. The intensity of the green color
is higher at the surface facing the feed compared to the bulk of the membrane and increases again towards the dense surface. The high intensity on the feed side denotes accumulation of protein aggregates caused by the presence of tannic acid.

**Fig. 4.8.** Intensity profiles for the cross-sectional CLSM images of casein/casein-FITC binding (Fig. 4.7) within an asymmetric 0.65 μm PES membrane at a depth of 4 μm, after filtering a solution comprising 25 mg/L casein and 150 mg/L tannic acid. Profiles are for samples taken after processing 125 mL permeate volume (●), 250 mL permeate volume (○), and 500 mL permeate volume (▼). Error bars represent the standard deviation of the intensity measurements.

Fig. 4.9 shows the CLSM images of the PES membranes that processed the mixture of casein (25 mg/L), tannic acid (150 mg/L), and β-cyclodextrin (50 mg/L). After collecting 250 mL of permeate, little protein has accumulated on the surface facing the feed compared to the case of the casein-tannic acid binary mixture. Only after 500 mL of
permeate has been collected do we see significant accumulation of casein on the surface. It appears that 50 mg/L of β-cyclodextrin in solution is an appropriate amount of polysaccharide to minimize the aggregation of protein by polyphenol.

Fig. 4.10 shows the CLSM images of the PES membranes that processed the ternary mixture of casein (25 mg/L), tannic acid (150 mg/L), and β-cyclodextrin (200 mg/L). Shown are the individual and overlaid fouling profiles for casein and β-cyclodextrin at different levels of fouling. Our expectation was that a higher concentration of the polysaccharide would disrupt the interactions between casein and tannic acid more effectively than the 50 mg/L concentration of polysaccharide. Rather, higher levels of casein and β-cyclodextrin accumulated within the membrane when using the polysaccharide at a higher concentration. This result was consistent with the observed acceleration in flux decline, and may be attributed to the excess of β-cyclodextrin (relative to what is needed to bind tannic acid), along with formation of a larger complex, as measured by DLS, that may be attributed to ternary casein-β-cyclodextrin-tannic acid aggregates. Thus, adding more polysaccharide to a casein-tannic acid mixture to disrupt their interactions is counterproductive. Fig. 4.11 shows the CLSM images of the PES membranes that processed the mixture of casein (25 mg/L), tannic acid (150 mg/L), and β-cyclodextrin (25 mg/L). There is an increase in the color intensity compared to the previous case using 50 mg/L β-cyclodextrin, indicating a higher amount of casein-FITC and β-cyclodextrin-RITC within the membrane. Also, accumulation of casein on the membrane surface facing the feed is observed even at early stages of the filtration (125 mL permeate volume).
Fig. 4.9. Cross-sectional CLSM images of casein/casein-FITC and β-cyclodextrin/β-cyclodextrin-RITC binding within an asymmetric 0.65 μm PES membrane at a depth of 4 μm. Feed comprised 25 mg/L casein (1:20 fluorescently labeled to non-labeled protein), 150 mg/L tannic acid, and 50 mg/L β-cyclodextrin (1:20 fluorescently labeled to non-labeled polysaccharide). The dense surface is on the LEFT of all images. TOP row images are for samples taken after processing 125 mL permeate volume: casein (LEFT), β-cyclodextrin (MIDDLE), superimposed image of casein and β-cyclodextrin (RIGHT). SECOND row images are for samples taken after processing 250 mL permeate volume. BOTTOM row images are for samples taken after processing 500 mL permeate volume. The scale indicator is 10 μm in diameter.
Fig. 4.10. Cross-sectional CLSM images of casein/casein-FITC and β-cyclodextrin/β-cyclodextrin-RITC binding within an asymmetric 0.65 μm PES membrane at a depth of 4 μm. Feed comprised 25 mg/L casein (1:20 fluorescently labeled to non-labeled protein), 150 mg/L tannic acid, and 200 mg/L β-cyclodextrin (1:20 fluorescently labeled to non-labeled polysaccharide). The dense surface is on the LEFT of all images. TOP row images are for samples taken after processing 125 mL permeate volume: casein (LEFT), β-cyclodextrin (MIDDLE), superimposed image of casein and β-cyclodextrin (RIGHT). SECOND row images are for samples taken after processing 250 mL permeate volume. BOTTOM row images are for samples taken after processing 500 mL permeate volume. The scale indicator is 10 μm in diameter.
Fig. 4.11. Cross-sectional CLSM images of casein/casein-FITC and β-cyclodextrin/β-cyclodextrin-RITC binding within an asymmetric 0.65 μm PES membrane at a depth of 4 μm. Feed comprised 25 mg/L casein (1:20 fluorescently labeled to non-labeled protein), 150 mg/L tannic acid, and 25 mg/L β-cyclodextrin (1:20 fluorescently labeled to non-labeled polysaccharide). The dense surface is on the LEFT of all images. TOP row images are for samples taken after processing 125 mL permeate volume: casein (LEFT), β-cyclodextrin (MIDDLE), superimposed image of casein and β-cyclodextrin (RIGHT). SECOND row images are for samples taken after processing 250 mL permeate volume. BOTTOM row images are for samples taken after processing 500 mL permeate volume. The scale indicator is 10 μm in diameter.
From these observations, 25 mg/L of β-cyclodextrin (4:1 polyphenol/polysaccharide) in solution appears to be insufficient to sequester all or the majority of tannic acid in solution, and there is still significant protein-polyphenol aggregation.

