Development of dual functional DNA/RNA nanostructures for drug delivery

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DEVELOPMENT OF DUAL FUNCTIONAL DNA/RNA NANOSTRUCTURES FOR DRUG DELIVERY

by

Vibhav A. Valsangkar

A Dissertation
Submitted to the University at Albany, State University of New York
in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy

College of Arts & Sciences
Department of Chemistry
May 2020
Dedicated to आई, बाबा and आज्जी, आज्जो
ABSTRACT

In addition to the traditional biochemical functions, DNA and RNA have been increasingly studied as building blocks for the formation of various 2D and 3D nanostructures. DNA has emerged as a versatile building block for programmable self-assembly. DNA-based nanostructures have been widely applied in biosensing, bioimaging, drug delivery, molecular computation and macromolecular scaffolding. A variety of strategies have been developed to functionalize these nanostructures. The major advantage is that DNA is a very stable molecule and its base pairing properties can be easily utilized to control and program the formation of desired nanostructures. In addition, some of these DNA/RNA nanostructures have been shown to have special properties in targeting cancer cells. In this project, we have utilized 2D and 3D DNA/RNA nanostructures as the platform to demonstrate a facile “Click” strategy to incorporate functional ligands into these nanostructures. Specifically, the DNA three-point star tile (3PST), RNA three-way junction (3WJ) and DNA tetrahedron (TET) are selected as the targets. Using solid phase oligo-nucleotide synthesis, we incorporated the desired modifications into the respective nanostructures. The DNA/RNA strands, with various lengths and sequences, could be annealed in specific ratios to generate homogeneous nanocomplexes from couple of hours (3WJ) to maximum 48 hours (TET). We modified the DNA/RNA with 2’-O-propargyl groups and observed the formation of the desired nanostructure post functionalization through the modifications. The addition of an azido-modified metal chelating ligand or fluorescent tag as well did not affect the assembly formation. Such modified complexes have great potentials to be used as general delivery platforms for metals or drugs. We further demonstrate proof-of-concept dual functionality nucleic acid nanostructures, where we use click chemistry for the payload attachment on the nanostructures and use light as a trigger to release an attached moiety. For controlled release, we incorporated a photocleavable linker in the strands that can be activated by UV. We confirmed the dual functionality and the viability of this strategy for the application of drug delivery on nucleic acid nanostructures of different size and materials, from simple junctions up to complex assemblies.
DECLARATION

I, Vibhav A. Valsangkar, declare that this thesis titled, “Development of Dual Functional DNA/RNA Nanostructures for Drug Delivery”, and the work presented in it are my own. Studies discussed in Chapter 2 and Chapter 3 have been either published or accepted in the following citations and I am the lead author of the works.


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<table>
<thead>
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<th>Definition</th>
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<tr>
<td>2′-OP</td>
<td>2′-O-propargyl nucleotide</td>
</tr>
<tr>
<td>3PST</td>
<td>Three Point Star Tile</td>
</tr>
<tr>
<td>3WJ</td>
<td>Three Way Junction</td>
</tr>
<tr>
<td>A</td>
<td>Adenosine</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic Lateral Sclerosis</td>
</tr>
<tr>
<td>C</td>
<td>Cytidine</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>CuAAC</td>
<td>Copper catalyzed Alkyne–Azide Cycloaddition</td>
</tr>
<tr>
<td>CuBr</td>
<td>Copper Bromide</td>
</tr>
<tr>
<td>CUGBP1</td>
<td>CUG-binding protein 1</td>
</tr>
<tr>
<td>DI</td>
<td>De-ionized</td>
</tr>
<tr>
<td>DM1</td>
<td>Myotonic Dystrophy type 1</td>
</tr>
<tr>
<td>DM2</td>
<td>Myotonic Dystrophy type 2</td>
</tr>
<tr>
<td>DMPK</td>
<td>dystrophia myotonica protein kinase</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>FAM</td>
<td>Fluorescein</td>
</tr>
<tr>
<td>G</td>
<td>Guanosine</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>IR</td>
<td>InfraRed</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal Titration Calorimetry</td>
</tr>
<tr>
<td>Lig. 2</td>
<td>Ligand 2</td>
</tr>
<tr>
<td>MBNL1</td>
<td>Muscle Blind-like 1</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotide</td>
</tr>
<tr>
<td>Oligos</td>
<td>Oligonucleotides</td>
</tr>
<tr>
<td>PA</td>
<td>2′-OP Adenosine</td>
</tr>
<tr>
<td>PAGE</td>
<td>Poly Acrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>PC</td>
<td>2′-OP Cytidine</td>
</tr>
<tr>
<td>PCL</td>
<td>Photocleavable linker</td>
</tr>
<tr>
<td>PG</td>
<td>2′-OP Guanosine</td>
</tr>
<tr>
<td>PPG</td>
<td>Photocleavable Protecting Group</td>
</tr>
<tr>
<td>pRNA</td>
<td>packaging RNA</td>
</tr>
<tr>
<td>PTX</td>
<td>Paclitaxel</td>
</tr>
<tr>
<td>PU</td>
<td>2′-OP Uridine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Rf</td>
<td>Retention factor</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RNP</td>
<td>Ribonucleoproteins</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface Plasmon Resonance</td>
</tr>
<tr>
<td>T</td>
<td>Thymidine</td>
</tr>
<tr>
<td>tBuOH</td>
<td>Tertiary Butanol</td>
</tr>
<tr>
<td>TET</td>
<td>Tetrahedron</td>
</tr>
<tr>
<td>TET extd</td>
<td>Tetrahedron extended</td>
</tr>
<tr>
<td>Tm</td>
<td>Melting Temperature</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated Region</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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ACKNOWLEDGMENT

It’s been six years since I started my PhD program here at the University at Albany, SUNY. The whole of my doctoral journey has been a paradigm shifting experience. From coming to a new country, adjusting to a new culture and education system, to pursuing science and becoming more skilled and independent at doing so—at every step there have been so many people I need to thank, without whom I couldn’t have achieved this dream of mine.

First and foremost, I would like to express my most sincere gratitude to my mentor, Professor Jia Sheng. In my opinion, one of the biggest factors in successfully completing your doctoral degree is having a good relationship with your mentor. Jia has been the most understanding, helpful mentor one can ever ask for. He is very passionate about science and he has always inspired all of us in the lab to unravel the mysteries of the nature by studying science. I’ve been through many ups and downs during these years; I could get through all of those difficult times easily because I knew Jia believed in me and always supported me. Right from day one of my rotation, Jia gave me very exciting research ideas and very intriguing projects to work with. He taught me many things which are helpful not just in science but in life in general. I am very thankful to Jia that he gave me the opportunity to be part of his lab, for inspiring me to study science, giving me a different perspective towards looking at things, for giving me wonderful ideas and projects to work on, and for being there for me during both my highs and my lows. I thank you from the bottom of my heart.

I am deeply grateful to Dr. Ken Halvorsen, my co-mentor. I started working with Ken as a collaborator on several of my projects. Eventually the collaboration became mentorship. He is always very approachable. He helped and trained me on the application of sophisticated analytical tools which became an integral part of my research. He always gave me constructive criticism, which helped me in analyzing and fine-tuning my approach towards my experiments. Thank you very much, Ken, for everything that you have done for me.

I would like to thank my doctoral committee members. Prof. Jayanti Pande, right at the
beginning of my PhD program I took your Biochemistry class and that laid a good foundation for
the research I conducted during my PhD work. You’ve always looked after me, ensuring I was
doing well. I thank you for your support and suggestions. I want to thank Prof. Maksim Royzen
for his collaborations and guidance for my projects. Max has been actively involved in a lot of
projects in our lab. He always comes to the lab with a smile and puts a smile on our faces with his
witty comments. I would like to thank Prof. Mehmet Yigit for keeping interest in my well-being
and helping me out with some good collaborations in my projects as well as for his guidance.

I want to thank my collaborators Prof. Xing Wang and Dr. Megan Kizer for their contribu-
tion and ideas for the DNA nanostructure functionalization and release project. I thank Prof. Bijan
Dey and Dr. Paromita Dey for their collaboration and help with the cell studies for the pathogenic
RNA repeat expansion related project. I’ve learnt a lot from both of you. Being a chemistry stu-
dent, I didn’t get much exposure to biological experiments and tools. Both of you have taught me
and trained me for the same. I sincerely thank you for that. I would like to thank Prof. Andrew
Berglund and Tammy Reid for their collaboration with testing potential drug candidates in neurode-
generative disorders such as myotonic dystrophy. I would like to thank Prof. Jeremy Feldblyum
and Mengwen Yan for their help with Atomic Force Microscopy. I want to thank Prof. Alan Chen
and Dr. Sweta Vengavati for their help in Molecular Dynamic simulations for the human alpha-
thrombin and modified thrombin binding aptamer interactions. I would like to thank Prof. Jiafeng
Cai and Songyi Xue for their collaboration and providing us with a library of peptidomimetics as
potential drug candidates against pathogenic RNA repeat expansions. I want to thank Dr. Vladimir
Ermolenkov for training me on various analytical tools such as Isothermal Titration Calorimetry,
Circular Dichroism spectrometry, and Fluorescence spectrometry. I want to thank Dr. Kim De-
Weerd for training me on the plate scanner as well as the Typhoon scanner. I would like to thank
Dr. Maneesh Kumar for training me in cancer cell culture.

Fundamentals of understanding and loving science were rooted in me by my teachers and
professors during my undergraduate and graduate studies back in India. I would like to thank
Dr. Vikas Vaidya, Dr. Sonali Trivedi, Dr. P. A. Sathe, and Mrs. S. Sathe for teaching me the basics
of chemistry during my undergraduate years and making me curious to learn the science further by pursuing higher education. I would like to thank Dr. Rohini Shivabalan for teaching me about zoology, taking us zoology students on very informative and inspiring excursions in variety of forests in India, and for making us aware that we are just small part of this vast nature and we need to conserve it, preserve it, and value it. I cannot thank Dr. R. T. Sane enough, as he was my mentor who taught me so much about science and how to pursue it. He was a brilliant scientist himself and he inspired me and many others to harness the skills to become someone like him. I want to thank Dr. B. B. Vakil and Dr. Naomita Dhume for their guidance and words of encouragement throughout my master’s degree.

I’ve always been blessed by presence of wonderful people around me; may it be family, friends, or relatives. I am very lucky to be surrounded by such people throughout my life who always care for me, love me and shower me with their blessings. I would like to take this opportunity to thank all of these people. I would like to thank my childhood buddies— my schoolmates Atul, Mithilesh, Advait and Nikhil. You guys have been there for me through all these years, we have grown up together, and now we are all pursuing our respective careers. You always helped me get through tough times and always put a smile on my face. I would like to specially thank Reji. You’ve always listened to all my nagging without complaints and put up with me through all these years. You are a true friend and I am grateful for all that you’ve done for me. I would like to thank Rupal for being there for me. I want to sincerely thank Anagha for being a kind, caring, and understanding friend and for always supporting me. I would like to thank Amrisha from the bottom of my heart for being a constant in my life for more than a decade. We’ve had our ups and downs, but we always managed to keep the bond strong. You’ve always been a big part of my life and I will always cherish this friendship.

I made some very good friends after coming here to Albany, they were the reason I could settle down in this new place quite easily without getting homesick. I would like to sincerely thank Phensinee; both of us started our PhD journey together in the Sheng lab. We’ve had some really good discussions related to science as well as sociopolitical issues and I enjoyed them a lot.
You’ve given me useful suggestions regarding my experiments and helped me from time to time. I’ve found a good friend in you. I would like to thank Dr. Song Mao from the bottom of my heart. Song you’ve been an integral part of our lab. You’ve helped and taught me so much. I’ve also enjoyed our football-related discussions and watching the games together. I would like to give a special thank you to Dr. Arun R. Chandrasekaran. Arun, after Jia and Ken, you are the person who has guided me the most in this whole journey. You are associated with most of my projects. You introduced me to this beautiful world of nucleic acid nanotechnology, and I cannot thank you enough for all that you’ve done for me during all these years. I would like to thank Dr. Lifeng Zhou, Dr. Jibin Punnoose for their help and suggestions in my experiments. I would like to thank Dr. Fusheng Shen, Dr. Yaning Tang, Dr. Ying Wu, Dr. Rui Wang, Johnsi, Goh Woon, Ya Ying, Jinxi, and Zhihua for being wonderful lab-mates and creating a friendly and healthy working environment in the lab. I would like to thank Parisa for being wonderful friend and for supporting me. I would like to thank Sangeetha, Sweta, Jibin, and Pradeep for being amazing hosts to number of parties and for the numerous delicacies that I’ve had at your respective places. I want to give a special thanks to Sarasi, we became such good friends during these six years. Everything from getting our daily coffee, going to gym together, to going for movies and brunches/dinners; I have so many fond memories with you over these past few years. You were always there for me, caring for me, looking after me, even after you graduated. I am really thankful that I found a friend like you. I would also like to thank Brian Gabriel from the bottom of my heart. Brian, you have helped me from my day one in the chemistry department from getting the teaching assistantship to all the little doubts and questions I’ve bothered you with for the last six years. I am truly thankful for everything.

