

**Instrument development for high sensitivity
size characterization of lipid vesicles and
other biological macromolecules via Taylor
dispersion analysis**

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“Commit to the Lord whatever you do, and he will establish your plans.” Proverbs 16:3

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Abstract

Just as humans communicate with other humans, the cells in our bodies communicate with each other through various, often complex, mechanisms. Cell-to-cell transmission of small molecules, lipids, proteins, peptides, or nucleic acids can be mediated by extracellular lipid vesicles called exosomes. Exosomes have been found to play a role in the delivery of regulatory molecules from one cell to another, serving as a universal communication mechanism. Currently, there is an emerging focus on characterizing exosome communication dynamics. Understanding exosome mechanisms of cell-to-cell communication requires accurate measurements of the spatiotemporal and chemical dynamics of exosome secretion. No current analytical approach offers the appropriate combination of spatial, temporal, and chemical resolutions needed to understand the dynamics of exosome communication at the tissue or organ level. The research outlined in this dissertation aims to bridge the gap between bulk fluid analysis of exosome cargo and single cell visualization of individual exosomes at a single point in time. To achieve better understanding of the dynamics of exosome secretion, there is a critical need for new analytical technologies. Without meeting this need, the roles of exosome communication in human health cannot be thoroughly understood. The long-term goal of this research is to develop tools to characterize communication between cellular networks, and model mechanisms of disrupted pathways with significance to neurological disorders. The primary objective is to develop an assay for characterizing temporal and chemical dynamics of exosome secretion.

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Chapter 1

Introduction

1.1 Biological Motivation and Analytical Challenge

1.1.1 Extracellular communication

Our cells communicate through various, often complex, mechanisms. Cellular communication can be both localized, where cells directly interact with one another, or distant, where various types of cargos are transported through the extracellular fluid to target cells. Endocrine signaling occurs when small molecules, lipids or proteins are released from one cell through the bloodstream often to bind receptors on a target cell, inducing intracellular cascades of response signals [1, 2]. Eukaryotic cells can also release membrane vesicles that transport encapsulated small molecules, proteins, or oligonucleotides directly to the intracellular space of a recipient cell, providing more complex routes of communication. Different classifications of extracellular vesicles exist based on their size and biogenesis. One type of vesicle, known as microvesicles, can be generated by the budding of the plasma membrane [3]. Apoptotic bodies are other types of vesicles generated by the random blebbing of the plasma membrane during cellular apoptosis. The smallest classification of extracellular vesicles is referred to as exosomes, which begin as intraluminal vesicles found in multivesicular bodies within the cell and are secreted into the extracellular space. For some diseases, the value may be found in certain proteins or microvesicles. For most diseases, including various types of cancer, there has been extreme interest and value in studying the secretion and communication dynamics of exosomes.

1.1.2 Exosome biogenesis

Exosome biogenesis, illustrated in Fig. 1.1, begins with the inward budding of cellular endosomes called multivesicular bodies (MVBs). MVBs have several possible fates, including fusion with lysosomes or fusion with the plasma membrane [4, 5]. Fusion with lysosomes results in the degradation of encapsulated materials, while fusion with the plasma membrane of the cell causes the release of intraluminal vesicles into the extracellular space [4]. These released vesicles are called exosomes and they differ from other extracellular vesicles, such as apoptotic bodies or shedding vesicles, in two key ways. First, exosomes have a narrow size distribution ranging from 50 - 100 nm in diameter, as compared to the broad 50 nm – 2 μ m diameter range of other

extracellular vesicles [6]. Second, exosomes often carry specific membrane protein biomarkers, the most common of which is the tetraspanin CD63 [7, 8]. Early hypotheses that exosome release is a mechanism only for cellular waste removal were disproved when exosomes originating from lymphocyte B cells and carrying antigenic peptides were shown to activate T cell immune responses [9]. This discovery, along with the ubiquity of exosomes in eukaryotic cells, sparked interest in characterizing the role of exosomes in endocrine communication.

1.1.3 Exosomes as diagnostic biomarkers

The inward budding of cellular endosomes results in the potential for incorporation of various components found in the cytoplasm [10]. These components may include, but are not limited to, proteins, lipids, or oligonucleotides. Fusion of MVBs with the cellular membrane facilitates the release of exosomes containing various types of cargo, under both normal and abnormal biological conditions. This ability facilitates the transport of important regulatory molecules to or from specific tissues or organs. Because of their ability to transport regulatory materials from cell to cell, exosomes may also serve as a vehicle for disease-associated cargo. Exosomes have recently been identified as potential sources of disease biomarkers due to their diverse range of encapsulated materials, including various forms of RNA [8, 11]. This is especially promising since a variety of RNA subtypes regulate gene expression. Small interfering RNA (siRNA) and microRNA (miRNA) have shown involvement in RNA interference, which silences regulatory genes and can lead to the progression of disease states [12]. Moreover, sequencing mRNA and miRNA found in exosomes from a biofluid can help identify the tissue of origin, aiding in accurate diagnostics. Recently, more attention has been focused on exosomes containing single and double stranded DNAs, specifically tumor-specific oncogenic mutant DNA [13]. DNA species can also be present on the surface of exosomes. This is important diagnostics and therapeutics because the role and function of exosomes is dependent on the originating cell or tissue status [14]. Exosomal surface proteins, such as tetraspanins, may also be viewed as potential disease biomarkers. For example, elevated expression of CD81 is associated with severe fibrosis and inflammation, making it a useful biomarker for the diagnosis and/or treatment of hepatitis C [14]. Based on the variety of materials that exosomes can transport from one cell to another and the cell type from which they

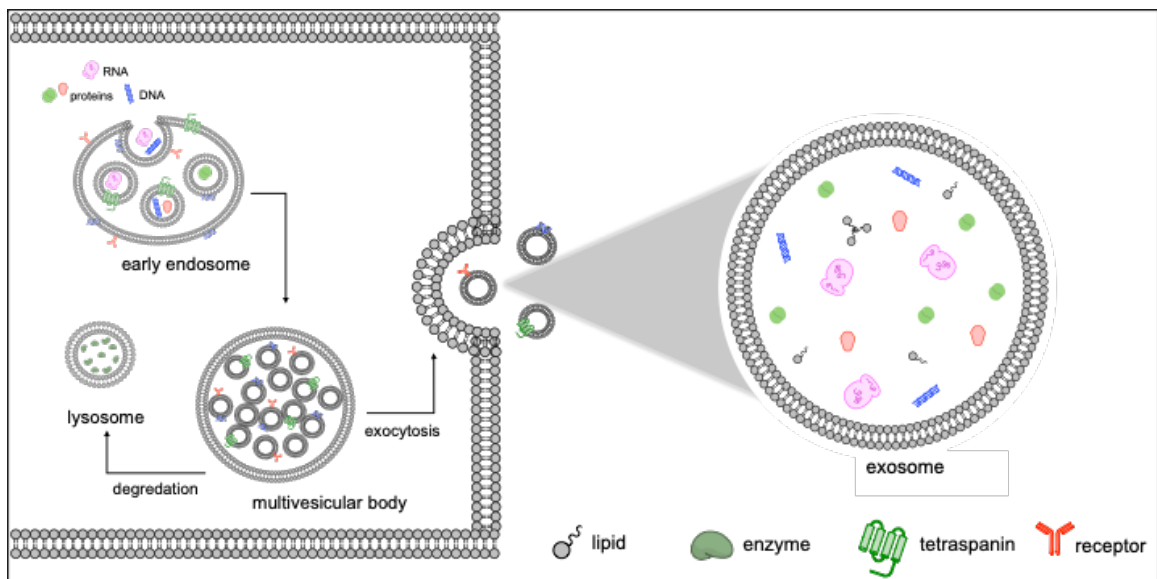


Figure 1.1: Illustration of exosome biogenesis, including generation via inward budding of early endosomes and release by exocytosis of multivesicular bodies. Potential surface markers and intracellular contents are also illustrated.

were released, the endocrine roles of exosomes are likely broad and are not well understood.

1.1.4 Exosome Characterization

1.1.4.1 Biophysical characterization

There are currently several techniques that have been employed for exosome size characterization. One approach to characterize the size range of exosomes is nanoparticle tracking analysis (NTA). NTA is an optical microscopy technique that involves the observation of the displacement of particles or molecules undergoing Brownian motion by video imaging [15]. Particle movement can then be correlated to size, concentration, and phenotype [14]. Another method for evaluation of exosomal size distribution is dynamic light scattering (DLS). The working principle behind this method is that light passes through a sample of particles suspended in solution. As a result of the Brownian motion of the particles, the light is scattered and produces scattering intensities relative to the position of the particles. Time-dependent fluctuations in these scattering intensities is monitored and the decay rate of the time correlation function is used to ultimately determine hydrodynamic radius of the particles in solution [16]. DLS offers size determinations of particles as small as 1 nm [14], rapid analysis times, and a simple sample preparation protocol. However, the ultimate limitation of DLS is its bias towards larger particles and aggregates, as signal intensity is proportional to the radius raised to the 6th power according to the Rayleigh approximation [17]:

$$\%I_a = \frac{a^6 N_a * 100}{N_a a^6 + N_b b^6} \quad (1.1)$$

where N_a and N_b are particles with radius a and b , and $\%I_a$ is the intensity-weighted distribution for particles with radius a . Thus, detection sensitivity is limited when characterizing small particles.

High resolution imaging techniques can also be employed for exosomal size characterization. For example, Transmission Electron Microscopy (TEM) provides structural information through visualization of size and morphology. Here, a beam of electrons interact with the particle sample as it passes through, producing secondary electrons and forming an image that is magnified and focused with special lenses onto an imaging device [14]. The biggest limitation to this method

is the sample preparation, as samples must be fixed, dehydrated, and placed under vacuum for imaging. While this technique offers high-resolution visualization of particle diameters and shapes, electron beams may alter morphology or even damage the sample.

In addition to size and structure, other biophysical properties of exosomes, such as the presence of surface proteins, may also be characterized by molecular approaches. One common method for this is flow cytometry, where particles with fluorescent labels are sized based on the degree of light scattering from a laser source. While conventional flow cytometers do not allow size detection below 300 nm, newer generations offer improved resolution that allows size detection of extracellular vesicles, like exosomes, smaller than 300 nm [14].

1.1.4.2 Biochemical characterization

The most common approach to characterizing exosome membrane and intra-vesicular proteins is mass spectrometry (MS)-based analysis of exosomes purified from bulk biofluids. In this approach, proteomic signatures are identified by comparing spectra to protein sequence databases [18]. MS has allowed for extracellular vesicle protein profiling for several diseases, including various types of cancer [19]. Though MS offers high chemical resolution, the biggest limitation for MS methods is the bulky instrumentation and required large sample volumes (ca. 10 mL) [20].

An alternate approach for evaluating and quantifying membrane surface and intra-vesicular proteins in exosomes is Western blotting. This involves lysis, gel electrophoresis, and transfer to blotting membrane for protein detection via antibodies [20]. The primary disadvantage with this technique is the extensive sample preparation, which often hinders reliability and reproducibility [21].

1.1.5 Analytical Challenge

Though there is a variety of biophysical, molecular, immunoaffinity and microfluidic approaches to characterize exosomes [22], each technique comes with its own set of limitations for the study of exosome secretion dynamics at the tissue or organ level with appropriate spatiotemporal resolution.

The majority of challenges with current techniques stems from the rigorous procedures involved in isolation and purification of exosomes from bulk fluids. Exosome isolation is challenging due to their small, specific, and narrow size distribution. The most conventional isolation technique is ultracentrifugation, which requires multiple cycles of centrifugation steps, high centrifugal forces, and larger sample volumes [14]. Limitations in sensitivity and limits of detection, as with MS techniques, necessitate the pre-concentration of exosomes from large volumes of biofluids by centrifugation techniques [23], ultimately limiting temporal and spatial resolutions. This is particularly limiting in cell and tissue perfusion experiments, where lengthy perfusion times or large tissue samples would be needed to collect sufficient quantities of secreted exosomes for mass spectral analysis. High sensitivity analyses that eliminate the need for exosome purification and pre-concentration are needed to achieve the spatiotemporal resolution required to understand the dynamics of exosome communication.

In contrast to the poor spatial resolution of bulk fluid analysis by mass spectrometry, microscopy can provide subcellular spatial resolution in studying exosome secretion. For example, high-resolution imaging such as Scanning Electron Microscopy (SEM) and TEM are established tools for analyzing particle size, morphology, and morphological processes at the single cell level. Van der Pol *et al.* evaluated several methods commonly used in single vesicle analysis and determined that TEM provided the greatest imaging resolution [24]. However, TEM offers limited chemical resolution, and high spatial resolution comes at the expense of limited imaging area. Thus, such methods do not offer the appropriate spatial resolution to study populations of cells together in cultured tissues and are not suitable for characterizing dynamic processes in living cells. Optical microscopy methods can extend imaging techniques to investigate dynamic living systems but similarly are limited spatially to a small number of cells. Van der Pol *et al.* also discuss exosome characterization challenges inherent in optical techniques, such as non-linear relationships between exosome size and intensity of scattered light [25]. Studying small populations of cells by imaging techniques obfuscates emergent properties of tissue and organ-level biological systems. Understanding these emergent properties is essential to determining the mechanisms of exosome communication that underlie normal and diseased biology.

Existing techniques allow the identification of exosome contents from bulk fluids, and the observation of secretion processes at the single cell level, but characterizing the spatiotemporal dynamics of exosome secretion from complex biological systems, such as primary tissue cultures, is not readily achieved with current technology. Thus, there is a critical need to bridge the gap of spatial and temporal scale between bulk fluid analysis of exosome cargo and single-cell imaging of exosome secretion.

1.2 Liposome application for exosome characterization in solution

1.2.1 Introduction

Previous sections have discussed the biological motivation and importance behind the primary objective of this dissertation, which is to target exosomes in bulk biological fluids for analysis of their contents to understand their endocrine roles in cellular communication. Appropriate levels of spatial, chemical, and temporal resolution for biochemical and biophysical characterization of exosomes cannot be readily achieved with current technologies. In Chapter 2, exosome content characterization is explored by the development of an analytical assay that utilizes the safe and selective delivery of assay reagents to biological exosomes in solution, without the need for purification. The delivery of assay reagents is facilitated by synthetically prepared lipid vesicles (liposomes) and is discussed in greater detail in Chapter 2.

1.2.2 Liposome Applications and Preparation

Lipids are composed of polar (hydrophilic) head groups and hydrophobic fatty acid tails. In water, lipids self-assemble so that the hydrophilic head groups are directed toward the aqueous phase and the hydrophobic tails are oriented towards each other, forming a fluid-filled, spherical lipid vesicle, or liposome. Because of their structure, liposomes have the unique ability to encapsulate and embed various types of molecules, drugs, or nanoparticles. Thus, they are commonly used as carriers for drug delivery systems and biosensors [26]. Because of this, liposomes have proven

applications in analytical separations [27, 28, 29, 30], electrochemical biosensors [31, 32, 33, 34], cosmetics [35, 36, 37], food research [38, 39], pharmaceuticals and clinical trials [40, 41, 42], and much more.

Liposomes are classified based on their size and lamellarity: small unilamellar vesicles (SUVs, 25 – 50 nm), large unilamellar vesicles (LUVs, 100 nm – 1 μm), giant unilamellar vesicles (GUVs, 1 – 200 μm), multilamellar vesicles (MLVs, 0.1 – 15 μm) and multivesicular vesicles (MVVs, 1.6 – 10.5 μm) [26] (Fig. 1.2). The formulation of liposomes is dependent on various techniques including, but not limited to, thin film hydration, reverse phase evaporation, detergent dialysis, freeze-thaw, membrane extrusion, heating, and sonication [43]. The selected method used for the preparation of liposomes described in this dissertation is a combination of freeze-thaw, sonication, and membrane extrusion.

First, a liposome suspension is prepared via the hydration of a lipophilic powder. In solution, lipids spontaneously form a heterogeneous mixture of liposomes. To reduce lamellarity and induce encapsulation, freeze-thaw cycling is performed. This method involves freezing the liposome solution with a dry ice bath or liquid nitrogen followed by thawing at a temperature higher than the lipid phase transition temperature [44, 45]. During this process, freezing disrupts the lipid bilayer and thawing induces vesicle fusion, resulting typically in large unilamellar vesicles. Large, multilamellar liposomes can be disrupted to produce small, unilamellar liposomes (ca. 15 - 50 nm in diameter) by sonication. To prepare liposomes of a specific size, membrane extrusion is then performed. This method involves passing the liposome solution through a porous membrane with defined pore size ranging from low nanometer to micrometer diameters. After several passes through the membrane, a homogeneous solution of liposomes is produced.

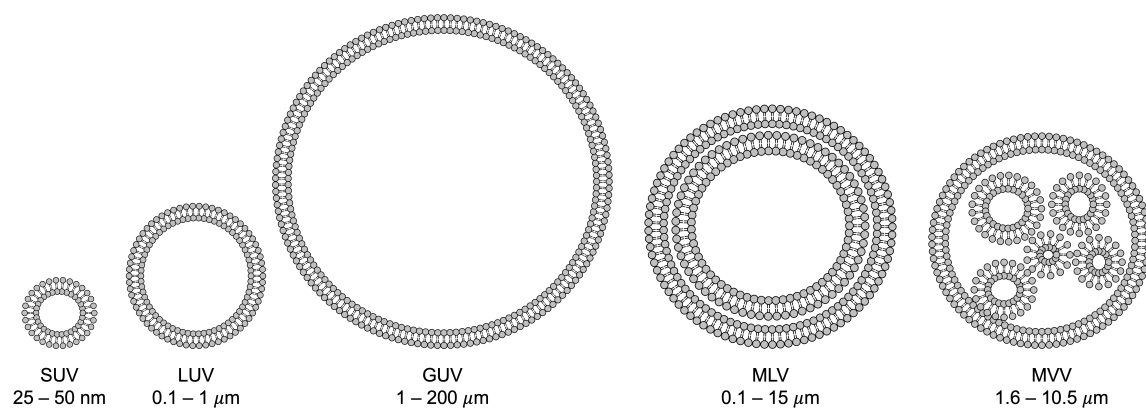


Figure 1.2: Illustration of size and lamellarity of the different classifications of liposomes.

1.2.3 Analytical Challenges

The preparation and application of liposomes encapsulating analytical reagents will facilitate selective detection of exosome membrane proteins and high sensitivity detection of exosome contents. Chapter 2 discusses a model system used for molecular recognition-driven vesicle fusion and the analytical challenges that arose from preliminary experiments. As the chapter summary highlights, the main challenge for the developed system involved the inability to fully understand our system. This redirected focus to making size-based characterizations of low-volume samples.

1.3 Taylor dispersion analysis for high sensitivity size characterization

Dynamic light scattering (DLS) and size exclusion chromatography (SEC) are gold standard methods for size characterization of macromolecules and particles. However, both warrant analytical challenges for characterizing the size of low-volume, low-concentration biological samples. In Chapters 3 and 4, the need for more accurate, sensitive, and straightforward tools for size characterization is discussed in depth. Taylor dispersion analysis, a promising analytical tool for size characterization, is reviewed extensively in Chapter 3, and the development of a 3D printed instrument for TDA is presented in Chapter 4.

1.3.1 Theory

Taylor dispersion analysis (TDA), first described in 1953 [46], enables direct determination of molecular diffusion coefficients across a wide dynamic range of hydrodynamic radii. TDA is advantageous over the more common sizing methods described above because it requires no calibration, purification, or knowledge of sample concentration [47]. Furthermore, TDA can be performed within small diameter fused silica capillaries, requiring only sub-nL sample volumes. Taylor dispersion itself is a phenomenon that occurs under well controlled pressure driven laminar flow where molecules of different size behave differently within the parabolic velocity profile. An injected sample plug spreads out axially, which is observed by band broadening. The behavior

of the analyte and degree of band broadening that results is directly related to its size. It is important to note that TDA is conducted under well-controlled flow conditions in which only band broadening from radial diffusion is considered and contributions from longitudinal diffusion is neglected. The first flow condition requires the rate of advection to be substantially greater than the rate of diffusion, which is satisfied when the Peclet number (Pe) is greater than 69 [48], defined by the following equation [49]:

$$Pe = \frac{uR_c}{D} \quad (1.2)$$

where u is the linear flow rate, R_c is the radius of the capillary, and D is the molecular diffusion coefficient. The second flow condition requires the sample to remain in flow long enough to observe sufficient band-broadening due to Taylor dispersion [46]. This condition is satisfied when $\tau > 1.4$, where τ is defined as:

$$\tau = \frac{Dt_R}{R_c^2} \quad (1.3)$$

where t_R is the requisite residence time of the sample plug in flow. If two detection points are employed, band broadening under continuous flow can be compared and D can be determined by [50]:

$$D = \frac{R_c^2(t_2 - t_1)}{24(\sigma_2^2 - \sigma_1^2)} \quad (1.4)$$

where t_1 and t_2 are the peak arrival times at detectors 1 and 2, respectively, and σ_1^2 and σ_2^2 are the peak variances as observed at detectors 1 and 2, respectively. From here, D can be transformed to hydrodynamic radius (R_H) by the Stokes-Einstein relation [51]:

$$R_H = \frac{k_B T}{6\pi\eta D} \quad (1.5)$$

where k_B is the Boltzmann constant, T is temperature, and η is the dynamic viscosity of the solution.

Principles, practical considerations, and applications of TDA are discussed in greater detail in Chapter 3.

1.3.2 Detection modes

1.3.2.1 UV-Vis absorbance

The most common detection mode used for TDA has been UV-Vis absorbance. For absorbance detection, a continuous light source, such as a deuterium, hydrogen, or halogen lamp, with power P_0 is directed through a detection cell or window. The intensity of light transmitted is reduced to P and used to calculate the absorbance of the sample solution. There is a linear relationship between the sample's concentration (c) and corresponding absorbance (A), given by Beer's law [52]:

$$A = -\log(T) = \log\left(\frac{P_0}{P}\right) = \varepsilon b c \quad (1.6)$$

where T is transmittance, ε is molar absorptivity, and b is the pathlength of the detection cell or window.

Absorbance detection is advantageous due to its versatility to a variety of molecules that absorb at a specified wavelength, label-free. However, label-free detection modes are inherently less selective, which could be disadvantageous when analyzing mixtures or analytes that have broad absorbance spectra. Another major disadvantage for absorbance detection in this setting is the pathlength, which is dependent on capillary diameter (typically 25 - 100 μm). According to Beer's law, absorbance decreases with pathlength. Additionally, absorbance is also proportional to sample concentration. Thus, absorbance detection modes often lead to sensitivity issues when analysis of low concentration samples is desired.

1.3.2.2 Fluorescence detection

For improved sensitivity and selectivity of target molecules, fluorescence detection has been employed for TDA, yet is not a widely adopted detection mode for this technique. Fluorescence methods typically use light sources that are monochromatic or highly tunable, such as mercury lamps, light-emitting diodes (LEDs), and lasers. These more intense light sources are necessary to provide a high number of photons with the specific wavelengths needed to induce fluorescence of a molecule. During fluorescence, high energy photons excite the electrons of a fluorescent molecule from their native ground state to a higher energy excited state upon absorbance. These electrons

lose vibrational energy and emit secondary photons at a lesser energy and longer wavelength on their way back down to the ground state. Unlike absorbance detection, fluorescence detection allows specificity by targeting a specific fluorescent molecule with its excitation wavelength. Fluorescence signal intensity can be dependent on the photophysical properties of the molecule, such as quantum yield [53]. Fluorescence quantum yield (Φ) is described by the ratio of emitted photons to absorbed photons:

$$\Phi = \frac{\text{emitted photons}}{\text{absorbed photons}} \quad (1.7)$$

Along with quantum efficiency, fluorescence intensity (F) is dependent on power of the excitation beam absorbed by the molecule, estimated by [52]:

$$F \approx \phi (P_0 - P) \quad (1.8)$$

where P_0 is the incident beam power and P is the power after transmittance. Substituting a form of Beer's law into this approximation equation, we get

$$F \approx \phi A P_0 \approx \phi \epsilon b c P_0 \quad (1.9)$$

where A is absorbance, ϵ is molar absorptivity, b is pathlength, and c is concentration. From this it is apparent that fluorescence intensity is also directly proportional to the sample concentration and the pathlength of the system.

Of the existing excitation sources, laser-induced fluorescence (LIF) detection is most easily adaptable to small pathlength systems, such as capillaries or microfluidic devices, as laser beams exhibit low divergence and are easily focused on small detection volumes with high irradiation [54]. Because of this, LIF limits of detection are lower than most other detection systems.

A primary benefit of fluorescence detection over absorbance detection is its high sensitivity resolution in low background noise. However, background signal can still potentially dominate, for example, via scattered excitation. In order to maximize signal-to-noise ratio, careful consideration must be made to reject sources of background via optical filtering. For a simple fluorescence

detection system, a bandpass filter is needed to collect the desired emission wavelength while blocking wavelengths that lie outside its range. Despite this effort, small percentages of unwanted light can still be transmitted through the filter and collected by the detector, contributing to background. The relative amount of light able to pass through a filter can be described by a property of the filter called optical density (OD). The OD value of a filter indicates how much optical power is reduced from the light source and is related to transmission (T) by [55]

$$OD = \log_{10}\left(\frac{1}{T}\right) \quad (1.10)$$

or

$$T = 10^{-OD} \quad (1.11)$$

where T represents a percentage. The higher the OD value, the less transmittance of wavelengths outside of the filter range. Nonetheless, further filtering can add to the efforts of rejecting excess background. If ever the background noise is substantial enough to exceed the dynamic range of the detector, a neutral density (ND) filter can be added to reduce the overall intensity of light reaching the detector. A common source of background is Raman scatter of the buffer or solvent. To reduce this contribution, short-pass or long-pass filters can be incorporated, so long as Raman scatter is being blocked and the desired emission wavelength is accepted. With the addition of the above mentioned filters, wavelengths outside of the bandpass filter wavelength range and Raman scattering are highly attenuated, but laser scatter may still be a significant hindrance. To reject excess laser scatter, a high OD notch filter can be added to filter out the wavelength specific to the laser. This also reduces background by reducing the shot noise and flicker noise associated with a laser source [53]. Additionally, spatial filtering methods may also be used in order to attenuate the beam of the laser source to reduce scatter.