Fig. 4.12 shows the comparison of intensity and concentration profiles for Figs. 4.9–4.11. As expected, the intensity and the mass of foulants within the membranes increase with permeate volume. Also, as expected from the flux data and visual inspection of the CLSM images, the lowest intensity and therefore mass of foulants corresponds to the case for 50 mg/L β-cyclodextrin. In most cases, the intensity and mass of foulants were similar for 200 mg/L or 25 mg/L of β-cyclodextrin in solution; although, in some cases, the intensity of the former was higher.
Fig. 4.12. Intensity profiles for the cross-sectional CLSM images of casein/casein-FITC and β-cyclodextrin/β-cyclodextrin-RITC binding (Figs. 4.9-4.11) within asymmetric 0.65 μm PES membranes at a depth of 4 μm, after filtering these solutions: (○) 25 mg/L casein (1:20 fluorescently labeled to non-labeled protein), 150 mg/L tannic acid, 50 mg/L β-cyclodextrin (1:20 fluorescently labeled to non-labeled polysaccharide); (●) 25 mg/L casein (1:20), 150 mg/L tannic acid, 25 mg/L β-cyclodextrin (1:20); and (▼) 25 mg/L casein (1:20), 150 mg/L tannic acid, 200 mg/L β-cyclodextrin (1:20). TOP row images are for samples taken after processing 125 mL permeate volume: casein (LEFT), β-cyclodextrin (RIGHT). SECOND row images are for samples taken after processing 250 mL permeate volume. BOTTOM row images are for samples taken after processing 500 mL permeate volume. Error bars represent the standard deviation of the intensity measurements.
4.3.4. Fouling mechanisms

CLSM images presented in section 4.3.3 show us where the foulants tend to accumulate within the membrane and help us to better understand or justify the trends observed in the flux measurement results. It is also our interest to understand the fouling mechanisms that lead to the results observed in the CLSM images and flux plots. We begin by analyzing the flux data presented in Fig. 4.1 with the Hermia model for constant pressure filtration (eq. 4.1).

\[ \frac{\partial^2 t}{\partial V^2} = k \left( \frac{\partial t}{\partial V} \right)^n \]  

(eq. 4.1)

In eq. 4.1, \( t \) and \( V \) are the filtration time and cumulative permeate volume (m\(^3\)), respectively. \( \frac{\partial t}{\partial V} \) is the reciprocal of the permeate volumetric flow rate; \( \frac{\partial^2 t}{\partial V^2} \) is defined as the resistance coefficient, or the rate of change of the instantaneous resistance to filtration with respect to permeate volume; and \( k \) and \( n \) are two model parameters, where \( n \) depends on the fouling model or mechanism (\( n = 0 \) for cake filtration, \( n = 1 \) for intermediate blocking, \( n = 2 \) for complete blocking, and \( n = 1.5 \) for standard blocking).

