Mechanistic Analysis of Four-Way DNA Junctions and Cytokine-Binding Aptamers for Therapeutic Interventions

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Mechanistic Analysis of Four-Way DNA Junctions and Cytokine-Binding Aptamers for Therapeutic Interventions

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

By

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Roaa Mahmoud

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<td>Double-Strand Break</td>
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ABSTRACT

DNA is inherently dynamic and topologically diverse and can fold into many different structures. Besides the canonical Watson-Crick structure, other higher-order structures such as G-quadruplexes (G4), i-motifs (iM), and four-way DNA junctions are possible. Although these high-order DNA structures are known to form transiently, they are important due to the crucial roles they play in many cellular processes including DNA replication, recombination, and repair. Among these DNA structures, 4-way junctions (also known as Holliday junctions, HJ) which are formed during the repair of double-strand DNA breaks (DSBs) and interact with proteins have garnered significant attention due to their central role in DNA damage repair. Therefore, dissecting the role of HJs, their topological properties, and their interactions with the HJ-binding proteins and small-molecule ligands is not only critical for understanding the mechanism of repair but also for deciphering the therapeutic potential of HJs.

Besides the inherent conformational dynamics, the HJs can migrate along the DNA axis through progressive base-pair rearrangements between the homologous DNA strands, called branch migration (BM). BM is conserved among organisms and is essential to stabilize recombination intermediates, which occurs by avoiding the reversal of strand exchange leading to the faithful repair of DSBs; however, molecular insights into the BM process including kinetics, the effects of microenvironments, and the role of HJ-binding proteins are poorly understood. Along this line, we have investigated the effects of cell-mimic solvent compositions and molecular crowding on the BM process by varying the concentration of cosolutes, namely dimethyl sulfoxide (DMSO) and polyethylene glycol (PEG). Using a single-molecule technique called fluorescence resonance energy transfer (FRET) and a mobile HJ analog capable of migrating, we have demonstrated that BM is significantly affected by cosolutes. The kinetic analyses of the FRET data
showed that the BM is enhanced under crowded environments. Furthermore, due to the importance of HJ in maintaining the genomic integrity of highly replicating cancer cells, there is a growing interest in exploiting HJ for cancer therapy via drug targeting. Therefore, we have expanded this project to investigate the binding of a small drug-like molecule to the HJ. Using both immobile and mobile junctions, we determined that the ligand called “VE-822” alters HJ conformations and dynamics, suggesting a potential application in therapeutics.

On the therapeutic side, we are also pursuing the engineering of cell surfaces with biomolecules for targeted applications. My project dealt with cytokines such as tumor necrosis factor-alpha (TNFα) for potential islet transplant therapy in the treatment of type 1 diabetes (T1D). Diabetes is one of many diseases that are life-threatening, affecting millions of individuals at different ages. T1D is characterized by a lack of insulin production due to the destruction of insulin-producing beta cells by the innate immune system. One novel therapeutic approach that has recently emerged as an alternative to exogenous insulin administration is islet cell transplantation (ICT). However, one major challenge in ICT is a considerable loss (50-80%) of the transplanted tissue mass due to inflammation and islet cell death post transplantation. To protect islets from inflammation, we used DNA aptamers to block TNFα and improve islet function. Using an aptamer-based single-molecule FRET (smFRET) coupled with in-vitro functional assays, we have evaluated the anti-inflammatory potential of a group of DNA aptamers by probing their binding interaction with TNFα and their ability to alleviate cytokine-induced islet cell death. Our analyses demonstrated the high potential of aptamers as anti-inflammatory agents in islet transplant therapy.
CHAPTER 1 – INTRODUCTION

1.1 Non-B DNA structures

Since the discovery of the double helical structure by Watson and Crick in 1953, our knowledge of DNA structure and topology has significantly evolved. DNA is a structurally flexible molecule with the ability to exist in many different conformations depending on the nucleotide sequence composition and other factors including hydration environment, solution ionic strength, binding of biomolecules such as proteins and/or ligands, etc.\textsuperscript{1,2} In physiological environments, the canonical double helix B-DNA is the most commonly adopted form. However, when genetic information is being accessed and processed during DNA metabolic processes such as DNA replication, transcription, and recombination, other higher-order DNA structures can form.\textsuperscript{3,4} Some of the important higher-order structures include triplex (triple helix), slipped (hairpin) DNA, G-quadruplex, i-motif, and cruciform or four-way junction (Figure 1.1).\textsuperscript{5–7} The formation of these structures is highly dependent on the DNA sequence, most of which are typically formed from repeat sequences.\textsuperscript{8} For example, cruciform DNA typically forms from inverted repeats, which are characterized by symmetrical sequences on complementary strands, triplex (or triple helix) DNA favorably forms from mirror repeats of purine and pyrimidine sequences, slipped (hairpin) DNA occurs from direct repeats, and i-motif and G-quadruplex occur from C-rich and G-rich sequences, respectively.\textsuperscript{5,9–12}

Recently, the importance of higher-order DNA structures has increasingly been recognized due to their key regulatory roles in determining genetic stability and their potential as therapeutic targets for the treatment of various genetic diseases.\textsuperscript{1,5} Out of the many structures that can form, four-way DNA junctions have attracted considerable interest due to their critical roles in genetic recombination and DNA damage repair pathways.\textsuperscript{13–15} The best-known four-way junction is the
Holliday junction (HJ). While the structural stability and therapeutic potential of the higher-order structures have been well characterized, such as G quadruplexes and i-motifs, the role of HJs in DNA repair including their conformational stability and interactions with proteins and small molecule ligands is yet to be elucidated. Understanding the dynamics of HJs and how they are regulated by repair proteins will not only provide new molecular insights into the recombination mechanism but will also open up new avenues for developing novel therapeutic strategies targeting the HJs.

![Table of DNA conformations and sequence requirements](image)

**Figure 1.1.** Some example noncanonical DNA conformations and their corresponding sequence requirements (taken from ref 5).
1.2 DNA damage and repair

DNA is constantly exposed to damage. It has been estimated that cells sustain approximately $10^4 – 10^5$ DNA lesions per day.\textsuperscript{20-22} Among the different types of DNA lesions, double-strand breaks (DSBs) are arguably the most detrimental and life-threatening.\textsuperscript{20,23,24} DSBs typically arise either from endogenous factors such as the cell’s own reactive oxygen species (metabolic by-products) and stalled replication forks, or from exposure to environmental agents including ionizing radiation and clastogens.\textsuperscript{23,25,26} To protect the cells from damage, multiple repair pathways exist among which homologous recombination (HR) plays a crucial role in repairing DSBs and guarding the genome against carcinogenic instability (described below in more detail).\textsuperscript{27-29} However, it was shown that defects in HR can lead to unreppaired or mis-repaired DSBs, causing an accumulation of oncogenic mutations and the progression of degenerative diseases and cancer.\textsuperscript{26,30-33} The HJ is a central intermediate in HR whose biological function is highly reliant on conformational dynamics and interactions with repair proteins;\textsuperscript{34-36} therefore, it is critical to dissect the mechanics and dynamics associated with the HJ at the molecular level to determine the origin of repair defects, which can potentially lead to the development of new therapeutic interventions.

1.3 Homologous recombination (HR)

Homologous recombination (HR) is essential for genetic exchange and diversity, telomere maintenance, and DNA repair.\textsuperscript{37-39} HR can be divided into three major phases: (i) pre-synapsis, which is the resection of the damaged DNA to form single-stranded overhangs to allow for strand invasion; (ii) synapsis, which is the strand exchange and pairing of homologous strands; (iii) and post-synapsis, which is the resolution or uncoupling of joint DNA molecules.\textsuperscript{20,40} These
fundamental stages of HR are known to be conserved across all organisms, except different repair proteins are involved. For example, in prokaryotes (e.g., *E. coli*), DNA is first resected at the break site by the action of nucleases and helicases (RecBCD complex) to produce 3’ tailed single-stranded DNA (ssDNA) overhangs (Figure 1.2). Next, nucleoprotein filaments of RecA (or Rad51 in eukaryotes) assemble onto the exposed ssDNA overhangs.\textsuperscript{41,42} The filament then starts its search for homology in the intact sister chromatid and pairs with a homologous section of the dsDNA template. The strand pairing and the subsequent ATP-driven disassembly of RecA leave behind a cross-strand DNA intermediate known as the HJ (Figure 1.2).\textsuperscript{43} The formation and processing of the HJ are critical steps to ensuring a successful repair of DNA. Briefly, the RuvA protein recognizes and binds the HJ and then recruits RuvB that acts as a motor to pull the DNA strands out through the junction.\textsuperscript{44,45} This allows the HJ to migrate along the DNA axis and exchange genetic information through a critical process called BM, further
discussed later.\textsuperscript{46,47} Finally, the HJ is resolved back into recombinant DNA molecules by helicases, thus restoring the genetic integrity.\textsuperscript{44,48}

1.4 Conformational dynamics of Holliday Junction (HJ)

Holliday junctions (HJs) are four-way DNA structures formed by joining of two duplex DNA molecules during homology-directed repair.\textsuperscript{49} HJs perform their biological function by mainly relying on conformational dynamics and interactions with proteins. Previous studies have shown that isolated HJs in solution are highly dynamic with the ability to adopt various conformational states.\textsuperscript{46,50–52} The two main classes of conformation include an open (extended) form and a more compact stacked-X form (Figure 1.3A).\textsuperscript{53} The switching between the different conformations, however, is known to be highly dependent on the ionic environment owing to the negatively charged nature of the DNA backbone. For example, in low salt environments such as in the absence

![Figure 1.3](image.png)

**Figure 1.3.** Representative structures of the HJ in an open (unstacked) form and stacked-X form (A) with the two possible stacking conformers (iso-I and iso-II) depending on the choice of stacking partners (B). The open form is more prevalent in low salt environments whereas the
stacked form is stabilized in high Mg$^{2+}$ environments. The equilibrium represented by the double-sided arrows demonstrates conformational preference with and without Mg$^{2+}$ (adapted from refs 53 and 56).$^{53,56}$

of divalent ions (e.g., Mg$^{2+}$), the HJ adopts an open form where the DNA duplex arms are extended assuming a near square planar geometry with an exposed junction center, which is thermodynamically more favorable as it minimizes the electrostatic repulsion between the arms (due to the negative charges from phosphate groups, Figure 1.3A).$^{50,51,54}$ On the other hand, in the presence of charge shielding ions (Mg$^{2+}$) at concentrations $\geq$ 100 µM, the helical arms of the HJ fold onto one another by coaxial stacking into a more compact stacked conformation due to the electrostatic shielding effects of Mg$^{2+}$ ions (Figure 1.3A).$^{54,55}$ The stacking of helical arms also generates two stereochemically equivalent conformers which differ in the choice of arms for stacking partners, namely iso-I and iso-II (Figure 1.3B).$^{50,55,56}$ Experimentally, it was shown that the HJ can dynamically interconvert between the stacking conformers through an intermediate open form.$^{46,51}$ The relative stability and population of the two stacked forms is largely dictated by the base sequence at the junction core and not by the ionic environment.$^{50,57,58}$ More importantly, in addition to conformational stacking, when the HJ is in the open conformation it can undergo sequential base pair exchanges at the crossover (junction) point which represents another dynamic property called branch migration (BM).$^{59,60}$

1.4.1 Branch migration (BM)

BM is a critical process during genetic recombination and DNA damage repair. Essentially, it is the movement of the crossover or junction point along the DNA axis through progressive base-
pair rearrangements, resulting in the extension of the heteroduplex arms and the transfer of genetic information between the exchanging DNA molecules. This is a spontaneous process that favorably occurs when the HJ is in its open form through base-pair rearrangements between regions of homologous sequences in the two exchanging strands (Figure 1.4A). The extent and efficiency of BM is important for stabilizing the repair intermediate (HJ), ensuring efficient genetic exchange, and the successful dissociation of the HJ into recombinant DNA molecules. Failure to remove the HJ due to a deficiency in the process has been associated with DNA damage and cell death. Therefore, it is critical to determine the mechanistic details of BM such as how fast or slow it is (i.e., kinetic parameters), how it is regulated by proteins (e.g., Ruv proteins in E. coli), and how it is affected by the surrounding environments.

Panyutin and coworkers have previously investigated BM and found that the rate is highly sensitive to metal ions (such as Mg$^{2+}$), where it is dramatically accelerated at Mg$^{2+}$ concentrations between 100 – 300 µM. Specifically, it was reported that one BM step takes ~300 ms in the presence of high Mg$^{2+}$, but is 1000 times accelerated in the presence of Na$^+$ than Mg$^{2+}$. This was attributed to the structure of HJ where the base stacking at the crossover point is disrupted in the absence of Mg$^{2+}$ resulting in increased sampling of the open form that enables BM (Figure 1.3A). Furthermore, they have also shown in another study that spontaneous BM is impeded by single-base mismatches in the DNA sequence (insertion or deletion), and the effect was found to be more pronounced in the presence of Mg$^{2+}$. However, despite these important findings revealing that HJ structure and sequence composition are limiting factors in dictating the rate of BM, there is limited information on the dynamics and kinetics associated with the BM process especially under biomimetic environments. This is particularly relevant as BM is a process that involves the exchange of base pairs through a series of hydrogen bond breakage and reformation
at the crossover point connecting the two DNA strands (Figure 1.4B).\textsuperscript{63} Also, given the effects of sequence context on HJ structural variations as described previously,\textsuperscript{61,65} it makes it plausible to assume that BM is affected by the local microenvironment. Therefore, it can be hypothesized that, in addition to ionic conditions, BM is likely to be sensitive to changes in the solution composition.

![Figure 1.4](image_url)

**Figure 1.4.** (A) Schematic of a HJ model in the open form with a homologous (inverted repeat) sequence of bases on opposite arms to allow BM. (B) zoomed-in illustration of base-pair rearrangements at the crossover point. Arrows indicate the movement of the bases at the junction (boxed) to form new pairs with complementary bases on the opposite strand. This process requires breaking and reforming new hydrogen bonds between the exchanging bases (adapted from ref 62).\textsuperscript{62}

A vast majority of previous reports on BM were done in dilute ionic environments, which are very far from the biologically relevant conditions.\textsuperscript{59,61,63,64} The intracellular environment is known to be molecularly crowded. In addition to the large macromolecules such as proteins, DNAs, RNAs, etc., small hydrophilic molecules (osmolytes and metabolites) are also highly concentrated in the cell (altogether accounting for ~40\% of the solution mass).\textsuperscript{66–68} This significantly affects the
physicochemical properties of the intracellular environment including the dielectric constant, diffusion and activity of water as well as the affinity of ions for DNA. Consequently, the thermodynamic and kinetic properties of nucleic acids and biomolecular processes inside the cell are different from those predicted in dilute conditions. Therefore, there is a lack of knowledge regarding the dynamics and kinetics of BM under biologically relevant environments. Motivated by this, we developed a mixed-solvent system using cosolutes to investigate BM of a mobile HJ analog under biomimetic environments. We revealed new insights on the kinetics of BM using our simplified biomimetic platform (discussed in Chapter 3), which may eventually prove useful in understanding the biological roles of HJ, the proteins that bind to it, and in targeting the HJ for therapeutic applications (discussed later in Chapter 4).

In addition, the lack of kinetic insights about BM generally stems from the lack of experimental techniques that are sensitive to conformational dynamics. Most of the previous studies used ensemble tools such as X-ray scattering, NMR, and gel electrophoresis to probe HJ dynamics, but these are limited due to ensemble averaging and the need for synchronization of BM dynamics. Also, given that BM is characterized as a one dimensional random-walk process with an equal probability of migrating in either direction along the homologous sequence on a timescale of micro- to milliseconds, techniques with high spatial and temporal resolution are needed. In this regard, single-molecule microscopy techniques enable real-time observation and tracking of the dynamics of individual molecules with high resolution and without the need for ensemble averaging. Therefore, to probe BM under biologically relevant conditions, in this project we have developed an experimental platform using a mobile HJ analog and single-molecule FRET, which allows real-time monitoring of these fast dynamic events (see section 1.8 for a full description of FRET).
1.5 Targeting the Holliday junction for therapy

Besides the biological importance of developing a mechanistic understanding of BM for DNA repair, this process also bears therapeutic significance especially in the field of cancer therapy. Generally, most cancer cells are deficient in the cellular pathways that govern the DNA damage response, which is viewed as a therapeutic advantage to exploit against cancer cell proliferation.\textsuperscript{73,74} More importantly, since DNA repair is a commonly used mechanism for cancer therapy resistance, this in turn provides a rationale for exploiting the repair system to sensitize cancer cells to anticancer agents (such as radiotherapy and chemotherapeutic agents).\textsuperscript{74} Among the several repair pathways, HR is one of the most important DNA damage repair pathways in cancer cells. Further, HR is preferentially employed during the S and G2 phases of the cell cycle (i.e., when a homologous DNA template is available), and therefore the outcomes of repair deficiency are known to be more significant in dividing cancer cells than in non-dividing cells of a healthy tissue.\textsuperscript{75–77} Therefore, it may be possible to make cancer cells more sensitive to DNA damaging agents by artificially interfering with the HR pathways.

Previous efforts have mainly focused on targeting the repair proteins associated with HR using various small-molecule inhibitors.\textsuperscript{78–80} However, due to the functional redundancy of the repair proteins where they play multifaceted roles to ensure genomic stability in cancer cells,\textsuperscript{81} the development of HR inhibitors aimed at targeting proteins remains a challenge. Alternatively, focusing on non-protein targets in HR can have a greater effect for the treatment of cancer. In that regard, artificially targeting the central intermediate of HR, that is the HJ, can open a novel therapeutic approach. Given the importance of the formation and resolution of HJ in genetic recombination and DNA damage repair, it is deemed an attractive target for cancer therapy. From
a structural viewpoint, the central cavity of HJ (binding pocket ~250 angstrom) makes it an ideal druggable target that can accommodate suitable ligands, thus exhibiting an underlying potential in drug design. Therefore, there has lately been a growing interest in developing ligands that target HJs for therapeutic interventions.

