ABSTRACT

Title of Dissertation:	GENE-ENCODING DNA ORIGAMI AND BIOMOLECULAR CONDENSATES AS POTENTIAL DELIVERY PLATFORMS FOR NUCLEIC ACID THERAPEUTICS
	Angelica Rose Galvan, Doctor of Philosophy, 2025
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Nucleic acid therapeutics are extremely powerful biomedical technologies that have the potential to save millions of lives worldwide. However, at present, there are only about 37 nucleic acid therapeutics which are approved by the FDA, as compared to more than 10,000 approved pharmaco-chemical drugs, highlighting the immense potential to develop these therapeutics in terms of their application space and complexity. In order to enable the development of safer, more effective and longer-lasting nucleic acid therapeutics for genetic diseases, this dissertation establishes the use of a pure cell-free transcription-translation (TXTL) system for benchtop studies and uses this to evaluate the design and expression of gene-encoding DNA origami NPs (Chapter 3) and establishes the use of de novo peptide coacervates as a nucleic acid delivery platform that can protect gene templates (such as plasmids, linear DNA and DNA origami) against exogenous nuclease degradation (Chapter 4).

GENE-ENCODING DNA ORIGAMI AND BIOMOLECULAR CONDENSATES AS POTENTIAL DELIVERY PLATFORMS FOR NUCLEIC ACID THERAPEUTICS

by

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“I love shapes because everything and everyone in the world is generally made out of the same stuff (in slightly different amounts), but how the stuff is assembled and put together determines what we will become. Shapes matter. It’s the complexity in the simplicity of this idea—structure and its relationship to function—that I keep discovering and re-discovering in every system that I study, whether it is bridges, batteries or biology, which never ceases to amaze me. It feels like being kissed by the universe and being enraptured by the magic of its little secret over and over again. How could I not fall in love with the platonic solids? Shapes matter. Shapes make us who we are.
Shapes have the power to change the world.”

–Angelica Rose Galvan

Dedication

For the shapes;

For the love of learning;

For the heart of humanity that lies inside all of us;

For the magic of science, engineering, and research.

For 15-year-old Angelica; for 28-year old Angelica; and for all of the Angelicas in between. Thank you for having the courage to pursue this dream; for having the dedication and discipline to finish this goal, no matter what; and for having the courage to fight for your authentic self and your magical spaces as fiercely, bravely and unapologetically as you have. You are lovable. You are enough.

And for my village.

Acknowledgements

As I close this chapter in my life, I would like to take the time to express my gratitude towards my academic village of friends, colleagues and mentors because I couldn't have gotten here without them. However, before I do that, I'd like to start by sharing one of my life philosophies with you, which I like to call the "crystalline moments analogy".

In materials science and engineering, a thermoplastic polymer is a type of material which is composed of non-crosslinked polymer chains. Because the chains are long and coily, they have the ability to form different types of structures inside of a bulk polymer material. In general, there are two distinct types of regions which are formed inside of a bulk thermoplastic polymer—crystalline regions and amorphous regions. The crystalline regions are the sections in which the polymers align to create an ordered structure. The amorphous regions are the sections in which the polymer chains are disordered and have no structure. The mechanical properties of a bulk thermoplastic polymer varies depending on the ratio of crystalline v. amorphous regions and can be tuned by controlling the structures which are formed.

However, in the words of Prof. Robertson,

"Polymers are like people. They remember their history, but these memories fade with time."

If polymers are like people, then I am a polymer. If I am a polymer, then my life is a polymer. If I were represent all of the time in my life using a thermoplastic polymer system, it would have crystalline and amorphous regions. The crystalline regions represent the ordered bits of time, the "crystalline moments" which I will remember for a long time. The amorphous regions represent the disordered bits of time, the moments which will soon settle into a hazy blur and join the tapestry of the past, which I may or may not remember.

As someone who has been pursuing a Ph.D. for 46% of her existence, what is the meaning of my Ph.D.? What is the meaning of my life?

The crystalline moments which I've shared with all of you.

So, as I sit on the precipice of the next phase of my career, I'd like to lock in the crystalline moments which I've shared with the people who have shaped my life throughout my journey to pursue this Ph.D. May the magic continue to live on in you, me and all of us, always.

Jennifer M. Roden – Ms. Roden is the first person who showed me the magic of science, engineering and research, and the reason why I decided to pursue a Ph.D. when I was 15 years old. When I went around asking the teachers in my high school to help me computer model the platonic solids for my science fair project, Ms. Roden was the only one who said yes. I used to hang out with Ms. Roden in her office during my lunch period, and she was the first person who showed me how to do research, the essence of which is to 1) ask a question and 2) find an answer. While Ms. Roden was teaching me how to computer model using Autodesk Inventor, she used to tell me stories about her wife Maggie, her adopted kids, and how she had just mastered out of her Chemistry Ph.D. at Northwestern University to teach high school. Back then, I had no idea what any of that meant. I just loved that she was someone who had really great answers to a lot of my questions because I had many questions about the world and life as a child. I also loved that she was someone who encouraged me to keep pursuing my curiosities because everyone else thought that they were weird. Ms. Roden didn't think that my love for the shapes was weird. She always encouraged me to keep asking questions and she guided me on how to find answers to the questions which she didn't have answers to. She always reminded me

that I could achieve anything for as long as I keep that love in my heart, and she was right. I'm glad that I could finally finish this Ph.D. with shapes for the both of us.

Kelly L. Johnson – Ms. Johnson was my high school English teacher and favorite teacher I've ever had. She's the reason why I became a Gates Millennium Scholar and the reason why I am a writer. She's the person who taught me how to write, how to think, how to listen, and eventually, how to speak with confidence. Without Ms. Johnson, I wouldn't have writing, and without writing, you probably wouldn't be reading these words. Writing has saved my life many times. She is a teacher who has shaped my life so much that I still keep realizing new ways of how much she has impacted my life up to now. There is no way I could ever explain how much Ms. Johnson means to me, so I'll just share one of my favorite lessons from her with you as an example.

When I was struggling to write a personal essay once, I asked Ms. Johnson, "how do I find my point in the writing?" She responded by telling me that the word "essay" is derived from the French word *essayer*, which means "to try", so I will find my point through the writing process itself. I will find the point by trying through writing.

I guess the writing process is very similar to the scientific method in that way. How do you find the point? How do you find meaning in life? How do you find the answer? Essayer. Perhaps you can try. I've been doing this ever since Ms. Johnson taught me, and it still works. It's like magic.