For the detailed discussion of the underlying assumptions and mathematical development of eq. 4.1, please refer to the publication by Hermia [38]. The fouling mechanism occurring during a filtration (\( n \) parameter) is obtained from the Hermia model equation by plotting on a logarithmic scale \( \frac{\partial^2 t}{\partial V^2} \) versus \( \frac{\partial t}{\partial V} \).

By analyzing the flux data from Fig. 4.1 with the Hermia model, we observed values for the \( n \) parameter greater than 2 (the maximum value for the Hermia model)
during early stages of filtration of the binary and ternary mixtures (permeate volume less than 125 mL). Other researchers have observed $n > 2$ during studies on the fouling of microfiltration membranes with interconnected pores [19, 39]. Based on these earlier studies, we attribute the steep initial slope in the $\log(\partial^2 t/\partial V^2)$ versus $\log (\partial t/\partial V)$ plots to the fact that liquid can flow under and around any blocked pore due to highly interconnected pore structure of the PES membranes used in this study. Also, it was observed that the slope decreased throughout the course of the filtration, and, at the end of the experiment, the $n$ parameter was close to 1 for casein/tannic acid, casein/tannic acid/β-cyclodextrin (200 mg/L) and casein/tannic acid/β-cyclodextrin (25 mg/L) mixtures, indicating intermediate pore blocking as the fouling mechanism. Towards the end of the filtration of the casein/tannic acid/β-cyclodextrin (50 mg/L) mixture, the $n$ parameter was close to 1.5, indicating that the mechanism of fouling is standard blocking. The results from the Hermia model analysis are coherent with the observations in CLSM image analysis and flux measurements. The 50 mg/L concentration of β-cyclodextrin (2:1 polyphenol to polysaccharide) in the casein-tannic acid mixtures is capable of decreasing or preventing the protein-polyphenol aggregation and thereby changing the fouling mechanism from intermediate pore blocking to standard pore blocking, where the smaller aggregates present in this mixture adsorb on the surface of the pore walls leading to pore constriction over time. While in the case of the binary casein-tannic acid mixture, the larger aggregates are capable of blocking the pores, consistent with the definition of intermediate blocking.
4.4. Conclusions

CLSM has proved to be a useful tool for visualizing the fouling within asymmetric membranes when filtering casein, tannic acid and β-cyclodextrin mixtures. By using our cross-sectional CLSM imaging protocol, we have overcome the limit of depth of penetration and obtained quantitative information on the masses of protein and polysaccharide deposited throughout the entire thickness of asymmetric microfiltration membranes at different degrees of fouling.

Evidence of association between the protein casein and the polyphenol tannic acid was obtained from flux and DLS data, as well as CLSM images. The effect of adding the polysaccharide β-cyclodextrin to the casein-tannic acid mixture was studied. Polysaccharides are known to disrupt protein-polyphenol interactions, and a 2:1 polyphenol to polysaccharide ratio was most effective for limiting flux decline associated with casein-tannic acid aggregates. Ratios below or above this ‘sweet spot’ were less effective at preventing severe, rapid flux decline. Information on the fouling mechanisms occurring during microfiltration was obtained by analyzing flux data with the Hermia model, and it was found that, at the end of the filtration experiments, the dominant fouling mechanism was intermediate pore blocking for the cases where significant fouling was observed (casein/tannic acid, casein/tannic acid/β-cyclodextrin 200 mg/L, and casein/tannic acid/β-cyclodextrin 25 mg/L); whereas, standard pore blocking was observed for the mixture containing a 2:1 polyphenol to polysaccharide ratio (casein/tannic acid/β-cyclodextrin 50 mg/L). The results from the Hermia model analysis
are coherent with the qualitative and quantitative CLSM image analysis of the CLSM images.