For example, Segall’s group have designed a set of hexapeptides and investigated their binding to HJ. These peptide ligands, of which peptide WRWYCR showed the highest potency, were found to bind to the open form of HJ and prevent repair proteins from binding to the junction (Figure 1.5A). Additionally, WRWYCR was shown to inhibit DNA unwinding by RecG helicase as well as HJ resolution by the RuvABC protein complex. However, although their biochemical activities have been characterized, there are some challenges associated with the efficacy of these ligands, limiting further applications. For example, it was found that the activity of WRWYCR is dependent on the formation of a dimeric structure via disulfide bonds (Figure 1.5B). This is not desirable considering the highly reducing cellular environments due to the susceptibility of the disulfide bond to reduction, leading to the inactive monomer form of the peptide. Moreover, other previously reported small-molecule ligands that bind HJ which include acridine dimers, Pt complexes, cyclic thioureas and guanidines, etc. have shown a high affinity for dsDNA, resulting in high toxicity levels due to nonspecific binding. Therefore, given these limitations, advancements in discovering new classes of compounds are currently underway in the field of HJ targeting.
More recently, a novel compound called Berzosertib (VE-822) known for its inhibitory effects against ATR kinase, a key regulator of the DNA damage signaling pathway, was identified as an effective HJ stabilizer and inhibitor (Figure 1.6A).\(^{90-93}\) VE-822 was screened from a DNA damage and repair library of 160 compounds and it was the only compound that was able to effectively promote the assembly of HJ (Figure 1.6). For example, using electrophoretic mobility shift assay (EMSA) of either unannealed (37 °C) or annealed (97 to 37 °C) HJs at various VE-822 concentrations (0 – 50 µM) resulted in clear band shifts from unassembled DNA to higher molecular weight bands (Figure 1.6B). This study reported that the half maximal effective concentration of VE-822 (EC\(_{50}\)) was \(~7.6\) µM for unannealed and \(~18.2\) µM for annealed HJs (Figure 1.6B).\(^93\) However, the structural effects of VE-822 binding to HJ and its impact on the BM activity remain elusive. Furthermore, the previous study mainly used ensemble assays (gel electrophoresis, fluorescence quenching, CD, etc.) which lack the ability to provide information about the conformational dynamics associated with the ligand-HJ interaction that would be easily accessible through single-molecule analyses. Therefore, using our single-molecule FRET platform

**Figure 1.5.** (A) Structure of WRWYCR peptide dimer docked into the Cre-\(\text{lox} P\) HJ. Stacking interaction between the amino acids W1a, Y4a, W1b and W3b and the HJ bases (highlighted in orange) are depicted in the structure. (B) WRWYCR dimeric form shown in the trans-conformation (taken from ref 84).\(^{84}\)
(see section 1.8 for an overview), we sought to probe the binding of VE-822 to HJ and its effect on the BM activity, which is essential for further characterization of the therapeutic role of this compound and for prompting further molecular design and drug discovery (discussed later in Chapter 4).

Figure 1.6. (A) Screening and identification procedure of the HJ-binding ligand VE-822 along with its structure. (B) Assembly of HJs (unannealed or annealed) upon treatment with VE-822 at increasing concentrations via EMSA (taken from ref 92).92

Along this therapeutic research line and via collaborative efforts, our group is also pursuing engineering of cell surfaces with biomolecules such as DNA aptamers for targeted applications. My project specifically dealt with biomolecular targeting of cytokines such as tumor necrosis factor-alpha (TNFα) for potential islet transplant therapy in the treatment of type 1 diabetes (T1D). T1D is an autoimmune disease that poses a major healthcare concern worldwide. According to the statistics of a recent modeling study, T1D cases were estimated to be over 8 million in the year 2021, which is predicted to increase up to 17.5 million cases by 2040.94 Further, according to the international diabetes federation (IDF), around 1 million young individuals (<20 years) are diagnosed with T1D worldwide with an anticipated annual increase by more than 100,000
T1D leads to lifelong insulin deficiency caused by the autoimmune destruction of pancreatic beta cells that produce insulin. These beta cells form a significant component of the islets of Langerhans which are located in the pancreas (~60-80%). Moreover, this autoimmune disease is also associated with hyperglycemia (high blood sugar) and severe long-term complications (such as blindness, kidney failure, heart disease, etc.), which can reduce the quality of life and result in a substantial financial burden for patients and health care systems. Currently, insulin replacement therapy is the mainstay of T1D treatment. This usually requires lifelong administration of insulin through daily injections or pumps as well as constant monitoring of glucose levels and intensive diet treatments. However, although this treatment method is good at keeping T1D under control, it is only for a short period of time, and it does not provide physiological regulation of blood glucose. This is largely due to the problems associated with the pharmacokinetics of insulin due to insulin absorption issues. Additionally, inconsistent glucose lowering effects have been reported due to insulin resistance and the lack of the prepared insulin analogues to fully replicate the biological functions of endogenous insulin. Together, coupled with the fact that exogenous insulin treatments can be very costly with billion dollars spent yearly on health care, there is an urgent need to identify alternative therapeutic approaches to restore glycemic control with long-term insulin independence.

1.6 Islet cell transplant (ICT) therapy

Among recent advancements in the treatment of T1D, one promising approach as an alternative to exogenous insulin treatment is to restore the lost beta cell mass either by a whole pancreas transplantation or by transplanting only the islets of Langerhans also known as islet cell transplantation (ICT). Although transplanting the pancreas has been shown to result in overall high survival rates among diabetic patients, this procedure involves major surgery and
prolonged hospitalization. Alternatively, ICT offers a noninvasive approach to treat T1D without
the need for major surgery or hospitalization, and is also independent of exogenous insulin. In fact,
the results of a study comparing the efficiency of islet transplantation to the intensive insulin
therapy showed that 84% of the ICT recipients had restored normoglycemic levels 6 months post
transplantation, whereas none of the patients receiving insulin treatment showed such
improvement. In ICT, the islets are extracted from the pancreas of the donor after which they
are purified, processed and transferred (or infused) into the intraportal vein and throughout the
liver of the recipient. With the current advances in donor selection, islet isolation methods, and
immunosuppressive treatments, achieving long-term insulin independence has been reported for
more than 50% of ICT recipients. However, despite the promising outcomes of ICT in terms
of enhancing the quality of life of T1D patients suffering from issues with blood glucose control,
one major limitation of this technique is the significant loss of more than 50-80% of the cell mass
during and post islet infusion mainly due to an innate reaction called instant blood-mediated
inflammatory reaction (IBMIR). This reaction is elicited when islets come in direct contact
with blood, and is characterized by the activation of coagulation and complement systems and
infiltration of leukocytes into the islets. It has been shown that the common underlying cause
behind the impaired function of islet engraftment and that largely contributes to IBMIR is the
secretion of inflammatory mediators known as proinflammatory cytokines (Figure 1.7). in fact,
clinical data have revealed elevated levels of cytokines such as interleukin (IL)-1β, tumor necrosis
factor (TNF)-α, and interferon (IFN)-γ immediately after islet transplantation. This in turn
results in the disruption of the integrity of islets and the rejection of the transplanted cells by the
immune system. Importantly, TNF-α induces central mediators of this inflammatory reaction
including nuclear factors (NF), cell surface molecules, and proteins like IL-1β and others.
Therefore, TNFα constitutes a key therapeutic target for the treatment of transplant rejection and enhancing T1D therapy.

**Figure 1.7.** Schematic representation of the different factors that trigger an inflammatory reaction in islet grafts. Highlighted are the main causes of inflammation representing obstacles in clinical transplant, which include donor factors, islet isolation procedures, peri-transplant reactions (IBMIR), and post-transplant factors (hypoxia, complement activation, coagulation, etc.). Taken from ref 110.110

Previous reports have shown that inhibition of the cytokine TNFα using immunosuppressive drugs can lead to a significant improvement in graft survival and function.117 The most common TNFα inhibitors reported to date include monoclonal antibodies (e.g., infliximab), humanized antibodies (e.g., adalimumab), and TNFα receptor fusion proteins (e.g., etanercept).116,118 Despite that these conventional inhibitors were found to be pharmacologically effective in inhibiting TNFα and enhancing the viability of the transplanted islets,117,119 their systematic clinical use is limited due to their high costs and increased immunogenic effects leading to nonspecific suppression of the immune response and increased risk of infection.120 For example, it was demonstrated that using high doses of etanercept (> 10 µg/ mL) can lead to reduced human islet function and integrity, which works against suppressing transplant rejection.121 Therefore, the shortcomings of
the current immunosuppressive protocols have inspired the development of new strategies to circumvent immunogenic reactions and protect the morphological and functional aspects of the transplanted islet tissue.

One major approach introduced recently is the surface modification or engineering of islet cells to protect them against IBMIR. To date, several surface-modification approaches have been identified using biocompatible polymers. The idea behind this technique is to form a protective layer on the islet cell surface by introducing various functional groups on the surface either through covalent binding to amino groups on membrane proteins, hydrophobic interactions between amphiphilic polymers and lipid bilayers, or electrostatic interactions of polyions (Figure 1.8). For example, polyethylene glycol (PEG) conjugated with N-hydroxyl succinimide (NHS) can be covalently linked to the cell membrane of islets. However, this modification is known to be difficult to control due to unselective conjugation of PEG with all cell-membrane proteins, thus disturbing membrane protein function. Another example of surface modification via electrostatic interactions involves the use of anionic or cationic polymers such as polyethyleneimine (PEI) and polyallylamine (PAA). However, the cell membrane is quite vulnerable and easily attacked in this method and therefore most cationic polymers cause severe cytotoxic effects and damage to islet cells. Hence, there is a need for alternative cell-surface modifying molecules that are capable of inhibiting cytokine-induced inflammatory responses while overcoming the unwanted immunogenic reactions. To this end, cell-surface engineering using synthetic nucleic acids known as aptamers can serve as a powerful tool for targeted therapy.
Aptamers are a special class of biomolecules that have emerged as powerful agents for molecular recognition in the fields of diagnostics and therapeutics. They are defined as short single-stranded DNA or RNA sequences (~20-100 nucleotides) that can fold into secondary and tertiary structures, allowing them to bind to various targets (small molecules, proteins, peptides, etc.) with high affinity and specificity just like an antibody-antigen interaction.\textsuperscript{128–130} Aptamers are therefore considered the chemical counterparts of antibodies, however, they have the advantage of being relatively smaller in size, less expensive, and highly specific with minimal immunogenic effects.\textsuperscript{129,131} Moreover, unlike antibodies that suffer from potential batch-to-batch variations and nonreproducible performance, aptamers are unique in the way they are identified which is through

\textbf{Figure 1.8.} (a) Chemical modification of cell surface with synthetic polymers either by covalent bonding, hydrophobic or electrostatic interactions, and by layer-by-layer method. (b) Immobilization of bioactive molecules to the cell surface via polymer-cell membrane interactions (taken from ref 122).\textsuperscript{122}

\textbf{1.7 Aptamers}

Aptamers are a special class of biomolecules that have emerged as powerful agents for molecular recognition in the fields of diagnostics and therapeutics. They are defined as short single-stranded DNA or RNA sequences (~20-100 nucleotides) that can fold into secondary and tertiary structures, allowing them to bind to various targets (small molecules, proteins, peptides, etc.) with high affinity and specificity just like an antibody-antigen interaction.\textsuperscript{128–130} Aptamers are therefore considered the chemical counterparts of antibodies, however, they have the advantage of being relatively smaller in size, less expensive, and highly specific with minimal immunogenic effects.\textsuperscript{129,131} Moreover, unlike antibodies that suffer from potential batch-to-batch variations and nonreproducible performance, aptamers are unique in the way they are identified which is through
an in-vitro selection process called SELEX (systematic evolution of ligands by exponential enrichment). This aptamer selection process consists of an iterative cyclic procedure (5-16 rounds) that is carried out in vitro and thus it allows to adjust experimental conditions as needed to achieve the best performance of the desired aptamer (Figure 1.9). Briefly, a synthetic nucleic acid library of ~$10^{15}$ different sequences is first incubated with the target of interest, followed by removing the unbound sequences, and finally amplifying the bound molecules (Figure 1.9). Additionally, aptamer synthesis offers flexible and site-specific chemical modifications that enhance their affinity, in-vivo stability, and diversify their applications.

Figure 1.9. Schematic representation of the in-vitro aptamer selection process called SELEX. The cycle consists of three main steps: incubation of the library (ssDNA or RNA) with the target, separation of unbound from bound sequences, and amplification of the best-bound sequences by PCR for DNA or RT-PCR for RNA libraries. After repeating the process (5-16 cycles), the enriched library is cloned and sequenced (taken from ref 130).
Due to the unique advantages of aptamers as described above, they have gained a broader attention in biomedical applications and targeted therapy.\textsuperscript{136} For example, aptamer-based surface engineering of mammalian and bacterial cells has been previously employed to visualize membrane structures, modulate the biological functions of membrane-bound proteins, and detect bacterial pathogens and protein biomarkers for cancer diseases.\textsuperscript{136–138} Therefore, given their applicability as cell-surface modifying agents, we have investigated the potential of a group of SELEX-generated aptamers for use as anti-inflammatory and immune modulating agents for islet cell engineering to protect against cytokine-induced inflammation and cell death. To this end, we have designed a single-molecule approach based on the smFRET technique (described below) to probe the binding of aptamers to the cytokine TNF\(\alpha\). This is important for determining the ability of our aptamer-based approach to block inflammatory responses associated with this cytokine for islet transplant therapy and T1D treatment. In this project, the bioactivity of these aptamers was also investigated using in-vitro functional assays in collaboration with another research group (Kanak group from VCU surgery department). In addition, NMR studies for TNF\(\alpha\) structural verification were also conducted in collaboration with the Fuglestad group (VCU chemistry department). The collective results from the single-molecule analyses that we performed along with the NMR and in-vitro assays conducted by our collaborators are delineated in detail later in Chapter 5.\textsuperscript{139}

1.8 Single-molecule fluorescence resonance energy transfer (smFRET)

Fluorescence resonance energy transfer (FRET) is a powerful technique that allows studying biomolecular dynamics and tracking even asynchronous processes at the single-molecule level.\textsuperscript{140,141} FRET is a nonradiative energy transfer process that occurs between a donor and an
acceptor fluorophore due to long-range dipole-dipole interactions between the excited electronic state of the donor and the ground state of the acceptor (Figure 1.5A).\textsuperscript{141,142} For FRET to efficiently occur, a few requirements must be satisfied: (i) significant spectral overlap between the donor emission spectrum and the acceptor excitation spectrum (Figure 1.5B) and (ii) appropriate distance between the donor and acceptor such that they are in close vicinity with a distance range of 1-10 nm.\textsuperscript{143,144} Compared to ensemble methods, smFRET largely overcomes ensemble-averaging and allows the detection of transient intermediates as well as individual species in heterogeneous and dynamic biomolecular complexes (e.g. nucleic acids and proteins).\textsuperscript{140} In addition, the ability to reliably extract quantitative kinetic and conformational data from smFRET measurements serves as an asset for complex biological processes (such as BM).

![Figure 1.10](image.png)

**Figure 1.10.** (A) a simplified Jablonski diagram illustrating the FRET mechanism. (B) excitation and emission spectra of donor and acceptor highlighting the spectral overlap requirement between donor emission and acceptor excitation for FRET to occur. (taken from ref 141).\textsuperscript{141}

The FRET efficiency can be used as a spectroscopic ruler to report on distances within the molecular scale and it is related to the inter-dye distance using the following equation:
\[ E_{\text{FRET}} = \frac{1}{1 + \left( \frac{R}{R_0} \right)^6} \]  

(1)

where \( E_{\text{FRET}} \) is the efficiency of energy transfer, \( R \) is the inter-dye distance, and \( R_0 \) is the distance corresponding to 50\% energy transfer. Therefore, FRET is highly sensitive to small changes in the inter-dye distance. Given all these advantages, we used smFRET to study HJ conformational dynamics (such as BM), HJ-binding small molecules, and binding interactions between cytokine-specific aptamers and TNF\( \alpha \) for both biological and therapeutic interventions.
CHAPTER 2 – METHODS

All the materials and methodologies outlined below apply to all projects. Further information specific to each project is covered in the corresponding Chapters 3 – 5.

2.1 DNA construct assembly

DNA constructs were rationally designed from synthetic DNA oligonucleotides (see Appendix 1 and 2 for sequence details for DNA constructs used in Chapters 3-5) using the principle of base-pair hybridization of complementary sequences. All DNA strands utilized in the projects described in Chapters 3-5 were purchased from Integrated DNA Technologies (IDT, Coralville, IA) and 100 µM stocks dissolved in double sterile water were stored at -20°C. To assemble the DNA constructs, the constituent single-stranded DNA (ssDNA) oligonucleotides were thermally annealed at ~1 μM concentrations (unless otherwise specified, Chapter 5) in an aqueous 1× TAE-Mg²⁺ buffer (40 mM tris(hydroxymethyl)aminomethane (Tris), 20 mM acetic acid, 1 mM ethylenediaminetetraacetic acid (EDTA), 12.5 mM MgCl₂, pH 7.4). Thermal annealing was executed by heating the solution at 95°C and gradually ramping the temperature down to 4°C in a thermal cycler, T100, Bio-Rad (Table 2.1) as described in our previous publications.¹⁴⁵,¹⁴⁶ This temperature ramping procedure helps to first melt and break any secondary structures formed by the ssDNA oligonucleotides and then slowly promote the formation of the most thermodynamically favorable structure at lower temperatures.

**Table 2.1.** Thermal annealing program used for the self-assembly of DNA constructs.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>5</td>
</tr>
</tbody>
</table>
2.2 Prism-based Total Internal Reflection Fluorescence (pTIRF) microscopy

Single-molecule imaging and fluorescence measurements were done using a custom-built prism-based total internal reflection fluorescence microscope (pTIRFM). The pTIRF setup (Fig. 2.1) offers a high signal to noise ratio (S/N) with minimal interference from background fluorescence enabling single-molecule studies. The excitation sector shows two lasers: a 532 nm (CrystaLaser, CL-532) and a 639 nm (Coherent Technologies, CUBE laser) that are collimated through a
dichroic mirror and are then directed through a focusing lens into the prism. An evanescent field is created at the interface between the quartz slide and the buffer as a result of the total internal reflection of the laser beams within the prism, whose intensity exponentially decays at a depth of a few hundred nanometers from the slide surface (typically 100-200 nm). This in turn allows the efficient excitation and imaging of only those fluorescent molecules that are within a few hundred nanometer of the surface. In the emission sector, the fluorescence emission from Cy3 (green; donor) and Cy5 (red; acceptor) fluorophores are first captured by an inverted objective and then routed to an optosplit II through which the collected fluorescence is split into green and red emissions to detect Cy3 and Cy5 emission intensities, respectively. Finally, the fluorescence emission output is directed into an electron multiplying charge coupled device (EMCCD) camera where it is fed into a computer for signal processing.