I would sincerely like to thank Nandini, Aishwarya, Mounika, Pavani, Shiren, Saurabh, and Abhishek for being amazing friends and making my life outside of the lab very entertaining and fun. I have many fond memories with all of you. I feel lucky to have made so many close friends here in Albany.

I want to thank Anirudha dada from the bottom of my heart. Coming here to the US, so far away from home was never worrisome to me because I knew you were here. You personally came
with me to Albany and helped me settle into this new place and get acclimatized. Thank you very much for being there for me. I sincerely want to thank Arshad. You’ve been my flat mate and a good friend for more than four years now. I have enjoyed all the board game sessions, jamming sessions, Indian food take outs, your Christmas gifts and the delicious meals that you cooked. Thank you for being so kind, helpful and considerate.

I want to thank from the bottom of my heart Prathamesh, Omkar, Dipti, and Mamta. You guys are not just my friends but my family here in the US. I always felt safe and comfortable knowing you all are there for me. You’ve looked after me and cared for me throughout these years. I cannot thank you guys enough for all that you have done for me.

Both my maternal and paternal grandparents have showered me with unconditional love and support since my childhood. I want to thank them all from the bottom of my heart. All your blessings have made it possible for me to achieve this. I would like to thank my younger sister, Vaidehi, for caring for me so much, for supporting me morally and emotionally, whenever I was in need of it, and for taking such good care of my parents and my grandparents while I was pursuing this degree over here. I want thank Milind mama, Gauri mami, Aditi attya, Sandeep kaka for being my well-wishers and for your blessings. I want to thank Soumitra, Rohan, Saudamini, and Ishaan for being the most amazing cousins.

Last but not the least, I want to thank आई (mother), Mrunal Valsangkar and बाबा (father) Amit Valsangkar for everything that you have done for me throughout my life. You’ve made many sacrifices so that I can achieve whatever I want. You both are my role models. You have inspired me to be a good human being through your actions. You have always been very understanding, supportive, and loving parents. The journey of my PhD wasn’t an easy one. Both of you always boosted my morale and prepared me to overcome all the hurdles. I am out of words to describe my gratitude towards both of you. Thank you for everything.
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CHAPTER 1

Introduction

1.1 Nucleic Acids as Materials

Over the last three decades, there has been an emerging novel perspective towards nucleic acids (DNA/RNA) beyond their biochemical functions for potential applications as materials/polymers. DNA and RNA are natural biopolymers that have well-defined basic structure and conformations and can form hydrogen bonds in order to form base pairing within two molecules. The nucleic acids being nanoscale molecules and with such characteristics, are good candidates to be used as materials to build nanoscale structures. With thorough understanding about the structure and the base-pairing properties of these molecules, one can manipulate the nucleic acid interactions. The programming of the sequences helps in achieving a rational design of intricate nanostructures of various sizes\(^1\). DNA/RNA nanotechnology provides a versatile approach towards formation of complex nanoscale devices. The thermodynamics of DNA base pairing can be well predicted and measured, the cost of DNA synthesis is comparatively low, and the ability of DNA to self-assemble into the nanoassemblies in an enzyme-free environment makes DNA nanotechnology successful\(^2,3\).

Over time, different techniques/approaches have been developed and fine-tuned for the formation of various sizes and shapes of these nucleic-acid-based nanostructures (Fig. 1.1)\(^4-7\).

The use of DNA is more common than the use of RNA. Recently, however, there have been several research groups who have started focusing development of RNA-based nanostructures as well. The RNA, although structurally very similar to DNA, has its own characteristics, such as being able to form non-canonical base pairing or having predominant single stranded stable secondary structures. These traits could benefit in achieving certain geometries or shapes using RNA as material. There are several functional RNA such as aptamers\(^8,9\), ribozymes\(^10\), riboswitches\(^11,12\), and other non-coding RNAs\(^13-15\), which can be incorporated into the initial design to achieve functionality into these RNA nanostructures\(^6,16\).
Figure 1.1: Structural foundations of structural DNA nanotechnology and representative examples (each panel described left to right). Seeman’s original proposals to use immobile DNA junctions to create self-assembling arrays (A) and self-assembled 3D DNA lattices (B) as scaffolds to organize macromolecules into crystalline lattices. (C) DNA nanostructure motifs used to create periodic 2D arrays and 3D crystal (top, helical structures of the motifs; bottom, AFM images of the assembled 2D arrays and optical image of the 3D crystal): double-crossover DNA tile, 4×4 DNA tile, 6×4 DNA tile, and tensegrity triangle DNA tile. (D) Polyhedral DNA nanostructures: molecular models of a DNA cube, DNA tetrahedron, DNA dodecahedron, and DNA biprism. (E) Algorithmic self-assembly based on double-crossover tiles: Sierpinski triangles and binary counter. (F) DNA origami nanostructures (top, schematic drawings of the structures; bottom, corresponding AFM or TEM images): 2D DNA origami smiley face, 3D DNA origami in the shape of a gear, curved single-layer 3D origami in the shape of a vase, and DNA origami gridiron. (G) Complex nanostructures produced using the single-stranded DNA tile strategy. Reprinted with permission from Zhang, F.; Nangreave, J.; Liu, Y.; Yan, H. Structural DNA Nanotechnology: State of the Art and Future Perspective. Journal of the American Chemical Society 2014, 136, 11198–11211, DOI: 10.1021/ja505101a. Copyright © 2014 American Chemical Society. Further permissions related to the material excerpted should be directed to the ACS.
There is a vast variety of DNA/RNA nanostructures and with that come variety of applications. The field has always been motivated by the applications of these nanostructures in various areas such as medicine, forensics, bioelectronics, etc.

These nanostructures are providing a solid platform for pharmaceutical applications. One of the major application for these nucleic acid nanostructures is for efficient drug delivery. Wide range of research is being done to bring these nanostructures in human trials as viable drug delivery agents in the near future. These nanostructures have proven very effective in drug delivery for ranging from RNAi to anticancer drugs. Other than drug delivery these molecules are looked at for biosensor-based applications as well. DNA-based biosensors utilize DNA strands as probes for sensing targets and the processes of target recognition are usually in two ways, (1) Hybridization of probes with DNA or RNA targets, based on which a wide range of sensors have been designed, including probes for in vitro DNA damage detection, (2) association of probes with targets or sub-units related to specific properties of targets. For example, various sensors have been developed on the basis of association of aptamers with proteins or small molecules and the intercalation of molecules to DNA double helix. Unlike common biosensors based on enzyme or antibodies, DNA-based biosensors with high sensitivity can be prepared with low cost and high assembly efficiency. DNA-based computation is another upcoming branch related to this field. DNA circuits rely on components that are mechanistically simple and rationally designed at the molecular level, which provides a high degree of control over the reaction pathway. New orthogonal components can be designed simply by changing sequence, which makes it easy to increase system size in a modular fashion. Most dynamic DNA devices have a relatively small DNA footprint compared with systems assembled from genetically encoded proteins.

DNA/RNA are looked at as materials for all these applications because of their programmability. With solid phase oligonucleotide synthesis being well established, customization of the component strands through various modifications has become much easier. Our lab focuses on nucleic acid modifications, both natural and synthetic. The nucleic acids modifications can be on the base, on the sugar or on the backbone. These modifications on the component DNA strands can be
beneficial to the nanostructure formations. The modifications can play vital roles such as providing thermodynamic stability to the assembly, the modifications can help the with protection against nuclease degradation, can provide structural support and rigidity to the nanostructures\textsuperscript{33–35}.

Most importantly these nucleic acid modifications can provide us means of functionalizing these nanostructures in order to provide them with applications. The modifications on the nucleic acid nanostructures can help facilitating different types of chemistries for various applications ranging from payload attachment, payload detachment, disassembling the nanostructure, to provide structural rigidity etc.\textsuperscript{36,37}. Hence, in past two decades, it has been well established that DNA as well as RNA can be used as materials in the field of nanotechnology.

1.2 Fundamentals of DNA/RNA nanotechnology

The discovery that DNA is the prime genetic molecule, carrying all the hereditary information within chromosomes, immediately focused attention on its structure. It was hoped that knowledge of the structure would reveal how DNA carries the genetic messages that are replicated when chromosomes divide to produce two identical copies of themselves. The DNA was found to have a duplex form with a helical structure. The connection between two DNA strands is through hydrogen bonding between the complementary bases of the nucleotides (A-T; G-C). RNA, which at first glance appears to be very similar to DNA, has its own distinctive structural features. It is principally found as a single-stranded molecule. Yet by means of intra-strand base pairing, RNA exhibits extensive double-helical character and is capable of folding into a wealth of diverse tertiary structures, such as non-classical base pairs\textsuperscript{38}, base-backbone interactions\textsuperscript{39}, and knot-like configurations\textsuperscript{40}.

These structural properties of the nucleic acids play a crucial role in their biochemical functions, but from material science point of view as well these unique characteristics of the nucleic acids can be utilized to build nanostructures.
Figure 1.2: Self-assembly of branched DNA molecules to form larger arrangements. A four-arm branched junction made from four differently colored strands is shown on the top. There are 5’ sticky ends on each strand, labeled (clockwise from the left) X, Y’, X’, and Y; the sticky ends are indicated by small extensions from the main strand, the arrow head end of the strands represent the 3’ end. The primed sticky ends complement the unprimed ones. The image on the right shows how four of these junctions can self-assemble through this complementarity to yield a quadrilateral. The unused sticky ends in the quadrilateral could be used to generate a lattice in two dimensions (2D) and in 3D.

There are three major factors governing DNA nanotechnology: hybridization, stably branched DNA and convenient synthesis of designed sequences. The diameter of the double helix is about 2 nm, and the helical pitch is about 3.5 nm; hence, construction involving DNA is fundamentally an exercise in nanoscience and nanotechnology. While considering the design two important factors are the primary motif and the sequence of the component strands. The motif will be the primary building block in order to achieve a larger assembly (Fig. 1.2). Another key factor to consider during the motif design is the polarity of the component DNA strands. The crossover operation of the strands can be done between strands of similar polarity or opposite polarity. Sequence symmetry minimization is a parameter that will govern the success of the assembly formation as well as the percent yield of the nanostructure. The key to any successful molecular engineering approach is to design the components of a construction not just so that they are capable of yielding the product,
but at the same time make sure that no byproduct will be competitive with the desired output structure. Ultimately, we must estimate the thermodynamics of all possible sequences and select the one most likely to lead to the intended product. There are software that help with such estimations such as Uniquimer 3D. In general to come up with a rational design there are several software commercially available, for example Cadnano, Autodesk Maya, BioBlender, etc.

In order to achieve the structure formation there are two approaches, which are most used: a) Hierarchical self-assembly approach; and b) DNA origami approach.

The hierarchical self-assembly approach starts with a primary motif formation. The component strands come together in specific ratios and at specific concentrations to form the primary motif. This primary motif then further leads to a higher ordered structure formation. The self-assembly of this type is usually one pot reaction.

The design and programming of the component strands is done in such a way, that apart from the region of the strands involved in the direct formation of the primary motif, there is an overhang region left. This is known as the sticky end region. The higher ordered structure formation takes place through sticky end cohesion amongst the primary motifs.

DNA origami-based approach is more recently developed compared to the hierarchical self-assembly. Origami is the art of folding, which precisely is what happening in DNA origami. In DNA origami, there are two sets of strands, 1) Staple strands; 2) Scaffold strands. The staple strands are typically hundreds in number and range approximately from 10 to 60 nucleotides in length. The scaffold strand is much larger than the staple strands and range approximately from several hundred to few thousand nucleotides in length. These staple strands help fold the scaffold strand into the desired shape through binding to the complimentary regions on the scaffold strand (Fig. 1.3).
1.3 DNA/RNA nanostructures as drug delivery vehicles

As discussed earlier, one of the major applications for these DNA/RNA nanostructures is drug delivery. There are few factors to be considered while considering the drug delivery application, the nanostructure must be stable in the biological fluids, the nanostructure should successfully reach the target, the nanostructure must be taken up by the cells etc. The studies done with different type of non-DNA/RNA nanoparticles suggest the size ranges acceptable for specific targets, for example, nanoparticles of size less than 15nm can cross the blood brain barrier, through intrapulmonary administration, non-cationic particles with a size range of 6–34 nm were reported.
to be trafficked rapidly to local lymph nodes, lipid and lipid-like material based nanoparticles with a size less than 100 nm can cross liver fenestrae and target hepatocytes, through inhalation or intravenous administration, nanoparticles can be delivered to the lungs of the size larger than 5 mm are more likely retained in the lungs for prolonged periods of time etc. DNA nanotechnology enables the precise assembly of well-defined nanostructures with sizes ranging from a few nanometers to over 100 nm. So nanoparticles suitable for various targets can be achieved by DNA/RNA nanotechnology.