Lastly, CLSM allows the direct visualization, location and quantification of foulants (individually) within microfiltration membranes. Additionally, CLSM imaging of the fouled membranes, along with the flux decline plots and analysis of the flux data with fouling models, helps in the description and understanding of the root cause(s) of fouling. Another advantage of using CLSM imaging in the study of MF membrane fouling is that it provides direct visual information on how individual foulants deposit within the membranes. This information can be used to infer the fouling mechanism(s) when fouling models that are based on assumed membrane structure, such as the Hermia model, do not apply or fail to provide physically meaningful information (e.g., during the early stages of filtration in this study).

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Lab. Images were taken on equipment housed in the Clemson Light Imaging Facility, Department of Biological Sciences, Clemson University.
4.5. References


5.1. Conclusions

The overall goal of my PhD research was to develop advanced imaging protocols for the characterization of microporous membranes. I have developed innovative cross-sectional imaging protocols that produced defect-free images and enabled the visualization of the membrane throughout its entire thickness for the characterization of its morphology and fouling occurring in beverage clarification applications. Specifically, the focus of my research was to exploit a high-potential, emerging imaging technique called Confocal Laser Scanning Microscopy and overcome its limit on depth of penetration that has been restricting it from a wider application, rendering it sub-utilized in membrane studies and providing, in some instances, misleading results. In this study, the combination of the proposed cross-sectional imaging technique along with software analysis provided reliable information regarding the morphology of microfiltration membranes and gave visual insight on the fouling that these membranes experience during application. Through my dissertation, I have developed straightforward, wide-applicability imaging protocols and have applied them along with image analysis for the characterization of the morphology of microfiltration membranes and to study fouling profiles of biological foulants in wet membrane structures. The results obtained from its application can be conclusive or complementary to the results obtained by other characterization tools. Even though this study has been applied to MF membranes, it
should not be limited to them and can be expanded to other kind of membranes like UF, NF, RO, etc. Even though the resolution limits of the confocal microscope could prevent morphology characterization of these membranes, it is still possible the characterization of fouling in these membranes.

In the first phase of the project, I developed a protocol for the cross-sectional imaging of symmetric and asymmetric membranes: staining, cryo-sectioning, imaging and image analysis. It was demonstrated that concerns about surface artifacts due to sample preparation were avoided by imaging just below the surface. The cross-sectional imaging protocol developed in this study provided defect-free images of the full membrane thickness. I identified the theoretical and experimental limits of resolution for the state-of-the-art confocal microscope used in the study. I found that the experimental limit of resolution could deviate from the theoretical limit due, but not limited to, loss of scattered light and photobleaching, which were influenced by the selection of fluorescent dye used to label the membrane. By analyzing the x-y CLSM images, it was found that, due to the limit on depth of penetration, about 70% of the membrane thickness was not within the reach of the CLSM. An equation describing this phenomenon was presented and the parameters for this equation were determined for the fluorescently labeled symmetric membrane. I performed the analysis of the CLSM cross-sectional images and quantified the porosity at different depths within the membranes. Also, I observed that the porosities at different depths within the symmetric membrane were statistically the same, which was expected for this kind of membrane and validated my protocol. I showed that the porosity measurements, based on the CLSM image analysis, were not
affected by the selection of the fluorescent dye. These results showed that my cross-sectional CLSM imaging protocol is an effective tool to provide reliable morphology information of the whole membrane thickness.

In literature research presented in Chapter 1, I highlighted how confocal microscopy is a versatile technique for the characterization of membranes for different applications. Thus, the focus of the second phase of my project was to extend the application of the cross-sectional imaging protocol previously developed to characterize fouling and better understand the effect of membrane-solute and solute-solute interactions on membrane fouling. Through the application of the protocol, I located and quantified the amount of foulants within asymmetric microporous membranes in the wet state after the filtration of solutions containing them. I also demonstrated that the flux measurements of the single component and binary components synthetic feeds of casein and dextran presented different fouling behaviors when they were alone compared to when they were mixed in solution. Single component dextran solution was highly fouling despite its relative low molecular weight (10 kDa); whereas, the single component casein solution and the binary mixture of casein and dextran did not show significant fouling.