Figure 2.1. Schematic diagram showing the excitation (a), the focusing and beam positioning (b) and the emission (c) sectors. Green and red lines represent 532 nm and 639 nm lasers, respectively (taken from ref 147).
2.3 Flow-cell assembly and surface functionalization

The standard quartz slides (75 × 26 × 1 mm) needed for flow cell assembly were first drilled diagonally with a diamond-coated bit using a Dremel multitool and cleaned by scrubbing with an Alconox paste and successively rinsing with deionized water, acetone and ethanol. The slides were then flamed using a propane torch for 20 sec on both sides to remove any fluorescent contaminants and subsequently submerged in a boiling aqueous solution containing 4% hydrogen peroxide and 4% ammonia for 30 min. The flow cells were constructed as described in a published protocol. In Figure 2.2, a parafilm into which the sample chamber was cut diagonally to enclose the drilled holes was first placed on the microscope slide and then a glass coverslip (24 x 60 mm, Fisher Scientific) was placed over the parafilm. The whole assembly was then sealed by heating at 98°C for 5 min on a hot plate. 200 μL plastic pipette tips were cut to ~ 1-inch long, inserted into the drilled holes, and plumbed with tubing (0.02 in. ID, 0.06 in. OD, Cole-Palmer) – both the pipette tips and tubing were affixed using Double Bubble Quick-Set epoxy from Hardman Adhesives. To prepare the slides for single-molecule imaging, they were first functionalized with 1 mg/mL biotinylated BSA (incubation time ~ 5 min) followed by 0.2 mg/mL streptavidin (incubation time ~ 2 min). The flow cell was then flushed with 1× TAE-Mg²⁺ buffer (pH 7.4) to wash away unbound streptavidin. The functionalized flow cells were stored at 4°C if not used immediately after preparation.
Prior to fluorescence imaging, the functionalized flow cells were initially photobleached by using both the green (λ = 532 nm, ~32 mW) and red (λ = 639 nm, ~22 mW) lasers through the custom-built pTIRF microscopy\textsuperscript{148} to remove any background fluorescence. Once the buffer channel has been exposed to the laser and an adequate path photobleached, the slide was incubated for 45 sec with ~20-50 pM HJ construct dispersed in 1× TAE-Mg\textsuperscript{2+} and a 1× oxygen scavenging (OSS) buffer solution (2 mM Trolox, 5 mM PCA, and 50 nM PCD)\textsuperscript{150}, after which it was flushed with 1× TAE buffer to remove unbound HJ molecules. Data acquisition was done after incubating the slide with an imaging buffer (see Chapters 3-5 for more details on the buffer composition). Briefly, the Cy3-labeled molecules were continuously excited with a 532 nm green laser and the fluorescence emissions from both the Cy3 and Cy5 fluorophores were simultaneously recorded for the green and red channels (512 × 256 pixels) using an EMCCD camera (iXON 897, Andor). FRET movie recordings were conducted at a specific sampling rate (i.e., time resolution) depending on the temporal resolution at which the processes under study occur. The presence of an active FRET
pair (i.e., both Cy3 and Cy5) on individual molecules was confirmed at the end of each movie by directly exciting the Cy5 fluorophores with a 639 nm laser. All single-molecule experiments were performed at room temperature (23°C).

2.5 Data acquisition and processing

FRET movies from the single-molecule experiments were processed into trace files using IDL and MatLab scripts obtained from the smFRET data acquisition and analysis package, which was available for download from the Ha Lab (https://cplc.illinois.edu/research/tools). Briefly, the .pma files generated by the Single.exe software were fed into IDL which then processes them and creates trace files that are recognized by MatLab written scripts. Upon running these scripts, the generated intensity-time and FRET traces were manually screened for subsequent analysis provided that they satisfied the following criteria: (i) single-step photobleaching of fluorophores; (ii) presence of both Cy3 and Cy5 signals; and (iii) total fluorescence intensity of Cy3 and Cy5 exceeding ~100 per frame. The FRET efficiency ($E_{\text{FRET}}$) was calculated using the following equation:

$$E_{\text{FRET}} = \frac{I_A}{I_D + I_A}$$

(2)

Where $I_A$ and $I_D$ denote the background-corrected fluorescence intensity of acceptor and donor, respectively.\textsuperscript{151,152}
CHAPTER 3 – SINGLE MOLECULE ANALYSIS OF DNA BRANCH MIGRATION UNDER BIOMIMETIC ENVIRONMENTS

3.1 Introduction

Genomic DNA is continuously exposed to a wide range of carcinogens and other intra- and extracellular factors that are detrimental to DNA, leading to the development of various DNA lesions including the harmful double-strand breaks (DSBs).\textsuperscript{23,24} DSBs are arguably the most deleterious forms of DNA damage, which primarily arise from exposure to reactive oxygen species (cell’s own metabolic products) and exogenous agents such as ionizing radiation or clastogens.\textsuperscript{26,153} To maintain genetic integrity, organisms have evolved multiple regulatory pathways among which homologous recombination (HR) plays a crucial role in repairing DSBs.\textsuperscript{28,29,38} Although HR is a high-fidelity repair mechanism, defects in the pathway can cause DSBs to be mis-repaired or even unrepaired, undermining the integrity of the genome and causing a variety of human diseases, such as degenerative diseases, immunodeficiency, and particularly cancer.\textsuperscript{30,154,155} While HR has been extensively studied,\textsuperscript{14,20,27,30,41,156} the molecular insights/kinetics of the repair steps and molecular mechanism(s) by which different DNA-repair proteins operate inside cells are not fully understood. Therefore, it is crucial to dissect the steps involved in the recombination process at the molecular level to identify the origin of repair defects and develop potential genomic tools to target the HR pathway for therapeutic applications.

One of the critical steps during HR is the strand exchange between homologous DNA molecules that leads to the formation of a cross-strand DNA intermediate called the Holliday junction (HJ).\textsuperscript{35,41,157} Because of their critical roles in HR, HJs are known to be the central intermediates of the DSB repair pathway. A number of studies have been performed using both
the isolated and genomic HJs toward understanding their molecular properties. For example, previous studies suggested that the isolated HJs can adopt two types of conformational dynamics, namely the flip-flop motion, also known as stacking conformer exchange, and the movement of the crossover point along the DNA axis as a result of base-pair rearrangement, i.e., branch migration (BM).\textsuperscript{14,46,51,60} Stacked conformations of HJs are known to be stabilized by divalent metal ions (primarily Mg$^{2+}$), and the isomer transition rates are reduced in the presence of high salt concentrations. On the other hand, the open form of the junction that mediates conformer exchange and allows BM is more prevalent in the absence of divalent cations such as Mg$^{2+}$.\textsuperscript{56,59,63,158} While the ionic effects on HJ properties by Mg$^{2+}$ and Na$^+$ have already been established, there is limited information about the kinetics and dynamics of BM – especially under intracellular environments. Therefore, the focus of this article is to determine the effect of cosolutes on BM by conducting single-molecule experiments on a mobile HJ analogue in biomimetic environments.

Analysis of HJ dynamics and BM under intracellular environments such as mixed-solvent and molecularly crowded systems can serve as a suitable platform not only to determine the kinetic behavior of these processes inside the cells but also to understand the role of HJ-binding proteins in HR.\textsuperscript{48,159–162} Recent findings have shown that the abundance of various ions, metabolites, osmolytes, and macromolecules (e.g., proteins, lipids, nucleic acids, etc.) in cellular milieus can contribute to the dielectric and crowding properties of the cell – thus impacting the kinetics and dynamics of various biological processes.\textsuperscript{66,163,164} In particular, these intracellular factors can directly and indirectly affect the conformational dynamics of nucleic acids by changing the activity of water and generating what is known as the excluded-volume effect.\textsuperscript{66,165–167} Since BM of HJs is a key step in HR that stabilizes this cross-strand intermediate and determines the extent of genetic exchange between homologous sequences, it is important to determine how this process operates
in biomimetic environments. For example, BM involves a series of hydrogen-bond dissociation/association events between the base pairs of exchanging DNA sequences, making it highly sensitive to changes in water activity.\textsuperscript{63,158} Thus, probing the effects of water depletion and macromolecular crowding on BM can provide an insightful view of the recombination mechanism.

Herein, we employed single-molecule Fluorescence Resonance Energy Transfer (smFRET)-based analyses to investigate BM under biomimetic conditions.\textsuperscript{152,168} Inspired by the previous studies showing that certain organic cosolvents are capable of disrupting the short-range and long-range water-water interactions, which in turn affects the thermodynamics of DNA structures and associated processes,\textsuperscript{68,169} we first tested a binary mixture of dimethyl sulfoxide (DMSO) and water to determine the dynamics and kinetics of BM at the single-molecule level. Interestingly, our synthetic, mobile HJ exhibited an enhanced BM in the presence of DMSO. Likewise, we used polyethylene glycol (PEG 6000), a water-soluble polymer, as an artificial crowding agent to simulate intracellular crowding.\textsuperscript{170,171} Compared to the BM kinetics without PEG, we observed a significant increase in kinetics in the presence of PEG. In addition, our kinetic data showed that mixed solvent and crowded systems maintain equilibrium between BM steps allowing a bidirectional movement of the junction, presumably due to changes in the DNA-water environment. Taken together, through systematic single-molecule analyses of a model HJ construct with a region of homologous sequence, we have revealed interesting kinetic data governing the BM process. These single-molecule kinetic analyses have provided insights into the BM process, which would otherwise be difficult to discern using ensemble studies due to averaging.\textsuperscript{141} Further, the single-molecule platform demonstrated here can be adapted to investigate other recombination DNA intermediates and study the role of HJ-binding proteins
which may inspire the development of HJ-binding small molecules (e.g., drugs) for potential therapeutic interventions.

3.2 Materials and Methods

Chemicals. Tris(hydroxymethyl)aminomethane (Tris), ethylenediaminetetraacetic acid disodium salt (EDTA), acetic acid, potassium chloride, glycerol, biotinylated bovine serum albumin (bBSA), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were all purchased from Fisher Scientific. Biotium Gel Red 10000X/DMSO and protocatechuate 3,4-dioxygenase (PCD) were also purchased from Fisher Scientific, where PCD was suspended in a pH 8.0 PCD buffer (100 mM Tris-HCl, 50 mM KCl, 1 mM EDTA, 50% glycerol), sterile filtered using a 0.2 μm filter (Thermo Fisher Scientific), and stored at −20 °C until needed. Magnesium chloride hexahydrate and poly(ethylene glycol) (PEG 6000) were purchased from Arcos Organics. Sodium chloride, streptavidin, protocatechuic acid (PCA), 30% ammonia/water, hydrogen peroxide, and agarose were purchased from VWR. Dimethyl sulfoxide (DMSO, HPLC grade, 99.9+) was purchased from Alfa Aesar.

DNA constructs. DNA strands needed for preparing the DNA constructs containing a homologous region labeled mobile or a nonhomologous region that is labeled immobile for the control construct (described later) were custom-designed (see Appendix 1 and Fig. 3.1). The constituent DNA strands that make up the mobile and immobile HJ analogs were assembled by thermal annealing at 1 μM concentration. The detailed annealing protocol is described in Chapter 2.
Electrophoretic Mobility Shift Assay (EMSA). In addition to the single-molecule analysis (described below), the formation of the HJ construct was also characterized by using native agarose gel electrophoresis (Figure 3.2). A 2% agarose gel was prepared at 30 mL total volume by mixing agarose, 1× TAE, and deionized water. The mixture was heated in a microwave for 45 s, after which 3× Gel Red (Biotium) was added for precast staining. A 100 bp ladder was used as a molecular weight (MW) marker which was injected along with strand R (44-nucleotide ssDNA control), a two-strand control (strands R and B), a three-strand control (strands R, B, and T), and

Figure 3.1. DNA construct design of the mobile HJ. The HJ construct consists of four custom-designed ssDNA sequences that hybridize to one another as depicted. A homologous region containing six base pairs (bps) on the top and bottom arms was incorporated to enable branch migration of this region. The Cy3 and Cy5 fluorophores were labeled internally on opposite arms outside the 12-bp homology region to allow for a broader range of FRET efficiency. B, T, L, and R represent the bottom, top, left, and right strand respectively.
the HJ construct into their designated wells. The gel was cast, immersed in a buffer system containing 2 mM MgCl₂, 1× TAE, and 69 mM NaCl (pH 7.4), and run at 65 V for 60 min before imaging using a UV transilluminator.

![Agarose gel characterization of the formation of the HJ using a native electrophoretic mobility shift assay (native-EMSA). Lane 1: DNA molecular weight (MW) marker. Lane 2: 44-nucleotide (nt) ssDNA (Strand R). Lane 3: Strands R & B annealed. Lane 4: Strands R, B & T annealed to give an incomplete 3-way junction. Lane 5: complete HJ construct. Slower migration of the band in lane 5 compared to that in lane 4 confirms the successful assembly of the HJ construct. The slightly faster but less intense band in lane 5 could be because a small fraction of the construct was not fully assembled (this does not cause an issue in single-molecule experiments as only the fully-assembled molecules containing both the Cy3 and Cy5 fluorophores were selected and analyzed). The gel was stained with Gel Red (precast) prior to running it at 65 V for 60 min. The image was taken using a UV-Vis transilluminator.]

**Figure 3.2.**

**Single-Molecule Fluorescence Microscopy and Imaging**

Before conducting the single-molecule imaging experiments, the microscope slides were assembled, and flow cells were surface functionalized for DNA immobilization as outlined in Chapter 2. Briefly, the flow cells were incubated with ~20 pM HJ construct that was dispersed in...
1× TAE-Mg\textsuperscript{2+} buffer containing 10 mM Mg\textsuperscript{2+} and 1× OSS (2 mM Trolox, 5 mM PCA, and 50 nM PCD)\textsuperscript{150} for ~1 min, after which it was flushed with 1× TAE-Mg\textsuperscript{2+} buffer to remove unbound HJ molecules. The imaging buffer for the (+) cosolvent systems consisted of 1× TAE buffer, 1× OSS and the desired concentration of the cosolvent (DMSO or PEG) at 150 mM NaCl. For the (-) cosolvent control system, the same buffer composition was used except that no cosolvent was added. Data acquisition was done after incubating the slide for ~5-10 min with the imaging buffer as previously described.\textsuperscript{172} Briefly, the Cy3 labels were excited with a 532 nm green laser using our TIRFM setup (see Chapter 2 for more details) and the fluorescence emissions from both the Cy3 and Cy5 fluorophores were recorded via an EMCCD camera at 30 frames per second (30 ms exposure time at a camera gain of 260).

**Single-Molecule Data Analysis.** Recorded movies from the single-molecule experiments were processed using IDL and MatLab scripts as described in Chapter 2. Briefly, the intensity-time and FRET traces were manually screened and selected for further analysis provided that they met the following requirements: (i) single-step photobleaching of fluorophores; (ii) evidence of active Cy3 and Cy5 signals; (iii) total fluorescence intensity of Cy3 and Cy5 exceeding ~100 per frame and (iv) anticorrelated fluorescence intensity of Cy3 and Cy5 for dynamic molecules. The FRET traces were truncated to the first 20 to 30 s observation time for subsequent analysis.

**Hidden Markov Model (HMM) and transition rates.** The HMM analysis was performed using the HaMMy program provided by the Ha lab (available at http://bio.physics.illinois.edu/) to determine the underlying states for FRET trajectories under each buffer condition and extract transition rates between FRET levels.\textsuperscript{173} Only dynamic traces with clear anti-correlation of the donor and acceptor intensities were chosen at each experimental condition for a given observation time (~20-30 sec time window) and were fit globally to calculate the interconversion rates. The
HMM was initialized using 3, 4, and 5 states, but the fits with higher than 3 states did not completely match up with the raw FRET traces, suggesting that at large there were three clear FRET states. Single-molecule time-binned FRET traces at 150 mM Na⁺, 2% DMSO, and 30% PEG were analyzed for the state-to-state transitions corresponding to BM and the truncated 30 s intervals of the HMM-fitted traces are shown in the Results and Discussion section. Transition density plots (TDP, a program also released by the Ha lab and coworkers) were constructed to conduct kinetic analysis on each set of molecules and find the on- and off-rates \((k_{on} \text{ and } k_{off})\) for each FRET transition under various buffer conditions. Briefly, this program identifies genuine FRET populations and their transitions and provides two-dimensional plots of final vs initial FRET states showing as a heat map wherein the frequency with which a specific FRET transition occurs is reflected by the intensity of the spots. Kinetic rates and standard deviations are derived from the fit parameters of the Gaussian-fitted histograms generated by TDP from all the transition rates that match the selected transition criteria.

**Free Energy (ΔG) Calculation.** The change in free energy (ΔG) of the FRET transitions in various DMSO and PEG environments was calculated as follows:

\[
\Delta G = -RT \ln(K_{eq}),
\]

where \(R\) is a constant equivalent to 8.3145 kJ/mol, \(T\) is the room temperature (23 °C), and \(K_{eq}\) is the equilibrium transition rate.

### 3.3 Results and Discussion

**Designing the HJ Construct for Single-Molecule Analysis**

A schematic of the HJ design is depicted in Figure 3.3 (a more detailed schematic is shown in Fig. 3.1). The four-way junction was assembled from four custom-designed single-stranded DNA (ssDNA) oligonucleotides as described earlier in the Methods. Although the confirmation of HJ
formation comes from single-molecule imaging (described below), it was also verified by running a native 2% agarose gel (Fig. 3.2). Briefly, compared to the control 44-nucleotide DNA strand and the incomplete 3-way junction, the HJ exhibited slower mobility indicating that it was successfully assembled.

