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FROM MICRO- TO NANO-SCALE: APPLICATIONS OF SOLID-PHASE ENZYMATIC REACTORS FOR BIOPOLYMER DISASSEMBLY

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Chemistry

by
Nyoté Jaedah Angelou Oliver-Calixte
B.S., Xavier University of Louisiana, 2007
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To my family, for their unwavering support my entire life and especially during the pursuit of this dream. Your love and encouragement helped make this dream a reality.

In Honor of:
Pamella J. Oliver

In Memory of:
Helen Mary King
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List of Abbreviations and Acronyms

CE  Capillary Electrophoresis
COC  Cyclic Olefin Copolymer
Da   Damkohler Number
DNA  Deoxyribonucleic Acid
dNMP Deoxynucleotide Monophosphate
dsdsDNA Double-Stranded Deoxyribonucleic Acid
EDC  3-(3-dimethylaminopropyl) carbodiimide
EOF  Electroosmotic Flow
FIB  Focused-Ion Beam Milling
FITC Fluorescein isothiocyanate
IMER Immobilized Microfluidic Enzymatic Reactor
$k_2/k_{cat}$ Catalytic Turnover
$K_m$ Michaelis Menten Constant
L    Contour Length
LIF  Laser-Induced Fluorescence
mM  Millimolar
MTC  Mass Transport Coefficient
NER  Nanofluidic Enzymatic Reactor
NHS  N-hydroxysuccinimide
NIL  Nano-Imprint Lithography
P    Persistence Length
PMMA Poly (methylmethacrylate)
Pt   Platinum (platinum wire)
R    Radius of gyration
SEM  Scanning Electron Microscope
ssDNA Single-Stranded Deoxyribonucleic Acid
TBE  Tris-borate EDTA
$T_g$ Glass transition temperature
UV   Ultraviolet
$V_{max}$ Maximum Reaction Velocity
WCA  Water Contact Angle
XPS  X-Ray Photoelectron Spectroscopy
$\lambda$-Exo Lambda Exonuclease
$\lambda$DNA Lambda Deoxyribonucleic Acid
$\mu_{EOF}$ Electroosmotic Flow
$\mu_{EP}$ Electrophoretic Mobility
$\mu_M$ Micromolar
Abstract

The process of immobilizing enzymes onto solid supports for bioreactions has some compelling advantages compared to their solution-based counterpart including the facile separation of enzyme from products, elimination of enzyme autodigestion, and increased enzyme stability and activity. We report in this work, the immobilization of λ-Exonuclease (λ-Exo) onto poly(methylmethacrylate) (PMMA) micro- and nano-pillars populated within a fluidic devices for the micro and nanoscale on-chip digestion of double-stranded DNA. Enzyme immobilization in both studies was successfully accomplished using EDC/NHS coupling to carboxylic acid functionalized PMMA micropillars. Our micro-scale results suggest that the reaction efficiency for the catalysis of dsDNA digestion using λ-Exo, including its processivity and reaction rate, were higher when the enzyme was attached to a solid support compared to the free solution digestion. We obtained a clipping rate of $1.0 \times 10^3$ nucleotides s$^{-1}$ for the digestion of λ-DNA (48.5 kbp) by λ-Exo. We suggest that the kinetic behavior of this solid-phase reactor could be described by a fractal Michaelis-Menten. Preliminary nano-scale λ-Exo immobilization experiments reveal potential enzymatic activity changes as observed in reduced digestion rates (≈303 nucleotides s$^{-1}$). Further studies will deduce reasoning for these observed differences. Simulation of the nanofluidic reactors reveal kinetic behavior to be mass transport limited, a result not expected due to the reduction in reactor dimensions. Nonetheless, the results from these studies work will have important ramifications in new single-molecule DNA sequencing strategies that employ free mononucleotide identification. As a step towards this goal, an investigation of the dynamics of DNA in these irregularly shaped structures has been performed.
1 Applications of solid-phase bioreactors

1.1 General Introduction

Solid-phase bioreactors have emerged as useful tools in industrial\textsuperscript{1-2} and chemical manufacturing.\textsuperscript{3} These scalable reaction chambers have contributed to numerous advances in micro-scale chemical\textsuperscript{4} and biological engineering,\textsuperscript{5} nanotechnology,\textsuperscript{6} and single-molecule enzymology.\textsuperscript{7} The use of these reactors has grown over the last 20 years because they can be functionalized with a wide variety of molecules allowing for numerous reactions to be performed with the resultant products more efficiently compared to their solution-phase counterparts.\textsuperscript{8} Solid-phase bioreactors provide users with a wide range of features that can be tailored to individual experiments to increase throughput and maximize reaction efficiencies.\textsuperscript{9} These highly customizable bioreactors can be designed for specific enzymatic reactions using a wide variety of solid-phases,\textsuperscript{10} immobilization chemistries, and reaction scales.

Creation of solid-phase bioreactors can include any number of biomolecules including proteins,\textsuperscript{11} peptides,\textsuperscript{12} drugs,\textsuperscript{13} nucleic acids,\textsuperscript{14} carbohydrates,\textsuperscript{15} lipids\textsuperscript{16} and conjugates.\textsuperscript{17} However, particular attention has been given to protein-based solid-phase bioreactors, more specifically enzymatic solid-phase bioreactors.\textsuperscript{18} Enzymatic reactors are regarded as highly desirable because catalytic reactions can be closely controlled, products easily separated, and if desired, processed downstream.\textsuperscript{19} Also, immobilized enzyme-based bioreactors have contributed to areas like biomedical diagnostics;\textsuperscript{20} examples include carbonate dehydratase utilized for artificial lungs,\textsuperscript{21} immobilized urease chambers for the study of artificial kidneys,\textsuperscript{22} and solid-phase studies using asparaginase for leukemia treatment.\textsuperscript{23}
Solid-phase enzymatic processes could allow for multi-enzyme reactions to be more easily separated, where for reactions like α-amylase, amyloglucosidase, and glucose isomerase must occur simultaneously to convert starch into oligosaccharides, fructose, and glucose. The use of solid-phase reactors containing inulinase enzyme facilitates the one-step hydrolysis of inulin to generate a 95% pure fructose product. The additional benefit of immobilizing inulinase enzyme is an increase in its thermostability due to conjugation, thus allowing for this reaction to take place at a temperature that prevents contamination on an industrial scale. With such a wide applicability, solid-phase bioreactors have immense potential in chemical, biomedical, and industrial fields. Large-scale industrial applications utilize solid-phase reactors in settings where typically a large processing capacity is required. However, many smaller scale applications benefit from advances in enzymology. The integration of multiple research platforms (i.e microfluidics, nanofluidics) to solid-phase enzymatic reactions has the potential to revolutionize analytical and biotechnology based fields. One such development, immobilized microfluidic enzyme reactors (IMERs), which employs solid-phase immobilizations within microfluidic devices offering even wider capabilities for solid-phase enzymatic studies. The work reported in this document seeks to examine the utility of for solid-phase immobilized micro- and nanofluidic enzyme reactors for nucleic acid structure elucidations and the parameters that affect their functionality and efficacy on both the micrometer and nanometer scales. This review will examine solid-phase enzymatic reactors from a broad perspective, in hopes to highlight the benefits of the work conducted herein.
1.2 Immobilization of Enzymes

The immobilization of enzymes involves the restriction of protein movement onto a specific surface. With the use of enzymes as a billion dollar industry, it becomes very advantageous and cost-effective to immobilize enzymes as an alternative to free solution enzymatic reactions. The benefits of immobilization involve increased control of the enzymatic reaction. Also, fast separation of enzyme from the reaction mixture mitigates the need to purify products post-reaction. Moreover, immobilized enzymes have improved stability against changes in temperature, pH, and solvents as well as less harmful affects from contaminants and impurities.

1.1.2 Activity and Stability Enzyme

One reason homogenous enzymatic reactions can show reduced activity is aggregation of enzymes in solution leading to inaccessibility of the active site due to crowding from other enzyme molecules. In contrast, activity of enzymes upon immobilization can be increased for numerous reasons. For instance, surface attachment of enzymes can prevent enzyme inhibition activities and auto-digestion of other free enzyme molecules in solution. Static enzymes also provide protection from the environment due to immobilization. The process of immobilization also induces rigidification of the enzyme, where enzyme activity in some instances increases because of less deformation of the enzymes primary, secondary, and tertiary structures.
1.3 Methods of Immobilization

There are a vast number of chemistries utilized for immobilization of biomolecules to solid surfaces. However, the method chosen must not compromise the enzyme structure, catalytic activity, and not make the active site inaccessible. In the following sections, the utility and drawbacks of 4 modes of immobilization (carrier binding, covalent, entrapment and encapsulation) will be discussed (see Figure 1.1).

1.3.1 Carrier Binding

Carrier binding is regarded as a general classification for the non-covalent attachment of enzymes, where the physical nature of the biomolecule (size, surface area, and chemical composition) mediates selection of the carrier or solid-phase selected for immobilization. This method can include physi-sorption, affinity tag modification, and protein-protein interactions for surface conjugation.
1.3.2 Physical Adsorption

Physical adsorption of enzyme onto a solid-phase substrate is the simplest method for immobilization and typically involves a reversible reaction between the substrate and enzyme. Advantages of this non-covalent attachment method are that it is reversible, which can be a desired parameter depending on the application. This method is facile and in some cases, retains all enzymatic activity because no chemical changes are incurred with regard to the enzyme. This method is also economical, as it reduces the need for other reagents and linker chemistries. Conversely, disadvantages of this method include detachment of enzyme from the support (i.e., leaching), which then leads to contamination of the product. Moreover, overloading of enzyme onto the solid-phase due to non-specific binding can lead to reduced activity due to blocked or distorted active sites. Liu et al.\textsuperscript{30} described an adsorption-immobilized enzymatic reactor for the proteolysis of various proteins using electrostatically trapped trypsin. In this work, (see Figure 1.2), silica microbeads were coated with poly(diallyldimethylammonium chloride), PDDA, or poly(styrene sulfonate), PSS, and were used to trap trypsin and proteins onto the surface through electrostatic interactions. The authors found that high proteolytic efficiency could be achieved only if the microbeads adsorbed both proteases and proteins. Once the proteases were confined within a small area, the enrichment lead to high proteolytic activity.
Figure 1.2 Silica microbeads coated with poly (diallyldimethylammonium chloride) (PDDA) or poly (styrene sulfonate) (PSS) were used to trap trypsin and proteins on the surface through electrostatic interactions in order to improve digestion efficiency. (Reproduced from *Talanta* 2013, 110, 101–107, with permission).

1.3.3 Poly Histidine Tags

A polyhistidine tag is a highly specific non-covalent attachment method utilizing bivalent ions of Co$^{2+}$ and Ni$^{2+}$ to attach targets to acid activated surfaces. This high affinity method requires that the enzyme used for attachment possesses a N- or C-terminal hexa-histidine (His6) tag to capture bivalent ions. The surface of the substrate used for immobilization must be activated with nitrilotriacetic acid (NTA) or iminodiacetic acid (IDA). Few enzymes to-date have been immobilized using this method,$^{31}$ yet its site-directed immobilization has great potential for nano-scale devices. With this method, you can control the enzyme conjugation such that they confer a specific orientation, thus limiting probability of enzyme denaturation.$^{32}$ One recent application of this site-specific immobilization is in the work by Abdul Halim and co-workers,$^{33}$ they developed an IMER using His6-tagged transketolase (TK) and transaminase (Tam) enzymes immobilized onto nickel-nitrilotriacetic acid (Ni-NTA) agarose beads that were packed
into tubes to enable multi-step enzyme reactions for the synthesis of chiral substrates for pharmaceutical applications. Their results concluded that the synthesis of chiral pharmaceutical intermediates was successful. Also, the His-tag immobilization method used provided a stable enzyme and was cost-effective due the low material cost and reusability of the IMER.

1.3.4 Biotin/avidin (streptavidin)
Biotin/avidin (streptavidin) chemistry is still regarded as one of the strongest protein-ligand bonds known. Avidin is a biotin-binding protein has the affinity to bind up to four biotin molecules and is stable and functional over a wide range of pH and temperature. Furthermore, avidin is adaptable to widespread chemical modification with little to no effect on function, making it useful for the detection and purification of biotinylated molecules in various conditions. This interaction yields near covalent bond strength with good stability at extreme pH values, heat, denaturants, and proteolysis. Also, enzyme immobilization mediated with this chemistry yields stronger conjugation of enzyme than any other non-covalent method. The disadvantage of this method is that it can be difficult to perform; large proteins could have a negative effect on enzyme due to steric restrictions, and the fact that avidin/streptavidin has the propensity to non-specifically adsorb to surfaces. Contrary to these challenges, Boehm and co-workers recently developed a flow microreactor enabling the small-scale evaluation of synthetic enzyme pathways, immobilization and compartmentalization strategies, and reaction conditions as seen in Figure 1.3. In this work, they compared a packed-bed microreactor to an open microchannel, each coated with avidin-based proteins for conjugation to biotinylated enzymes, β-galactosidase (βgal), glucose oxidase (GOx), and horseradish
peroxidase (HRP). Their study suggested that the packed bed microreactor yielded better efficiency as compared to the open-channel microreactor.

![Figure 1.3](image)

**Figure 1.3** Two fundamental strategies for enzyme compartmentalization within a microfluidic channel. (A) Packed bed microreactor: Enzyme-coated microbeads are introduced into a microfluidic channel by pump-driven pressure to form a microcolumn for continuous flow substrate conversion. (B) Microchannel reactor: Channel walls within a microfluidic chip are directly coated in enzyme for continuous flow substrate conversion. (Reproduced from *Lab on a Chip* 2013, 13(17):3426-3432, with permission).

### 1.3.4.1 Alternative Non-Covalent Attachment Methods

Alternative non-covalent bonding methods are available and protocols are evolving in protein conjugation. One method recently developed used potassium fluoride as a non-covalent support for a carboxylic acid containing a pyrrolidine substituent. The immobilization is carried out by simply treating the enzyme in dichloromethane (DCM) solution with non-anhydrous potassium fluoride (KF). In this work, researchers present the first use of strong hydrogen bonding for the immobilization of enzymes providing a cheap, practical, and convenient way to non-covalently attach biomolecules.
1.3.5 Covalent Bonding

Covalent bonding is the strongest linkage of enzyme to substrate and most commonly used for reliable, robust attachment. This technique can be facilitated through conjugation between functional groups on the surface of a solid-phase and the enzyme. Advantages of this approach include minimal leaching of enzyme from the surface, increased thermal stability, and easily interfaced with the desired substrate. One major disadvantage of this chemistry is that it can reduce enzyme activity as a result of conformational changes in the enzyme structure once immobilized.

1.3.5.1 Advances in Covalent Immobilization Chemistries

Many immobilization chemistries exist for the covalent attachment of enzymes to solid-phase substrates. These chemistries, including 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-Hydroxysuccinimide (NHS), EDC/NHS, Imine, epoxide, click chemistry, enzyme fusion proteins, DNA, photo-immobilization, and ionic liquids\(^{36}\) have been thoroughly discussed in recent reviews.\(^ {37-38}\) Nonetheless, the development of new approaches for biomolecule immobilization can provide enhanced activity and wider applicability, thus the focus of this section will be to highlight recent advances and improved methodologies for protein immobilization to solid surfaces.

In recent work by Chen and co-workers, NaCl salting-in and microwave irradiation were combined to drive thermolysin molecules into a mesoporous support to obtain efficient immobilization of the target enzyme.\(^ {39}\) This work was observed to have a 4.5-fold increase in activity versus thermolysin enzyme immobilization using non-microwave immobilization techniques, and a 1.6-fold increase versus free enzyme. Additionally, the thermal stability of the immobilized thermolysin was significantly improved. When incubated at 70°C degrees, there was no reduction in activity by 3.5 h, whereas free
thermolysin lost most of its activity within 3 h. Immobilization also protected thermolysin against organic solvent denaturation. This microwave-assisted immobilization technique when combined with sodium chloride salting-in, could be applied to other sparsely soluble enzymes because of its simplicity and high efficiency.

Another covalent immobilization technique for the immobilization of trypsin used a one-step reaction and a low-energy electron beam. The authors successfully demonstrated the immobilization of trypsin on poly(ethersulfone) and poly(vinylidene fluoride) microfiltration membranes (see Figure 1.4). The membranes were dipped into an aqueous solution of trypsin followed by electron beam (EB) treatment. This novel immobilization chemistry was achieved through utilizing the radicals and activated species formed by electron beam irradiation, which can undergo various reactions, to induce trypsin cross-linking between the membranes. This EB cross-linkage method demonstrated that the resulting membranes showed a significantly higher enzymatic activity as compared to trypsin immobilization per adsorption on the membrane surface.

![Figure 1.4 Procedure for the coupling of trypsin on polymer membranes using electron beam (EB) irradiation](Reproduced from Reactive and Functional Polymers 2013, 73 (5), 698-702, with permission).
1.3.6 Cross-linking

Cross-linking is an approach that uses either chemical or physical means to join enzyme molecules into a complex structure. This is achieved through a covalent linkage between the enzymes through a bi- or multifunctional reagent, such as dimethyl suberimidate, dimethyl adipimidate, SMCC [succinimidyl trans-4-(maleimidylmethyl)cyclohexane-1-carboxylate], or formaldehyde. The major advantage to this method is the increased enzyme stability due to restriction of enzyme subunits, yet aggregation of enzyme can become a limitation of this method. Additional disadvantages of this type of chemistry are the harsh reagents required. Lastly, in some cases cross-linking can limit or wholly reduce the activity of the enzyme.

Aissaoui and co-workers\textsuperscript{41} studied the effects of cross-linkers on the operation and stability of immobilized glucose-6-phosphate dehydrogenase (G6PDH) onto salinized surfaces. This work examined bi-functional linkers with different reactive functional groups (aldehyde, isothiocynate, isocynate) and varying with aromatic or aliphatic spacers. These linkers could be modified in three ways 1) reactive groups on either end (for attachment of enzyme to salinized surface), 2) nature of the linker (aliphatic or aromatic) and 3) geometric (meta or para). Their results concluded that although immobilization was achieved via random multipoint attachment, it was observed that higher numbers of anchoring points stabilized the three-dimensional structure of the enzyme. The study revealed that the catalytic activity and thermostability of G6PDH depended on the number of attachment points. Also they observed that the steric hindrance (due to meta-orientation of the aromatic ring) of the cross-linker at the interface could greatly reduce enzyme activity. This work demonstrated the importance
of low interfacial steric hindrance for successful multipoint attachment by the usage of cross-linkers for immobilization.

1.3.7 Entrapment

Entrapment of enzymes consists of a free enzyme that has been confined within a gel. A lattice-type entrapment is a matrix (gel or polymeric substance) that contains pores to allow the desired materials to pass through while enzyme remains confined within the medium. The porosity of the matrix must be controlled to prevent leakage of enzyme while allowing for passage of substrate and subsequent product. An advantage of this method is that high enzyme loads can be achieved. However, this method is diffusion limited due to the lattice, so transport of substrate to enzyme and product diffusion away from enzyme becomes difficult to control.

1.3.7.1 Microcapsule

Microcapsules for entrapment consist of membrane-like matrices that contain enzymes large enough so that they cannot pass though the pores, yet small substrates and products can. Encapsulation allows for the simple, low cost immobilization of enzyme with the added versatility by being able to be designed to entrap a large variety of biomolecules. A disadvantage of encapsulation is that the formation of products can be too rapid for their passage out of the capsule. Recent work by Piwonski and co-workers (Figure 1.5) took advantage of this phenomenon. In this work, the authors studied product inhibition at the single-molecule level where individual horseradish peroxidase (HRP) molecules were entrapped within surfacetethered lipid vesicles and their reaction with a fluorogenic substrate was investigated (see Figure 1.5).
Figure 1.5 An individual enzyme molecule within a lipid vesicle. A large unilamellar vesicle encapsulating a single horseradish peroxidase (HRP) molecule is attached to a glass-supported lipid bilayer using biotin-avidin chemistry. Externally added Amplex Red substrate molecules penetrate the vesicle and react with the enzyme, leading to creation of the fluorescent product resorufin that remains trapped in the vesicle interior. (Reproduced from Ref.7, Proceedings of the National Academy of Sciences 2012, 109 (22), E1437-E1443, with permission).

While the substrate readily penetrated into the vesicles, the charged product (resorufin) accumulated within the vesicles. Individual enzyme molecules were found to stall when small amounts of product accumulated within the reactor. Bulk enzymology experiments verified that the enzyme was non-competitively inhibited by resorufin. The reaction velocity of individual enzyme molecules and the number of product molecules required for their complete inhibition were broadly distributed and dynamically disordered. The authors suggested that these parameters were correlated. Their results suggested that enzymes have evolved to correlate fluctuations at structurally distinct functional sites.
1.4 Factors Influencing Enzymatic Reactions on Solid Surfaces

The process of attaching proteins to solid-phases requires a firm understanding of the factors that influence enzymatic activity and the conditions needed for optimal reaction efficiency. These factors, including pH, temperature, and the properties of the solid-phase material (and environment) must be carefully controlled and counterbalanced to maintain, and/or improve enzyme activity within bioreactors. These components become more critical as reactor dimensions are decreased from macro- down to micro- and nano-scale as the localized environment of the enzyme changes.

1.4.1 pH

Enzymes, like most biological materials, function best within an optimal pH range. pH can have a great effect on reaction performance such as the enzyme’s ability to bind to its solid-phase substrate as well as its structure and ionization. pH is also important when attempting to bind to the solid substrate because the desired functional groups within the protein for immobilization must be readily accessible to facilitate its linkage to the solid support. Furthermore, pH conditions can greatly influence enzyme structure and can lead to reordering or unfolding of the protein’s primary, secondary and tertiary structure. This can allow for distortion of the enzyme to the point of activity loss, especially if the active site is no longer available. Verma et. al.\textsuperscript{43} recently demonstrated that beta-glucosidase when immobilized resulted in a pH shift for optimal activity when compared to the free enzyme.\textsuperscript{43} The enzyme was covalently bonded to nanoparticles because the high surface area of the nanomaterial yielded increased enzyme loading. They observed that the optimum pH for beta-glucosidase activity was higher than the native enzyme and they concluded that this could be due to the interactions between the charged groups of the enzyme molecules and solid support. The results of their
study concluded that covalent bonding of beta-glucosidase onto nanoparticles resulted in a slightly decreased access of substrate to the enzyme’s active site.

1.4.2 Temperature

It has been shown that enzymes may exhibit increased thermostability upon binding to a solid support leading to reduced denaturation of enzyme at increased temperatures and/or harsh reagent conditions. This concept was explored by Rivera-Burgos et. al., 44-45 where they wanted to determine if native proteins could be digested without disulfide reduction by: (i) Enhancing the unmasking of cleavage sites through elevated reaction temperatures; and (ii) increasing trypsin concentration by use of an IMER. To confirm, transferrin was chosen as a model protein for these studies due to its resistance to trypsin digestion. Trypsin digestion can be regarded as less efficient if the cleavage sites are “masked” due to the presence of the protein backbone and protein folding.46 For this reason, disulfide reducing agents are employed. However, in this study temperature was found to increase sequence coverage or efficiency of trypsin due to temperature induced unfolding of the protein, thus making more cleavage sites available for clipping. The results showed >70% sequence coverage in the peptides identified when non-reduced transferrin was digested at 60°C as opposed to lower temperatures studied (27, 37 and 45°C). Results from this study suggested that temperature could be used to reveal additional cleavage sites the path of subsequent digestion depended strongly on the type of treatment used to open protein structures up for proteolysis.