The change in fouling behavior for the mixed solution was attributed to association between casein and dextran, which was supported by increases in the apparent casein micelle size seen in DLS measurements. Intensity at different depths was quantified, and, based on these results, I generated intensity versus depth plots for the cross-sectional CLSM images. By using calibration plots, I was able to relate the intensity at different depths within the membranes to the amount of foulant present. Based on the results from
this study, it was demonstrated that CLSM image analysis is an effective complementary tool for the understanding of fouling. When combined with flux and DLS measurements, it was possible to better describe the fouling process and its root cause. Also, CLSM image analysis validated the results of flux data analysis with the Hermia model and provided a basis to hypothesize the fouling mechanism when the Hermia model failed to provide physically meaningful results. I demonstrated the applicability of the cross-sectional CLSM protocol to characterize membrane fouling and understand the root causes of fouling. Thus, this information can be used as feedback in the membrane design loop to tailor or modify the membrane structure or surface chemistry on those regions prone to foul.

Finally, I extended the application of the cross-sectional imaging protocol to the characterization of membrane fouling by ternary mixtures containing proteins, polyphenols and polysaccharides, emulating a beverage clarification process. Concentration of protein (casein) and polyphenol (tannic acid) were kept constant and the concentration of polysaccharide (β-cyclodextrin) was varied. Differentiation between foulants was achieved by using different fluorescent labels. Through this study, I showed that cross-sectional CLSM of the fouled membranes agreed with flux measurements. I also was able to determine the location of the foulants and quantify their concentrations throughout the entire thickness of the membrane. In a comprehensive set of experiments, I demonstrated that there is evidence of association between casein and tannic acid through DLS measurements, flux measurements and CLSM images. Furthermore, using flux data and CLSM images, I showed the effect of different concentrations of the
polysaccharide on the casein/tannic acid association. The results suggested that there was an appropriate concentration of polysaccharide to prevent the casein/tannic acid aggregation and recover the flux. The results obtained from CLSM image analysis were consistent with the flux data and results from Hermia model, proving once again the value of my protocol for the characterization of fouling as a complementary tool to the techniques implemented in this study along with CLSM.

Imaging techniques have been limited by surface artifacts, limited depth of imaging, imaging conditions that affect the sample features of interest, inability to differentiate between foulants, among others. During my PhD research, I was able to develop a versatile cross-sectional imaging protocol that overcomes these limitations, and I applied it to the effective characterization of membrane morphology and fouling.

5.2. Recommendations

As future work, I would suggest the application of the protocol in a more in-depth characterization of membrane morphology. For instance, a challenge that remains is the characterization of pore-size distribution data from the CLSM image analysis. It would be very interesting and useful to perform a comparative study between the information obtained from CLSM, microscopy techniques like SEM; and bubble-point, mercury intrusion, and liquid/gas permeability methods. Additionally, porosity and porometry data obtained from these techniques can be used to predict performance parameters and a comparison of the predicted and experimental performance data should be done in order to assess which technique provides the best prediction.
The versatility of CLSM and the cross-sectional imaging protocol proposed in this dissertation make possible their application to studies other than morphology and fouling characterization. For instance, it can be used in the characterization of surface chemistry. It would be interesting to perform a study to visualize how uniformly the binding groups are distributed throughout a membrane adsorber after surface modification by graft polymerization, as is used extensively by members of the Husson Group. Such knowledge may provide opportunities to improve modification strategies and increase membrane binding capacities. The experiment could be done with an appropriate fluorescent dye that specifically reacts covalently with the binding groups in the grafted polymers.