Kent E. Wardle – Dr. Wardle was my mentor when I interned in the Nuclear Engineering Division at Argonne National Lab the summer I graduated high school. He was the person who taught me how to 3D print using FDM and SLA 3D printers and use different types of materials, which is the reason why I ended up studying materials science and engineering at the University

of Michigan. I loved that he let me print as many Pokemons and M Blocks as I wanted using the little 3D printer in my office. Dr. Wardle used to 3D print custom name plates for everyone in his lab group, so he's the one who got me into making name plates. That's why I liked to 3D print or laser etch name plates for my graduate student friends whenever one of them defended their Ph.D. back when I was an undergrad. Now, I finally get to etch my own name plate :)

Alex Hopke – Alex was the postdoc who I worked with when I did an internship at Massachusetts General Hospital in Boston. He is the first biologist who I ever worked with, and I remember being very impressed that he was a biology triple-major. Alex taught me how to isolate neutrophils from human blood and set up immune cell assays with bacteria or fungi. Alex used to tell me about all kinds of animals like zebrafish and his pet tarantulas, and he spoke very fondly of his wife who he met in the same Ph.D. lab group. Whenever something in our lab broke or didn't function properly, I remember that Alex was always extremely calm and it impressed me very much because he didn't even need to drink coffee or juice or anything to make himself feel better. I am grateful to Alex for introducing me to the world of biology and immunology in a very nice way that was not too overwhelming for my engineering brain.

Kendell M. Pawelec – Kendell was my postdoc mentor friend in Sakamoto Lab at the University of Michigan. She's the reason why I decided to pursue a Ph.D. in bioengineering. I really enjoyed working with her in the lab. It never felt like work. Looking back, I think that working with Kendell in Sakamoto Lab was the most fun research experience I ever had. I love that Kendell gave me the freedom to explore and try out my own ideas. I also loved that she was an artsy musical scientist, like me. No one has ever inspired me to create so much art, science and music in my life as much as Kendell has. She created such a welcoming environment in our lab through our tea time chats, her candy jar, her baked treats, etc. that Sakamoto Lab became

my favorite place in the world. Working with Kendell in the lab taught me how to be confident in my abilities to become an independent researcher, and I will always be grateful to her for giving me that space. I really miss doing research with her.

Susan H.C. Brown – Susan is my Posse mentor and deliverer of harsh truths that I need to learn in life. I don't see Susan as often as any of my other mentors, but whenever Susan teaches me a lesson, it's usually good enough to occupy me for about a year or two. She's very excellent at helping me understand things in life which are difficult for me to understand and accept. She's one of the few people in my life who can out-stubborn me. I love her, and I will forever be grateful to her for being the person who finally convinced me to start doing therapy while I was in undergrad. I will never forget the day when Susan read my personal statement for graduate school and told me "that's good". Hearing that Susan liked my essay felt like more of an accomplishment than when I actually received my acceptance offer to do a Ph.D.

Luciana Nemțanu – Luci was my mentor when I worked as a peer facilitator in the UROP office in undergrad. I like to call her my social science and humanities mentor to differentiate from all of my research mentors, and I love that she challenges me to see the world in different ways. I have a lot of fond memories sitting on the blue couch in her office and asking for advice about research, teaching, life, etc. She's the person who helped me gain the confidence to believe that I could be a good teacher because she gave me the freedom to explore teaching in the way that I wanted to, but she was always there to guide me whenever I had questions, and I really appreciated that. I am also grateful for Luci's husband Bogdan and her daughter Aida because I have a lot of fond memories playing tennis and hanging out with them. I love how much they love food, music, storytelling and life as much as I do. I'm never bored when I'm hanging out with their family, and I love it!

Sandra Gregerman – Sandy is my other Posse and UROP mentor who is always there to help me find resources and/or connect me with people whenever I need something, and I appreciate her for being this person in my life. She was especially helpful when I moved to the DMV area for graduate school because she gave me a lot of really great suggestions for coffee shops and restaurants, most of which I still go to today. She's the reason why the Takoma Bev Co became my go-to writing spot and I've had all of my greatest life epiphanies during grad school at the Bev Co, so I'm grateful to Sandy for introducing me there. Whenever I was working in a coffee shop that was suggested by Sandy, it made me feel less lonely and homesick in grad school.

Aaron H. Morris – Aaron was my postdoc mentor in Shea Lab. He was the first person to introduce me to the world of immune-engineering and the first person to teach me about animal work, so I'm grateful to him for that.

Emmanuelle A. Marquis – Prof. Marquis was my favorite professor in undergrad. I will never forget when she took a walk with me in the rain and explained how the math in thermo is like the notes and scales in a piece of music, but the translation of the sound into meaning and understanding that process—that's the why and that's what I was missing in thermo. I loved that she was a musical human and that she knew how to explain engineering math in a way that I could understand. I couldn't have graduated undergrad without her, and I am grateful to her for helping me understand the math.

Keara T. Saud Greatwood – I met Keara when I was a first-year undergrad and she was a first-year graduate student at the University of Michigan. I don't really know how to put our relationship into words because at various times of my life, she's felt like a best friend, a colleague, a mentor and sometimes even a sister. She understands me more than most of the people that I know and whenever Keara's around, the world feels magical. She's just one of the

most incredible human beings I have ever met and also one of the most amazing scientists I have ever met, I'm actually crying as I type this because I feel so much respect and love for her. She's one of my favorite people and I just feel so lucky to have her in my life.

Colin N. Greatwood – Whenever I'm feeling anxious and a little scared to do something, like cold emailing people and networking, I just think to myself "what would Colin do?" and usually that's the right answer. He's a very good role model for practicing confidence, and it has come in very handy as I'm networking with people in the field of patent law, so I'm grateful to have someone who is such a good example for that.

Arushi Gupta – I used to call Arushi was my lab mom in Sakamoto Lab because she was always very caring and attentive towards everyone. I will never forget the evening when we were both in lab and I told her the story about my mom and she told me the story about her cousin, and then my life was changed forever. I also don't really know how to explain my relationship with Arushi because it's difficult to put into words. I am grateful to her for teaching me so much about Indian culture and for being the reason that I finally went to go visit India. She's just one of my favorite humans and I love her very much.

Joseph M. Valle – Joey is one of my friends from the Sakamoto Lab and I admire him very much for writing half of his Ph.D. thesis as an autoethnography. I also admire him very much for being brave enough to fight for what he believes is right.

Corin L. Bowen – Corey is one of my graduate student friends from aerospace engineering at the University of Michigan. I wish that I had gotten to know her sooner while I was in undergrad. However, I feel extremely grateful for all of the times we've chatted about engineering education throughout my time in graduate school. I also feel extremely grateful for the time that I visited

her and her family in LA after I defended my Ph.D. and we hung out at a llama farm. That was really cool! And I love baby Emmie!