1.5 Types of Solid-Phases for IMERS

The type of solid-phase chosen for an IMER relies heavily on its intended application, the attachment chemistry and the method to be used for analysis. There are many classes of supports that can be used for solid-phase attachment such as, natural
polymers like cellulose,\textsuperscript{47} and collagen,\textsuperscript{48} inorganics like glass\textsuperscript{49} and silica,\textsuperscript{50} synthetic polymers like polycarbonate\textsuperscript{51} and poly (methyl methacrylate),\textsuperscript{52} or other proteins.\textsuperscript{53} In order for these materials to be used as supports for bioreactors, they must possess several properties; good chemical and physical stability and functional groups for attachment of the target protein.\textsuperscript{54} Also, the solid phase must possess reduced protein affinity to prevent non-specific adsorption onto its surface, unless adsorption is desired.\textsuperscript{38} Lastly, it must minimize undesired surface effects that can alter behavior of protein causing distortion, subunit misordering, or changes in protein affinity for its substrate.

Glass and other inorganic materials like silica and alumina often show good mechanical properties, such as flow resistance, wettability, and thermal stability.\textsuperscript{55} They are often resistive to attack from organic solvents and foreign biomolecules making them attractive for an IMER.\textsuperscript{56} A study conducted by Richter and co-workers,\textsuperscript{57} utilized a microfluidic reactor containing glass beads for the cross-linkage of HPR enzyme and xantin oxidase enzyme. These devices, while easy to prepare, were limited to the use of a few enzymes due to denaturation. A PDMS/Glass based IMER was used by Gao et al.,\textsuperscript{58} for the absorption of trypsin enzyme for protein cleavage. This device was also easy to prepare, but was less efficient and experienced leakage at high flow rates. Disadvantages of using glass or similar materials for an IMER include its fragile nature and the costly fabrication processes.\textsuperscript{59}

In contrast, natural organic polymers such as cellulose and collagen are suitable options for IMERs because they are cheap and highly abundant.\textsuperscript{60} However, these materials often require adsorption-crosslinking for enzyme attachment and can suffer
from diffusional limitations. In this study, 78% activity of invertase was retained post-immobilization. The author observed no change in optimal pH, yet the enzyme produced better stability over a wider pH range. The optimal temperature for invertase increased to 60°C degrees yielding better thermostability compared to the solution-phase counterpart. Immobilization did not alter enzyme affinity for its substrate and resulted in a four-fold longer activity compared to the free invertase enzyme.

An alternative to organic polymers is the use of synthetic organic solid-phases (also known as thermoplastics) because they offer the widest applicability due to their highly tunable synthetic composition, which allows for custom tailoring to any enzymatic process. A work by Sakai-Kato et al., demonstrated the use of a trypsin-encapsulated monolith that was fabricated in situ on a PMMA microchip to produce an integrated bioreactor that can perform enzymatic digestion, electrophoretic separation and detection in one chip. Also, Qu and co-workers employed a PMMA based IMER for the cross-linkage of trypsin within a silica monolith. The benefit of this device was the increased stability of trypsin under denaturation conditions. In general, these materials are often uncompromised by microbial attack, making them useful for medical devices where sterility is necessary. Synthetic polymers also retain numerous reaction capabilities such as ion exchange, basic and acidic groups. Activation of these supports to create functional groups for covalent attachment of enzymes can also be achieved using for example oxygen plasma, ultra-violet irradiation, microwave radiation, or chemical modification. Usage of a synthetic polymer for an IMER was exhibited by Cerdeira Ferreira et al.; they developed an IMER in poly(methyl
methacrylate), PMMA, for the detection of glucose. The microchannels of the reactor were chemically modified with polyethyleneimine (PEI), which showed good effectiveness for the immobilization of glucose oxidase (GOx) using glutaraldehyde as the crosslinking agent. The hydrogen peroxide generated by the enzymatic reaction was detected in an electrochemical flow cell localized outside of the IMER using a platinum disk as the working electrode. The results were in close agreement with those obtained by the classical spectrophotometric method.

1.6 Fabrication of Micro- and Nano-Scale Solid-Phase Reactors

In-vivo-like study of various biological phenomena and processes that occur at the molecular level require the ability to assess enzymatic activities at relevant length scales (~2-100 nm). However, these systems are usually studied in macroscopic environments in dilute conditions- conditions unlike the high concentration environment in which these reactions naturally occur. These highly concentrated environments create effects known as macromolecular crowding, and many researchers add crowding agents such as Ficoll and polyethylene glycol (PEG) to mimic these effects in-vitro. However, these conditions can be mimicked in the absence of crowding agents if microfluidic/nanofluidic dimensions can be tailored to the reaction. To achieve this, experimental systems should have representative sizes of several micrometers (cell ~10-30 µm) down to a few nanometers (single deoxyribonucleotide monophosphate, dNMP ~2-3 nm) to mimic native environmental conditions of the biological systems to be investigated. When adapting bioreactors to study these systems, fabrication technologies have become available to make micro- and nanoscale devices enabling the study of biochemical processes within these dimensions (Figure 1.6). These fabrication techniques can include a variety of methods, but
depending on the length scale desired, devices can be fabricated with micro- and/or nanomachining techniques. Photolithography followed by silicon wet/dry etching, doping, deposition, lift-off and other patterning methods enable the production of micro-scale patterns on a variety of substrates including glass, quartz and silicon. In addition, e-beam lithography, Focused-Ion Beam (FIB) milling, Atomic Force Microscope (AFM) and Transmission Electron Microscope (TEM) can produce nanostructures on these materials.

Figure 1.6 Graphical representations of various analytes and the relevant Micro- and Nano-machining scales used to evaluate these types of samples. (Reproduced from Madou, M; 2002, 2nd Edition, CRC Press, pp. 752, with permission).

Fabrication of structures in synthetic polymers (also known as thermoplastics) utilizes hot embossing, injection molding, compression molding, and thermal forming or casting for microstructure formation. Nano-imprint Lithography (NIL) has the capability
to build structures in thermoplastics from the millimeter to sub-10 nm range. This technique utilizes a master (usually Si) that is milled/etched with the desired nanostructures and then replicated (creating a stamp) with a material preferably containing a low young’s modulus and similar expansion coefficient to the desired solid-phase material. One of the major advantages of NIL is the repeatable usage of this stamp and the diverse choice of solid-phase materials. Additional benefits of this process are that structures can be made in various substrates by adjusting only the imprinting temperature and mixed-scale fluidic structures can be created in a single step.

1.7 Fluidics of IMERS

Fluidics, whether micro- or nano-scale, describe the behavior of fluids confined to a small volume element. In general, microfluidics refers to the manipulation of fluids by micro-scale components. In microfluidics, fluid transport can be carried out using hydrodynamics or electrokinetics and can be used to introduce samples and/or reagents through the IMERs. When hydrodynamics are employed, mechanical pumps drive fluids and microvalves determine the flow direction. For electrokinetics, an electric field is applied across the fluidic network and material is driven either through its intrinsic electrophoretic behavior (i.e., charged particle) or the surface’s electroosmotic flow.

Nanofluidics is the study of the behavior, manipulation, and control of fluids confined to structures with nanometer length scales (1-100 nm). Fluids confined in these structures exhibit physical behavior not observed in larger structures because the characteristic scaling lengths of the fluid (e.g. Debye length, hydrodynamic radius) very closely coincide with the dimensions of the nanostructure itself. Fluidic properties that are altered in nano-confined environments include viscosity, thermodynamic properties, and chemical reactivity of biomolecules at the solid/liquid interface.
The contrast between microfluidics and nanofluidics is particularly compounded when electrokinetics are used at the nanoscale. Electrified heterogeneous phases induce an organized charge distribution near the surface known as the electrical double layer (EDL). Devices with nanometer structures can have an EDL that may span the nano-confined space, resulting in changes in the composition of the fluid and the related properties of fluid motion. In addition, nano-confined reactors can have considerably larger surface-to-volume ratios compared to micro-scale reactors where the dominance of counter-ions allow for maneuvering biomolecules with selective polarity along the channel wall to achieve unusual fluidic manipulation patterns not possible in micrometer structures.

1.7.1 Reactor Modes

Practical considerations for IMER design depend on the mode in which the experiment is performed in terms of the fluidics. Reaction modes most frequently involve batch or continuous flow processes. The best way to determine the reactor type necessary for an experiment is to establish if the immobilized enzyme requires a static interaction with its substrate or can undergo a steady influx of substrate for generation of product, which defines batch or continuous reactors, respectively.

1.7.1.1 Batch Versus Continuous Flow

In macro-scale applications, batch reaction modes are most commonly employed because they are well suited for the production of valuable products. Due to its static state, batch reactors most frequently require agitation of some kind to the reaction mixture to ensure efficient conversion of substrate to product. Advantages of batch, as opposed to continuous flow reactions, include higher conversion per unit volume in a single use, and better homogeneity in the product once the reaction is complete.
Micro-scale applications using batch reactors utilize reduced sample volumes, which can be advantageous, yet can require complex valving to confine the sample.

Under continuous flow operational modes, a constant stream of substrate is introduced into the IMERs and products generated are collected at the IMER outlet. These reactors can also have higher mixing rates compared to batch reactors and tend to be implemented more frequently in the study of enzyme kinetics. In the work of Bhangale and co-workers, an enzyme packed microreactor was compared with a batch reactor to determine the effects of reaction water content on immobilized Candida Antarctica Lipase B (CALB) efficiency for end-group functionalization (See Figure 1.7). These experiments were conducted in either dried toluene, designated as the “dry” system, or with water saturated toluene, which is referred to as the “water saturated” system in the study. The authors observed that the average molar mass versus conversion plots for “dry” and “water saturated” conditions were similar, yet under “water saturated” conditions in the batch reactor, had an average molar mass that was much lower than the packed bed reactor. Moreover, higher end-group functionalization was achieved for polymerizations in the (packed) continuous flow IMER (0.75 to ≥0.98) as compared to the batch reactor (0.2). Their results concluded that the packed microreactor design resulted in effectively “dry” conditions even when reactants are “water saturated”.
1.7.2 IMER Efficiency as a Function of Scale

Examination of immobilized enzyme reactions on the micro- and nano-scale IMERs will depend on differences in the information required and the nature of the experiment. In some instances, reactor conditions mimic behavior found in-vitro analysis. For example, acid phosphatase was covalently immobilized onto poly(methyl methacrylate) (PMMA) beads with an epoxy linker using a micro-scale IMERs was examined by Babich and co-workers. The authors observed that after immobilization, 70% of the activity was retained and the immobilized enzyme was stable for months. With this micro-scale IMER, the authors produced several phosphorylated products from the corresponding primary alcohol using either a fed-batch or a continuous-flow packed-bed reactor.

In the work by Grzelakowski and co-workers demonstrated the first successful application of polymer vesicles as an immobilization platform for studying acid phosphatase inside nano-reactors.
This unique application involved immobilization of nanoreactors on a glass substrate where acid phosphatase was surface confined within the nano-reactor by employing biotinylation though conjugation to streptavidin.

**1.7.3 Multi-scale Reaction Control**

Micro- and nano-scaled IMERs offer better reaction control as opposed to larger scale reaction systems due to better heat transference within smaller dimensions and higher surface-to-volume ratios for reducing diffusional barriers. However, the dynamics of biological reactions can be altered due to scaling affects. Wang *et. al.*\(^\text{82}\) reported nanofluidic devices coupled to an electrochemical detector for the study of GOx reaction kinetics confined in nano-space (see Figure 1.8). In this study, GOx species were chemically immobilized onto the surface of nanochannels catalyzing the oxidation of glucose as it flowed through the channels, where the enzyme reaction product, hydrogen peroxide, produced an anodic current. The steady state current at various glucose concentrations was used to evaluate GOx activity under different confinement length scales. The results showed significant nano-confinement effects that were dependent on the channel size, as compared to bulk studies. This observation demonstrates the importance of spatial confinement on GOx reaction kinetics. Their work also found that this confinement effect is dependent on the size of nanospaces where the enzyme reaction occurs, that is, as the confinement space decreases from 140 to 80 nm, the GOx activity decreases; while the reaction rate is accelerated. They suggest that this decreased GOx activity may be due to the change of enzyme conformation and steric hindrance of the catalytic site in the nanochannels. This change in orientation could render the active site inaccessible and produce decreased activities as observed in this work.
Figure 1.8 Schematic layout of the nanofluidic-chip fabrication process: a) UV light-irradiated PC under a PET photomask; b) pattern transfer of the photomask to the PC plate surface; c) GOx immobilization on the nanochannel; d) sealing PDMS with two reservoirs (s and t, for substrates) to PC with nanochannels reversibly; and e) formation of the final nanofluidic chip. f) SEM image of the nanochannel formed after 120 min of UV irradiation, about 110 nm in depth. The symbol r denotes the end-channel electrochemical detection point. Total length: o–r = 8 mm, s (or t)–o = 4 mm. The single nanochannel was achieved by exposing a PC substrate to UV irradiation at a lamp-to-plate distance of 2.0 cm (Reproduced from ChemPhysChem 2012, 13 (3), 762-768, with permission).

1.8 IMERs for the Study of Proteins

Enzymatic reactors for protein investigations have been a major contribution to the field of proteomics. Study of proteins that are expressed by various cells, tissues, and organisms can provide important information, especially in disease biomarker identification. Yet, these molecules prove to be difficult to identify, characterize and quantify due to post-translational modifications they can undergo. Therefore, the processing pipeline for proteins requires careful selection including the method of pretreatment, or catalytic disassembly. Typically, pretreatment of protein samples can be laborious, yet the advent of IMERs for proteolytic digestion provides increase efficiency, reduced auto-digestion and easy post-processing due to decreased contamination from enzyme.
1.8.1 Biomolecules Used for Protein and Peptide Analysis

Trypsin, a common proteolytic enzyme, is a serine protease that cleaves the carbonyl side of a protein at the basic residues lysine and arginine creating peptides. In proteomics, trypsin digestion is a major component of preparing proteins identification and quantification by mass spectral (MS) analysis. Unfortunately, the proteolysis step is the slowest step of the proteomics process by an order of magnitude.\textsuperscript{45} However, immobilization of this enzyme for protein digestion has made this reaction widely efficient, due to enhanced mass transport and decreased auto-digestion. Recently, work by Xiao \textit{et al.},\textsuperscript{83} using a trypsin IMER based on aptamers was developed and applied to protein digestion (See Figure 1.9).\textsuperscript{83} The authors took 25 single-stranded DNA aptamers that were specific for trypsin after SELEX (Systematic Evolution of Ligands by EXponential enrichment) and subsequently grafted them to an amino-modified silica surface with glutaraldehyde. The results concluded that when compared to in-solution digestion, the aptamer-based IMER exhibited similar results for protein identification, yet achieved a much shorter digestion time (~30 min). The system, when compared with an IMER using covalent enzyme immobilization, demonstrated more rapid and convenient immobilized as well as the ability to re-immobilize the enzyme for reactivation of the reactor. This superior reactor demonstrated that an IMER can be employed to reduce proteolysis times for proteoform analysis and aptamers could become a more widely used method for enzyme immobilization.
1.9 IMERs for DNA and RNA Studies

Enzymatic action on DNA and RNA can serve a significant purpose for sequencing and single nucleotide polymorphism (SNP) identification. One major benefit is that enzymes can synthesize or degrade these molecules, yet only those that produce small homogenous fragments will be most useful. Enzymes like polymerases, exonucleases, and endonucleases meet this requirement due to their innate ability to add or remove bases, typically one-by-one.

1.9.1 Common Biomolecules Used for DNA/RNA Examination

Polymerase is an enzyme that synthesizes DNAs and RNAs from nucleotide monomers. More specifically, DNA and RNA polymerases are used to assemble these polymers by copying a template strand using base-pairing interactions. In 2011, Lim and co-workers\textsuperscript{84} published the covalent immobilization of Taq DNA polymerase within mixed self-assembled monolayers (SAMs) through amide bonds between the enzyme and the carboxyl group within the SAM.\textsuperscript{84} They observed that the activity of the immobilized enzyme was $\sim$70\% of the solution-phase reaction by masking the active
site of the Taq DNA polymerase prior to immobilization. The activity observed with protected immobilized enzyme was \(~20\) times higher than that observed with randomly immobilized enzyme. In addition, optimizing the carboxyl group concentration in the mixed monolayer controlled the number of immobilization bonds. In a more recent study, Lim and co-workers further developed the method of oriented and activity-preserved immobilization of biologically active proteins based on concepts of active-site masking and kinetic control. They observed that minimal requirements for the masking DNA molecule were found to be a 5'overhang with 5-7 nucleotides and a double-stranded region of 11-13 bp to retain \(~70\)% of the enzyme activity. They found that the amplification range of protected immobilized (PIM) Taq DNA polymerase was \(>1.2\) kb.

Exonucleases are enzymes that work by cleaving nucleotides one at a time from the end (exo) of a polynucleotide chain resulting in a hydrolyzing reaction that breaks phosphodiester bonds at either the 3' or 5' end. While these enzymes have been suggested for sequencing upon immobilization, they also possess unique applications as demonstrated by Snyder et. al., where an exonuclease enzyme was reversibly immobilized onto a relief-patterned stamp to ablate ssDNA monolayers (See Figure 1.10). The authors showed that by using an immobilized catalyst, the lateral resolution of micro-contact printing depended only on the length and flexibility of the tether (<2 nm) as opposed to diffusion (>100 nm). The data showed the percent ablation was 70% as determined via confocal fluorescence microscopy.
Figure 1.10 Confocal microscopy images of Acrylamide-NTA surfaces with selectively patterned ExoI and resultant fluorescently labeled ssDNA patterned by enzymatic microcontact printing. Glass slides functionalized with isothiocyanate were incubated with 5′-hexylaminated, 3′-fluorescently labeled DNA and printed with an NTA-stamp bearing exonuclease I. Stamp dimensions are exactly reproduced on the DNA monolayer. (Reproduced from *The Journal of Organic Chemistry* 2007, 72 (19), 7459-7461, with permission).

Another important nucleic acid enzyme is the endonuclease, which cleaves phosphodiester bonds in the middle (endo) of the phosphodiester bond at sequence specific sites. Xu and co-workers demonstrated the feasibility of a single-molecule microfluidic approach for both sequencing and obtaining kinetic information for restriction endonucleases of dsDNA.\(^8^7\) In this method, a microfluidic device was designed to trap, hold, and linearize double-stranded (ds) DNA to which a restriction endonuclease was pre-bound sequence-specifically. They noted that their accuracy in determining the location of the recognition site was comparable to or better than other single molecule techniques due to the fidelity with which they could control the linearization of the DNA molecules.

On the nano-scale, Price and associates\(^8^8\) demonstrated the first successful enzyme-catalyzed reaction of a nucleic acid substrate within the confines of nano-engineered polymer capsules assembled by a layer-by-layer (LbL) process. In this work,
co-encapsulation of DNase I and dsDNA enabled the degradation of the DNA and high-throughput monitoring of the encapsulated, fluorescently labeled DNA provided a novel means of measuring the kinetics of the reaction. In contrast to other encapsulation techniques, polymer capsules enable the simple diffusion or exclusion of reaction components primarily on the basis of size, as well as simple control of the reaction through the use of chemical stimuli.

From an RNA perspective, RNase (ribonuclease) mapping by nucleobase-specific endonucleases combined with mass spectrometry (MS) is a powerful analytical method for characterizing ribonucleic acids, such as transfer RNAs. Typical free solution enzymatic digestion of RNA samples result in a significant amount of RNase being present in the sample solution analyzed by MS. In some cases, the RNase can lead to contamination for the high performance liquid chromatography and MS analysis steps. However, work by Butterer et. al.,\textsuperscript{89} compared several different approaches for reducing or eliminating contaminating RNase from the digested RNA sample before LC-MS analysis using immobilized RNases. They observed that immobilization of RNases were found to be effective, with no enzyme carryover into the digested sample detected, biological activity was retained and were re-usable. These researchers demonstrate that the use of immobilized RNases provided a simple approach for eliminating enzyme contamination in mass spectrometry-based RNase mapping experiments. In addition, Ponomareva et. al.\textsuperscript{90} produced IMERs by the covalent attachment of ribonuclease A to macroporous methacrylate-based monolithic supports. In this study, enzyme immobilization was carried out by direct covalent binding as well as through attachment via a polymer spacer and methods. Their results found that introduction of a polymeric
long-chain spacer between the surface of the monolith and the enzyme molecule was proven to have a positive effect on the ribonuclease IMERs. RNase bound to the chemically modified support through the use of spacer exhibited higher activity than enzyme directly bound to the surface.

1.9.2 Processivity and High Throughput Devices for DNA Disassembly

Processivity is an enzyme's ability to catalyze "consecutive reactions without releasing its substrate". This trait of enzymes is desirable to exploit in the hopes of increased read lengths for sequencing applications. Pacific Biosciences proposed one application of processive enzymes for sequencing where they presented single-molecule, real-time sequencing. The platform introduced several interesting features including immobilization of a DNA polymerase, real-time monitoring of the synthesis process and potential read lengths of several kilobases.

A year later Korlach and co-workers, developed a single-molecule real-time sequencing device that relied on DNA polymerase immobilized at the bottom of a zero-mode waveguide (ZMW) nano-structure (Figure 1.11). They asserted that this structure was critical because it provided a confined optical observation space of ~100 zeptoliters, enabling minimization of background noise, parallelization, and monitoring of single-molecule DNA polymerization. In this study, the DNA template was allowed to diffuse into the ZMV in the presence of primer and nucleotides with fluorescent labels attached to the phosphate chain. Single-molecule, real-time (SMRT) sequencing has high error rates, mainly due to failure to detect all incorporations leading to indels.
Figure 1.11 Principle of single-molecule, real-time DNA sequencing. (A) Experimental geometry. A single molecule of DNA template-bound Φ29 DNA polymerase is immobilized at the bottom of a ZMW, which is illuminated from below by laser light. The ZMW nanostructure provides excitation confinement in the zeptoliter (10\(^{-21}\) liter) regime, enabling detection of individual phospholinked nucleotide substrates against the bulk solution background as they are incorporated into the DNA strand by the polymerase. (B) Schematic event sequence of the phospholinked dNTP incorporation cycle, with a corresponding expected time trace of detected fluorescence intensity from the ZMW. (1) A phospholinked nucleotide forms a cognate association with the template in the polymerase active site, (2) causing an elevation of the fluorescence output on the corresponding color channel. (3) Phosphodiester bond formation liberates the dye-linker-pyrophosphate product, which diffuses out of the ZMW, thus ending the fluorescence pulse. (4) The polymerase translocates to the next position, and (5) the next cognate nucleotide binds the active site beginning the subsequent pulse. (Reproduced from Science 2009, 323 (5910), 133-8, with permission, Copyright 2014).