1.3.3 TDA instrumentation

TDA instrumentation primarily involves a capillary with one or two detection regions and a flow source. Currently, there are few automated capillary systems commercially available that

have sizing capabilities based on Taylor dispersion. The Viscosizer TD (Malvern Panalytical) and the Fida 1 (Fidabio) are two popular instruments, which operate using Poiseuille's law and Taylor dispersion to separately determine viscosity and hydrodynamic radius. To make these measurements, the Viscosizer TD has been reported to use 7 μL per injection for samples ranging from 2 - 5 mg mL^{-1} [56]. Despite the availability of these automated systems, TDA is most commonly performed within capillary electrophoresis (CE) instrumentation. This is primarily due to the reduced analysis times, improved precision of flow rate control, and low sample consumption. The evolution of TDA towards commercial CE instrumentation has presented several engineering challenges, including the integration of dual-point detection. Though there have been some advances to address these challenges [57, 58], the use of CE instrumentation for TDA is also limited to the expense of additional detection modes aside from routine UV-Vis absorbance. While it is most common for TDA to be performed using UV-Vis absorbance, TDA can also be coupled to other detection modes such as fluorescence detection [58], mass spectrometry [59, 60], refractive index [61], and backscattering interferometry [62].

Ultimately, TDA can be easily and simply implemented into a variety of scientific studies, which may be acquiescent to the development of low-cost, open-source hardware that will enable adoption of this analytical method. To deliver improved instrumentation for performing TDA in capillaries, the development and optimization of a 3D printed TDA instrument is discussed later in Chapter 4.

1.3.3.1 3D printing

3D printing has emerged as a transformative manufacturing and fabrication tool for countless applications. The process involves printing complex structures and geometries one layer at a time using a variety of methods, materials, and equipment [63]. The ability to fabricate customized products often with complex geometries at a low cost makes 3D printing desirable for various industries such as construction, dentistry, biomedical, microfluidics. Some common methods for 3D printing include: fused deposition modelling (FDM), selective laser sintering (SLS), inkjet

printing, and stereolithography (SLA).

Developed in 1986, SLA is the earliest commercially available solid freeform technique [64], and is the selected 3D printing method used in this research. Stereolithography uses UV-light to locally polymerize and solidify UV-active acrylic or epoxy-based resin layer-by-layer. Depending on the apparatus, a laser beam or LCD screen patterns the structure to a build platform from the bottom up. Once the first layer is UV-cured to the platform, the next layer is patterned and cured to the first, until the 3D structure is complete. While most fabrication techniques offer resolution between 50 - 200 μm , commercial SLA products allow fabrication down to 20 μm and even sub- μm for two-photon initiation setups [64].

1.3.4 Immunoassays

One primary application of the developed assay for exosome characterization outlined in Chapter 2 and the TDA instrument described in Chapter 4 is the utility of immunoassays for highly selective detection of exosome membrane proteins. Molecular recognition of exosome membrane proteins will likely be carried out with antibody-conjugated liposomes. Additionally, selective targeting of exosome membrane proteins via antibodies can be monitored by TDA with the developed instrument. These potential avenues will be explored and discussed in more detail in Chapter 5.

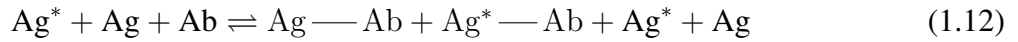
1.3.4.1 Principles

The basic principle behind an immunoassay is based on the highly specific molecular recognition of antigens and antibodies [65], and the corresponding reversible binding reaction. Here, the antibody binds to the antigen to form an immunocomplex. Types of immunoassays can be broken down into labelled and unlabelled assays, depending on the measurability of the binding reaction [66]. For a labelled immunoassay, either the free antibody or antigen is labelled and measured against the formed immunocomplex. Selecting which species to label depends on the desired measurement or technique used for detection and quantification, and can be broken down into

competitive and non-competitive systems.

1.3.4.2 Competitive vs Non-competitive

In competitive immunoassays, the unlabelled target antigen competes with a labelled similar antigen to bind the same antibody. Once an equilibrium is reached, the amount of bound, labelled immunocomplex is inversely proportionate to the concentration of the unlabelled, target antigen present in the sample [67]. This concept can be described by the following:



where Ag is the antigen, Ab is the antibody, and $*$ is the label (Fig. 1.3A).

In non-competitive immunoassays, an excess amount of labelled antibody binds the target antigen and forms a bound, labelled immunocomplex. Here, the amount of bound, labelled immunocomplex is directly proportionate to the concentration of target antigen in the sample [67]. This concept can be described by the following:



where Ag is the antigen, Ab is the antibody, and $*$ is the label (Fig. 1.3B).

1.3.4.3 Introduction to TDA Immunoassays (TDA-IA)

Though there are no specific molecular biomarkers that distinguish exosomes from other extracellular vesicles, exosome membranes are often enriched with certain tetraspanin proteins, the most common of which are CD9, CD63, and CD81 [13]. Thus, they can be utilized for the detection and quantification of exosomes via the combination of TDA and an immunoassay (TDA-IA).

The average immunoglobulin G (IgG) antibody, found in blood, is approximately 5 - 6 nm in hydrodynamic radius [68, 69] while the exosomes we are interested in measuring are around 25 - 50 nm in hydrodynamic radius, and the expected radius of the immunocomplex is similar to

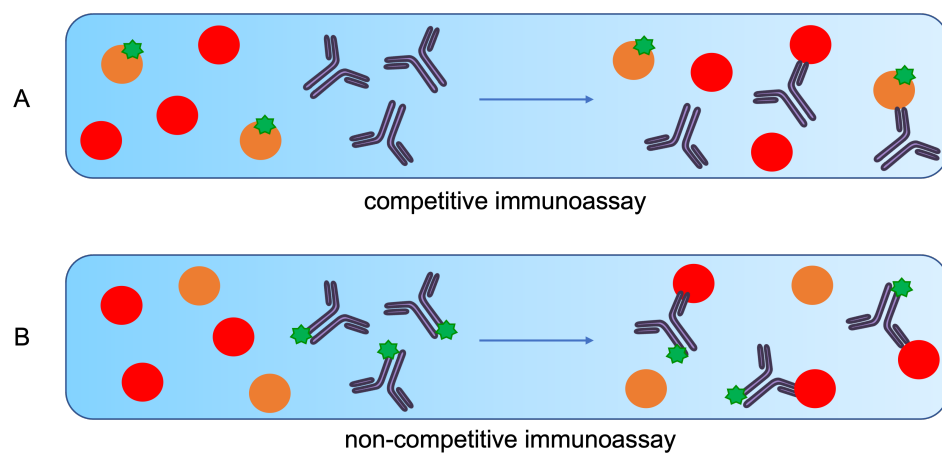


Figure 1.3: Illustration of competitive vs non-competitive immunoassays.

that of the exosomes (ca. 25 - 55 nm). Thus, the proposed immunoassay will employ the non-competitive format, where the antibody is labeled and the amount of exosomes (antigen) can be directly measured based on the amount of formed antibody-exosome complex. This format offers an advantage over a competitive format because a distinct difference in size measurements can be made from free, labeled antibody and bound, labeled complex. If the competitive format were employed, there would be a need for distinguishing between two species of similar hydrodynamic radius (free exosome vs antibody-exosome complex). Thus, the concept of this TDA-IA involves size characterizations of a sample of a fluorescently labeled antibody specific to one of the above mentioned exosome membrane proteins and of this antibody added to a sample of purified exosomes.

Further discussion of this TDA-IA is included in Chapter 5.3. There, a potential design of experiments will be outlined and preliminary data will be provided.

1.4 Conclusion

This chapter provides an introduction to the biological background and motivation of this research, analytical challenges, and methodology used in the following chapters. The concepts described above will work together towards a common research goal: developing cost-effective analytical tools that allow the characterization of exosome size and contents without the need for purification in limited volume, low concentration biological samples. In Chapter 2, an outline of the progress made towards this goal will be described, while a description of analytical challenges that arose will be given. In Chapter 3 we review fundamental principles and progress in the area of TDA, since this technique forms an important part of our approach to circumvent the challenges described in the previous chapter, as well as to contribute to current size characterization technologies. In Chapter 4, the development of a 3D printed instrument for performing TDA in fused silica capillaries is discussed. Finally, Chapter 5 will conclude this dissertation with a discussion of future work that is needed to progress the research. Preliminary results for future work will also be presented and discussed.

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Chapter 2

Towards Development of Fluorescence Assays in a Model of Molecular Recognition-driven Vesicle Fusion

2.1 Abstract

The primary limitation in current approaches in exosome isolation and purification is the inability to distinguish between other extracellular vesicles and molecules in biological fluids. Additionally, membrane proteins that commonly enrich the surface of exosomes cannot be used as universal biomarkers for exosomes. More accurate methods and tools for characterization of exosome contents is necessary for understand their communication dynamics. This chapter describes the development of a novel analytical assay utilizing synthetic lipid vesicles to deliver assay reagents to exosomes in bulk biofluids, without the need for purification. Preliminary work is provided and discussed, as well as potential pitfalls and how they may be remedied.

2.2 Introduction

The importance of establishing a universal, reliable, and reproducible isolation and characterization method for exosome research has been discussed. The greatest limitation in current approaches in exosome isolation is the inability to distinguish between the different populations of extracellular vesicles (i.e. exosome, apoptotic bodies, microvesicles, etc.) with diameters less than 200 μm [1]. Because of this limitation, analyses are typically performed on these extracellular vesicles, rather than a purified population of exosomes. An added dimension of distinguishing between exosomes and other small extracellular vesicles is the targeting of specific tetraspanin proteins (most commonly CD63, CD81, and CD9) as identifying biomarkers [1]. It is important to note that these proteins are also expressed on the surface of the cell and other types of extracellular vesicles, and are also not present in all exosomal membranes. Thus, a single biomarker does not exist to selectively identify an exosome from all other vesicles. Because of these limitations, there is currently no established isolation method for exosomes from other extracellular vesicles of similar size or biological components of similar composition. A more accurate method for exosome isolation and characterization would provide a better understanding of the endocrine roles of exosomes, further aiding diagnostic and therapeutic applications [2].

2.2.1 Research Objective

Eliminating the need for purification of exosomes from bulk biological fluid poses the challenge of distinguishing between molecules that are encapsulated by exosomes and un-encapsulated molecules of similar composition that are present in the extracellular environment. To address this challenge, we propose a mechanism for the delivery of exosome contents to the intraluminal volume of synthetic lipid vesicles that encapsulate assay reagents. Analytical assays can take place within the encapsulated volume without interacting with extracellular molecules. The success of this approach will depend on optimized assay conditions and the efficacy of molecular recognition and detection modes. These factors will serve as the basis for the development of fluorescence assays in a model of molecular recognition-driven vesicle fusion. This assay will utilize the fusion of exosomes with synthetically prepared lipid vesicles that encapsulate analytical reagents. Vesicle fusion can facilitate encapsulated analytical reactions and high sensitivity detection of vesicle contents. Small unilamellar vesicles (SUVs) with diameters on the order of 10 - 100 nm readily fuse with one another to reduce energetic strain caused by the high degree of membrane curvature. When the vesicles fuse, their contents mix, enabling analytical reactions to assay encapsulated biomolecules. This system can be functionalized for molecular recognition of exosome membrane proteins, offering specificity for assaying exosome contents. Vesicles can be prepared synthetically to encapsulate, for example, fluorescamine, which reacts with primary amines to form a fluorescent product. Thus, when the synthetic vesicle and exosome fuse, directed by molecular recognition processes, their contents mix and produce a quantitative fluorescence signal.

2.2.2 Spontaneous Vesicle Fusion

For two vesicle membranes to fuse, they must be brought into close proximity, make initial contact, and have enough energy to overcome the fusion energy barrier [3]. A vesicle's inclination to fuse with another vesicle or membrane is dependent on lipid composition and the surface curvature at the point of contact [4]. Vesicle curvature is dependent on diameter, as smaller diameter vesicles (i.e. 50 nm) have greater surface curvature and a higher energy strain than larger diameter vesicles (i.e. 1 μ m). In a study of vesicle fusion simulation models, Smeijers *et. al* observed that the connection between two vesicles (large or small) was made when the intervesicle distance was

between 1.3 - 1.7 nm [4]. After initial contact, spontaneous fusion is initiated by one of several accepted pathways. One pathway, deemed the stalk-pore hypothesis, suggests that the outer monolayer of the vesicles form a stalk, which expands radially to connect the inner monolayer of the vesicles [5]. A fusion pore is formed and grows until fusion is complete. Other hypotheses argue over the simultaneous versus independent formation of the stalk and fusion pore.

2.3 Preliminary Development

2.3.1 Design of model system

The primary objective of this research was to analyze exosome contents by safely delivering synthetic vesicles that encapsulate assay reagents to exosomes *in situ*. This system was modeled by preparing two sets of vesicles that encapsulate analytical reagents that mix to produce a measurable signal upon vesicle fusion. The design of the model system addresses two key points: modelling molecular recognition and modelling detection. Molecular recognition of exosomal surface proteins, with antibodies or other molecular recognition approaches, were modeled by exploiting the strong protein-ligand interaction of a biotin-avidin complex. Avidin is a protein derived from eggs of oviparous vertebrates [6] that has 4 binding domains and a strong affinity to biotin. To prepare vesicles for molecular recognition, lipids with biotinylated head groups were incorporated into the vesicle membrane. An avidin molecule was then able to bind up to four biotin-conjugated vesicles. The biotin-avidin interaction facilitated driving the bound vesicles to close proximity and high vesicle surface tension induced fusion. To model detection of potential exosomal contents, the vesicles were prepared to encapsulate two separate assay reagents to produce a fluorescent signal once combined. It is known that exosomes may contain specific RNA subtypes [7, 8], and single- and double-stranded DNA [1], thus the selected model assay reagents include DNA and an intercalating dye. When combined, SYBR green intercalates and binds the minor groove of the DNA backbone. As a result, a fluorescent signal ($\lambda_{ex} = 497$ nm, $\lambda_{em} = 520$ nm) was observed. The complete model system consisted of two prepared sets of vesicles. SUVs with diameters of 50 nm were prepared with encapsulated salmon sperm DNA; these are referred

to here as exosome models (EM). SUVs with 100-200 nm diameters encapsulating the DNA-intercalating fluorescent dye SYBR green are referred to here as reagent vesicles (RV). Formation of RV-avidin-EM complexes brings vesicles into close proximity, promoting spontaneous vesicle fusion. When vesicles fuse, their contents mix, enabling analytical reactions to assay encapsulated biomolecules (Fig. 2.1). This system can be modified for molecular recognition of biologically derived exosomes, offering specificity for assaying exosome contents.

2.3.2 Reagents and sample preparation

A model system of exosome molecular recognition and vesicle fusion has been developed. Giant multilamellar vesicles (GMVs) that encapsulate assay reagents were formed by the freeze-thaw method using a buffered aqueous solution (TBS) containing DOPC (20 mg mL⁻¹), 1% w/w Biotinyl cap-PE (25 mg mL⁻¹), and assay reagents. GMVs were converted to SUVs with specific diameters by membrane extrusion. Vesicle solutions were then purified by size exclusion columns with Sephadex G50 media.

2.3.3 Results

Early progress has been made towards the long-term goal of optimizing several experimental variables in the model system, including choice of analytical reagents, vesicle diameter, purification methods, and concentration of added avidin. In the proximity vesicle fusion assay, measured signal depends on the type of chemical reaction utilized. Initially, a chemiluminescence-based approach was selected to provide high signal-to-noise ratios and therefore low limits of detection compared to, for example, a fluorescence quenching assay. The chemiluminescence reaction involved three reagents: luminol, horse radish peroxidase (HRP), and hydrogen peroxide. Since only two vesicle populations are prepared in the assay, two of the reagents had to be encapsulated together. This presented several challenges, such as encapsulation inefficiency and undesired reaction between co-encapsulated reagents. Since the experimental setup included a high sensitivity photon-counting photomultiplier tube (PMT) and changes in light intensity were being measured, difficulty in creating an appropriately dark workspace arose, resulting in excess

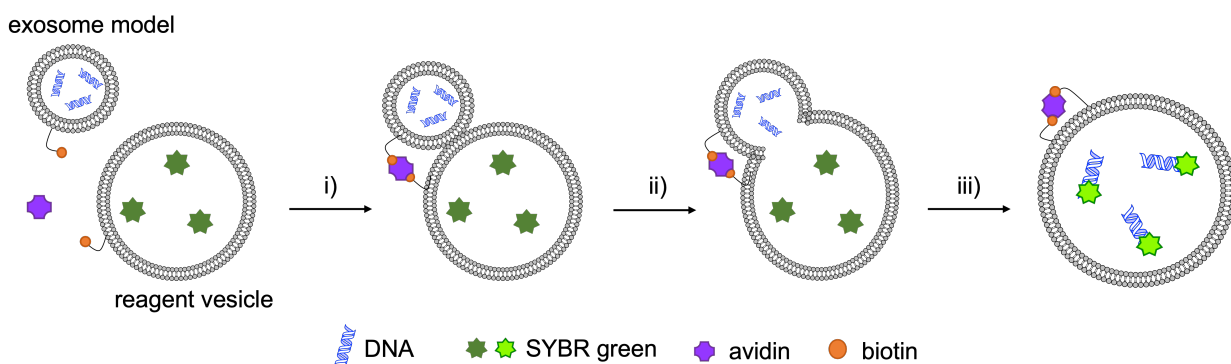


Figure 2.1: EMs (50 nm) are synthetically prepared to encapsulate salmon sperm DNA while RVs (100-200 nm) are synthetically prepared to encapsulate SYBR green. Addition of avidin drives biotin-labeled vesicles together (i). Direct contact initiates vesicle fusion (ii). Vesicle fusion results in content mixing and fluorescent signal for detection (iii).

background noise from stray light. To address these challenges a fluorescence-based approach was devised in which only two reagents are needed, and spectral resolution can be used to reduce the magnitude of collected stray light.

Fluorescence measurements were carried out within a polystyrene 96-well plate positioned on the stage of an inverted epifluorescence microscope (Eclipse Ti-U, Nikon, Newton, NJ), equipped with an LED excitation source (DC2200, Thorlabs, Newton, NJ), and photon counting PMT (H7828-50, Hamamatsu, Hamamatsu, Japan). Changes in fluorescence intensity were collected via LabView (National Instruments, Austin, TX). Two populations of synthetic vesicles were prepared, EMs encapsulating salmon sperm DNA (2 mg mL^{-1}) to model exosome oligonucleotide content, and RVs encapsulating SYBR green, a concentration-sensitive oligonucleotide intercalating fluorescent dye. Due to challenges in quantifying EMs and RVs in purified solution, all experiments were conducted using aliquots from the same purified RV and EM solutions in the same ratios. These were each purified by centrifugation, and $30 \mu\text{L}$ each of purified EM and RV solutions were added together into a single well giving an increase in background fluorescence signal which may be due to the carryover of small quantities of unencapsulated DNA and SYBR Green or spontaneous vesicle fusion/lysis (Fig. 2.2A, $t = 10\text{s}$). While measuring fluorescence intensity ($\lambda_{ex} = 482 \pm 9\text{nm}$, $\lambda_{em} = 520 \pm 12\text{nm}$), 30 mL of avidin ($100 \mu\text{M}$) was added (Fig. 2.2A, $t = 180\text{s}$) and the fluorescence intensity monitored versus time (Fig. 2.2B). Upon addition of avidin, fluorescence intensity increased, suggesting proximity-induced vesicle fusion and content mixing. A series of control experiments (Fig. 2.2C) were performed to demonstrate that increased fluorescence intensity is not the result of avidin (shown in green) or the independent combination of EVs with avidin (red) or RVs with avidin (black).

2.4 Discussion

Though an increase in fluorescent signal was observed upon the addition of avidin to the EM and RV mixture, there are several analytical challenges that arise due to the sample preparation and purification approaches. The major limiting step in our procedure is the purification of vesicles via centrifugation. First, vesicles were prepared by freeze-thaw cycles to encapsulate the assay

reagents (i.e. DNA and SYBR green). The vesicles underwent several wash steps involving water, chloroform, and methanol before centrifugation. Centrifugation of the suspension formed a pellet, and the supernatant was removed and replaced with fresh buffer repeatedly to remove any unencapsulated material. This is potentially problematic because the procedure is similar to ethanol precipitation, a commonly used technique for concentrating and desalting nucleic acid preparations in aqueous solutions. Thus, the formed pellet from centrifugation could contain any unencapsulated DNA. If there is also any unencapsulated SYBR green in our second population of prepared vesicles, contribution to the fluorescent signal could be observed from unencapsulated DNA-SYBR green interactions. Furthermore, SYBR green is membrane-permeable, as its structure contains a positive charge and hydrophobic aromatic rings [9]. Thus, SYBR green has the potential to permeate back into the buffer environment, embed in the membrane of the vesicles, or even permeate through the membranes and into an adjacent vesicle containing DNA. This would contribute fluorescent signal to leakage and/or vesicle aggregation.

Understanding the conditions of our model system is crucial in proceeding with the development of our assay. We must be able to make characterizations that help distinguish between vesicle fusion (Fig. 2.3a), aggregation (Fig. 2.3b), or content leakage (Fig. 2.3c). One distinctive characteristic of these different system configurations is size. Thus, a highly sensitive analytical method for making size-based determinations from low-volume samples is necessary in understanding the status of our proposed analytical assay.

2.5 Conclusion

The work described in this chapter focuses on early progress made towards developing a novel assay for exosome analysis without the need for purification and with the potential for detecting exosome contents without getting interactions from other extracellular vesicles or molecules of similar composition. A proximity vesicle fusion assay was proposed with the central hypothesis that vesicle fusion combined with molecular recognition of exosome membrane proteins can

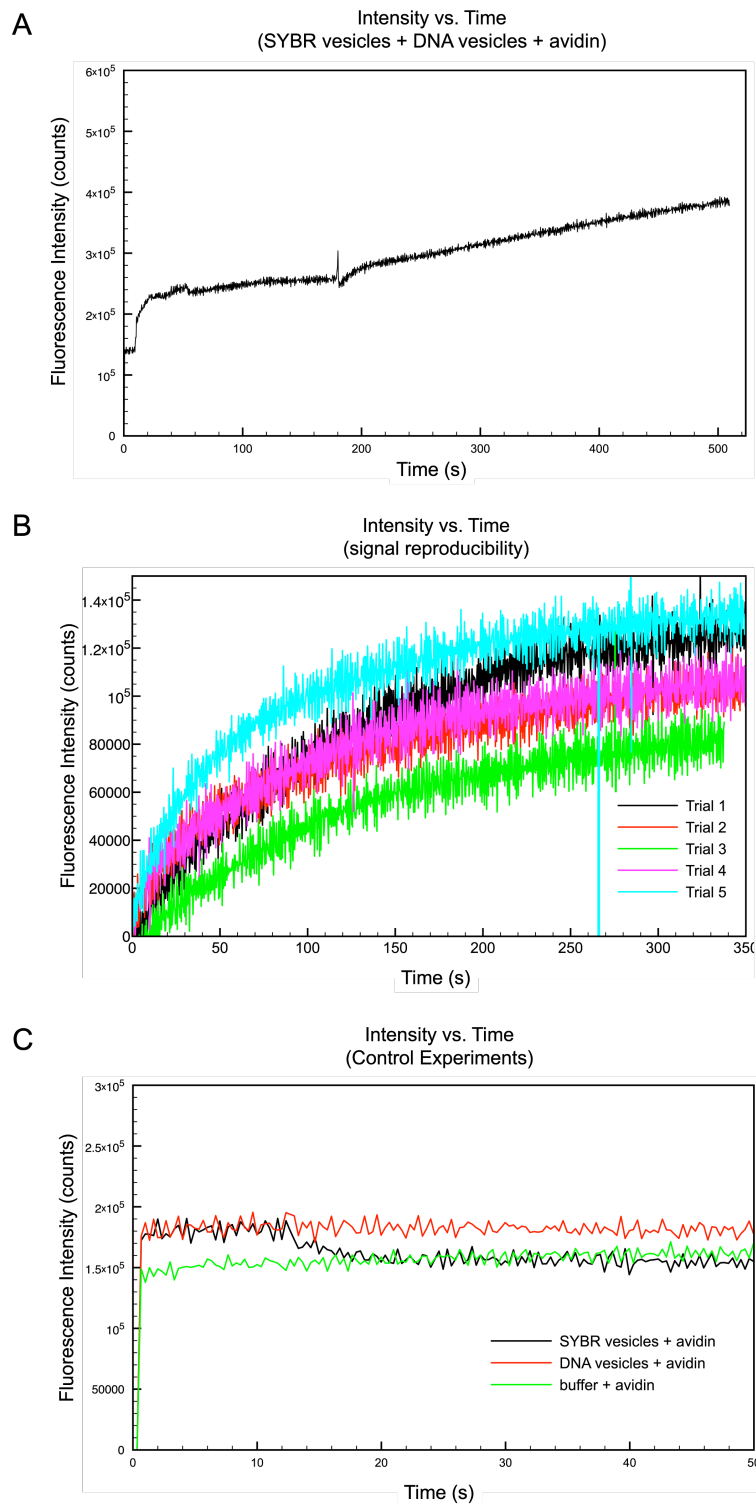


Figure 2.2: A) Fusion and fluorescence demonstration upon avidin addition to reagent vesicles, B) reproducibility of signal increase, and C) graph of control experiments, where signal increase is not due to avidin or the combination of assay vesicles with avidin.

facilitate encapsulated analytical reactions and high sensitivity detection of vesicle contents, enabling specific assay of biomolecules encapsulated by exosomes. Early progress was made towards the development of a model system of exosome molecular recognition and vesicle fusion using synthetically prepared lipid vesicles encapsulated with analytical reagents. However, efficiency with which reagents remained encapsulated within lipid vesicle reagent carriers presented a challenge to assay sensitivity, proving difficulty in isolating which variables were contributing to signal. This significant analytical challenge allowed us to look at our system from a new perspective—to make size-based determinations so that we could observe vesicle fusion, aggregation, or leakage, each phenomenon arising from a different composite state of the vesicle system resolvable by hydrodynamic radii. This presented a critical need for the development of a sensitive, selective, and straightforward analytical tool for characterizing the state of the proximity vesicle fusion assay. Chapters 3 and 4 describe the fundamental principles of size characterization by TDA, and the development of a 3D-printed instrument for the size-based characterization of lipid vesicles.