Kori Maria Maxie – Kori was my only undergrad friend in the materials science and engineering department at the University of Michigan, and I am so grateful to her for being my friend. She was my homework buddy and we used to study for exams together. I miss the times when we used to watch the Bachelor and drink hot chocolate + Bailey's. I visited her in Portland after I defended my Ph.D., right before she was about to move to Dallas and I'm grateful that she let me visit her and Zazu before she moved out. I feel really proud of her and what she's accomplished in her career and life.

Isabella E. Rios – Bella is my fellow Posse scholar and friend. We weren't very close in undergrad, but I love how much we've gotten to know each other as I was in grad school. Bella was always there for me whenever I needed someone to rant to or cry with, and she gives really excellent advice. We're kind of like each other's therapist friend, I think, and I'm really grateful to have someone like that in my life. Bella was the person who helped me practice my dry-runs the day before my defense and she helped moderate the presentation on Zoom, and I feel extremely lucky to have someone who I could trust and rely on, like Bella.

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whenever I was struggling to write my proposals and all of the time she's taken to give me advice about life in general. I love thinking out loud with Abby!

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Kimberly M. Stroka – Dr. Stroka was first my co-advisor and then became my full advisor when Igor left. I am grateful to her for being patient and understanding of me whenever I was going crazy, especially during my defense. I know that I'm not always the easiest person to be around, but I greatly appreciate all of her help and support.

The Greatwoods in Richmond, VA – Thank you for having me over at your house for Thanksgiving and Christmas. Even though I only spent a couple of days with ya'll, it always did a lot to help me recover from the stress of grad school, so thank you for welcoming me into your home.

Esra Oktay – Esra was my aPCR buddy in Veneziano Lab and fellow musical human. I think that she has the coolest Ph.D. thesis ever, and I love that she was the lab DJ. I miss the times when I've driven back home from the lab with her and the times when we've explored around Alexandria and attended concerts together.

Igor L. Medintz – Thank you for letting me do my Ph.D. in your lab.

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And last but not least, thank you to my mom, **Araceli E. Galvan**, for buying me a car that I could learn how to drive on the highway and for all of the support and love she's given me throughout my life; thank you to my dad, **George Q. Galvan**, for teaching me how to love learning; and thank you to my sister, **Hazel Rose Galvan**, for encouraging me and believing in me during the times in my life when I didn't know how to believe in myself. I love you.

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Chapter 1: Introduction

1.1 Nucleic acid based therapeutics

The astounding success of nucleic acid based COVID vaccines has demonstrated the potential power of nucleic acid therapeutics to save millions of lives worldwide. However, at present, there are only about 37 nucleic acid therapeutics which are approved by the Food and Drug Administration (FDA), as compared to more than 10,000 pharmaco-chemical drugs which are approved [1-3]. Most of these nucleic acid therapeutics have been approved within the past decade, highlighting the great opportunity to develop current technologies and expand on their potential applicability to help treat other diseases.

Although the most widely known nucleic acid therapeutics are COVID vaccines, this technology was initially developed with the promise to provide solutions for genetic diseases, not infectious diseases [4]. **Table 1.1** shows that 27 out of 37 FDA approved nucleic acid therapeutics are used to treat genetic diseases; 8 out of 37 are used to treat infectious diseases; and 2 out of 37 are used to treat degenerative diseases. Genetic diseases, which are developed from the expression of abnormal proteins due to genetic mutations in the genome, can have very debilitating effects for people. Even those which are caused by simple mutations (like point mutations or single nucleotide variants), can lead to severe physiological effects (e.g. the formation of sickle-shaped red blood cells which have inefficient oxygen delivery and increased clotting (sickle cell anemia); severe intellectual disability, physical and skeletal abnormalities (Bohring-Opitz syndrome); overgrowth of skin, connective, brain and other tissues (Proteus syndrome); or severe mental retardation, multiple congenital malformations, skeletal abnormalities (Schinzel-Giedion syndrome)) [5-8]. It is a great feat that our knowledge and

understanding of biology and genetics has developed enough to enable us with the technologies to elucidate disease mechanisms and identify which specific mutations in specific genes cause the development and progression of a disease. However, we are still lacking the effective tools that we can use to directly target, replace and/or edit the genetic mutations which cause these diseases. Currently, the standard treatment for most genetic diseases only function to delay disease progression and/or to minimize the effects of the disease, highlighting the significant need for the development of technologies which can be used to provide more permanent, long-term solutions.

Fortunately, one of the most effective tools that can be used to prevent the expression of abnormal proteins from genetic mutations is the use of exogenous nucleic acids. Our understanding of the use of nucleic acids to design therapeutics is new and evolving; however, the premise of current technologies is to use exogenous nucleic acids, which are delivered into cells in order to modulate gene expression by adding, inhibiting, replacing or editing at the RNA or DNA level, with the aim of creating a desired therapeutic effect [1]. The defining feature in the design of a nucleic acid therapeutic is the type of nucleic acid used because it is determined by 1) the specific step in the process of gene expression which is intended to be targeted for modulation, 2) the location where this step occurs, and 3) how this step is intended to be modulated.