An effort to achieve nanopore sequencing is carried out by Oxford Nanopore.\(^92\) In one rendition of their approach, exonuclease sequencing employs a modified \(\alpha\)-hemolysin nanopore with an immobilized exonuclease, which is situated within a synthetic membrane with high electronic resistance. As DNA enters the membrane, the exonuclease cleaves off single nucleotides and feeds them sequentially into the nanopore. Each individual nucleotide can then be identified from their distinct electrical signal as they transitorily move through \(\alpha\)-hemolysin nanopore; the characteristic electrical signal gives a unique signal for nucleotide identification.
Figure 1.12 a) Experimental setup for the exonuclease experiments. Nucleotides liberated by the enzyme are detected by the WT-(M113R/N139Q)-6(M113R/N139Q/L135C)-1-am6amDP1βCD pore. b, Residual pore current histogram of nucleotide binding events for a ssDNA containing G, A and C. c, Residual pore current histogram of nucleotide binding events for a ssDNA containing G, T and C. Experimental conditions: 200/500 mM KCl, 25 mM Tris HCl, pH 7.5, 1 mM MgCl2, 5 µM ssDNA and 80 U exonuclease I, at +180 mV and room temperature (Reproduced from *Nat Nanotechnol* 2009, 4 (4), 265-70, with permission).

1.10 Conclusion

The technologies and applications reviewed in this work support the growing interest in the integration of immobilized enzymes into microfluidic and nanofluidic systems. As demonstrated here, these systems offer wide applicability to study heterogeneous enzymatic reactions on multiple scales. From these works examined we observe that the benefits of IMERs as reaction mediums include high customization, reduced sample volumes and reagent consumption, higher throughput, enhanced enzyme stability and reduced cost due to reusability. Some of these benefits have been realized in the creation of immobilized λ-Exonuclease (λ-Exo) reactors for the digestion of dsDNA. For this application effect of IMER dimensions will be investigated for the eventual integration of this powerful bioanalytical into a nano-scaled DNA sequencing device.
1.11 References


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2 Immobilization of Lambda Exonuclease onto Polymer Micropillar Arrays for the Solid-Phase Digestion of dsDNAs

2.1 Introduction

Recently, solid-phase bioreactors have found interesting applications in the areas of single-molecule enzymology, biochemical manufacturing and nanotechnology. A sub-group of solid-phase bioreactors called Immobilized Microfluidic Enzymatic Reactors (IMERs) comprises systems in which an enzyme is immobilized within the channels of a microfluidic device. There are several advantages associated with these systems as opposed to their homogeneous (liquid-based) counterparts. These include enhanced stability and activity of the tethered enzyme relative to the enzyme in free solution, reduced interference from catalytic enzymes during the analysis phase of the assay and reusability of the enzyme. In the case of proteolytic enzymes such as trypsin, immobilization of the enzyme can prevent autodigestion as well. The reported success in the attachment of enzymes onto solid supports stems from the availability of several enzyme/solid surface attachment chemistries. Based on the plethora of available attachment chemistries, solid supports such as silicon, glass, or polymers, can be selected to accommodate the pendant functional groups available on most proteins and the fabrication strategies used to produce the fluidic devices associated with IMERs.

Of the numerous chemical strategies for protein attachment, many rely upon reactions between functional groups within the protein (amine and/or carboxylic acids) and complementary groups on the solid surface. In general, non-covalent and covalent attachment chemistries have been used to immobilize proteins to solid surfaces.

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surfaces with the latter reported to provide more robust linkages, hence, less susceptibility to detachment or denaturation.\textsuperscript{3} If the interactions between the protein and support are not carefully designed, there is a tendency to produce reactors possessing randomly oriented proteins with some orientations providing inactive forms.\textsuperscript{21} Attachment chemistries involving the use of affinity tags such as poly-His and glutathione s-transferase have been shown to eliminate issues with random attachment of enzymes to solid surfaces; nevertheless, they form chemical bonds that can become unstable over time or after multiple usages of the reactor.\textsuperscript{3} A recent study suggested that limiting the surface functional group density of a substrate can induce single site-attachment minimizing the generation of multi-site attachment potentially deactivating the immobilized biomolecule.\textsuperscript{22}

In the past decade, polymer substrates have become beneficial for the design of biological reactors due to their exceptional biocompatibility, widespread surface functionality, attachment chemistries that are relatively stable over a wide range of pH values and the ease of surface activation for the generation of functional scaffolds for protein attachment.\textsuperscript{23-26} In many cases, polymer substrates can exhibit glass-like properties, such as high optical transparency and low autofluorescence, and provide production of low-cost fluidic devices with good fidelity, appropriate for \textit{in vitro} diagnostics.\textsuperscript{27-29} Some polymeric materials which possess the aforementioned characteristics are poly(methylmethacrylate) (PMMA)\textsuperscript{27} and cyclic olefin copolymer (COC).\textsuperscript{30} In particular, PMMA has been a substrate of choice in the design of fluidic devices for biological assays due to its favorable biocompatibility,\textsuperscript{27} excellent optical properties and simplicity in the surface modification techniques that can be employed.\textsuperscript{24}
Previously, we have shown that IMERs can be generated using PMMA substrates for the proteolytic digestion of proteins.\textsuperscript{31-32} PMMA IMERs with immobilized trypsin have shown enhanced enzyme stability, high reaction rates and the absence of trypsin autodigestion, thereby simplifying protein identification using mass spectrometry.\textsuperscript{31,32}

Exonucleases, which cleave double-stranded deoxyribonucleic acids (dsDNA) or single-stranded DNA (ssDNA) along the phosphate backbone to generate mononucleotides, are involved in biological processes such as replication, repair and recombination.\textsuperscript{33} Lambda-Exonuclease (\lambda-Exo), isolated from lambda bacteriophage, is a toroidally shaped processive enzyme composed of three identical subunits with a tapered pore active site; 30 Å diameter on one face for entry of dsDNA and 15 Å diameter on the opposite face for the exit of ssDNA.\textsuperscript{34-36} \lambda-Exo, which digests only dsDNAs with phosphorylated 5' ends, has been suggested to possess a processivity of \(~3,000\) nucleotides in free-solution and generate an intact ssDNA byproduct with an electrostatic ratchet digestion mechanism.\textsuperscript{37} Though its clipping rate is highly variable, single-molecule measurements have revealed an average value of \(~1,000\) nt s\(^{-1}\).\textsuperscript{38} The digestion properties of \lambda-Exo offer several unique applications.\textsuperscript{39} For example, \lambda-Exo has been suggested to be useful in single-molecule DNA sequencing strategies,\textsuperscript{40} one format of which involves the exonuclease and an \(\alpha\)-hemolysin nanopore. Previous simulation and experimental reports have suggested the use of immobilized \lambda-Exo for the systematic clipping of DNA into mononucleotides with each unit identified via a molecular-dependent flight time through nanochannels.\textsuperscript{41-42} The unique capabilities of \lambda-Exo and its immobilization onto solid supports can serve as a useful tool in the design of biosensors directed towards the sequence analysis of DNAs.\textsuperscript{10}
A recent study demonstrated the digestion of dsDNA by λ-Exo with the dsDNA electrostatically anchored onto the substrate surface and the enzyme introduced in free solution and allowed to randomly bind to the free end(s) of the anchored dsDNA.\textsuperscript{43-45} Single-molecule fluorescence studies, with a fluorescently stained dsDNA target revealed that λ-Exo digestion occurred in three modes; (i) Incomplete digestion at only one end of the dsDNA molecule; (ii) full simultaneous digestion at both ends; and (iii) incomplete digestion at both ends.\textsuperscript{43} While this single-molecule enzyme study provided valuable information concerning the catalytic action of λ-Exo, it did not address the scenario in which the enzyme was immobilized and the dsDNA target was present in free solution.\textsuperscript{40} A report by Perkins \textit{et al.}, which showed the successful immobilization of a single λ-Exo apoenzyme/dsDNA complex onto a quartz substrate, revealed that the activity of λ-Exo remained comparable to the free solution digestion.\textsuperscript{46}

In this work, we report the first IMER involving λ-Exo as the immobilized enzyme for the digestion of dsDNA. λ-Exo was immobilized onto a PMMA device consisting of an array of micropillars populating a bioreactor. This device geometry allowed for an increased enzyme load and reduction in the diffusional kinetic barriers associated with open-channel IMERs.\textsuperscript{47} The immobilization was accomplished using 3-(3-dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide (EDC/NHS) coupling chemistry for the conjugation of λ-Exo to UV-generated carboxylic acids on the PMMA surface. Atomic Force Microscopy (AFM) revealed the absence of non-specific attachment (physisorption) of the enzyme to the polymer surface indicating that the enzyme was only attached covalently to the surface. Capillary electrophoresis using laser-induced fluorescence detection (CE-LIF) of the digestion products provided
information on the lengths of the fragments remaining after digestion. Fluorescence microscopy studies of YOYO-1 stained dsDNA allowed for real-time observation of the digestion from which the enzyme clipping rate and apparent processivity were deduced.

2.1 Materials and Methods

2.1.1 IMERs fabrication

The IMERs used in this study consisted of a 1.4 mm x 24 mm polymer microchannel populated with 3,600 microposts, each 50 µm tall and 100 µm in diameter. In brief, the fabrication and assembly of this device included hot embossing using a HEX03 hot embossing system (JenOptik, Jena, Germany), which created the microstructured devices. The devices were replicated from a brass mold master that was fabricated using a micromilling machine (Kern, MMP Feinwerktechnik, Murnau-Westried, Germany). The PMMA IMERs consisted of a microreactor bed, which was comprised of a channel (24 mm long and 1.4 mm wide) containing 3,600 micropillars (100 µm height, 100 µm diameter and 50 µm pillar-to-pillar spacing) and a total surface area of 116.9 mm² with a 2.9-µL volume. A schematic of the device and a SEM can be found in Figure 2.1A and 2.1B, respectively. Post-processing of the microfluidic device included mechanically drilling reservoirs for sample introduction followed by cleaning the device and cover plate with isopropyl alcohol, rinsing with ddH₂O for debris removal and finally, drying in an oven at 70ºC. Ultraviolet (UV) radiation of the immobilization beds using a 254-nm UV light with a power density of 16.0 mW/cm² for 15 min was performed so as to activate the polymer surface by producing carboxylic acid groups that could be later functionalized with the enzyme following cover plate assembly.

Thin PMMA sheets, 0.125 mm thick, were used as cover plates to enclose the fluidic network of the IMERs by thermal fusion bonding. The PMMA substrate and cover
plate were sandwiched between two borosilicate glass plates (McMaster, Atlanta, GA, USA) and clamped together prior to insertion into a convection oven. The thermal bonding of the PMMA IMERs was performed at 101°C for 20 min.

![Diagram](Image)

Figure 2.1 (A) Schematic showing the layout of the IMERs used for λ-Exo digestion of double-stranded DNA. The reactor bed was populated with micropillars that were 100 µm in diameter. The IMERs was made from the thermoplastic, PMMA, via hot embossing. (B) SEM of the IMERs fabricated via hot embossing from a brass-molding tool. (C) Schematic showing the immobilization of λ-Exo onto a PMMA substrate. The substrate was activated by ultraviolet radiation to generate surface confined carboxylic acid groups. This was followed by EDC/NHS coupling chemistry to covalently attach the enzyme to the substrate during an incubation period, which was carried out overnight at 4°C.

### 2.2.2 Enzyme immobilization onto PMMA IMERs

λ-Exo was anchored onto the IMERs' surfaces using EDC-NHS coupling chemistry previously outlined by our group for the immobilization of amine-containing biological entities onto photo-activated PMMA substrates. λ-Exo was provided with a 10X reaction buffer (670 mM glycine-KOH, pH 9.4, 25 mM MgCl₂, 0.1% (v/v) Triton X-100), which was purchased from Fermentas Life Sciences (Glen Burnie, MD). No purification steps were performed prior to use. Following thermal fusion bonding of the cover plate
to the substrate, succinimidyl ester intermediates were formed to facilitate enzyme attachment. This was carried out by filling the reactor bed with a solution containing 200 mM 3-(3-dimethylaminopropyl) carbodiimide (EDC), and 50 mM N-hydroxysuccinimide (NHS) in 0.1 M 2-(4-morpholino)-ethane sulfonic acid at pH 5.1 (MES, Fisher Biotech, Fair Lawn, NJ) for 15 min at room temperature. The EDC/NHS reagents were then hydrodynamically displaced with a solution consisting of 0.03 µg/µL λ-Exo enzyme; the reaction was allowed to proceed overnight at 4°C. The enzyme-functionalized device was then rinsed with 1X λ-Exo reaction buffer to remove all unbound reagents from the PMMA surface. The schematic for this reaction is depicted in Figure 2.1C.

2.2.3 Digestion studies of dsDNA

Duplexed λ-DNA (48,502 bp), purchased from New England Biolabs (Ipswich, MA), was incubated in the enzyme-modified IMERs for various reaction times. The desired reaction times were achieved by hydrodynamic pumping (PHD2000 syringe pump, Harvard Apparatus, Holliston, MA) a λ-DNA solution through the IMERs at an appropriate flow rate. An experimental control, which involved the introduction of a solution containing λ-DNA into the IMER bed in the absence of immobilized enzyme, was performed. The control revealed that there was neither a loss nor breakage of the dsDNA from non-specific adsorption onto the reactor wall or shearing, respectively. On-chip enzymatic reactions were temperature controlled at 37°C via a custom-built thermocouple heating stage. The effluent was collected at the device outlet for downstream analyses with CE and bulk fluorescence measurements.
2.2.4 Fluorescence measurements of IMER-digested dsDNA

PicoGreen intercalating dye (Life Technologies, Grand Island, NY) was used to determine the amount of intact dsDNA remaining after passage through the IMERs. PicoGreen shows high specificity for binding to dsDNA with a 1,000-fold fluorescence enhancement after intercalation to dsDNA. Because the dye displays minimal amounts of fluorescence upon binding to ssDNA (<10% that of dsDNA) and does not bind to mononucleotides with an associated fluorescence increase, it is suitable for determining specifically the dsDNA content from a λ-Exo reaction, which should consist of ssDNA, dsDNA and mononucleotides. The DNA staining dye was added post-digestion to avoid perturbation in enzymatic activity of λ-Exo that may result from nuclear staining. The dye-labeled samples were excited at 480 nm and fluorescence spectra (490 – 700 nm) were collected and analyzed using a Fluororolog-3 spectrofluorimeter (Horiba JobinYvon, Edison, NJ) and DataMax Software 2.20. Kinetic data was acquired using a PicoGreen-stained digested dsDNA sample at varying input concentrations (20 µg/mL – 5 µg/mL) using a 60 s reaction time with 4.96 pmol of surface-bound enzyme.

2.2.5 Capillary Electrophoresis-Laser Induced Fluorescence

Digestion products and the HIND III sizing ladder were analyzed using a home-built CE instrument with LIF detection. Bare fused silica capillaries from Molex Polymicro Technologies (Phoenix, AZ) were used for the CE (total length = 33 cm, 20 cm effective length) and possessed a 50 µm internal diameter. The CE columns were preconditioned with 0.1 M NaOH for 30 min and rinsed by flushing with 0.5X TBE buffer (pH 8.3). Finally, the capillary surface, prior to the electrophoretic separations, was treated with a dynamic coating containing 2% (w/v) polyvinylpyrrolidone (PVP, Mr = 40,000; Sigma
Aldrich St. Louis, MO) in 0.5X TBE and a sieving matrix of methylcellulose (Sigma Aldrich) that was 0.5% (w/v) in 0.5X TBE buffer (pH 8.3).

Figure 2.2 Schematic of the in-house built CE-LIF system utilizing a 532 nm, 20 mW excitation laser with edge filter and 560 nm long pass filter, 532 nm dichroic filter and SPCM-AQR single photon counting module. A 40X high numerical aperture (NA = 0.85) microscope objective was used to focus the laser beam onto the capillary and collect the fluorescence.

Sample introduction was performed by electrokinetic injection at 10 kV for 180 s. DNA digestion products of λ-Exo were electrophoresed at a field strength of 303 V/cm and fragment size analysis was determined by comparing the results to the Hind III λ-DNA sizing ladder purchased from New England Biolabs (Ipswich, MA). CE data was acquired and analyzed using a custom designed LabView 6.1 program (National Instruments, Austin, TX) and Origin 8.7 software (OriginLab Co., Northampton, MA), respectively.
The LIF detector depicted in Figure 2.2 was configured in an epillumination format containing a 532 nm, 20 mW excitation laser (LaserGlow Technologies, Toronto, Ontario, Canada), XF 3085 edge filter (Horiba Scientific, Middlesex, UK), 3RD560LP 560 nm long pass filter (Omega Optical, Brattleboro, VT), a 532 nm dichroic filter (550DRLP, Omega Optical) and a SPCM-AQR single photon counting module (Perkin Elmer Optoelectronics, Waltham, MA). A 40X high numerical aperture (NA = 0.85) microscope objective from Nikon (Natick, MA) was used to focus the laser beam onto the capillary and collect the fluorescence. Prior to CE, the dsDNA was stained with Sytox Orange (547/570, Life Technologies, Grand Island, NY).

2.2.6 Enzyme quantification

The amount of \( \lambda \)-Exo in solution was determined using a spectrophotometric assay (Pierce 660 nm protein assay kit, Thermo Fisher Scientific; Rockford, IL). Table 2.1 shows the absorbance loss for the enzyme, \( \lambda \)-Exo, loaded onto a photo-activated PMMA IMERs bed. The amount of \( \lambda \)-Exo in solution was determined using a spectrophotometric assay and consisted of measuring the 660 nm absorbance both before and after passing through the activated bed in the presence of EDC/NHS. The amount (pmol) of enzyme used for Beds 1-3 was 75, 90 and 100, respectively. The enzyme was suspended in 25 \( \mu \)L of reaction buffer containing EDC/NHS and was pumped through the reactor bed at a rate to allow for a reaction time of \(~15\) min. The effluent was collected and its absorbance measured without dilution. The absorbance difference was ascribed to material covalently immobilized to the reactor surface.
Table 2.1 660 nm absorbance values for protein immobilization

<table>
<thead>
<tr>
<th>Bed</th>
<th>Pre-Fill Absorbance</th>
<th>Post-Fill Absorbance</th>
<th>Absorbance Difference</th>
<th>Calculated pmoles lost to Immobilization</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.128</td>
<td>0.123</td>
<td>0.005</td>
<td>3.25</td>
</tr>
<tr>
<td>2</td>
<td>0.147</td>
<td>0.124</td>
<td>0.004</td>
<td>4.96</td>
</tr>
<tr>
<td>3</td>
<td>0.16</td>
<td>0.158</td>
<td>0.002</td>
<td>6.40</td>
</tr>
</tbody>
</table>

Table 2.2 shows the percent of digestion as a function of enzyme load onto the bed. A 50 µg/mL solution of λ-DNA was introduced into the IMERs and allowed to react for 60 s. The effluent from the bed was stained with PicoGreen to determine the amount of dsDNA remaining following passage through the IMERs. Following staining, the effluent fluorescence was measured using a Fluorolog fluorescence spectrometer.

Table 2.2 Percent digestion at various enzyme surface concentrations

<table>
<thead>
<tr>
<th>pMoles of Enzyme</th>
<th>3.25</th>
<th>4.96</th>
<th>6.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Enzyme Digestion</td>
<td>96.49 ± 1.75</td>
<td>94.69 ± 2.85</td>
<td>84.74 ± 8.80</td>
</tr>
</tbody>
</table>

Table 2.3 provides data on the percent of dsDNA digestion as a function of reaction time within the IMERs device. This data was collected following the same procedure as that described for Table 2.2. The amount of enzyme immobilized to the bed was 4.96 pmol.

Table 2.3 Percent digestion at various reaction times

<table>
<thead>
<tr>
<th>Reaction Time (s)</th>
<th>60</th>
<th>300</th>
<th>1200</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Enzyme Digestion</td>
<td>95.01 ± 2.69</td>
<td>94.40 ± 1.53</td>
<td>90.20 ± 0.22</td>
</tr>
</tbody>
</table>

2.2.7 AFM characterization of PMMA/λ-Exo surfaces

To deduce the surface coverage and possible orientation of the enzyme, cleaned PMMA sheets (1.7 cm x 1.7 cm squares, 3 mm thick) were activated with UV light and
incubated with a λ-Exo solution overnight at 4°C in the absence and presence of EDC/NHS coupling reagents. Samples were rinsed with reaction buffer, ddH$_2$O, and gently dried with compressed air prior to AFM analysis. Surface characterization was performed using an Asylum Research MFP3D AFM at a 1.00 Hz scanning rate in AC (tapping) mode. At this scanning frequency, we speculate that there would be negligible damage of the immobilized enzyme from the tapping force exerted by the tip.

### 2.2.8 Real-time digestion analysis using fluorescence microscopy

The microscope used in this study was a Zeiss Axiovert 200M inverted microscope (75 W Xe lamp, Zeiss, Germany) fitted with a 100x/1.3 NA oil-immersion microscope objective and an Andor iXon3 EMCCD camera (20 fps acquisition rate). A custom mount was machined to hold the assembled IMERs onto the microscope stage. All images were acquired using MetaMorph Advanced 7.7.6.0 Software (Molecular Devices LLC, Sunnyvale, CA) and analyzed using ImageJ (National Institutes of Health, Bethesda, MD).

PMMA IMERs were modified as previously described. λ-Exo reaction buffer (glycine-KOH in ultra-pure water at pH 9.4, 0.1% (v/v) Triton X-100, reagents purchased from Sigma Aldrich) was prepared without the co-factor (Mg$_2^+$). Following enzyme attachment, λ-DNA stained with YOYO-1 in a 1:50 dye-to-base pair ratio was introduced into the reactor in a Mg$_2^+$ free buffer to allow for the generation of the necessary enzyme/DNA complexes. Next, the reaction buffer (1X) containing 2.5 mM MgCl$_2$ was introduced into the IMERs to initiate the enzymatic reaction after which the system was heated to 37°C with real-time monitoring of the digestion process. Reagent introduction into the IMER was achieved using a PHD2000 syringe pump (Harvard Apparatus,
Holliston, MA). The pump was connected to the inlet and outlet reservoirs of the IMERs through peak tubing sealed via epoxy with the inlet tube connected to a syringe using a leur-lock connector.

2.3 Results and Discussion

2.3.1 Enzyme attachment and characterization

To determine if λ-Exo was covalently attached to the activated PMMA surface of the IMERs reactor bed, we performed spectrophotometric analysis (660 nm) using a protein quantification kit. In this analysis, an aliquot of the reaction solution containing the enzyme, before and after running through the IMERs, was evaluated. The differences in the pre- and post-filling absorbance values were used as an indicator of the amount of enzyme remaining on the reactor surfaces. A calibration plot ($R^2 = 0.992$) using the UV absorbance intensities of a protein calibration standard was used to determine the amount (in pmols) of enzyme attached to the polymer surface for three different input amounts (75, 90 and 100 pmol). The amount of enzyme immobilized range from 3.25 to 6.40 pmol, yielding a reaction efficiency of 4.3 – 6.4%. Based on the available surface area of the IMERs bed (1.17 cm$^2$), the surface concentration (pmol/cm$^2$) was determined to range from 2.78 to 5.47. The fact that the surface concentration increased over the concentration range studied indicated that the surface was below monolayer coverage (see Table 2.1 for results).