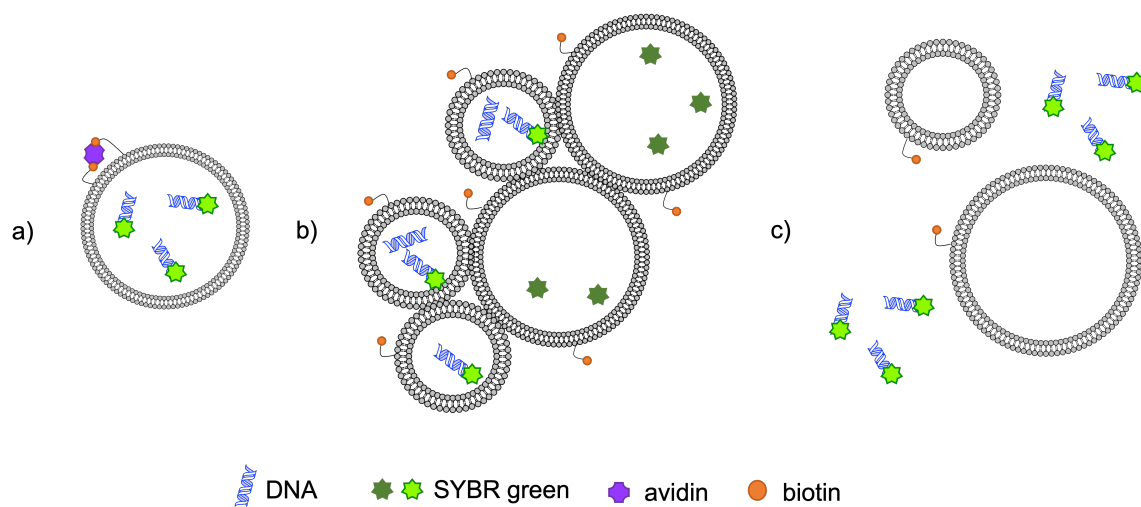


Figure 2.3: An illustration of potential avenues for observed fluorescence signal: (a) Fusion and fluorescence upon avidin addition, (b) fluorescence signal due to vesicle aggregation, and (c) fluorescence signal due to leakage of assay contents.

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Chapter 3

Taylor Dispersion Analysis in Fused Silica Capillaries: A Tutorial Review

A version of this chapter was originally published by Meagan R. Moser and Christopher A. Baker. Only minor modifications were made.

Meagan R. Moser and Christopher A. Baker, “Taylor Dispersion Analysis in Fused Silica Capillaries: A Tutorial Review” *Anal. Methods*, 2021, **13**, 2357-2373.

3.1 Abstract

Biological and pharmaceutical analytes like liposomes, therapeutic proteins, nanoparticles, and drug-delivery systems are utilized in applications, such as pharmaceutical formulations or biomimetic models, in which controlling their size is often critical. Many of the common techniques for sizing these analytes require method development, significant sample preparation, large sample quantities, and lengthy analysis times. In other cases, such as DLS, sizing can be biased towards the largest constituents in a mixture. Therefore, there is a need for more rapid, sensitive, accurate, and straightforward analytical methods for sizing macromolecules, especially those of biological origin which may be sample-limited. Taylor dispersion analysis (TDA) is a sizing technique that requires no calibration and consumes only nL - pL sample volumes. In TDA, average diffusion coefficients are determined via the Taylor-Aris equation by characterizing band broadening of an analyte plug under well-controlled laminar flow conditions. Diffusion coefficient can then be interpreted as hydrodynamic radius (R_H) via the Stokes-Einstein equation. Here, we offer a tutorial review of TDA, intended to make the method better understood and more widely accessible to a community of analytical chemists and separations scientists who may benefit from the unique advantages of this versatile sizing method. We first provide a tutorial on the fundamental principles that allow TDA to achieve calibration-free sizing of analytes across a wide range of R_H , with an emphasis on the reduced sample consumption and analysis times that result from utilizing fused silica capillaries. We continue by highlighting relationships between operating parameters and critically important flow conditions. Our discussion continues by looking at methods for applying TDA to sample mixtures via algorithmic approaches and integration of capillary electrophoresis and TDA. Finally, we present a selection of reports that demonstrate TDA applied to complex challenges in bioanalysis and materials science.

3.2 Introduction

Designing and producing drug delivery systems, therapeutic proteins, nanoparticles, and biopolymers requires fast and straightforward characterization of these products via high accuracy and high sensitivity analytical methods. Many standard methods for solution-phase size characterization require extensive method development, tedious sample preparation, large sample volumes, lengthy analysis times, and/or expensive instrumentation. One widely used size characterization method is dynamic light scattering (DLS), which can, in some cases, be performed with limited sample preparation and offer rapid analysis times. Complex samples such as blood plasma have been analyzed by DLS with preparation as simple as dilution, filtration and/or centrifugation [1, 2]. However, signal intensity in DLS is proportional to the analyte radius raised to the 6th power [3], which makes detection sensitivity particularly challenging when characterizing small particles. For the same reason, DLS is sensitive to issues of analyte aggregation [3]. These qualities are often disadvantageous when interrogating the size of small proteins, especially in the presence of larger proteins or aggregates. Hawe *et al.* measured a range of concentrations (0.05 – 50 mg mL⁻¹) of various peptides and proteins [4]. While DLS accurately sized the antibody drug adalimumab (5 - 6 nm) at the lowest concentration, sizing the peptide oxytocin (0.8 nm) was not possible at low concentrations due to the influence of dust or excipients in the sample. In this case, DLS provided inaccurate and inconsistent hydrodynamic radius (R_H) determinations for oxytocin ranging from 6.9 – 130 nm. In the same work, DLS was utilized to observe aggregation in heat-stressed formulations of protein samples. With a 5°C increase in temperature, a model IgG antibody showed an increase from $R_H = 7.5$ nm to $R_H = 22$ nm by DLS, whereas TDA measured a modest increase from $R_H = 6.5$ nm to $R_H = 7.5$ nm under identical conditions. This disagreement was attributed to the bias of DLS towards large particles, in which the largest particles or aggregates dominate the overall scattering intensity, thus resulting in inaccurately high R_H determinations.

Size exclusion chromatography (SEC) is another method often employed for size determination. SEC requires precise calibration with appropriate size standards, and is susceptible to deleterious effects of analyte-column interactions [5]. Pacáková *et al.* found that melittin, a strongly basic peptide found in bee venom, displayed deleterious retention on a hydrophilic SEC

column, which they attributed to the peptide's inability to form a predominantly hydrophilic shell due to the uneven distribution of exterior hydrophilic residues [6]. Overcoming these deleterious analyte-column interactions required method optimization such as the addition of an organic solvent to the mobile phase and pH optimization. Ricker *et al.* explain that SEC columns can exhibit electrostatic effects when the mobile phase ionic strength is low, and hydrophobic effects when ionic strength is high [7]. Either case can result in deleterious retention of analytes, which can result in peak deformation and, ultimately, inaccuracy of size determinations by SEC. They observed the effect of mobile phase ionic strength on SEC of three mouse myeloma antibodies using a silica-based stationary phase material. At low ionic strength, the least basic antibody had no net positive charge, and was therefore unaffected by adsorption to the anionic silanols at the stationary phase surface. However, at high ionic strength, the antibody was retained due to hydrophobic effects. This work demonstrates the careful consideration for method development that is needed for accurate size determinations by SEC.

Various micro- and nanoscopic imaging techniques such as transmission electron microscopy (TEM), scanning electron microscopy, and atomic force microscopy have been applied to particle sizing [8, 9, 10, 11, 12, 13, 14]. However, field of view ultimately limits the throughput of analysis for any imaging technique applied to molecular or particle sizing.

Taylor dispersion analysis (TDA) is a powerful analytical method for size characterization that addresses many of the shortcomings of the more common methods described above. Size determinations by TDA are absolute, requiring no calibration nor prior knowledge of sample concentration [15]. TDA conducted in fused silica capillaries requires only sub-nanoliter sample volumes, and the method can be applied to sizing analytes from small molecules [16, 17, 18, 19, 20] to micron-scale particles and complexes [21, 22]. TDA has evolved from studying gaseous diffusion coefficients in large tubes [23, 24, 25] to TDA measurements of therapeutic proteins [26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36], drug delivery systems [26, 30, 37, 38, 39, 40, 41, 42], nanoparticles [43, 44, 45, 46, 47, 48, 49, 50, 51, 52], mixtures [15, 44, 53, 54, 55, 56, 57, 58, 59], synthetic polymers [30, 60, 61, 62, 63, 64], and more. While TDA has been discussed in other reviews of physiochemical characterization methods in specific application areas[65, 66, 67, 68,

69, 70], to our knowledge no recent review has combined a tutorial discussion of the underlying principles and practical considerations of TDA with an outline of the breadth and depth of modern TDA applications. Here, we offer a tutorial review of TDA, intended to make the method better understood and more widely accessible to a community of analytical chemists and separations scientists who may benefit from the unique advantages of this versatile sizing method.

3.3 Principles of TDA

First described by Taylor in 1953 [23], TDA enables the direct determination of diffusion coefficients across a wide range of hydrodynamic radii ($\text{\AA}$ – μm). TDA is a mathematical framework for analyzing dispersion that results from the interaction between the parabolic velocity profile of pressure driven laminar flow in a cylindrical tube and the radial diffusion of analytes across that velocity profile. A sample plug injected into flow spreads axially due to the combined effects of convection and diffusion, which is observed as band broadening (Fig. 3.1). Diffusion occurs both radially and longitudinally, however TDA is conducted under well-controlled flow conditions (discussed in more detail below) that ensure the contribution to band broadening from longitudinal diffusion is negligible. As a result of the parabolic velocity profile, the initial velocity of any individual analyte molecule or particle is a function of its starting radial position within the injected sample plug. As radial diffusion proceeds, each molecule or particle samples the full range of flow velocities in the parabolic profile over the duration of flow, resulting in an ensemble average velocity across all particles in the sample population.

Analytes with low diffusion coefficients move slowly across the parabolic velocity profile such that two analyte particles starting with disparate velocities remain separated within their respective flow streams for a longer duration as compared to the behavior of analytes with higher diffusion coefficients. The net result is a broad distribution of velocities about the population mean velocity for analytes with low diffusion coefficients, which is observed as a high degree of band broadening. Conversely, molecules with relatively high diffusion coefficients move rapidly across the parabolic velocity profile, experiencing a narrower distribution of velocities about the population mean, thus a lower degree of band broadening is observed.

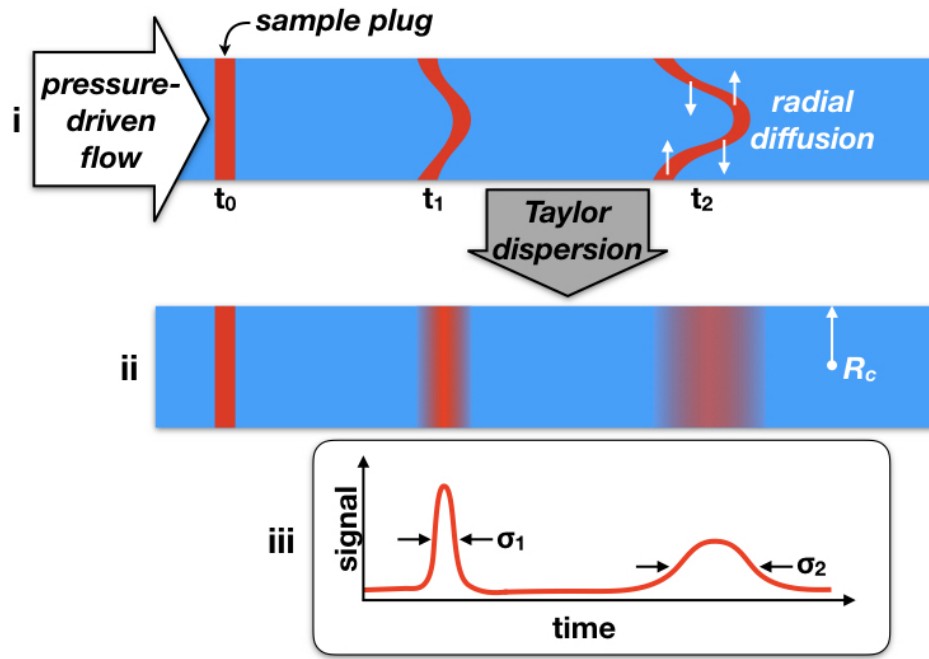


Figure 3.1: Illustration of Taylor dispersion, and the corresponding variables utilized in TDA. Under the parabolic velocity profile of pressure-driven flow, and neglecting any effects of diffusion, a sample plug would deform as illustrated (i). Considering the effects of diffusion only in the radial direction gives Taylor dispersion, yielding the band profiles illustrated (ii). The evolution of Taylor dispersion can be observed as band broadening of a peak measured at multiple locations in the flow path (iii). The variables t_1 , t_2 , R_c , σ_1 , and σ_2 are the same variables utilized in Equation 3.13.

It is worth noting here that the relationship between diffusion coefficient and band broadening observed by TDA may seem counterintuitive to many separations scientists more accustomed to considering longitudinal diffusion. By the mechanism of longitudinal diffusion, higher rates of diffusion result in increased band broadening, which is the opposite of what is described above. Nevertheless, when considering only radial diffusion in TDA measurements, molecular diffusion coefficients (D) can be determined by observing the degree of band broadening as follows [71, 72]:

$$D = \frac{R_c^2 t_d}{24\sigma^2} \quad (3.1)$$

where R_c is the channel radius, t_d is the average elution time, and σ^2 is the peak variance. D can be used as a structural descriptor of the analyte when transformed to R_H via the Stokes-Einstein relation [73]:

$$R_H = \frac{k_B T}{6\pi\eta D} \quad (3.2)$$

where k_B is the Boltzmann constant, T is temperature, and η is the dynamic viscosity of the solution.

For TDA to yield accurate determinations of D , precise control of flow is required to meet two requisite conditions known as the Taylor conditions. First, TDA considers radial diffusion while neglecting longitudinal diffusion. For this to be possible, the rate of advection must be significantly greater than the rate of diffusion, which is widely considered to be satisfied when the Peclet number (Pe) is greater than 69 [74]. Pe is defined for a cylindrical channel as [72]:

$$Pe = \frac{u R_c}{D} \quad (3.3)$$

where u is the linear flow rate. In his original work [74], Taylor defined the following inequality as a requirement to satisfy eqn (3.1):

$$\frac{4L}{R_c} \gg \frac{u R_c}{D} \gg 6.9 \quad (3.4)$$

where L is the length of the channel. In that work, a ratio of 1:10 between the inequalities was considered requisite, which gives the accepted Taylor condition $Pe \geq 69$. Cottet *et al.* demonstrated that longitudinal diffusion can be neglected even in conditions where $Pe < 69$, defining requisite flow velocity as [56]:

$$u \geq \frac{D}{R_c} \sqrt{\frac{48}{\epsilon}} \quad (3.5)$$

where ϵ is the relative error in determination of D . By this analysis, Taylor's condition of $Pe \geq 69$ gives a relative error due to neglecting longitudinal diffusion of 1%, whereas Cottet and coworkers suggest that a relative error of 3% can be tolerated, leading to the requisite condition $Pe \geq 40$.

The second Taylor condition describes the duration for which a sample plug must remain in flow in order to observe sufficient band-broadening effects due to Taylor dispersion. Taylor's original work described the condition as follows: "the time necessary for appreciable effects to appear, owing to convective transport [must be] long compared with the 'time of decay' during which radial variations of concentration are reduced to a fraction of their initial value through the action of molecular diffusion" [23]. As such, the requisite residence time (t_R) of the sample plug in flow is influenced by D and R_c . Residence time can be normalized for these factors to give a dimensionless time factor (τ) defined as:

$$\tau = \frac{Dt_R}{R_c^2} \quad (3.6)$$

Taylor expresses eqn (3.6) as the inequality [74]

$$D \gg \frac{R_c^2}{4t_R} \quad (3.7)$$

Taking a 1:10 ratio as satisfying Inequality (3.7), the following condition satisfies minimum required

$$t_R \geq 2.5 \frac{R_c^2}{D} \quad (3.8)$$

Substituting t_R in eqn (3.6) with Inequality (3.8), we can obtain the minimum required value for τ :

$$\tau = \frac{Dt_R}{R_c^2} = \frac{D(2.5\frac{R_c^2}{D})}{R_c^2} \geq 2.5 \quad (3.9)$$

Alternative minimum τ values have also been reported. In the same work that studied alternative minimum Pe conditions, Cottet *et al.* demonstrate that the minimum t_R can also be expressed as a function of ϵ as follows[56]

$$t_R \geq \frac{3R_c^2}{80D\epsilon} \quad (3.10)$$

Presuming an acceptable ϵ of 3%, Inequality 3.10 becomes:

$$t_R \geq \frac{1.25R_c^2}{D} \quad (3.11)$$

Substituting t_R in eqn (3.6) with Inequality (3.11), we can obtain the minimum required value for τ :

$$\tau = \frac{Dt_R}{R_c^2} = \frac{D(1.25\frac{R_c^2}{D})}{R_c^2} \geq 1.25 \quad (3.12)$$

There is some disagreement on appropriate limiting value of τ . Several works report the condition $\tau > 1.4$ [45, 72, 75, 76]. However, to our knowledge, no mathematical basis for alternate limiting τ values has been offered with the rigor of either Taylor or Cottet's solutions shown above.

3.4 Practical Considerations for TDA Measurements

Several practical considerations arise from the requisite Taylor conditions. First, eqn (3.6) shows that analysis times can be dramatically reduced by reducing R_c . This has motivated the use of fused silica capillaries (typ. 10 - 250 μm i.d.) as flow systems for TDA. As a result, capillary electrophoresis (CE) instrumentation has become a prominent tool in the continued development and application of TDA. Second, although TDA is a method for determining D , target values of D are needed to define the requisite Taylor conditions. Since the Taylor conditions are met by exceeding thresholds (i.e. $\tau \geq 2.5$ and $Pe \geq 69$), a priori knowledge of D is not required. Instead, careful consideration is needed to bracket an appropriate range of D values for a given analysis, which can be used to deduce the limiting values of operating parameters u , R_c , and t_R that satisfy

the Taylor conditions. Finally, it is impractical to consider the two requisite Taylor conditions independently when designing the parameters of a TDA experiment. For example, Pe must exceed a value of 69 but is given no theoretical upper bound, which suggests there is no upper bound of the operating parameter u and therefore flow velocity should be maximized. While u is not given an upper bound on the basis of theory, increasing u requires increasing capillary length in order to achieve a value for t_R to satisfy $\tau > 2.5$, and impractical capillary lengths can quickly arise from poorly chosen u values. Thus, practical limitations require a careful selection of operating parameters to meet the Taylor conditions for an appropriately bracketed range of D . With careful consideration, and several iterative calculations of eqn (3.3) and eqn (3.6), selecting appropriate and practical operating conditions for TDA is not prohibitively laborious. Still, making available purpose-built calculation tools for establishing appropriate operating parameters would benefit researchers currently utilizing TDA and may foster more widespread adoption of the technique.

Practical and theoretical limitations exist beyond the scope of appropriately selecting operating parameters to meet the requisite Taylor conditions. For example, the TDA principles described above assume that the sample plug remains under continuous flow for the full duration of t_R . In practice, this can be difficult or impossible to achieve, especially when utilizing CE instrumentation. The act of injecting a sample plug via conventional CE injection methods necessarily gives discontinuous flow velocities because flow must be stopped to bring a sample vial to the capillary inlet and stopped again to return a buffer vial to the capillary inlet before flow recommences for the TDA procedure. A key to overcoming this limitation has been the use of dual-detector schemes [16, 17, 77, 78], in which band broadening is compared between two detection points positioned on the flow path to achieve the appropriate t_R value within the volume between the detection points. In this way, band broadening evolves while under continuous flow between the two detection points, and any contribution to overall band broadening from discontinuous flow in the sample injection process is accounted for in the initial observation at the first detection point. Dual-detector TDA utilizes a modified form of the Taylor-Aris equation as follows [76]:

$$D = \frac{R_c^2(t_2 - t_1)}{24(\sigma_2^2 - \sigma_1^2)} \quad (3.13)$$

where t_1 and t_2 are the peak arrival times at detectors 1 and 2, respectively, and σ_1^2 and σ_2^2 are the peak variances as observed at detectors 1 and 2, respectively.

Chamieh *et al.* compared the performance of TDA in single- and dual-point detection configurations [53]. In their work, TDA of monodisperse albumin proteins and polydisperse polymer standard samples was performed utilizing a commercial CE instrument equipped with a 60 cm x 50 μm i.d. fused silica capillary and UV absorbance detector. For single-point detection, band broadening was analyzed for signals collected at three detector positions (effective capillary lengths: 8.5 cm, 24.5 cm, and 51.5 cm). For dual-point detection, signals were analyzed with detection points positioned at 24.5 cm and 51.5 cm. Unsurprisingly, single-point detection TDA resulted in overestimates of R_H when the injection volume was a significant fraction ($> 1\%$) of the total effective capillary volume. Utilizing previously reported mathematical corrections[17] for the effects of pressure ramping and the finite volume of the injection plug, R_H determinations by single-point detection TDA were not statistically different from those determined by dual-point detection, provided injection volume remained $< 1\%$ of effective capillary volume. However, dual-point detection TDA is arguably preferable, as it does not require any mathematical corrections or presumptions of dynamic flow conditions which may be difficult to observe. To facilitate precision TDA, technologies have been developed to achieve dual-detector configurations in commercial CE instrumentation for UV absorbance[17] and fluorescence detection[16] modes.

TDA analysis times are significantly reduced by decreasing R_c , which has motivated the use of fused silica capillaries. Precise control of applied pressures to a capillary flow system can be achieved with modern commercial CE instrumentation, making these instruments well suited for TDA. Williams and Vigh leveraged the integration of CE and TDA in a commercial instrument by first separating analytes by CE then switching to pressure driven flow to perform TDA on the separated analytes [79]. One critical challenge in using a commercial CE instrument was described by Sharma *et al.*, who described the effects of the initial ramp in flow velocity that occurs upon pressure application [72]. This non-uniform velocity profile introduces significant error in determinations of D . To circumvent this effect, ramp rate can be characterized and corrected for

mathematically. The ramp rate of the CE instrument must be determined and used to convert the observed residence time to the ideal residence time by the equation:

$$t_R = \frac{t_{R,obs} + \sqrt{t_{R,obs}^2 - 16L_T L_D \eta / R_c^2 r_i}}{2} \quad (3.14)$$

where L_T is capillary length, L_D is tube length from inlet to detector, and r_i is the rate of increase of applied pressure. When there is no initial velocity ramp, $t_R = t_{R,obs}$ and D can be determined simply by eqn (3.1). However, a velocity ramp leads to errors in the measured values of D if not corrected using eqn (3.14). With increased $t_{R,obs}$ the effect of r_i becomes negligible, and as $t_{R,obs}$ approaches infinity, any error in D would approach zero. Thus, the effect of the ramp is greater at lower $t_{R,obs}$. To demonstrate this, the error in D determinations for phenylalanine was characterized as a function of $t_{R,obs}$. Errors greater than 50% were observed for $t_{R,obs} < 50s$ whereas $< 1\%$ error was observed for $t_{R,obs} > 100s$. Correcting for flow velocity ramp by eqn (3.14), reduced the error at all $t_{R,obs}$ evaluated to $< 2\%$. While this correction was straightforward for frontal analysis, zonal analysis required additional corrections to account for the finite width of the injection plug. The additional correction required the calibration of observed peak variance (σ_{obs}^2) vs injection volume (V_i) at each t_R . Thus, correcting for a velocity ramp in zonal analysis can add substantial additional work to the overall workflow.

The operating principles of TDA are conceptually similar to those of hydrodynamic chromatography (HDC), although these are mechanistically distinct modes of analysis. Cottet and coworkers characterized the relationship between R_H , applied pressure, and capillary diameter to elucidate the conditions under which HDC mechanisms interfere with the accurate interpretation of TDA results [80]. In Figs. 3.2A and 3.2B, the red lines correspond to experimental conditions of constant analyte size (vertical line) or constant mobilizing pressure (horizontal lines), and the dots represent the conditions illustrated in Figs. 3.2C and 3.2D. When 250 nm polystyrene nanoparticles (PS NPs) were analyzed by TDA utilizing a 50 μm i.d. fused silica capillary under mobilization pressures from 7 – 550 mbar, peak shape became distorted at and above 90 mbar (Fig. 3.2C). Interestingly, the conditions with observed peak distortion corresponded to $\tau \leq 1.25$, which fails to satisfy the requisite Taylor condition. When various sizes of PS NPs ($R_H = 110$

nm, 250 nm, 500 nm) were analyzed by TDA utilizing 25 μm i.d. fused silica capillary and a fixed mobilizing pressure of 28 mbar, the effects of HDC reduced elution times of 250 nm and 500 nm PS NPs (Fig. 3.2D). Importantly, HDC affected the variance of the NP peaks even without appreciable effects on mean elution time, which adversely affected the accuracy in determinations of D . The authors identified an upper limit on the ratio of R_H to capillary radius (R_C) as a function of ϵ given by:

$$\frac{R_H}{R_c} = 0.17\epsilon \quad (3.15)$$

In many cases, especially when designing TDA experiments for the analysis of macromolecular constructs, this constraint will warrant careful consideration when selecting appropriate operating parameters to meet the Taylor conditions.