	Trade name	Drug name	Approval year	Disease indication	Disease type	Developing Company
1	Macugen™	(pegaptanib)	2004	Neovascular Age-related Macular Degeneration (AMD)	Degenerative	Eyetech Pharmaceuticals, Pfizer Inc.
2	Izervay™	(avacincaptad pegol)	2023	Age-related Macular Degeneration (AMD)	Degenerative	Iveric Bio Inc., Astellas Pharma Inc.
3	Glybera™	(alipogene tiparvovec)	2012[†2017]	Familial lipoprotein lipase deficiency	Genetic	uniQure biopharma B.V.
4	Kynamro™	(mipomerson)	2013[†2018]	Homozygous Familial Hypercholesterolemia (HoFH)	Genetic	Ionis Pharmaceuticals
5	Exondys 51™	(eteplirsen)	2016	Duchenne Muscular Dystrophy (DMD)	Genetic	Sarepta Therapeutics
6	Spinraza™	(nusinersen)	2016	Spinal Muscular Atrophy (SMA)	Genetic	Ionis Pharmaceuticals
7	Strimvelis™	(GSK2696273)	2016	Adenosine Deaminase Deficiency (ADA-SCID)	Genetic	GSK plc
8	Luxturna™	(voretigene neparvovec-rzyl)	2017	RPE65-linked Retinal dystrophy	Genetic	Spark Therapeutics
9	Onpattro™	(patisiran)	2018	Hereditary Transthyretin Amyloidosis (hATTR)	Genetic	Alnylam Pharmaceuticals
10	Tegsedi™	(inotersen)	2018	Hereditary Transthyretin Amyloidosis (hATTR)	Genetic	Ionis Pharmaceuticals
11	Waylivra™	(volanesoresen)	2019	Familial Chylomicronemia Syndrome (FCS)	Genetic	Ionis Pharmaceuticals, Akcea Therapeutics
12	Givlaari™	(givosiran)	2019	Acute Hepatic Porphyria	Genetic	Alnylam Pharmaceuticals
13	Milasen™	(milasen)	2019	Batten Disease	Genetic	Boston Children's Hospital
14	Vyondlys 53™	(golodirsen)	2019	Duchenne Muscular Dystrophy (DMD)	Genetic	Sarepta Therapeutics
15	Waylivra™	(volanesorsen)	2019	Familial Chylomicronemia Syndrome (FCS)	Genetic	Akcea Therapeutics, Ionis Pharmaceuticals
16	Zolgensma™	(onasemnogene abeparvovec-xioi)	2019	Spinal Muscular Atrophy (SMA)	Genetic	AveXis (now Novartis Gene Therapies)
17	Zynteglo™	(betibeglogene autotemcel)	2019[suspended]	Sickle Cell β-thalassemia Disease	Genetic	Bluebird Bio, Inc.
18	Oxlumo™	(lumasiran)	2020	Primary hyperoxaluria type 1 (PH1)	Genetic	Alnylam Pharmaceuticals
19	Viltepso™	(viltolarsen)	2020	Duchenne Muscular Dystrophy (DMD)	Genetic	NS Pharma, Inc.
20	Amondys 45™	(casimersen/SRP-4045)	2021	Duchenne Muscular Dystrophy (DMD)	Genetic	Sarepta Therapeutics
21	Leqvio™	(inclisiran)	2021	Heterozygous Familial Hypercholesterolemia (HeFH)	Genetic	Novartis AG, Alnylam Pharmaceuticals
22	Amvuttra™	(vutrisiran)	2022	Hereditary Transthyretin Amyloidosis (hATTR)	Genetic	Alnylam Pharmaceuticals
23	Qalsody™	(tofersen)	2023	Amyotrophic Lateral Sclerosis (ALS)	Genetic	Ionis Pharmaceuticals, Biogen Inc.
24	Casgevy™	(exagamglogene autotemcel)	2023	Sickle Cell β-thalassemia Disease	Genetic	Vertex Pharmaceuticals, CRISPR Therapeutics
25	Lyfgenia™	(lovotibeglogene autotemcel)	2023	Sickle Cell β-thalassemia Disease	Genetic	Bluebird Bio, Inc.
26	Qalsody™	(tofersen)	2023	Amyotrophic Lateral Sclerosis (ALS)	Genetic	Biogen Inc.
27	Rivfloza™	(nedosiran)	2023	Primary hyperoxaluria type 1 (PH1)	Genetic	Dicerna Pharmaceuticals Inc., Novo Nordisk A/S
28	Vyjuvek™	(beremagene geperpavec)	2023	Epidermolysis bullosa	Genetic	Krystal Biotech Inc.
29	Wainua™	(eplontersen)	2023	Hereditary Transthyretin Amyloidosis (hATTR)	Genetic	AstraZeneca plc
30	Vitravene™	(fomiversen)	1998[†2002]	Cytomegalovirus (CMV) Retinitis	Infectious	Ionis Pharmaceuticals
31	Heplisav-B™	(hepatitis B vaccine)	2017	Hepatitis B	Infectious	Dynavax Technologies
32	Jcovden™	(Ad26.COV2.S)	2021	SARS-CoV-2	Infectious	Johnson & Johnson
33	Comirnaty™	(tozinameran)	2021	SARS-CoV-2	Infectious	BioNTech SE, Pfizer Inc.
34	Convidecia™	(Ad5-nCoV)	2021	SARS-CoV-2	Infectious	CanSino Biologics Inc.
35	Vaxzevria™	(AZD12222, ChAdOx1 nCoV-19)	2021	SARS-CoV-2	Infectious	Oxford University, AstraZeneca plc
36	Spikevax™	(mRNA-1273, elasomeran)	2022	SARS-CoV-2	Infectious	Moderna Inc.
37	Cyfendus™	(anthrax vaccine)	2023	Anthrax	Infectious	Emergent BioSolutions Inc.

Table 1.1. List of FDA approved nucleic acid therapeutics. Data compiled with permission from references [1, 2, 9, 10].

Table 1.2 shows that majority (22 out of 37) of currently approved nucleic acid-based treatments for genetic disease utilize antisense oligonucleotides (ASOs), short interfering RNAs (siRNAs) and aptamers which bind to mRNA and other molecules and prevent the effects of genetic mutations by inhibiting the second step of gene expression (translation of mRNA into an abnormal protein) [1, 2, 9, 10]. These treatments provide only temporary, short-term solutions for genetic diseases because they do not directly target the cause of the problem. The most direct way to prevent genetic disease is to edit or replace the genetic mutations which are causing the disease inside of the chromosome. This highlights the significant need to develop technologies which can be used for gene replacement and editing in order to provide more permanent, long-term solutions for genetic diseases.

	Trade name	Drug name	Approval year	Disease type	Nucleic acid
1	Macugen™	(pegaptanib)	2004	Degenerative	Aptamer
2	Izervay™	(avacincaptad pegol)	2023	Degenerative	Aptamer
3	Kynamro™	(mipomerson)	2013[†2018]	Genetic	ASO
4	Exondys 51™	(eteplirsen)	2016	Genetic	ASO
5	Spinraza™	(nusinersen)	2016	Genetic	ASO
6	Tegsedi™	(inotersen)	2018	Genetic	ASO
7	Milasen™	(milasen)	2019	Genetic	ASO
8	Vyondlys 53™	(golodirsen)	2019	Genetic	ASO
9	Waylivra™	(volanesorsen)	2019	Genetic	ASO
10	Viltepso™	(viltolarsen)	2020	Genetic	ASO
11	Amondys 45™	(casimersen/SRP-4045)	2021	Genetic	ASO
12	Qalsody™	(tofersen)	2023	Genetic	ASO
13	Qalsody™	(tofersen)	2023	Genetic	ASO
14	Wainua™	(eplontersen)	2023	Genetic	ASO
15	Vitravene™	(fomiversen)	1998[†2002]	Infectious	ASO
16	Cyfendus™	(anthrax vaccine)	2023	Infectious	CpG DNA
17	Heplisav-B™	(hepatitis B vaccine)	2017	Infectious	PS-DNA
18	Onpattro™	(patisiran)	2018	Genetic	siRNA
19	Waylivra™	(volanesoresen)	2019	Genetic	siRNA
20	Givlaari™	(givosiran)	2019	Genetic	siRNA
21	Oxlumo™	(lumasiran)	2020	Genetic	siRNA
22	Leqvio™	(inclisiran)	2021	Genetic	siRNA
23	Amvuttra™	(vutrisiran)	2022	Genetic	siRNA
24	Rivfloza™	(nedosiran)	2023	Genetic	siRNA

Table 1.2. List of FDA approved nucleic acid therapeutics that are not designed for gene expression. Data compiled with permission from references [1, 2, 9, 10].