Successful attachment of λ-Exo onto PMMA was further confirmed by AFM analysis. From the AFM scan depicted in Figure 2.3A for the activated-PMMA/λ-Exo reaction performed in the absence of the EDC/NHS coupling reagents, there was no indication of the presence of surface features consistent with the size of the λ-Exo enzyme. This confirmed that physisorption of enzyme onto the activated polymer surface did not occur.
under these reaction conditions. AFM images acquired from the PMMA surface in which the EDC/NHS coupling reagents were used revealed the presence of surface features consistent in height with λ-Exo (Figure 2.3B). Substrates containing covalently attached λ-Exo had an average RMS roughness of 1.58 ±0.18 nm as compared to 0.34 ±0.01 nm for surfaces without enzyme. Further AFM scans of the EDC/NHS/λ-Exo functionalized PMMA surface over a 15 µm x 15 µm area (Figure 2.3C) revealed surface features that possessed an average height of 14.3 ±2.3 nm (see Figure 2.3D). This value is similar to the reported dimensions of λ-Exo measured from x-ray crystallography at angles α = β = γ = 90° (15.6 nm x 15.6 nm x 13.1 nm).36

Figure 2.3 Tilted view of a 3 x 3 µm AFM scan of a PMMA surface following UV activation and incubation with 7 µg/mL λ-Exo enzyme without (A) and with (B) EDC/NHS coupling reagents. (C) A 15 x 15 µm phase image of a PMMA surface incubated with 7 µg/mL λ-Exo enzyme with EDC/NHS coupling. Surface AFM analysis revealed an RMS roughness of 1.58 ±0.18 nm. (D) Histogram of the height of features on the activated PMMA surface and subsequently functionalized with λ-Exo determined by taking an AFM line scan across each immobilized enzyme and measuring the maximum height of the feature.
Although it is difficult to directly visualize the orientation of the immobilized enzyme on PMMA, the closeness of the average measured height of each feature to the protein crystallographic size indicates that the enzyme is primarily oriented with its access pore normal to the polymer surface. Though qualitative, this data indicated that the UV dose and enzyme concentration used for the immobilization reaction did not lead to surface cross-linking. Cross-linking may result in the enzyme laying parallel to the surface making its pore inaccessible to dsDNA based on surface steric considerations. This data also confirmed that the conjugation of the complete homotrimer was achieved with little if any dissociation into its monomer units.\textsuperscript{56}

After UV-activation of the PMMA surface, the carboxylic acid group density was measured using a Toluidine Blue assay. For a UV dose of 16.0 mW/cm\textsuperscript{2} for 15 min, a carboxyl surface density of 32 pmol/cm\textsuperscript{2} was obtained; a value 10-fold higher than the \textlambda-Exo surface concentration stated above.\textsuperscript{52, 57} The Toluidine Blue assay, while effective for approximate surface carboxylate quantification, has the propensity to label carboxylic acid groups below the substrate surface where enzyme attachment is not possible due to inaccessibility issues.\textsuperscript{52}

\textbf{2.3.2 Surface enzyme activity}

Figure 2.4 shows the fluorescence spectra of a free solution \textlambda-Exo digestion of \textlambda-DNA and one carried out in the IMERs for the same effective reaction time (60 s). The control for this experiment consisted of a 50 \mu g/mL \textlambda-DNA stock solution exposed to an enzyme-free reactor for 60 s, which was collected at the outlet of the reactor and measured to determine if any loss of dsDNA resulted from transport through the reactor. To determine the extent of DNA digestion in the IMER, PicoGreen was added to the
digestion products from the IMER and the free solution reaction. The amount of dsDNA remaining after digestion was monitored using fluorescence microscopy. As can be seen from Figure 2.4, the amount of fluorescence observed from the digestion products of the free solution was higher than that from the IMERs. This indicated that more dsDNA was remaining for the free solution digestion compared to the IMERs digestion. For the reactions carried out here, peak area analysis of Figure 2.4 revealed that 91.7% of the dsDNA was digested for the IMERs compared to 83.3% for the free solution digestion.

Figure 2.4 Plot of fluorescence intensity for a λ-DNA stock solution, free enzyme digestion and the effluent from an IMERs digestion. The emission spectra were taken from 490 to 700 nm with 480 nm excitation. The spectrum labeled in black depicts the intensity of the λ-DNA stock. The blue line represents the spectrum of the IMERS digestion and the red line was that for the free solution digestion. For the IMERs digestion, the amount of immobilized enzyme was 4.96 pmol. For the λ-DNA stock, the IMERs was free of immobilized enzyme. In all cases, the solutions were incubated with PicoGreen following the reaction.
To evaluate the effects of enzyme surface concentration on the activity of the immobilized enzyme, experiments were conducted in which the reaction time and dsDNA substrate concentrations were kept constant and the enzyme surface concentration used for the digestion varied (Table 2.2). Our data revealed that ~96% digestion of λ-DNA was achieved when loading the IMERs with 3.25 pmols of enzyme (Table 2.2) with a slight decrease in digestion efficiency at higher enzyme loads (~85% at a load of 6.20 pmol). However, over the range of λ-Exo surface loads investigated, no statistical difference in the percent λ-DNA digestion was observed at the 95% confidence level.

Next, experiments were performed to carefully determine the effect of changing the reaction time of the dsDNA with the immobilized enzyme on the digestion efficiency. The IMERs were exposed to λ-DNA for 60, 300 and 1200 s, which was controlled by changing the linear velocity of the input λ-DNA through the reactor. The reactor generated digestion efficiencies >90% for all reaction times investigated (Table 2.3).

2.3.3 Analysis of λ-Exo reaction products using CE-LIF

As shown in the electropherogram obtained for the digestion products of the IMERs, the Hind III sizing ladder and intact λ-DNA (Figure 2.5), there was the absence of peaks corresponding to the intact λ-DNA following IMERs digestion suggesting that most of the λ-DNA was digested. Electrophoretic analyses of λ-Exo digestion products using the IMERs were accomplished by adaptation of published separation protocols with slight modifications. In Figure 2.5, an electropherogram of the digestion product(s), the Hind III sizing ladder, and λ-DNA transported through an enzyme-free IMERs are shown. Using methylcellulose as the sieving matrix, baseline resolution of five Hind III peaks
were observed with co-migration of the 2027/2322 bp and 4361/6557 bp fragments. The peak at 125 bp was not observed because its fluorescence intensity was below the detection limit of the LIF system. Using the same CE conditions, the dsDNA remaining after an IMER’s reaction was analyzed yielding a peak of ~7 kbp in size when compared to the Hind III sizing ladder (see top electropherogram of Figure 2.5). Previous work performed on λ-Exo digestion of surface immobilized λ-DNA produced fragments equivalent to ~19 kbp. As can be observed from our data, the digestion reactions were absent of peaks corresponding to intact λ-DNA suggesting that the majority of the λ-DNA was digested upon passage through the IMERs containing immobilized enzyme.

Figure 2.5 Electropherograms of the Hind III ladder, λ-DNA (passed through an enzyme-free IMERs), and product(s) of the IMER digestion. The dsDNA was stained in a 1:5 dye/bp ratio with Sytox orange (547 nm); the staining was accomplished using the effluent from the IMERs. Following staining, the effluent was then introduced into the electrophoresis capillary via 10 kV electrokinetic injection and the CE was carried out using a capillary coated with 2% PVP. The sieving matrix consisted of 0.5% methylcellulose solution in 0.5X Tris-Borate EDTA buffer and the applied separation field strength was 303 V/cm.
This is consistent with the data shown in Tables 2.2 and 2.3 and Figure 2.4. The digestion reaction will proceed until; 1) the end of the dsDNA molecule is reached; 2) the enzyme dissociates into its monomers; and/or 3) the DNA is expelled from the enzyme.\textsuperscript{36} Furthermore, it is possible that a dsDNA molecule after threading through the pore of λ-Exo could be partially digested, disengaged and re-engaged with another enzyme molecule within the IMER and undergoing further digestion from its complementary phosphorylated strand. Because the CE results indicated that the dominant dsDNA fragment size remaining was ~7 kbp, this indicated an apparent processivity of ~41 kbp if the re-engagement of the dsDNA molecule, which the CE-LIF results cannot determine, is ignored.

2.3.4 Reactor reusability

We also tested whether the enzyme could be used for subsequent rounds of digestion by running different batches of λ-DNA through the reactor with different resident times (see Figure 2.6). For the initial reaction, the digestion efficiency was found to be 95% for a 60 s reaction (see Table 2.3). The reactor was then washed with buffer and a second round of digestion was undertaken by infusing λ-DNA through the IMERs. For the second round (60 s reaction time), the digestion efficiency dropped to 80% and for the third round, 53%.

2.3.5 Real-time digestion of λ-DNA

The digestion of a single dsDNA molecule with an immobilized enzyme was studied in real-time using fluorescence microscopy. λ-Exo was immobilized onto PMMA using EDC/NHS coupling chemistry and a solution of YOYO-1 stained dsDNA in a 1:50 dye to base-pair ratio was introduced into the enzyme reaction buffer (glycine-KOH in ultra-
pure water at pH 9.4, 0.1% (v/v) Triton X-100) without Mg\(^{2+}\). Previous work by Kang et al. revealed that stained dsDNA with a 1:50 dye-to-base pair ratio has comparable digestion rates to unstained dsDNA in the presence of \(\lambda\)-Exo.\(^{43}\)

Figure 2.6 Percent digestion of \(\lambda\)-DNA at 60, 300 and 1200 s for three uses of the same reactor. \(\lambda\)-DNA in presence of reaction buffer was introduced to 4.96 pmol of enzyme immobilized to the reactors at 37ºC and over 3 usages to determine reactor short-term reusability. Squares – first use; Circles – second use and triangles – third use of IMER.

Real time monitoring of the enzyme-threaded DNA’s fluorescence was conducted. Some \(\lambda\)-DNA molecules within the microscopic region were observed to be immobile at one end due to complexation with the \(\lambda\)-Exo enzyme, and freely moving at the opposite end due to shear forces. Uncomplexed DNAs remained in the bulk flow and eventually disappeared from the field-of-view. Following complexation, the reaction buffer containing the necessary Mg\(^{2+}\) co-factor for \(\lambda\)-Exo was added to initiate clipping and the reaction was monitored in real time as depicted in Figure 2.7 under non-flow conditions.
Figure 2.7 (A-D) Fluorescence still images for the real-time digestion of dsDNA using λ-Exo covalently immobilized to a PMMA substrate configured in the IMER device. (E-H) The corresponding fluorescence intensity line plots taken from the still images shown in A-D. In these cases, the line plot was secured from a horizontal line that crossed the section in the still image containing the stained DNA molecule. (I) Graphical depiction of the relative fluorescence intensity of a single dsDNA that was digested by an immobilized λ-Exo molecule as a function of reaction time, where possible pausing events were seen in each inset. Immobilization of λ-Exo was accomplished using EDC/NHS onto a PMMA substrate. The λ-DNA was labeled in a 1:50 dye/bp ratio with YOYO-1. The fluorescence intensity was measured in the presence (black) or absence (red) of the enzyme cofactor, Mg$^{2+}$. The dotted line for the intensity profile in the presence of Mg$^{2+}$ indicates the time at which the cofactor was infused into the IMER.
To ascertain that the reduction in fluorescence intensity was a result of digestion and not photobleaching or photonicking, control experiments were performed by exposing an enzyme/DNA complex to the excitation light in the absence of Mg$^{2+}$. Under these conditions, minimal amounts of fluorescence were lost during the time course of the experiment (60 s).

When the reaction was fortified with Mg$^{2+}$ ions, there was an observed decrease in the bulk fluorescence of the λ-DNA/λ-Exo complex (Figure 2.7). There were two regions in the Mg$^{2+}$ fortified enzymatic reaction where the fluorescence intensity remained relatively constant for a short period of time indicating that the digestion paused. According to previous work, pauses are likely sequence-dependent; λ-Exo has the propensity to pause in regions with GGCGATTCT sequences, which includes GGCGA 5-bp motif. This study also suggested that sequences associated with pausing could also be contained within a GGCGATTCT domain. Upon examination of the sequence of λ-DNA, it was determined that two regions within its sequence contained the first 7 of the 9 bases within the GGCGATTCT motif at 37,701 bp and 43,372 bp positions. This is consistent with the pauses we observed in the fluorescence intensity profile shown in Figure 2.7. The fluorescence intensity was monitored until the signal strength became indistinguishable from the background.

Fluorescence measurements were used to determine the size of the smallest detectable dsDNA fragment stained using a 50:1 base-pair to dye ratio. From the calibration plot, the smallest detectable fragment was 4.6 kbp (see Figure 2.8). The average digestion rate was determined based on the total number of base-pairs for λ-DNA (48,502 bp) minus the size of the smallest detectable fragment (4.6 kbp) and the
time required for the fluorescence to reach baseline. This calculation yielded a digestion rate of \(1.0 \times 10^3 \pm 100\) nt/s \((n = 4)\), a value similar to that reported by Kang et al. for electrostatically immobilized dsDNA.\(^{43}\)

![Figure 2.8](#)

Figure 2.8 Fluorescence intensity as a function of DNA length (bp) to determine the smallest observable DNA fragment using a 1:50 dye/bp stained standards (staining dye was YOYO-1). The standards consisted of lambda and T4 duplexed DNA. DNA fragments as small as 4.6 kbp could be detected based on the linear plot shown (95% confidence interval; \(R^2\) value of 0.9973). The fluorescence was measured using the Zeiss inverted microscope fitted with an EMCCD camera.

We also estimated the degree of processivity from a single digestion event (see Figure 2.7) and the shortest fragment we could observe using fluorescence (4.6 kbp, Figure 2.8). As seen in Figure 2.7, a single \(\lambda\)-DNA molecule engaged with the immobilized \(\lambda\)-exonuclease resulted in a complete loss of fluorescence indicating that the remaining fragment was <4.6 kbp in length; the apparent processivity based on this analysis would be >40 kbp.
This number is in close agreement to that seen by the CE-LIF data (see Figure 2.6). Our value was approximately 10-fold higher than previous reports for free solution digestions using λ-Exo.\textsuperscript{38,43}

The higher apparent processivity of the solid-phase reactor relative to the free-solution case could be attributed to increased stability of the enzyme when anchored to the support. Previous reports have shown that enzyme attachment may prevent the dissociation of λ-Exo into its monomer units during a digestion event that terminates the enzymatic reaction and thus, limits its processivity.\textsuperscript{56} In addition, the processivity observed could be associated with a dependence on dsDNA substrate length;\textsuperscript{34} as the dsDNA length increases from 0.5 to 23 kbp, it is less likely the enzyme will dissociate from the DNA.\textsuperscript{34} We note that the processivity reported here was labeled as apparent because of the indirect evidence used to secure this value. As discussed above, the CE-LIF data did not account for re-engagement of a partially digested λ-DNA molecule. In addition, the single-molecule fluorescence observations could not account for digestion at both ends of a single dsDNA molecule.

\textbf{2.3.6 Kinetic description of immobilized λ-Exo}

Attempts to correlate the kinetic behavior observed for IMER digestions to the classical Michaelis-Menten (MM) model or the Lilly-Hornby model for packed solid-phase reactors have been unsuccessful,\textsuperscript{60-61} due to limitations associated with the MM model that assumes free diffusion and thermodynamically driven collision of enzyme/substrate. This is not the case for IMERs in which molecular mobility of the enzyme is restricted due to immobilization. Also, the model for continuous-flow enzymatic reactors, when the Lilly-Hornby model was applied, was determined to be
insufficient due to the strong reaction dependence on flow rate of substrate through the reactor.\textsuperscript{61} Alternatively, the IMER reactions can be described by fractal-like MM kinetics.\textsuperscript{62-64} Furthermore, enzymatic reactions involving polymerized substrates like DNA can exhibit characteristics of fractal/MM kinetic behavior.\textsuperscript{65}

To explain the kinetic behavior of our system, the fractal MM model proposed by Xu was used, which modifies the classical MM approach by incorporating a fractal contribution offering a more detailed explanation of MM like behavior.\textsuperscript{65} From this formalism, a fractal dimension, $f$, in the rate coefficient was used to account for the fractal behavior observed using enzyme concentration $[E]_{a,b}$, and time $t_{a,b}$, where $a$ and $b$ denote two different concentrations and times as seen from equation 1:

$$\frac{\log [E]_{a}}{\log [E]_{b}} = 1 - f$$

For a solubilized enzyme acting on a polymer substrate processively, the reaction may be considered as 1-dimensional with $f = 0.5$. For an immobilized enzyme acting on a soluble substrate, the reaction can be considered 2-dimensional with $f \sim 0.3$. The system depicted in our work involves both cases and was determined to have a theoretical $f$ value of $\sim 0.7$ based on equation (1) and values obtained from experimental data. We then plotted our experimental data at various $\lambda$-DNA concentrations as a Lineweaver-Burke (double reciprocal) plot using the fractal formalism by Xu et al. shown in equation 2:

$$\frac{1}{\nu} = \frac{K_{m}'}{k'_{2't-\tilde{f}[E]}} \frac{1}{[S]} + \frac{1}{k_{2't-\tilde{f}[E]}}$$

where $\nu$ is the initial rate of the reaction and its corresponding change (difference in concentration divided by reaction time), $[E]$ and $[S]$ are the enzyme and substrate
concentrations, respectively, where [E] was determined from the 660 nm colorimetric assay for protein quantification as previously mentioned, \( t \) is reaction time, \( k'_2 \) is the enzyme turnover rate, and \( K'_m \) is the Michaelis constant; the primes indicate modified Michaelis’ constants based on the fractal behavior of our system (see equation 2). As can be seen in Figure 2.9, the double reciprocal plot for immobilized \( \lambda \)-Exo digestion of dsDNA was linear and yielded \( k'_2 = 5.24 \text{ s}^{-1} \). This turnover rate was found by taking the reciprocal of the intercept from the double reciprocal plot depicted in Figure 2.9 and incorporating the enzyme concentration, fractal number, and reaction time as noted in equation 2.

![Double Reciprocal plot](image)

Figure 2.9 Double Reciprocal plot depicting fractal-like Michaelis-Menten kinetics of \( \lambda \)-Exo based on equation (2). Experiment parameters were: \([S] = 1.5, 1.8, 2.1, 5, 3, \text{ or} 6.6 \times 10^{-6} \text{ mM}; [E] = 4.96 \times 10^{-4} \text{ mM}; f = 0.7; K_m = 4.8 \times 10^{-6} \text{ mM}; \text{ and } k_2 = 5.24 \text{ s}^{-1}. \) These values were determined by extrapolation of \( 1/v = (K_m/V_{max})/[S]+1/V_{max}, \) where the initial rate was calculated by the difference in concentration as a measure of the fluorescence of the remaining dsDNA divided by the reaction time (60 s).
Taking the reciprocal of the intercept and multiplying it by the slope of the line from Figure 2.9 yielded $K'_m$, which was found to be $4.8 \times 10^{-6}$ mM. According to Berg et al., enzymes that have upper limit catalytic efficiencies, $k'_2/K'_m$, on the order of $10^8$ to $10^9$ M$^{-1}$ s$^{-1}$ are said to have attained kinetic perfection, which means that the reaction they catalyze occurs as quickly as the reactants diffuse to the enzyme. From Figure 2.9, the catalytic efficiency for our system was determined to be $1.1 \times 10^9$ M$^{-1}$s$^{-1}$. According to Berg et al, this suggests that the catalysis is restricted only by the rate at which the enzyme encounters substrate in the system. The efficiency of our solid-phase reactor as denoted by $k'_2/K'_m$ when compared to a homogenous digestion ($0.9 \times 10^9$ M$^{-1}$s$^{-1}$), indicated a 17% increase in catalytic efficiency for duplex disassembly into individual mononucleotides for the IMERs.

2.4 Conclusion
In this study, we demonstrated for the first time, to the best of our knowledge, the attachment of a processive exonuclease (λ-Exo) to a PMMA solid phase reactor configured in a microfluidic device (IMERs). The covalent attachment to a photo-activated PMMA support was accomplished using EDC/NHS coupling chemistry, which utilized carboxylic acid groups that were generated by UV-activation of the PMMA surface. The data presented in this work suggested that λ-Exo, when immobilized onto a solid support with controlled carboxylic acid surface density, adopted primarily a single-point attachment configuration with the pore of the enzyme accessible to dsDNA. The IMERs demonstrated increased efficiency as determined by the $k'_2/K'_m$ value when compared to a homogenous digestion of dsDNA. In addition, our IMERs exhibited an increased apparent processivity compared to a homogeneous reaction and displayed fractal-like enzyme kinetics due to the heterogeneous nature of the IMER and the
processive digestion of dsDNAs. The findings secured in this study will provide important information on strategies for immobilizing exonuclease enzymes to solid supports for potential applications in single-molecule DNA sequencing.\textsuperscript{40-41}

2.5 References


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3 Investigation of Hybrid Polymers for Creation of Immobilized Nanofluidic Enzymatic Reactors

3.1 Introduction
Spatially confining biomolecules exists extensively in living systems. Confinement reduces some expanded configurations of the unfolded chain, leading to the equilibrium between the unfolded state and native state.\(^1\)\(^-\)\(^4\) Enzymes take part in various biological reactions in living systems and perform their functions in crowded cellular environments and/or confined spaces, including the chaperonin cavity, the proteosome, interiors of ribosomes, and translocation. Very high concentrations of macromolecules may take up as much as 30% of the intracellular space.\(^5\) This crowding and the resultant confinement may substantially manipulate the space available for enzyme reactions, and affect the behavior of enzyme molecules. Experimental and theoretical studies have been performed to mimic the intracellular environment by adding crowding agents, such as Ficoll, dextran, and polyethylene glycol (PEG), into the buffer solution of enzymes. The presence of these crowding agents, which do not participate directly in a particular biological process, can decrease the amount of free space and the volume of solvent available to the protein and thus, can significantly affect the dynamics and conformation of the enzyme. It has been demonstrated that molecular crowding could stabilize the folded (native) state of an enzyme, restrain the dissociation of multiple enzymes, increase the effective concentrations of the enzyme molecules, and modulate the affinity between the enzyme and the substrate while inducing alterations in the enzyme structure.\(^6\)\(^-\)\(^11\) Therefore, the investigation of enzyme reaction kinetics in confined space mimicking the conditions in living systems is of great significance. Up to now, various reports on enzyme reactions have been performed in dilute solutions containing only
one or just a few different types of biomolecules in vitro, which are different from the crowded environments under in vivo conditions.

Enzymatic assays occurring in confined spaces can be classified as heterogeneous or homogeneous reaction systems. In a heterogeneous reaction system, enzymes are usually immobilized onto nanostructured materials\textsuperscript{12-18} or walls of microchannels\textsuperscript{19-22} as well as, single or arrays of nanochannels.\textsuperscript{23-25} For microchannel and nanochannel reactors, enzymes can be immobilized onto channel walls allowing the enzymatic reactions to occur in a continuous flow format. As nanobioreactors, immobilized nanofluidic enzymatic reactors or (NERs), can render biomolecules more mechanically robust, thermally stable, and more easily separable from the reaction media. Furthermore, NERs formed from nanochannel technologies can be used to independently analyze small reaction volumes, which will extend biochemical reactions to a level of rapid, parallel analysis. However, the application of an immobilized enzymatic reactor in nano-scale dimensions still faces challenges. The largest challenge faced in NER systems is the limited reaction rate, which is a result of slow diffusion of the substrate to the enzymes within the nano-scale dimensions.\textsuperscript{26-27} The most common approach to address this challenge is to induce a driving force in the system that will impel solution through the nanochannels via hydrodynamic or electroosmotic force.\textsuperscript{28-29} This action will result in accelerating the mass transport within these confined spaces and subsequently increase the formation of enzyme-substrate complexes.\textsuperscript{25}

Several studies have demonstrated the use of \(\lambda\)-Exonuclease (\(\lambda\)-Exo) enzyme, in free-solution\textsuperscript{30-36} for the processive degradation of double-stranded DNA into single-
stranded DNA and mononucleotide products. This homogenous method, while suitable for assays where product purity is non-essential, limits the biosensor applicability of this enzyme due to detection inference. However, when immobilized, the desired enzymes have been proven to reduce interference during the analysis phase of the assay. To demonstrate a heterogeneous analysis, a report by Perkins et al., showed the successful immobilization of a single λ-Exo apoenzyme/dsDNA complex onto a quartz substrate. In recent work, we demonstrated the attachment of λ-exonuclease onto a PMMA solid phase reactor configured in a microfluidic device (IMER). The results of this work demonstrated the unique capabilities of λ-Exo and its immobilization onto micro-scale solid supports, which can serve as a useful tool for biosensors directed towards the sequence analysis of DNAs. To realize the potential of DNA sequence analysis using immobilized exonuclease enzymes, it is critical to understand the fundamentals of the immobilization process within nano-confined environments and the effects of immobilization on the enzyme kinetics when poised within a reactor of nano-scale dimensions.