Another important consideration is the potential for analyte-capillary adsorption, which will introduce peak asymmetry in the resulting elution profile, or taylorgram. Latunde-Dada *et al.* observed asymmetric peaks with pronounced tailing in varying concentrations of lysozyme (1 - 20 mg mL^{-1}) in a standard 75 μm i.d. capillary [81]. As an attempt to mitigate the effect of peak tailing on R_H determinations, they developed a constrained fitting algorithm to isolate dispersive components of the concentration profiles from the solute-capillary interacting components. This mathematical correction yielded improved accuracy in R_H determinations for high concentration (i.e. 5 - 20 mg mL^{-1}) lysozyme samples. Interestingly, at 1 mg mL^{-1} lysozyme, both free and constrained fits gave inaccurate R_H determinations (4.5 ± 0.3 nm and 3.33 ± 0.03 nm, for free and constrained fits, respectively as compared to previously reported values of 1.89 - 2.05 nm [82, 83]). The authors postulated that these inaccuracies resulted from a much greater fraction of total sample engaged in adsorptive interactions at the capillary wall and, thus, subject to a net flow velocity that did not meet the Taylor criteria. Here, we suggest that adverse adsorption effects may warrant adapting common practices from CE methods, such as capillary surface modification or buffer additives [61, 84, 85], for use in TDA measurements. Such efforts will require careful

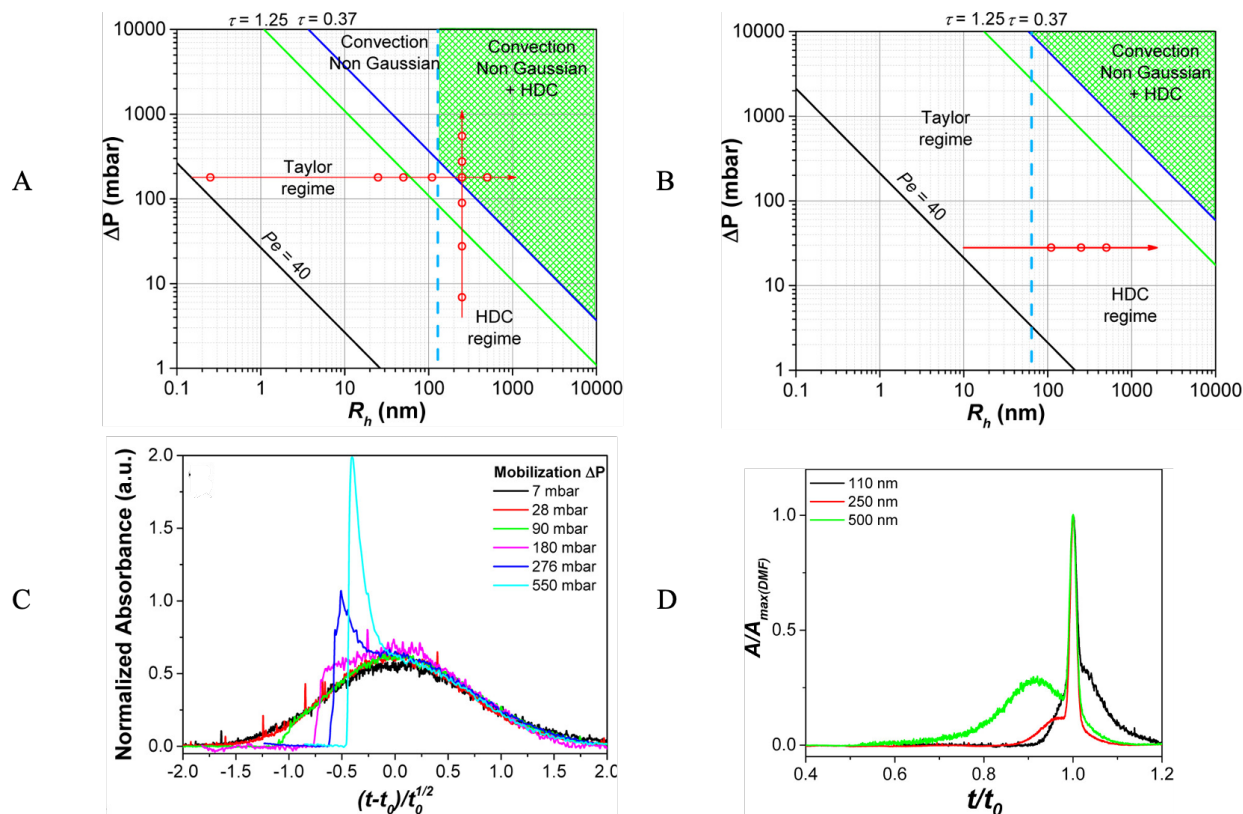


Figure 3.2: Illustration of the conditions under which HDC mechanisms interfere with the accurate interpretation of TDA results for A) 50 μm i.d. and B) 25 μm i.d. capillaries. Red lines correspond to experimental conditions of constant analyte size (vertical) or constant mobilizing pressure (horizontal lines), and the dots represent experimental conditions investigated. Taylorgrams obtained from PS NPs with C) constant analyte size at various mobilizing pressures and D) constant mobilizing pressure with various analyte sizes. Note that deviations from gaussian peak shape arise when experimental conditions enter the HDC regime, illustrated as shaded green region in A and B. Adapted with permission from: Chamieh J, Leclercq L, Martin M, Slaoui S, Jensen H, Ostergaard J, et al. Limits in Size of Taylor Dispersion Analysis: Representation of the Different Hydrodynamic Regimes and Application to the Size-Characterization of Cubosomes. *Anal Chem.* 2017; 89(24):13487-93. Copyright (2017) American Chemical Society.

consideration and characterization of the effects on solution viscosity to yield accurate calibration-free size determinations.

Though the principles of TDA were first described in the early 1950s, modern capillary flow systems have enabled this technology to be leveraged more recently as a powerful technique for size determination in various application areas and across various modes of analysis. For example, in addition to UV absorbance [17] and fluorescence detection [16, 86] modes, TDA has been coupled to mass spectrometry [87, 88], refractive index detection [62], and backscattering interferometry [89]. To this point, we have considered TDA of pure substances. TDA leverages tools common to separation science, and therefore it is interesting to examine how the practice and principles of TDA become more complex in the context of sample mixtures.

3.5 Algorithmic Approaches to TDA of Mixtures

TDA is applicable to both monodisperse and polydisperse samples [53, 55]. For monodisperse samples, TDA results for the determination of R_H are directly analogous to those of DLS, which is a standard method for particle size characterization [55]. Conventionally, TDA gives weight-averaged or number averaged R_H values when utilizing mass- or concentration-sensitive detection modes, respectively [15]. Thus, the resulting R_H determinations for polydisperse samples can differ from the harmonic z-averaged R_H value obtained by DLS [90]. This incongruency with DLS has motivated various regression and statistical analysis approaches to improve the performance of TDA for mixtures and polydisperse samples.

Common peak fitting methods for non-Gaussian peak shapes have been applied to TDA data for the analysis of polydisperse samples. Deviations from a strict Gaussian peak shape were observed at the apex and base of taylorgrams produced from a mixture of third and fifth generation dendrigraft poly-L-lysine, complicating the determination of appropriate peak variance in the taylorgram [53]. In the case where Gaussian fits cannot be applied to taylorgrams of polydisperse samples, peak variance can be determined via an integration-based algorithm. In that work, peak variance was determined by integrating the whole signal of the resulting non-gaussian peaks across the time interval using the following equation:

$$\sigma^2 = \frac{\int h(t)(t - t_d)^2 dt}{\int h(t) dt} = \frac{\sum_m^{i=n} h_i(t_i - t_d)^2(t_{i+1} - t_i)}{\sum_m^{i=n} h_i(t_{i+1} - t_i)} \quad (3.16)$$

where $h(t)$ is detector response, t_i is elution time for a given point i , t_d is the average elution time, n and m are the starting and ending points considered for the integration. This integration method yielded a weight-average R_H determination. Furthermore, this method depended heavily on the selection of the boundaries for integration, defined by the variables n and m . Several boundaries were considered defined by cutoff lines ranging from 0 - 2% of the peak apex (Fig. 3.3A). Fig. 3.3B compares the results of the integration method (data points) with the results of conventional gaussian fitting (horizontal dashed lines). A general trend of reduced mean R_H with increased cutoff percentage was observed. Comparison of the mean R_H values obtained from the integral and gaussian fitting methods revealed no significant difference and agreement with 95% confidence. However, for third and fifth generation dendrigraft poly-L-lysine (G3 and G5), poor agreement was observed between the Gaussian fitting and integration methods at all cutoff percentages. The authors attributed this to Gaussian fitting being poorly suited for the irregular peak shapes of G3 and G5. To prevent contributions from noise influencing the integration method, the cutoff line corresponding to $4 \times \sigma_{noise}$ was defined as the appropriate threshold, which in this work corresponded to 0.75% of the peak apex (Fig. 3.3B, vertical dashed line).

Data analysis methods have been developed to extract multiple constituent R_H values from the taylorgrams of mixtures [15, 55, 56]. In one example, three independent data analysis methods were applied to monitoring a polymerization reaction by TDA [55]. In the first method, the degree of conversion was determined by comparing integrated areas in the taylorgrams before and after the polymerization reaction. In the second method, the taylorgrams for each component in the reaction mixtures were recorded and fitted as Gaussian curves, and the taylorgram for the reaction mixture was deconvolved by fitting as the sum of Gaussian curves for the mixture components. The third method subtracted the Gaussian contribution of the monomer mixture from the taylorgram of the reaction mixture and fitted the reduced signal iteratively to extract the polymer contribution. Three standards of polyacrylamide (PAM) with varying weight average molar masses and acrylamide (AM) monomer were analyzed by TDA individually and as AM/PAM (10:90 v:v) mixtures for

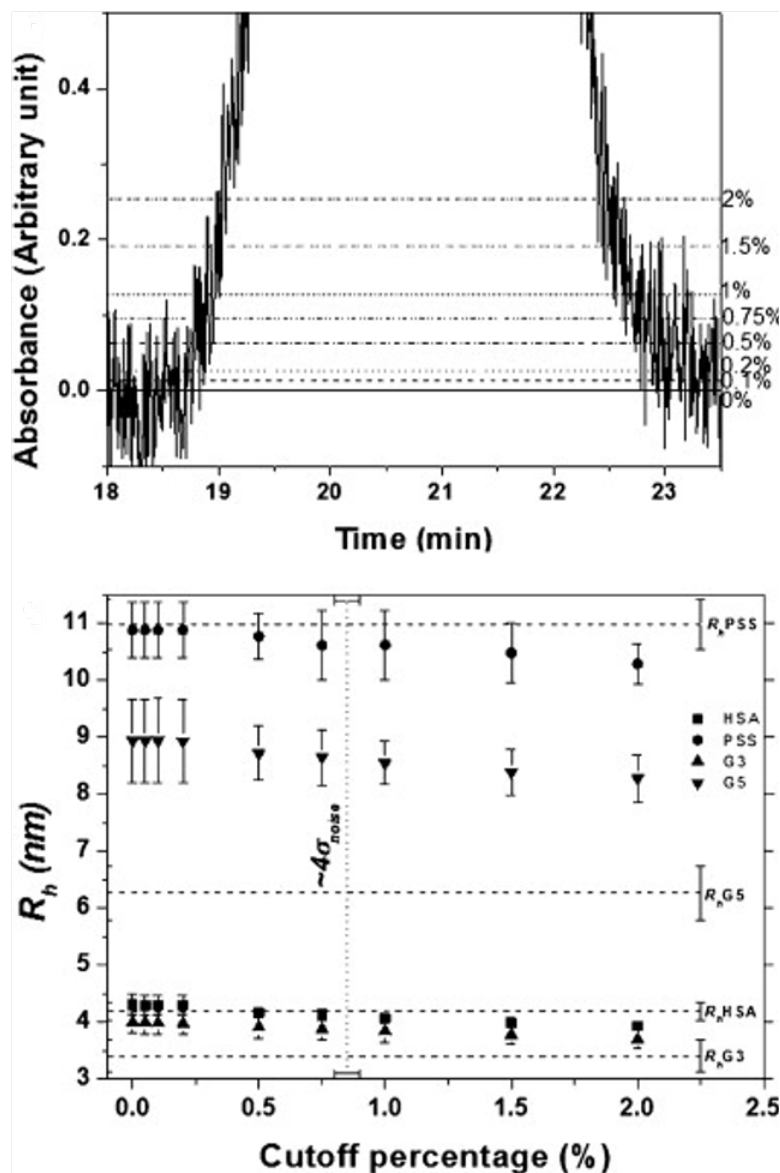


Figure 3.3: Graphical representation of variance determination by peak integration. A) Integration boundaries are represented by cut-off lines given as percentage of the peak apex (horizontal dashed lines); and B) resulting trends in R_H measurements as a function of integration cut-off boundaries. The limiting cut-off boundary was determined to be 0.75% (vertical dashed line), which represented $4 \times \sigma_{noise}$. R_H measurements by the integration method (data points) are compared to standard Gaussian peak fitting values (horizontal dashed lines). Reprinted from Journal of Chromatography A, Vol. 1241, Joseph Chamieh, Herve Cottet, Comparison of single and double detection points Taylor Dispersion Analysis for monodisperse and polydisperse samples, 123-127, Copyright (2012), with permission from Elsevier.

each molar mass standard to mimic a polymerization medium. Peak profiles in AM/PAM mixtures were shown to be the sum of the contributions from each component, as illustrated in Fig. 3.4A. All three data analysis methods determined $R_H = 0.22$ nm for AM, while R_H values for AM/PAM agreed across all methods to within 0.5 nm. TDA was performed on aliquots of an acrylamide polymerization reaction mixture at several time points, as illustrated in Fig. 3.4B. The three methods were separately utilized to determine R_H for the resulting PAM, and all methods agreed to within 4%.

Cipelletti described a cumulant analysis method for determining the polydispersity of moderately complex sample mixtures [54]. By the cumulant method, the logarithm of any taylorgram can be expanded into a cumulant series, in which the first cumulant (Γ_1) is directly related to the mean of the gamma distribution of D . The authors showed that polydispersity could be characterized by evaluating the deviation from linearity in the plot of the cumulant series. In further work, Cipelletti *et al.* described the constrained regularized linear inversion (CRLI) approach for determining probability density functions (PDFs) of D from taylorgrams [44]. This added additional constraints to the standard least-squares fit to overcome the difficulties of an infinite set of PDF solutions that fit the taylorgram function. D averages were determined by CRLI approach that agreed to within 10% of the expected values utilizing both simulated and experimental data, and up to 10% error was observed for determinations of polydispersity indices of various polystyrenesulfonate samples and mixtures.

Latunde-Dada *et al.* described an algorithmic approach to deconvolution of taylorgrams of mixtures based on initial parameter estimations as seed values for fitting by least squares regression [57]. In that approach, a system of equations describing the relative contributions of various peak amplitudes and variances can be solved using initial parameters derived from the second derivative, integral, and double integral of the net taylorgram signal. The authors demonstrated the utility of their approach in various use cases, including 2, 3, and 4 component mixtures of related and unrelated analytes, and with various conditions of a priori knowledge of analyte radius. In each case, hydrodynamic radii in good agreement with nominal reported values were determined for all

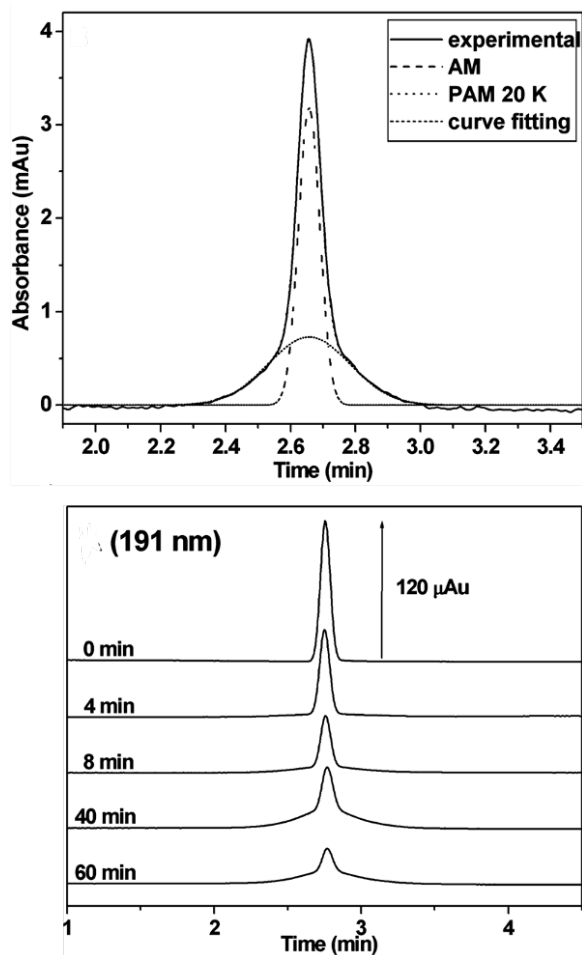


Figure 3.4: A) Taylorgram of an AM/PAM standard mixture with UV absorbance detection (solid line). The signal was deconvoluted by fitting to the sum of two gaussian curves (dashed lines) . B) TDA time course profile of acrylamide polymerization with UV absorbance detection at 191 nm. Signal analysis using three methods based on conservation of mass of sample injection, deconvolution via gaussian fits, and deconvolution via monomer contribution subtraction results in R_H determinations that agree to within 4%. Adapted with permission from: Cottet H, Biron JP, Cipelletti L, Matmour R, Martin M. Determination of Individual Diffusion Coefficients in Evolving Binary Mixtures by Taylor Dispersion Analysis: Application to the Monitoring of Polymer Reaction. Anal Chem. 2010; 82(5):1793-802. Copyright (2010) American Chemical Society.

mixture components.

These examples illustrate that fitting and deconvolution algorithms can be applied to interpreting taylorgrams of sample mixtures. Although no theoretical upper limit has been proposed for the number of mixture components that can be handled by these methods, the mathematical complexity and uncertainty inherent to such approaches suggests that they are best reserved for relatively simple mixtures. Applications requiring increased resolution and peak capacity will benefit from the common instrumentation shared by both CE and TDA, which facilitates their online integration.

3.6 Integrating CE and TDA

As we previously discussed, dual-point detection is an effective approach to circumventing challenges of non-uniform or discontinuous flow velocities in TDA, but it presents an engineering challenge of integrating two detection points within the confines of commercial CE instrumentation. Chamieh *et al.* implemented a dual-point UV detection approach by looping the capillary inside of a standard CE capillary cassette such that it passed the UV detection point twice before exiting the cassette (Fig. 3.5A) [17]. This required modification of the instrument's detection interface to allow both detection windows on the looped capillary to overlap within the same interface. Fig. 3.5B (top) compares the taylorgrams obtained for 75 μM HSA using the unmodified interface (gray trace) and the modified interface (black trace). A 10-fold decrease in sensitivity was observed when using the modified detection interface, which the authors attributed to the removal of a spatial filtering slit which typically prevents transmission through the capillary in regions outside of the capillary inner diameter. However, reduced sensitivity did not adversely affect the observed peak variance or elution profiles of sufficiently concentrated samples, as observed by the normalized data in Fig. 3.5B (bottom). Dual-point UV detection was utilized to determine R_H at two mobilizing pressures (30 and 50 mbar, respectively) for 10 mM caffeine ($R_H = 0.462 \pm 0.013$ nm and 0.436 ± 0.017 nm), 75 μM BSA ($R_H = 4.10 \pm 0.12$ nm and 4.13 ± 0.13 nm), and 75 μM HSA ($R_H = 4.19 \pm 0.09$ nm and 4.25 ± 0.14 nm) and in all cases were found to agree with literature values to within 5%.

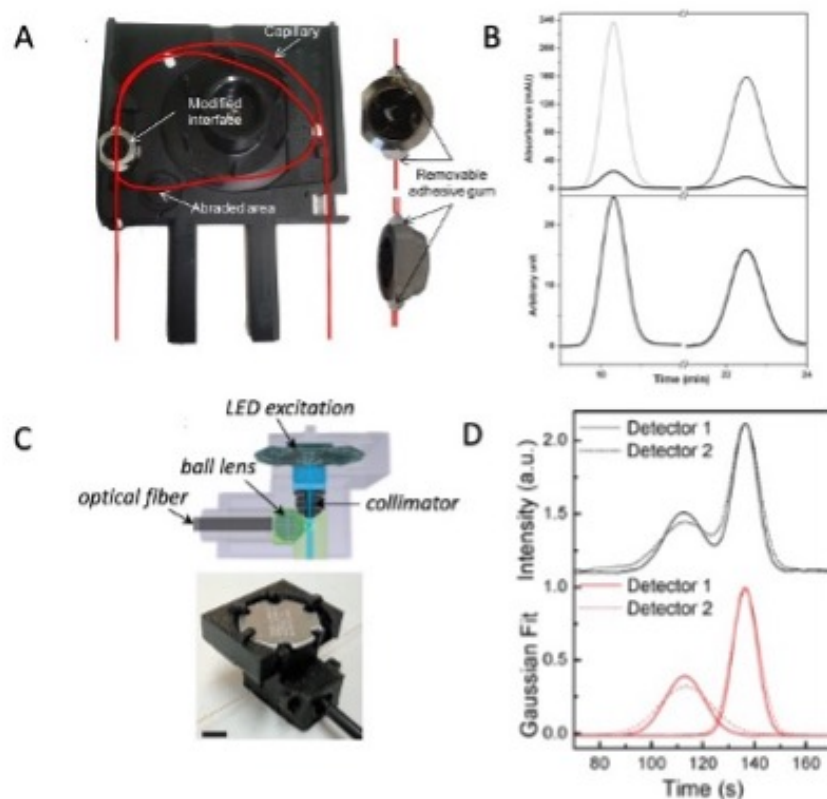


Figure 3.5: A) Illustration of a looped capillary within a standard CE instrument cassette for double detection TDA with UV absorbance detection. B) Raw data (top) and normalized data (bottom) Taylorgrams of HSA by conventional single-point UV absorbance detection interface (gray trace) and modified double detection interface (black trace). Note a dramatic reduction in sensitivity (ca. 10-fold) for the modified double detection interface. Normalized data shows no substantial difference in peak variance or elution time between detection methods. Adapted from Journal of Chromatography A, Vol. 1235, Joseph Chamieh, Farid Oukacine, Herve Cottet, Taylor dispersion analysis with two detection points on a commercial capillary electrophoresis apparatus, 174-177, Copyright (2012), with permission from Elsevier. C) Schematic illustration and photograph (scale bar is 5 mm) of a miniature 3D printed fluorescence detector for two-point fluorescence detection in a commercial CE instrument. D) Raw (top) and Gaussian fitted (bottom) data from CE-TDA of FITC-BSA conjugation reaction. TDA determinations of R_H enabled peak assignments of FITC-BSA (first peak) and free FITC (second peak). Adapted with permission from: Casto LD, Do KB, Baker CA. A Miniature 3D Printed LED-Induced Fluorescence Detector for Capillary Electrophoresis and Dual-Detector Taylor Dispersion Analysis. Anal Chem. 2019; 91(15):9451-7. Copyright (2019) American Chemical Society.

Fluorescence is often utilized as a detection mode in CE because it overcomes pathlength limitations of UV-absorbance detection in small diameter capillaries, but the integration of dual-point fluorescence detection in commercial CE instrumentation is challenging. Our group developed a miniature LED-induced fluorescence detection system for CE that was sufficiently compact to enable the integration of two detectors within the cassette of a commercial CE instrument [16]. The 3D printed design, shown in Fig. 3.5C incorporated an LED excitation source, bandpass excitation filter, pinhole collimator, and emission-collecting ball lens. Operating conditions such as LED current, and PMT gain control voltage were optimized via multivariate analysis to yield a detection limit of 613 ± 13 pM for fluorescein. The system was used to monitor the progress of a fluorescent bioconjugation reaction between fluorescein isothiocyanate (FITC) and BSA, and the integration of CE-TDA with fluorescence detection was shown to enable standard-free identification of peaks in the CE separation. Fig. 3.5D shows the overlay of signals obtained from both detectors (solid and dashed black traces) and the corresponding Gaussian fits (solid and dashed red traces) at reaction time = 2 min. TDA of the CE-separated zones provided R_H values of 4.4 nm and 0.54 nm for peak 1 and peak 2, respectively, allowing the assignment of these peaks to FITC-BSA and free FITC, respectively.

CE and TDA have been used together to monitor reaction progress in other systems. Affinity CE is widely utilized for studying biomolecular interactions, and in this regard CE-TDA can offer advantages for elucidating biophysical and functional properties of binding systems. Østergaard and Jensen demonstrated the first application of CE-TDA for the simultaneous characterization of protein-ligand binding and protein R_H in two separate affinity systems [76]. Advancing fronts were used to obtain D and R_H values for free ligands, α_1 -acid glycoprotein (AGP) and human serum albumin (HSA), and for propranolol-ligand complexes to investigate and quantify their interactions. Differences in the degree of binding between propranolol-AGP and propranolol-HSA were significantly different, showing a trend in agreement with previous literature. Further, Liu *et al.* monitored surface functionalization of dendrigraft poly-L-lysines (DGL) [91]. TDA was used to determine the R_H of the polypeptides and of a click reaction product. While the results confirmed the reaction, TDA gives a weight average R_H which does not provide information on reagent or product purity nor homogeneity. CE was used separately from, and complimentary to,

TDA to characterize the reaction mixture components. Comparison of electropherograms of the clicked product spiked with the starting materials and intermediate compounds clearly illustrated the absence of these reaction components in the final product. Similarly, Deschamps *et al.* characterized the size of an ionic polydiacetylene by TDA while monitoring its polymerization process by CE [92]. R_H values for the polymer were reported as 1.77 nm and 1.9 nm by TDA and DLS, respectively, where the small difference was attributed to sample polydispersity. CE results were used in this study to determine degree of polymerization, polydispersity index, and number average molar mass. CE is also well-suited for online integration with TDA, which combines in a single analysis the high resolving power of CE with R_H determinations by TDA [93]. A mixture can be electrophoretically separated with high resolution while experimental traces from the same detector are recorded and used for TDA. Several groups have reported success in on-line integration of CE-TDA for monitoring of bioconjugation reactions [16, 76], nanoparticle characterization [49, 50, 78, 94], characterization of charged complexes [93, 95], and more.

Characterizing the 3D structure of proteins and biomolecules is critical in understanding their biological function. Xu and coworkers developed mobility capillary electrophoresis (MCE) to circumvent challenges associated with common structural analysis techniques [96, 97]. MCE combines CE with suppressed electroosmotic flow and TDA to enable determinations of R_H and effective ionic charge from a single experiment. MCE has been demonstrated in combination with mass spectrometry and molecular dynamics simulations for 3D protein structural analysis from solution-phase samples. MCE has been applied to a variety of proteins and protein mixtures, under native conditions and non-native pH conditions [98, 99].

The characterization of size and function in non-biological systems by CE-TDA has also been reported. Leclercq and Cottet proposed a methodology for the characterization of polyelectrolyte complexes in which a CE separation of constituents followed by TDA allows for the determination of charge stoichiometry and R_H , respectively [95]. Oukacine *et al.* utilized the high separation performance of CE in conjunction with the absolute size determination of TDA for the determination of R_H of a bimodal mixture of nanolatexes (56 and 70 nm in size) [78]. First, a baseline separation of the two nanolatexes by CE was required before TDA could be performed.