Table 1.3 shows that 13 out of 37 utilize single-guide RNA (sgRNA), single stranded RNA (ssRNA), single stranded DNA (ssDNA) and double stranded DNA (dsDNA), which are used for gene expression, replacement and editing, which can be used to provide more permanent, long-term solutions for genetic diseases [1, 2, 9, 10]. Of these 13 therapeutics, eight are used for the treatment of genetic disease. Half of these treatments are administered *in vivo* and the other half are administered *ex vivo*. Also, seven out of eight of these treatments utilize viral vectors (lentivirus, adeno associated virus, gamma-retrovirus, herpes simplex virus) for delivery. Therapeutics which are designed to be administered *ex vivo* are limited to only be able to treat cells which can be easily explanted and implanted again (e.g. blood cells), highlighting the significant need to develop more therapeutics which can be administered *in vivo* and used for a wider range of cellular targets. In addition, therapeutics which utilize viral vectors for delivery pose a high safety risk to infect cells with unedited viral genome which could potentially cause the development of other diseases, highlighting the significant need for the development of non-viral platforms for the delivery of nucleic acids for gene replacement and editing.

	Trade name	Approval year	Disease type	Nucleic acid	Vector
1	Glybera™	2012[†2017]	Genetic	ssDNA	Adeno Associate Virus 1 (AAV1)
2	Luxturna™	2017	Genetic	ssDNA	Adeno Associate Virus 2 (AAV2)
3	Zolgensma™	2019	Genetic	ssDNA	Adeno Associate Virus 9 (AAV9)
4	Jcovden™	2021	Infectious	dsDNA	Adenovirus Vector (AdV)
5	Convidecia™	2021	Infectious	dsDNA	Adenovirus Vector (AdV)
6	Vaxzevria™	2021	Infectious	dsDNA	Adenovirus Vector (AdV)
7	Strimvelis™	2016	Genetic	ssDNA	Gamma-retrovirus
8	Vyjuvek™	2023	Genetic	dsDNA	Herpes Simplex Virus (HSV-1)
9	Zynteglo™	2019[suspended]	Genetic	ssRNA	Lentivirus (BB305)
10	Lyfgenia™	2023	Genetic	ssRNA	Lentivirus (BB305)
11	Onpattro™	2018	Genetic	siRNA	Lipid nanoparticle
12	Comirnaty™	2021	Infectious	mRNA	Lipid nanoparticle
13	Spikevax™	2022	Infectious	mRNA	Lipid nanoparticle

Table 1.3. List of FDA approved nucleic acid therapeutics that are designed for gene expression. Data compiled with permission from references [1, 2, 9, 10].

To the best of our knowledge, there is currently no FDA approved, non-viral nucleic acid therapeutic which can be used for the safe and effective gene delivery, expression, replacement and/or editing for genetic diseases *in vivo*. Therefore, in order to enable the development of safer, more effective and longer-lasting nucleic acid therapeutics for genetic diseases, this dissertation establishes the use of a pure cell-free transcription-translation (TXTL) system for benchtop studies and uses this to evaluate the design and expression of gene-encoding DNA origami NPs (Chapter 3) and establishes the use of de novo peptide coacervates as a nucleic acid delivery platform that can protect gene templates (such as plasmids, linear DNA and DNA origami) against exogenous nuclease degradation (Chapter 4).

Chapter 2: Background

The goal of this thesis was to advance new materials—gene-encoding DNA origami nanoparticles (Chapter 3) and coacervates (Chapter 4)—in order to improve on the technologies available for the delivery of nucleic acid therapeutics; and advance a new platform—cell-free transcription-translation systems (TXTLs) (Chapters 3 and 4)—in order to expand on the technologies available for studying gene expression. This chapter provides background information on these new materials and this new platform.

2.1 Gene-encoding DNA origami nanoparticles

2.1.1 Structural DNA nanotechnology

In the field of biology, deoxyribonucleic acid (DNA) is most commonly known as the fundamental building block of life because it is the molecule that encodes the genetic information which determines the structure and function of all living organisms. Thus, the focus for a majority of biology research is to elucidate understanding of how the genetic information encoded in DNA affects the structure and function of cells in biological systems [11]. However, one of the new technologies which we will discuss in this thesis, gene-encoding DNA origami nanoparticles, is derived from a field that is at the intersection of biology, chemistry and materials science, and primarily views DNA from a materials perspective rather than a biological one—structural DNA nanotechnology [12]. Structural DNA nanotechnology recognizes that DNA has certain properties enabled by its chemical structure, like DNA hybridization, DNA branching and DNA crossovers, which allows us to exploit it as a versatile, self-assembling biomolecular construction material [12-18]. This field was pioneered by Nadrian (Ned) Seeman with the goal of assembling DNA molecules into 3D lattices [13, 19-21]. However, today this

field has grown to establish the assembly of various complex 2D and 3D DNA architectures, including 2D arrays, 3D lattices, 3D polyhedra, DNA crystals and DNA origami [21-24]. Although each complex DNA structure has different individual components (e.g. branched junctions, DNA tiles, DNA bricks) that it uses as building blocks and different principles for assembly (e.g. sticky end assembly or scaffold/staple assembly), most DNA architectures utilize the same properties for their construction.

Among the technologies which have mushroomed from the field, the advancement of DNA origami structures has been one of the most popular and fastest growing. The design and assembly of DNA origami structures are based on the principles which Ned Seeman established for the assembly of DNA lattices [13, 19, 20]. Both DNA lattices and DNA origami structures take advantage of the properties of DNA hybridization, DNA branching, and DNA crossovers for their construction. Therefore, in order to enable a better understanding of DNA origami structure design and assembly, the next sections give an overview of these properties.