When designing the construct, one of the DNA strands was labeled with a biotin at the 5′-end to enable tethering of the HJ molecules to the streptavidin-functionalized flow cell (Fig. 3.3A). In this study, we incorporated a homology region comprising 12 base pairs (bps) of A-T repeats (6 bps on each side of the junction) into the HJ design to enable BM along the A-T repeat sequence. The rationale for this design came from the previous study which showed that the BM was promoted when inverted repeat sequences with a low G-C content were used.47 Due to the presence of mobile homology sequences, the junction is expected to undergo a bidirectional migration with a possibility of visiting many FRET states; the two extreme configurations corresponding to the lowest (herein called HJ\(_{TB}\)) and the highest (HJ\(_{LR}\)) FRET states are shown in Fig. 3.3A. In addition, opposite arms of the junction can elongate depending on the extent of base-pair rearrangement or the size of BM steps (Fig. 1B). Monitoring such a dynamic property is possible because a FRET pair was attached internally on opposite arms outside the homology region (to avoid any unforeseen effects of fluorophores on BM) such that FRET efficiency is more sensitive to BM than to isomerization between isoI and isoII (inter-dye distance does not change during the flip-flop motion, Fig. 3.4).174
Figure 3.3. Experimental design for the smFRET analysis of BM. (A) Experimental setup for TIRF analysis. The biotin-labeled HJ is immobilized onto the functionalized microscope slide via biotin/streptavidin interaction. HJ$_{LR}$ represents the open form with extended left and right helical arms, whereas the HJ$_{TB}$ represents the open form with extended top and bottom helical arms resulting from BM. The two extremes, HJ$_{LR}$ and HJ$_{TB}$, correspond to the highest and lowest FRET efficiency states, respectively. (B) Schematic elucidation of HJ structural configurations for what could be expected from an ideal stepwise BM. Estimated inter-dye distance ($R$) and FRET efficiencies for 2-bp steps of the open form conformation are shown for each configuration. These configurations are simplified and may not completely agree with the experimental FRET states observed in Fig. 3.5. BM from 1 through 7 assumes 2-bp steps (1 bp rearrangement per each homologous arm).
In addition, the construct was designed to have a ~20 bp non-homologous region on each arm to avoid migration off the ends. Based on the DNA construct and assuming an ideal BM with 2-bp steps, the change in the FRET efficiency is expected to vary between ~0.2 - ~1.0 (Fig. 3.3B) where the fluorophores move away from each other causing the FRET efficiency to drop and vice versa. The expected FRET values due to BM were estimated based on the known value of $R_0$ (5.4 nm)$^{175,176}$ for the Cy3 and Cy5 pair and assuming extended arms at ~90°, a B-form geometry of dsDNA with a width of ~2 nm, and a base-pair contour length of 0.34 nm.$^{177}$ However, it is important to note that this estimation is without considering the local microenvironments which may cause the inter-dye distances to be underestimated and the experimental FRET to vary. The relationship between FRET efficiency ($E_{\text{FRET}}$) and the inter-dye distance ($R$) is given by:

$$E_{\text{FRET}} = \frac{1}{1 + (\frac{R}{R_0})^6} \quad (2)$$

Where $R_0$ is the inter-dye distance corresponding to 50% FRET efficiency. The annealed HJ constructs were imaged for BM using a prism-based TIRF microscope (Fig. 3.3A) after immobilization of the HJ molecules on the microscope slides (see Materials and Methods for details). An oxygen-scavenging system (OSS) was added to the buffer in all experiments to retard the blinking and photobleaching of fluorophores.$^{150}$ All buffer conditions were tested at a constant ionic strength of 150 mM Na$^+$ and a pH of 7.4 to mimic the intracellular ionic environment. Since the interconversion rates between stacked structures ($iso$I and $iso$II, Fig. 3.4) are slowed down upon divalent-ion binding such as Mg$^{2+},^{46}$ no Mg$^{2+}$ ions were added to the imaging buffer to ensure sufficient sampling of the open form and increase the chances of BM.
DMSO Affects BM in a Concentration-Dependent Manner

To validate the design of the mobile HJ, we probed the BM properties in various solvent compositions (Fig. 3.5). The hypothesis was that altering the solvent composition would influence key solvent properties such as dielectric constant, viscosity, and water activity, which may affect DNA topology, stability, and ultimately the BM process. To this end, we utilized DMSO, an aprotic and amphiphilic organic molecule that is widely used in cell biology, as a cosolvent to study the effect of mixed-solvent systems on BM. All experiments were performed in a 150 mM Na\(^+\) buffer (pH 7.4). Some typical intensity-time traces as well as the corresponding FRET-time traces for three representative dynamic molecules obtained at 2% DMSO (v/v) are shown in Figure 3.5. These results showed that the HJ undergoes a series of transitions between different FRET states, especially in traces 2 and 3, with well-defined FRET values that fall within the expected range of BM. For example, in trace 2 the HJ molecule first adopted a medium FRET (~0.5) for almost 2s and then the efficiency increased to ~0.7 where the molecule resided for a longer period.

Figure 3.4. Conformational switching of the mobile HJ. In our labeling schemes (fluorophores being on the opposite arms), the isomers – iso\(_1\) and iso\(_{II}\) – are expected to exhibit a similar FRET efficiency due to having the same inter-dye distances. Whereas the open form is expected to show a variable FRET efficiency due to branch migration.
of time (~4s), after which it dropped back to a lower FRET state (~0.3) and the transitions kept repeating. Another remarkable observation is the significant increase in the anticorrelated intensity changes and the FRET states over time upon the addition of DMSO (Fig. 3.5B) as compared to the cosolvent-free environment (Fig. 3.5A), showing that the BM process is promoted by DMSO. This observation is consistent with the previous studies that the DMSO creates a water-depleted environment that destabilizes dsDNA and noncanonical DNA structures. We surmised that the weakening of hydrogen bonding and ultimately the base-pairing by DMSO resulted in increased BM.

Figure 3.5. Typical intensity-time traces and respective FRET trajectories for the dynamic population at different buffer conditions. All conditions – with and without cosolvent – were tested using 150 mM Na+. (A) Traces in the absence of a cosolvent. A slow and occasional BM was observed as a single or multi-step FRET change. The symbol (*) represents the photobleaching events so as not to confuse them with the anti-correlated intensity changes. (B) Traces in 2% DMSO (v/v). BM was seen as multi-state anti-correlated intensity changes that were relatively faster than in the cosolvent-free environment. (C) Traces in 30% PEG (w/v). Multi-state intensity traces were more rapidly changing than in the cosolvent-free and 2% DMSO environments.
In addition, the effect of DMSO on BM was systematically investigated by titrating DMSO. We assessed this by examining the fraction of dynamic molecules (approximately molecules showing evidence for BM) at different concentrations of DMSO (Fig. 3.6A). The % dynamic molecules at a given concentration of DMSO was calculated by dividing the number of single molecules that exhibited dynamic FRET for the given observation time (~30 s) by the total number of single molecules (both static and dynamic) and multiplying the value by 100. Because the photobleaching of fluorophores is a stochastic process and depends on the solution environments, to make a fair counting across various buffer conditions, we chose the 30 s time window to assign molecules to either a dynamic or a static group. Notably, we observed a clear upward trend for the proportion of dynamic molecules as the DMSO concentration was increased from 0 to 2%, of which 2% DMSO yielded the highest dynamic molecules (44 ± 3%). However, there was a slight decrease in the fraction of dynamic molecules (by ~4%) when the DMSO concentration was increased to 3%. This observation was rather surprising, and the decrease in BM is possibly due to the lower stability of newly formed base pairs at higher concentrations of DMSO, thus hindering BM.

**Figure 3.6.** Kinetic analysis of BM as a function of DMSO concentration. (A) Percentage of dynamic molecules under different DMSO environments. 0% DMSO refers to the ionic
environment control (150 mM Na+, (−) DMSO). Error bars represent the standard deviation (SD) from three different subsets of molecules collected under the same buffer condition containing more than 150 single molecules in total. (B) Equilibrium constant ($K_{eq}$) defined as $k_{on}/k_{off}$ of various FRET transitions was determined by the hidden Markov model (HMM) analysis of the FRET traces, wherein $k_{on}$ denotes the rate of junction migration to higher FRET states and $k_{off}$ denotes the rate of migration to lower FRET states. The higher the $K_{eq}$, the more favored the low-to-high FRET transition and vice versa. These rates were determined from more than 225 transitions for each DMSO concentration (227, 407, and 442 transitions for 1, 2, and 3% DMSO). Error bars represent standard deviations which were calculated by using propagation of errors.

To investigate whether the decline in the fraction of dynamic molecules is consistent at DMSO concentrations greater than 3%, we ran experiments at 5% and 10% DMSO and noticed that the trend was more or less steady with a more statistically significant drop in the dynamic percentage (Fig. 3.6A). It is, however, important to note that a significant percentage of molecules were static within the observation window for each tested concentration of the cosolvent. Some example traces with no clear evidence of BM within the 30 s time window under different buffer environments (i.e., static) are provided in Figure 3.7. The molecules that we assign as static did not show any dynamics during the first 30 s of observation. However, some of these molecules exhibited some dynamics outside this time window after a long static FRET state. Although it is not clear what triggers such a long stalling of BM, it might be due to the misalignment of base pairs in the mobile region during the rearrangement process. Some example molecules showing dynamics after a long pause are shown in Figure 3.8. However, the counting of dynamic molecules within a specified time-window (30 s) in all experiments still gives a relative comparison of how various buffer environments influenced the BM. Overall, regardless of the concentration of DMSO tested, the fraction of dynamic traces was higher in the presence of DMSO than in the DMSO-free system (25 ± 3%, Fig. 3.6A), and the dynamics of FRET transitions were much higher in DMSO, suggesting that DMSO–water binary mixtures facilitate BM.
Figure 3.7. Typical intensity-time traces and corresponding FRET trajectories for the relatively static molecules of the mobile HJ at different buffer conditions. (A) Traces in the absence of a cosolvent. High-FRET states were mainly observed as in traces 2 (~ 0.75) and 3 (~ 1.0) with a small fraction showing low-FRET states as in trace 1 (~ 0.25). (B) Traces in 2% DMSO (v/v). The vast majority of the static traces exhibited high-FRET efficiency (~ 0.75 - 1.0) as shown in the three example traces. (C) Traces in 30% PEG (w/v). Most of the static molecules showed high-FRET states as in traces 1 (~ 0.85) and 2 (~ 1.0), while some other molecules showed low FRET (~ 0.2, trace 3). All conditions were tested at 150 mM Na⁺.

To look at the effects from a kinetic perspective, we analyzed the dynamic FRET traces at 1%, 2%, and 3% DMSO using a well-known kinetic analysis algorithm for single-molecule FRET traces called Hidden Markov’s Model (HMM, described later in the Chapter). This analysis showed that the traces on average consisted of at least three clear FRET states, ~0.8, 0.6, and 0.2, with varying state-to-state transition rates and frequencies compared to the (-) cosolvent condition.
This is largely consistent with the overall FRET states shown in the histograms generated from all of the dynamic traces under each condition (Fig. 3.9), which showed three most prominent FRET states. More importantly, fitting the data with HMM enabled us to extract quantitative information of BM kinetics at different DMSO concentrations. While the raw on- and off-rates \((k_{on} \text{ and } k_{off})\) for the prominent FRET transitions are provided in Figure 3.10, the equilibrium constant \(K_{eq} \quad (K_{eq} = k_{on}/k_{off})\) is used to provide an easy overview of how similar the on- and off-rates are (Fig. 3.6B). In fact, the more similar the rates are, the closer the value of \(K_{eq}\) to 1, which is an important parameter in the context of bidirectional BM as observed here. Figure 3.6B shows the \(K_{eq}\) between the FRET states for 1, 2, and 3% DMSO. As the DMSO concentration increased, there was no effect observed for the 0.2–0.8 transition which was far from equilibrium.

**Figure 3.8.** Typical intensity-time traces and corresponding FRET trajectories showing dynamics after a long static FRET state. All presented traces were taken from the 2% DMSO (v/v) data. A similar behavior was also observed in (-) cosolvent and (+) PEG buffer environments.
However, the $K_{eq}$ for the 0.2–0.6 transition was unequivocally enhanced (toward an equilibrium) at higher DMSO levels while the $K_{eq}$ for the 0.6–0.8 transition was $\sim 1$. This implies that the small-step mid–high FRET transitions are more favorable with a bias toward the FRET state of $\sim 0.6$ at

**Figure 3.9.** Histograms from all dynamic molecules for the mobile construct under 1× TAE buffer with 150 mM Na$^+$ (A) and the same buffer with 2% DMSO (B) and 30% PEG (C). All histograms were fitted with a three-peak Gaussian function to determine the mean FRET values and their corresponding populations. The FRET vs time data corresponding to the first 20 s observation time for the given experimental condition were combined without averaging, and the data were binned to a 0.05 FRET value before plotting the histogram. All histograms show three major FRET states with mean values of $\sim 0.1$–0.2, $\sim 0.6$–0.7, and $\sim 0.8$–0.9, which corroborates the findings obtained from HMM analysis.

However, the $K_{eq}$ for the 0.2–0.6 transition was unequivocally enhanced (toward an equilibrium) at higher DMSO levels while the $K_{eq}$ for the 0.6–0.8 transition was $\sim 1$. This implies that the small-step mid–high FRET transitions are more favorable with a bias toward the FRET state of $\sim 0.6$ at
higher DMSO levels. This is also evident from the change in the raw rates as a function of DMSO concentration (Fig. 3.10A), where despite the slight variations in rate among the DMSO conditions, the off rates were relatively higher than the on rates for the 0.2–0.8 compared to the other two transitions. The high preference for the 0.6 FRET level, which actually represents half migration, might be due to a perfect balance of two extremes (horizontal and vertical sliding of the junction arms as depicted in the schematic in Figure 3.3). This may indicate the stochastic nature of BM where a forward and backward movement of the crossover (branch) point along the DNA axis is possible. 63,158

Briefly, the Hidden Markov Model (HMM) allows determining the interconversion rates between the FRET states by quantifying dwell times of various FRET states before transitioning to other states. In this analysis, we considered only the dynamic FRET traces under different cosolute environments that showed clear anticorrelation and distinct transitions between different FRET states. Single-molecule traces were truncated at the time point before fluorophore photobleaching occurred and globally fitted with HMM (Fig. 3.11). The most likely sequence of underlying states was determined by the HMM analysis which assumes a limited number of states and assigns the acquired data to one of those states to reproduce the hidden state transition trajectory. A transition density plot (TDP) algorithm was also applied in conjunction with HMM, which enabled finding the idealized FRET levels, the number of transitions for each pair of FRET levels, and the corresponding transition rates using a two-dimensional Gaussian function (TDP plots are shown in Figure 3.12). 60,173 Due to the reversibility of the FRET transitions or conformational changes of the HJ molecule, a mirror symmetry relative to the diagonal is observed in the TDPs. Each pair of mirrored spots represents one type of reversible transitions. It is
important to note that there can only be a maximum of \( n(n - 1) \) possible transitions mapped on a TDP, where \( n \) represents the number of discrete states found in the HMM analysis.\(^{173}\)

**Figure 3.10.** Raw rate constants (\( k \)) extracted from HMM analysis of FRET state transitions. (A) Transition rates between the FRET states, 0.2, 0.6, and 0.8, under 1\%, 2\%, and 3\% DMSO (v/v). (B) Transition rates between the same FRET states under 10\%, 20\%, and 30\% PEG (w/v). The on-rate (\( k_{on} \)) denotes the rate of a low-to-high FRET transition whereas the off-rate (\( k_{off} \)) denotes the rate of a high-to-low FRET transition. Error bars represent the standard deviations of the transition rates which were obtained from the HMM analysis of the dynamic traces under each cosolute condition.
Figure 3.11. HMM analysis of the mobile HJ. Only a small section of the globally fitted single-molecule traces obtained at each buffer condition is shown (blue traces). Please note that on average all buffer environments caused the HJ to exhibit the same three main FRET states (~0.8, ~0.6, and ~0.2) but with varying dwell times and transition rates. Equilibrium rates between the three FRET states obtained from the HMM analyses (green traces) of the DMSO and PEG environments are presented in Fig. 3.6B and Fig. 3.16B, respectively.

Figure 3.12. Representative Transition Density Plots (TDPs) highlighting the most prevalent transitions within a population of molecules in 2% DMSO (A) and 30% PEG (B). The plots were weighted by the number of times a particular FRET transition occurs. Brighter spots indicate a
larger number (frequency) of transitions. Please note that the program does not yield a scale (color) bar as an index of the frequency of transitions. The total number of dynamic molecules used in the TDP plots was 45 and 31 for DMSO and PEG conditions, respectively.

The three clear states observed upon HMM analysis (Fig. 3.11) suggested that the mean step-size for BM is usually of several bps as reported previously.\textsuperscript{59,174} To determine if there were multiple small steps within the seemingly single FRET states of the mobile junction, we designed a control construct incapable of migrating, which we call an immobile junction and carried out a more thorough analysis of the FRET traces. This immobile construct was expected to show a FRET value of ~ 0.7-0.8 (Fig. 3.13). The FRET analysis of this control construct with and without the addition of DMSO indeed showed a static FRET state with an overall mean FRET value of ~ 0.8, verifying that the BM was not possible in either case (Fig. 3.14A and B). This result also verified that the FRET dynamics observed for the mobile junction in DMSO were indeed due to BM and not due to cosolute effects on DNA or fluorescence intensity of the dyes. Interestingly, when we compared the overall FRET distribution of the immobile construct with the 0.8 FRET state of the

![Figure 3.13](image-url)  

**Figure 3.13.** Schematics of the mobile HJ (Left) and immobile HJ control construct (Right) after modifying some of the bases. The immobile control was custom designed to break the homology
formed by the repeated AT sequence in the BM construct. The mutated nucleotides in the immobile construct are shown in red. This conformation is expected to show ~ 0.7-0.8 FRET efficiency.

Mobile construct under the same buffer environment (2% DMSO), we found that the latter had a much wider FRET distribution (Fig. 3.15). This observation indicated that there were some small-step migrations (perhaps single bp steps) within the seemingly static FRET levels in the BM construct which were not easy to observe or be picked by the HMM fitting.

**Figure 3.14.** Typical intensity-time along with FRET traces and all-molecule histograms for the immobile HJ control under 1× TAE buffer with 150 mM Na⁺ (A) and the same buffer with 2%
DMSO (B) and 30% PEG (C). Histograms were fitted with a one-peak Gaussian function to determine the mean FRET values and their corresponding populations. The mean FRET efficiency with and without cosolute were identical. All data were recorded at 30 ms exposure time.

**Figure 3.15.** Histograms from all molecules for the immobile (A) and mobile HJ (B). All experiments were done under the 2% DMSO buffer environment. Histogram in (A) is taken from Fig. 3.14B for comparison purposes. Only the 0.8 FRET state of the dynamic molecules from the mobile (BM) construct was selected to plot the histogram in (B) to directly compare the FRET distribution of the immobile construct and the seemingly static 0.8 FRET level shown by the mobile construct.
Taking these observations into account, it is clear that the dynamics/kinetics of BM are significantly enhanced by DMSO, which is likely the result of the dehydration effects of DMSO.\textsuperscript{68,179,180} For example, organic cosolvents have been found to change the equilibria in telomeric G-quadruplex (interconversion between parallel and antiparallel structures) because of the release of water molecules upon G-quadruplex formation.\textsuperscript{179} Likewise, in the case of the HJ, a similar situation could be occurring where the equilibrium of BM is affected by dehydrated conditions. In addition, statistical geometry analyses by MD simulations suggested that pure water clusters are disfavored in the presence of DMSO and that interactions between water and DMSO lead to various types of water–DMSO complexes of different stoichiometries depending on the concentration of the cosolvent.\textsuperscript{180,181} Our data support this view as it showed that the higher amount of DMSO (≥3\%) leads to a decrease in the dynamics of the HJ as compared to the optimal 2\% DMSO concentration (Fig. 3.6A), presumably due to the formation of more complex water–DMSO clusters of the (DMSO)$_2$:H$_2$O type with varying water activities and dehydration effects than the DMSO:(H$_2$O)$_2$ type favored at lower cosolvent concentrations.\textsuperscript{181} Further studies using computational modeling such as molecular dynamics would be useful to expand on the data presented here to better determine the origin of the heterogeneity in BM dynamics.