Also, the protocol developed in this dissertation for the cross-sectional CLSM imaging should be extended to characterization of UF, NF, RO membranes. Although, CLSM has a limit of resolution around 0.2 µm, making it impossible to characterize morphology directly for membranes with small pore sizes, it is possible to characterize fouling, performance and surface chemistry on those membranes.

It is suggested to extend the fouling characterization studies by using different kinds of membranes to get information regarding the role of the membrane structures and materials on fouling when processing proteins/polyphenols/polysaccharides mixtures. Also, it is suggested to perform a comparative study between the estimated amounts of foulant contained within the membrane based on CLSM image analysis and calibration curves and the results from a mass balance.
It was explained in Chapter 3 that a possible cause for not observing FRET was that the distance between the fluorescent tags was too far due to the relatively big size of the foulants and/or not enough extent of labeling of casein-FITC and dextran-Alexa Fluor® 594. If this happens, FRET will not be observed even though there is association happening between protein and polysaccharides. It is recommended to perform the labeling of the foulants in the laboratory instead of purchasing them already labeled to obtain a higher extent of labeling. Additionally, testing should be done to determine if higher extent of labeling affects the fouling behavior of proteins and polysaccharides, if tests reveal that fouling behavior is affected then it should be determined the maximum extent of labeling that does not significantly affect the behavior fouling of the proteins and polysaccharides. This will allow more in-depth exploration of FRET as a tool to explain interaction between components.

In the fouling characterization study presented in Chapter 4, only proteins and polysaccharides were fluorescently labeled and the polyphenol was not labeled. I suggest developing a labeling protocol for polyphenols (since they are not commercially available) and perform a more complete fouling study where the three components are labeled and visualize them within the fouled membrane. Also, it was mentioned in Chapter 4 that a possible mechanism by which the polysaccharide prevents the protein/polyphenol aggregation at the sweet spot concentration is by encapsulating the polyphenol. Labeling the polyphenol will allow to prove if the proposed mechanism is occurring. Also, it will allow us to understand what is happening at the concentrations of polysaccharide above and below the sweet spot. For instance, it was also mentioned that at the concentration
above the sweet spot, a ternary complex forms. By labeling the polyphenol, it will be possible to prove if the proposed mechanism is correct.

Multiphoton microscopy is an analog technology to confocal microscopy and it offers advantages over the confocal microscope such as higher depth of penetration and less photobleaching. Just recently, a multiphoton microscope has become available on campus. The protocols developed in this dissertation can be used in studies that use multiphoton microscopy instead of CLSM. Photobleaching can affect results that involve the quantification of intensity. Then, multiphoton microscopy is a suitable alternative to CLSM when the researcher is dealing with a system prone to photobleaching. For example, it is recommended to use multiphoton microscopy when using 5-DTAF as fluorescent dye since this fluorochrome is highly prone to photobleaching.
Appendix A

Sonication effect on dextran flux data

Fig. A-1. Permeate flux evolution for sonicated dextran (25 mg/L) (●).
Appendix B

Flux data for casein/dextran mixtures at different pH and ionic strength conditions

Fig. B-1. Permeate flux evolution for 50:50 (w/w) casein-dextran mixture (25 mg/L) at 0.125 ionic strength and three pH conditions (7, 6.25, 5.5).
Fig. B-2. Permeate flux evolution for 50:50 (w/w) casein-dextran mixture (25 mg/L) at 0.25 ionic strength and three pH conditions (7, 6.25, 5.5).
Appendix C

Description of FRET and immunoprecipitation assays

Fluorescence resonance energy transfer (FRET) experiments

In this study, fluorescein isothiocyanate (FITC) was used as a donor and tetramethyl isocyanate (TRITC) was used as an acceptor for the FRET experiment. Three binary component solutions were prepared with a final mixture concentration of 25 mg/L comprising 50:50 (w/w) protein-polysaccharide in phosphate buffer solution.