2.1.2 DNA chemical structure and conformation

Double-stranded DNA (dsDNA) is a molecule which is comprised of two antiparallel polynucleotide chains which bind to form a linear, double-stranded helix, shown in **Figure 2.1c and d**. The polynucleotide backbone is composed of five-carbon sugar molecules and phosphate groups which are arranged in an alternating fashion, sugar-phosphate-sugar-phosphate, shown in **Figure 2.1b and c**. The phosphate groups function as the binder for sugar molecules through the formation of phosphodiester bonds, which specifically join the 5' carbon of one sugar molecule to the 3' carbon of another sugar molecule, shown in **Figure 2.1e**. This is what determines the directionality of the polynucleotide's orientation. Nitrogenous bases, adenine, thymine, cytosine and guanine, are attached as side groups on the 1' carbon of each sugar molecule on the

backbone via a glycosidic bond. Each component of the polynucleotide which includes the sugar-phosphate backbone and an attached nitrogenous base is known as a nucleotide [11]. The conformation of the nitrogenous base on the sugar molecule (syn v. anti) determines the overall structure of the dsDNA helix, and it can exist in three different conformations (A-DNA, B-DNA, Z-DNA), shown in **Figure 2.2a** [25-27]. However, the predominant form of dsDNA is B-DNA, which is formed when the nucleotide bases adopt the anti conformation. B-DNA is a right-handed double helix with a 2 nm diameter and 3.4 nm height (10.5 bases) per turn, shown in **Figure 2.1d**. [11, 27, 28].

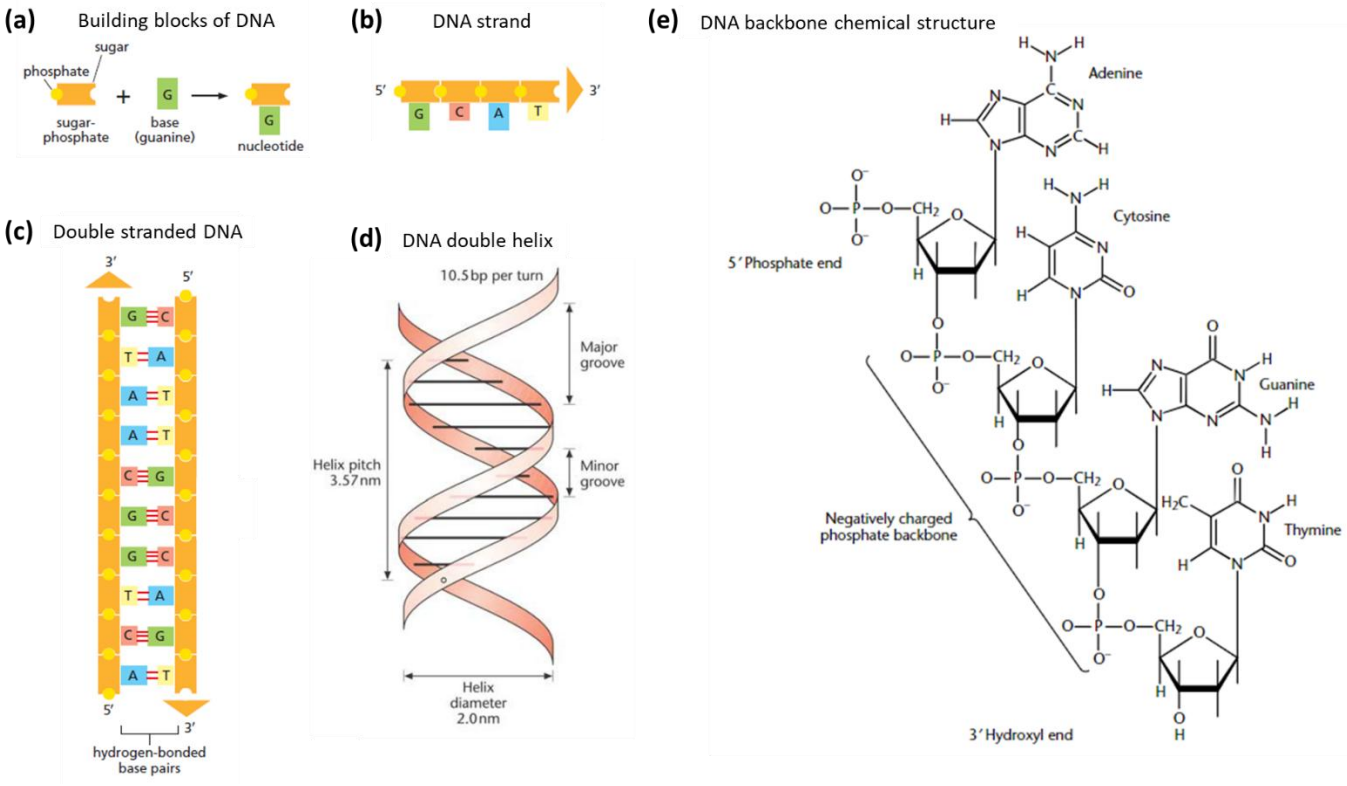

Figure 2.1. DNA structure and conformation. (a) building blocks of DNA, (b) DNA strand, (c) double-stranded DNA, (d) DNA double helix, (e) DNA backbone chemical structure. Adapted and reprinted with permission from references [11, 27].

2.1.3 DNA hybridization

DNA hybridization is the process in which two single-stranded (ss) DNA molecules bind together to form one dsDNA molecule. DNA hybridization is driven by a phenomenon known as base pairing, which is a specific type of hydrogen bonding that only occurs between specific pairs of nitrogenous bases located on directionally opposite ssDNA molecules. Among the four nitrogenous bases—adenine (A), thymine (T), cytosine (C) and guanine (G)—adenine and thymine (AT) are base pairs, and cytosine and guanine (CG) are base pairs [11]. Due to the arrangement of potential hydrogen donors and acceptors on each nitrogenous base, there is a specific number of hydrogen bonds and a specific pattern of hydrogen bonding which can be formed by each base, resulting in each base having only one unique base pair. It is not possible for a base to pair with itself. The hydrogen bonding patterns of base pairs can be influenced by the conformation of bases on the polynucleotide backbone and these are described by different rules. The Watson, Crick and Franklin base pairing rules describe all four nitrogenous bases in the anti conformation, resulting in the formation of two hydrogen bonds in the AT base pair and three hydrogen bonds in the CG base pair [28]. However, the Hoogsteen base pairing rules describe nitrogenous bases A and T in the syn conformation and bases C and G in the anti conformation, resulting in the formation of two hydrogen bonds for both the AT base pair and the CG base pair [29, 30]. Although it is possible for both of these base pairing conformations to occur during DNA hybridization, the conformation of Watson, Crick and Franklin base pairs are generally more stable than the conformation of Hoogsteen base pairs [30-32].

In order for DNA hybridization to occur, base pairing must occur across an entire sequence of complementary base pairs between two directionally opposite ssDNA molecules, not just between one complementary base pair. These are known as complementary sequence

domains [24, 33]. The longer and more unique the complementary sequence domain is between directionally opposite ssDNA molecules, the stronger and more specific they will be at hybridizing with one another. We can control the conformation of molecular assemblies by tuning the length and location of complementary sequence domains on individual ssDNA molecules [13].