**PEG Affects BM in a Concentration-Dependent Manner**

Molecular crowding is common in all living organisms, and biological processes are known to be optimal under such intracellular crowding.\textsuperscript{182} Therefore, understanding how the crowded environment affects the properties of biomolecules and associated molecular mechanisms is critical toward understanding the biological processes. Specifically, in the context of homologous recombination, the knowledge of how the crowded environment affects the BM process has
remained elusive. In this study, using poly(ethylene glycol) (PEG 6000) as a model crowding agent, we sought to determine how cell-mimicking crowding affects the BM process. Our choice of PEG as a crowding agent comes from the fact that it is highly soluble in water, easy to handle, and possesses no net charge, making it suitable for exclusively studying the effects of macromolecular crowding. Typical dynamic traces obtained in PEG are shown in Figure 3.5C. In contrast to the (−) cosolvent and 2% DMSO buffer conditions, the exchange in the donor–acceptor intensities was more rapid with multiple short-lived intermediate FRET states occurring in the presence of 30% PEG (w/v), such as in the 15–25 s time window (trace 1, Fig. 3.5C). These large, multistate excursions in FRET efficiency were distinguishable from the background signal as they were visually sharper and more pronounced than the typical noise-induced peaks. Interestingly, the disappearance of the well-defined FRET states (plateaus observed in Figures 3.5A and B) and the emergence of the rapid and transient FRET states (Fig. 3.5C) might suggest an accelerated rate of BM under crowding.

In addition, systematic titration of PEG concentration (Fig. 3.16A) showed a constant increase in the dynamic fraction from 0 to 30% PEG, with 30% being the optimal concentration. This indicated that BM was preferentially enhanced in 30% PEG, which is the cosolute

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**Figure 3.16.** Kinetic analysis of BM as a function of PEG concentration. (A) Percentage of dynamic molecules under different PEG conditions. 0% PEG represents the ionic environment control (150 mM Na+, (−) PEG). Error bars were obtained from three different subsets of molecules.
for the same buffer condition containing more than 150 molecules in total. (B) The equilibrium constant ($K_{eq}$) for various FRET levels was determined by using HMM fitting of the FRET traces as in Figure 3.6. These rates were determined from more than 300 transitions for each PEG concentration (304, 487, and 466 transitions for 10, 20, and 30% PEG). Inset represents the equilibrium constant for the 0.2 to 0.8 transition as a function of PEG concentration. Standard deviations (SD) in $K_{eq}$ were determined by using propagation of error.

concentration that most closely mimics intracellular crowding and is most effective in altering the conformational equilibria of non-canonical DNA forms.$^{184,185}$ We did not use concentrations beyond 30% because of the higher background fluorescence observed, making it difficult to identify single molecules in the FRET movies. More importantly, upon HMM analysis, we found that the dynamic population at 30% PEG overall exhibited three distinct FRET states with similar efficiency values (~0.2, ~0.6 and ~0.8) as those obtained in the cosolvent-free and DMSO buffer systems (Fig. 3.11). This finding also agrees well with the mean FRET values determined by the Gaussian fitting of the histograms (Fig. 3.9). However, the HMM-fitted data showed a clear distinction in the dynamics obtained at each buffer condition, where the traces in the molecularly crowded environment (30% PEG) were more rapidly changing relative to the (−) cosolvent condition (Fig. 3.11). Notably, these dynamic features were absent in the immobile control with and without PEG (Fig. 3.14A and C), supporting that the observed dynamics in the mobile junction are the result of BM and not due to cosolute effects on the dye intensity. More interestingly, a comparison of the 0.8 FRET-state distribution of the immobile control with that of the mobile construct in 30% PEG did not show any significant difference especially in the width of the histogram (Fig. 3.17), indicating the absence of small-step migrations within the static 0.8 FRET state of the mobile HJ, which is the opposite of what we observed with 2% DMSO (Fig. 3.15). These results indicate that compared to PEG, DMSO might have a higher propensity of destabilizing DNA base pairing and hence promoting branch migration.
In addition to visualizing the underlying FRET states, HMM allows determining the interconversion rates between different states. Taking advantage of this, we performed a quantitative kinetic analysis of the dynamic FRET traces and acquired transition rates for the three discernible FRET states (raw rates are shown in Figure 3.10B). Figure 3.16B shows the equilibrium constant ($K_{eq}$) between each pair of FRET states under 10%, 20%, and 30% PEG. The kinetics obtained for each type of transition was significantly different; $K_{eq}$ for the 0.2–0.8 and 0.6–0.8 transitions decreased as a function of PEG concentration, whereas it increased for the 0.2–0.6 transitions. These equilibrium constants are also clear from the change in the raw rates as a function of PEG concentration (Fig. 3.10B). These results suggest a few insights: first, the large migration (0.2 to 0.8 FRET state) is not highly favorable as shown by the low $K_{eq}$ values under all conditions (similar to DMSO data), and second, the BM construct predominantly favors a mid-FRET state under molecularly crowded conditions, which is nearly the center point of the highest and the lowest FRET states. The tendency to populate the mid-FRET state – an equilibrium position (half migration) – of this mobile junction was also observed under the optimal DMSO environment (Fig. 3.6B). In fact, our analysis of the free energy change ($\Delta G$) for the three types of FRET transitions under various DMSO and PEG environments revealed that the 0.6–0.8 transition possesses the smallest average $\Delta G$ value, and thus it supports that the small-step mid- to high-FRET transitions are more favorable than the large-step transitions (see the Methods section for $\Delta G$ calculation).
Taken together, a few postulations can be made about these observations. First, comparable to intracellular macromolecules, large-sized PEG (PEG 6000, 30%) occupies a large proportion of volume in solution, limiting the space accessible to biomolecules and generating the so-called “excluded volume effect”. This consequently reduces the degrees of freedom available for the

Figure 3.17. Histograms from all molecules for the immobile (A) and mobile HJ (B). All experiments were done under the 30% PEG buffer environment. Histogram in (A) is taken from Fig. 3.14C for comparison purposes. Only the 0.8 FRET state of the dynamic molecules from the mobile (BM) construct was selected to plot the histogram in (B) to directly compare its FRET distribution to that of the immobile construct.
HJ, possibly leading to an increased sampling of the open form that promotes BM.\textsuperscript{47,63} Second, the crowding-induced reduction in the water activity and dielectric constant might be facilitating reactions accompanied by the release of water, including the exchange of H bonds between the bases during BM, as well as by decreasing the affinity of cations for DNA, leading to destabilization and enhanced BM.\textsuperscript{174,183} This is because of the formation of cosolute–solvent interactions that dominate the free-floating water molecules in solution, resulting in decreased hydration near the DNA surface and a lower number of ions bound (see Figure 3.18 for a simplified illustration). This may also relate to the preference toward the mid–high FRET transitions as the BM approaches a state of equilibrium where the exchange of bps at the crossover point is balanced. However, it is important to note that the kinetics of BM may vary depending on the homologous sequence (or core sequence) of the junction being used. This is because the minor groove of an AT-pair-containing duplex is narrower with potentially more restrictively bound water molecules (i.e., minor groove spine of hydration) than a GC-rich duplex,\textsuperscript{50,186} possibly causing it to be more affected by cosolute-induced dehydration. Regardless, the single-molecule analyses demonstrated that the BM construct used here serves as a platform to delineate the effects of solution environments on BM.
In summary, through FRET-based single-molecule analysis of BM of DNA HJ, we have revealed environment-dependent BM dynamics. First, the solution composition modeled by various DMSO concentrations showed enhanced dynamics of BM in a concentration-dependent manner. However, the dynamics of BM slightly decreased at concentrations greater than 2% DMSO presumably due to the formation of more complex DMSO–water interactions that influence water-mediated events.\textsuperscript{178,187} Similarly, BM kinetics was affected even more under crowded environments (simulated using PEG 6000) possibly due to the increased viscosity and volume-exclusion effects. Second, both dielectric and crowding effects favored the FRET state representing the equilibrium point between the highest and lowest FRET states, suggesting that BM is possible in the forward and backward directions under such environments. With the cosolute-dependent kinetic behaviors of BM that we determined using smFRET analysis, future

\textbf{Figure 3.18.} Proposed simplistic model of how pure vs mixed aqueous systems change the BM property of the HJs. Reduced water activity and dielectric constant are predicted in mixed-solvent solutions due to cosolute-solvent interactions, leading to an enhanced BM.

\section*{3.4 Conclusions and Future Work}

In summary, through FRET-based single-molecule analysis of BM of DNA HJ, we have revealed environment-dependent BM dynamics. First, the solution composition modeled by various DMSO concentrations showed enhanced dynamics of BM in a concentration-dependent manner. However, the dynamics of BM slightly decreased at concentrations greater than 2% DMSO presumably due to the formation of more complex DMSO–water interactions that influence water-mediated events.\textsuperscript{178,187} Similarly, BM kinetics was affected even more under crowded environments (simulated using PEG 6000) possibly due to the increased viscosity and volume-exclusion effects. Second, both dielectric and crowding effects favored the FRET state representing the equilibrium point between the highest and lowest FRET states, suggesting that BM is possible in the forward and backward directions under such environments. With the cosolute-dependent kinetic behaviors of BM that we determined using smFRET analysis, future
work will be directed toward unraveling the roles of HJ-binding proteins, such as the *E. coli* protein RuvA, in a biomimetic context which may inspire the development of potential therapeutic interventions utilizing HJs and associated BM properties.
CHAPTER 4 – SMALL-MOLECULE TARGETING OF HOLLIDAY JUNCTION FOR THERAPEUTIC INTERVENTIONS

4.1 Introduction

As discussed in Chapter 3, double-strand DNA breaks (DSBs) are highly deleterious with the potential of predisposing individuals to develop certain cancer diseases. To counteract the genotoxic lesions, cells have evolved a set of mechanistic pathways for DNA damage signaling and repair known as the DNA damage response (DDR).\textsuperscript{188,189} While healthy cells are able to utilize and take advantage of the full DDR capacity, precancerous and malignant cells are usually prone to losing one or more of the DDR capabilities during their development, and therefore their survival is highly reliant on the remaining repair pathways for protection against DNA damage.\textsuperscript{73,74} This cancer-specific repair deficiency provides a great opportunity to artificially exploit the DDR dependency of cancer cells in order to sensitize them to anticancer therapeutics.

In this regard, we have focused on targeting the homologous recombination (HR) pathway that is employed during DSB repair and has a significant effect on the genomic stability and proliferation of cancer cells.\textsuperscript{74,190,191} Given that the HJ structure is a key intermediate in homology-directed repair whose regulation ensures the accuracy of strand exchange and maintains the stability of genome, it constitutes a potential target for cancer treatment. Hence, in this chapter we sought to exploit the inherent HJ dynamic properties for drug targeting.

In the literature, several compounds and small molecules have been reported to bind to HJs.\textsuperscript{8,87,90} For example, a set of peptide ligands have been discovered by Segall and coworkers including the hexapeptide WRWYCR which was shown to bind the open form of the junction with nanomolar potency.\textsuperscript{82,83,192} Despite its effectiveness, it was found that dimerization of the peptide
via disulfide bridging is necessary for its activity, which can be a limitation for in vivo studies due to the highly reducing cellular environments.\textsuperscript{83,85} Alternatively, Segall’s group designed a single chain analogue of WRWYCR that lacks a disulfide linkage and has the following amino acid sequence WRWYRGGRYWRW.\textsuperscript{193} Although this peptide analogue was active even in the presence of reducing agents such as dithiothreitol (DTT), it exhibited increased nonspecific DNA binding reflected by the high levels of toxicity against eukaryotic cells.\textsuperscript{90} This too can be a limitation when it comes to targeted therapy, since high levels of selectivity and specificity (i.e., low toxicity profile) represent a key aspect of drug design and development. Furthermore, another class of chemical compounds that are classically known as DNA binders include acridine-based molecules whose DNA binding is characterized by insertion into Watson-Crick base pairs.\textsuperscript{34,194,195} One example is the acridine dimer called 9-aminoacridine-4-carboxamide designed by Brogden et al whose structural analysis revealed that it binds to the HJ in an intercalative fashion.\textsuperscript{87} However, despite that their binding modes have been investigated, the fact that their bioactivities have not been studied in depth and that they exhibit a relatively high affinity for dsDNA renders them less suitable for HJ targeting. Therefore, advancements in the selectivity of four-way junction binding compounds and the discovery of new potential HJ binders are underway in the field of DNA targeting.

More recently, Yin et al discovered that the previously established drug VE-822 (also known as Berzosertib) commonly used for ATR inhibition (a signaling pathway involved in DDR) can promote the formation of HJ.\textsuperscript{34,90,92,93} VE-822 was screened from a DNA damage and repair library of 160 compounds and demonstrated inhibitory effects in HR via HJ stabilization.\textsuperscript{93} In fact, previous gel and fluorescence-based assays have shown that the compound VE-822 promotes the assembly of HJ and facilitates the formation of different HJ branch migrations.\textsuperscript{34,90,93} Moreover,
circular dichroism (CD) and docking studies have revealed that the compound might have the potential to convert the HJ structure into the stacked form.\textsuperscript{93} Despite these important findings, the techniques that have been used to characterize the binding of VE-822 to HJ are exclusively based on ensemble (or bulk) measurements, which are not highly sensitive to the conformational dynamics and kinetics of biomolecular interactions due to signal averaging.\textsuperscript{141,142,196} Taking this into account and the fact that VE-822 exhibits low levels of binding to ds and ssDNA (\(K_d \geq 50\mu\text{M}\)) compared to the HJ (\(K_d \sim 8.64\mu\text{M}\)), we sought to investigate the binding interaction of VE-822 with HJ substrates and the associated kinetics at the single-molecule level using our well-established FRET-based platform.\textsuperscript{148} Our preliminary smFRET data suggest that the VE-822 ligand binds to the HJ substrates used in this study. More interestingly, the FRET traces of the mobile HJ analog indicated that the ligand stabilizes specific conformations of the HJ and affects its dynamics, suggesting that this ligand may be used as a HJ stabilizer.

4.2 Materials and Methods

4.2.1 HJ construct assembly and smFRET imaging

For the single-molecule binding experiments, different HJ constructs (immobile and mobile) were used which have nucleotide sequences that are identical to those used in Chapter 3 (Appendix 1). The HJ construct assembly was done by thermal annealing of the constituent strands as discussed previously in Chapter 2. Initially, to verify the binding of VE-822, the single-molecule binding experiments were conducted using surface-functionalized slides and a high concentration of VE-822 (10 \(\mu\text{M}\), which is higher than the literature reported \(K_d \sim 8.64\mu\text{M}\)). Briefly, the HJ construct was injected onto the slide at a final concentration of \(\sim 20 \text{pM}\), flushed with a wash buffer (1\(\times\)TAE-Mg\(^{2+}\) with OSS), and incubated with 10 \(\mu\text{M}\) VE-822 for 20 min. It is important to note that VE-822 (solution form) was procured from Selleck Chemicals at a stock concentration of 10
mM in DMSO; however, for dilution purposes, double sterile water was used to dilute the solution immediately before injecting into the flow channel. For the titration experiments with the mobile HJ, the concentration of VE-822 was varied and the FRET data was acquired after injecting the imaging buffer which consisted of an aqueous 1×TAE buffer with OSS, 2 mM Mg$^{2+}$, and the desired concentration of ligand VE-822. Chapter 2 provides a more comprehensive description of the smFRET imaging and data analysis portions of the experiments.

4.3 Results and Discussion

4.3.1 Single-molecule VE-822 binding analysis using an immobile HJ

First, to validate the binding of VE-822 to HJ, the same immobile HJ construct designed and tested in Chapter 3 was used (Appendix 1). This junction has a similar sequence to that of the mobile HJ except that the core nucleotides have been altered to restrict BM and lock the junction in one conformation (Fig. 3.13). The intention was to test the immobile HJ first as it lacks the ability to branch migrate, therefore it will allow to only look at the binding. Notably, this immobile HJ is labeled with a FRET pair (Cy3 and Cy5) on opposite arms instead of adjacent arms to emphasize the structural effects of the ligand on HJ while eliminating any interference from the iso-I – iso-II conformational switching of the junction. The single-molecule experiments were carried out using the same experimental procedure delineated in the Methods section 4.2.1. Briefly, the immobilized HJs were incubated with 10 µM VE-822 at a low Mg$^{2+}$ concentration (2 mM Mg$^{2+}$) after which FRET movies were recorded. The single-molecule FRET traces in the absence of the ligand showed a FRET efficiency of ~0.8, which matches the expected FRET of the construct (Fig. 4.1). However, upon the addition of ligand, the FRET efficiency decreased to ~0.6 (Fig. 4.1). This is also clear from the FRET histograms that show a shift in the mean FRET value.
from ~0.85 to ~0.67 after adding VE-822 (Fig. 4.2, ligand structure provided in the inset). Moreover, the histogram with the ligand displayed a wider FRET distribution compared to the one without the ligand (Fig. 4.2). Although these observations do not provide structural insights into the binding interaction, we predict that these changes in the FRET signal should be a result of the ligand altering HJ conformations. In fact, previous molecular docking studies postulated that this ligand binds to the central groove of the HJ altering its conformations.\(^{93}\)

**Figure 4.1.** Representative intensity-time traces and corresponding FRET traces for the immobile HJ labeled on opposite arms in the absence (left panel) and presence (right panel) of VE-822. Data were collected in a low Mg\(^{2+}\) buffer (2 mM Mg\(^{2+}\)) at 100 ms time resolution. Ligand binding experiments were performed using 10 µM VE-822.
Next, we examine the effect of VE-822 on the structural dynamics of HJ using a mobile analog. The mobile HJ used here is analogous to the one used in Chapter 3 for the BM study. This junction has a short homologous sequence of AT repeats (6 nts) on opposite arms flanked by a pair of fluorophores to enable BM (Fig. 3.1). Single molecule data were collected independently at different concentrations of VE-822 using the same experimental procedure outlined in section 4.2.1. Control experiments without the ligand were also performed in a similar manner. Figure 4.3 shows the intensity-time and FRET traces of representative HJ molecules in the absence and presence of VE-822. Without the ligand, the traces exhibited a high FRET efficiency of ~0.9 with little to no FRET transitions in a low Mg\textsuperscript{2+} buffer environment. Under these buffer conditions, the junction is expected to predominantly adopt an open form, possibly one where the fluorophores
are closest to one another due to the high-FRET efficiency observed experimentally. Alternatively, the high FRET could also be the product of multiple open conformations adopted by the HJ whose FRET efficiencies are averaged out under low Mg\textsuperscript{2+} conditions. On the other hand, the traces in the presence of VE-822 (10 µM) were distinct from the control data, where we clearly observed a dynamic FRET pattern spanning multiple FRET states (mainly 0.3, 0.6, and 0.9). This suggests that the compound affects the HJ dynamics possibly by stabilizing specific conformations giving rise to multiple FRET states. This in turn may allude to a potential therapeutic role of this ligand in BM and DNA repair.

![Figure 4.3](image.png)

**Figure 4.3.** Typical intensity-time and FRET traces of the mobile HJ in the absence (left) and presence (right) of the compound VE-822 at 10 µM. All data were collected in a low Mg\textsuperscript{2+} buffer (2 mM Mg\textsuperscript{2+}) at 100 ms time resolution.