In this work, we utilized polymers for the development of a hybrid nano-scale reactor for the immobilization of λ-Exo enzyme for dsDNA digestion. λ-Exo was immobilized onto a PMMA device consisting of an array of nanopillars populating a bioreactor. Polymer substrates were characterized to ensure proper carboxylate functionalization and device bonding, critical components of nano-scale device fabrication. Device geometries were investigated for increased enzyme load and ease of DNA introduction into the reactor through electrokinetic forces. The immobilization was accomplished using 3-(3-dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide (EDC/NHS).
coupling chemistry via UV-generated carboxylic acids on the PMMA surface. Dye conjugation and UV spectral analysis confirmed that the enzyme was covalently attached to the polymer surface. Bonding of these nanofluidic reactors took place post-enzyme attachment, so temperature based analyses on the enzyme activity were carried out. Fluorescence microscopy studies of YOYO-1 stained dsDNA allowed for real-time observation of the nano-scaled digestion. Simulated kinetic analyses provided insight on the mass transport effects at nano-scale dimensions.

3.1.1 Nanoconfinement Effects on Enzymes

3.1.1.1 Activity differences observed through kinetic behavior in the nanoscale

Several groups have reported improved catalytic activities for enzymes confined in nanostructures. In these findings, researchers attributed improvements in catalytic activity to the confinement of the enzyme in the nano-scale environments. These studies observed increased immobilization efficiency, higher enzyme loading, and increased enzyme stability at nano-lengths as opposed to bulk measurements. Conversely, a recent work by Wang et. al. observed that confinement effects are dependent on the size of the nano-space where the enzyme reaction occurred. In their study using Glucose Oxidase (GOx), they observed that as the confinement space decreased from 140 to 80 nm the GOx activity decreased while the reaction rate was accelerated. They attributed this observed decrease in GOx activity to the possible change of enzyme conformation and steric hindrance of the catalytic site in nanochannels.

3.1.2 Benefits of Nano-Scaled Enzymatic Reactions

The benefits of using nano-scale structures for immobilization are the reduced diffusional distances and the maximized functional surface area for enzyme loading. In
general, nanostructures for enzyme immobilization can improve enzymatic efficiency because of the reduced system dimensions. For nanoparticles, Jia et al. observed that surface attachment to smaller particles provided larger surface areas for enzyme attachment, thus providing higher enzyme load per unit mass of particles. A recent review by Ansari et al. noted that nanoparticles (and other nano-structures) can strongly influence the mechanical properties of a biomolecule like stiffness and elasticity and provide biocompatible environments for enzyme immobilization. For these reasons, immobilization of enzymes on/in nanostructures has great potential in practical applications such as biosensors, bioremediation, and bioconversions.

3.2 Materials and Methods

3.2.1 NERs fabrication

The immobilized nanofluidic enzymatic reactor (NER) used in this study consisted of two 98 µm x 1.16 µm reactors populated with a linear array of nanopillars each 130 nm tall and 900 nm in diameter. Each reactor utilized PMMA sheets (T_g ~104°C), which were purchased from Good Fellow (Berwyn, PA). Cycloolefin copolymer sheets, COC 6017 (T_g ~ 170°C) were used as the back plate for fabricating the nanoimprinting stamp and COC 8007 (T_g ~ 75°C) were used as the cover plate, both of which were purchased from TOPAS Advanced Polymers (Florence KY). Si <100> wafers were purchased from University Wafers (Boston, MA). Anti-adhesion monolayer of (Tridecafluoro – 1,1,2,2 – Tetrahydrooctyl) Tricholorosilane (T-Silane) was purchased from Gelest, Inc. Fluorescein Isothiocyanate (FITC) salt and 10X Tris Borate EDTA (TBE) buffer were purchased from Sigma-Aldrich (Saint Louis, MO). All required dilutions were performed using 18 MΩ/cm milliQ water (Millipore technologies) and all measurements were performed at 25°C unless specified otherwise.
Figure 3.1 Process scheme for the fabrication and assembly of the thermoplastic-based nanofluidic devices. (A) Fabrication of the Si master, which consisted of micron-scale access channels and the nanochannels; (B-C) Fabrication of the protrusive polymer stamp in a UV-curable resin from the Si master; Generation of the fluidic structures in the thermoplastic substrate from the UV-curable resin stamp by thermal imprinting and (D) bonding of the substrate with the low T_g cover plate to build the enclosed mixed-scale thermoplastic fluidic device.

Previously, we have reported the development of nanoslits and nanochannels in polymer substrates following a single step fabrication scheme that is based on nanoimprint lithography (NIL) \(^{44-45}\). Figure 3.1 shows the steps involved in the development of the nanofluidic devices. Device fabrication involves four key steps: (i) Fabrication of a Si master with nanochannels using FIB lithography; (ii) fabrication of the UV-resin stamp with COC back plate; (iii) thermal imprinting into a thermoplastic substrate; and (iv) sealing of the fluidic channels with a low T_g cover plate. The Si master was developed by initially patterning two V-shaped access microfluidic channels, 55 µm wide, 12 µm deep 1.5 cm long into the Si <100> wafer using standard
photolithography followed by anisotropic etching with 50% KOH. Next, nanofluidic channels were patterned across the microchannels by Focused-Ion Beam (FIB) milling using a Helios NanoLab 600 Dual Beam instrument (FEI Company). SEM images of the Si master for the device in this study are shown in Figure 3.2. This design featured an entrance taper at the microchannel/nanochannel interface to facilitate the entry of dsDNAs when electric fields were applied. This NERs device contained an array of pillars, symmetrically aligned that were 900 nm in diameter with a height of 130 nm. Spacing between the pillars and sidewalls is also 130 nm. The sidewall spacing were the basis for the mass transport kinetics simulated due to the complex NER design.

Figure 3.2 Immobilized nanofluidic enzymatic reactor (NER) design where a) design schematic of device nanochannel/microchannel interface that includes a taper for facile entry of dsDNAs b) SEM of design master in Si with discrete pillar formations illustrated in inset c). FITC sealing test of the design in a polymer substrate to ensure fluidic channels are not hindered by structural deformities due to master/stamp fabrication or issues with demolding.

The FIB spot size (beam current), sputtering rate and dwell time were carefully controlled to ensure that the desired channel dimensions were designed. Following this, an anti-adhesion monolayer of T-Silane was coated on the Si master from the gas
phase in a desiccator under vacuum for 2 h to facilitate the demolding process. The structures on the Si master were then carefully transferred into a UV-curable resin polymeric blend, containing 68 wt% TPGA as the base, 28 wt% TMPA as the crosslinking agent and 4 wt% Irgacure 651 as photo-initiator) coated onto a cyclic olefin copolymer (COC) back plate via UV-NIL to produce polymer stamps with protrusive structures. To achieve this, the Si master (mold) was initially coated with the UV resin by dispensing with a pipette, followed by gentle pressing of the COC back plate on the resin-coated master to ensure complete filling of the resin into mold cavities. This was followed by exposure to a 365 nm UV light (10 J/m²) through the COC back plate for 5 min in a CL-100 Ultraviolet crosslinker. After curing, the UV-curable resin was gently demolded from the Si master to get the negative copy on the UV-curable resin. Next, the patterned UV-curable resin was used as the stamp to hot emboss into a 3 mm-thick PMMA sheet (Lucite CP) (2 cm x 2 cm) with access holes for reservoirs drilled prior to embossing. The imprinting was performed at a pressure of 1910 kN/m² for 120 s with the top and bottom plates maintained at a temperature of 125°C using the Hex03 hot embosser (JenOptik). Pressure was applied after 30 s preheating of the stamp and the substrate at the desired molding temperature and was maintained during the imprinting process until the system was cooled to 45°C. Upon cooling, the PMMA replica was easily demolded from the UV-resin stamp. A 120 µm thick COC 8007 sheet was used as the cover plate. Both the patterned PMMA sheet and cover plate were pre-activated with oxygen plasma at 50 W for 35 s and 7 sccm gas flow rate. Device assembly was performed immediately at 70°C for 900 s under a 680 kN/m² pressure. Device fidelity was determined based on FITC sealing tests as shown in Figure 3.2d.
3.2.1.1 Activation of polymer surfaces for functionalization and bonding

Initial challenges with this study resulted from PMMA’s lack of ultra violet transparency. This substrate, while highly biocompatible and extensively characterized for microfluidic and nanofluidic applications,\textsuperscript{46-49} the limited transmissivity of 254 nm light prevented carboxylate functionalization of devices assembled prior to enzyme attachment. In addition, PMMA nano-scale devices when assembled in the absence of oxygen plasma or UV irradiation to lower the glass transition temperature $T_g$, are prone to collapse because of high temperatures necessary for device enclosure.\textsuperscript{44} These facts led to the investigation of low $T_g$ (75°C) COC 8007 materials that would facilitate the transmission of UV light through thin cover plates of assembled devices and prevent structural deformation during enclosure.

Utilization of COC 8007 devices satisfied the UV transmission parameters, but could not be assembled in the absence of oxygen plasma modification. Also, bond strength for COC-COC based devices, while more robust than PMMA-PMMA nano-devices, occasionally experienced cover plate detachment. Therefore, we developed a PMMA-COC low temperature hybrid-bonding scheme useful for the assembly of thermoplastic-based nanoslits and nanochannels. With this scheme, we addressed the most notable challenge associated with the use of thermoplastic substrate materials for the fabrication of nanofluidic devices – the relatively small Young's modulus associated with these materials, which makes cover plate assembly to the patterned substrate difficult due to cover plate collapse and/or nanostructure deformation using either thermal or chemical assembly to enclose the fluidic network. We found that this approach not only aided in achieving low temperature device assembly with high tensile strength, but also
contributed to effective functionalization of the nanochannel surface with carboxyl (hydrophilic) functional groups, which proved necessary in subsequent experiments.

3.2.1.2 NER substrate characterization

To investigate the UV transmission and carboxylate group formation on the COC 8007 surface, water contact angle (WCA) measurements and X-ray photoelectron spectroscopy (XPS) were used. Sessile water contact angle results were obtained using a VCA Optima instrument (AST Products). The measurement required depositing 2.0 µL of deionized (DI) water onto the COC 8007 substrate. The left and right contact angles of the water drops were measured immediately after placement on the polymer surface, followed by collecting the image and measuring the contact angle using the manufacturer's software. Each value reported was the average of a minimum of three measurements secured at separate positions on any given substrate.

For XPS measurements, C1s and O1s photoelectron signals were acquired using an Axis Ultra DLD X-ray photoelectron spectrometer (Kratos Analytical) under ultra-high vacuum conditions (10⁻⁸ to 10⁻¹⁰ Torr) with a monochromatic Al Kα X-ray source, 20 eV pass energy, 93 s acquisition time, 400 ms dwell time, and 20° electron take-off angle. Given an inelastic mean free path of 3 – 4 nm, 95% of the resultant signal originated 9 – 12 nm from the surface.⁴⁸

3.2.2 Enzyme immobilization onto nanofluidic enzymatic reactors (NERs)

The NERs were created via covalent attachment of λ-Exo onto photo-activated PMMA substrates as previously described.⁴⁹ λ-Exo was provided with a 10X reaction buffer (670 mM glycine-KOH, pH 9.4, 25 mM MgCl₂, 0.1% (v/v) Triton X-100), which was purchased from Fermentas Life Sciences (Glen Burnie, MD). When necessary, enzyme purification steps were performed prior to use to remove enzyme storage buffer.
Prior to thermal fusion bonding of the COC 8007 cover plate to the substrate, carboxylic acid moieties were generated in the NERs through a UV dose of 20.76 mW/cm$^2$ for 15 min.

![Figure 3.3 Schematic showing the functionalization of the NERs used for λ-Exo digestion of double-stranded DNA. The reactor bed was populated with nanopillars that were 900 nm in diameter. a) The NERs was made from the thermoplastic, PMMA, via nano-imprint lithography (NIL). b-d) Schematic showing the immobilization of λ-Exo onto a PMMA substrate. The substrate was activated by UV radiation to generate surface confined carboxylic acids. This was followed by EDC/NHS coupling to covalently attach the enzyme to the substrate during an incubation period, which was carried out overnight at 4°C) inset illustrating NER modified with enzyme; f) actual NER device.](image)

Enzyme functionalization was carried out by exposing the NER bed to a solution containing 200 mM 3-(3-dimethylaminopropyl) carbodiimide (EDC), and 50 mM N-hydroxysuccinimide (NHS) in 0.1 M 2-(4-morpholino)-ethane sulfonic acid at pH 5.1 (MES, Fisher Biotech, Fair Lawn, NJ) for 15 min at room temperature, where succinimidyl ester intermediates were formed to facilitate enzyme attachment. λ-Exo enzyme (0.03 µg/µL) was allowed to react overnight at 4°C in the presence of water to hydrate the reaction chamber and prevent reagent evaporation.
The enzyme-functionalized surface was then gently rinsed with ultrapure 18 MΩ/cm milliQ water and dried at 60°C for 5 min prior to bonding. The schematic for this reaction is depicted in Figure 3.3.

3.2.2.1 Characterization of PMMA/λ-Exo surfaces

To determine if λ-Exo was covalently attached to the activated PMMA surface of the NER’s reactor bed, we performed spectrophotometric analysis of PMMA at each modification step. Absorption of chemical species was measured using an Ultrospec 4000 UV/Vis spectrophotometer (Pharmacia Biotech). Spectral scans between 200-800 nm were collected for PMMA substrates activated with UV light, modified with EDC/NHS, and λ-Exo enzyme. Absorbance values occurring between the spectral range of 260-280 nm were overlaid to assess surface changes at each modification step. Immobilization parameters were repeated using the nanoreactor and immobilization was verified by dye-labeling of the primary amine groups present in λ-Exo using AlexaFluor 488 TFP ester dye (Life Technologies, Grand Island, NY) in 25-fold excess of enzyme. Epifluorescence was monitored using Zeiss Axiovert 200M inverted microscope (100 W HBO lamp, Zeiss, Germany) fitted with a 100x/1.3 NA oil-immersion microscope objective and an Andor iXon3 EMCCD camera. A custom mount was machined to hold the assembled NERs onto the microscope stage. All images were acquired using MetaMorph Advanced 7.7.6.0 Software (Molecular Devices LLC, Sunnyvale, CA).

3.2.3 Temperature dependence on enzyme digestion of dsDNA

Effect of temperature on enzyme activity was assessed using PicoGreen intercalating dye (Life Technologies, Grand Island, NY) to determine the amount of intact dsDNA remaining after exposure to immobilized λ-Exo. Enzyme functionalized substrates were exposed to room temperature at 25°C or 80°C for 15 min prior to
introduction of dsDNA for subsequent digestion at 37°C. The DNA staining dye was added post-digestion to avoid perturbation in enzymatic activity of λ-Exo that may result from nuclear staining. The dye-labeled samples were excited at 480 nm and fluorescence spectra (490 – 700 nm) were collected and analyzed using a Fluorolog-3 spectrofluorimeter (Horiba JobinYvon, Edison, NJ) and DataMax Software 2.20.

3.2.4 Real-time nano-scale digestion analysis using fluorescence microscopy

Duplex λ-DNA (48,502 bp), purchased from New England Biolabs (Ipswich, MA), was incubated in the enzyme-modified NERs at a constant voltage of 0.65 V. The reaction times were determined by conjugation of λ-DNA with enzyme upon introduction to the NERs. An experimental control, which involved the introduction of a solution containing λ-DNA into the NER bed in the absence of immobilized enzyme, was performed. The control revealed that there was neither a loss nor breakage of the dsDNA from non-specific adsorption onto the reactor wall or shearing, respectively. Nano-scale enzymatic reactions were temperature controlled at 37ºC via a custom-built thermocouple heating stage. However, due to reaction scale product quantification was not performed.

The microscope used in this study was a Zeiss Axiovert 200M inverted microscope (100 W HBO lamp, Zeiss, Germany) fitted with a 100x/1.3 NA oil-immersion microscope objective and an Andor iXon3 EMCCD camera (30-50 fps acquisition rate). A custom mount was machined to hold the assembled NERs onto the microscope stage. All images were acquired using MetaMorph Advanced 7.7.6.0 Software (Molecular Devices LLC, Sunnyvale, CA) and analyzed using ImageJ (National Institutes of Health, Bethesda, MD).
Hybrid NERs were modified as previously described. Following enzyme attachment and device enclosure, λ-DNA stained with YOYO-1 in a 1:50 dye-to-base pair ratio was introduced into the reactor in enzyme reaction buffer fortified with Mg$^{2+}$ for immediate DNA digestion. NERs system was heated to 37ºC with real-time monitoring of the digestion process. Buffer reagents were introduced into the NER through capillarity and applied electric field (50 V/cm).

3.3 Results and Discussion

3.3.1 Hybrid polymer characterization

Hydrophobicity changes in COC 8007 as a result of UV irradiation and oxygen plasma exposure were assessed via sessile water contact angle (WCA) measurements (see Table 3.1). The water contact angle for native COC 8007 substrate was 78.68 ±2.31°. Because COC is composed entirely of saturated hydrocarbons, this polymer is more hydrophobic than PMMA, which contains ester moieties producing a lower water contact angle compared to COC 8007. Upon UV irradiation, activation of the surfaces was apparent as the wettability increased; water contact angles decreased to 49.73 ±1.28° after UV-irradiation and 62.53 ± 2.20° for oxygen plasma exposure. When a COC cover plate was placed on top of the 8007 substrate and irradiated with UV, the contact angle decreased to 66.38 ±0.68°. This data suggested that UV irradiation through the cover plate could effectively activate the COC substrate, but the degree of the activation was reduced in comparison to UV modification without the cover plate.

Also, we observed that oxygen plasma treated COC 8007 (62.53 ± 2.20°) experienced comparable changes in hydrophobicity as compared to UV irradiation with the COC cover plate. Further analysis of cover plate influence on wettability of COC 8007 revealed that changes in cover plate grade (i.e., norbornene content), changed the
contact angle, yet no linear correlation with % norbornene and the degree of wettability were observed. These studies have initiated another ongoing study concerning the surface charge density when our hybrid devices are irradiated through the cover plate.

Table 3.1 Sessile water contact angles (WCAs) for COC 8007 for native state and UV and oxygen plasma exposure (top); water contact angles for COC 8007 when exposed to UV through various polymer cover plates.

<table>
<thead>
<tr>
<th>COC Grade</th>
<th>T_g (°C)</th>
<th>Norbornene Content (%)</th>
<th>Pristine WCA</th>
<th>Oxygen plasma treatment WCA</th>
<th>UV treatment WCA</th>
<th>UV treatment (through cover plate) WCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>8007</td>
<td>75</td>
<td>65</td>
<td>78.68±2.31</td>
<td>62.53±2.20</td>
<td>49.73±1.28</td>
<td>66.38±0.68</td>
</tr>
</tbody>
</table>

To determine the degree of carboxylic acid formation in COC 8007, X-ray photoelectron spectroscopy (XPS) was used. XPS spectra for pristine, UV and oxygen plasma modified COC 8007 are seen in Figure 3.4a. As can be observed, O1s peaks for pristine COC are minimally represented as expected for hydrocarbons like COC. The spectra in Figure 3.4a demonstrated that as COC was modified, oxygen-containing groups were generated. To determine which groups were generated, XPS C1s spectra were deconvoluted to calculate the percent of the C1s signal arising from carboxyl groups (see Figure 3.4b). Strong signals from carboxyl containing functionalities were observed for oxygen plasma and UV-activated COC. These results suggest that oxygen plasma and UV activated COC contain comparable carboxylic acid (COOH) surface densities, but in comparison to contact angle results, UV had increased wettability.
Figure 3.4 XPS data of COC 8007; a) relative intensity differences in the C1s and O1s peaks for pristine, oxygen plasma, and UV modified 8007 polymer. Deconvoluted C1s peaks for COC 8007 after pre- and post-surface modification corresponding to the presence of COOH are shown in b).

To confirm that there were no differences in -COOH generation, we observed the atomic concentration for C1s and O1s peaks for both activation conditions as shown in Table 3.2. Our results indicated that oxygen plasma and UV irradiation generated comparable carbon and oxygen concentrations; therefore, changes in wettability may not be due to atomic compositional differences. This observation could be due to surface roughness differences as a result of oxygen plasma treatment as opposed to UV-activation, which generates lower RMS surface roughness compared to plasma activation.

Table 3.2 XPS C1s and O1s peak atomic concentrations for 8007 after oxygen plasma treatment and UV exposure with and without a coverplate

<table>
<thead>
<tr>
<th>Grade</th>
<th>Atomic concentration (%)</th>
<th>O/C ratio</th>
<th>% increment*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C1s</td>
<td>O1s</td>
<td></td>
</tr>
<tr>
<td>8007 (75°C, 65%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Pristine</td>
<td>93.61</td>
<td>6.39</td>
<td>0.0682 ± 0.0052</td>
</tr>
<tr>
<td>2 Oxygen plasma treatment</td>
<td>80.21</td>
<td>19.79</td>
<td>0.2469 ± 0.0168</td>
</tr>
<tr>
<td>3 UV treatment</td>
<td>80.24</td>
<td>19.76</td>
<td>0.2474 ± 0.0460</td>
</tr>
<tr>
<td>4 UV treatment (through cover plate)</td>
<td>85.78</td>
<td>14.22</td>
<td>0.1668 ± 0.0422</td>
</tr>
</tbody>
</table>
3.3.2 Enzyme attachment and analysis

To determine if λ-Exo was covalently attached to the activated PMMA surface of the NER, we performed spectrophotometric analysis. In this investigation, scans of UV-activated, EDC/NHS coupled, and enzyme modified PMMA were taken (see Figure 3.5a). The differences in the absorbance spectra at each step were used as an indicator of the degree of conjugation at each modification step.

Successful attachment of λ-Exo onto PMMA was confirmed by labeling the remaining primary amines within the protein post-immobilization. From the epifluorescence images in Figure 3.5c-d for the activated-PMMA reactor in the absence of the EDC/NHS coupling reagents and enzyme, there was no indication of the presence of fluorescence. This confirmed that the dye-labeling agent used was specific to the primary amine groups in the enzyme and not conjugated to the activated polymer surface (see Figure 3.5d).