Payload attachment is the first step towards designing the DNA/RNA-based nanostructures. The choice of cargo attachment depends on factors such as tunable stability of the drug–carrier complex and responsiveness to internal, environmental or remote triggers for controlled delivery. Usually for small molecules such as some of the anti-cancer drugs (e.g., doxorubicin) and metal complexes, intercalation is the method of loading in the duplex region of the nanostructure. DNA polyhedra, cage-like DNA nanostructures and DNA origami-based structures (such as triangular, tubular, and cylindrical shapes) loaded with anthracyclines (class of anti-cancer drugs) by intercalation showed improved tumor localization and toxicity compared to the free drug. For attachment of larger molecules chemical methods such as biotin-streptavidin interaction etc. can be incorporated in the design. The release of the payload can be achieved through external stimuli such as pH change, strand displacement or spontaneous release such as by DNAse degradation.

We developed a similar approach to use nucleic acid modifications to functionalize the nucleic acid nanostructures to be used for the drug delivery application. We intended to control the attachment and the detachment of the payload (drug molecule) through different chemistries, which can be applied to the nanostructures through specific nucleic acid modifications.

In our study, we are focusing on the nanostructures based on the self-assembly of the component strands. We selected three structures to demonstrate our strategies of functionalizing these nanostructures for the drug delivery application.
A) Phi29 DNA packaging motor

B) pRNA 2° structure

C) pRNA 3WJ 2° structure  

pRNA 3WJ crystal structure

Figure 1.4: (A) The side-view of the hexametric structure of phi29 DNA packaging motor. (B) Predicted secondary structure of the phi29 packaging RNA (pRNA) with the 3WJ motif outlined and the pRNA-3WJ and pRNA-3WJ secondary structure with solved crystal structure. Reprinted with permission from Binzel, D. W.; Khisamutdinov, E. F.; Guo, P. Entropy-Driven One-Step Formation of Phi29 pRNA 3WJ from Three RNA Fragments. *Biochemistry* 2014, 53, 2221–2231, DOI:10.1021/bi4017022. Copyright © 2014 American Chemical Society. Further permissions related to the material excerpted should be directed to the ACS.

RNA three-way junction (3WJ), DNA three-point star tile (3PST), and DNA tetrahedron (TET) were the three models we chose to work with. These three models were picked based on literature review. These three structures were shown to have potential as drug delivery agents. While considering the models we picked different size, shape, material, and design in order to show the versatility of our approach.

The RNA 3WJ is the simplest and smallest with respect to the design and size compared to the rest of the two structures (Fig. 1.4). It is a two-dimensional (2D) structure made up of three component strands of the lengths 16, 18 and 20 nucleotides (nt). The RNA 3WJ model is inspired from a naturally existing motif. This motif exists in packaging RNA (pRNA) of the bacteriophage...
phi29 dsDNA packaging motor. The motif is extensively studied by Prof. P. Gou from Ohio State University. In various publications he has shown that the nanostructure is very stable and capable to be used as drug delivery vehicle. This is only RNA-based nanostructure in our project.

The DNA 3PST is a hierarchical self-assembly based 2D motif. It consists of three component strands with lengths 21, 42 and 78 nt respectively (Fig. 1.5, 2.1). This structure has been published and well characterized. Unlike the RNA 3WJ, it has never been used for the drug delivery purpose before. This structure is a primary motif, which leads to hierarchical self-assembly of higher ordered 3D structures through sticky end cohesion. We modified the 21 and 48 nt long strands to have blunt ends instead of the having sticky ends so that we could restrict the structure formation to 3PST. The sticky ends on the 21 and 48 nt long strands would lead to formation of 3D DNA tetrahedron.

![Diagram](https://example.com/diagram.png)

**Figure 1.5:** Three different types of DNA single strands stepwise assemble into symmetric three-point-star motifs (tiles) and then into polyhedra in a one-pot process. There are three single-stranded loops (coloured red) in the centre of the complex. The final structures (polyhedra) are determined by the loop length (3 or 5 bases long) and the DNA concentration. This figure is Fig. 1 (Page 198) from He et al., 2008, available at 10.1038/nature06597, reproduced with permission (Fig. B.5). Copyright © 2008, Springer Nature.
The DNA tetrahedra form when solutions containing the DNA tetra-strands are mixed and slowly cooled from 95°C to room temperature. The size of the closed structures is concentration-dependent. At sufficiently low DNA concentration, pseudoc-continuous DNA duplexes form. By cryo-electron microscopy (cryo-EM), we observed tetrahedron structures (Fig. 2e), the observed edges are 16 nm long, assuming 0.33 nm per base pair for the pitch and 2 nm for the diameter of a DNA duplex, respectively.

To provide direct evidence for the self-assembly of DNA into tetrahedron shapes, we used dynamic light scattering (DLS) and non-denaturing polyacrylamide gel electrophoresis (PAGE). DLS measures the physical sizes of the dissolved DNA strands, while PAGE provides a direct measurement of the physical sizes of the dissolved DNA. The results from these experiments are consistent with the formation of tetrahedra.

We also used atomic force microscopy (AFM) and cryogenic transmission electronic microscopy (cryo-EM) to visualize the tetrahedra. AFM images show uniform-sized, triangular particles with a base length of 5 nm and a height of 110 nm. Cryo-EM images reveal circular features with uniform sizes (Fig. 3b) and apparent hydrodynamic radii of 24.0 nm for the assembled objects.

The final structure that we used in our project is DNA tetrahedron. This is the largest structure amongst all three structures. The size of it is approximately 14 nm. This structure is hollow from within and has six arms/edges. These edges are formed by sticky end cohesion amongst four 3PST. We chose this structure as the tetrahedron-shaped nanostructures have shown good potential in the drug delivery application.

Our approach is to use the nucleic acid modifications to facilitate the payload (drug molecules, fluorophore, aptamers, etc.) attachment onto the nanostructures as well as to trigger the controlled detachment of the payload upon reaching the target.
CHAPTER 2

Functionalization of the DNA nano structures for Payload attachment through ‘Click’ chemistry


2.1 Introduction

The nanoscale features of DNA and its specific molecular recognition properties, as well as easy synthesis and modification, have made it a highly programmable biopolymer building block for the self-assembly of functional nanoscale materials, devices and machines. Some examples of DNA-based constructions include DNA objects, two- and three-dimensional lattices, DNA polyhedra and larger, complex shapes using DNA origami. Such DNA-based nanostructures have been used as biosensors, in biomolecular computation, as scaffolds for biomolecules and in biomedical applications including drug delivery. Over the last two decades, tremendous efforts have been made to incorporate functional guests into DNA for the construction of hybrid DNA nanoarchitectures. These include DNA–protein, DNA–RNA, DNA–small ligands, and DNA–nanoparticle hybrids. These additional functionalities can diversify the complexity of DNA assemblies and fine-tune the overall chemical or physical properties of DNA nanostructures for wider applications. Some of the strategies developed for the modification of DNA with functional moieties include synthesis of thio-DNAs to link gold nanoparticles with DNA strands, streptavidin–biotin conjugation, and biorthogonal strategies through click reactions. The compatibility of different functionalities with the DNA
self-assembly property is a key factor to fabricate new hybrid systems. In this study, we report the use of 2’-O-propargyl-modified nucleotides, which have been used for constructing several fluorescent probes\textsuperscript{100,101}, as a tool for the facile incorporation of small molecules into different positions of a designed DNA motif through a click reaction.

\section*{2.2 Results and Discussion}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure2.jpg}
\caption{Design and sequence of the three-point star tile.}
\end{figure}

For this study, we chose the three-point-star motif\textsuperscript{102} (Fig. 2.1), a three-arm planar structure that is composed of seven DNA strands where each of the three arms is a four-arm junction (Fig. 2.1). The motif is three-fold symmetric, thus requiring three unique strands for assembly: a long central strand (strand L, red), three identical medium strands (strand M, green) and three identical short strands (strand S, blue). Three T5 loops at the center prevent the helices from stacking on one another. The advantage of this motif was, that the same motif could lead to the formation of three different higher ordered structures through sticky end cohesion\textsuperscript{73}.

This motif has previously been used for the assembly of two-dimensional arrays\textsuperscript{102}, closed three-dimensional polyhedra\textsuperscript{73} and for the encapsulation of nanoparticles and proteins\textsuperscript{103}. We used
copper catalyzed alkyne–azide cycloaddition (CuAAC) to functionalize the DNA three-point-star motif. The Click chemistry is known for its biorthogonal nature. In the reaction, the alkyne forms a covalent linkage with the azide by formation of triazole, making it a viable functionalization strategy to facilitate the attachment of the payload on to the modified DNA strands. We started our study with keeping tetrahedron (TET) as the desired structure for our study. The 3PST was the primary motif, which leads to the Tetrahedron formation through sticky end cohesion (Fig. 1.3). The TET is assembled with four 3PST coming together. The TET formation is very much dependent on the ration of the three component strands L, M, and S (1 : 3 : 3 respectively) as well as the concentration of the strands. The motif was designed in such was that just by tweaking ratio and concentration of the component strands it would yield different 3D structures with the same starting material. We were able to form the native TET quite easily (Fig. 2.2). We then tried identifying suitable locations to incorporate the 2’-O propargyl modification. The nanostructure assembly is sensitive to changes in the primary design. Modified nucleotide would mean addition of new functional groups, which were not considered in the initial design.

Figure 2.2: DNA TET formation on non-denaturing PAGE. The red box indicates the band representing TET.
Figure 2.3: Click functionalization of a DNA tile. (a) Assembly of the three-point-star tile and a molecular model (inset). (b) 2′-O-propargyl modification and alkyne–azide click strategy. (c) The four modified tiles used in this study. Tiles M1 and M2 have 2′-O-propargyl modifications at two different sites on strand M. Tiles S1 and S2 have 2′-O-propargyl modifications at two different sites on strand S.

Figure 2.4: Design and sequence of the 2′-O-propargyl modified three-point star tiles.
Hence, the location of the modification was a crucial factor for the nanostructure to assemble successfully. For our study, the total number of modifications on the nanostructure would correspond directly to the loading capacity for the particular version of the nanostructure, as we intended to apply click chemistry as the means of payload attachment. We modified strands M and S to contain a $2'$-$O$-propargyl group at specific locations on the sequence (Fig. 2.4). The $2'$-$O$-propargyl modification and the overall strategy to functionalize a DNA strand via click chemistry are shown in Fig. 2.3 b. The strategy of functionalizing nanostructures with the help of a $2'$-$O$-propargyl group has its advantages. Propargyl residues with click groups introduced in the sugar residue of nucleosides most likely lie outside the helix and have a relatively small perturbation on the stability of the duplex structure\textsuperscript{104,105}. Due to the simplicity, efficiency, high yield and facile nature of the click reactions, many azide-derivatized functional ligands are now commercially available or can be synthesized according to the needs\textsuperscript{74,106}. Hence, without changing the underlying DNA tile, this modification will allow the attachment of different kinds of functional ligands, ranging from fluorescent tags to anti-cancer drugs. We synthesized two variations each of the strands M and S containing the $2'$-$O$-propargyl modifications at two different sites on the sequence and assembled four species of the three-point-star motif using these modified strands (Fig. 2.3 c, 2.4). The three-fold symmetry of the design then provides us with three sites in case of either one of the modified strands used or six sites in case of both the modified strands used, for the attachment of the ligand at 3PST level, these numbers would be four times in case of TET.

We first wanted to see the TET assembly formation with these modifications in place. We tried the TET assembly with the medium strand modifications. We could only observe partial TET assembly on the non-denaturing PAGE profile, for both locations of modification on the M strand (Fig. 2.5). We then tried several versions of TET with all four modified strands, by combining them in different combinations. This would tell us which combinations were able to successfully assemble into the TET and we could then work with those for further studies.
Figure 2.5: DNA TET formation on non-denaturing PAGE with M1 (lane 6) and M2 (lane 7). The bands in these lanes indicate incomplete TET formation.

Figure 2.6: DNA TET formation on non-denaturing PAGE with different combinations of the modified strands. A) assembly concentration 500nM B) assembly concentration 750nM.
The results were quite discouraging as we could not see a complete TET formation in any of the combinations. The usual concentration required for the TET assembly formation in this design is 75nM. We even tried increasing it to 500 nM and 750 nM (Fig. 2.6 A, B). This led to disappearance of the control native TET band as well, at the same time it did not help in any of the modified TET combinations for both strand M and S. As a final variable to play with, we did titration with changing modified strand M ratio, while keeping the other two strand ratios constant. We wanted to see if due the modification, the assembly required either less or more of the modified strand instead of the standard 1 : 3 : 3.

The results indicated that at higher ratios, i.e. 1 : 4 : 3 and 1 : 5 : 3, for both M1 and M2 modifications, there was TET formation observed (Fig. 2.7). But the band intensity was quite low, which meant the yield of the complete TET was very low for both the ratios. We also observed multiple bands under the TET band, which were representing the incomplete assemblies. In general, from all the preliminary studies, there were two evident conclusions, 1. The modifications at the designed locations were affecting the TET formation, 2. Although, we could not form the TET

![Figure 2.7: Titration of modified strand M ratio in the TET formation. A) non-denaturing page showing titration for M1 TET, B) non-denaturing page showing titration for M2 TET. Red box indicates +ve control native TET.](image-url)
successfully, we certainly observed the 3PST i.e. the primary motif formation in majority of the cases in presence of the modification. Hence we decided to limit this study to 3PST as the model to demonstrate the ‘click’-based functionalization for 2’-O propargyl modified DNA nanostructure.