To investigate whether this dynamic FRET outcome is concentration dependent, we next carried out smFRET experiments at lower VE-822 concentrations using the same buffer and experimental conditions as described before. Interestingly, when the concentration of VE-822 was
lowered by half (10 to 5 µM), the FRET dynamics were significantly different (Figs 4.4). Notably, the traces showed a distinct dynamic FRET pattern with faster transitions between the states compared to the 10 µM VE-822 data (Fig. 4.3). Moreover, the percentage of dynamic molecules (% dynamic molecules) significantly decreased from ~77% down to ~29% as the concentration of the compound was lowered. This percentage was determined based on single molecule counting by dividing the number of dynamic molecules by the total number of single molecules (both static and dynamic) for the same buffer condition. Overall, these results demonstrate the effects of VE-822 on HJ are concentration dependent with the possibility of affecting BM dynamics and kinetics. Therefore, this ligand might have applications in cancer therapy.

Figure 4.4. Typical intensity-time and FRET traces of the mobile HJ with the compound VE-822 at 5 µM. All data were collected in a low Mg²⁺ buffer (2 mM Mg²⁺) and at 100 ms time resolution.
Future work: Binding and structural analyses of VE-822-HJ complex

With the finding that VE-822 binds to HJ in a concentration dependent manner, the concentration range of VE-822 can be extended to acquire smFRET data at various concentrations and ultimately determine the binding affinity of the ligand. Briefly, concentration-dependent binding data will need to be collected at various concentrations of ligand (0 – 100 µM) as described in section 4.2.1. The ligand-bound population of HJ molecules can then be plotted against the concentration of VE-822 where the data is fitted with an appropriate model (most likely single-exponential fit) to determine the dissociation constant ($K_d$). One major challenge that might be encountered in these titration experiments is the background fluorescence at high VE-822 concentrations that could negatively affect the analysis of single-molecule traces. In the event of high background fluorescence noise, one can optimize the microscope parameters (laser intensity, exposure time, etc.) to minimize the fluorescence interference from the ligand. Additionally, to

Figure 4.5. Preliminary data showing the percentage of dynamic molecules as a function of VE-822 concentration. The concentration of VE-822 was varied (5, 7.5, and 10 µM) and dynamic molecules observed under each condition were recorded. The number of total molecules collected for each concentration was 82, 91 and 111, respectively. Error bars denote standard deviations from three different sets of molecules for each condition. All data were collected in a low Mg$^{2+}$buffer (2 mM Mg$^{2+}$) and at 100 ms time resolution.
further investigate the role of VE-822 in BM, the same single-molecule experiments in the presence of junction-binding proteins (such as RuvA) can be conducted. Briefly, this can be done by incubating the HJ with the compound before adding RuvA and recording FRET movies. Control experiments can be done similarly without the protein or the compound. It is expected that in the presence of both the protein and compound the FRET dynamics will be higher than in controls. This data can then be analyzed using the Hidden Markov Model (HMM) described in Chapter 3 to extract transition rates (binding rate – $k_{on}$ and unbinding rate – $k_{off}$). These rates can be used to calculate the equilibrium constant ($K_{eq}$) to describe how effectively the ligand binds to the junction.

In parallel, bulk fluorescence assays (such as fluorescence quenching) can be carried out to examine the binding. Briefly, this can involve testing a HJ labeled with a fluorescent dye and a quencher against a range of VE-822 concentrations and recording the fluorescence. It is expected that as the junction assembles, the fluorescence intensity of the dye decreases due to quenching. Control experiments can be performed using HJs either labeled only with a single-fluorescent dye (i.e., no quencher) to confirm that VE-822 does not quench the fluorescence or pre-annealed to confirm the role of VE-822 in assisting HJ assembly. To complement the bulk assays, circular dichroism (CD) and computational studies such as molecular dynamic (MD) simulations can also be conducted (through collaborations) to study the structural effects of VE-822 binding. For example, in CD the concentration of the VE-822 compound can be varied while fixing the concentration of the junction and CD spectra can then be recorded over a wavelength range of 220-320 nm. Control experiments can be done similarly except conformationally locked HJs without the ligand will be used to compare against the CD spectra of the ligand-bound HJs.
4.4 Conclusions and Future Work

Previous studies used bulk assays such as electrophoretic gels and fluorescence quenching to characterize the ability of VE-822 (Berzosertib) to promote the assembly of immobile HJ analogs, but they lacked conformational information. Using smFRET, we have demonstrated that this ligand alters the HJ conformational dynamics. Even more importantly, we were able to analyze the binding of VE-822 to different HJ substrates (both immobile and mobile), providing insights into the behavior of individual molecules. Our data indicated that VE-822 might bind HJ presumably by interacting with the central groove of the junction as described previously.93 Furthermore, using a mobile HJ analog capable of migrating, we showed that VE-822 affects HJ dynamics in a concentration dependent manner. The unique FRET dynamics suggested that VE-822 stabilizes multiple HJ conformations having different branch points (or core sequences). In the future, it will be interesting to pursue the effects of VE-822 on HJ conformational dynamics in the presence of HJ-processing proteins such as E.coli RuvA and human Rad51B proteins. Altogether, our analysis provided insights into the potential therapeutic role of VE-822 in DNA repair which can inspire the development of new drug molecules that target HJ toward advancing the field of cancer therapeutics.
CHAPTER 5 – APTABINDING OF TUMOR NECROSIS FACTOR-α (TNFα) INHIBITS ITS PROINFLAMMATORY EFFECTS AND ALLEVIATES ISLET INFLAMMATION

5.1 Introduction

Type 1 diabetes (T1D) is a chronic autoimmune disorder that is characterized by a lack of insulin due to beta cell destruction and it can affect millions of individuals at different ages. In the United States, the prevalence of T1D has increased to 1 in 300 with an incidence rate increasing annually by ~ 2%-5% worldwide. Exogenous insulin administration using pumps is commonly used to attain glycemic control, however, there are issues with the efficiency that lead to life-threatening complications. Alternatively, novel therapeutic approaches independent of exogenous insulin are currently emerging, among which islet cell transplantation (ICT) is considered a promising treatment for severe T1D. Although ICT can replace the lost beta-cell mass in T1D patients, one major problem encountered is the massive loss (50%-80%) of the transplanted islet tissue due to an innate immune reaction called the instant blood-mediated inflammatory reaction (IBMIR). IBMIR is mediated by proteins produced by islet cells such as tissue factor (TF), macrophage chemotactic protein 1 (MCP-1) and Interleukin-8 (IL-8). IBMIR is an innate immune response that involves the activation of inflammatory pathways and the coagulation cascade that are mediated by a group of proinflammatory cytokines/chemokines, leading to leukocyte infiltration and loss of islets. Importantly, the plasmatic levels of several proinflammatory cytokines are significantly increased soon after islet infusion due to IBMIR. One key modulator
of such inflammation is the tumor necrosis factor α (TNFα) whose dysregulation or excessive activation has been linked to multiple inflammatory and autoimmune diseases.\textsuperscript{202–204} Given its pivotal role in inflammation and islet cell death post-transplantation, TNFα has gained considerable interest as a potential therapeutic target for treating inflammation. Clinical reports have shown that post-transplant introduction of a TNFα inhibitor significantly improves graft survival outcomes.\textsuperscript{117} Therefore, we sought to suppress the inflammatory responses by blocking TNFα, which can serve as a means to protect islets and maintain graft function in the treatment of T1D.

Previously, TNFα-related therapeutic applications utilized conventional inhibitors including monoclonal antibodies (e.g., infliximab, adalimumab, and golimumab) and soluble TNF receptor fusion proteins (e.g., etanercept).\textsuperscript{205–208} For example, two drugs, namely etanercept and anakinra, were evaluated for their synergistic effects in blocking cytokine-induced islet cell inflammation.\textsuperscript{119,209} However, despite improved graft functional outcomes, protein therapeutics are limited by high production costs and non-specific immunogenic effects that arise due to their systemic use.\textsuperscript{202,210,211} Alternatively, strategies that aim to engineer the islets prior to the transplantation would be more effective in improving the islet functions than the systemic administration of anti-inflammatory agents. For instance, chemical surface modifications of islets with heparin or low molecular weight dextran sulfate have been studied for their inhibitory effects against the innate IBMIR process.\textsuperscript{124,212} Although this approach is simple to implement, it only leads to marginal suppression of IBMIR due to the propensity of these chemical agents to bind to nonspecific proteins and surfaces.\textsuperscript{213} Therefore, there is a need for a novel approach that enables an effective blocking of the pro-inflammatory effects of TNFα without having to deal with the harmful side effects of systemic administration.
In this regard, surface modification of islets using anti-inflammatory aptamers can potentially serve as a tool to suppress IBMIR-induced pathways during islet transplantation. Briefly, aptamers are short sequences of single-strand DNA or RNA that bind to specific target molecules with high affinity and specificity owing to their unique 3-dimensional structures.\textsuperscript{130,214–216} Compared to commercial inhibitors such as monoclonal antibodies and receptor fusion proteins, aptamers offer unique advantages in many different aspects. For example, aptamers are smaller in size, less expensive, and elicit minimal immunogenicity.\textsuperscript{214,217} The facile chemical synthesis of aptamers along with the variety of chemical modifications available have significantly enhanced their affinity to targets and their \textit{in vivo} stability.\textsuperscript{134,214,216} Furthermore, instead of a simple binding, the detection triggered by conformational changes of an aptamer upon binding to the specific target offers more selective targeting.\textsuperscript{134,218} Aptamers specific to a target are normally screened using a combinatorial library of random DNA/RNA sequences via a process called systematic evolution of ligands by exponential enrichment (SELEX).\textsuperscript{130,133,217} Using this method, recent studies have identified ssDNA aptamers specific to TNFα (see Table S1 for sequence details).\textsuperscript{219,220} Taking advantage of these findings, we sought to determine the aptamers’ potential to prevent inflammatory signaling in islets post-transplantation. Specifically, we designed a DNA construct comprising the TNFα-specific aptamer and evaluated the aptamer’s ability to bind and block TNFα through single-molecule fluorescence resonance energy transfer (smFRET).\textsuperscript{168,221} We also assessed its ability to block inflammatory signaling in human islet cells using \textit{in-vitro} molecular assays.

We started this study by testing an aptamer\textsuperscript{219} (herein called monoApt1) against TNFα using our smFRET construct which showed a concentration-dependent response. Since TNFα is known to be biologically active as a non-covalently bound homotrimer that exhibits three binding sites
for its natural receptor, we then characterized the binding of bivalent and trivalent forms of the aptamer. The hypothesis was that having more than one binding site would increase the binding affinity due to multi-site binding. Contrary to our expectation, we found that the bi- and trivalent forms do not significantly enhance the binding relative to the monovalent aptamer. Given that single-site binding was more effective, we also tested another literature-reported aptamer (monoApt2) using the same approach and found that it performs slightly better than monoApt1. The tested aptamers showed sensitivity to as low as 5-10 pM TNFα with dissociation constants ($K_d$) in the nanomolar (nM) range, and they are specific to the target even in a mixture of other cytokines. The FRET-based single-molecule analyses along with the functional studies looking at the TNFα blocking efficacy of these aptamers support that the TNFα monoaptamers tested here are promising for surface-engineering of islets to minimize IBMIR. Further, the single-molecule mechanistic study and the functional assay developed here can be directly used to study other pro-inflammatory proteins using corresponding aptamers.

5.2 Materials and Methods

5.2.1 Chemicals and Proteins

Acetic acid, magnesium chloride, potassium chloride, glycerol, tris(hydroxymethyl)aminomethane (Tris), ethylenediaminetetraacetic acid disodium salt (EDTA), biotinylated bovine serum albumin (bBSA), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were all purchased from Fisher Scientific. Protocatechuate 3,4-dioxygenase (PCD) was also purchased from Fisher Scientific where it was suspended in a pH 8.0 PCD buffer (100 mM Tris-HCl, 50 mM KCl, 1 mM EDTA, 50% glycerol), sterile filtered using a 0.2 μm filter (Thermo Fisher Scientific), and stored at −20 °C. Sodium chloride, streptavidin, and
protocatechuic acid (PCA) were purchased from VWR. Liberase MTF C/T and thermolysin used in the isolation of human islets were purchased from Roche. Prodo islet media standard PIM(S) and recovery medium PIM(R) with human serum AB and glutamine/glutathione additives required for the culture of human islets were purchased from Prodo labs. Human albumin was purchased from Baxter. Humulin-R U-100 was purchased from Eli Lilly. Heparin and cefazolin were purchased from Hospira. DAPI was purchased from Invitrogen, Life Technologies. Hank’s buffered saline solution was purchased from Corning. Cold storage/purification, priming, dilution, wash, perfusion and 2mM HEPES solutions were purchased from Mediatech. Belzer UW cold storage solution was purchased from Bridge to Life. 10% povidone iodide solution was purchased from Medichoice. Quick-RNA™ Miniprep Plus Kit was purchased from Zymo Research, CA, USA. Fetal bovine serum was purchased from Gibco Laboratories, MD, USA. Dulbecco’s Modified Eagle medium high and low glucose, penicillin-Streptomycin solution, phosphate buffered saline, propidium iodide, dithizone, optiprep, fine chemicals, and reagents were acquired from Sigma Aldrich. Bovine serum albumin (BSA) and native human factor VIIa (FVIIa) were purchased from Thermo Scientific and Abcam, respectively. Tumor necrosis factor alpha (TNFα) and interleukin-1 beta (IL-1β) were in-house expressed and purified as described below.

5.2.2 Recombinant TNFα and IL-1β preparation and NMR

Production of TNFα followed the general strategy previously published, with modifications.\(^{223}\) The codon-optimized gene encoding TNFα was synthesized and inserted into a pET-28a(+) vector, provided by GenScript USA Inc. The expression product contains an N-terminal poly-histidine affinity tag and TEV protease cleavage sequence. The plasmid was transformed into BL21(DE3) E. coli cells, which were stored as glycerol stocks and grown in 50 mL of an M9 minimal media starter culture overnight. The starter culture was used to seed 1L of M9 minimal media containing
ammonium-\textsuperscript{15}N chloride for NMR analysis, which was grown to an OD\textsubscript{600} of 0.8 then induced with 1 mM IPTG for 3 hours at 37 °C. Cells were harvested by centrifugation with supernatant discarded. Cells were resuspended in a lysis buffer containing 100 mM Tris, pH = 7.4, 300 mM NaCl, 1% (V/V) Triton-X 100, 0.1 mg/ml lysozyme, EDTA-free HaltTM protease inhibitor cocktail (Thermo Scientific), and 2 mM DTT. Resuspended cells were lysed on ice for 90 minutes, with gentle mixing throughout, followed by sonication. The cell lysate was centrifuged, supernatant was separated and loaded onto a 5 ml gravity column of Nickel-NTA affinity resin, preequilibrated with wash buffer (100 mM Tris, pH 7.4, 300 mM NaCl, 50 mM Imidazole, 2 mM DTT). Three-column volumes of wash buffer were used to eliminate contaminated proteins, followed by elution of the polyhistidine-tagged TNFα using five-column volumes of elution buffer (100 mM Tris, pH 7.4, 300 mM NaCl, 300 mM Imidazole, 2 mM DTT). The polyhistidine tag was removed via dialysis with an optimized TEV protease, uTEV2Δ, produced in-house.\textsuperscript{224} The plasmid encoding uTEV2Δ was a gift from Alice Ting (Addgene plasmid # 135456). Dialysis was performed in PBS buffer containing 5 mM Imidazole and 2 mM DTT. Cleaved TNFα was separated from polyhistidine-tagged uTEV2Δ, cleaved poly-histidine tag peptides, and remaining uncleaved TNFα using Nickel-NTA purification. Flow-through and wash containing cleaved TNFα was collected and quantified by Bradford assay, with SDS-PAGE used to confirm purity. Protein NMR of \textsuperscript{15}N-labeled confirmed properly folded and trimeric TNFα. \textsuperscript{1}H-\textsuperscript{15}N TROSY spectrum was collected on \textsuperscript{15}N-labeled TNFα to confirm the protein is properly folded and in the trimeric state. Sample conditions were 450 µM protein in NMR buffer consisting of 1X PBS buffer, pH = 7.4, 2 mM DTT, 0.02% (w/v) sodium azide, with 10% D\textsubscript{2}O added as a lock solvent. Spectrum was collected at 37 °C on a 700 MHz Bruker Avance III spectrometer equipped with a room temperature probe. Spectrum was collected with 1024 x 64 complex points and 32 scans.
Production of IL-1β followed the same protocol outlined above for TNFα, using a pET-28a(+) vector encoding IL-1β with an N-terminal polyhistidine tag with a TEV protease site.

5.2.3 DNA constructs

The aptamer constructs were assembled by thermally annealing the single-stranded oligonucleotides (see Appendix 2 for sequence details) at 1 μM concentration in 1× TAE-Mg\(^{2+}\) buffer, pH 7.4 (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, 12.5 mM Mg\(^{2+}\)) in a thermal cycler (Table 2.1 in Chapter 2). Please note that the aptamer and blocker strands were added in excess (3 and 2 μM respectively) relative to the three common strands (1 μM) to optimize aptamer incorporation into the construct.

5.2.4 Electrophoretic Mobility Shift Assay

A 7.5% SDS polyacrylamide gel was prepared with 0.1% ammonium persulfate, 0.375 M Tris-HCl, 10 μL of tetramethylethylenediamine (TEMED), 0.1% sodium dodecyl-sulfate (SDS), and double sterile water to reach a final volume of 10 mL. A 2× SDS loading buffer was also prepared with 100 mM Tris-HCl, 4% SDS, 20% Glycerol, 0.003% Coomassie, and double sterile water to a final volume of 5 mL. The protein samples were premixed with 0.5% glut, incubated at room temperature for 90 min, followed by the addition of 2× SDS loading buffer containing 0.1 M dithiothreitol (DTT) to quench reactions and to reduce the proteins for gel analysis. The control samples were treated similarly except that they did not contain glut – double sterile water was added instead followed by the addition of the same loading buffer. All samples were heated at 95 °C for 8 min in a thermal cycler before loading onto the gel. The HiMark prestained protein standard of 30-460 kDa was used as a molecular weight marker. The gel was run for 45 min at 95 V, stained with Coomassie brilliant blue for 20 min, followed by destaining overnight in deionized water containing methanol and acetic acid.
5.2.5 *Single-molecule fluorescence microscopy*

**Flow cell assembly and surface functionalization**

The flow cells used for running the single-molecule experiments were assembled using materials such as quartz slides, parafilm, coverslips, and tubing as described in Chapter 2 and surface functionalized prior to imaging.\(^{69,226,227}\)

**Sample preparation and imaging**

After functionalizing the flow cell, it was photobleached as described in Chapter 2 using our custom-built prism-based TIRF microscope to remove the fluorescent impurities and prepare the slide for imaging.\(^{226}\) The DNA construct (~ 20 pM) was dissolved in an imaging buffer of 1× TAE with 150 mM NaCl and an oxygen scavenging system (4 mM Trolox, 10 mM PCA, 100 nM PCD),\(^{150}\) and the solution was then injected into the flow cell and incubated for ~45 sec. Upon immobilization of the construct on the slide, the flow cell was washed with the imaging buffer to get rid of unbound molecules, followed by a 20-min incubation with the desired amount of the protein target (or nontarget protein(s) for assessing the specificity of the aptamer) and helper strand prepared in the same imaging buffer. Control experiments were carried out in a similar manner either with no protein and helper strand, only the helper strand, or only the protein to account for any positive background signal. Single-molecule FRET data was acquired by continuous excitation of the Cy3 fluorophores and recording the fluorescence emission intensities of Cy3 and Cy5 at 100 ms time resolution (10 frames/sec). More details can be found in Chapter 2 section 2.4.