![Figure 3.5 Immobilization of λ-Exo](image)

Figure 3.5 Immobilization of λ-Exo where a) absorbance spectra are overlaid to confirm modification at each step; b) SEM image of a nano-pillared Si master with <200 nm pillar spacing, c) fluorescence image of nano-pillared device in PMMA that had been incubated with AlexaFluor 488 dye in the absence of enzyme and d) in the presence of enzyme to verify covalent enzyme attachment onto the nano-pillars.
Temperature influence on enzyme activity

We were interested in determining the temperature stability of λ-Exo following immobilization to the PMMA surface. Table 3.3 shows the percent digestion of λ-DNA when exposed to immobilized λ-Exo after incubation at 25°C and 80°C for 15 min. The control for this experiment consisted of incubating the NER PMMA at 80°C in the absence of enzyme for 15 min followed by incubation with 50 µg/mL λ-DNA at 37°C for 5 min. After each digestion, the NER effluent was measured to determine if any loss of dsDNA resulted from digestion. To determine the extent of DNA digestion, PicoGreen was added to the effluent. The amount of dsDNA remaining after digestion was monitored using fluorescence microscopy. The fluorescence intensities were converted to DNA concentrations (ng/mL) using a linear calibration plot for λ-DNA.

<table>
<thead>
<tr>
<th>Experimental Parameter</th>
<th>Enzyme Temperature (°C)</th>
<th>Reaction Temperature (°C)</th>
<th>Remaining dsDNA (ng/mL)</th>
<th>Percent Digestion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dsDNA Stock</td>
<td>n/a</td>
<td>37</td>
<td>1067.3 ± 339.0</td>
<td>-</td>
</tr>
<tr>
<td>PMMA Substrate No Enzyme</td>
<td>80*</td>
<td>37</td>
<td>1020.5 ± 306.3</td>
<td>-</td>
</tr>
<tr>
<td>PMMA w/ Enzyme</td>
<td>80</td>
<td>37</td>
<td>672.2 ± 8.6</td>
<td>62.98</td>
</tr>
<tr>
<td>PMMA w/ Enzyme</td>
<td>25</td>
<td>37</td>
<td>632.2 ± 56.2</td>
<td>59.23</td>
</tr>
</tbody>
</table>

As can be seen from Table 3.3, the amount of λ-DNA remaining in the effluent showed that λ-Exo, even after exposure to 80°C, yielded comparable digestion efficiency as to that of the reaction performed at room temperature (25°C). This indicated that λ-Exo activity was maintained at 80°C, conditions that typically
deactivated the free-solution form of λ-Exo. This finding proved critical for the subsequent reactions carried out in this work as NER fabrication requires heating the enzyme-functionalized device close to 70°C, which is required for assembly of the NER device. This observation was supported by reports in the literature that suggest immobilization improves enzyme thermostability.\textsuperscript{50-52}

### 3.3.4 Real-time digestion of λ-DNA

Nano-scale digestion of a single dsDNA molecule with an immobilized enzyme was studied in real-time using fluorescence microscopy. λ-Exo was immobilized onto PMMA using EDC/NHS coupling chemistry and a solution of YOYO-1 stained dsDNA in a 1:50 dye to base-pair ratio was introduced into the enzyme reaction buffer (glycine-KOH in ultra-pure water at pH 9.4, 0.1% (v/v) Triton X-100) containing 2.5 mM MgCl\(_2\). Previous work by Kang \textit{et al.} revealed that stained dsDNA with a 1:50 dye-to-base pair ratio has comparable digestion rates to unstained dsDNA in the presence of λ-Exo\textsuperscript{30}. Ultra-dilute (3 fM) λ-DNA concentrations were utilized in this work to make sure that single molecules were monitored.

Under these conditions, DNAs became immobile when they were threaded into the pore of λ-Exo even when electric fields were used to drive DNA into the enzyme-modified regions of the NERs. To ascertain whether the reduction in fluorescence intensity was a result of digestion and not photobleaching or photonicking, control experiments were performed by exposing dye-labeled DNA molecules to the excitation light in the presence of Mg\(^{2+}\) at zero field strength. These control parameters were selected because the co-factor, Mg\(^{2+}\), that is necessary for λ-Exo activity could influence the electrokinetic transport properties of the DNA molecules. To benefit our work, a
recent study on the effects of DNA-binding proteins and enzyme co-factors on the conformation and extension of nanoconfined DNA has been performed.\textsuperscript{53} This work suggested that DNA-binding proteins shorten the DNA when used with dyes like YOYO-1 due to interaction of the dye with the protein. However, they observed no noticeable effect as a result of the presence of Mg\textsuperscript{2+}.

Figure 3.6 Still images containing stained DNA molecule during enzymatic digestion inside the NER device. In these cases, a line plot was secured from a horizontal line that crossed the section in the image. The corresponding fluorescence intensity line plots taken from the still images.

In this study, fluorescence measurements were used to determine the rate of digestion of dsDNA by taking horizontal line plots across still images of the real-time digestion (see Figure 3.6). The digestion rate obtained was determined based on the total number of base-pairs for \(\lambda\)-DNA (48,502 bp) minus the size of the smallest detectable fragment (4.6 kbp) and the time required for the fluorescence to reach baseline. This calculation yielded a digestion rate of \(~303\ \text{nt/s},\) a value nearly 3-fold lower in comparison to our microreactor studies (\(~1000\ \text{nt/s}\)).\textsuperscript{39}
The digestion rate for this enzyme is highly variable in the literature,\textsuperscript{30, 33, 35} which may account for the observed differences. Potential reasons for the activity differences observed here could be due, in part, to changes in reaction buffer pH when electric fields are applied.\textsuperscript{54} These buffer changes can alter the enzymatic activity even when immobilized because the reactivity of functional residues within the enzyme is strongly influenced by pH.\textsuperscript{55} In general, enzyme reaction rates are higher at more alkaline pH (\textlambda-Exo pH = 9.4). Therefore, lowering the pH below the pKa of accessible amino groups will decrease the enzyme reaction rate, as protonated amino groups can be regarded as unreactive.\textsuperscript{55}

3.3.4.1 Effects of the electric field on \textlambda-Exo activity

3.3.4.1.1 Enzyme activity in NER

Currently, limited information is known about the activity of \textlambda-Exo under the influence of electric fields, especially in an immobilized state. The applied electric field may result in a change in pH inside the nanochannel and consequently in enzyme reaction rate. Electrokinetic transport is typically produced from both the electrophoretic mobility ($\mu_{EP}$) of the molecule being driven through the nanochannel or nanoslit and the bulk electroosmotic flow ($\mu_{EOF}$).

This relationship is depicted as:

$$\mu_{App} = \mu_{EP} + \mu_{EOF}$$

where $\mu_{EP}$ is the electrophoretic mobility of the species (DNA), $\mu_{EOF}$ is electroosmotic flow and $\mu_{app}$ is the apparent electrophoretic mobility.

Electroosmotic flows (EOFs) are highly dependent on the surface charge of the material and this is where polymers distinguish themselves from glass or fused silica-based substrates; the surface charge for polymers depends on the identity of the
surface material (PMMA_{EOF} in nanochannels = 1.02 \times 10^{-4} \text{ cm}^2/\text{Vs}). Because the enzyme and often the surface onto which the enzyme is attached carries an electrical charge, an electrical double layer (EDL) extends into the solution adjacent to the surface. The transport of the charged substrate and product species occurs by migration under the influence of the electrical field, as well as by diffusion. The electrical charge on the surface arises from the dissociation of acid and basic groups of the enzyme and surface, and is consequently affected by the pH in the bulk solution.\textsuperscript{56}

Another factor that may affect the local pH of the reaction system and therefore the surface charge on the enzyme is the release of H\textsuperscript{+} ions during the enzymatic reaction. In addition, the local pH determines the intrinsic rate constants of the enzymatic reaction, through dissociation of the active sites that participate in the enzymatic catalysis.\textsuperscript{56} The charge at the active site is as a result of the dissociation equilibria of the acid and basic sites, however, all surface groups including those not participating in the catalytic process contribute to the surface charge.\textsuperscript{56} Nevertheless, transport of the substrate and products is a result of both migration due to electric field and mass transport via diffusion. The latter part of this work will examine theoretical predictions of diffusion in nanoconfined spaces.

3.3.4.1.2 DNA dynamics in NER

The NER design used in this work employed dimensions smaller than its natively coiled state (radius of gyration, R) and approached sizes near the molecule’s persistence length (25-100 nm). Recent reviews by Reisner \textit{et al.} \textsuperscript{57} and Dorfman \textit{et al.} \textsuperscript{58} have characterized the transport dynamics and nano-confinement of DNA and these principles will be revisited for further discussion in Chapter 4 of this document. For our results, we tested the applicability of our NER design by translocating DNA across the
device under constant electric field (50 V/cm). This step was necessary to ascertain the NER functionality—could DNA enter and exit the device and at which field strengths would best facilitate this translocation. Also, this experiment served as a control for the NER device in which no enzyme was immobilized and determine if DNAs under constant field strengths (0.65 V) would become non-mobile. Using an enzyme free nanoreactor, we filled the device with a λ-DNA solution containing 4% β-mercaptoethanol in 0.5X TBE buffer (final concentration = 7.5 pM). We observed DNA that frequently traveled alongside the walls of the nanochannel or frequently became hooked around a single pillar as shown in Figure 3.7a. These observations lead us to investigate the field distribution within this geometry via COMSOL (data not shown). This data revealed that the symmetric array of nanopillars in our design contained field free regions that occurred between the axial lengths of each nanopillar. We studied single molecule dynamics by analyzing translocation frames, and we observed that the DNA can “sit” in these field free regions approximately 400 ±103 ms before extending its free ends around either side of the pillar and eventually translocating along the nanochannel walls to exit. Under constant field strengths, any DNAs that encountered the field free region took this hooked conformation and quickly stretched and relaxed to exit along the channel walls.

This observation proved critical to our real-time digestion study as DNAs that were threaded to λ-Exo, even under constant field strength, did not continue along the channel walls but instead remained immobile. To consider which re-design parameters to potentially integrate into our device, we observed that conformational changes in the DNA are common in electrophoretic separations of DNAs using nanopillars.
Figure 3.7 a) Still fluorescence images of λ-DNA conformation in our NER device with 900 nm pillars with 130 nm spacing. b) Conformation patterns observed by Baba et al.,\textsuperscript{59} demonstrating a well balanced DNA conformation and thus different electrophoretic migration behavior than due to radius of gyration of the DNA molecules.

In work by Baba et al.,\textsuperscript{59} they predicted that there are two patterns (Pattern-1 and Pattern-2, see Figure 3.7b) for the insertion of DNA from the nanopillar region into a nanopillar-free region. Pattern-2 indicated that the distribution of DNA in this configuration has well-balanced conformation because both edges of DNA were simultaneously drawn around the nanopillars into the pillar-free regions. Conversely, pattern-1 indicated that the distribution of DNA was inserted into the nanopillar region without forming a well-balanced conformation. This observation revealed that the residence time of pattern-2 is longer than that of pattern-1. From this study, they were able to observe DNA electrophoretic migration behavior at the single molecule level. This report compared the electrophoretic migration behavior of a single λ-DNA molecule and a single T4 DNA molecule within the nanopillar region. In their observation, the T4
DNA chain frequently got hooked on the nanopillars, where its “extending arm” pulls out the shorter arm, and then relaxes into a more compact conformation. This type of motion is widely observed in conventional gel or polymer matrices like agarose or polyacrylamide $^{60}$. Although the T4 DNA molecule showed periodic conformational changes, λ-DNA molecule tended to keep a compact conformation during electrophoresis even in this serial array pattern where the collisions with pillars are unavoidable. They suggested that these two distinct types of behavior within the array pattern could be understood by considering the radius of gyration of a DNA molecule. When a DNA molecule, which has larger radius of gyration than the nanopillar spacing, enters the nanopillar region, it is forced to deform to adapt its size to the nanopillar spacing. This observation supported the dynamics observed in our NER, as the nanochannel spacing in this design was 130 nm, a width much smaller than the radius of gyration for λ-DNA (~550 nm). Our results suggested that the orientation of DNA around nanopillars can be carefully manipulated based on nanochannel spaces utilized in the design if they occur below the target molecules radius of gyration.$^{61}$

3.3.5 Simulated kinetic description of nano-scale immobilized λ-Exo

Many factors must be taken into account in order to attempt a description of the kinetics of immobilized enzymes. Kinetics in these heterogenous systems are governed by two parameters: 1) The intrinsic enzyme catalysis reaction; and 2) mass transport due to diffusion. In the microenvironment; the immediate vicinity of the immobilized enzyme, the concentrations of the species that influence the intrinsic kinetics differ from those in the bulk solution. These concentration disproportions result from electrostatic and other interactions between the solid-phase surface and the substrate or other species as well as from the presence of diffusional resistances. These diffusional
resistances can be two-fold: a) Diffusional resistances for the external transport of substrate and products between the bulk solution and the external surface of the enzyme-modified surface, which are affected by the hydrodynamics; and b) diffusion resistances for the internal transport of these species within pores. Internal diffusion proceeds in parallel with the chemical reaction, whereas external diffusion occurs in series with the actual reaction step. As a result, theoretical approaches used to analyze the interaction of the enzyme-catalyzed reaction with external and internal diffusion are different. Therefore, the effects of external diffusion on the rate of a reaction catalyzed by an enzyme immobilized on a nonporous solid surface are examined in this work.

Due to the limited reaction volume of the NER device, kinetic assessments via remaining DNA quantification could not be performed. Therefore, simulation of mass transport effects as a function of nanochannel dimensions was performed using a BIASimulation program (BIAcore). In this work, real-time reaction parameters were coupled to the observed influence of mass transport as nanochannel spacing within the design decreased. To obtain the most accurate theoretical values, we took into account the electroosmotic flow (EOF) for PMMA nanochannels which would move in the opposite direction of DNA mobility (1.02 x10^{-4} \text{ cm}^2/\text{Vs}) and this was used to account for flow rate in the system using the equation: \( Q = VA \), where \( Q \) is the volumetric flow rate, \( V \) is linear velocity- in this instance EOF, and \( A \) is cross-sectional area. This volumetric flow rate was calculated for the reaction conditions using cross-sectional areas of each channel (130 nm, 100 nm, and 50 nm). Also, other theoretical parameters that were incorporated include the diffusion coefficient for DNA and the concentration range for
DNA, a range that was based on our real-time analyses (5 pM – 80 pM). The data output and calculations based on these parameters is summarized in Table 3.4.

We found that the mass transfer coefficients (MTCs) became larger as channel dimensions decreased. This suggests that the partition of substrate and product occurs faster as dimensions decrease, as to be expected. We then calculated the Damkohler numbers based on the MTC for each channel width, based on the following equation:

\[
Da = \frac{\text{maximum rate of reaction}}{\text{maximum rate of external diffusion}} = \frac{V_m}{k_L[S_b]}
\]

where \(V_m\)' is the maximum reaction rate (g/m^2s), \(k_L\) is the MTC, and \([S_b]\) is the bulk substrate concentration. For these theoretical calculations, \(V_m\)' and substrate concentration \([S_b]\) (DNA), were obtained from our microreactor data (Chapter 2). \(V_{\text{max}}\) was calculated from the relationship relating \(V_{\text{max}}\) to \(k_{\text{cat}}\) and enzyme concentration \([E]\), as follows:

\[
V_{\text{max}} = k_{\text{cat}}[E]
\]

Bulk substrate concentration was taken from this study as 50 µg/mL of dsDNA. Based on these parameters and as shown in Table 3.4, Damkohler numbers (Da) of 5.59, 5.13, and 3.97 were obtained for nanochannels of 130 nm, 100 nm, and 50 nm in width, respectively. In theory, the boundary conditions imposed by the NER should limit the diffusional distances, making them very short, and thus reaction limited. Damkohler numbers represent the overall kinetics of a reaction as the fraction of intrinsic enzymatic rate contributions to mass diffusional rate contributions. Therefore, when \(Da >> 1\), external diffusion is rate limiting, when \(Da << 1\), the reaction rate is limiting, and when \(Da \approx 1\), contributions from external diffusion and reaction rate are comparable. From our data, we see that the kinetics in these systems are diffusion limited, yet it is interesting
to note that Da numbers decrease with channel dimension. Furthermore, reaction rate increases with decreased nanochannel widths.

Table 3.4 BlAsimulation kinetic parameters to estimate mass transport contribution to overall rate of reaction under various nano-scaled dimensions.

<table>
<thead>
<tr>
<th>Channel Dimensions (nm)</th>
<th>V\text{EOF} (m/s)</th>
<th>D (m²/s)</th>
<th>k_L (m/s)</th>
<th>Da</th>
<th>V\text{RXN} (g/m²/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>130</td>
<td>5.1x10³</td>
<td>9.61x10⁻¹²</td>
<td>4.463x10⁻³</td>
<td>5.59</td>
<td>0.22</td>
</tr>
<tr>
<td>100</td>
<td>5.1x10³</td>
<td>9.61x10⁻¹²</td>
<td>4.871x10⁻³</td>
<td>5.13</td>
<td>0.24</td>
</tr>
<tr>
<td>50</td>
<td>5.1x10³</td>
<td>9.61x10⁻¹²</td>
<td>6.137x10⁻³</td>
<td>3.97</td>
<td>0.31</td>
</tr>
</tbody>
</table>

Based on these values in Table 3.4, the reaction in each case is mass transport limited. However, this would seem counterintuitive at the scales investigated. Nonetheless, these trend observed in Da numbers from the are logical because the substrate (dsDNA) has to travel less distance to reach enzyme as channel dimensions are reduced. This also means that product (ssDNA) does not need to travel far to remove itself or be removed from the enzyme surface. A depiction of this phenomenon is illustrated in Figure 3.8. The kinetics observed here also suggests that smaller channel sizes decrease the contributions from the bulk substrate concentration \([S_b]\), thinning this concentration to mimic substrate surface concentrations \([S_s]\) (see Figure 3.8c). Thus, making the system more and more strongly reaction limited, \([S_b] \approx [S_s]\).

These results, coupled to the real-time digestion studies conducted provide insight as to the parameters that effect enzymatic digestion in nano-scaled reactors. These data suggest that smaller nanochannel dimensions can more closely mimic intrinsic reaction rate limited kinetics for immobilized λ-DNA.
Figure 3.8 Mass transport kinetics were estimated by BIAsimulation software where our NER design a) was estimated to have Damkohler numbers (Da) of 5.59, 5.13 and 3.97 at b) 130 nm, 100 nm, and 50 nm spacing, respectively. c) Illustration of bulk $[S_b]$ and surface $[S_s]$ substrate concentrations where as dimensions decrease, bulk contributions to the overall kinetics decrease. These data suggest that as nanochannel dimensions decrease, the bulk contributions make the system less influenced by diffusion and more dependent on intrinsic properties of the enzyme.

Accordingly, the findings secured in this study may serve as important for designing strategies to immobilize $\lambda$-Exo enzymes to nano-scale solid supports for potential applications in single-molecule DNA sequencing.\textsuperscript{62-63}

3.4 Conclusion

In this work, we characterized COC materials for the development of a hybrid nanoscale reactor for the immobilization of $\lambda$-Exo for dsDNA digestion. The COC characterized in this study was confirmed to have carboxylic acid groups to ensure proper carboxylate functionalization and device bonding via (WCA) and XPS measurements. The immobilization was accomplished using (EDC/NHS) coupling chemistry and dye conjugation and UV spectral analysis confirmed that the enzyme was
covalently attached to the polymer surface. The device geometry investigated was easily fabricated and DNA introduction into the reactor through electrokinetic forces was facile even though occasional DNA hooking around the nanopillars was observed. Bonding of these nanofluidic devices took place post-enzyme functionalization and temperature analyses confirmed that the enzyme activity was maintained for immobilized λ-Exo. Preliminary fluorescence microscopy studies of YOYO-1 stained dsDNA allowed for real-time observation of the nano-scale digestion, where we observed a digestion rate of ~303 nt/s, a value lower than our microreactor. Lastly, simulated kinetic analyses at various nanochannel widths provided insight on the mass transport effects in these reactors; as dimensions decreased the kinetics observed were less diffusion limited.

3.5 References


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4 Transport Dynamics of Biopolymers through Irregularly Shaped Nanochannels

4.1 Introduction
Nanopores are versatile label-free sensors for the detection and analysis of single molecules and particles that uses label-free resistive-pulse sensing for monitoring the transport of biomolecules through the pore.\textsuperscript{1} Efforts have been invested toward developing nano-scale approaches for DNA sequencing using nanopores, which can read the DNA sequence from electrical signatures of mononucleotides as they translocate through the pore’s nano-confined environment.\textsuperscript{2-6} In most studies, the 3-dimensional nanopore consists of α-hemolysin, which is a proteinaceous membrane channel protein produced by the bacterium, \textit{S. aureus}. DNA translocation through α-hemolysin nanopores,\textsuperscript{6-7} and artificial solid-state nanopores,\textsuperscript{8-9} has been shown to provide structural information about a molecule and pores in the 10-500 nm range are well suited for analyzing physiochemical characteristics of single molecules.\textsuperscript{1} The thickness of these nanopores influences the nucleotide base resolution of the sequence analysis; increased pore thickness makes probing of a single molecule’s local structure more difficult during a translocation event.\textsuperscript{10} Nonetheless, the advantages of nanopore technology as a DNA sequencing approach include; (1) the ability to sequence large DNA fragments (≥50 kbp); (2) does not require the use of amplification or sub-cloning techniques; (3) does not require deoxynucleotides or dideoxynucleotides that are fluorescently labeled; (4) small input DNA sample sizes are required, on the order of 1 x 10\textsuperscript{8} copies for whole genome sequencing and; (5) the rate at which DNA sequence information can be obtained could provide near real-time readout.
In spite of the compelling discoveries emanating from biological nanopores, the use of this technology has some challenges: (1) Its mechanical and chemical stability are in many cases, problematic; (2) it has a fixed pore size that allows transduction of only selected types of molecules and; (3) the ability to manufacture high-density arrays of such nanopores can be difficult. This has led to efforts in using synthetic nanopores\textsuperscript{9,11} that can be fabricated with 1 – 50 nm sizes in polymer or silicon nitride membranes using electron or ion beams.\textsuperscript{12-13} The attractive feature of synthetic nanopores is the ability to adopt different readout modalities, such as the use of transverse electrodes decorating the synthetic pore to monitor perturbations in tunneling currents or conductance changes induced by the mononucleotides.\textsuperscript{14-17}

Nanochannels, which consist of in-plane two-dimensional nanostructures with widths/depths $\leq 100$ nm, are attractive tools for the study of single molecules and have been used for DNA sizing/extension, restriction mapping, protein/DNA binding and separation. These applications are facilitated by the 2-dimensional structure of nanochannels, which generally have axial lengths equal to or greater than the analyte’s fully extended (contour) length, especially when coupled to width and depth dimensions that are less than the radius of gyration.\textsuperscript{18} For this reason, the linearization of DNA in nanochannels has been found to be useful in the investigation of DNAs for direct-read sequencing.\textsuperscript{19} The stretching of single DNA molecules allows for the interrogation of sequence specific motifs and organizational structure of genomes.\textsuperscript{20}

The elongation experienced by DNA biopolymers through confinement in nanochannels mitigates the random motion of the DNA that is observed in nanopores, thus lessening the noise present in ionic current measurements using these devices.\textsuperscript{21}
In addition, nanochannels allow slower translocations and the ability to fabricate multiple sensing spots along the channel, parameters that improve the read-out resolution. Furthermore, nanochannels can be fabricated to possess high optical clarity for real-time microscopic analysis and flows controlled via Debye length variation.