In order to demonstrate motif formation and eliminate potential higher ordered self-assembly, we designed the tiles to have blunt ends (Fig. 2.4). Without the sticky ends the 3PST would not lead to the formation of higher ordered assemblies.

A bipyridine azide ligand (Fig. 2.8, A) was selected as the functional moiety to be attached onto these strands via click chemistry. This bipyridine ligand could be used as a metal chelating ligand due to its robust redox stability and ease of functionalization. These ligands have also been used as precursors for helical assembly, chiral molecular recognition, luminescent devices, and other applications in photonics, optoelectronics and functional nanomaterials. Post click reactions, the DNA strands were purified by preparative denaturing PAGE. The desired bands were cut from the gel and the extracted for further studies. Polyacrylamide non-denaturing gel electrophoresis was used to analyze the formation of the three-point star motif containing modified DNA strands (Fig. 2.8 C, D).

Modification of strand M did not affect the formation of the motif, with the band corresponding to the modified tile migrating similarly to its native counterpart (Fig. 2.8, C, D lane 4), although the assembly of this motif is not fully completed as shown by the lower bands in the gel. Moreover, the click-based addition of the ligand also did not seem to interfere with motif formation (Fig. 2.8, C, D lane 5). However, the motif formed using the modified strand S seemed to have more incomplete structures, possibly due to the incorporation of this short strand being perturbed by the attachment of the ligand (Fig. 2.8, C, D lane 7). While complex formation with the modified strands was evident from gel electrophoresis, we further tested the success of the click reaction by using an azido-modified fluorescent tag (6-FAM-azide) instead of the bipyridine ligand (Fig. 2.11). The use of FAM-dye has two purposes: (i) it serves as a confirmation of click-based incorporation (discussed below) and (ii) functionalizing such a moiety on DNA tiles has additional uses.
Figure 2.8: Assembly of the DNA tile with a bipyridine ligand. A) ‘Click scheme with the structure of bipyridine ligand. B) Lane identities for both gels in C) and D); lane 1: single strand L; lane 2: Strands L+ M (1 : 3 ratio); lane 3: Native tile with L+M+S (1 : 3 : 3 ratio); lane 4: Tile with M1 modification (c), Tile with S1 modification (d); lane 5: Tile with M1 modification clicked with bipyridine (c), Tile with S1 modification clicked with bipyridine (d); lane 6: Tile with M2 modification (c) Tile with S2 modification (d); lane 7: Tile with M2 modification clicked with bipyridine (c), Tile with S2 modification clicked with bipyridine (d). Tiles with modifications on strand M (tile M1) and strand S (tile S1) assemble properly and migrate similarly to the unmodified native tile. The tile with the bipyridine ligand clicked on modifications on strand M is stable while the bipyridine ligand clicked on strand S destabilizes the structure.
For example, FAM-dyes have previously been used in DNA tiles for selective detection of cancer biomarkers\textsuperscript{112} to characterize reversible switching of DNA devices\textsuperscript{113}, to study DNA circuits\textsuperscript{114}, and to monitor co-localization of drug-loaded DNA origami structures\textsuperscript{59}. After the click reaction, we tested the success of the reaction on the single strands (Fig. 2.9) on analytical scale denaturing PAGE and then purified them with preparative scale denaturing PAGE.

We had to adjust the basic ratio of the assembly formation while incorporating our functionalized ligand containing DNA strands. We again performed titration for the varying ratio of the functionalized strands while keeping the other two strand ratios constant (Fig. 2.10).

The assembly of the tile was not affected by incorporation of the modified strand M even after the FAM was clicked on (Fig. 2.11 C, D lanes 3 and 4). We imaged the gel both under UV ($\lambda = 260$ nm, top panel) and at the excitation wavelength of FAM ($\lambda = 495$ nm, bottom panel). Incorporation of the FAM via click was confirmed by a fluorescent band only in the presence of the clicked strand (Fig. 2.11 D lane 4). We found that clicking of the FAM on strand S did not affect tile formation (Fig. 2.11 C, D lane 8), which contrasted with tile destabilization upon clicking of the bipyridine ligand. This is possibly due to the bipyridine ligand affecting the helicity of the DNA duplex, while the incorporation of the FAM molecule is tolerated.

We analyzed the stability of the motifs that contained the modified strands with and without the ligand by UV-Vis thermal melting and circular dichroism experiments. UV-melting data showed that the modified as well as clicked complexes had similar melting temperatures to that of the native complex (Fig. 2.13). This suggests that the stability of the overall structure is not affected by the addition of 2'-O-propargyl modification even after further addition of the ligand to the DNA strand. Circular dichroism experiments also resulted in similar profiles for the native, modified and clicked tiles (Fig. 2.12).
Figure 2.9: The denaturing PAGE profiles of clicked strands S and M. a,c are the images taken at $\lambda = 260$ nm for strand M and S, which show all the DNA in the gel including control native strands; b,d are the images taken at $\lambda = 495$ nm, which is the excitation wavelength for FAM. These images show only the FAM functionalized bands.

Figure 2.10: Strand titration for assembly of (a) Tile M1 and (b) Tile S1.
Figure 2.11: Assembly of the DNA tile with the FAM dye. Results of tiles assembled using modified strand M clicked with the FAM dye. The top panel shows a gel imaged under UV ($\lambda = 260$ nm): the modified (lane 3) and clicked tile (lane 4) migrate similarly to the native tile (lane 5). The bottom panel shows the same gel imaged at the excitation wavelength of FAM ($\lambda = 495$ nm). The fluorescence band corresponding to the clicked tile (lane 5) confirms incorporation of FAM into the tile.
Figure 2.12: Circular dichroism data for native, modified and clicked tiles.

![Circular dichroism data](image)

Figure 2.13: Thermal melting profiles of tiles with modifications on (a) strand M and (b) strand S. (c) Melting temperatures of different modified and clicked species.

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2.3 Conclusion

We have shown successful formation of the three-point star motif with the incorporation of 2′-O-propargyl-modified DNA strands. We also functionalized the DNA strands to contain a bipyridine ligand and a FAM dye via click chemistry and demonstrated successful assembly of the motif. The click-based strategy provides a relatively quicker, more efficient and simpler method to functionalize the nanostructure. The location of modification in the DNA sequence plays a crucial role in the successful formation of the tile; despite this limitation, the DNA three-point-star motif can associate via sticky ends to form higher ordered structures such as a tetrahedron.

While incorporation of the 2′-O-propargyl group followed by clicking of the bipyridine ligand did not affect motif formation, modifications at these specific positions seemed to impede the assembly of higher order structures (Fig. 2.14). In these situations, the incorporation of the modified nucleotide might affect the assembly of the motif and prevent proper sticky end cohesion.
between motifs that are important for hierarchical self-assembly. Further studies will include an analysis of the effect of the position of the modification and the use of other possible click groups to functionalize these nanostructures. Such functionalized structures have the potential to be used in drug delivery because of their efficient internalization into cells \(^{74,106}\), and enhanced resistance to enzymatic digestion compared to linear dsDNA \(^{115}\). Adding to the already existing library of strategies to functionalize nanostructures such as antigen–antibody interactions \(^{116}\), aptamer-based conjugation \(^{91}\), polyamide recognition \(^{117}\), triplex forming oligonucleotides \(^{118,119}\), and chemical crosslinking \(^{120}\), click-based functionalization provides an alternative route to the covalent attachment of guest molecules. Click chemistry has previously been used for enhancing the stability of DNA origami structures \(^{121}\), attachment of molecules on origami \(^{122}\) and in the conjugation of tumor-penetrating peptides onto DNA nanostructures \(^{75}\). Most of these cases contained terminal modifications on DNA while the method we report can be used for internal modifications and further attachment of guests. Specifically, 2′-O-propargyl modifications can be used for intra-and inter-strand crosslinking to stabilize DNA nanostructures \(^{105}\) and to provide angular control in artificially branched DNA nanostructures \(^{123}\). In addition, DNA complexes containing 2,2′-bipyridine-based nucleosides have been shown to aggregate forming higher order structures \(^{124}\); thus click-based functionalization might provide a route to the hierarchical assembly of larger DNA nanostructures in the presence of suitable metal ions. Moreover, this strategy can also be used to introduce functionality into a DNA nanostructure without modifying the underlying DNA tile. We needed to improve or change the design in order to apply this strategy to higher ordered nanostructures (3D).

2.4 Materials and methods

Oligonucleotides:

The native oligonucleotides were purchased from Integrated DNA Technologies (https://www.idtdna.com/site). The modified oligonucleotides were synthesized on Oligo-800 DNA synthesizer using routine phosphoramidite procedures, removed from the support, deprotected and
purified by HPLC. Phosphoramidite monomers and other reagents were obtained from Chemgenes Corporation.

**Complex formation:**

Oligonucleotides for each tile were mixed stoichiometrically in 1X TA/Mg2+ (40 mM Tris base (pH 8.0), 20 mM acetic acid and 12.5 mM magnesium acetate) and annealed from 95°C to 25°C over two days.

**Non-denaturing PAGE:**

Complexes were run on a non-denaturing 6% polyacrylamide gel in 1X TA/Mg2+ at 4°C. The final concentration of the tiles was 75 nM. Gels were post-stained with ethidium bromide and imaged using BioRad Chemi Doc. Gels with FAM-complexes were imaged on Amersham (GE) Typhoon 9400 Imager at $\lambda=495$ nm.

**UV melting:**

Thermal melting profiles for the complexes were determined by measuring their UV absorbance at 260 nm with a Cary 300 Bio UV-Visible Spectrophotometer. The final concentration of the tile was 250 nM. Samples were heated at a rate of 0.5 °C min$^{-1}$ and Tm values were determined from the first derivatives of the melting profiles using the software provided with the machine.

**Circular dichroism:**

DNA complexes were annealed to a final concentration of 500nM for circular dichroism studies. CD spectra of the samples were recorded on a Jasco J-8150 CD spectrometer.

**Click reaction:**

2′-O-propargyl modified DNA oligonucleotides (0.38 mM, 200 μL in H2O) and azido-bipyridine (10 mM, 114 μL, H2O) were placed in a 1.5 mL vial. In a separate vial, 17 μL CuBr solution (100 mM in DMSO/tBuOH 3 : 1) and 34 μL CH$_3$CN solution (100 mM in DMSO/tBuOH 3 : 1) were mixed and added to the DNA solution. The mixture was shaken at room temperature overnight.
before being evaporated to near dryness in a Speed-Vac at 65°C. Sodium acetate (0.3 M, 100 μL) was then added and the suspension was stirred for 1 h before 1 mL of ethanol was added. The vial was vortexed well and stored in a freezer (-80°C) for 1 h before centrifugation for 15 min at 13000 rpm. The supernatant was carefully removed from the DNA pellet. 70% cold ethanol (~20°C) was used to wash the pellet for three times. Finally, the pellet was left drying on air and dissolved in DI water.

Oligonucleotide sequences (written 5’ to 3’):

Strand L (78-nt): AGG CAC CAT CGT AGG TTT TTC TTG CCA GGC ACC ATC GTA GGT TTT TCT TGC CAG GCA CCA TCG TAG GTT TTT CTT GCC

Strand M (42-nt): AGC AAC CTG CCT GGC AAG CCT AC GAC ACG GTA ACG ACT

Strand S (21-nt): TTA CCG TGT GGT TGC TAG TCG

Strand M1 (42-nt): AGC AAC CTG CCT GGC AA(PG)CCT ACG ATG GAC ACG GTA ACG ACT

Strand M2 (42-nt): AGC AAC CTG CCT GGC AAG CCT AC(PG)ATG GAC ACG GTA ACG ACT

Strand M1-F (42-nt): AGC AAC CTG CCT GGC A(PA)G CCT ACG ATG GAC ACG GTA ACG ACT

Strand M2-F (42-nt): AGC AAC CTG CCT GGC AAG CCT(PA)CG ATG GAC ACG GTA ACG ACT

Strand S1 (21-nt): TTA CCG T(PG)T GGT TGC TAG TCG

Strand S2 (21-nt): TTA CCG TGT(PG)GT TGC TAG TCG

Strand S1-F (21-nt): TT(PA)CCG TGT GGT TGC TAG TCG

Strand S2-F (21-nt): TTA CCG TGT GTG TGC T(PA)G TCG

Strand MB (42-nt): CGA CTA GCA ACC TGC GTG GCA AGC CTA CGA TGG ACA CGG TAA

Strand SB (21-nt): AGT CGT TAC CGT GTG GTT GCT
CHAPTER 3

Stimuli responsive release of the payload from functionalized DNA/RNA nanostructures


3.1 Introduction

Nanoscale structures built from DNA/RNA have potential applications as nanocarriers for targeted drug delivery. Site-specific attachment of guest molecules and controllable release are two parameters required for such drug delivery carriers. For attachment, scientists have used intercalation, crosslinking, ligand receptor interactions and hybridization. For release, DNA/RNA nanostructures can be stimulated to undergo a conformational change by molecular triggers such as other nucleic acids, small molecules, enzymes, or environmental factors like pH. Adding to this list, a variety of chemical groups can be incorporated into oligonucleotides to enable control by light. Previously, researchers have used light to control DNA branch migration reactions, operate a DNA nanomachine, perform site-specific cleavage on DNA origami, reconfiguration of nanostructures, and to release molecules from DNA nanostructures.
3.2 Results and Discussion

In this study, we show proof-of-concept dual functionality in nucleic acid nanostructures where we incorporate both attachment and release chemistries within a single nanostructure. As a model system, we attached a guest molecule (6-fluorescein azide, FAM) via click chemistry, and show release of the attached moiety using a photocleavable linker that is cleaved by UV light (Fig. 3.1).