5.2.6 *Single-molecule data analysis*

The same procedure outlined in Chapter 2 section 2.5 was followed to analyze the FRET data, with slight modifications. The FRET traces were truncated to the first 6 s of observation time (i.e.,
60 frames) for further data analysis using the Origin software. Specifically, FRET histograms were plotted by feeding the truncated files for each experimental condition into Origin and fitting them with a two-peak Gaussian function. The fitting parameters allowed us to determine the area under the curve (AUC) for the assigned FRET populations and the fraction of high-FRET molecules (see section 5.3 for more details). The standard deviations in the TNFα binding curves were calculated by randomly assigning molecules collected for the same experimental condition into three individual group sets and obtaining the high-FRET fraction using the AUC determination method. Approximately 100 or more molecules were used to construct the histograms at various TNFα concentrations. The dissociation constant ($K_d$) for all aptamers was determined using the binding curves of % high FRET vs TNFα concentration. The data analysis software Origin was used to determine the $K_d$ by fitting the data with the built-in Hill-1 equation provided below,\textsuperscript{228,229} where $HF$ is % High FRET, $HF_{\text{max}}$ is the maximum % High FRET, $HF_0$ is the % High FRET in the absence of TNFα and $K_d$ is the dissociation constant.

$$HF = HF_0 + \frac{(HF_{\text{max}} - HF_0)[TNF\alpha]}{K_d + [TNF\alpha]}$$

For data fitting, we assumed a binding stoichiometry between the aptamer and protein of 1:1 for the monoaptamer constructs. As for the biApt1 and triApt1 constructs, we assumed all possibilities (1:1 and 1:2 for biApt1 and 1:1, 1:2, 1:3 for triApt1). For those where more than one stoichiometry ratio was used, the extracted $K_d$ values were averaged out.

5.2.7 Cell Culture

Mouse βTC6 cell line was obtained from the American Type Culture Collections (ATCC, VA, USA). βTC6 cells were cultured in a medium consisting of high glucose Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and 1% Penicillin-Streptomycin reagent. The cell line was cultured at 37 °C in a humidified atmosphere of 5% CO₂. The medium
was replaced with a fresh medium every other day. The cells were used for subsequent MTT assay after reaching 70% confluence.

5.2.8 Human islet isolation

Human pancreatic islets were isolated from the pancreas obtained from adult deceased organ donors through local organ procurement organizations. The studies were exempted by IRB due to the use of tissues from deidentified deceased donors. Islet isolation was performed using a modified Ricordi method. Briefly, after decontamination of the pancreas with antibiotics, the collagenase enzyme solution was perfused into the pancreas through the main pancreatic duct. After sufficient perfusion, the pancreas was cut into smaller pieces and placed into the Ricordi chamber to facilitate digestion of the pancreas by enzymatic recirculation and mechanical dissociation. Islet cells were identified by dithizone staining. Digested tissue was collected, recombined and washed to remove any trace enzymes. Islets were purified with continuous density gradient using a COBE 2991 cell processor and OptiPrepTM density gradient medium. Islet yield was evaluated by counting a fraction of the islet prep using dithizone staining and then converted to a standard number of total islet equivalents (IEQ). One IEQ corresponds to 150 µm in diameter.

5.2.9 Islet culture

After isolation, islets were cultured in Prodo recovery media for 24-48hrs. Then, the islets were maintained in Prodo’s standard culture medium with 5% Human AB serum, 1% Glutamine and Glutathione, 1% penicillin and streptomycin. Islets were maintained in an incubator at 37 °C with a 5% CO₂ atmosphere for 24 and 72 h. Islets were cultured at a density of 30,000 IEQ/Flask. The cultured human islet cells were subsequently used for viability assessment and qPCR studies.
5.2.10 Assessment of viability

Cell viability was determined by MTT assay based on the detection of mitochondrial dehydrogenase activity in living cells. Briefly, the βTC6 cells (0.01×10⁶ cells/well/mL) were cultured in a 96-well culture plate. When the cells reached over 70% confluency, they were treated with different concentrations of monoApt1 and monoApt2 (0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50 and 100 μM) respectively and Etanercept (ETA) (100 μM) for 48 h. After the indicated timeframe, 25 μL of MTT (0.5 mg/mL) solution was added to each well and then incubated for 4 hr at 37°C followed by DMSO (100 μL/well). The absorbance was measured at 490 nm using a Varioskan Flash microplate reader. The percentage cell viability in each group was calculated. The in vitro determination of cell viability was observed at 48 h in human islets after treatment with proinflammatory cytokines in the presence or absence of anti-inflammatory compounds (MonoApt1, monoApt2, or ETA). 4′,6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) stains were used to determine the amount of viable and non-viable cells, respectively. Isolated islets were pretreated with monoApt1 and monoApt2 (0.05, 0.5, 5, 50 and 100 μM) respectively and Etanercept (100 μM) for 1 hr before challenging the cells with the pro-inflammatory cytokine cocktail (IL-1β, TNFα and IFNγ). Control islets were untreated with the cytokine cocktail, monoApt1 and monoApt2. The islets were incubated with DAPI (10 μg/mL) and PI (20 μg/mL) for 10 min at 37 °C before imaging via fluorescent microscopy. Fluorescent micrographs were merged in Image J (National Institutes of Health, Bethesda, MD), and the PI-positive area (dead cell) was divided by the DAPI-positive area (Total cell) to provide a calculation of islet viability. At least 10 islets per aliquot were used.
5.2.12 Statistical analysis

Statistical analysis was performed with GraphPad Prism6 (GraphPad Software, La Jolla, CA, USA). Statistical significance for more than two groups was determined by one-way analysis of variance (ANOVA). Differences were considered significant when p values were less than 0.05.

5.3 Results and Discussion

5.3.1 Assembly of aptamer construct and working principle

The aptamer construct is composed of 5 single-stranded DNA (ssDNA) oligonucleotides (Appendix 2) that were rationally designed to incorporate aptamers based on the complementary base pairing\(^\text{231}\) (Fig. 5.1). The construct was assembled by thermally annealing the 5 strands,\(^\text{69,146,227}\) two of which are labeled with a donor (Cy3) or an acceptor (Cy5) fluorophore to enable FRET. A biotin tag was also incorporated into the Cy3-labeled strand at its 5'-end to allow surface-immobilization of the DNA construct on the microscope slide passivated with biotin-BSA and streptavidin (Fig. 5.1). Additionally, the construct includes a blocker strand (B1) which partially hybridizes with both the Cy5 strand and the aptamer. It is important to note that the aptamers were extended at the 5'-end by 6 nucleotides (ACT GCT) for a stable hybridization with the B1 strand (i.e., the hybridizing portion of the aptamers includes the 6-nt extension in addition to 9 nts of the aptamer sequence, which make a total of 15 nts). This extension was designed to incorporate an aptamer without blocking a significant portion of it and to reduce non-specific binding. Further, the use of B1 instead of a fluorophore-labeled strand to incorporate the aptamer is a cost-effective approach when multiple aptamers are to be tested, as this strategy allows the use of same labeled strands for different aptamer constructs.
As shown in Figure 5.1, when TNFα is absent, the fluorophores on the DNA construct are far from one another adopting a conformation of low-mid FRET efficiency. However, when TNFα is introduced along with the helper strand H1 (fully complementary to B1), both the aptamer and B1 get displaced from the construct forcing it into a high-FRET conformation. Since H1 is fully complementary to B1, it displaces B1 from the construct through a process called toehold-mediated strand displacement (TMSD). With the B1 strand displaced, the Cy5-labeled arm is now free to bind to its complementary part of the Cy3-labeled arm (16 + 8 = 24 bp binding region plus 12 nts loop in between), closing the two arms onto each other and producing a high-FRET signal.

5.3.2 Gel Electrophoresis and NMR analysis of TNFα for subunit determination

Prior to conducting single-molecule experiments to ensure successful binding of the aptamer to TNFα, the trimeric nature of the protein was first examined using gel electrophoresis and NMR (Figs 5.2A and B). Previous studies have shown that TNFα is active as a homotrimer in solution with three potential binding sites for TNF receptors. Therefore, it was important to confirm the formation of a trimer as this will be used later to test the binding of bivalent and trivalent forms of monoApt1 (see Binding Affinity section). To verify this, we first ran a denaturing SDS-PAGE gel based on TNFα crosslinking via glutaraldehyde (glut). In this crosslinking strategy, multiple units of TNFα are covalently linked and thereby cannot be separated even under denaturing environments. As shown in Figure 5.2A, the band corresponding to TNFα alone (lane 4) shows a molecular weight of ~17 kDa, which is equivalent to a TNFα monomer. However, when glut was preincubated with TNFα, the band clearly shifted to a higher molecular weight of ~51 kDa confirming the formation of a trimer (lane 5). Streptavidin was used as a control protein and showed expected results without (~13 kDa, lane 2) and with (26 kDa representing dimer with very
faint bands ~39 kDa and ~55 kDa corresponding to trimer and tetramer respectively, lane 3) glutaraldehyde. Similarly, the NMR experiments demonstrate that the TNFα produced here forms a trimeric complex (Fig. 5.2B). Preliminary observations of $^{15}$N-labeled TNFα using $^{15}$N-HSQC experiments yielded broad lines and a low signal-to-noise ratio, suggesting that a large complex is formed. If the ~17 kDa form of TNFα was predominant, narrow line shapes and higher signal intensity would have been observed. The use of $^{15}$N-TROSY enhances the signal of larger proteins and complexes. Collection and observation of the $^{15}$N-TROSY spectrum indicated a significant narrowing of line widths and increased signal intensity in most peaks, consistent with the large, ~51 kDa trimeric complex (Fig. 5.2B). Comparison to a previously published spectrum indicates $^{15}$N-TROSY peaks are generally positioned where expected for the TNFα homotrimer.

5.3.3 Single-molecule analysis of TNFα binding

First, the DNA construct at ~20 pM dispersed in 1× TAE buffer was injected into the flow cell to immobilize the constructs onto the surface-coated slide via biotin-streptavidin interaction (Fig. 5.1). The unbound molecules were then washed off using an imaging buffer containing an oxygen scavenging system (OSS), which helps retard the photobleaching and photoblinking of fluorophores. The FRET movies were recorded using a prism-based total internal reflection fluorescence microscope. Movies were acquired by continuously illuminating the slide with a 532 nm laser (Fig. 5.1) while simultaneously recording Cy3 and Cy5 emissions using an EMCCD camera.
Toward the end of each movie (after ~1000 frames), the red laser (639 nm) was turned on to excite Cy5 labels to confirm an active FRET pair in the DNA construct, which also confirms the full assembly of the construct. The binding experiments were performed by injecting the same imaging buffer containing a mixture of protein and H1 strand into the flow cell followed by ~20 minutes of incubation before recording movies. The recorded FRET movies were processed using previously established IDL and MATLAB scripts to get intensity-time and FRET efficiency traces.\textsuperscript{237,231} Intensity-time traces were later manually screened to only choose single molecules confirmed by the presence of both the Cy3 and Cy5 fluorophores and the single-photobleaching

\textbf{Figure 5.1.} The DNA construct is labeled with either a Cy3 or Cy5 fluorophore to enable FRET. The biotin tag incorporated at the 5’ end of the Cy3 strand enables surface immobilization of the DNA construct via biotin-streptavidin interaction. In the absence of the protein of interest or H1 strand, the construct adopts a low-to-mid FRET state but it is changed to a high-FRET state when both the aptamer and B1 are displaced in a two-step process – (i) aptamer forms complex with protein and (ii) the exposed B1 strand hybridizes with H1 forming H1-B1 duplex.
events for the fluorophores. In this study, we first investigated the binding of monoApt1 and TNFα and compared it with its bivalent and trivalent versions.

Figure 5.2. (A) SDS-PAGE verifying TNFα trimer via glutaraldehyde (glut) cross-linking. Lane 1: HiMark ladder; Lane 2: Streptavidin (2µg); Lane 3: Streptavidin (2µg) + 0.5% glut; Lane 4: TNFα (2µg); Lane 5: TNFα (2µg) + 0.5% glut. All samples were preincubated at room temperature for 90 min, after which a 2× SDS loading buffer containing DTT was added to quench crosslinking reactions. The gel was run for 45 min at 95 V and the bands were visualized by staining in Coomassie blue. (B) ¹H-¹⁵N TROSY NMR spectrum of TNFα for further verification of the trimer. The spectrum was collected at 700 MHz and 37 °C. The use of TROSY resulted in a much higher quality spectrum compared to a ¹H-¹⁵N-HSQC spectrum, indicating that TNFα is trimeric in these conditions. Note: NMR data were collected by the Fuglestad group. (C) Single-molecule FRET efficiency traces. Five representative traces in the absence and presence of target [TNFα + H1] are shown. The traces showed a mid-FRET state in the absence of both TNFα and H1 (blue). In the presence of TNFα + H1, however, a high-FRET state was observed (magenta) indicating a binding event. Intensity-time traces from which these FRET traces were obtained are shown in Fig. 5.3. The data presented belongs to monoApt1.

To validate that the DNA construct works, it was initially tested it in the absence of TNFα as a negative control experiment. The FRET efficiency traces clearly showed a relatively static mid-FRET state of ~0.5 in the absence of a target, however, upon the addition of TNFα + H1, the population shifted to ~0.8 states (Fig. 5.2C), demonstrating a successful binding. The corresponding intensity-time traces of each of the 5 molecules obtained with and without TNFα are shown in Figure 5.3.
To quantify binding as a function of TNFα, FRET efficiency histograms were plotted for each aptamer tested (Fig. 5.4, see Methods for details). As expected, the histograms showed two distinct FRET populations. The ~0.5 and 0.8 FRET states correspond to the target unbound and target bound populations respectively (further explanation later). To determine the TNFα-bound population, the histograms were then fitted with a two-peak Gaussian function and the percentage distribution of the bound population was calculated using the area under the curve (AUC) according to the following equation:

$$\% \text{High-}E_{\text{FRET}} = \left[ \frac{AUC \text{ high-}E_{\text{FRET}}}{(AUC \text{ high-}E_{\text{FRET}} + AUC \text{ low-}E_{\text{FRET}})} \right] \times 100$$

A set of control experiments for each aptamer was carried out (highlighted region, Fig. 5.4). Although unexpected, a small high-FRET population (~10% or less) was observed for each control denoting the experimental background. This observation is attributed to a possible small fraction of the annealed DNA constructs with missing aptamer, which can directly assemble into a high FRET conformer. Similar results were observed when either the H1 strand or TNFα alone was added, indicating that the presence of either of them does not induce a significant high-FRET signal and that the high-FRET population that is greater than ~10% is indeed contingent upon the presence of both H1 and TNFα simultaneously. This was corroborated by conducting experiments with both H1 and TNFα introduced simultaneously into the system, which yielded a concentration-dependent increase in the high-FRET population. It is important to note that the presence of a small high-FRET population in controls does not negatively affect our analysis of TNFα binding, since the high-FRET population from the TNFα binding can be easily corrected for the background when needed.
5.3.4 Analysis of aptamer binding affinity

The binding affinity of aptamers toward TNFα was determined via titration experiments where we varied the concentration of TNFα. As described earlier, the high-FRET population expressed as %high-FRET was determined from the FRET efficiency histograms (Fig. 5.4) at each concentration of protein. As can be seen from the binding curve in Figure 5.5A, the %high-FRET

**Figure 5.3.** Typical intensity-time traces of five representative molecules in the presence and absence of target (TNFα + H1). Without the target, the intensity of Cy3 and Cy5 was almost overlapping, leading to a mid-FRET efficiency (see Fig. 5.2C for FRET traces). Upon addition of target, there was a clear separation of the Cy3 and Cy5 intensities, which translated into a high-FRET state (see Fig. 5.2C). All traces belong to the monoApt 1 construct. Experiments were conducted at room temperature (23 °C).
increased with protein concentration and reached a plateau at approximately 4 µM of TNFα for monoApt1. Given the three binding sites of TNFα for its natural receptor, we sought to test bi- and trivalent forms of this aptamer which we call biApt1 and triApt1, respectively. Our hypothesis was that increasing the number of binding sites would result in a more stable binding interaction due to multi-site binding. Figure 5.6 shows the predicted folded structures for all aptamers tested which indicate that the hairpin is the most likely binding motif for mono-, bi-, and triApt1. While biApt1 yielded a virtually similar binding curve to monoApt1, triApt1 reached a plateau at a much lower high-FRET fraction (Fig. 5.5). These observations suggest that (i) multi-valency does not improve binding efficiency most likely due to a one-to-one interaction between the aptamer and protein molecules and (ii) an inefficient dissociation of triApt1 from the construct (causing early plateau) is a result of non-competitive binding to this aptamer as it has 3 binding sites for TNFα and the protein does not have to displace the aptamer for the formation of a bound complex. In contrast, the data shows that biApt1 gets displaced by the protein as efficiently as monoApt1 despite having 2 binding sites, which is possibly due to steric reasons as the bulky complex does not fit well without having the aptamer dissociated from the construct. In parallel, we also tested another literature-reported TNFα-aptamer\textsuperscript{220}, which we call monoApt2 (mFold-predicted secondary structure is shown in Fig. 5.6D), in the exact same manner as we did for Apt1. Interestingly, this aptamer showed a much better binding affinity than monoApt1.
Figure 5.4. Single-molecule FRET efficiency histograms for monoApt 1 (A), biApt 1 (B), triApt 1 (C), and monoaApt 2 (D). The area shaded in yellow represents control experiments with the DNA construct alone (top row), construct + H1 strand (second row), and construct + 10.5μM TNFα (third row). The various concentrations of TNFα used are displayed on each histogram. The
concentration of H1 was constant (1 µM) in all the experiments in which it was used. All histograms were fitted with a two-peak Gaussian function from which the area under the curve (AUC) was determined. Histograms were created using 100 or more molecules at each protein concentration.