A UV detector and capillary with three detection windows in a looped configuration was used in this study. Reported values of D were in good agreement with values obtained by TDA of the two nanolatexes individually, ultimately demonstrating CE-TDA as a suitable approach for the characterization of mixtures of nanoparticles similar in size.

3.7 Applications of TDA to Bioanalysis

Analysis of biological and pharmaceutical compounds can take advantage of the low sample volumes of TDA, since sample availability in these cases can be limiting [54]. Such compounds include therapeutic peptides and proteins [26, 27, 28, 29, 31, 32, 33, 34, 35], drug delivery systems [30, 37, 38], and lipids [39, 40, 41, 42]. Diffusion and transport through tissue mimics, hydrogels, and capillaries has also been modeled for biological applications [30, 31, 100].

Hulse *et al.* demonstrated TDA for the size characterization of therapeutic proteins and their respective aggregates [28]. 60 nL of 10 mg mL^{-1} bovine serum albumin (BSA) were prepared and analyzed in less than 3 minutes using a commercial TDA instrument. R_H was determined to be 4.18 nm with an RSD of 0.24%, which was in agreement with previously reported values of 3.3 - 4.3 nm. Aggregation was induced in two samples of BSA via heat stress. Comparing average R_H values of BSA determined by DLS and TDA, it was determined that greater repeatability was achieved with TDA, indicated by $< 1\%$ RSD as compared to 7.09% RSD by DLS in each aggregated sample.

Høgstedt *et al.* assessed protein-protein and peptide-peptide interactions (PPIs) by TDA [27]. PPIs can be characterized by the diffusion interaction parameter, k_D , which is observed as the slope of the linear fit in a plot of D vs analyte concentration. A comparison of TDA and DLS for characterizing PPIs in model peptides was not possible since, the authors reported, DLS lacked appropriate detection sensitivity for the model peptides. Therefore, TDA and DLS were compared for characterizing PPIs for the proteins α -lactalbumin and HSA with highly comparable results. The higher sensitivity of TDA enabled observation of repulsive and attractive PPIs in a set of three model peptides. Furthermore, Latunde-Dada *et al.* proposed a method for obtaining concentration dependent diffusion coefficients and k_D in a single measurement by measuring dispersion as a

function of concentration along the front of a sample slug [18]. For the application to caffeine and BSA solutions, the values and signs of k_D as well as the values of D were in good agreement with literature values and DLS results.

Protein-ligand interactions have also been studied by TDA coupled to mass spectrometry (TDA-MS). Hong *et al.* investigated noncovalent interactions of lysozyme and cytochrome C with tri-N-acetylchitotriose [87]. A home-built sample introduction system utilizing branched capillary channels and constant pressure pumping enabled two-point detection TDA-MS via electrospray ionization. Peaks corresponding to the protein, ligand, and the protein-ligand complex were well resolved by MS, and effective charges were determined. Ion chromatograms from the mass spectra were used for R_H determinations by TDA. TDA results showed a 6.963% and 7.53% increase in R_H of lysozyme and cytochrome C, respectively, after incubation with the ligand, indicative of protein-ligand binding.

Nanoscale hydrogels are utilized as drug delivery systems, enabling spatial, temporal, and stimulus-controlled drug release. Size characterization of these nanogel delivery systems is important, since size distribution influences in vivo diffusion, biodistribution, and ultimately the biological fate of these drug delivery vehicles [30]. Several studies have been successful in characterizing these drug delivery systems via TDA. Ibrahim *et al.* have worked to characterize the size and effective charge of a polymeric nanogel by TDA and CE, respectively [30]. Four copolymer nanogels were sized by TDA, resulting in an RSD of $< 2.2\%$ for all R_H values, while effective ionic charge (z_{eff}) was determined for each nanogel from electrophoretic mobility and R_H . Jensen *et al.* have also studied hydrogel matrices as drug delivery systems, but as a subcutaneous tissue mimic [31]. A UV imaging method was combined with TDA to visualize and characterize diffusivity and self-association behavior of insulin within an agarose hydrogel matrix in real-time. At various concentrations, insulin monomers and hexamers were easily distinguished by TDA. Low concentrations (0.1 and 0.2 mM) and pH (3.0) resulted in $R_H = 1.5 \pm 0.1$ nm, representing the monomeric form of insulin, and high concentrations (1 mM) and moderate pH (7.4) resulted in $R_H = 3.0 \pm 0.1$ nm, indicative of the insulin hexamer. The authors reported that

DLS was less sensitive to these small changes in hydrodynamic radius.

Ye *et al.* demonstrated the application of TDA to characterizing D and R_H values of drug substances in water and various pharmaceutical media (acetonitrile, methanol, isopropyl myristate, medium chain triglyceride, and propylene glycol), along with simultaneous measurements of solvent viscosity [37]. Relative solvent viscosity measurements were made using the two detection windows and water as a reference viscosity standard by the following equation:

$$\eta = \frac{\eta_{water}(t_{2,s} - t_{1,s})}{t_{2,water} - t_{1,water}} \quad (3.17)$$

where η is the relative solvent viscosity, η_{water} is water viscosity, $t_{1,s}$ and $t_{2,s}$ are the times at which the analyte in the solvent reaches detection windows 1 and 2, respectively, and $t_{1,water}$ and $t_{2,water}$ are the times at which the analyte in water reaches detection windows 1 and 2, respectively. Viscosity was determined for all solvents with RSD < 1%. This work highlights the importance of considering solvent viscosity in R_H determinations, since D significantly decreases with increasing solvent viscosity, which can lead to overestimation of R_H . Simultaneous determination of solvent viscosity and D by TDA offers a promising approach to circumventing this problem.

Surfactant micelles or microemulsions are used as drug delivery systems to improve the solubility and bioavailability of drugs with poor water solubility. Formulations of these lipid-based excipients can be complex and, as with other drug delivery systems, their size characteristics will impact the efficacy of the drug delivery system. Chamieh *et al.* have made significant contributions to the characterization of micelles and microemulsions by TDA. TDA is well suited to this purpose because it is less sensitive than DLS to deleterious effects from aggregates, and variation in viscosity of micellar solutions can readily be accounted for. The effect of concentration and temperature on the size of commercial self-emulsifying pharmaceutical excipients was investigated by TDA and results compared to DLS measurements. The two excipients studied, Labrasol® [40] and Gelucire®44/14 [39], were found to have opposite trends in behavior based on concentration and temperature; Labrasol® microemulsions showed a decrease in the measured R_H (90 nm to 6 nm) with increased concentration, while Gelucire®44/14 showed an increase in measured R_H

(1 nm to 5.5 nm) with increased concentration. The authors postulate that this opposite trend was due to coacervation in low concentrations of Labrasol® and increase in viscosity of higher concentrations of Gelucire® 44/14.

An often-important criteria for drug delivery systems is the ability to keep the loaded drug inside the prepared emulsion or droplet solution in the gastrointestinal tract, where digestive enzymes are present. In continuation of their previous studies, Chamieh *et al.* monitored the size of excipients during in vitro lipolysis under conditions simulating the gastrointestinal tract [41]. Digestion of the excipients were monitored by TDA at several time points in the degradation process. Similar to their previous work, an opposite behavior was observed, as Labrasol® droplets decrease in size (Figs. 3.6A and 3.6B), due to the disappearance of coacervates that are unable to solubilize the hydrophobic fluorescent marker and Gelucire® droplets increase in size during lipolysis (Figs. 3.6C and 3.6D) due to the increase in micelle size, maintaining solubilizing capacity. TDA was shown to be effective for size analysis of microemulsions and their behavior under digestive conditions. TDA is also an effective method for quantifying peptide drugs released from lipidic self-emulsifying drug delivery systems. The role of electrolyte ionic strength on the release of two therapeutic peptides, leuporelin and desmopressin has also been characterized [26].

3.8 TDA Applications in Nanomaterials Characterization

Physiochemical properties of inorganic nanoparticles (NPs) are primarily dependent on their size, and TDA has proven well-suited for characterizing NPs. Sizing of NPs by TDA has been well-studied and has demonstrated utility [43, 44, 45, 46, 47, 48, 49, 50, 51]. NP size characterization by TDA has been compared to TEM imaging techniques and found to result in comparable particle size values. For example, Balog *et al.* studied superparamagnetic iron oxide NPs (SPIONs), gold (Au) NPs, and silica (SiO_2) NPs and compared TDA to TEM in determination of particle radius [43]. The reported radii of SPIONs, Au NPs, and SiO_2 NPs were 7.6 nm, 33.8 nm, and 44.0 nm, respectively, by TDA, and 6.7 ± 1.1 nm, 28.0 ± 4.7 nm, and 39.3 ± 6.2 nm, respectively, by TEM.

Sizing is also useful in characterizing the modification of NPs with adsorbed functional components such as enzymes. Høldrich *et al.* investigated pepsin coated gold nanoparticles by

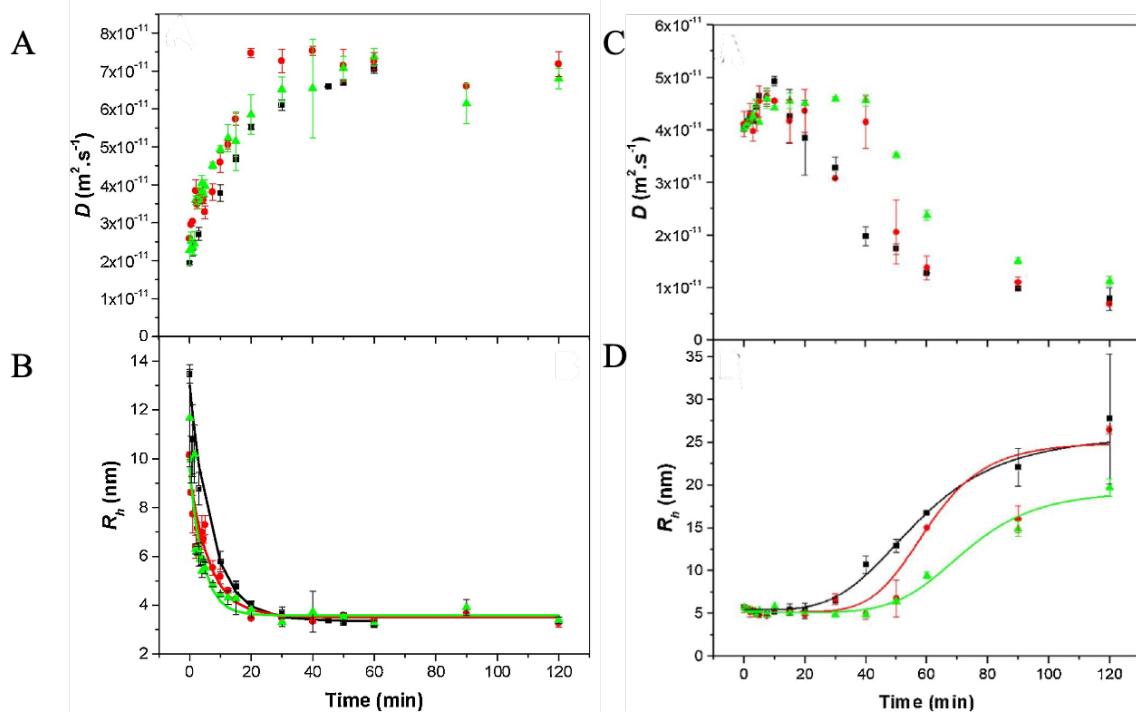


Figure 3.6: Graphical comparison of diffusion coefficients and hydrodynamic radii of Labrasol® and Gelucire® droplets during lipolysis at 37°C. A) D measurements of Labrasol® droplets are shown to increase and B) corresponding R_H values decrease exponentially before reaching a plateau. C) D measurements of Gelucire® droplets are shown to decrease and D) corresponding R_H values increase sigmoidally. Adapted from International Journal of Pharmaceutics, Vol. 537, Joseph Chamieh, Habib Merdassi, Jean-Christophe Rossi, Vincent Jannin, Frederic Demarne, Herve Cottet, Size characterization of lipid-based self-emulsifying pharmaceutical excipients during lipolysis using Taylor dispersion analysis with fluorescence detection, 94-101, Copyright (2018), with permission from Elsevier.

DLS and TDA [47]. Pepsin was adsorbed onto synthesized gold nanoparticles (GNPs), with a DLS measured diameter of 44.1 ± 0.3 nm, at varying concentrations to obtain a range of thicknesses of the adsorbed layer. The average hydrodynamic diameter of pepsin-coated GNPs by DLS was reported as 64 ± 2 nm, where the GNPs synthesized with different concentrations of the pepsin coating solution were not significantly different. This suggests that DLS was unable to successfully distinguish the small differences in hydrodynamic diameter of the pepsin-GNP bioconjugates as compared to bare GNPs (Fig. 3.7A), while TDA successfully resolved increases in R_H due to pepsin adsorption as small as < 2 nm with a high degree of repeatability and accuracy (Fig. 3.7B).

Size characterization is also important for understanding organic materials, for observing polymer synthesis and degradation, ligand binding, and monitoring reaction progress. The utility of UV and fluorescence detection modes for TDA are discussed previously in this review. In many situations pertinent to organic synthesis, however, molecules may exhibit neither significant UV absorption nor fluorescence. Refractive index (RI) detection is a potential alternative in these cases, and RI detection has been used for TDA performed with HPLC instrumentation, but these examples required detection volumes on the order of $10 \mu L$, which eliminates the key advantages of small sample volumes and reduced analysis times in TDA [62, 101, 102]. Saetear *et al.* developed a backscattering interferometry (BSI) approach to improve RI detection in TDA with nanoliter sample volumes [89]. A selection of poly- and monosaccharides, which present a significant detection challenge due to low UV absorbance, were characterized by TDA using the BSI technique in a commercial CE instrument. Determinations of D gave an average RSD of 2%, demonstrating high repeatability in the TDA-BSI analyses of non-UV absorbing molecules. In continuation of this study, Leclercq *et al.* investigated a UV-photooxidation (UV-POD) detection mode for TDA of polysaccharides and compared results to the BSI method, as detection sensitivity of TDA-BSI is relatively low ($LOD \approx 50 - 80 \text{ mgL}^{-1}$) [22]. R_H values for a selection of polysaccharides were determined by TDA-BSI and TDA-UV-POD with an average relative difference in R_H between the two detection modes of 2% and an RSD below 3%. Additionally, detection sensitivity of TDA-UV-POD for pullulans and dextrans was greater than that of TDA-BSI ($LOD = 40 \text{ mgL}^{-1}$ and $50 - 60 \text{ mgL}^{-1}$, respectively). These works expand

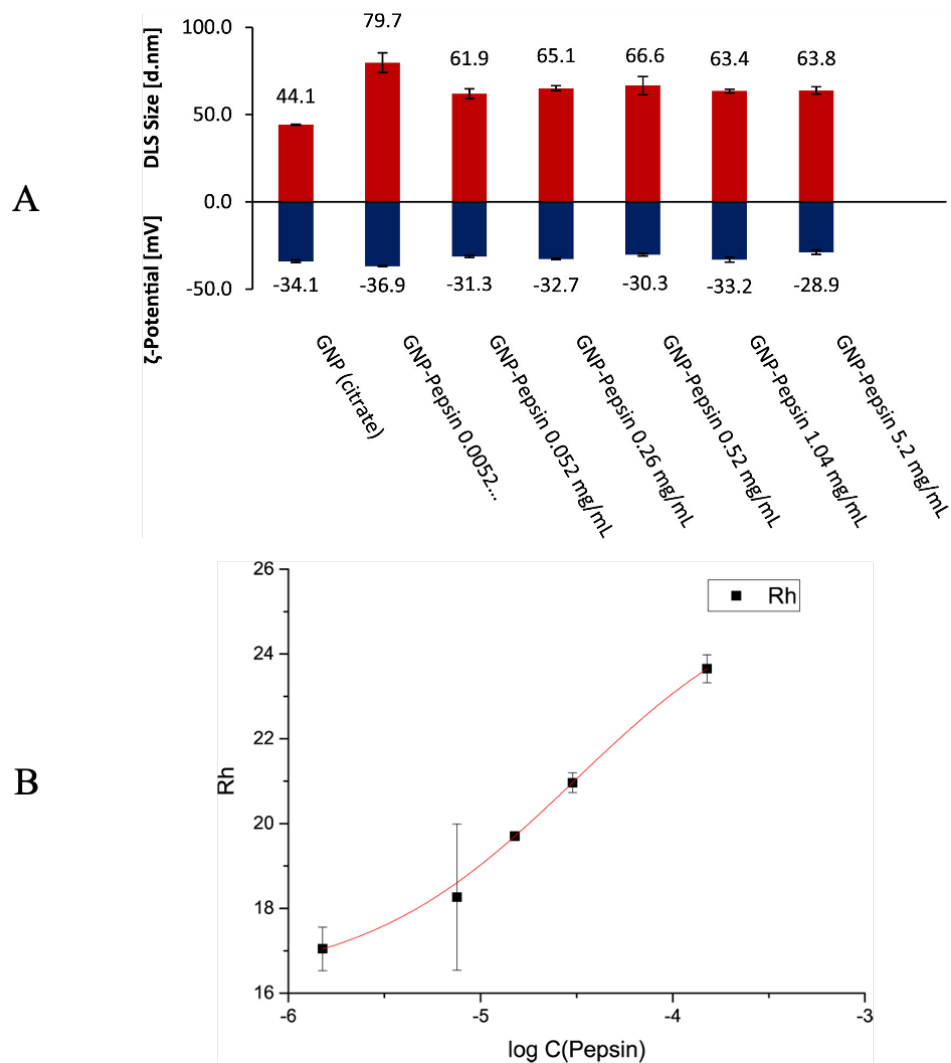


Figure 3.7: Comparison of A) DLS measurements for hydrodynamic diameters and B) TDA measurements for R_H of pepsin-coated GNPs at varying concentrations of pepsin. While increasing diameter of pepsin-functionalized GNPs was expected with increasing pepsin concentration, DLS did not resolve the small size differences of bioconjugates prepared at various pepsin concentrations. TDA resolved the trend of increasing R_H with increasing pepsin concentration. Adapted from Talanta, Vol. 167, Markus Holdrich, Siyao Liu, Markus Epe, Michael Lammerhofer, Taylor dispersion analysis, resonant mass measurement and bioactivity of pepsin-coated gold nanoparticles, 67-74, Copyright (2017), with permission from Elsevier.

the applications of TDA to applications requiring universal detection modes.

Characterizing the degradation products of biopolymers is essential for understanding the fate of these materials in biomedical applications. The application of TDA to monitoring hydrolytic degradation of a fifth generation dendrigraft poly-L-lysine (DGL G5) has been described [60]. Using three different approaches – curve fitting, cumulant series expansion, and constrained regularized linear inversion - R_H values were obtained at different degradation times. The R_H value for the enzymatic degradation of DGL n corresponds to the R_H of the $n - 1$ generation, elucidating structural and behavioral information about the polymer and enzyme, such as reaction kinetics and degradation process. Other biopolymers, such as natural rubber [103], polyplexes [21], and the influence of ionic strength[104] on these systems have also been studied by TDA.

3.9 Conclusion

In this review, we aimed to illustrate TDA as a powerful sizing technique that offers comparable sizing performance with reduced sample consumption and often improved detection sensitivity as compared to more common sizing techniques such as DLS, SEC, or various imaging approaches. First, we offered a tutorial on the fundamental principles that allow TDA to achieve calibration-free sizing of analytes across a wide range of R_H , with an emphasis on the reduced sample consumption and analysis times that result from utilizing fused silica capillaries. We continued by highlighting relationships between operating parameters, such as u and R_c , and the critically important Taylor conditions. Our intention was to acquaint those seeking to utilize TDA with the careful consideration needed to design effective TDA experiments. Our discussion continued by looking at methods for applying TDA to sample mixtures, first via algorithmic approaches, then by looking at the integration of CE and TDA. Finally, we presented a selection of reports that demonstrate TDA applied to complex challenges in bioanalysis and materials science.

TDA is a particularly attractive analytical method because it achieves calibration-free sizing across an impressive dynamic range (ca. $\text{\AA}$ - μm) while utilizing nL sample volumes and straightforward and accessible instrumentation. We see a valuable opportunity to expand the

adoption of this technology within the broader separation science community. Meeting that opportunity requires effort in a few areas. First, wider dissemination of the principles and capabilities of TDA are needed, towards which we offer the current tutorial review. Second, navigating the relationship between operating parameters and meeting the Taylor conditions is a barrier to entry for those with little or no prior experience in TDA. This barrier would be substantially lowered by making available adaptable, open-source calculation tools. Third, although TDA can be readily achieved with commercial CE instrumentation, the cost of these instruments is not trivial, and their capabilities far exceed the minimum requirements for typical TDA analysis. Therefore, those without a CE instrument may not be inclined to acquire one for the sole purpose of TDA, and those with a CE instrument may find its broader capabilities put to more efficient use for other purposes. A few TDA-specific instruments are commercially available, but the cost may be yet another barrier to entry. Ultimately, TDA is a mechanistically simple measurement to implement, which may be amenable to the development of low-cost, open-source hardware that will enable wide adoption of this powerful analytical method. We believe that wider adoption of TDA will enable new dimensions of analysis across various sub-fields of analytical chemistry and measurement science.

Currently, there are few automated capillary systems commercially available that have sizing capabilities based on Taylor dispersion. Because of its inherent ability to control pressure flow within a capillary, TDA is commonly performed within commercial CE instrumentation. However, as described in Chapter 1, the use of CE instrumentation comes with its own set of limitations for dual-point fluorescence detection. Chapter 4 will describe the development of a novel 3D printed instrument for performing TDA in fused silica capillaries and high sensitivity laser-induced fluorescence detection.

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Chapter 4

A 3D Printed Instrument for Taylor Dispersion Analysis with Two-point Laser-induced Fluorescence Detection

A version of this chapter was originally published by Meagan R. Moser, Claire M. Smith, and Christopher A. Baker. Only minor modifications were made.

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4.1 Abstract

Precise control of the size of various types of biological and pharmaceutical compounds is often critical. Standard methods for size characterization, such as dynamic light scattering, are ultimately sample intensive and do not provide the sensitivity needed for low volume, low concentration biological systems. There is a need for more sensitive, accurate, and straightforward analytical tools for size analysis. Taylor dispersion analysis (TDA) is a proven analytical method for size determination without the need for calibration and utilizes only nL - pL of sample. In TDA, average diffusion coefficients are determined by characterizing band broadening of an analyte under well-controlled laminar flow conditions, allowing for the determination of hydrodynamic radius. Here, we describe the design and optimization of a 3D printed instrument for TDA with dual-point laser-induced fluorescence (LIF) detection. The instrument utilized a fully 3D printed eductor as a vacuum source for precise and stable pressure driven flow within the capillary, evidenced by a linear response in generated static pressure to applied gas pressure ($R^2 = 0.997$) and a 30-fold improvement in stability of static pressure (0.05% RSD) as compared to a standard mechanical pump (1.53%). Design aspects of the LIF detection system were optimized to maximize S/N for excitation and emission optical axes, and high sensitivity was achieved by an 80 pM limit of detection for the protein R-phycoerythrin and low-nM limits of detection for three additional fluorophores. The utility of the instrument was demonstrated via sizing of R-phycoerythrin at pM concentrations, and evaluation of synthetic liposome preparations.

4.2 Introduction

Macromolecular products for biological and pharmaceutical applications, such as liposomes, nanoparticles, engineered proteins, and drug delivery systems, can require precise control over product size to achieve desired material properties. The design and development of new macromolecular materials can benefit from the availability of simple, rapid, sensitive, and high accuracy analytical methods for size characterization. Many common size characterization techniques require extensive method development, tedious sample preparation, large sample volumes, or lengthy analysis times [1]. Two widely used methods for size characterization are dynamic light scattering (DLS) and size exclusion chromatography (SEC). DLS provides rapid measurements with limited sample preparation, but is biased in sensitivity towards large particles and aggregates [2]. For example, Hawe *et al.* used DLS to measure a range of concentrations (0.05 - 50 mg mL⁻¹) of several proteins and peptides [3]. Though DLS successfully sized the antibody drug adalimumab (5 - 6 nm) at the lowest concentration, it was not possible to size the peptide oxytocin (~ 0.8 nm) at lower concentrations. This was due to the influence of dust or excipients in the sample, which resulted in inaccurate and inconsistent hydrodynamic radius (R_H) determinations ranging from 6.9 - 130 nm for oxytocin. SEC requires calibrations and is susceptible to analyte-column interactions dependent on mobile phase composition [4]. Ricker and Sandoval reviewed various operational parameters, such as column length, flow rate, sample volume, and mobile phase conditions, that affect SEC separations [5]. They demonstrated the susceptibility of SEC methods to deleterious effect related to mobile phase ionic strength and pH while analyzing mouse myeloma antibodies with differing charges. At pH 7, the strongly basic antibody showed broadening and non-specific retention in the column at lower ionic strengths (20 and 50 mM) while the weakly basic antibody begins to broaden and be retained at higher ionic strengths (600 and 1000 mM). Such electrostatic and hydrophobic effects exhibited in SEC columns ultimately result in inaccuracies in measured R_H . Though various solvent additives and organic modifiers have been explored and employed for SEC separations to suppress adsorption and hydrophobic interactions [4, 6, 7, 8, 9], extensive method development is still often required to obtain accurate sizing.

Taylor dispersion analysis (TDA) offers similar sizing capabilities to DLS and SEC, while utilizing nL sample volumes and without the need for size-based calibration or comparison to size standards. First described in 1953 [10], TDA enables direct determination of molecular diffusion coefficients across a wide dynamic range of hydrodynamic radii from Å to μm . Taylor dispersion arises from the interplay of radial diffusion within a cylindrical flow channel and the parabolic velocity flow profile of pressure-driven laminar flow in such flow systems. As a result, a sample zone will spread out axially, the degree of which can be observed by characterizing band broadening of a sample zone under continuous flow. Diffusion coefficient is inversely proportional to an analyte's hydrodynamic radius, and therefore the rate of band broadening by Taylor dispersion is influenced directly by the analyte's hydrodynamic radius. TDA is a mathematical framework for extracting diffusion coefficients directly from the observed rates of band broadening due to Taylor dispersion.