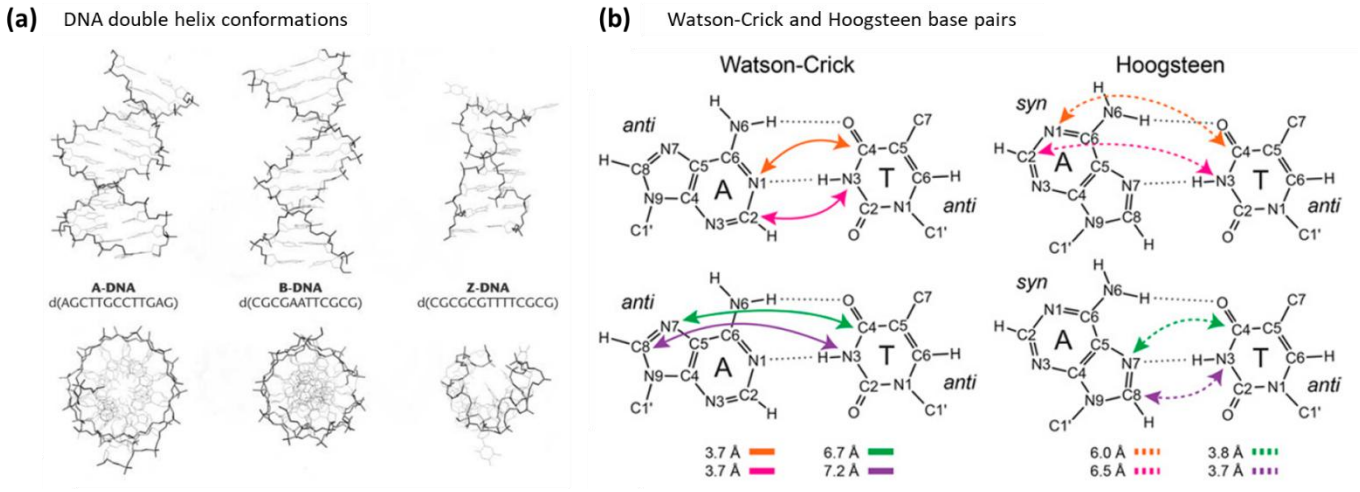

Figure 2.2. Helix conformations and complementary base pairs. (a) DNA double helix conformations and (b) Watson-Crick and Hoogsteen base pairing. Modified and reprinted with permission from references [27, 29].

2.1.4 Branched junctions, sticky ends and lattices

There are many different simple structures which can be made using DNA hybridization. The simplest type of DNA structure that one can make is a linear DNA structure. A linear DNA structure is composed of two or more individual ssDNA molecules which are hybridized together to form a linear structure, shown in **Figure 2.3a**. Linear structures can be formed with continuous or discontinuous components [13]. A more complex type of DNA structure that one can make is a branched DNA structure. Branched DNA is a type of DNA structure in which three or more individual segments can be identified around a single point, known as a branch point, shown in **Figure 2.3b**. These individual segments can include helical dsDNA, ssDNA or

individual unhybridized nucleotides, and they do not have to enclose the branch point. Branched DNA can be formed with continuous or discontinuous segments [34-36]. An example of a branched DNA structure which naturally occurs in biology is the DNA replication fork. However, a more specific type of branched DNA structure that one can make is a branched junction. A branched junction is a branched DNA structure which is composed of continuous and rotationally symmetrical helical dsDNA segments, shown in **Figure 2.3b and d**. Branched junctions can be made with three or more segments, but the most common type of DNA branched junction used in the field of structural DNA nanotechnology is a four-way branched junction. Four-way branched junctions are assembled by hybridizing four individual ssDNA molecules to form a structure that looks like a four-way road intersection, shown in **Figure 2.3d** [19, 37, 38]. An example of a four-way branched junction structure which naturally occurs in biology is the Holliday junction structure which is formed during the process of homologous recombination. Branched junctions are like the unit cells of metallic or ceramic crystal lattices because they are the fundamental unit which is repeated to determine the overall structure of a DNA lattice. Branched junctions are the tile pieces which are assembled into 2D DNA arrays and the block pieces which are assembled into 3D DNA arrays [39-42]. 2D DNA arrays are typically made using four-arm branched junctions and 3D DNA arrays are typically made using six-arm branched junctions with segments that are designed to extend in Cartesian coordinate directions.

The assembly of DNA branched junctions is achieved by incorporating a specific structural motif into the ends of its segments, shown in **Figure 2.3c**. This structural motif is known as a sticky end. A sticky end is an unhybridized ssDNA end of a dsDNA molecule. A blunt end is a fully hybridized end of a dsDNA molecule. Both linear and branched DNA can be

assembled together using sticky ends. The branched junctions which are used to form 2D arrays and 3D lattices are assembled together using sticky ends [13, 19, 38, 43].