The ‘Click’ chemistry approach was previously established using the 3PST.

In this study, we also demonstrate the versatility of our approach using three nucleic acid nanostructures that vary in size, dimensionality and composition: an RNA three-way junction, a DNA motif (3-point-star), and a 3D DNA object (tetrahedron). To create our designed nanostructures, we synthesized DNA and RNA strands containing both a 2′-O-propargyl (2′-OP) modified nucleotide and a photocleavable linker (PCL)\textsuperscript{139}. The 2′-OP modified strand allows the attachment of an azidomodified payload via click chemistry (Fig. 3.1 a) while the PCL group allows light-triggered release of the cargo (Fig. 3.1 b). We have earlier designed oligonucleotides with 2′-OP as functional units to attach moieties to DNA nanostructures\textsuperscript{140}. The PCL used in this study is commercially available and can be site-specifically incorporated into any DNA or RNA sequence. Further, the PCL is rapidly cleaved using near-UV while also leaving a 5′-phosphate that allows downstream processing of oligonucleotides and assembled structures.

3.2.1 Nanostructure design exploration and optimization

We witnessed very poor to almost no TET formation in majority of the cases. So, we wanted to address this issue before proceeding with the study. Our hypothesis was the location of modification is in the middle of the assembly which might be adding structural strain when the assembly is trying to form a higher ordered structure (TET). At the same time, we wanted to add another functionality into the existing design to achieve release of the payload.
Figure 3.1: Functionalization of oligonucleotides. (a) Incorporation of 20-O-propargyl groups in oligonucleotides facilitate attachment of cargo (fluorescein-azide, yellow circle) through click chemistry. (b) Photocleavable linkers incorporated in strands allow light-triggered release of attached cargos.
3.2.1.1 DNA Tetrahedron

Initially we changed the location of the modification to the sticky ends of the medium (M) and small (S) strand. Sticky ends are responsible for the TET formation, so we needed to strengthen the sticky end cohesion if we were to add two modifications in the sticky end region. To address that, we extended the sticky end region from five nucleotide long to eight nucleotide long (TET extd). We positioned our modification in the sticky end region such that two 2′-OP modified nucleotides (one on strand M and other on strand S) are placed in middle of the sticky end region. To either side of these 2′-OP nucleotides we placed the PCL groups within the sticky end region. This would lead to release of the payload including the two 2′-OP upon exposure to UV. Simultaneously we also re-designed strand M1 from our previous study to have two PCL groups on either side of the 2′-OP modified nucleotide (TET M1PCL2) as a comparative study.

We first tested the formation of TET extd and TET M1PCL2 on a non-denaturing PAGE (Fig. 3.2). We observed a band much higher than the control TET band for TET extd and a very

![Figure 3.2: TET formation on non-denaturing PAGE Native, Native extended, M1PCL2.](image-url)
faint band for TET M1PCL2. We wanted to further investigate if this system will give us similar assembly formation even after the payload attachment. We did the click functionalization of the 2’-OP strands for the respective TETs. We then tested the release of FAM post UV exposure (Fig. 3.3).

![Figure 3.3: TET extd formation post click reaction and FAM release post UV exposure. The lanes 6,7 indicate the TET extd with PCL and FAM before (B) and after (A) exposure to UV.](image)

Here we clearly observed in the FAM specific image that the TET extd with PCL and FAM before UV exposure, the band was visible and matched the Rf of that in the UV image. Post UV exposure the band almost disappeared which was observed in the UV image as well and an intense band at the bottom of the gel appeared. These observations were consistent with our hypothesis. As the payload was placed in the sticky end region, the TET assembly should get affected post UV induced photolysis. The release of FAM was responsible for the intense bottom band in the gel. These results were encouraging. As the next step we wanted to estimate the amount of time for the UV exposure to achieve maximum release. We did a time point series exposure of UV to the TET extd PCL F assembly.

We clearly observed the release increased with increasing time (Fig. 3.4). At 5 mins of UV
exposure we achieved maximum release. We kept the nanostructures under UV exposure up to 10 mins, but the amount of release was similar to that at 5 mins.

We couldn’t achieve reproducibility with this extended sticky end version of the TET. All the experiments done so far were from a single batch of assembly. We couldn’t achieve a successful assembly formation in our successive attempts. In the first TET extd assembly formation test, we observed that the band for TET extd was much higher than the control TET. This observation gave us some indication regarding this assembly might not be a TET shape. We twitched the original design to suit our purpose without taking proper technical assistance of software which are used for the original design. The extension of the sticky ends might have reduced the structure stability and as a result we couldn’t get the assembly to work.

We also tested the formation of TET structures with internal modifications as continuation of the work explained in Chapter 2, we synthesized component strands for the DNA tile and tetrahedron that position the 2′-OP and PCL modifications within the nanostructures on assembly. In the case of DNA tile, incorporation of modifications within DNA nanostructure did not signifi-
cantly affect formation of the structure (Fig. 3.5). However, when we used these tiles to assemble the DNA tetrahedron, we found that the assembly yields were lesser for the structure with internal modifications. The PCL is similar to an additional nucleotide in the structure and could thus affect the twist of the tile arms, resulting in destabilization of the assembly.

Figure 3.5: Comparison of external vs internal modifications in the formation of nanostructure. (a) Design and (b) gel analysis of assembly of DNA three-point-star; (c) design and (d) gel analysis of assembly of DNA tetrahedron.
3.2.1.2 RNA Three Way Junction

This was a new structure introduced in our study. Although RNA 3WJ is a simpler assembly in terms of design compared to 3PST and TET, the material is different (Fig. 1.4, 3.9). The assembly consists of three strands. Based on our observations in the DNA-based nanostructures, we understood that the modifications would affect the assembly formation if they are located within the structure. The 3WJ is open structure at the end of each arm (Fig. 3.9). We designed two versions for two of the component strands to have the 2’-OP modification as well as the PCL towards the 5’ end and towards 3’ end. We tested various combinations of the modified strands to form the 3WJ to see which combinations yield us the complete functionalized assembly at the same time we tested the UV triggered release for each version.

The analysis involved single as well as doubly modified 3WJ formations (Fig. 3.6). Although, the release of FAM from 3WJ was successful in all the versions, only few versions could assemble into complete 3WJ. The RNA 3WJ with strand 1 5’ PCL with FAM showed the best assembly formation. Hence that was chose for the further studies.

3.2.2 Optimized dual functionalized DNA/RNA nanostructures

Post optimization of the design for each nanostructure, we started with validated clicking of the cargo (FAM) and UV-triggered cleavage of the single strand (\(\lambda = 254\) nm) using denaturing polyacrylamide gel electrophoresis (PAGE) (Fig. 3.7 and Fig. 3.8).

We clearly observed that the oligo strand containing only the PCL without the payload, before and after UV exposure showed difference in Rf due to the loss of few nucleotides from the chain post photolysis (Fig. 3.7). The same oligo strand post click reaction also showed different Rf and a band in FAM specific image confirming the success of the payload attachment. Finally, this PCL and payload containing oligo strand was exposed to UV and it again clearly showed different Rf post UV exposure and the FAM specific image showed significant reduction in intensity of the band confirming the release of the payload. To further test the exposure time required for maximum
Figure 3.6: Comparison of native 3WJ with versions of 2'-OP/PCL modified 3WJ. (A), (C) are image taken at 260nm; (B), (D) are images taken at 495nm.
Figure 3.7: Denaturing PAGE profile of single strand showing incorporation of PCL, FAM (post click reaction), and dual functionality.

Figure 3.8: Denaturing PAGE functionalized single strand showing release of FAM over different UV exposure times. The FAM gel images are false colored for clarity.

release, we exposed the payload containing strand to UV for different time points and understood that more than 80% release took place at around 5 mins. (Fig. 3.8) Using this strategy, we tested functionalization of three different nucleic acid nanostructures: an RNA three-way junction (3WJ), a DNA 3-point-star tile (3PST), and a DNA tetrahedron (TET).

The RNA 3WJ is part of the packaging RNA (pRNA) of the bacteriophage phi29 dsDNA packaging motor, previously shown to be very stable and a viable drug delivery vehicle. The
3WJ has three component RNA strands of similar lengths (Fig. 3.9 a, b), one of which we modified near the 5’ terminal with both 2’-OP and PCL. We assembled the 3WJ using equimolar concentrations of each strand and confirmed its proper formation with and without the modification using non-denaturing PAGE (Fig. 3.9 c, d). We found that the incorporation of the modified oligo in the 3WJ was lower than other structures, possibly due to its effect on junction formation (Fig. 3.9 c, d). Stability of nucleic acid junctions increases with the length of the arms and increasing the base pairing on each of the arms could lead to better assembly yields and minimize the effect of the modifications.

The second model system is a DNA 3-point-star tile (DNA tile), a three-fold symmetric DNA motif where each arm is a four-arm junction (Fig. 3.10 a, b). The DNA tile is comprised of three strands: a large (L), medium (M) and a short (S) strand in 1 : 3 : 3 ratio (Fig. 2.1). During the studies from our design optimization, we observed that position of modification does play a role in the successful formation of the functionalized assembly. In this study were having an additional modification. Hence to avoid complications during the assembly of the nanostructures, we modified strand S with an 8-nucleotide single stranded extension and incorporated both 2’-OP and PCL functional groups in this region. This extension region stays outside of the main assemble and doesn’t play role in any of the crossovers taking place during the assembly. We annealed the structure and again confirmed correct formation using non-denaturing PAGE (Fig. 3.10 c, d). The DNA 3PST formation with both the modifications and payload took place successfully. We could also clearly observe the band representing payload carrying 3PST in the FAM specific image as well.

Our third model system is a DNA tetrahedron (TET), a 3-dimensional DNA object that is hierarchically self-assembled from the 3-point-star tiles (Fig. 3.11 a)\textsuperscript{140}. This structure is assembled from similar strands as the DNA tile, but the arms of the tile were extended to contain 5 nucleotide sticky ends that allow the tiles to assemble into higher order structures (Fig. 3.11 a). By controlling the concentration of the strands, the tetrahedron can be formed as the predominant product. On assembly, the single stranded extension on the tile remains outside the tetrahedron and thus the
Figure 3.9: a) RNA 3WJ native design; b) RNA 3WJ PCL PU design; c) RNA 3WJ formation gel (at 260nm); d) RNA 3WJ formation gel (at 488nm)
**Figure 3.10:** a) DNA 3PST native design; b) DNA 3PST PCL PG design (blunt-version); c) DNA 3PST formation gel (at 260nm); d) DNA 3PST formation gel (at 488nm)
Figure 3.11: a) DNA 3PST PCL PG design (sticky-ended); b) DNA TET formation gel (at 260nm); c) DNA TET formation gel (at 488nm)
modifications do not interfere with assembly of the DNA tetrahedron. We assembled both native tetrahedron and a modified tetrahedron (containing 2’-OP and PCL modifications) and checked their formation using non-denaturing PAGE (Fig. 3.11 b, c).

Having successfully assembled the structures with the modified oligonucleotides, we next tested light-triggered release of the fluorophore cargo with UV exposure. We exposed each of the three structures to UV (254 nm) for various times up to 16 min to achieve photocleavage of the PCL and measured the results using non-denaturing PAGE. Gel profiles showed decreasing FAM intensity of the nanostructure band with increasing UV exposure time and appearance of an additional band near the bottom of the gel corresponding to the released FAM (Fig. 3.13). Native nanostructures without the modifications were not affected by UV exposure at 16 min (Fig. 3.12). To estimate the rate of release, we quantified the intensity of the bands corresponding to FAM.

For the DNA tile and tetrahedron, we observed more than 75% release taking place by 4 minutes of UV exposure, which is consistent with the efficiency we observed in single strand cleavage (Fig. 3.13 c). Complete release of the FAM was achieved in 16 min for the DNA tile and tetrahedron while 80% release was observed for the RNA 3WJ at the same time point. As a step toward
utility in drug delivery, we tested the stability of these nanostructures and the release mechanism in complex biological fluids to see if physiological conditions affect the nanostructures or its stimuli-responsive release.