Several groups have reported the analysis of DNA chains transported through nanochannels mostly fabricated in glass or fused silica as a means to study the transport dynamics. Glass possesses a well-characterized surface chemistry, with high surface wetting (hydrophilicity), non-conductivity, rigidity, and minimal surface defects, in addition to non-deformability at high pressures and proven top-down fabrication techniques. However, quartz and glass based materials are generally harder to process as substrates for nanochannel fabrication due to the requirement of sophisticated instrumentation. Also, it is very difficult to form high aspect ratio structures in glass and quartz substrates, which poses certain challenges for sample introduction and device integration. In addition, glass has a tendency to slowly dissolve in aqueous solution at high pH values, making long-term reliability problematic in these devices, especially for nano-scale devices.

Conversely, polymers serve as an attractive alternative to glass-based substrates due to the wide range of physiochemical properties available, low-material cost and a variety of fabrication methods that can be employed to make the necessary structures even on the nanometer length scale. One recent paper investigated the dynamics of dsDNA stretching in square PMMA nanochannels with channel cross-sectional dimensions (250 nm) much larger than the persistence length of dsDNA (50 nm).
In this work, the transport properties of single DNA molecules when poised in irregularly-shaped nanochannels will be studied. The information obtained from these experiments will provide insight as to the equilibrium conformations and behavior of dsDNA biomolecules when translocated through nanoconstructs made from thermoplastics. Studies focusing on the translocation of dsDNA molecules through irregularly-shaped nanochannel geometries and entropic barriers have been reported using glass-based devices. In this work, we will examine the effects of various nanochannel geometries, entropic trap sizes, and the overall effects of electric fields within these distinct structures on the translocation of λ-DNA through PMMA/COC nanochannels.

4.1.1 Nanoconfinement Dynamics of DNA

Nano-scaled dimensions occurring below the radius of gyration can stretch DNA to near its full contour length for a variety of applications. However, the forces required to introduce DNA into nanochannels (i.e., hydrodynamic, electrophoretic, etc.) can result in folded, compressed, or stretched conformations. Thus, equilibrium models of confinement rely on critical nanochannel dimensions to characterize the conformation of these polymer chains. The contour length \( L \) is defined, as the ‘length’ a DNA molecule would occupy if extended end-to-end with no thermal fluctuations acting upon it. Quantitatively, \( L \) is equal to the total number of base pairs in the dsDNA molecule, \( N \), multiplied by the (average) spacing, \( h \), of a base pair (0.34 nm for B-state duplexed DNA). Unlabeled λ-DNA (\( N = 48.5 \) kbp), has \( L = 16.5 \) μm and genomic DNA (\( N = 6 \) Gbp) has a contour length of 2 m.

It should be noted that translocation studies require the use of fluorgenic labels for imaging and as a result, can induce increased contour lengths due to intercalation from
the staining dye.\textsuperscript{40} Alternatively, chain conformations in bulk, such as circular chains where this end-to-end length cannot be delineated, can be described by the radius of gyration, $R$, which provides a measure of the chain distribution around the average chain center-of-mass position as depicted in Figure 4.1. For instance, $\lambda$-DNA in its randomly coiled conformation has $R = 550$ nm.\textsuperscript{41}

![Figure 4.1 Illustration depicting the end-to-end length denoted as the radius of gyration ($R$), persistence length ($P$), effective width ($w$) and wall–DNA depletion width ($\delta$). (Reproduced from Rep. Prog. Phys. 2012, 75 106601 with permission).](image)

Figure 4.1 Illustration depicting the end-to-end length denoted as the radius of gyration ($R$), persistence length ($P$), effective width ($w$) and wall–DNA depletion width ($\delta$). (Reproduced from Rep. Prog. Phys. 2012, 75 106601 with permission).

The persistence length ($P$) is a measure of polymer chain flexibility and denotes the length at which the molecule loses elasticity and can be treated as a rigid object.\textsuperscript{38} Another physical factor to consider for DNA in nanoconfined environments is the effective width of the DNA ($w$).\textsuperscript{38} This constant describes the effective interaction range of a folded DNA (Figure 4.1) and is often under-estimated by adding the intrinsic chain width to two-times the Debye length.\textsuperscript{39} $w$ is strongly influenced by ionic strength and as a result, highly variable if careful considerations are not taken. Wall depletion, $\delta$, has ionic strength dependence due to salt concentrations and electrostatic interactions. The
implications of these dynamics are compounded at smaller and smaller nanochannel dimensions and occur in several stages of polymer confinement in a channel of diameter, $D$. In a scenario where the channel dimension, $D$, $>> P$, the slightly elastic chain will only “weakly feel” the effect of confinement and the chain dynamics will embody a self-avoiding system. However, when $D \leq P$ the “bending” energy will rapidly increase, causing entropy to decrease because the restricted semi-elastic polymer can no longer coil. As illustrated in Figure 4.2, when the polymer moves into increasingly confined spaces there is an evolution in the physics from a slightly compressed polymer coil to a highly elongated chain. These dynamic changes in polymer behavior due to confined environments have been described by two well-known scaling theories proposed by De Gennes$^{42-43}$ and Odijk.$^{44-45}$

In the confinement model proposed by De Gennes, DNA is described as a “self-avoiding” polymer where each chain can be regarded as “blobs” having an average diameter that is equal to $D$ (Figure 4.2). This model assumes that $D$ is much greater than the persistence length of the DNA taking on a spherical blob conformation. When channel size drops below the critical channel width, $D^*$, the De Gennes theory transitions in free energy to the extended De Gennes regime as depicted in Figure 4.2. Also, as nanoconfinement amplifies in the extended De Gennes regime, self-avoidance effects are overcome as channel dimensions approach the persistence length of the polymer. On the other hand, the Odijk scaling argument is applied to DNA in a confined environment where $D << P$. In this model, it is proposed that DNAs are “deflected” by the walls assuming rigid conformation. However, at the classic Odijk regime a transition occurs where the DNA chain is folded back on itself assuming a conformation where it
can still coil but dimensions are so small this coiling is a rare occurrence.\textsuperscript{46} This phenomenon can be described as hairpin turns with tight bends near the wall and the average distance between these bends regarded as the global persistence length, $G$.\textsuperscript{46}

Figure 4.2 Overview of the physical regimes in nanochannel confinement. In the De Gennes regime, the conformation consists of a string of isometric Flory blobs. At $D = D^{**}$, there is a transition to the extended De Gennes regime characterized by elongated blobs that are at the cross-over between ideal and Flory behavior. At $D = D^* = 2P$, the polymer enters a ‘transition’ regime, characterized by isolated hairpin backbends with a global persistence length $G$. For $D < P$, hairpins are frozen out and the polymer can only store contour through a series of successive deflections with the wall characterized by the deflection length $\lambda$, the ‘classical Odijk’ regime (Reproduced from Rep. Prog. Phys. 2012, 75 106601, with permission).

4.1.2 Translocation Dynamics and DNA Analysis

The physics of stretching DNA via entropically driven dynamics and compression through nanoscale dimensions are of particular interest because of the ability to study these elongated DNA molecules for restriction mapping, DNA-protein interactions, and other physical behaviors.\textsuperscript{47-48} Biopolymers like DNA and RNA cross through a number
of barriers in vivo to execute various biological functions, which in turn emphasizes the numerous applications of DNA translocation including sequencing,\textsuperscript{49-51} entropic sorting,\textsuperscript{34} and as sieves\textsuperscript{52} for pharmaceutical and food industries.\textsuperscript{53} Translocation involves the electrokinetic passage of biomolecules from one region of a nanochannel to another, where this movement induces complex transport dynamics that are dependent on the nanochannel dimensions. Therefore, the study of DNA molecules within unique geometries and dimensions as presented herein will provide critical insights into the application of nanochannels for DNA sequencing.

The translocation of DNA chains through nanochannels occurs in four phases: 1) Locating the entrance to the nanochannel; 2) entering the nanochannel; 3) passage through the nanochannel; and 4) ejection of DNA from the channel.\textsuperscript{53} One aspect of DNA translocation in nanochannels is the ability to influence the migration and conformational behavior of its polymer chains through changes in nanochannel size, introduction of flow, and electric field manipulation. The unique aspect of this work is the ability to perturb the migration and conformational behavior of these biomolecules through the introduction of entropic traps and impenetrable pillars to create non-uniformity in the electric field. The method designed here allows for constant assay conditions to be used (i.e., field strength, buffer composition, and nanochannel sizes) while manipulating the DNA via differences in field discontinuity due to trap and pillar design elements. These characteristics would offer the ability to perform multiple nanoscaled reactions, in parallel, with minimal sample contamination, reduced fabrication time, and the ability to interrogate multiple reactions in real-time. Therefore, this work will examine DNA translocation through irregular nanostructures at various lengths and
entropic barriers in the hope to understand the changes in field discontinuity as design parameters are altered.

4.2 Materials and Methods

4.2.1 Design Optimization

Figure 4.3 Illustration of irregular nanochannel geometry to be investigated, where probing parameters include variation of trap size, pillar geometry, effective distance from trap to pillar and effect of field strength on DNA mobility throughout these unique nanostructured devices. These investigative considerations could potentially provide critical entropic force information that will aid reactor functionality once enzyme is included.

The irregular nanochannels utilized in this work contain the design parameters depicted in Figure 4.3. The initial design consisted of a mixed scaled device with anisotropically dry-etched micro-feed channels and a nano-scale channel array milled into a silicon master using Focused-Ion beam (FIB) lithography. The device had an input of ~300 nm that tapered to ~50 nm, an entropic trap of ~500 nm (diameter), and an impenetrable barrier (pillars) of 350 nm (diameter) with an outlet of 100 nm. The input taper was used to promote DNA entry into the entropically confined nanochannel, which possessed dimensions near the dsDNA's persistence length (~50 nm). Following fabrication of the Si master, the nano-structures were transferred to a resin stamp using
UV nano-imprint lithography (UV-NIL) as described by Wu et. al.28 SEMs of the Si master and resin stamp were acquired by thin film platinum deposition (30 s) using a deposition current of 20 mA yielding a 2.5 nm layer of platinum on the surface of the stamp as shown in Figure 4.4.

Figure 4.4 SEMs of a) silicon master of the nanofluidic device; and b) protrusive resin stamp. The stamp was comprised of a TP70 UV curable resin with a COC back plate that was irradiated for 20 s at a UV intensity of 1.8 W/cm². The resin stamp consisted of tapered channels ranging from 50 to 300 nm, with a nanochannels <50 nm, which contains a pillar ~350 nm in diameter and 150 nm in height, and entropic trap ~500 nm in diameter.

It was determined that dry etching induced surface roughness in the microchannel sidewalls with an orientation perpendicular to the direction of demolding.54 This roughness compromised the integrity of the stamp, and as a result a new device design was secured using anisotropic wet etching, which produced smoother microchannels with roughness parallel to the direction of demolding making the process less deleterious on the stamp.54

Si masters consisted of micron-scale access channels, which were fabricated using standard photolithography, followed by anisotropic wet etching with 50% KOH solution. Arrays of connecting irregularly designed nanochannels were fabricated using Focused
Ion Beam (FIB) milling using AutoCAD design software. Si masters were subsequently used to construct protrusive resin stamps, which were made from a UV-curable resin polymeric blend containing 68 wt% tripropylene glycol diacrylate (TPGDA) as the base, 28 wt% trimethylolpropane triacrylate (TMPA) as the crosslinking agent and 4 wt% Irgacure 651 (as photo-initiator) coated onto a COC back plate.\textsuperscript{28} Thermal imprinting was used to transfer the nanofluidic structures into PMMA from the UV-curable resin stamp and the device was sealed with a PMMA or COC cover plate using low-temperature plasma-assisted bonding to build the enclosed mixed-scale polymer nanofluidic device. NIL conditions were carefully selected to ensure that patterns from the resin stamp were transferred with high fidelity and minimum deformation into the polymer substrate.

### 4.2.2 Feed channels to keep DNA in a stretched configuration

Figure 4.5 shows SEMs of the second version of the Si master, cured UV-resin stamp and thermally imprinted PMMA with an array of nanochannels. The nanofluidic device possessed the following dimensions: Nanopillar \(\sim 800\) nm surrounded by a nanochannel that was \(40 \times 40\) nm in width and depth; entropic trap that was \(\sim 600\) nm diameter and \(200\) nm deep with varied trap-pillar distances as displayed in Figure 4.5.

Translocation of DNA across this design required that the biopolymers transition from the micro-scaled feed channels into the nano-confined regions of the device. To increase the capture rate of DNA into the nanofluidic structures, slight tapers populated with pillars were included at the microchannel/nanochannel interface.\textsuperscript{55} Others have observed that DNA molecules in close proximity to the nanochannel entrance are injected into the nanofluidic device at low rates if an abrupt interface is employed.\textsuperscript{7, 56-57}
Therefore, a secondary 1-D microfeed channel was constructed to better aid in the feed rate of DNA into our nanofluidic device. Because the mixed-scale access channel possessed an aspect ratio >1, structure collapse in this region was pervasive and thus, we placed large pillars into the feed channel region (Figure 4.6) to prevent collapse.
In addition to feed channel collapse, another issue that needed to be resolved was the frequency of prolonged trapping of DNA at various locations within the nanoconfined domains. Steps to address this issue are delineated in Section 4.3.1.1. The final PMMA nanofluidic device had nanochannel widths of 50 nm. SEMs of this device are shown in Figure 4.7, where pillar to trap distances varied from 30 to 9 µm. For each distance, entropic trap sizes were varied as well (400, 300, 200, 100 and 75 nm) with the pillar diameter kept constant at 1.34 µm.

The UV resin stamp possessed a thermal expansion coefficient similar to PMMA (6 × 10⁻⁵/°C) and was chosen due to its capability to fabricate micro- and nano-scale patterns via UV-NIL and reduce thermal stress following thermal-NIL production of the nanofluidic device.⁵⁸-⁵⁹ A single UV resin stamp was used for thermal imprinting for up to 20 times without any noticeable damage to the structures.

Figure 4.7 SEM image of the nanoscale device with a single off-set (and symmetric) pillar 1.34 µm in diameter that is separated from entropic traps ranging from 400nm down to no trap with ~50nm spacing. This device hosts a pre-stretch region to facilitate injection of the DNA into the nanochannels.
PMMA devices were sealed using plasma assisted low temperature thermal fusion bonding. Initial studies utilized PMMA substrates with PMMA cover plates of 125 µm in thickness. We discovered that hybrid bonding afforded superior optical clarity and facilitated better tracking of DNA through our nanofluidic devices. The structural fidelity of these channels was evaluated by introducing 5 mM fluorescein isothiocyanate (FITC) solution in 0.5X TBE buffer into the enclosed fluidic network; fluorescence indicated successful sealing of the nanofluidic network.

Figure 4.8 a) SEM image showing an array of the device with 1.3 µm diameter nanopillar (see insert), entropic traps ranging from 0 nm to 400 nm diameter with a spacing of 9.18 µm. b) and c) shows the results obtained from the sealing test for the hybrid (PMMA - COC) device and PMMA - PMMA devices, respectively, acquired at 500 ms exposure time. The obscurity of the nanochannel/nanopillar region in the PMMA-PMMA devices is due to possible collapse of the device during assembly.

As shown in Figure 4.8, the fluidic channels did not show any signs of leakage. Also, it can be observed that the optical clarity gained by hybrid bonding of low Tg COC to the higher Tg PMMA provided better examination of DNA dynamics through these devices because channel deformation was minimal compared to that found in the PMMA-PMMA
devices. Using low thermal bonding temperatures (70°C for COC-PMMA, 80°C for PMMA-PMMA) also minimized the amount of surface reorganization of the polar functional groups following plasma treatment.

4.2.3 Experimental Conditions

All images were collected and analyzed using DNA samples (λDNA - 48.5 kbp, New England Biolabs) as received. The DNA was fluorescently stained with YOYO-1 intercalating dye (Invitrogen) at 5:1 base-pair-dye ratio in a 0.5X Tris-Borate EDTA buffer (45 mM Tris, 45 mM Borate, 0.5 mM EDTA) with the addition of 4% v/v of β-mercaptoethanol as a radical scavenger to minimize photoinduced damage (photobleaching and photonecking). Stock solutions were allowed to equilibrate at room temperature overnight prior to dilution for experiments. Experiments were run using solutions having a DNA concentration of 0.5 µg/mL. The stained DNA molecules were imaged with an inverted microscope (Axiovert 200 M, Carl Zeiss, Thornwood, NY), which was equipped with a Zeiss 100 x/1.3 NA oil immersion objective and illuminated with a 100 W mercury-vapor lamp with the appropriate filter set (Ex: 450–490 nm and Em: 515–565 nm). A custom mount was machined to hold the assembled nanofluidic device onto the microscope stage for optimal image acquisition. Images were acquired using an iXon3 897D EMCCD camera (Andor Technology) collecting approximately 50-200 frames s⁻¹ and analyzed using MetaMorph Advanced 7.7.6.0 software (Molecular Devices LLC, Sunnyvale, CA) and ImageJ 1.46 (National Institutes of Health, Bethesda, MD). All buffer solutions were filtered using a 0.2 µm filter and degassed with an ultrasonicator prior to use.

Fluidic channels were rinsed with a series of solutions prior to the DNA translocation experiments. First, a binary mixture of methanol/ultrapure water (50% v/v) was
introduced into the fluidic channels. Next, the chip and reservoirs were filled with ultrapure water to rinse the previous solution from the fluidic network. This was followed by adding the appropriate buffer solution to the chip. Each rinsing step was done for 5 – 10 min. Prior to the translocation experiment, buffer solution in one of the microchannels was replaced with a buffered solution containing the stained dsDNA. DNA molecules were electrokinetically driven through the nanochannels by immersing Pt electrodes in the reservoirs on either side of the nanochannel and applying a DC bias voltage using a variable voltage power supply. The recording frame rate and electric field strength were used to compute the transport parameters.

4.3 Results and Discussion

4.3.1 Importance of Entropic Trap Component

DNA behavior in nano-confined geometries can produce the stretching and controlled positioning of DNA in nanofluidic devices. Furthermore, nanoscale confinement induces changes in DNA free energy and accordingly, entropic forces associated with this confinement can induce interesting behavior, such as the “storage” of DNA due to these forces. Consequently, our design included a component to trap the DNA through entropic forces. The DNA can be ejected from this trap when sufficient energy is applied in the form of electrokinetic energy by applying a particular applied electric field.

4.3.1.1 Variations in Entropic Trap

Entropic trapping of DNA has been used in the electrophoretic separation of large DNA molecules. For our work, the entropic trapping component serves as a critical element for facilitating the synchronized introduction of DNA into a smaller confined element. Earlier designs of irregularly shaped nanochannels contained entropic traps
with dimensions equal to or greater than the radius of gyration, R, of λ-DNA ($R_{\lambda-DNA} \approx 550$ nm). This one-dimensional measure of DNA size is estimated from the molecule’s center of mass and calculated from its contour length as follows from equation (1): 

$$R_g = \frac{1}{6} \sqrt{LP} \quad (1)$$

where $L$ is the contour length of the polymer chain and $P$ is its persistence length.

Therefore, these devices (with entropic traps >500 nm) were observed to render λ-DNA immovable and required higher voltages to remove the DNA from this trap. To observe the distribution of the electric field across the entropic traps, electric field line mapping was performed on the nanochannels containing various trap diameters (1 µm-200 nm).

Figure 4.9 Illustration of Graphical representation of electric field strength versus entropic trap size where changes in the electric field denote changes in electric field strength.

In this simulation, the point of reference for electric field changes was the center of the traps as illustrated in Figure 4.9a. From this information, we were able to calculate
the change in electric field strength as these dimensions increased (see Figure 4.9). As can be observed, the change in electric field, $\Delta E$, in the 1 µm wide trap is larger compared to smaller trap diameters. This suggested that the field lines were dissipated across the trap as the trap radius increased. When the trap diameter was reduced to 200 nm (radius = 100 nm), the change in electric field from the center of the trap was smaller, suggesting more evenly distributed field lines across the entire width of the trap, indicating that the field was more uniformly distributed at this smaller trap size. This would suggest that smaller trap widths could better facilitate ejection of DNA.

We next calculated the volume of each entropic trap, using the volume equation for a cylinder. We also used the radius of gyration of DNA to find its volume ($8.71 \times 10^7$ nm$^3$) based on the equation of a sphere. A list of these values is presented in Table 4.1.

Table 4.1 Table of entropic trap volumes and calculated percent DNA occupancy.

<table>
<thead>
<tr>
<th>Entropic trap diameter (nm)</th>
<th>Volume (nm$^3$)</th>
<th>Percent DNA Occupancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>400</td>
<td>$1.26 \times 10^7$</td>
<td>19.6%</td>
</tr>
<tr>
<td>300</td>
<td>$7.07 \times 10^6$</td>
<td>8.1%</td>
</tr>
<tr>
<td>200</td>
<td>$3.14 \times 10^6$</td>
<td>3.6%</td>
</tr>
<tr>
<td>100</td>
<td>$7.85 \times 10^5$</td>
<td>0.9%</td>
</tr>
<tr>
<td>75</td>
<td>$4.42 \times 10^5$</td>
<td>0.5%</td>
</tr>
</tbody>
</table>

Because the volume of DNA was larger than the cylindrical volumes calculated for each entropic trap and the radius of gyration of λ-DNA, we subtracted the entropic trap volume from that of λ-DNA and divided the remaining volume by the original DNA volume. As can be observed in Table 4.1, only 20% of the DNA molecule occupied the 400 nm trap. These values decreased with decreasing trap diameters as expected. However, these percentages of DNA within the trap do not correlate with what we
observed via fluorescence microscopy observations; we found that the DNA nearly fully compressed into the entropic trap, which suggested that the field strengths being applied forced the DNA into these entropically unfavorable scenarios.

4.3.2 Off-set and Symmetric Pillar Geometries

We included in the nanofluidic design an impenetrable obstacle (i.e., pillar), which consisted of a cylindrical structure that the DNA would move around when translocating through the nanofluidic device. The position of this pillar, either symmetrical or off-set with respect to the fluidic channel, would determine which path the DNA would follow during the translocation event based on path resistance. We noticed that dsDNA moved equally in both directions around the pillar in the symmetric orientation. This was anticipated based on the fact that the channel area on either side of the pillar was of equal cross-sectional dimensions and as such, the electric fields should be similar in either direction. This pillared region of our device functioned as a voltage divider with two resistors in parallel, thus reducing the current in each parallel resistor and slowing the movement DNA once it reached the pillar. As such, we frequently observed DNA stationary at this pillar region due to the lower electric field strength.

We next investigated an offset geometry at the pillar region. In this design, DNA translocation was observed to bypass the region of the pillar where the field strength was significantly reduced. With the manipulation of the offset, we could control the frequency at which the DNA moved through the path with least resistance.

4.3.3 DNA Translocation

DNA translocation events monitored through the device shown in Figure 4.3 revealed some interesting phenomena. In this work, transport properties were evaluated in terms of duration of time spent in the entropic trap and apparent electrophoretic
mobility as channel length and trap dimensions were varied. The apparent electrophoretic mobility, $\mu_{ep}$, is a parameter in which the electrophoretic velocity, $v_{ep}$, of a molecule can be related to the electric field strength, $E$. This relationship can be illustrated as depicted in equation (2):

$$\mu_{ep} = \frac{v_{ep}}{E}$$ (2)

As can be observed in Figure 4.10a, at a constant entropic trap to pillar distance, as the entropic trap size decreased the apparent electrophoretic mobility of the DNA increased. The mobility of the DNA at smaller entropic volumes indicated that there were higher perturbations on the DNA’s mobility when it encountered smaller trap volumes. This is supported by the fact that as trap dimensions decreased, mobility values began to approach mobility values for DNA in straight polymeric channels that are not encumbered by entropic barriers along the length of the channel.