The binding affinities of the various aptamers tested were compared using dissociation constants (K_d). The K_d values were determined by fitting the TNFα binding data shown in Fig. 5.5A for each aptamer construct with the built-in Hill-1 equation in Origin (see Methods for more details).228,229

As shown in Figure 5.5B, the K_d value for monoApt1 was found to be 34 ± 22 nM which is higher than the previously reported K_d value of 7.0 ± 2.1 nM219 (see Table 5.2). This discrepancy in the binding affinity could be due to the restrictive binding of the aptamer to TNFα in our single-molecule strategy where the aptamer sequence is partially blocked by the blocker strand. Nonetheless, our binding analysis showed that the K_d of monoApt1 is much smaller than its bi- (K_d = 340 ± 471 nM) and trivalent (K_d = 862 ± 81 nM) forms, suggesting that monoApt1 is a better binder. A similar analysis for monoApt2 showed a K_d value of 0.02 ± 0.01 nM, which is significantly lower than all other aptamers tested possibly due to differences in their sequences and/or secondary structures (Fig. 5.6). For example, monoApt2220 is more G-rich and it has been indicated in the literature that G-rich sequences can adopt stable secondary structures called G-quadruplexes.238,239 When compared, monoApt1219 contains 4 GG repeats while monoApt2 contains G-rich stretches: GGGG, GGG, and GG, which emphasizes the argument that the higher binding affinity for monoApt2 may be coming from G-rich stretches. Interestingly, the K_d determined for monoApt2 is substantially lower than the previously reported K_d value220 (Table 5.2). One possibility for the higher K_d in the previous report could be due to the use of a nitrocellulose filter-based binding assay in which a high concentration of the protein can oversaturate the filter, limiting affinity measurements.240
Figure 5.5. Binding affinity analyses. (A) Binding curves demonstrating %high-FRET population as a function of TNFα concentration for all aptamers tested. The high-FRET populations come from Fig. 5.4. The error bars represent the standard deviations (SD) determined by randomly assigning molecules under the same protein concentration into 3 groups. (B) Individual binding curves for each aptamer fitted with the Hill-1 equation. The corresponding dissociation constants ($K_d$) are shown. It was assumed 1:1 binding interaction when determining $K_d$ values for monoaptamers. Whereas for biApt1 and triApt1, the $K_d$ is the average of two (1:1 and 1:2) and three possibilities (1:1, 1:2, and 1:3), respectively. The standard deviations for monoApt1 and monoApt2 were obtained from the fitting parameters of the Hill-1 function. For biApt1 and triApt1 as they have more than one fitting scenarios, the SDs were calculated using two and three $K_d$ values, respectively.
Figure 5.6. mfold-predicted secondary structures of monoApt1 (A), biApt1 (B), triApt1 (C), and monoApt2 (D). Note that the structures were generated using the UNA fold program with a folding temperature of 23 °C and ionic conditions of 150 mM [Na⁺] for the aptamer sequence without the nucleotide extension at the 5’ end. The ΔG values represent the Gibbs free energy of these predicted structures. The predicted structures of monoApt1 and monoApt2 shown here correspond to the lowest ΔG, whereas those of biApt1 and triApt1 are among the lowest ΔG values which are consistent with the secondary structure of monoApt1.
Table 5.1. Dissociation constants determined from previous studies and this study for monoApt1 and monoApt2.

<table>
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<th>Aptamer</th>
<th>$K_d$ (literature)</th>
<th>$K_d$ (this study)</th>
</tr>
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<tbody>
<tr>
<td>monoApt1</td>
<td>7.0 ± 2.1 nM (ref 219)</td>
<td>34 ± 22 nM</td>
</tr>
<tr>
<td>monoApt2</td>
<td>8 nM (ref 220)</td>
<td>0.02 ± 0.01 nM</td>
</tr>
</tbody>
</table>

5.3.5 Determination of specificity of binding

Specificity of binding is critically important when applying biomolecular interactions in biotechnology applications. Being able to specifically recognize the target in the large excess of non-target molecules is necessary for applying such technologies in real-life samples. Therefore, in order to assess the specificity of the aptamer construct designs that we developed here, we tested them by carrying out a series of experiments using saturating concentrations (10.5 µM) of a non-target protein bovine serum albumin (BSA) and other interfering cytokines, interleukin 1-beta (IL-1β) and coagulation factor VIIa (FVIIa). This was done by determining the high-FRET response of both monoApt1 and monoApt2 in the presence of each protein separately and in a mixture (Fig. 5.7). The H1 strand was added in all experiments conducted for the specificity study. Furthermore, a negative control with no proteins and an H1 strand was used to set the benchmark for the background FRET signal. Our results showed that the percentages of the high-FRET population arising from the individual non-target/interfering proteins are closer to the background (without TNFα) for both monoApt1 and monoApt2, and the p-value analysis showed that monoApt2 is less responsive to non-target proteins than monoApt1. Moreover, the resulting high-FRET population when using a mixture (BSA+ IL-1β + FVIIa + TNFα, each at 10.5 µM except FVIIa at 105 nM) is closer to the high-FRET signal obtained with TNFα alone for both aptamers. Hence, our data collectively demonstrate the specificity and selectivity of the developed method toward the targeted cytokine - TNFα.
5.3.6 Toxicity profile of monoApt1 and monoApt2 on βTC6 cell lines

After characterizing the ability of aptamers to bind the cytokine TNFα using our single-molecule platform, we followed these binding studies with in vitro functional assays (Figs. 5.8 and 5.9) through collaborative work with the Kanak Lab (Department of Surgery, VCU) to assess the anti-inflammatory activities of the aptamers in vitro using a n islet inflammation model. First, it is important to evaluate the toxicity profile of aptamers on beta cells and determine the optimal concentration that is tolerable for the islets. Thus, cytotoxicity of the aptamers was performed on mouse beta cell line βTC6 as a model system after treatment with monoApt1 and monoApt2 separately at different concentrations ranging from 0.01μM to 100μM using the MTT assay and the results are presented in Figure 5.8A (see Methods for more details). The viability was determined after treating the cells with the aptamers for 48 h (Fig. 5.8A). The viability of monoApt1-treated cells remained similar to control in all the concentrations tested, except a
significant decrease in viability was observed at 100 μM concentration, suggesting increased toxicity of monoApt1 beyond 50μM. Interestingly, the viability of monoApt2-treated cells was similar to the control group, but overall showed a better trend compared to monoApt1. The viability of monoApt2-treated cells at 100μM was significantly better than monoApt1-treated cells, suggesting little to no toxicity of monoApt2 towards beta cells. The cell viability of monoApt2 was also comparable with Etanercept (ETA 100μM) treated cells. Overall, we found monoApt2 to be more tolerable by beta cells at concentration ranges up to 100μM and performs similarly to ETA.

5.3.7 Alleviation of proinflammatory cytokine-induced cell death by TNFα aptamers

Cytokines such as TNFα, IL-1β and IFNγ have been linked to islet cell death due to apoptosis both in vitro and post-transplantation. It is therefore imperative to protect human islets from cytokine-induced cell death. Hence, monoApt1 and monoApt2 were next tested to protect human islets from proinflammatory cytokine-induced cell death (see Methods). Briefly, human islets were cultured in media containing cytokine cocktail (CC) in the presence or absence of aptamers. Human islets (1000IEQ/group) were pre-treated with monoApt1 or monoApt2 for 1 h and then exposed to cytokines in culture for 48 h. Cell viability was calculated by DAPI/PI staining after 48 h. MonoApt1 and monoApt2 were tested at various concentrations ranging from 0.05 to 100 μM in the presence of mild cytokine exposure (data not shown here) or stringent cytotoxic cytokine cocktail (CC) exposure (IL-1β+TNFα+IFNγ) (Fig 5.8B). Human islets exposed to a more stringent cytokine cocktail (CC) treatment demonstrated a modest reduction in the islet viability compared to controls (Fig. 5.8B). More importantly, monoApt1 treatment showed little-to-no improvement in the cell viability after 48 h of culture at any of the tested concentrations, however, Etanercept (ETA) and monoApt2 treatment at 100 μM showed significant improvement in viability.
when compared to CC-treated islets (Fig. 5.8B). Overall, these results suggest that monoApt2 is more effective than monoApt1 in protecting islets from TNFα-induced cell death, which is consistent with the much lower $K_d$ of monoApt2 that we determined from the single-molecule binding experiments (Fig. 5.5). Furthermore, these comparative analyses also show that the cell viability is equal to or better than the current gold standard treatment of ETA. For a more extensive report of all the in vitro functional assays conducted in this project, please refer to our publication.\textsuperscript{139}

\textbf{Figure 5.8.} (A) The cytotoxicity of monoApt1 and monoApt2 on βTC6 cells after an incubation period of 48 h compared with a control group (Ctrl), shown as 100%. Significance was determined by the one-way ANOVA test (see statistical analysis under Methods) followed with Dunnett’s multiple comparisons test (* $p < 0.01$ compared to the control group). Results are expressed as
mean ± SD (n = 2). (B) Islet viability after the 48-h treatment with monoApt1 (top) and monoApt2 (bottom) at different concentrations in the presence of cytokine cocktail (CC). Blue and red represent DAPI-stained all nuclei and propidium iodide (PI)-stained nuclei of necrotic cells, respectively. The mean viability (± SD) after 48 h of monoApt1 and monoApt2 treatment to human islets are shown on the right side of the corresponding panels. Results are expressed as mean ± SD (n = 3). Significance was determined by the one-way ANOVA test (* denotes P<0.05 and ** denotes P<0.01, significant as compared to CC). All these experiments were conducted by the Kanak group.

5.4 Conclusions and Future Work

The outcomes of islet transplantation can be significantly improved by addressing the issue of islet cell loss caused by post-transplant inflammation. One of the key cytokines that results in islet cell apoptosis post-transplantation is TNFα and it has been demonstrated clinically that introducing an antagonist for TNFα such as etanercept improves graft survival. Herein, we developed and utilized a novel single-molecule approach along with in vitro assays to characterize the ability of different aptamers in blocking the inflammatory cytokine TNFα, which can be useful for islet surface engineering for improved engraftment. Through our single-molecule analyses, we found that mono-aptamers (specifically monoApt2) bind more effectively to TNFα with significantly higher binding affinities than bi- and tri-aptamers. Furthermore, the in vitro cell viability analysis showed that monoApt1 and monoApt2 are safe for cells up to 50 µM and 100 µM, respectively, however, monoApt2 is more effective in preventing cytokine-mediated cell death and proinflammatory gene expression in human islets. The specificity and selectivity of both aptamers were assessed and verified in the presence of potentially interfering inflammatory cytokines. From the design perspective, the developed single-molecule strategy in combination with in vitro assays can be easily adapted to target other cytokines by implementing cytokine-specific aptamers. Hence, this study can be extended to target other relevant cytokines such as IL-1β and FVIIa for a more robust suppression of cytokine-induced inflammation.
CHAPTER 6 – CONCLUDING REMARKS

In project 1, using a single molecule FRET based platform, we have successfully investigated the dependence of HJ branch migration on the microenvironment using different solution compositions and crowded environments simulated by the cosolutes DMSO and PEG. We have shown that BM dynamics and kinetics are affected in a concentration-dependent manner under such biomimetic environments. Specifically, BM was enhanced in DMSO and PEG as buffer additives as compared to the cosolute-free environments. Additionally, from the kinetic analyses we demonstrated that the HJ preferably undergoes partial migrations through a few bp hops rather than full migration along the entire homology region, suggesting an equilibrated state of BM under these buffer conditions. The mechanistic insights that we gained into the BM process from these single-molecule analyses can lead to new perspectives on the functional roles of HJ-binding proteins and may open the door for therapeutic opportunities targeting the HJ and its dynamics. Therefore, future work will compromise single-molecule investigations of the mobile HJ-protein systems (e.g., RuvA protein) to determine their functional roles in BM.

In project 2, we conducted a preliminary evaluation of a HJ-binding ligand called VE-822. Using immobile and mobile HJ model constructs and adapting the single-molecule approach we developed in project 1, we showed that this ligand binds to the HJ and alters its conformation. Moreover, this ligand was also shown to affect the dynamics of the HJ by possibly stabilizing specific conformations, and thus it bears some therapeutic potential. These findings set the stage for future experiments using smFRET, CD and computational studies for a more thorough analysis of HJ-ligand binding interactions and structural effects. Once fully characterized, VE-822 has the potential to be used as a HJ stabilizer for therapeutic applications.
Finally, in project 3, we moved beyond the biological and therapeutic roles of HJ and instead targeted a critical cytokine called TNFα that is implicated in islet cell inflammation for the treatment of type 1 diabetes (T1D). Through collaborative work using single-molecule coupled with in-vitro studies, we were able to investigate the ability of DNA aptamers to bind and block TNFα and protect islets from cytokine-induced inflammation and cell death. Notably, through a systematic study, we showed that mono-aptamers bind more effectively to TNFα than bi- and tri-aptamers with high specificity, significant alleviation of islet inflammation, and protection against cell death, which was comparable to the gold standard treatment of the TNFα antagonist etanercept. Overall, in this project we were able to collaboratively demonstrate the high potential of using aptamers as anti-inflammatory agents for islet surface engineering to improve graft function. In the future, we hope to optimize our platform to target other cytokines such as IL-1β and FVIIa for their ultimate application as cell-surface modifying agents to treat islet inflammation.
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Pervushin, K.; Riek, R.; Wider, G.; Wuthrich, K. Attenuated T2 Relaxation by Mutual Cancellation of Dipole–Dipole Coupling and Chemical Shift Anisotropy Indicates an Avenue


APPENDIX 1

Custom-designed sequences for all the oligonucleotides used in constructing biotin-, Cy3-, and Cy5-labeled HJs. All the biotin- and fluorophore-modified DNA oligonucleotides were purchased HPLC purified. B, T, L, and R represent the bottom, top, left, and right strand respectively. Nucleotides that were mutated to break the sequence homology and form the immobile (IM) construct are shown in red.

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<th>Strand Name (IM)</th>
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<td>Strand T (IM)</td>
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<td>Strand R (IM)</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td>Strand L</td>
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APPENDIX 2

Sequences of DNA oligonucleotides used in the assembly of the aptamer constructs. The aptamers were extended at their 5’ ends by 6 nucleotides. The aptamer part of the sequences is underlined and references for aptamer sequences are provided. BiApt1 and triApt1 represent the bivalent and trivalent versions of monoApt1. A short section of polythymine chain (poly T, shown in bold) was inserted between each aptamer unit to serve as a flexible linker.

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CURRICULUM VITAE

EDUCATION

Ph.D. in Chemistry, Virginia Commonwealth University Aug. 2019 – May 2024
B.Sc. in Chemistry, American University of Sharjah, UAE Jan. 2015 – June 2019

SELECTED SKILLS

Single-molecule techniques

• Single-molecule fluorescence microscopy using total internal reflection fluorescence microscope (TIRF) and the fluorescence resonance energy transfer (FRET) technique.
• Single-molecule analysis using optical tweezers microscopy.

Biochemical and spectroscopic techniques

• UV-vis spectrophotometry
• Fluorescence spectroscopy
• Fluorometry
• High-performance Liquid Chromatography (HPLC)
• Circular Dichroism (CD)
• Electrophoresis Gel Mobility Shift Assays (EMSA)

Computer and software skills

• Microsoft office package
• Other software (MatLab, HaMMy, Origin Pro, Corel Draw, Zotero)

PROFESSIONAL EXPERIENCE

Graduate Research Assistant, Department of Chemistry, VCU May 2021 - 2024

Investigated DNA Holliday Junction (HJ) conformational dynamics and interactions with proteins at the single molecule level.

Probed binding interactions between inflammatory cytokine and DNA aptamers for therapeutic applications.

Served as the Chemical Hygiene Officer (CHO) and managed safety and upkeep of the lab

Graduate Teaching Assistant, Department of Chemistry, VCU Aug. 2019 - Dec.2023

Supervised and mentored students in the following courses:
• CHEZ 301: Organic Chemistry I Lab – Fall 2019, Spring 2020
• CHEM 102: General Chemistry II Recitation – Summer 2020-2021
• CHEZ 101: General Chemistry I Lab – Summer 2020
• CHEZ 302: Organic Chemistry II Lab – Fall 2021, Spring 2021
• CHEZ 102: General Chemistry II Lab – Spring 2022
• CHEZ 409: Instrumental Analysis Lab – Fall 2022, Spring – Fall 2023

PUBLICATIONS


Kaur, A.†; Mahmoud, R.†; Megalathan, A.; Pettit, S.; and Dhakal, S. Multiplexed SmFRET Nucleic Acid Sensing Using DNA Nanotweezers. *Biosensors* 2023, 13, 119. † = co-first author


PRESENTATIONS

Mahmoud, R. Mechanistic analysis of four-way DNA junctions and cytokine-binding aptamers for therapeutic interventions. Departmental research seminar, VCU, February 2024 (Oral)

Mahmoud, R.; Kalivarathan, J.; Castillo, A. J.; Wang, S.; Fuglestad, B.; Kanak, M. A.; and Dhakal, S. Aptabinding of tumor necrosis factor-α (TNFα) inhibits its proinflammatory effects and alleviates islet inflammation. VCU, August 2023 (Poster)


Mahmoud, R.; Dhakal, S. “Single-molecule analysis of DNA branch migration under biomimetic environments”, VCU, October 2022 (Poster)
Mahmoud, R. (contributing); Dhakal, S. “Single-molecule analysis of DNA branch migration under biomimetic environments”, ACS annual meeting, August 2022 (Poster)

Mahmoud, R. (contributing); Dhakal, S. “Branch migration of Holliday junction DNA”, ANPA Conference, July 2021 (Oral)

Mahmoud, R.; Dhakal, S. “Single-molecule analysis of DNA branch migration”, Virginia Commonwealth University, October 2021 (Poster)

Mahmoud, R. (contributing); Dhakal, S. “Single-molecule analysis of branch migration kinetics”, ACS annual meeting, April 2021 (Oral)

Mahmoud, R. “PROTACs: a novel therapeutic strategy based on induced protein degradation” Literature Seminar, Virginia Commonwealth University, March 2021 (Oral)

Mahmoud, R.; El-Kaderi, O. “Radioactive iodine capture and storage using activated carbon”, Senior project presentation, AUS, April 2019 (Oral)

FELLOWSHIPS/HONORS/AWARDS

Gerald and Susan Bass Scholarship
Chemistry, Virginia Commonwealth University – Aug. 2023

Departmental Summer Fellowship
Chemistry, Virginia Commonwealth University – Jun. 2022

Albert T. Sneden III Scholarship
Chemistry, Virginia Commonwealth University – May 2022

Magna Cum Laude Honors
Chemistry, American University of Sharjah – Jun. 2019

Dean’s List Honors

Outstanding Graduate Student Award
Chemistry, American University of Sharjah – May 2019

SERVICE AND OUTREACH

Chemistry Club Executive Assistant, American University of Sharjah, Jul. 2017 – May 2018
American Chemical Society (ACS) membership, Aug. 2016 – 2017
Subcommittee member of the ACS student chapter at the Sharakah festival, American University of Sharjah, 2017