We incubated the DNA tile in fetal bovine serum (10% FBS) and synthetic urine and shined UV on the samples. Results showed that the nanostructure is stable in these biological fluids and UV-triggered release is also functional as compared to release in buffer (Fig. 3.14). In FBS, full release was not achieved in 16 min, possibly due to the presence of other biological molecules in the serum. Further studies will establish the potential use of DNA-based structures in drug delivery, and their robustness to sustain in biological conditions. Assembly of such DNA nanostructures is very sensitive to the size and position of modification. In the examples above, we inserted modifications toward the outside of the structures so that they do not interfere with assembly.
Figure 3.13: (A–C) Non-denaturing PAGE of UV-triggered release of FAM from functionalized nanostructures A) RNA 3WJ, B) DNA 3PST, C) DNA TET. Gels were imaged under UV (260 nm) as well as FAM-specific wavelength (488 nm). The FAM gel images are false colored for clarity. Plots showing photo-release over time for each structure are shown below the gels. Experiments were done in triplicates.
**Figure 3.14:** UV release in biofluids. Release of FAM from a DNA tetrahedron in buffer, synthetic urine and 10% FBS. The FAM gel images are false colored for clarity.
3.3 Conclusion

Our study compares different nanostructures based on size, dimensions (2D and 3D) as well as starting materials (DNA and RNA) being used as stimuli responsive nanostructures. The proof-of-concept for dual functionality was established on these nanostructures by using click chemistry as means of functionalization and PCL as the means of controlled release. The click strategy provides quantitative, robust, and at the same time facile ways to attach a payload onto nanostructures. The PCL provides non-contact control over payload release for a variety of nanostructures. Our strategy can be expanded to specific control over different parts of a nanostructure by using different types photo-cleavable or photo-responsive groups. The use of UV as a trigger does pose some challenges and limitations including absorbance and potential damage of biological materials. The nitrobenzyl protective groups that remain after UV cleavage could also have a toxic effect, but this type of caging system has been widely applied in many biological systems\textsuperscript{141}. It is likely that the UV-released byproduct (nitrophenyl short oligos) might cause immune response; this aspect needs more systematic cellular studies measuring the overall efficiency and toxicity, which are currently undergoing in our lab. Future work may also focus on other wavelengths, especially those in the blue light\textsuperscript{142,143}, near-infrared (NIR) that have minimal absorbance by skin and tissue\textsuperscript{144}. With a number of wavelength-specific photoactive chemistries available\textsuperscript{145}, it could be possible to have DNA/RNA nanostructures that respond to multiple wavelengths. The programmability of such approaches coupled with the suite of chemical tools may one day enable complex functions such as selective release of drugs from multi-drug cocktails as well as sustained or controlled release based on the wavelength.

3.4 Future directions

As mentioned currently there is ongoing work to establish release with different source of light i.e. blue light, IR. As a future aspiration, we also intend to combine two or more wavelength specific triggers, in the same nanostructure to achieve release of the payload at two different time
points, which could make the carrier a multipurpose with two different payloads attached to the nanostructures being released at two different time points.

The blue light activated molecule was synthesized by the post-doctoral fellow in our group, Dr. Song Mao. The molecule is a derivative of hydroxymethyl coumarin, which has been used for photocleavable protecting group (PPG) \(^1\)\(^2\), \(^3\)\(^4\), \(^5\)\(^6\). The photocleavable molecule was derivatized to have the propargyl group as well as the payload to attached to it (Fig. 3.15). This complex can then be ‘clicked’ onto the nanostructure using a diazido linker.

![Chemical Structure](image)

**Figure 3.15:** Scheme for Synthesis of PTX-Coumarin Photocleavable Linker (6).

Synthesis of the target blue light photocleavable linker commenced with commercially available compound 1, undergoes oxidation in the presence of SeO\(_2\) to afford compound 2. The 2 was then reacted with propargyl bromide in the presence of activated Zinc, providing compound 3 with a free hydroxyl group. The 3 was then activated using 4-nitrophenyl chloroformate to obtain the activated carbonate 4. Transesterification of p-nitrophenoxy on 4 with PTX drug under the catalytic condition of DMAP afforded compound 5. The tether triple bond in 5 was coupling with 1,4-diazidobutane under Click reaction, delivering PTX-Coumarin Photocleavable Linker 6, which contains another azido group to coupling with DNA oligonucleotides.
We also switched our TET model with a different version of TET. We wanted to keep the tetrahedron shape consistent in our studies as the shape has been successfully used as drug delivery vehicle in other studies\textsuperscript{74,79,106,115}. We intend to do cell studies to study the performance of these nanostructures as delivery vehicles, for cell studies we need pure form of the functionalized nanostructures. For 3WJ and 3PST, scaling up of the synthesis wouldn’t affect the yield of the nano-assembly. On the other hand, the current TET design is hierarchical self-assembly based design, so it’s yield is dependent on the concentration (specific scale) of the synthesis. If we increase the scale, the output product would be something other than the TET as per the original design. Hence, we adapted to a new TET system which would we compatible with scaling up of the reaction.

Figure 3.16: Design of the new DNA TET 17. It has four component strands of equal lengths.

The TET 17 is termed such was as each of the vertex’s length 17 nucleotides\textsuperscript{148–150}. The approximate size of the vertex is 5.4nm. We tested the native assembly formation at three different scales (Fig. 3.17, a). Once we confirmed the assembly formation taking place successfully, we designed and synthesized 2′-OP modified versions of each of the four component strands. We placed the 2′-OP modification towards the 5′ end of the strands. We then tested the assembly formation with different combinations of the 2′-OP modified strands (Fig. 3.17, b).
We observed a successful formation of the TET 17 with a significant yield at 1µM, 2µM as well as 10µM scaled synthesis (Fig. 3.17, a). Encouraged by this result, we proceeded to try versions of 2′-OP modified TET 17 to see if the modifications have any effect on the assembly formation. We observed that all versions of the 2′-OP modified TET 17 showed a successful complete assembly formation. One interesting observation from this study showed us, that the TET 17 with all four component strands with 2′-OP modification had much less byproducts than the Native TET 17.

We are currently in process of functionalizing these modified versions of TET 17 with the Blue light activated molecule complexed with Paclitaxel (PTX) drug (anti-cancer). We could then be conducting release assays. Based on the success of the release assays, we intend to proceed with delivery studies on cancer cell lines.

Figure 3.17: A) The native TET 17 formation at three different scales; B) TET 17 formation with 2′-OP modified strands compared with native TET 17.
3.5 Materials and methods

Oligonucleotide synthesis:

Oligonucleotides were purchased from Integrated DNA Technologies (IDT) with standard desalting. DNA oligonucleotides with photocleavable linkers (PCL) were chemically synthesized at 1.0-μmol scales by solid phase synthesis using an Oligo-800 synthesizer. PCL phosphoramidites were purchased from Glen research and used as 0.1 M solution in acetonitrile. All the other reagents are standard solutions obtained from ChemGenes Corporation. After synthesis, the oligos were cleaved from the solid support and fully deprotected with AMA (ammonium hydroxide:methylamine = 1 : 1) at 65 °C for 30 min. The amines were removed by Speed-Vac concentrator before purification. The DNA strands were purified by reverse phase HPLC using a Zorbax SB-C18 column at a flow rate of 6 mL/min. Buffer A was 20 mM triethylammonium acetate, pH 7.1; buffer B contains 50% acetonitrile in 20 mM triethylammonium acetate, pH 7.1. A linear gradient from buffer A to 80% buffer B in 25 min was used to elute the oligos. The purified samples were concentrated, desalted and lyophilized to dry before re-dissolving to working buffers.

Copper catalyzed alkyne-azide cycloaddition reaction:

2′-O-propargyl-modified DNA oligonucleotides (0.38 mM, 200 μl in H2O) and azido-bipyridine (10mM, 114 μl, H2O) were placed in a 1.5 ml vial. In a separate vial, 17 μl CuBr solution (100 mM in DMSO/tBuOH 3:1) and 34 μl CH3CN solution (100 mM in DMSO/tBuOH 3:1) were mixed and added to the DNA solution. The mixture was shaken at room temperature overnight before being evaporated to near dryness in a speed-vac at 65 °C. Sodium acetate (0.3 M, 100 μl) was then added and the suspension was stirred for 1 h before 1 ml of ethanol was added. The vial was vortexed well and stored in a freezer (-80°C) for 1 h and centrifuged for 15 min at 13000 rpm. The supernatant was carefully removed from the DNA pellet. 70% cold ethanol (-20 °C) was used to wash the pellet three times. Finally, the pellet was left drying on air and dissolved in DI water.
Complex formation:

**RNA 3WJ**

The component strands were annealed in 1 : 1 : 1 ratio in 1x TMS buffer (50 mM TRIS pH = 8.0, 100 mM NaCl, 10 mM MgCl2), and annealed by heating to 95 °C and cooling down to room temperature by itself over 2 hours, followed by 4°C for 30 mins.

**DNA 3-point-star tile**

Strands L, M and S were mixed in 1 : 3 : 3 ratio in 1X TA/Mg2+ (40 mM Tris base, pH = 8.0; 20 mM acetic acid and 12.5 mM magnesium acetate) at a final concentration of 75 nM. The mixture was annealed slowly from 95°C to 20°C over two days.

**DNA tetrahedron**

Strands L, M and S were mixed in 1:3:3 ratio in 1X TA/Mg2+ at a final concentration of 75 nM. The mixture was annealed slowly from 95°C to 20°C over two days. For the modified DNA tetrahedron formation, strand S was replaced by strand S-t-PCL while keeping all the ratios and concentrations the same.

**DNA tetrahedron (TET 17)** Strands A, B, C and D were mixed in 1:1:1:1 (equimolar) ratio in 1X TA/Mg2+ at a final concentration of 1 µM. The mixture was annealed at 95°C for five mins, then kept on ice for five mins, finally stored at 4°C for 30 mins. For the modified DNA tetrahedron formation, each strand was replaced with the 2’-OP modified one based on the version of the modified TET while keeping all the ratios and concentrations the same.

**Non-denaturing PAGE**

RNA 3WJ complexes were run on 12% non-denaturing polyacrylamide gel in 1x TBM buffer at 20°C. The DNA tile and tetrahedron were run on non-denaturing 6% and 4% polyacrylamide gels respectively in 1X TA/Mg2+ at 4°C. Gels were post-stained with ethidium bromide and imaged using BioRad Chemi Doc. Gels with FAM-complexes were imaged on Amersham (GE) Typhoon 9400 Imager at λ = 488 nm.
UV release

The UV release experiment was conducted by placing the tubes containing the nanostructures in the ice bucket with open tops approximately 3 inches from the light source. The nanostructures were exposed to UV (254 nm) using a UV light source (handheld UV light Spectroline EF 240C with an output of 4 Watts). For release in biological fluids, the nanostructures were mixed to a final of 10% FBS and 10% synthetic urine, followed by exposure to UV for 16 minutes.

Oligonucleotide sequences (written 5’ to 3’):

RNA three-way junction

3WJ 1 (18-nt): UUG CCA UGU GUA UGU GGG
3WJ 2 (20-nt): CCC ACA UAC UUU GUU GAU CC
3WJ 3 (16-nt): GGA UCA AUC AUG GCA A
3WJ 1 PCL (19-nt): (PU)(PCL)U GCC AUG UGU AUG UGG G

DNA 3PST and DNA tetrahedron

Strand L (78-nt): AGG CAC CAT CGT AGG TTT TTC TTG CCA GGC ACC ATC GTA GGT TTT TCT TGC CAG GCA CCA TCG TAG GTT TTT CTT GCC
Strand M (42-nt): AGC AAC CTG CCT GGC AAG CCT ACG ATG GAC ACG GTA ACG ACT
Strand S (21-nt): TTA CCG TGT GGT TGC TAG TCG
Strand S-t-PCL (29-nt): TTA CCG TGT GGT TGC TAG TCG CCT C(PCL)AA(PG) A
Strand MB (42-nt): CGA CTA GCA ACC TGC GCA AGC CTA CGA TGG ACA CGG TAA
Strand SB (21-nt): AGT CGT TAC CGT GTG GTT GCT
DNA tetrahedron 17

Strand A (55-nt):
ACA TTC CTA AGT CTG AAA CAT TAC AGC TTG CTA CAC GAG AAG AGC CGC CAT AGT A

Strand B (55-nt):
TAT CAC CAG GCA GTT GAC AGT GTA GCA AGC TGT AAT AGA TGC GAG GGT CCA ATA C

Strand C (55-nt):
TCA ACT GCC TGG TGA TAA AAC GAC ACT ACG TGG GAA TCT ACT ATG GCG GCT CTT C

Strand D (55-nt):
TTC AGA CTT AGG AAT GTG CTT CCC ACG TAG TGT CGT TTG TAT TGG ACC CTC GCA T

Strand A-PC (55-nt):
A(PC)A TTC CTA AGT CTG AAA CAT TAC AGC TTG CTA CAC GAG AAG AGC CGC CAT AGT A

Strand B-PC (55-nt):
TAT (PC)AC CAG GCA GTT GAC AGT GTA GCA AGC TGT AAT AGA TGC GAG GGT CCA ATA C

Strand C-PC (55-nt):
T(PC)A ACT GCC TGG TGA TAA AAC GAC ACT ACG TGG GAA TCT ACT ATG GCG GCT CTT C

Strand D-PC (55-nt):
TT(PC) AGA CTT AGG AAT GTG CTT CCC ACG TAG TGT CGT TTG TAT TGG ACC CTC GCA T
CHAPTER 4

Targeting ‘Special’ RNA nanostructures: Identifying potential drug candidates against pathogenic RNA repeat expansions