![Graphical representations of a) the electrophoretic mobility of double stranded $\lambda$-DNA at various entropic trap diameters when the trap to pillar distance was held constant at 19 $\mu$m and b) linear relationship of the residence time $\lambda$-DNA spends in the trap as a function of the trap diameter with constant 19 $\mu$m distance.](image)

Figure 4.10 Graphical representations of a) the electrophoretic mobility of double stranded $\lambda$-DNA at various entropic trap diameters when the trap to pillar distance was held constant at 19 $\mu$m and b) linear relationship of the residence time $\lambda$-DNA spends in the trap as a function of the trap diameter with constant 19 $\mu$m distance.
An examination at constant nanochannel/trap distance (19 µm) and various entropic trap diameters (400-75 nm) revealed a strong linear dependence on barrier size to the residence time the DNA spent in the trap before ejecting itself into the nanochannel. As expected, Figure 4.10b depicts that as the entropic trap diameter (volume) increased, the residence of time DNA spent in the trap increased. This correlation was observed at \( R^2 = 0.99972 \) (\( n = 3 \) events).

For a 400 nm trap at 30, 19, and 9 µm nanochannel lengths, the time DNA spent in the trap was 0.438 s, 0.671 s, and 2.795 s, respectively. This indicated that at equal entropic trap dimensions (400 nm), the DNA residence time in the trap was longer when the length between the trap and the pillar decreased. This observation seemed counter intuitive, prompting further investigation. Analysis of the video frames suggested that the DNA was approximately the same size at each length investigated and differences in trap residence time were not due to different DNA sizes translocating through the channels. Therefore, we assert that the differences in DNA residence time in the trap arose from non-uniformity of the electric field due to trap positioning. This conclusion was based the device geometry for the 9, 19, and 30 µm trap to pillar spacing as can be observed in Figure 4.7. The component that changed was the trap position along the entire length of the nanostructure to vary the pillar to trap distance. This means when a given voltage is applied across this device, the strength of the field “felt” by the DNA could be different when the trap was further from the trap entrance. This in turn could describe why DNA remained in the 400 nm trap longer at shorter distances from the pillar (9 µm vs 30 µm).
From this data, we observed that the critical distances may not be between the trap and pillar, but that there may be more perturbation in the field when entropic trap distance from channel entrance was varied. Also, we concluded that due to this non-uniformity, electric field strengths could be higher when the channel was closer to the entrance, thus there was a high propensity of DNA to be ejected from constant trap sizes when nanochannel lengths were increased.

![Figure 4.11](image)

Figure 4.11 Representative series of frames showing the transport of λ-DNA that has been fluorescently stained in a 5:1 ratio and electrokinetically driven though an irregular nanochannel with a 30 µm trap to pillar spacing and contains an entropic trap of 400 nm.

We further studied a device that consisted of a 400 nm trap with a 30 µm spacing between the entropic trap and pillar. Using this device, it was observed that as the DNA entered the nanochannel from the entropic trap and moved across the channel that relaxation occurred (see Figure 4.11). When the dynamic extension lengths were calculated, it was determined that upon entry into the nanochannel, the DNA was extended by 30% assuming the contour length of the DNA was 20 µm due to 5:1 bp/dye labeling with YOYO-1. We noticed compression of the λ-DNA as it approached the
pillared region of the device. As seen in Figure 4.11, λ-DNA as it approached the pillar was extended by only 17%.

Extension lengths cannot be quantitatively compared to equilibrium models, but theoretical values can serve as a useful frame of reference. For this work, it was expected that at the current cross-sectional dimensions and buffer ionic strength used, we only observed dynamics occurring in the De Gennes regime. This observation was interesting because the entropic trap served not only to store DNA but to induce a drag on the dsDNA during its translocation to keep the dsDNA stretched to near its contour length irrespective of channel dimensions.

4.3.4 Nanopore Sequencing using Exonucleases

Nanopores have emerged as versatile sensors for the detection and analysis of single molecules and particles over the past decade.\textsuperscript{11, 62-67} Particularly, attention has been focused on developing nanopores for rapid sequencing of DNA using α-hemolysin and solid-state nanopores.\textsuperscript{62} However, nanopore technology for DNA sequencing is accompanied by a number of challenges:\textsuperscript{17, 68} (1) The translocation time through the pore are fairly short (1-20 µs per nucleotide) requiring the bandwidth of the readout electronics to function in the MHz range; (2) the readout resolution requires a pore thickness equal to or less than the single base spacing of DNA molecules, ~0.34 nm. Because the thickness of both synthetic and α-hemolysin pores is much larger (5-15 nm) than this spacing, multiple bases simultaneously reside within the pore. This need for smaller nanopore dimensions consequently excludes the use of larger nanopores (10-500 nm),\textsuperscript{65-66} which are relatively easy to fabricate, but require significantly improved signal-to-noise ratios.\textsuperscript{1, 57} Nonetheless, even if nanopores could be fabricated with this prerequisite thickness, the effective electric field read region would extend
approximately 1 pore diameter unit on either side of the pore.\(^6\)

3. The production of arrays of nanopores must be done in a high production mode reproducibly with the prerequisite size dimensions and at low-cost to accommodate the intended application.

4. High quality genomic DNA must be extracted from a diverse array of samples (blood, tissue, bone marrow, urine, saliva, etc) and then processed to produce DNA fragments (~50 kbp), which are used as the input for sequencing. The sample preparation and sequencing steps should be integrated into a single platform and operate in a basic turn-key mode to allow a broad user base.

Readout resolution limitations can be mitigated if nucleotides are physically separated from each other while maintaining their original order following clipping from the DNA, for example through the use of an exonuclease enzyme.\(^7\) This has been demonstrated to be feasible using a highly processive exonuclease enzyme, which sequentially clips individual nucleotides from an intact DNA fragment and directing these bases through an \(\alpha\)-hemolysin nanopore fitted with a cyclodextrin collar.\(^8\) In fact, Oxford Nanopore (www.nanoporetech.com) is developing this technology, which consists of the \(\alpha\)-hemolysin pore fitted with a cyclodextrin molecule and a chemically tethered exonuclease enzyme.\(^9\) Unfortunately, the single base identification efficiency using blockage currents is \(\sim 83\%\),\(^10\) and therefore, errors in sequencing using blockage currents alone may not generate the necessary sequencing accuracy required to identify mutations if single molecule DNA sequencing is to be used for diagnostics. Unfortunately, salt conditions required for optimum exonuclease activity could not be matched to conditions required for high accuracy base identification and thus, the
identification efficiency ranged from 90% to 99%. Therefore, additional base identification strategies must be considered.

Nanofluidic devices emanating from this study can serve as a unique tool for the understanding of DNA stretching, entropic forces exerted on biopolymers, and the controlled positioning of DNA for applications in DNA/RNA sequencing. The dynamics we observed also become critical as we seek to immobilize λ-exonuclease in this device for DNA digestion to produce mononucleotides that can be launched sequentially into a nanochannel for identification. This aspect, which serves as the foundation of our DNA sequencing platform, will address the key issue for using nanochannels to sequencing single biopolymers, like DNA or RNA, where each monomer unit from the biopolymer must modulate a signal in a specific and measurable way as it passes through the channel. For our design, the entropic trap can be used to store intact dsDNAs before ejection into a stretching channel to orient the DNA so that its 5’ end is facing a pillar decorated with the λ-exonuclease to produce mononucleotides that can be cleaved from the single DNA molecule and sent electrophoretically into an additional nanochannel for identification via its molecular dependent flight time through this channel.

4.4 Conclusion
For this work, we studied the dynamic transport properties of single DNA molecules when translocated across irregularly shaped nanochannels. The information obtained from this study suggested that the designed nanostructures can allow for the manipulation of DNA molecules with minimal external forces. The unique aspect of this work was the ability to perturb the migration and conformational behavior of these biomolecules through the introduction of entropic traps and impenetrable pillars to create non-uniformity in the electric field. The device design here suggested that
entropic barriers with dimensions less than the target molecules radius of gyration can be used for the effective storage of DNA. The introduction of pillars, whether symmetric or off-set can also halt or selectively direct the transport direction of DNA. Also this work revealed that careful positioning of entropic barriers along the length of the channel can alter its transport to slow DNA. Therefore, this work provides insights as to the changes in field discontinuity for DNA manipulation. This information will be applied for the realization of a nanoscale sequencing of single DNA and RNA molecules.

4.5 References


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5 Summary and On-Going Developments

5.1 Summary
To envision our DNA sequencing tool, solid-phase reactors using λ-Exo were created. Solid-phase bioreactors are regarded as attractive tools for performing high-speed and cost-effective reactions and have wide applicability in biosensors. This is especially true in the case of enzymatic reactors, as they are viewed as powerful tools in analytical processes and are established techniques.1 As discussed in Chapters 1 and 2, Immobilized Microfluidic Enzymatic Reactors (IMERs),1,2 possess several advantages as opposed to their homogeneous (liquid) counterparts: i) Reuse of the enzyme for subsequent analysis.3-5 This will allow the use of the nanosensor for multiple sequencing rounds without requiring a new sensor for each sequencing run. ii) Enhanced stability and activity of the enzyme when immobilized to a solid-support.6-8 iii) Decreased interferences from the catalytic enzyme during the readout phases of the assay. Because we are using a label-free readout strategy for the DNA sequencing tool, which monitors conductivity changes when a free dNMP travels through a pair of electrodes (see Figure 5.1), the presence of “free” enzyme could produce false positive results if it was allowed to perform its function in solution. Also, integration of an IMER in a microchip can provide enhanced reaction efficiency because of reduced diffusion distances, reduced analysis time and sample consumption, and increased automation.1,9

The unique aspect of this research project, as opposed to other IMER reports, is that the bioreactor will be comprised of a nano-confined space consisting of a single nanopillar decorated with multiple enzyme molecules configured in a reactor volume of nanometer scale (Nanofluidic Enzyme Reactor, NER). In addition, the nanoreactor will
perform its enzymatic function on a single DNA molecule to produce the prerequisite deoxynucleotide monophosphates (dNMPs) for feeding the nanosensor. Therefore, in Chapter 3, we investigated the effects of nanoconfinement on the enzyme to understand the fundamentals associated with the immobilization process, especially within nano-scaled environments, and the effects of immobilization on the enzyme kinetics when poised within a reactor of these dimensions.

Figure 5.1 Schematic of the nanosensor: a representation of the typical output of the nanosensor for transducing the flight-time of dNMPs through the nanochannel. The processing steps can include cell selection and lysis, solid-phase extraction of the target material (DNA or RNA), shearing of the selected molecular material and finally, the nanosensor (either single element or arranged in a high density array to increase throughput) to read the sequence from the selected molecular material. In all cases, the modules, including the nanosensor, is made from the appropriate polymeric material to suit the application need and the structures produced via micro- and nano-replication technologies. The nanosensor using electrical signatures to monitor the input of dsDNA, immobilized exonuclease to complex the dsDNA, entropic traps to stretch the dsDNA and identify the clipped dNMPs using flight-times through nanochannels.

5.2 On-Going Developments and Future Work

5.2.1 Background

Since its first inception, the human genome project has driven the understanding of chemistry, biology, medicine and evolution. This project has made it possible for
scientists to gain extensive understanding into processes like gene transcription and
gene variation as well as made it feasible to make genetic associations to inherited
diseases and identify genetic alterations in cancer.\textsuperscript{10-12} While genome sequencing has
evolved into methods that offer higher throughputs that could assist with improving
patient diagnoses, the ability to make the technology more accessible still remains a
challenge.\textsuperscript{10} A recent review stated that the ultimate goal is for sequencing to become
simpler and inexpensive so that it can routinely be employed as a general-purpose tool
throughout biomedicine.\textsuperscript{3} Accordingly, the project proposed here builds on this
foundation by expanding accessibility to DNA sequencing tools and makes these
technologies more affordable, while also increasing their throughput for ascertaining
important data in a variety of critical application areas, including \textit{in vitro} DNA diagnostics.

The DNA sequencing tool proposed herein is a combination of sequential DNA
disassembly using immobilized exonuclease enzymes and flight-time-based
identification of single dNMPs through a nanochannel sensor made from plastics by
means of low-cost replication. While this aim is ambitious, it will be realized through the
successful integration of several projects. An integral part of this technology platform
consists of a solid-phase bioreactor consisting of a nano-scale support structure to allow
the enzymatic digestion of intact dsDNA molecules into their individual monomer units
\textit{(i.e., dNMPs)}.

\textbf{5.2.2 Components for Nano-scale Device Integration}

\textbf{5.2.2.1 Single-Pillar Nanofluidic Enzymatic Reactor (NER)}

The results obtained in Chapters 3 and 4 for this work provide insight as to the
nanoconfinement effect of $\lambda$-Exo and DNA, two parameters that will be essential for
implementation of these biomolecules onto single nanopillars in our sequencing device.
Attaching λ-Exo onto a single nanopillar with optimal geometry will be essential for the successful direction of mononucleotides into our time of flight (ToF) nanosensor. In order to efficiently and effectively optimize the design of the transition region of the nanosensor and make appropriate choices for the strength of the electrical fields used to transport the individual dNMPs from the point of their release to the entrance of the nano-sensing channel, we will need to determine two things: 1) the wall interactions of the dNMPs between the pillar and sensor and 2) the geometries necessary for nanosensor design in this region between the pillar and entrance to the nanosensor. A recent work by our group answers the question of mononucleotide ToF effects due to wall interactions. Analysis of the effect of diffusion at realistic flow rates indicates that the dNMP velocity should be relatively high in order to get a reasonable minimum analysis time per dNMP. Therefore, the future work of this project will be optimizing the design of this reactor for effective dNMP transport into the ToF sensor.

5.2.2.2 Single-molecule Biopolymer Structure Elucidation using Time-of-Flight Nanosensor Arrays

5.2.2.2.1 Single-Molecule Fluorescence Tracking: Nano-electrophoresis

To understand scaling effects related to nanochannel electrophoresis, initial experiments were conducted in fused-silica capillaries utilizing conventional capillary zone electrophoresis (CZE) with UV detection (254 nm). CZE is a rapid and cost-effective approach to understand the electrophoretic mobility of a wide range of molecules. Also, since molecule-dependent flight times can be generated either through differences in electrophoretic mobilities and/or solute/wall interactions (CE and CEC). Buffer composition and pH were evaluated as experimental parameters that could possibly affect the electrophoretic mobility and separation of the four dNMPs. As seen in
Figure 5.2a, results revealed a mobility dependence on the pH of the buffer carrier electrolyte. At pH = 9.3, guanine (pKₐ = 9.24) has a q = -2 and was expected to possess the highest mobility. Thymine has a pKₐ of 10; therefore, it should demonstrate an increased electrophoretic mobility as the pH increased. Despite the obvious effect pH had on the separation, a complete separation was not observed for all 4 dNMPs. The addition of Mg²⁺ aimed to elucidate differences in mobility of the dNMPs through complexation with this inorganic cation which is a known phenomenon \(^{14}\); however, no effect was observed.

Figure 5.2 a) Effect of pH on the CZE separation of 4 dNMPs with 1 mM Mg²⁺ as BGE (1x TBE, ID 50 µM, L 56 cm, 25 kV, 90 mbar*sec, 254 nm. b) MEKC separation of 4 nucleotides (dAMP, dCMP, dTMP, dGMP) using EOF reversal with the addition of 6 mM CTAB to a buffer containing 75 mM AMP and 20 mM NaCl (pH = 9.0) (-20 kV, 90 mbar*sec injection, capillary length 56 cm, 50 ID, detection at 254 nm).

To improve the separation performance we observed when using CZE of the four dNMPs, micellar electrokinetic chromatography (MEKC) was investigated. MEKC is an attractive complement to CZE to illuminate differences in nucleotide mobility because it combines the separation mechanism of chromatography with the electrophoretic and electroosmotic movement of solutes. \(^{15}\) The addition of a cationic surfactant allows for a reversal of the electroosmotic flow (EOF) and thus, reduces the separation time, especially for anionic species such as the mononucleotides when using a negatively
charged capillary wall. As seen in Figure 5.2b, complete separation of the 4 dNMPs was observed at pH 9.0 with the addition of 6 mM cetyl trimethylammonium bromide (CTAB) which is above its critical micelle concentration of 0.92 to 1 mM. The elution order follows the predicted order based in increasing hydrophobicity. The large surface area of guanine available to interact with the micelle can explain the inversion of cytosine and guanine. This data is in agreement with molecular dynamic simulations of mononucleotide electrophoresis on the nanoscale observing hydrophobic wall interactions as a mechanism for separation.\textsuperscript{13} Furthermore, we were able to separate methylated-cytosine, which will allow for the identification of modified bases through TOF detection utilizing our DNA sequencing device.

Previously, we reported the fluorescence tracking of single molecules in polymer microchannels\textsuperscript{16} and single DNA molecules stained with intercalating dyes in nanochannels with widths well below the diffraction limit. Therefore, in future experiments, our group will investigate the electrophoretic properties of single mononucleotides by tracking them in an electric field moving through a polymer nanochannel. To conduct these experiments, our group will use a reporter molecule, in this case a single fluorescent molecule, such as ATTO532 that can be excited with the second harmonic of a Nd:YVO\textsubscript{4} laser but eventually single nucleotide detection will be label-less. Studies are currently being conducted in our group to assess electrophoretic mobility and diffusion coefficients of single particles without the use fluorescence labels.
5.2.2.2.2 Label-Free Single Particle Tracking in Nanoslits for Analysis of Electrophoretic Mobility and Diffusional Contributions

Molecule dependent flight times can be generated either through differences in electrophoretic mobilities and/or solute/wall interactions (CE and CEC). In this study we use our polymer nanoslit devices to model on-chip nano-capillary electrophoresis. Here we track single particles using silver nanoparticles (AgNPs) with dark field microscopy and investigate their different mobilities based on the size differences (see Figure 5.3).

Figure 5.3 Frames showing a single event for a bias voltage of 200 V/cm. The yellow arrows show the position of the single 60 nm AgNP traveling across the nanoslit from anode to cathode as a result of the applied electric field. Nanoslits were filled with a very dilute citrate buffer. The dimensions of the nanoslits were 100 µm in length and 150 nm deep.

In this work, the mobility of the AgNPs was extracted from the time it takes to travel through the nanoslits from tracking the particle as shown in Figure 5.3. Also other parameters were extracted using the mobility data such as variation, diffusion coefficient and plate number. From the preliminary results elucidated so far it can be concluded that the possibility of separating AgNPs based on their size using nanoslits at a specific
voltages is feasible. The advantage of using our device to perform this nano-capillary electrophoresis is that there is no need of adding extra reagents such as SDS as previously reported. The insights gain from this work will also aid the label-less detection of dNMPs for our nanosensor device.

5.2.2.2.3 Fabrication of ToF transduction prototype device in Fused Silica

In our nanosensor, the time of flight (ToF) of individual dNMPs through the nanochannel is measured by 2-pairs of electrodes that are positioned orthogonally to the electrically driven flow direction with the electrode gap equal to or less than the channel width. The label-less detection of single mononucleotide generated from exonuclease digestion will require only a signature be elicited when the dNMP travels through the electrode pair to determine the entrance and exit times through the nanochannel. For prototype experiments using the ToF transduction modality, we fabricated a device in fused silica due to the robust and well-established fabrication technologies currently available (see Figure 5.4). The fabrication process for producing this device involved four major steps; (i) Optical lithography to make micro-contact pads and the micro-scale fluidic channels; (ii) electron beam lithography and liftoff to make the nanoelectrodes; (iii) FIB milling of the substrate to build the nanofluidic network and produce the gap in the electrodes; and (iv) cover plate bonding to form the fluidic network.

The nanoelectrode size was ~50 nm x 45 nm and 1.8 µm in length (see Figure 5.4A). The entrance and exit nanoelectrodes were spaced 30 µm apart (see Figure 5.4B). It was critical to ensure that the nanochannel width and the electrode gap had dimensions below the reported persistence length of dsDNAs (50 nm). The fabricated width and depth for both nanogaps were ~30 nm and 50 nm, respectively (see Figure 5.4C).
Figure 5.4 SEMs of the molecular nanosensor made in glass via a combination of optical lithography, EBL and FIB. (A) and (B) show the device before FIB milling while (C) and (D) show the device following FIB milling. (A) High resolution SEM of the nanoelectrode that is 50 nm wide with a depth of 50 nm. The electrode is buried into the glass substrate using EBL and ICP-RIE to make trenches to accommodate the electrodes. Also shown are the Au microcontacts. (B) Low resolution SEM showing the location of the 30 µm length nanochannel. (C) High resolution SEM showing the 30 nm gap between electrodes. (D) Low resolution SEM showing the entire device. (E) SEM of the pillars populating the input funnel.

5.3 Conclusion

The first sequencing efforts of the Human Genome Project initiated worldwide interest in not only understanding the human genetic makeup but also how to identify gene abnormalities and signs of disease. While this interest has sparked cutting edge research and initiated steps towards achieving the $1000 genome, clinical applications have been slow to appear and thus have made this goal difficult to realize. The
sequencing tool proposed has the capability to enhance the foundation for genomic research by providing novel nano-scale instrumentation for the study of genetics and the diseases that arise from malfunctions in the genetic machinery. This single molecule-sequencing tool will allow for the elimination of amplification steps and the need for designing primers that typically do not work well for repetitive regions within the genome. Also, current single-molecule sequencing methods have the need for fluorescent labels for readout, which consequently requires detailed optical hardware.\(^{17}\) However, our tool will not require fluorescence and will allow for direct detection of mononucleotides in their native state through changes in solution conductance generated by a single dNMP resident between a pair of nano-scale electrodes. Lastly, the ease of use of this sensor, when all components of the project are realized, will remove the need for expensive equipment, high levels of scientific expertise and variations in the assay format.

The benefits of this project to society include better and more efficient Point-of-Care Testing and expanding the availability of genome sequencing testing to groups that could typically not afford it. Lastly, this technology can serve as a potential tool for clinical applications by searching the entire genome for sequence variations associated with a particular patient. Therefore, the immobilization of λ-Exo enzyme on a solid-phase poised within a nano-reactor will serve as a step towards realizing our on-chip DNA sequencing tool.

5.4 References


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Vita

Nyoté Jaedah Angelou Oliver-Calixte was born in Kansas City, MO to Pamella J. Oliver and Melvin Jordan. She attended Harold L. Holliday Montessori School until her acceptance into Lincoln College Preparatory High School. At Lincoln, Nyoté was an honor roll student, cheerleading captain, and Science Olympiad competitor. Her love of chemistry as a high school student motivated her to attend Xavier University of Louisiana for her undergraduate degree, where she majored in Chemistry ACS. At Xavier, Nyoté was President of NOBCChE, Secretary of Phi Lambda Upsilon Chemistry Honor Society and an active ACS chemistry club member. During this time, Nyoté became a member of the Gamma Alpha Chapter of Delta Sigma Theta Sorority Incorporated. Nyoté was a member of the NIH Minority Access to Research Careers (MARC) Scholars program and a recipient of one of Xavier’s highest awards, The William H. Mitchell Christian Leadership award. Eventually, Nyoté joined the LSU chemistry department in 2009, on a National GEM Consortium Fellowship, where she joined the Soper Research Group. During her time at LSU, Nyoté has been awarded a NSF East Asia and Pacific Summer Institutes Fellowship and a NIH Ruth Kirschstein F31 Award. She has authored one first author publication and has presented her work at national conferences. She will receive her doctorate degree in analytical chemistry at the December 2014 commencement.