TDA has evolved from studying gaseous diffusion coefficients in large tubes [11] to capillary-based solution phase measurements of small molecules [12, 13], macromolecular complexes [14, 15, 16], and even micron-scale particles [17]. In TDA, reduced tubing diameter is associated with reduced analysis times. The time required to generate a measurable change in dispersion for measurements of D is proportional to the square of the tubing radius. Thus, small molecule analytes flowing through a tube of 1 mm radius would require several hours to produce the same degree of Taylor dispersion observed in a 50 μm radius capillary in only minutes [18]. Reduced analysis times, improved precision of flow rate control, and low sample consumption have all motivated the use of capillary electrophoresis (CE) instrumentation for performing TDA.

Highlighting the need for continuous flow velocity in TDA, Sharma *et al.* described the effects of the initial ramp in flow velocity that occurs upon pressure application in CE instruments [18]. Discontinuous flow velocities resulting from stopping flow for sample injection introduced significant error in determining diffusion coefficients, requiring empirical mathematical corrections. Measurement errors as high as 40% have been attributed to these sub-optimal flow conditions [19]. To address these limitations, dual-point detector schemes have been developed that allow TDA to be conducted under continuous flow [12, 20, 21, 22]. Chamieh *et al.* addressed the engineering

challenge of integrating dual-point detection into commercial CE instrumentation by modifying the optical detection interface within an Agilent CE cassette such that the capillary could be looped within the cassette and pass the UV detection point twice [21]. Diffusion coefficients and corresponding hydrodynamic radii measurements for 10 mM caffeine, 75 μ M BSA, and 75 μ M HSA were in agreement with literature values to within 5%, however this detection scheme resulted in substantially reduced sensitivity for UV absorbance detection. Our group developed an LED-induced fluorescence detection system for integration into commercial CE instrumentation, enabling dual point detection with higher sensitivity than is possible by UV absorbance detection [22]. A miniature detection head was 3D printed to house an excitation source and excitation and emission optics which enabled the integration of two detectors within a single capillary cassette. Multivariate optimization of operating conditions, such as LED current, yielded a detection limit of 613 ± 13 pM for fluorescein. While LED-induced fluorescence offered improved sensitivity over UV absorbance detection, these detection limits were still modest in comparison to laser-induced fluorescence (LIF) detection, a common detection mode for high sensitivity CE assays.

The evolution of TDA towards commercial CE instrumentation has enabled fast analysis times and precise control over flow rates, but it has also presented engineering challenges, especially with regard to integrating dual-point detection. None of the existing approaches to dual-point detection in commercial CE instruments can achieve detection limits comparable to that of CE-LIF. Therefore the substantial expense of acquiring CE instrumentation is accompanied by a significant compromise in quantitative performance when applying that instrument to TDA. An improved approach to instrumentation for capillary TDA would offer precision flow control, dual point LIF detection, and, to improve accessibility and adoption of TDA, dramatically reduced cost compared to available commercial CE instrumentation.

To deliver improved instrumentation for capillary TDA, and to enable wider adoption of TDA as a sizing technique, in the present work we describe the design and optimization of a 3D printed instrument for TDA with dual-point LIF detection. The instrument utilized a fully 3D printed eductor as a vacuum source for precise and stable pressure driven flow within the capillary. An LIF detection system was developed based on a 3D printed architecture for holding

various optical elements in alignment with the capillary. Design aspects of the LIF detection system were optimized to maximize S/N, and the utility of the high sensitivity TDA instrument was demonstrated via protein sizing at pM concentrations, and evaluation of synthetic liposome preparations.

4.3 Theory

TDA is a powerful sizing technique that is surprisingly underutilized within the separation science community. Therefore, we offer here a brief primer on the underlying theory of TDA to facilitate implementation by others of the instrumentation described elsewhere in this work.

To enable direct determinations of diffusion coefficient by characterizing the rate of band broadening, two stipulations are placed on the experimental flow conditions, collectively termed the “Taylor conditions”. The first Taylor condition ensures that the rate of band broadening due to longitudinal diffusion is negligible compared to the rate of band broadening due to Taylor dispersion. To achieve this, the rate of advection must be sufficiently greater than the rate of diffusion, which is satisfied when the Peclet number (Pe) is greater than 69 [23], defined by the following equation [18]:

$$Pe = \frac{uR_c}{D} \quad (4.1)$$

where u is the linear flow rate, R_c is the radius of the capillary, and D is the molecular diffusion coefficient. Note that since this is a threshold condition, it does not require foreknowledge of D for a particular analysis, only a defined upper bound above which the accuracy of D measurements will diminish. Still, this condition warrants careful consideration when defining parameters for TDA experiments.

The second Taylor condition ensures that the sample remains in flow long enough to observe substantial band-broadening via Taylor dispersion [10]. This condition is satisfied when the unitless time parameter (τ) is greater than 1.4, with defined as:

$$\tau = \frac{Dt_R}{R_c^2} \quad (4.2)$$

where t_R is the requisite residence time of the sample plug in flow. This equation illustrates that by reducing R_c , analysis times are also reduced, which has encouraged the use of fused-silica capillaries in modern TDA applications.

The Diffusion coefficient (D) is determined directly from the observed rate of band broadening via the Taylor-Aris equation:

$$D = \frac{R_c^2 t_d}{24\sigma^2} \quad (4.3)$$

where σ^2 is the variance of the resulting peak that has undergone band broadening via Taylor dispersion. One counterintuitive consequence of the Taylor-Aris equation is that by Taylor dispersion low D corresponds to higher rates of band broadening. This is the opposite trend to that observed by longitudinal diffusion, and highlights the importance of satisfying the Taylor conditions.

Due to considerations discussed above, two-point detection is an effective way to improve accuracy in TDA by ensuring stable and continuous flow for the duration of t_R . Therefore, for two-point detection, the Taylor-Aris equation is modified as follows [24]:

$$D = \frac{R_c^2(t_2 - t_1)}{24(\sigma_2^2 - \sigma_1^2)} \quad (4.4)$$

where t_1 and t_2 are the peak arrival times at detectors 1 and 2, respectively, and σ_1^2 and σ_2^2 are the peak variances as observed at detectors 1 and 2, respectively. Using a spherical approximation of molecular geometry, D is transformed to hydrodynamic radius (R_H) via the Stokes-Einstein relation [25]:

$$R_H = \frac{k_B T}{6\pi\eta D} \quad (4.5)$$

where k_B is the Boltzmann constant, T is temperature, and η is the dynamic viscosity of the solution.

4.4 Materials and Methods

4.4.1 Reagents and materials

Rhodamine 6G 99% was ACROS Organics brand from Fisher Scientific (Suwannee, GA). R-Phycoerythrin was purchased from AnaSpec, Inc. (Fremont, CA), AlexaFluor 532 was purchased from Thermo-Fisher Scientific (Waltham, MA), and CF532 was purchased from Biotium (Hayward, CA). Phosphate buffered saline (PBS) was Fisher BioReagents brand and purchased from Fisher Scientific (Suwannee, GA). All aqueous solutions were prepared using DI water. Fused silica capillary was Polymicro brand from Fisher Scientific (Suwannee, GA).

Phospholipids, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Liss Rhod PE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL).

4.4.2 3D printing

An AnyCubic Photon-S Digital Light Processing Stereolithography (SLA) 3D printer was used to print the eductor with a Peopoly Moai resin (Matter Hackers, Burbank, CA) with a layer height of 0.1 mm, 80 s bottom layer exposure time, and 30 s exposure time to completion. A Form 3B SLA 3D printer was used to print the capillary cartridges, alignment tool, and fully functional instrument with Tough 2000 resin (RS-F2-TO20-01) using the default resin settings and a resolution set to 50 μm .

4.4.3 Eductor-driven pressure system

To characterize the vacuum pressure system, the eductor component of the instrument was 3D printed separately. The gas inlet barb fitting was connected to an in-house nitrogen line and the vacuum barb fitting was connected to a 5 ft length of 1/8" i.d., 1/4" o.d. vinyl tubing. The end

of the tubing was submerged into a beaker containing Milli-Q water to form a water column for static pressure measurements. For vacuum pressure characterization, the nitrogen line was set to varying pressures (2-10 psi) and mmH₂O was recorded by measuring the resulting level within the water column. The corresponding values of mmH₂O for each gas inlet pressure were converted to units of psi for calibration plots. Eductors are ultimately controlled via volumetric flow rate of the compressed gas source, thus the regulated gas pressure supply was calibrated to allow reporting of volumetric flow rate as an independent variable. A 1000 mL graduated cylinder was filled with water and inverted within a tank of water. The effluent of the nitrogen supply line was captured within the graduated cylinder at various regulated pressures (2 - 10 psi), and the rate of water evacuation from the graduated cylinder was recorded on video to determine the volumetric flow rate of nitrogen gas as a function of regulated gas pressure.

For pressure stability characterizations, video was recorded via smartphone camera of the water column level at 10 vacuum pressure settings. ImageJ was used to track the pixel location of the air-water interface over time, and a MatLab script written in-house was used to convert the sequence of tracked pixel locations to height of the water column in mm via a calibration image that included a ruler in the field of view for length calibration purposes.

4.4.4 LIF detection system

Two 532 nm collimated laser-diode-pumped laser modules were purchased from Thorlabs Inc. (CPS532, Newton, NJ). Laser excitation light was directed to the detection windows of the capillary via a 9.0 mm 20 mm FL N-BK7 plano convex lens (LA1472, Thorlabs Inc., Newton, NJ) and internally printed pinhole aperture. Fluorescence emission was collected via 6.0 mm N-BK7 ball lens (Edmund Optics Inc., Barrington, NJ) and 10 mm FL 25 mm diameter concave mirror (43464, Edmund Optics Inc., Barrington, NJ) for transmission to a Hamamatsu H7828-50 Photomultiplier Module fitted with a 0.9 OD neutral density filter (NE09B, Thorlabs Inc., Newton, NJ), 533/17 nm OD 6 notch filter (NF533-17, Thorlabs Inc., Newton, NJ), 580 ± 2 , FWHM 10 ± 2 nm OD6 bandpass filter (FB580-10, Thorlabs Inc., Newton, NJ), 550 nm OD6 longpass filter (FELH0550, Thorlabs Inc., Newton, NJ), 50 mm focusing lens (LA1131, Thorlabs Inc.,

Newton, NJ), and 532 nm OD5 notch filter. Data acquisition via a USB DAQ module (USB-6341, National Instruments, Austin, TX) with software written in-house in LabView (National Instruments, Austin, TX).

4.4.5 Validation of TDA using model fluorescent molecules

Taylor dispersion experiments were carried out in a 25.5 cm length, 100 μm i.d./360 μm o.d. fused-silica capillary with 15 cm window spacings. Samples of 20 nM AlexaFluor 532 and 10 nM, 5 nM, 1 nM, and 750 pM R-PE were prepared with 1X PBS buffer prior to the experiments. For each run, the capillary was filled with 1X PBS buffer via eductor-driven vacuum flow. Before sample introduction, buffer flow was stopped via pneumatic valve, which allowed the replacement of the buffer vial with the sample vial without introducing air bubbles into the capillary. Samples were injected by momentarily resuming eductor flow via the pneumatic valve. Flow was stopped again, and sample vial was replaced with buffer vial, before eductor flow was resumed for the remainder of the measurement. Each peak was fitted as a Gaussian function, and differences in arrival time and peak variance at the two detection points were used to calculate diffusion coefficient (eq. 4.4) and hydrodynamic radius (eq. 4.5). Values were tested for outliers using Grubb's test for outliers. At a confidence interval of 95%, 2 data points were eliminated as outlying values from data collected with 5 nM and 1 nM R-PE.

4.4.6 Lipid vesicle preparation

DOPC lipid solutions containing 1% w/w of Liss Rhod PE were prepared to a final lipid concentration of 2 mg mL^{-1} . Chloroform solvent was evaporated from the lipid mixture by N_2 stream for 10 minutes and placed on vacuum overnight. The dry lipid film was hydrated with PBS buffer for 1 hour. The buffer was prepared by adding 0.1% w/w bovine serum albumin (BSA) (Fisher Scientific) to 1X PBS. For sonicated vesicle preparation (diameter ca. 50 nm) the lipid solution was sonicated for 2 hours using a bath ultrasonic cleaner (Branson 2800). To produce vesicles of larger diameters, hydrated lipid solutions were subjected to 10 freeze-thaw cycles before 15 passes of extrusion through 200 nm pore diameter polycarbonate membrane filters at

room temperature using a mini extruder (Avanti Polar Lipids Inc., Alabaster, AL).

4.4.7 Sizing of synthetic lipid vesicles by TDA-LIF

Taylor dispersion experiments for the population of vesicles prepared via sonication were carried out in a 25.5 cm length, 100 μm i.d./360 μm o.d. fused-silica capillary with 15 cm window spacings. For the 200 nm vesicles prepared via membrane extrusion, experiments were carried out in a 35.5 cm length, 100 μm i.d./360 μm o.d. fused-silica capillary with 25 cm window spacings. The experimental procedure for sample injections and post-analysis were identical to that described above for model fluorescent molecules. Values were tested for outliers using Grubb's test. At a confidence interval of 99%, 1 data point was eliminated as an outlying value for data collected with the 50 nm vesicle population.

4.5 Results

Commercial CE instrumentation provides convenient and precise control of flow rates in capillaries, but significant barriers to adopting TDA exist including instrumental cost and the lack of available dual-point LIF detection. To reduce these barriers, we have developed a 3D printed instrument for TDA that combines accurate capillary positioning, precise flow control at low flow rates, and two-point LIF detection.

4.5.1 Capillary positioning

Precise control of capillary length and the distance between detection points are key to accurate measurements by TDA because they influence the precision of t_R and u , both of which are critical to satisfying the Taylor conditions. The instrument design utilized the geometric precision of 3D printing to achieve accurate detection window positioning via capillary cartridges designed for three specific detection window spacings: 10 cm, 15 cm, and 25 cm (Fig. B.1). To prepare a capillary cartridge with appropriately spaced detection windows, an accompanying 3D printed alignment tool was produced (Fig. B.1) to indicate precise locations of detector windows and

cutting locations (Fig. B.1, red arrows) at the capillary inlet and outlet. Capillary cartridges mounted to the alignment tool with the same dovetail joint utilized on the instrument body. Thus, once the capillary is prepared the cartridge is removed from the alignment tool, placed onto the instrument body, and secured with a 3D printed key (Fig. B.1, red box) to ensure that the placement of the detection windows aligns appropriately with excitation and detection optics within the 3D printed LIF detector system.

While the cartridge and alignment tool ensured appropriate detection window positioning in the longitudinal dimension of the capillary, lateral positioning of the capillary within the detection volume defined by the focal volumes of excitation and collection optics was also critical. Typical consumer grade 3D printing resolution is not sufficient to produce features small enough to define lateral positioning of the 360 μm o.d. capillary within the detection volume. While this is well within the capabilities of high cost, high resolution 3D printing, the present technology was intended to improve access to TDA, and therefore we designed the instrument with low cost commercial 3D printers in mind. A simple approach to achieve appropriate lateral positioning was the use of capillary tubing sleeves of the type utilized to interface capillaries with 1/16" fingertight fittings. Thus, the opening to accept the capillary into the 3D printed detectors was sized to fit the outer diameter of these tubing sleeves, and then sleeves were inserted to ensure that the capillary was centered on the intended axis through the middle of the detector (Fig. B.2).

4.5.2 Eductor-driven pressure system

Achieving accurate TDA with relatively short capillary lengths required precise and stable control of pressure-driven flow at low nL min^{-1} volumetric flow rates. At the core of the instrument design is a fully 3D printed eductor, designed to generate sub-ambient pressure at the capillary outlet and, therefore, drive pressure-driven flow through the capillary with an open and accessible capillary inlet. Eductors operate on the Bernoulli principle, and have been employed in various applications, including coupling digital microfluidics to electrospray mass spectrometry [26] and for the generation of liposomes [27]. The internal geometry of the 3D printed eductor can be seen in (Fig. 4.1A), and consists of two coaxial nozzles nested such that compressed air flowed through

the internal nozzle entrains flow through the external nozzle, generating modest vacuum pressure to induce flow within the capillary. As highlighted by the benefits of two-point detection for TDA, stable continuous flow rates are essential for accurate TDA. (Fig. 4.1B) illustrates static vacuum pressure vs. time for a mechanical diaphragm pump (blue) as compared to that achieved by the 3D printed eductor (black). A 30-fold improvement in stability of static pressure is observed for the 3D printed eductor (0.05% RSD) as compared to the mechanical pump (1.53% RSD). (Fig. 4.1C) shows that highly stable static pressures ($RSD < 0.1\%$) were produced with the eductor system across a range of static vacuum pressures from 0.2 to 1.7 psi below ambient pressure. Ultimately, the eductor system must provide precise control of vacuum pressures to allow flow conditions to be tuned to meet Taylor conditions for various analyses. (Fig. 4.1D) shows the resultant static vacuum pressure as a function of volumetric flow rate of compressed air applied to the eductor inlet ($R^2 = 0.997$). Precise control of static pressure produced by the eductor enabled precise control of flow rates in the capillary for TDA experiments.

4.5.3 Optimized design of the LIF detection system

To reduce the collection of scattered excitation light, the optical design collects fluorescence emission 90° from the excitation path as illustrated in Fig. 4.2A. Two LIF detectors of identical configuration are integrated into the instrument design. Each detector consisted of a housing to mount a low cost 532 nm diode laser module as an excitation source, and a concave mirror and ball lens as the primary optics for collecting fluorescence emission (Fig. 4.2C). The mirror and ball lens were positioned to collimate collected emission light, which was transmitted via a conventional 25 mm lens tube to a PMT for signal transduction. The PMT was fitted with a planoconvex lens to focus collimated emission light onto the PMT active area, and a 532 nm notch filter to selectively reduce the intensity of scattered excitation light. Despite the notch filter fitted at the PMT, aberrant scatter of the laser sources was a significant hurdle to achieving appropriate fluorescent detection limits with this detector design. Therefore, we set out to optimize the detector design to reduce scatter and improve overall S/N for detected fluorescence signals.

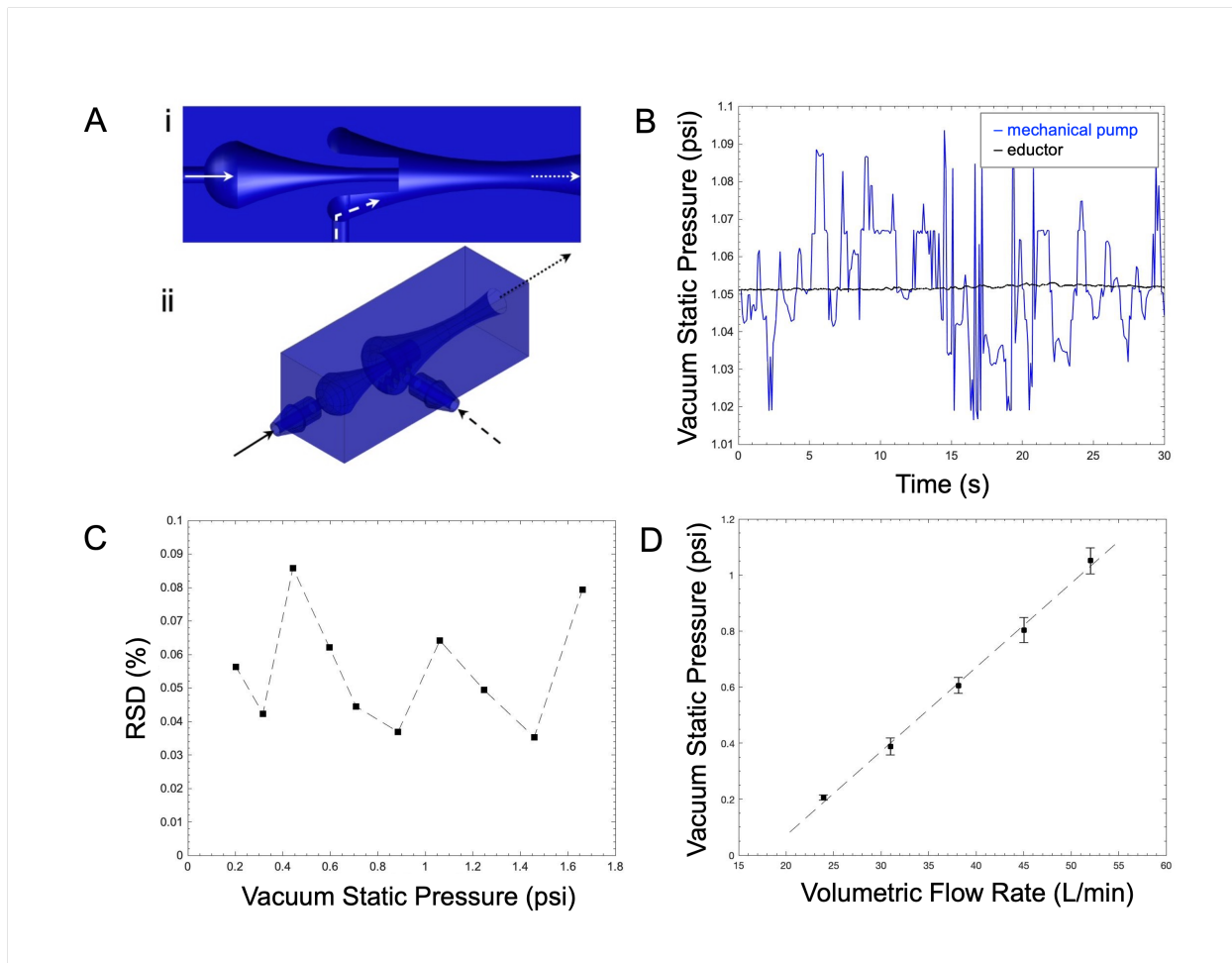


Figure 4.1: Eductor pressure characterization. (A) CAD illustrations of the eductor in cross section (i) and 3D with transparency (ii) to illustrate internal geometry. Arrows indicate the direction of flow for compressed air/ N_2 (solid arrows), entrained vacuum flow (dashed arrows) and exhaust flow (dotted arrow). (B) Comparison of vacuum pressure stability over time for eductor (black trace, 0.05%) and mechanical pump (blue trace, 1.53% RSD). (C) Relative standard deviation percentages in static pressure stability over a range of applied inlet gas pressures. Average RSD = 0.055%. (D) Calibration of the vacuum static pressure generated by the eductor for an applied inlet gas volumetric flow rate.

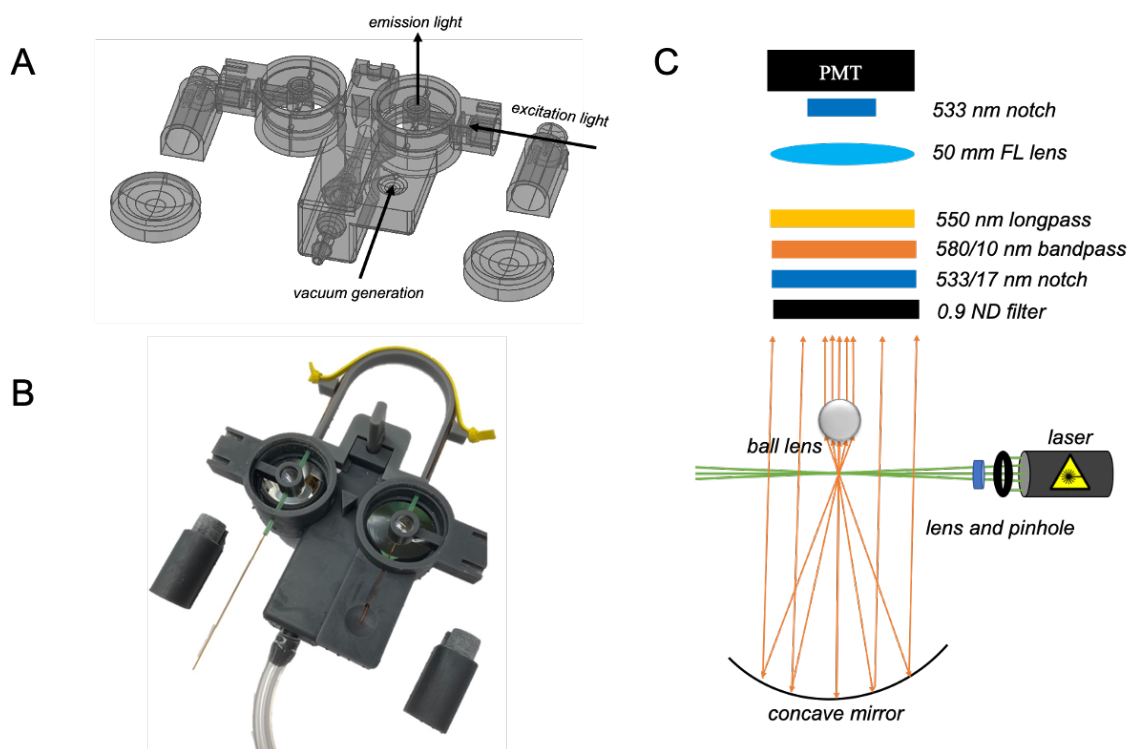


Figure 4.2: Design illustrations of 3D printed TDA instrument. (A) Rendering of 3D CAD model illustrating internal geometries. (B) Photograph of the fully assembled instrument, mirrors, ball lenses, capillary, and capillary cartridge. (C) Schematic illustration of the optical design in the optimized excitation and emission axes.

Using a 500 nM Rhodamine 6G solution as a model fluorophore, we observed the resultant S/N for various design configurations, and results of this optimization are illustrated in (Fig. 4.3). The emission axis was optimized by characterizing the serial addition of various optical filters (Fig. 4.3A and B). In addition to the PMT outfitted with a focusing lens (PCX, FL = 50 mm) and notch filter (533/17 nm, OD 6), the emission axis initially included the appropriate bandpass filter (580 ± 2 , FWHM 10 ± 2 nm OD6), however under this configuration the net signal of fluorescence and background exceeded the dynamic range of the PMT. Thus, the first design modification evaluated, termed “Design A”, added a neutral density filter (OD 0.9) to attenuate overall signal intensity to within the dynamic range of the PMT. This OD value was selected based on observing S/N with 500 nM Rhodamine 6G for various OD values (data shown in Fig. B.3). In an attempt to further selectively reduce the effect of laser scatter, Design B added a longpass filter (550 nm, OD6). Design C added an additional notch filter (532 nm, OD5), resulting in a dramatic decrease in background intensity and a commensurate increase in S/N. Since the majority of gains in S/N from design A to Design C appeared to be attributed to the additional notch filter, Design D evaluated simplifying the emission axis by removing the longpass filter. Surprisingly, this resulted in dramatic reduction of S/N, and led us to conclude that the maximum filtering approach (i.e. Design C) was optimal among the conditions evaluated. Although this implies that further filtering may have resulted in continued improvements of S/N, in our judgement the added cost of additional filtering was counter to the intended purpose of producing an accessible TDA instrument, and we anticipated that the sensitivity provided by Design C would be sufficient to achieve practical detection limits for various common fluorophores in this wavelength regime. Still, more work in this area may further improve S/N in this LIF detector design.