In addition to the drug-nanostructure conjugates, we are also interested in the RNA repeats expansions that could cause a series of currently untreatable neurodegenerative diseases. The RNA repeats also form highly ordered secondary structures that are similar to some DNA/RNA nanostructures, we can call them ‘special’ RNA nanostructures. Our goal is to discover small/macro molecule ligands that can bind the RNA repeat secondary structures and block their pathogenic activities. In our preliminary study, we have identified a set of polypeptide ligands as well as specialized peptidomimetic to bind with RNA expansions, r(CUG)×10 and r(CCUG)×6, which are responsible for causing Myotonic Dystrophy type 1 and type 2 respectively.\(^{151}\)

It has been estimated that from the whole of RNA population, approximately only 2% of it will be translated in to proteins.\(^{152}\) The remaining termed as noncoding RNAs comprise of micro RNAs (miRNA) and long noncoding RNA, which play different regulatory roles. There are several RNAs who’s functions are still unknown. The RNA are found in cells in the form of ribonucleoproteins (RNPs)\(^ {153,154}\), which are the functional forms of the corresponding RNA and can be in large numbers as there are variety of RNAs as well. Any sort of mutation with respect to RNPs can affect the function, and can have adverse effect on the cells\(^ {151}\). It has been identified that several neurodegenerative disorders occur due to certain RNA repeats mutating to a higher number in the transcriptome (Fig. 4.1). Several diseases such as Myotonic dystrophy, Huntington’s disease and spinocerebellar ataxias have their roots associated with pathogenic RNA repeat expansions\(^ {151,153,155,156}\). Within them, the genetic origin and overall pathogenesis of myotonic dystrophy type 1 (DM1) and type 2 (DM2) was established more than two decades ago. We are still unable to cure these disorders despite having a fair amount of information on the mechanism involved in the pathogenesis.
Figure 4.1: (A) Microsatellite expansions within transcribed regions with confirmed or potential RNA gain-offunction effects. The type and number of nucleotide repeats are indicated. (Fragile X-associated tremor ataxia syndrome, FXTAS; premature ovarian insufficiency, POI; Huntington’s disease, HD; spinocerebellar ataxias, SCAs; dentatorubral pallidolysian atrophy, DRPLA; spinal and bulbar muscular atrophy, SBMA; Huntington’s disease-like 2, HDL2.) (B) Sequestration of MBNL1 in myotonic dystrophy (DM1). (C) Activation of protein kinase C (PKC) by the expanded CUG RNA induces hyperphosphorylation of CUGBP1, which stabilizes the protein\(^\text{[51]}\). This figure is Fig. 3 (Page 785) from Cooper et al., 2009, available at 10.1016/j.cell.2009.02.011, reproduced with permission (Fig. B.6). Copyright © 2009 Elsevier Inc.

DM1 originates in a progressive expansion of an unstable CTG triplet repeat in the 3’- untranslated region (UTR) of the dystrophia myotonica protein kinase (DMPK) gene\(^\text{[57]}\). It’s been observed that healthy individuals have <37 CTG repeats, whereas DM1 patients carry between 50
and many thousands of repeating units. The level of two alternative splicing regulators, muscle blind-like 1 (MBNL1) and CUG-binding protein 1 (CUGBP1) are affected by the r(CUG)exp due to toxic gain of function effect. Myotonic Dystrophy type 2 (DM2) is a result of mutation in form of an expansion of an untranslated tetranucleotide CCTG repeat in the first intron of the ZNF9 gene on chromosome 3q.

There are several important differences between the two types, unlike DM1 patients, DM2 patients show predominantly proximal muscle weakness and only moderate muscle wasting. RNA CCUG repeats target large protein-protein complexes in the cytoplasm of DM2 cells\textsuperscript{151,158–161}. The goal is to develop small molecule ligands that can bind with pathogenic RNA repeats therefore interfere with the proteins from binding to the RNA and have the potential to be effective drugs to treat neurogenerative disorders such as Myotonic dystrophy, ALS, Huntington’s disease etc. by rescuing the splicing events and regulating the resulting protein expression.

Although this work is not directly in the same scope with our previous two chapters about the nucleic acid nanostructures as drug delivery vehicles, these RNA repeat expansion sequences can form specific secondary structures that are similar to those nanostructures. We have been working with a series of polypeptide ligands, gold-complexes and synthetic peptidomimetics. These ligands were screened against RNA repeat sequences that are already identified to cause certain neurodegenerative disorders.

The binding studies were predominantly be done with Isothermal Titration Calorimetry (ITC), Gel shift assay as well as Surface Plasmon Resonance (SPR). We synthesized the RNA repeats sequences in our lab using Solid Phase Oligonucleotide Synthesizer. Our aim was to target these RNA repeats forming secondary structures, which are attractive binding sites for proteins, by blocking or cleaving them with the help of the ligands. Then study the effect of the same in stem cells and eventually patient derived cells to understands the effect of binding on the phenotype level.

Through the screening of several molecules, one of the oligopeptide ligands (Ligand 2) showed binding against r(CUG)\times10 and r(CCUG)\times6. The repeats under study as assumed to
form stem-loop type of secondary structure\textsuperscript{159–161}. These types of secondary structures form grooves similar to that of DNA duplex. But the groves size might be much larger compared to DNA duplexes, hence small molecules show either weak or no binding to such RNA secondary structures\textsuperscript{162–165}. J. Pai et al. published a wonderful article in JACS in 2012 talking about screening of a library of peptide ligands against HIV related pathogenic RNA secondary structure\textsuperscript{166}. Inspired from the article, we decided to screen our neurodegenerative disorder related RNA repeat expansions against pool of macro molecules such as oligopeptides, peptidomimetics etc. Ligand 2 (Lig 2) is one of the oligopeptides from the library (Fig. 4.2), which showed strong binding against both r(CUG)\texttimes10 and r(CCUG)\texttimes6.

More recently, we received a pool of cyclic peptide-like compounds from our collaborator Prof. Jianfeng Cai at the University of South Florida. These are unique synthetic peptidomimetics, which they term as ‘AApeptides’. These peptidomimetics are inspired from chiral PNA backbone. These molecules can potentially form secondary structures through self-assembly\textsuperscript{167–169}. From our screening we also found one hit specifically against r(CUG)\texttimes10 repeat. The peptidomimetic showed substantial binding against the specific RNA repeat and we confirmed the binding even with gel shift assay.

![Figure 4.2: Ligand 2: LKKLLKQQKKQQKLKG; M.W.: 1936.47](image-url)
4.1 Results and Discussion

There have been several pathogenic RNA repeats identified associated with specific neurodegenerative disorders as mentioned earlier. Based on the information available and the capacity of the Solid Phase Oligonucleotide synthesizer, initially we designed the following sequences to synthesize (Table 4.1).

Table 4.1: Different RNA repeats with varying length of the repeat expansions synthesized for the binding studies against pool of ligands as potential drug candidates.

<table>
<thead>
<tr>
<th>RNA ID</th>
<th>(Sequence)×n</th>
</tr>
</thead>
<tbody>
<tr>
<td>VV1-92-A</td>
<td>(CAG)×6</td>
</tr>
<tr>
<td>VV1-92-B</td>
<td>(CAG)×8</td>
</tr>
<tr>
<td>VV1-92-C</td>
<td>(CAG)×10</td>
</tr>
<tr>
<td>VV1-92-D</td>
<td>(AUUCU)×6</td>
</tr>
<tr>
<td>VV1-92-E</td>
<td>(AUUCU)×8</td>
</tr>
<tr>
<td>VV1-92-F</td>
<td>(AUUCU)×5</td>
</tr>
<tr>
<td>VV1-92-G</td>
<td>(CUG)×10</td>
</tr>
<tr>
<td>VV1-92-H</td>
<td>(CCUG)×6</td>
</tr>
</tbody>
</table>

4.1.1 Isothermal Titration Calorimetry (ITC)

We started with testing the pool of oligo peptides ligands and gold metal complexes against the above-mentioned sequences on ITC. From the preliminary study, we obtained a only a single few hits. Only the Ligand 2 showed binding against both r(CUG)×10 and r(CCUG)×6. Then we optimized our conditions and test the same system (Lig 2 vs rCUGx10; Lig 2 vs rCCUGx6) with ITC to confirm the binding in both the systems.

Isothermal titration calorimetry (ITC) is a tool to study and characterize the thermodynamics from the binding of molecules.\textsuperscript{170,171} ITC analysis provides us details of the energy changes during the complex formation through binding, with giving us quantitative values of the binding affinity ($K_d$) and stoichiometry (n) of the reaction. So, ITC is a tool to measure heat change.\textsuperscript{172}
The top half of Fig. 4.3 shows the representative primary data from the calorimetric titration of 
$r$(CUG)$\times 10$ and $r$(CCUG)$\times 6$ against Lig. 2 at 20°C. Each heat burst curve in the figure corresponds 
to a single injection. These injection heats were corrected by subtracting the corresponding dilution 
heats derived from the injection of identical amounts of Lig. 2 into the buffer (water) alone. In 
Fig. 4.3 (bottom half) the resulting corrected heats are plot against the molar ratio. The data points 
in this panel reflect the experimental points and the continuous line represents the calculated best

![Figure 4.3: ITC plots showing the binding of Ligand 2 with $r$(CUG)$\times 10$ and $r$(CCUG)$\times 6$ and the sigmoidal curves plot based on the ITC data.](image-url)
fit to the data. It can be seen that the binding was characterized by exothermic heats in case of r(CUG)×10; both exothermic and endothermic in case of r(CCUG)×6. The ITC data were fit to a single site model as the integrated heat data showed only one binding event. We replicated this data in duplicates to confirm the binding. The result yielded a binding affinity for Lig. 2 vs r(CUG)×10 the average $K_d$ was found to be 322 nM; for the for Lig. 2 vs r(CCUG)×6 the average $K_d$ was found to be 392 nM (Fig. 4.3, bottom half). An enthalpy change ($\Delta H$) of $-41.71$ kJ/mol, an entropy (change in energy) contribution ($\Delta S$) of $-22.80$ J/mol·K for r(CUG)×10 and $\Delta H$ of $58.31$ kJ/mol, $\Delta S$ of $315.20$ J/mol·K for r(CCUG)×6 was observed. The large positive entropy term is suggestive of the disruption and release of water molecules on intercalation in case of r(CCUG)×6 and Lig. 2. The ‘n’ value representing the stoichiometry of the reaction was observed to 1, which indicates 1 : 1 ratio for the reaction. The positive $\Delta S$ indicates endothermic reaction, which can be an entropy driven reaction for r(CCUG)×6, on the other hand the negative $\Delta H$ indicates exothermic reaction, which can be enthalpy driven.

We checked the reproducibility of the results by conducting multiple runs of ITC. We found that the ligand showed binding in every run, which confirmed that there is binding between the RNA and the ligand. ITC experiment were carried out at room temperature, ligand and RNA were dissolved in deionized RNase free water. Post ITC experiments, gel shift assays were carried out and we confirmed the binding.

The peptidomimetics were put through similar screening process against all the RNA repeat expansions. Out of sixty compounds, one showed promising binding against r(CUG)×10 repeat. The AApeptide XSY 16 (Fig. 4.4) was the only compound to show any signs of binding in the entire lot. We tested XSY 16 with r(CUG)×10 on ITC.

In the primary attempt, the experiment wasn’t successful as there was a buffer mismatch between the titrant and the titrand. The XSY 16 stock solution was prepared in 100% DMSO, hence it caused a buffer mismatch as the RNA was prepared in water. The ITC peak intensity was beyond the software’s upper limit to be fit into the kinetic plot (Fig. 4.5). After adjusting the DMSO
concentration equally in both the titrant and the titrand, we conducted the experiment and got the desired binding curves. We did a triplicate of this set of experiments to confirm the result (Fig. 4.7).

The Ligand XSY 16 was specific when it came to bind with r(CUG)×10, unlike Ligand 2 it didn’t bind with r(CCUG)×6 repeat (Fig. 4.6). The result yielded a binding affinity for the for XSY 16 vs r(CUG)×10, the average $K_d$ was found to be 206 nM. An enthalpy change ($\Delta H$) of $-100.01$ kJ/mol, an entropy (change in energy) contribution ($\Delta S$) of $-202$ J/mol·K for r(CUG)×10. The stoichiometry of the reaction was observed to be 0.923, which indicates 1 : 1 ratio for the reaction.
Figure 4.6: ITC plot of XSY 16 with r(CCUG)×6.

Figure 4.7: ITC plots showing the binding of XSY 16 with r(CUG)×10 and the sigmoidal curves plot based on the ITC data. a), b), c) are trial 1, 2 and 3 respectively for the triplicate data.
4.1.2 Gel shift assay

After identifying the hits and confirming the binding of Ligand 2 on ITC, we moved to gel shift assay to further confirm the binding of ligand with the RNA. In order to observe the shift, we kept the RNA concentration in each well constant and kept adding increasing equivalents of the ligand.