Solution 1 comprised FITC-labeled casein and unlabeled casein in a 1:20 ratio dissolved in phosphate buffer solution. Solution 2 comprised TRITC-labeled dextran (4.4 kDa molecular weight, Sigma Aldrich, T1037) and unlabeled dextran (from *Leuconostoc menesteroides*, 5 kDa molecular weight, Sigma Aldrich, 31417) in a 1:20 ratio dissolved in phosphate buffer solution. Solution 3 comprised FITC-labeled casein and casein in a 1:20 ratio and TRITC-labeled dextran and dextran in a 1:20 ratio dissolved in phosphate buffer solution.

Solutions 1 and 2 were used as control samples to assess bleed-through. Solution 3 was used as the sample for the FRET experiment. Three membrane samples were prepared by filtering 500 mL of each solution.

FRET experiment consisted in exciting the donor (FITC) with the 488 nm laser. Light emitted from FITC is able to excite the acceptor (TRITC) and the CLSM microscope is configured to detect only the light emitted by TRITC. If TRITC is visualized when illuminating the sample with the 488 nm is indication that FRET is happening between FITC and TRITC.
**Immunoprecipitation assays**

In order to assess interaction between casein and dextran, we performed a modified immunoprecipitation assay. We utilized magnetic Dynabeads coated with anti-rabbit antibodies (M280 sheep anti-rabbit IgG, Life Technologies, Carlsbad, CA) and proceeded with the manufacturer’s recommended protocol with minor modifications. Briefly, beads were vortex mixed, and ~4.5 x 10^7 were removed from the stock bottle and washed two times with PBS. To coat the beads with antibody, washed beads were divided into two populations and resuspended in 1 ml PBS, supplemented with rabbit anti-casein antibody (Abcam, Cambridge, MA) (~3.2 µg, a ratio recommended by Life Technologies) or an equivalent volume of buffer (~6.5 µL) as a control. Beads were incubated overnight at 4°C with rotation. Coated beads were then washed three times in PBS. Each set of beads was further divided into two populations, and these beads (+/- anti-casein antibody) were each incubated with 500 µL of dextran-Alexa Fluor® 594 solution (12.5 µg/ml) or buffer (control) for one hour at room temperature with rotation. After washing three times with PBS, beads (~1 x 10^7, as recommended by the manufacturer) were added to the wells of a 96 well plate and read on a microplate fluorescence reader using excitation/emission wavelengths of 530/635 nm (BioTek Flx800, Winooski, VT).
Appendix D

Calibration curves for casein-FITC and dextran-Alexa Fluor® 594

Fig. D-1. Calibration curve for casein-FITC.
Fig. D-2. Calibration curve for dextran-Alexa Fluor® 594.
Table D-1. Amount of casein deposited within PES membrane as a function of depth at different degrees of flux decline. Feed solution: single component (25 mg/L casein); binary component (12.5 mg/L casein, 12.5 mg/L dextran); pH 6.8.

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Table D-2. Amount of dextran deposited within PES membrane as a function of depth at different degrees of flux decline. Feed solution: single component (25 mg/L dextran); binary component (12.5 mg/L casein, 12.5 mg/L dextran); pH 6.8.

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Appendix E

Flux data for casein/catechin mixture

![Graph showing permeate flux evolution for casein/catechin binary component mixture (25 mg/L and 150 mg/L, respectively).](image)

**Fig. E-1.** Permeate flux evolution for casein/catechin binary component mixture (25 mg/L and 150 mg/L, respectively).
Appendix F

Flux data for mixtures containing pectin or xanthan gum

![Permeate flux evolution for polysaccharide single-component and protein/polysaccharide/polyphenol ternary mixtures.](image)

**Fig. F-1.** Permeate flux evolution for polysaccharide single-component and protein/polysaccharide/polyphenol ternary mixtures.
Appendix G

DLS data for casein/tannic acid/β-cyclodextrin ternary mixtures

Fig. G-1. DLS data for casein/tannic acid/β-cyclodextrin ternary mixtures.
Appendix H

Calibration curve for β-cyclodextrin-RITC

Fig. H-1. Calibration curve for β-cyclodextrin-RITC.
Appendix I

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