The design of the excitation optical axis was also considered on the basis of maximizing signal intensity and reducing laser scatter. A 9 mm diameter plano-convex lens (FL = 20 mm) was positioned in the excitation path to focus the laser onto the capillary detection window, maximizing incident intensity for fluorescence excitation. We evaluated spatial filtering of the laser source with pinholes of various diameters (0.4-2.5 mm) as a means to reduce the intensity of scattered excitation light collected in the emission axis. The pinholes were positioned between the laser source and the focusing lens to effectively attenuate the beam diameter of the laser, and

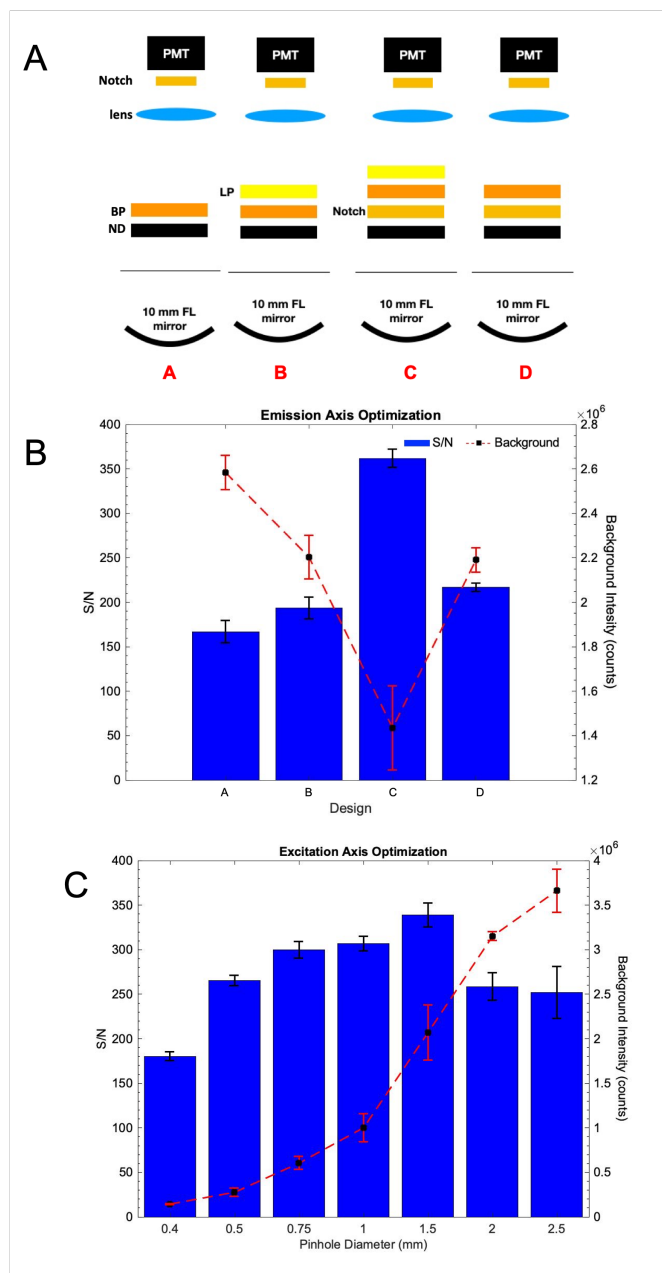


Figure 4.3: Excitation and emission axis optimization. (A) Design configurations included in S/N optimization labeled A through D. (B) Results of emission axis optimization, including comparison of S/N and background counts. (C) S/N and background counts as a function of pinhole diameter in the excitation axis. A pinhole diameter of 1.5 mm produced the greatest S/N ratio. At 2.5 mm pinhole diameter, 500 nM analyte concentration produced a detector response that exceeded the dynamic range of the PMT, therefore the data shown is a 2-fold extrapolation of data collected for an analyte concentration of 250 nM.

S/N and background intensity were investigated as a function of annulus diameter (Fig. 4.3C). Unsurprisingly, we observed increased background due to scatter with increasing beam diameter. Similarly, we observed increasing fluorescent signal with increasing beam diameter (data not shown), as the increased beam diameter represented an increase in excitation intensity at the focused detection point. Increases in both signal and noise were balanced at an optimal S/N with a pinhole diameter of 1.5 mm. This spatial filter was then incorporated into the final 3D printed geometry of the instrument.

4.5.4 LIF detection limits

To evaluate the efficacy of the LIF detection system for various applications, we produced calibration curves for 4 common fluorescent molecules compatible with 532 nm excitation: Rhodamine 6G, R-Phycoerythrin (R-PE), AlexaFluor 532, and CF532 (Fig. 4.4). In each case we observed a linear response over at least one decade of dynamic range. Importantly, these experiments were designed to characterize detection limits, and therefore further work is needed to evaluate the full linear and dynamic ranges for each fluorophore. The calculated LODs, based on linear regression of the calibration data, were 15 nM, 80 pM, 1 nM, and 5 nM, for Rhodamine 6G, R-Phycoerythrin (R-PE), AlexaFluor 532, and CF532, respectively. These detection limits offer the prospect of analyte sizing by TDA at low and sub-nM analyte concentrations.

4.5.5 Validation of TDA using model fluorescent molecules

The printed capillary cartridge and alignment tool were used to prepare a 100 μm i.d. capillary with 15 cm detection window spacings for a TDA demonstration on AlexaFluor 532 and R-PE. First, a flow rate calibration was performed with the selected capillary size and window spacing to determine the input eductor pressure needed to stay within the range of precise flow rate conditions (Fig. B.4). Operating at a flow rate within the TDA regime, sample plugs were injected into the capillary and the corresponding peaks were fitted to a Gaussian function (Fig. 4.5A). Figure 4.5A and 4.5B illustrates the differences in band broadening between AlexaFluor and R-PE, respectively. Raw data traces (gray) and Gaussian fits for detector 1 (red) and detector 2 (blue) are normalized

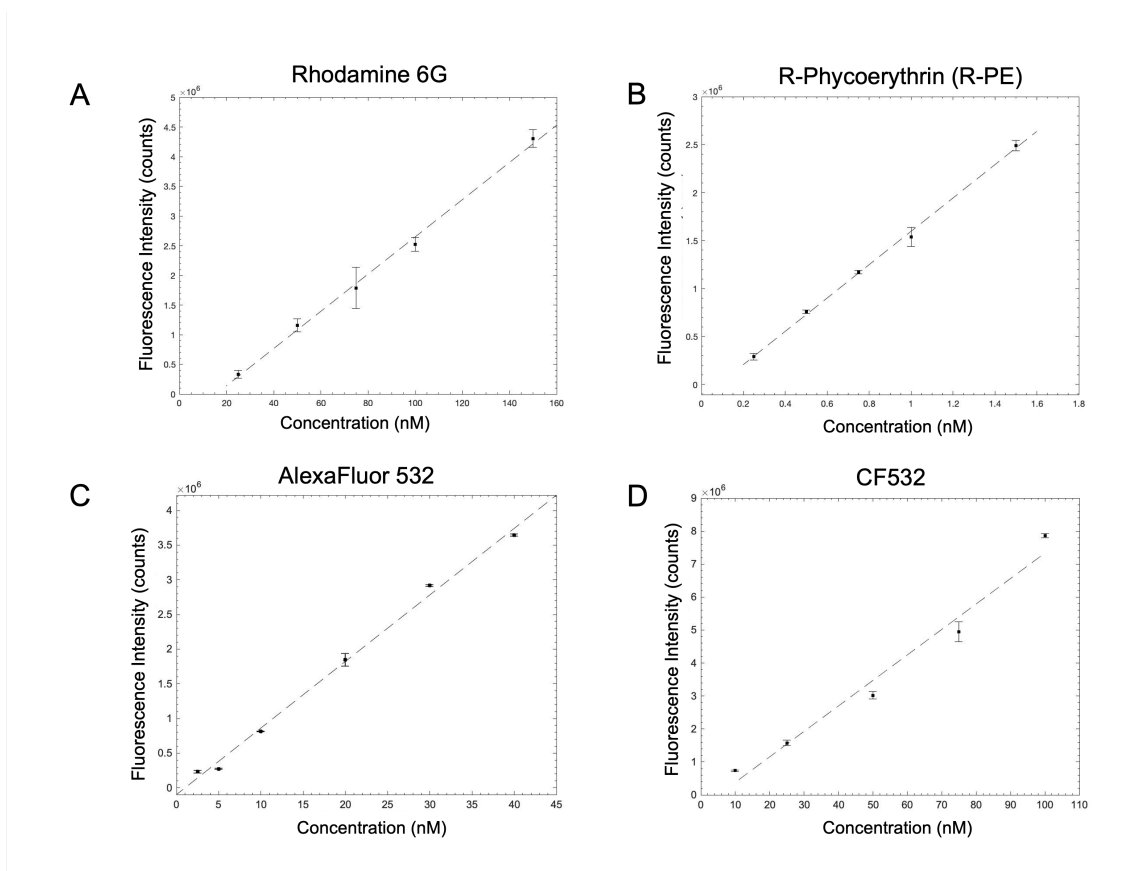


Figure 4.4: Concentration calibration for (A) rhodamine 6G, (B) R-Phycoerythrin (R-PE), (C) AlexaFluor 532, and (D) CF532. LODs were defined as $S/N = 3 \times$ standard deviation of the baseline, and determined by linear regression to be 15nM, 80pM, 1nM, and 5nM for A - D, respectively.

and peak the centers are overlaid to visualize the relative degree of band broadening. TDA of AlexaFluor 532 determined an R_H of 0.87 ± 0.35 nm (n=5). TDA was performed with 10 nM, 5 nM, 1 nM, and 750 pM R-PE providing R_H values of 5.48 ± 0.40 nm (n=11), 5.64 ± 0.3 nm (n=13), 5.75 ± 0.14 nm (n=12), and 4.87 ± 0.98 nm (n=14), respectively (Fig. 4.5B). We attribute the large variance in measured R_H at 750 pM to imprecision of the gaussian fit resulting from a relatively low S/N. As a result, the 750 pM data was statistically different from the 1 nM data at the 95% confidence level. Still, the median result of the 750 pM data set was 5.03 nm, which represents a 10.7% error from the population mean observed across all other concentrations (5.63 nm), and suggests some degree of efficacy in protein sizing even at sub-nM concentrations. TDA results were within reasonable agreement with MacColl *et al.*, who determined a hydrodynamic radius of 5.54 ± 0.18 nm for R-PE by DLS [28].

4.5.6 Sizing of synthetic lipid vesicles by TDA-LIF

To illustrate the utility of the TDA-LIF system towards potential pharmaceutical preparations, the instrument was applied to the sizing of synthetic lipid vesicles prepared by two different methods. First, vesicles prepared by sonication were analyzed using 100 μ m i.d. capillary with a 15 cm detection window spacing (Figure 4.6A). TDA, resulted in an R_H measurement of 22 ± 1 nm (n=4), consistent with previous reports of liposomes prepared in this manner (c.a. 7.5 - 25 nm [29]).

Equation 4.2 indicates that longer analysis times are required to meet the Taylor conditions for analytes with smaller diffusion coefficients. Thus, for TDA of liposomes prepared via extrusion through 200 nm track etched filter membranes, 25 cm detection window spacing was used to meet this criteria. TDA of extruded vesicles determined a R_H measurement of 107 ± 21 nm (n=3) was obtained, which agrees well with previous reports of extruded vesicles at or slightly larger than the diameter of extrusion pores [30].

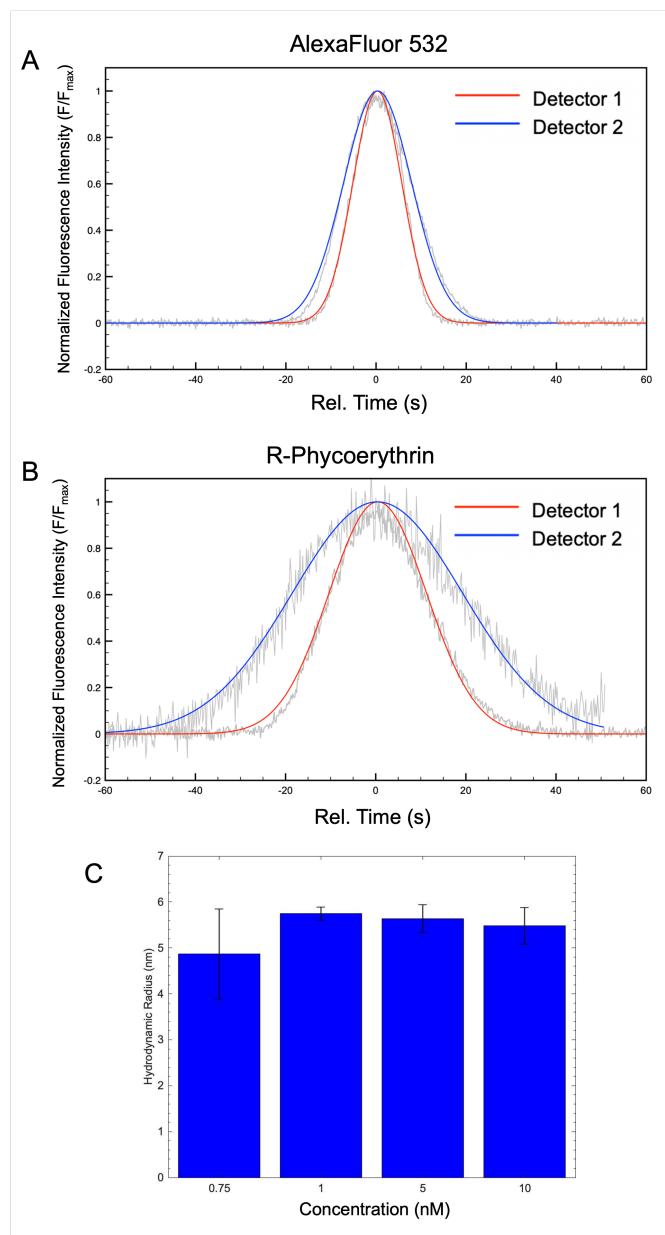


Figure 4.5: Dual-point detection TDA of (A) AlexaFluor 532 (20 nM); and (B) R-PE (5 nM). Raw data (gray) Gaussian fits (red and blue) are each normalized to a relative fluorescence scale, and the peaks are aligned on the time axis to illustrate relative differences in band broadening between the two model fluorophores. (C) Size determination for R-PE at various concentrations, illustrating protein sizing at low-to-sub nM concentrations.

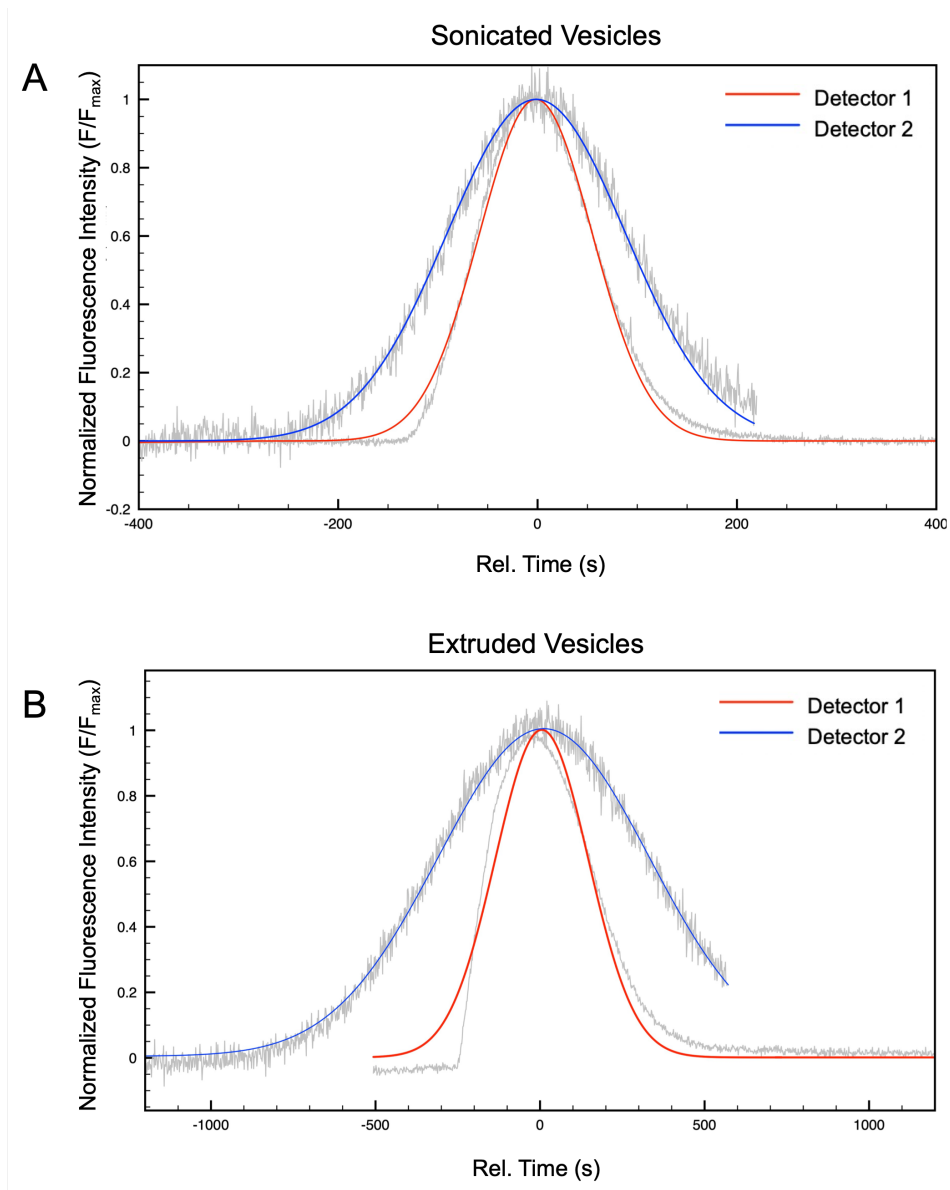


Figure 4.6: Dual-point LIF detection TDA of lipid vesicle preparations. (A) Vesicles prepared by sonication measure $R_H = 22 \pm 1$ nm; (B) Vesicles prepared by extrusion through 200 micron membrane pores measure $R_H = 107 \pm 21$ nm. Peaks are plotted in the same manner described for Figure 5.

4.6 Conclusion

Size characterization is an important analysis across many fields of materials science, and especially for the analysis of biomacromolecules and pharmaceutical preparations. Shortcomings of common methodologies like DLS and SEC, present a need for rapid, straightforward, sensitive, and inexpensive methods for size characterizations. TDA meets most of these needs, but it is commonly performed using expensive CE instrumentation with UV absorbance detection that substantially limits sensitivity. We have developed a low-cost 3D printed instrument for TDA in fused silica capillaries that enables two-point LIF detection with low nM to mid pM LODs. Still, further work in this area remains to achieve LODs competitive with the highest sensitivity LIF systems. The system relies on low cost 532 nm laser diode modules, which limits fluorophore selection to labels that are not optimum for the lowest possible fluorescent LODs. Additionally, the emission axis relies on multiple dielectric optical filters that significantly drive up the overall cost of the system. Future work in this area will focus on improved optical efficiency, and achieving more effective scatter rejection at reduced cost via spatial and spectral filtering.

The integration of an eductor-based pressure system enabled precision flow rate control with exceptional pressure stability, which is needed for accurate TDA. Since the pressure system is completely 3D printed and includes no moving parts, it presents a robust and exceptionally low cost approach to pressure driven flow for capillary-based analyses. Ultimately, the instrument was shown to be effective for protein sizing at low-to-sub nM concentrations, and was able to resolve size differences that result from various liposome preparation methods. We anticipate that making this instrument available via digital distribution of the 3D printable models will facilitate wider access to the powerful sizing capabilities of TDA.

4.7 Acknowledgements

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4.8 Supporting Information

SI for this article are provided in Appendix [B](#).

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Chapter 5

Conclusions and Future Work

5.1 Summary

New analytical tools are necessary for investigating spatiotemporal secretion dynamics of exosomes. A focus of this work has been towards the development of a novel proximity vesicle fusion assay for the determination and quantification of exosome contents. The analytical challenges addressed in this dissertation involve the lack of sensitivity and selectivity of common analytical techniques for the characterization of exosome contents and size, as well as the uncertainty in assay sensitivity, which proved difficulty in isolating which variables were contributing to signal. Taylor dispersion analysis (TDA) offers the advantage of solution-phase size characterizations in sub-nL volume samples without the need for calibrations or internal standards. These advantages are desirable for the volume-limited, low-concentration biological samples that are under investigation. The remainder of the work described within focuses on the application of TDA to our research goal and the development of new tools and assays that contribute to the utility of size-based measurements via TDA.

5.2 Proximity vesicle fusion assay

The primary objective of the research goals for this dissertation was to develop a novel assay in which vesicle fusion combined with molecular recognition of exosome membrane proteins would facilitate encapsulated analytical reactions and high sensitivity detection of vesicle contents, enabling specific assay of biomolecules encapsulated by exosomes. Early progress was made towards a proof-of-concept model system, but concerns surrounding which variables were contributing to signal were raised. For example, increased fluorescence signal could have been indicative of various states of the system: vesicle fusion and content mixing, vesicle aggregation, or vesicle content leakage. The uncertainty in our results led to the new perspective of analyzing our system by making size-based characterizations, which led our research in a new direction.

5.2.1 Preliminary results

Future work would include revisiting the original proximity vesicle fusion assay and characterizing the system using our developed 3D printed TDA instrument. Small changes in R_H determinations by TDA would confirm the state of the assay (i.e. fusion, aggregation, or leakage). Fusion of EMs (50 nm diameter) with RVs (200 nm diameter) would result in an R_H measurement slightly above 100 nm, vesicle aggregation would result in $R_H \gg 200$ nm, and content leakage would result in low R_H values that are comparable to that of the fluorescent marker encapsulated in RVs ($\ll 200$ nm).

The application of our TDA instrument for making size characterizations of the proximity vesicle fusion assay has been demonstrated by preliminary experiments. DOPC lipid solutions containing 1% w/w of Liss Rhod PE and 1% w/w of biotinyl cap PE were prepared to a final lipid concentration of 2 mg mL^{-1} . Chloroform solvent was evaporated from the lipid mixture by N_2 stream for 10 minutes and placed under vacuum for 3 hours. The dry lipid film was hydrated with PBS buffer for 1 hour. The buffer was prepared by adding 0.1% w/w bovine serum albumin to 1X PBS. The lipid solution was sonicated for 2 hours using a bath ultrasonic cleaner. Taylor dispersion experiments were carried out with a diluted sample (1:1 of initial preparation) in a 25.5 cm length, $100 \mu\text{m}$ i.d./ $360 \mu\text{m}$ o.d. fused-silica capillary with 15 cm window spacings. The experimental procedure for sample injections and post-analysis were identical to that described in Chapter 4.4.5. To the same vesicle sample, streptavidin was added with approximately a 1:12 molar ratio to the biotinylated lipid in the vesicle sample. TDA was performed on the vesicle-streptavidin system as described above. Results of the TDA experiments before and after the addition of streptavidin are shown in Fig. 5.1, where fitted peaks from both detection points are normalized and overlaid to visualize band broadening. Though a change in band broadening between detection points before and after the addition of streptavidin may not be visually obvious, the difference in R_H determinations is evident. TDA of the prepared population of vesicles determined an R_H of $25 \pm 6 \text{ nm}$ ($n=3$), while after the addition of streptavidin determined an R_H of $40 \pm 5 \text{ nm}$ ($n=3$).

If the vesicle population is assumed to have equal radii of 25 nm (mean value), the number of vesicles needed to fuse to produce a larger vesicle of 40 nm (mean value) radius can be estimated based on the respective volume or surface area of the spherical vesicles. However, given by the equations for volume (V) of a sphere

$$V = \frac{4}{3} * \pi * r^3 \quad (5.1)$$

and surface area (A) of a sphere

$$A = 4 * \pi * r^2 \quad (5.2)$$

where r is the radius, the volume and surface area of the vesicles will not grow at the same rate. If either case is considered over the other to determine the number of smaller vesicles needed to fuse to produce a larger vesicle, the system is necessarily left with excess lipid or excess aqueous environment is needed after fusion. More experiments are needed to resolve both potential mechanisms.