![Figure 4.8: A) Gel shift assay profile of Lig 2 vs r(CUG)×10; B) Gel shift assay profile of Lig 2 vs r(CCUG)×6. The values in the chart represent the number of equivalents for RNA and ligand in respective wells](image)

As per the design the RNA was kept at 1 equivalent and the ligand was mixed with the RNA in 0, 0.5, 1, 2, 5, 8, 10, 15, 20 equivalents for respective wells. The mixing of the RNA and ligand was done and the it was kept at RT for at least 15 mins before loading it in the gel for the binding interaction to take place. The volumes of both RNA and ligand for each well were kept constant so as to keep the dilution factor constant. In both r(CUG)X10 and r(CCUG)×6, we observed proper gel shift with increasing concentration of ligand. In r(CUG)×10 we observed a two-step shift, whereas in r(CCUG)×6 we observed a single step shift (Fig. 4.8).

We wanted to understand the binding mechanism of Lig. 2 with these RNA repeats, so to investigate that further at the same time develop a fluorescence based assay for detection of potential drug candidates for the pathogenic RNA repeats, we synthesized 5’ TAMRA labeled versions of r(CUG)×10 and r(CCUG)×6. We also purchased Lig. 2 with C-terminal FAM tag. We repeated the gel shift assays with these added modifications to the systems (Fig. 3.16, 4.10).
The RNA repeats with the 5’ TAMRA modifications showed similar binding patterns as the native RNA against native Lig. 2 (Fig. 4.9 a, b; Fig. 4.10 a, b). Contrary to these results, when we tested Lig. 2 with C-terminal FAM against native RNA repeats, it showed no binding at all (Fig. 4.9 c, d; Fig. 4.10 c, d).

Gel shift assays in case of the peptidomimetics, helped us with comparatively faster screening of the ligand pool, instead of doing ITC for individual ligand. We designed the gel shift assays to identify a hit while screening eight ligands against single RNA repeat at a time along with positive and negative controls in a single gel. We had already established the assay for ligand two. So, we had a positive control for the screening purpose. We chose a single concentration of the ligands for screening based on the Ligand 2 results. We kept the RNA and ligand volumes the same. For
the eight screening wells, 10 equivalents of individual ligands were mixed with 1 equivalent of RNA. First well was negative control and the final well was positive control (Fig. 4.11). Using this design, we screened all sixty of the AApeptides. We found a single hit against r(CUG)×10, which was XSY 16. The sixty ligands did not bind to any of the other RNA repeats.

We also synthesized fluorescence labeled r(CUG)×10 using DMT-6-FAM phosphoramidites. Once we identified XSY 16 as a hit, we carried out the same assay as ligand 2 with XSY 16 and both native and fluorescence labeled r(CUG)×10. Fluorescent RNA provided us additional confirmation about the binding when imaged under fluorescence specific filter (Fig. 4.12).
**Figure 4.11:** Fast screening of a subset of AApptides with r(CUG) × 10, with lane 1 as negative control and lane 10 as positive control, with lane 6 showing XSY 16 has a hit.

**Figure 4.12:** A) Gel shift assay profile of XSY 16 vs r(CUG) × 10 image at 260nm; B) Gel shift assay profile of XSY 16 vs r(CUG) × 10 at 488nm. The values in the chart represent the number of equivalents for RNA and ligand in respective wells.
4.1.3 Surface plasmon resonance (SPR)

Surface Plasmon Resonance is a phenomenon that occurs when polarized light hits a metal film at the interface of media with different refractive indices. SPR techniques excite and detect collective oscillations of free electrons (known as surface plasmons) via the Kretschmann configuration, in which light is focused onto a metal film through a glass prism and the subsequent reflection is detected\textsuperscript{173,174}.

We also used SPR to test the binding between ligand 2 and the confirmed RNA repeats as well as XSY 16 and r(CUG)×10. With SPR, we required very less amount of RNA sample, which was one of the major advantages. The sensor chip was coated with basic gold nanoparticles, in addition to that we also used streptavidin functionalized sensor chips. For standard gold chips, we synthesized 5’-thiolated RNA strands in order to immobilize them onto the sensor chips; for the Streptavidin functionalized sensor chips, we synthesized 5’-Biotinilated RNA. One more advantage of SPR, once we immobilized the RNA on a sensor chip, we could use the same sensor chip for multiple experiments involving the immobilized RNA.

![Figure 4.13: SPR analysis of 5’ Thiol r(CUG)×10 vs XSY 16 on standard gold sensor chip.](image)

The experiment with XSY 16 wasn’t very successful. Although we observed binding, there was bulk shift happening, which is visible in all the peaks in Fig. 4.13, at the same time the $K_d$ obtained from the kinetic analysis was far off from that of the same system tested on ITC. As
discussed earlier, XSY 16 stock solution was dissolved in 100% DMSO. We realized that the bulk shift is due to the presence of DMSO in the buffer system, as we had to adjust the DMSO in the running buffer equal to that of ligand concentrations, which were injected in the system so that we could avoid buffer mismatch. We are still trying to optimize the experimental conditions to see if we can get accurate binding kinetics.

The Lig 2 with both r(CUG)×10 and r(CCUG)×6, showed promising binding. We observed consistent curves in the kinetic analysis for both the systems. The average $K_d$ value for Lig 2 vs r(CUG)×10 was 1.19 µM (Fig. 4.14) and for Lig 2 vs r(CCUG)×6 was 1.24 µM (Fig. 4.15).

**Figure 4.14:** SPR analysis of 5’ Thiol r(CUG)×10 vs Lig 2 on Standard gold sensor chip in triplicates.

**Figure 4.15:** SPR analysis of 5’ Thiol r(CCUG)×6 vs Lig 2 on Standard gold sensor chip in triplicates.
4.2 Conclusion

We synthesized a number of pathogenic RNA repeat sequences and generated a pool of peptides and peptidomimetics as potential drug candidates against the pathogenic RNA repeats, based on literature survey. The idea was to use these ligands to bind to the RNA repeats as a blocking mechanism. We screened the pool of ligands against the RNA repeats using analytical techniques such as gel shift assays. Once a hit was identified, the binding was extensively studied with gel shift assays, ITC and SPR analysis. In our study we identified two ligands to be binding to the RNA repeats associated with Myotonic dystrophy type 1 and type 2. The ligand 2, which is an oligopeptide that has Leucine, Lysine and Glutamine rich sequence. It binds to both rCUGx10 (DM1) and rCCUGx6 (DM2) with respective $K_d$s in low nanomolar range. The peptidomimetic/ AApeptide XSY 16 showed promising binding to rCUGx10 (DM1) the $K_d$ to be in the low nanomolar range.

The approach that was used for the study can be applied for other functional RNAs as well. Using three different analytical techniques, binding studies can be thoroughly verified, and false positives and non-specific binding interactions can be avoided. We are currently in collaboration with Prof. Dey and Prof. Berglund for the cell studies, which would tell us about the effect of these ligands in patient derived cells at a phenotype level.

![Figure 4.16: Fluorescent labeled Ligand 2 incubated with muscle stem cells for 12 hours, stained with DAPI.](image-url)
We had begun the very basic testing by incubating the Fluorescence tagged Lig 2 with muscle stem cell. We clearly observed that the ligand was able to penetrate into the cells and even inside the nucleus to a certain extent as shown in Fig. 4.16. We are currently doing further testing with qPCR, fragment analyzer by targeting DMPK gene and monitoring the MBNL-dependent missplicing events. We are hoping for encouraging data from these studies. For the ideal complex systems with good binding constants, we are also trying to obtain high resolution crystals for these RNA-ligand systems to further understand their detailed binding mechanisms, which will not only provide the insights into the current RNA-ligand interactions, but also provide a new foundation for the further structure based rational optimization of new ligand for better binding affinities and specificities and eventually coming up with a potent drug for these neurodegenerative disorders.

4.3 Materials and Methods

Synthesis of RNA repeat oligonucleotide sequences:

RNA oligonucleotides were synthesized by standard solid phase synthesis using Oligo-800 DNA/RAN synthesizer. The native RNA phosphoramidites were purchased from ChemGenes Corporation. 5'-Thiol-Modifier C6 phosphoramidites were obtained from Glenresearch, DMT-6-FAM phosphoramidites were purchased from ChemGenes Corporation. RNA oligonucleotides were chemically synthesized at 1.0-μmol scale. The RNA phosphoramidites were used as 0.07 M solution in acetonitrile. All the other reagents are standard solutions obtained from ChemGenes Corporation. After synthesis, the oligos were cleaved from the solid support and fully deprotected with AMA (ammonium hydroxide:methylamine :: 1 : 1) at 65°C for 45 min. The amines were removed by Speed-Vac concentrator before purification. The RNA strands were purified by preparative PAGE. The desired bands representing the full-length RNA were cut. The gel was cut into small rectangular pieces and soaked in 800 μL of elution buffer (Ammonium acetate 500 mM; Magnesium acetate 10 mM; EDTA 2 mM dissolved in RNAse free water). Kept for overnight shaking at RT. The buffer layer was then transferred into fresh tubes and extracted with butanol. Finally, the RNA was precipitated using ethanol solution by keeping it in -80 degree Celsius for 3
hours. The precipitate was then dissolved in RNAse free water, desalted before using them for the experiments.

 Isothermal Titration Calorimetry:

 The RNA samples were annealed at 95°C for five minutes. The sample was allowed to cool down to room temperature gradually over 1 hour. The sample was stored in 4°C for 2 hours. Before the experiment, sample was brought up to the room temperature. The RNA was dissolved in RNAse free water. In case of ligand stock being dissolved specific solvent, adjustments were done such that both RNA and ligand are at same buffer conditions before initiating the ITC experiment. For Ligand 2 against r(CUG)×10 set up, RNA was 30 µM; 300 µL and against r(CCUG)×6 set up, RNA was 25 µM;300 µL. The Ligand 2 was 100 µM; 50 µL in both cases. For XSY 16 against r(CUG)×10 set up, RNA was 15 µM; 300 µL and the XSY 16 was 100 µM; 50 µL. The ITC was set up for 20 titrations with titrant volume set to be 2.5 µL for each round and the syringe rotation speed was set to be 350 rotations/min. Reference cell was always filled with fresh water or the experimental buffer of volume 300 µL. The experiment was carried out at the room temperature setting. The instrument used was TA instruments nano ITC. Kinetic analysis was conducted on NanoAnalyze software by TA instruments.

 Gel Shift assay:

 The gel shift assay was performed with non-denaturing PAGE. We used 12% polyacrylamide gels. The running buffer was 1x TBE (0.089M Boric acid, 0.002M ETDA sodium salt, 0.089M Tris). Gels were run at 200V for 40 mins at RT. For the Ligand 2 vs r(CUG)×10 and r(CCUG)×6, the RNA was 10 µM; 6 µL and the ligand volumes was 2 µL with the 0, 0.5, 1, 2, 5, 8, 10, 15, 20 equivalents respectively. For the XSY 16 vs r(CUG)×10 the RNA was 5 µM; 6 µL and the ligand volumes was 2 µL with the 0, 0.5, 1, 2, 4, 8, 16 equivalents respectively. The Gels were stained with Ethidium bromide staining and destained in water before imaging. The imaging was done on BioRad Chemi Doc. Gels with FAM-complexes were imaged on Amersham (GE) Typhoon 9400 Imager at $\lambda = 488$ nm.
Surface plasmon resonance:

The running buffer used for all the SPR experiments was 0.005% PBS: 0.1% BSA in RNase free water. This buffer was also used for the Ligand dilution and sample preparation. The RNA sample to be immobilized on the standard sensor chip was prepared in only 0.005% PBS buffer. After the sensor chip has been installed in the instrument and a good baseline is obtained the flow rate will be changed to 20 μL/min. Next 200 μL mixture of your ligand (Thiol modified oligos) and blocking molecule (most common one would be cysteamine concentration around 5 - 50 µg/mL and a start ratio of 3:1 for blocker:oligo) will then be injected into the system. After this injection we did a second injection of blocker alone to ensure the whole sensor is covered with the RNA and blocker. We looked for the baseline increases after this injection. If it did, then we did blocker injections until the baseline was the same after the injection is finished as it was before the injection. The buffer then was switched to 0.005% PBS: 0.1% BSA in RNase free water, flow rate was increased to 150 μL/min and system was let to equilibrate to this new buffer system for 30 mins until the baseline stabilized and flattened. The ligand injections were done after this.

The SPR instrument used was OpenSPR by Nicoya life sciences. The kinetic analysis was done with Tracedrawer software provided by Nicoya life sciences.
APPENDIX A

Formal Permissions for the Published Papers Included

A.1 Permission for Chapter 2


Click-based functionalization of a 2′-O-propargyl-modified branched DNA nanostructure

DOI: 10.1039/C6TB03277J

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**Figure A.1:** License for Chapter 2
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APPENDIX B

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