5.2.2 Future work

Though the preliminary data discussed above may suggest that our system is in the proposed condition of total vesicle fusion, there is a challenge of resolving vesicle fusion from aggregation. Two potential studies could lead to distinguishing between the two conditions: evaluation of overall size and evaluation of the fusion/aggregation rate. Evaluating the size determinations of the system would provide evidence suggesting fusion over aggregation, or vice versa. For example, if two 50 nm diameter vesicles aggregate, their combined diameter would be doubled, which R_H determinations would reflect. However, there is one other factor that might affect the size measurements. If two (or more) are aggregated, it is likely that the spherical approximation of their shape cannot be made. Since the Stokes-Einstein equation is a spherical approximation, R_H results may be less precise or even difficult to obtain in general. Furthermore, an experimental study that monitors the change, if any, in R_H measurements over time could be beneficial in

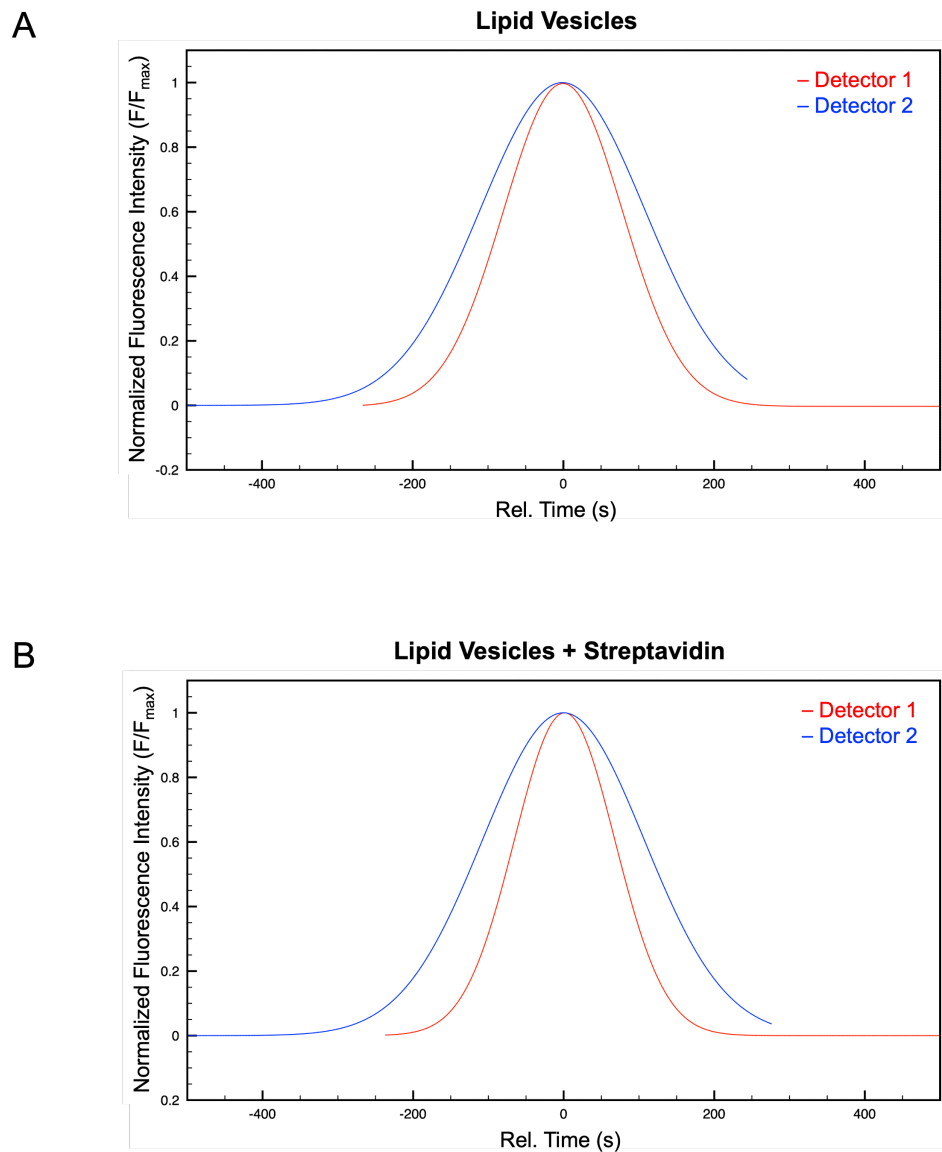


Figure 5.1: Preliminary results from a proof-of-concept vesicle fusion experiment. TDA was performed on rhodamine-labeled vesicles before (A) and after (B) the addition of streptavidin. The peaks shown are the raw data fits from each detector, normalized and overlaid.

distinguishing between fusion and aggregation, as fusion occurs at a faster rate (nanoseconds [1]) than aggregation (up to days [2]). Additionally, a standard addition calibration of the number of equivalents of streptavidin relative to the number of vesicles could be made. For instance, varying molar ratios of streptavidin to biotinylated lipids can be added to the vesicle sample and TDA performed to produce a plot of average R_H versus molar ratio of streptavidin added. With increasing molar ratios of streptavidin to biotinylated lipid, the average R_H should also increase until the amount of streptavidin is in excess, where the plot would plateau. If R_H results can predict the series of fusions (i.e. 1+1 up to 1+1+1+1) with increasing amounts of added streptavidin, it can be hypothesized that fusion is occurring over aggregation. Non-spherical aggregates would likely have less precise predictions. Predicting the number of vesicles that have fused may lead to additional challenges, as it is possible that the system may contain a mixed population of the number of fused vesicles, or even bound biotin per avidin protein. TDA can be coupled inline to separation modes, such as CE, to resolve these populations. Nonetheless, plenty of potential studies and experiments are possible and necessary to make determinations of the state of our assay.

In regards to our initial model system, if the proposed experiments confirm that the increased fluorescence signal observed when EMs, RVs, and streptavidin are mixed is due to vesicle fusion, then future experiments could be performed to complete the vesicle fusion assay with molecular recognition of exosome biomarkers. Here, synthetic vesicles would be functionalized with CD63/CD9/CD81 antibodies, combined with commercially purified exosomes, and the exosomes assayed by proximity vesicle fusion, demonstrating the application of the proximity vesicle fusion assay to characterizing exosome contents.

5.3 3D printed LIF detection TDA instrument

Chapter 4 describes the development of a 3D printed instrument for performing TDA in fused silica capillaries with the detection sensitivity of laser-induced fluorescence. A low-cost, 3D printed, eductor-based pressure system was developed and shown to produce nL min^{-1} flow rates with a 30-fold improvement in stability of static pressure (0.05% RSD) as compared to a common mechanical

pump (1.53% RSD). The instrument was found to have a detection limits of 15 nM, 80 pM, 1 nM, and 5 nM with Rhodamine 6G, R-Phycoerythrin, AlexaFluor 532, and CF532, respectively. Quantitative performance was characterized with R-Phycoerythrin and a TDA demonstration was performed.

5.3.1 Future work

Future directions include small modifications to the 3D print design of the instrument. The current instrument design involves a mirror and ball lens for emission collection from the capillary detection point. The curved mirror collimates light that is reflected in the opposite direction in which it originated. A small percentage of this light, directed toward the detector, is partially obstructed by part of the print engineered to hold the ball lens and by the capillary. The ratio of focal length to lens diameter is termed f-number and given by the equation

$$f/\# = \frac{f}{D} \quad (5.3)$$

where f is the focal length and D is the lens diameter. F-number can be related to numerical aperture (NA) by the following approximation

$$NA \approx \frac{1}{2f/\#} \quad (5.4)$$

Though focal length will decrease as the diameter of the ball lens decreases, the numerical aperture will remain the same. By incorporating a smaller diameter ball lens (1 - 2 mm) (Fig. 5.2), we could further limit the amount of light that is obstructed while collecting the same amount of light.

5.4 TDA-immunoassay

Our developed 3D printed instrument for performing TDA with high sensitivity via LIF detection could be used in a variety of applications. Ultimately, the future work outlined in Section 5.1 would allow us to re-evaluate the condition of our originally proposed proximity vesicle fusion assay. Additionally, there are other approaches to exosome characterization that can be made, such

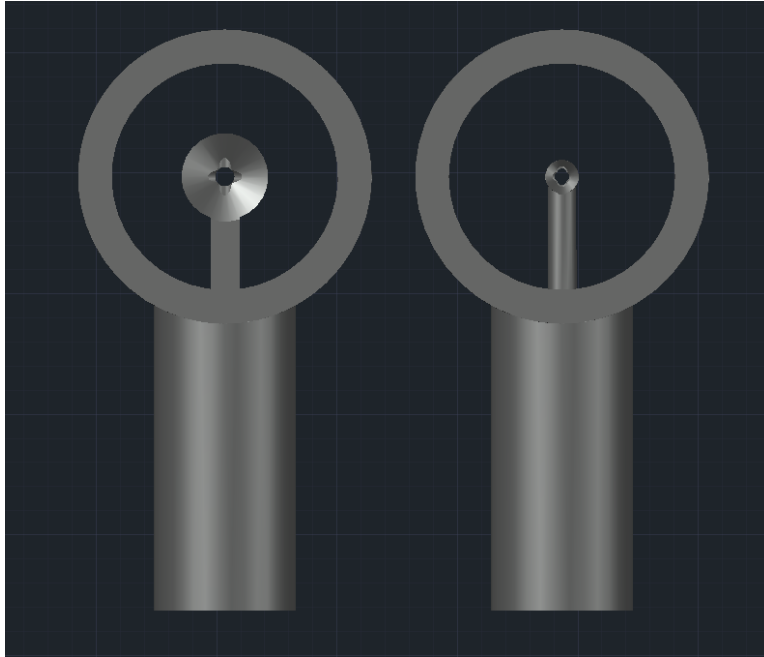


Figure 5.2: AutoCAD design comparison of 6 mm diameter ball lens holder (left) to 1 - 2 mm diameter ball lens holder (right). This illustrates the difference in obstruction of reflected, collimated emission light from the mirror towards the detector.

as a TDA-immunoassay (TDA-IA) as described in Chapter 1.4.4.2.

5.4.1 Preliminary results

A proof-of-concept experiment was performed with purified human preadipocyte exosomes (Zen-Bio, Inc., Research Triangle Park, NC) and a FITC-labeled CD9 mouse monoclonal antibody (Fisher Scientific). The experimental setup involved a looped 50 μm i.d. capillary so that two detection windows lined up on an inverted epifluorescence microscope (Eclipse Ti-U, Nikon, Newton, NJ), equipped with an LED excitation source (DC2200, Thorlabs, Newton, NJ), and photon counting PMT (H7828-50, Hamamatsu, Hamamatsu, Japan). Changes in fluorescence intensity were collected via LabView (National Instruments, Austin, TX). A 482 ± 9 nm LED excitation filter, and 520 ± 12 nm emission filter was used for excitation and emission of FITC-conjugated fluorophores. The low pressure driven flow rates necessary for TDA were achieved with a U-tube. Here, the U-tube was filled with ethylene glycol and fitted with a 3-way connection valve and tubing leading to the capillary outlet. Removal of the ethylene glycol from one leg of the U-tube generates a differential pressure between both legs, based on the difference in height, and produces a vacuum in the opposite leg from which the liquid was removed. This vacuum is responsible for the low pressure driven flow within the capillary. The vacuum pressure (in psi) was converted to volumetric flow rate within the capillary via a simple calibration experiment. Thus, experimental flow rates could be estimated based on the volume of liquid removed from the U-tube for a TDA experiment.

TDA was first performed on the CD9 antibodies to obtain a diffusion coefficient of $63.73 \pm 9 \mu\text{m}^2\text{s}^{-1}$ and corresponding hydrodynamic radius of 3.9 ± 0.6 nm. Antibodies were mixed with purified exosomes and TDA was performed to obtain a diffusion coefficient of $7.4 \pm 1 \mu\text{m}^2\text{s}^{-1}$ and corresponding hydrodynamic radius of 33.8 ± 5 nm. A comparison of the band broadening between detection windows for the pure antibodies (solid lines) and antibody-exosome mixture (dashed lines) are given in Fig. 5.3. Peaks from both detection windows are normalized and overlaid to illustrate band broadening, and both data sets are plotted side-by-side to illustrate the

difference in band broadening between the antibodies and antibody-exosome complex.

The increase in R_H between the pure antibodies and antibody-exosome mixture is indicative of antibody binding to the CD9 membrane protein on the exosomes.

5.4.2 Future work

Future work following the proof-of-concept experiment described above would involve using these size-based measurements to develop a complete and working immunoassay. A standard protocol would be to first perform TDA on the labeled antibodies alone, with a hydrodynamic radius (R_H) of 5 – 6 nm. Next, the antibodies are added to a sample of purified exosomes ($R_H = 25 – 50$ nm) and TDA is performed. Since the antibodies are now bound to the surface proteins of the exosomes, the measured R_H will correspond to the complex. Furthermore, TDA provides an average R_H for the entire sample, so the determined R_H for the mixture will lie between 25 – 50 nm, so long as all of the antibodies are bound. A complete development of the TDA-IA would involve the creation of a standard addition calibration representing a fraction of bound exosomes. When the concentration of exosomes is zero, we should expect the measured average R_H to be 5 – 6 nm, corresponding to the size of the antibody alone. When the concentration of exosomes is equal to the concentration of antibodies, we should expect an R_H measurement of 25 – 50 nm, corresponding to the size of the antibody-exosome complex. When the concentration of exosomes is less than the concentration of antibodies, we would expect the measured R_H to lie somewhere in between (5 – 50 nm), corresponding the average radius of bound and unbound antibodies. Such calibration plot is necessary to determine some degree of quantitation of the CD9 membrane protein present in the exosome sample, based on the fraction of bound exosomes. Future experiments could be modified to target other potential exosomal membrane or surface proteins, such as CD63 or CD81.

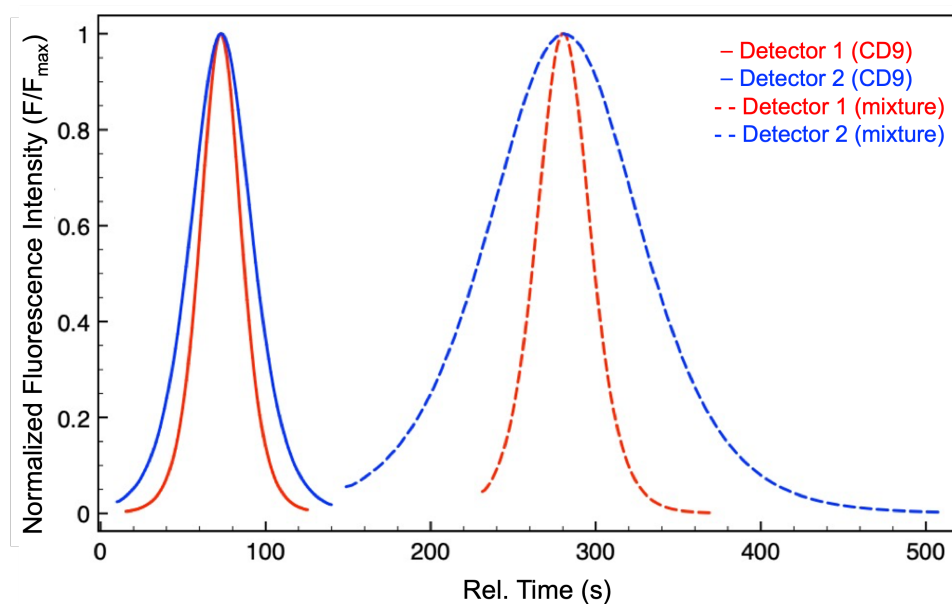


Figure 5.3: Preliminary data of TDA performed on CD9 antibodies (left) and antibody-exosome mixture (right). Peaks are normalized and overlaid to represent band broadening between both detection windows.

References

- [1] A. Smeijers, A. J. Markvoort, K. Pieterse, and P. Hilbers, “A detailed look at vesicle fusion,” *The Journal of Physical Chemistry B*, vol. 110, no. 26, pp. 13212–13219, 2006. [119](#)
- [2] F. B. Howard and I. W. Levin, “Lipid vesicle aggregation induced by cooling,” *International journal of molecular sciences*, vol. 11, no. 2, pp. 754–761, 2010. [119](#)

Appendices

A Additional Materials for Chapter 3

The printing rights submitted as an attachment to this dissertation are meant to accompany the article in Chapter 3, originally published in *Analytical Methods* by Meagan R. Moser and Christopher A. Baker.

B Additional Materials for Chapter 4

The following figures and table are meant to accompany the article in chapter 4, originally published in the Electronic Supplementary Information by Meagan R. Moser, Claire M. Smith, and Christopher A. Baker. Only minor modifications were made.

”A 3D Printed Instrument for Taylor Dispersion Analysis with Two-point Laser-induced Fluorescence Detection” *Submitted*, 2021

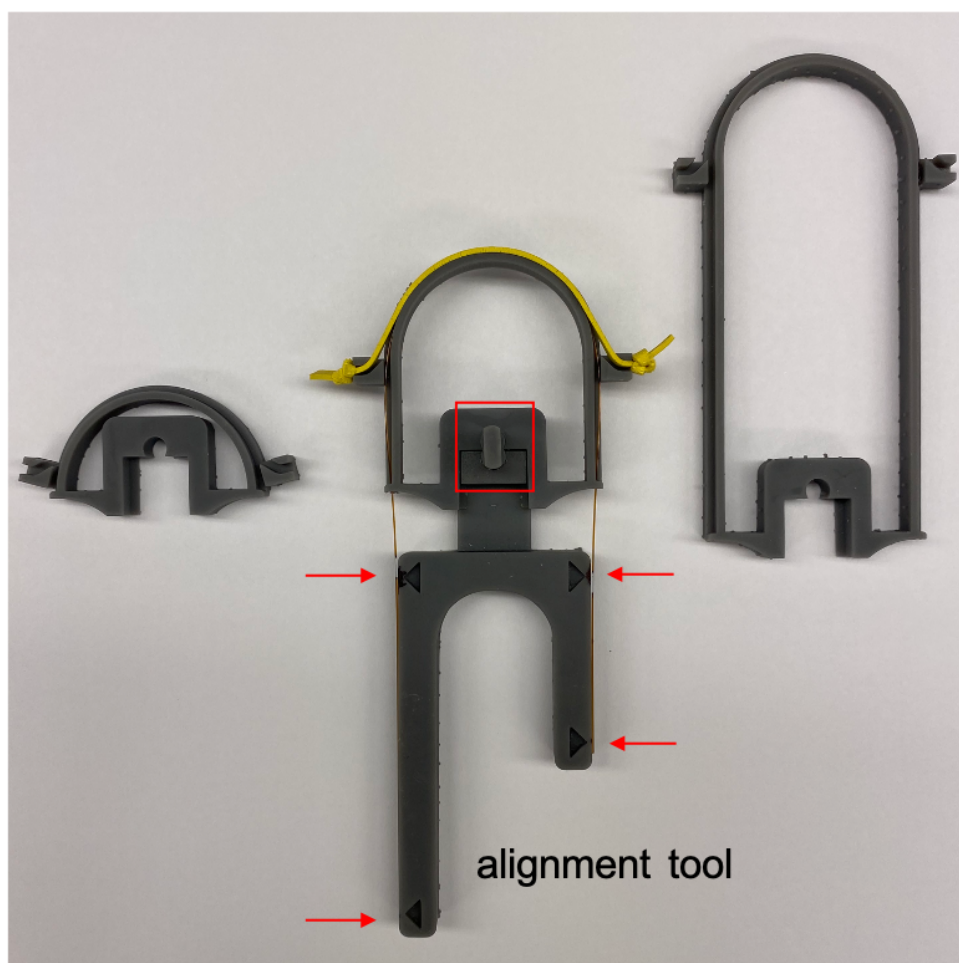


Figure B.1: Capillary cartridge and alignment tool. From left to right: 10 cm, 15 cm, and 20 cm window spacing cartridge. Middle cartridge illustrates use of alignment tool for window and capillary end markings (red arrows), secured with the 3D printed key (red box).

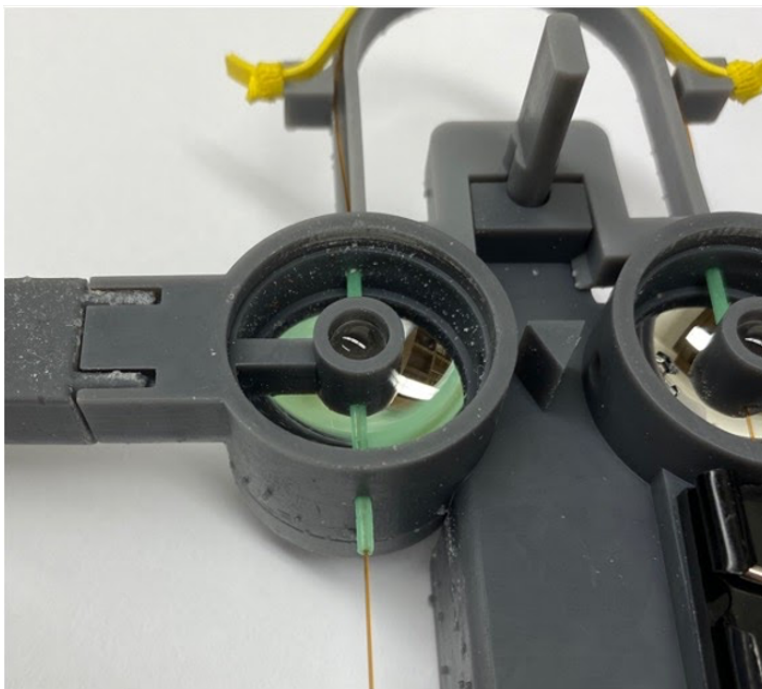


Figure B.2: Photograph of mounted capillary tubing sleeves (green) used to laterally hold the capillary in place. Sleeves fit on either side of the detection window on the capillary. The 3D print design was modified to tightly secure the tubing sleeves.

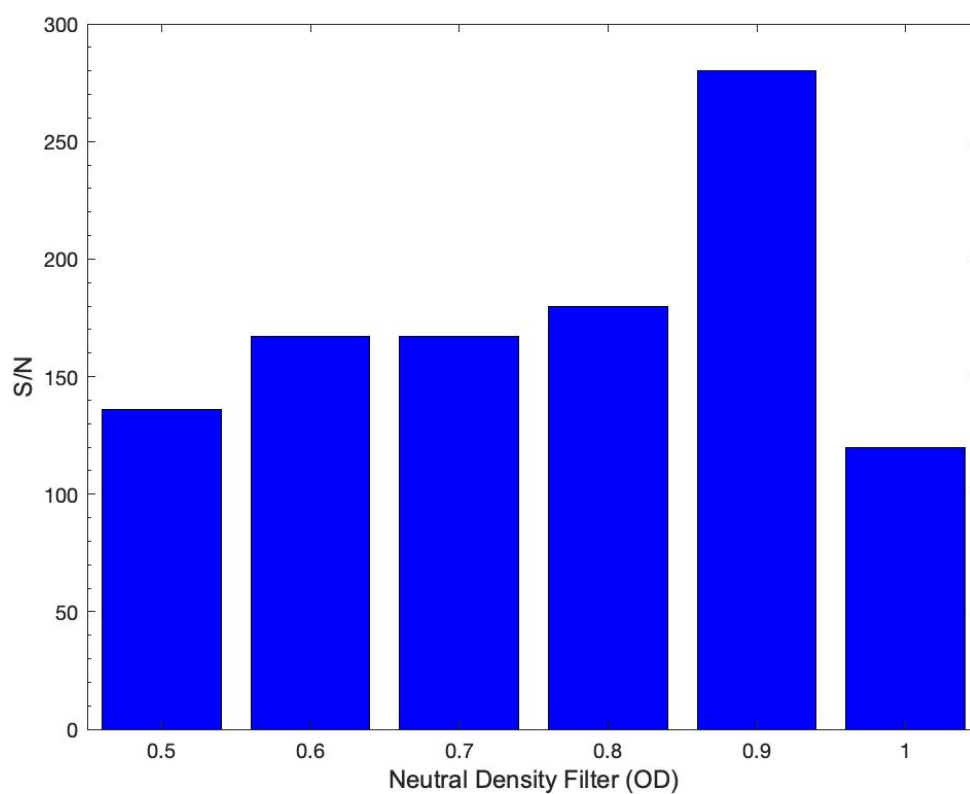


Figure B.3: Selection of neutral density filter. OD 0.9 provided the highest S/N value among the conditions observed. Further optimizations and experiments were performed using the OD 0.9 filter.

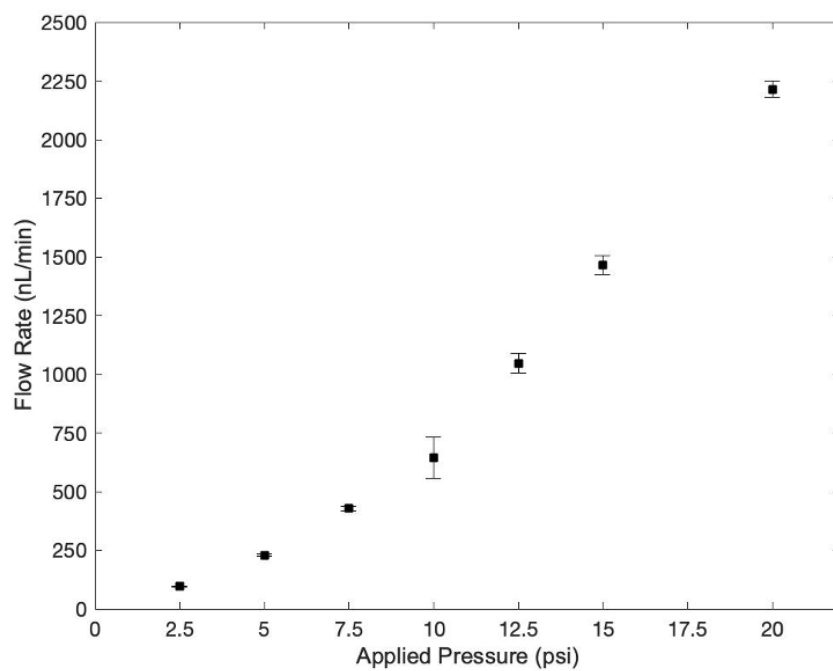


Figure B.4: Flow rate calibration for 100 μm i.d. capillary with 15 cm window spacing.

Vita

Meagan Moser, nee Rhyne, grew up in the small town of Greenback, Tennessee. There she attended Greenback High School from kindergarten through twelfth grade, and graduated Valedictorian in 2012. She then earned her Bachelor of Science in Chemistry from the University of Tennessee, Knoxville, graduating Summa Cum Laude in 2016. During her undergraduate career, she completed three semesters of research with Dr. Michael Best working on synthesizing lipid probes for the study of membrane interactions for drug delivery systems. It was this experience, along with upper-level laboratory courses, that inspired her interest in bioanalytical research of biophysiological systems. In August 2016 she remained in the Chemistry Department at the University of Tennessee, Knoxville and joined the research group of Dr. Christopher Baker. In the Baker Bioanalysis Lab (BBL) her research was motivated by the development of analytical tools for characterizing size and contents of extracellular vesicles. This work has resulted in peer-reviewed publications, presentations at national conferences and a first place award at a graduate research symposium. Throughout her time in the UTK Chemistry Department, she was involved with the Association of Chemistry Graduate Students, serving as Secretary and Career Development Chair. She also spent her time volunteering at local STEM related middle school events, such as the TN State Science Olympiad, UTK Forensic Chemistry Camp, Freedom Schools Program, and the Southern Appalachian Science and Engineering Fair. Meagan will receive her Ph.D. in Analytical Chemistry in December 2021.