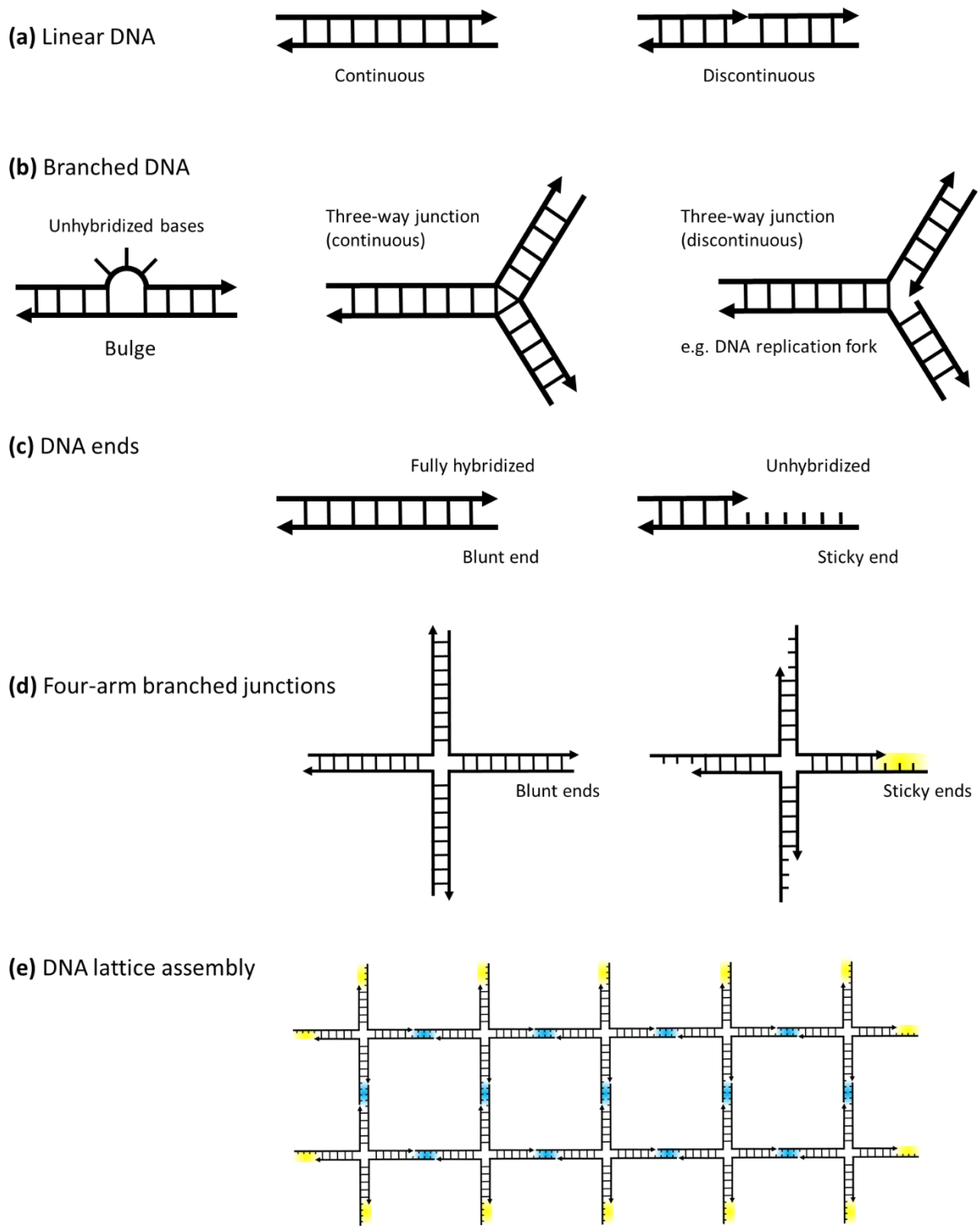

Figure 2.3. Types of DNA structures. (a) Linear DNA, (b) branched DNA, (c) DNA ends, (d) four-arm branched junctions and (e) DNA lattice assembly.

2.1.5 Crossovers and DNA origami

Like DNA lattices, DNA origami structures utilize the properties of DNA hybridization and DNA branching. However, DNA origami structures don't involve the use of branched junctions as tiles or blocks that are self-assembled using sticky ends. Instead, DNA origami structures involve the use of 1) one really long piece of ssDNA called a "scaffold", which can be linear ssDNA or circular ssDNA, and 2) many short ssDNA strands called "staples", and all of these components are hybridized together to form a complex structure. These structures are known as DNA origami nanoparticles (NPs), and their method of assembly was first designed by Paul Rothemund [44]. On a nanostructural level, the scaffold is the long piece of DNA that rasterizes the shape of a 2D structure and the staple strands are the short pieces of DNA that hold it all in place. In order to avoid creating tension in the DNA origami structure, the scaffold and staple strands must be designed to occur at points where there is a natural turn in the double helix. For a 2D nanostructure, scaffold turns can only occur every 10.67 bases, and the distance between crossover staple strands must occur every 16 bases [44]. On an intermolecular level, DNA origami scaffold and staple strands are designed to form four-arm branched junction structures which connect and hold individual dsDNA helices in place, like a rope which holds the individual pieces of a raft together. The four-arm branched junction structures which are found in DNA origami NPs are an isomerization of the four-arm branched junction used for DNA lattices, and they are known as crossovers because they are designed to cross over from one dsDNA helix to another [38, 45, 46]. The overall design of a DNA origami NP is determined by the crossover design between the scaffold and staple strands [44, 47, 48].

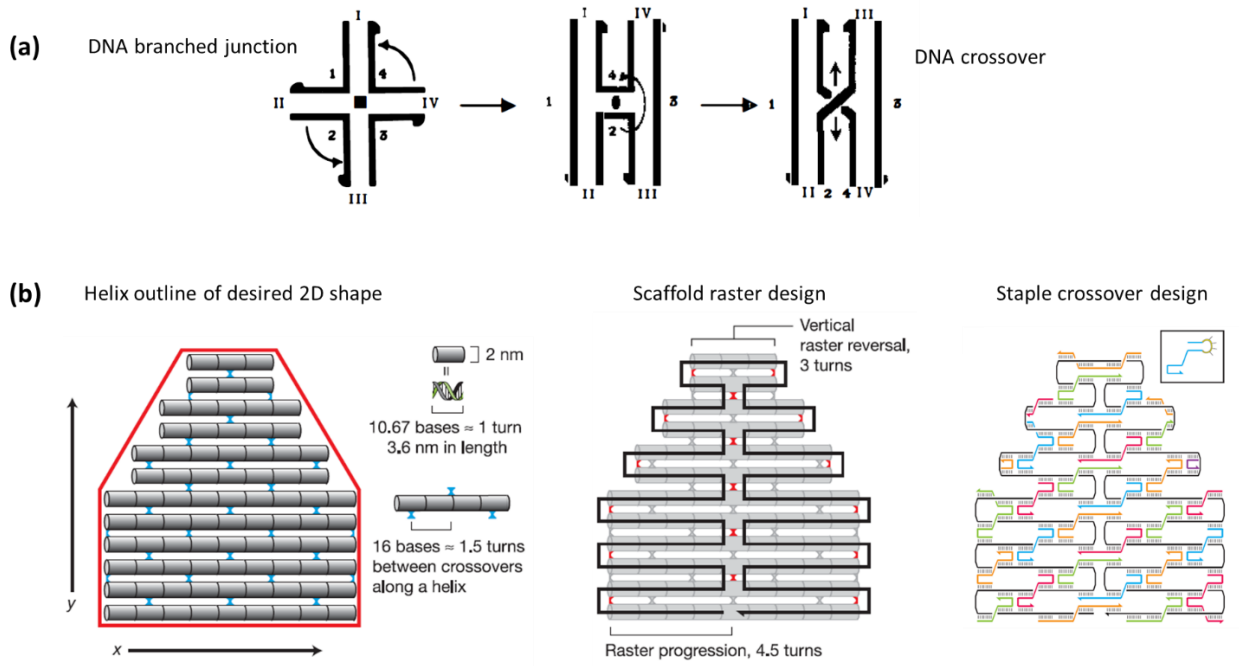

Figure 2.4. Components of DNA origami structures. (a) Isomerization of four-arm branched junction and (b) helix outline, scaffold raster design and staple crossover design of 2D DNA origami structure. Modified and reprinted with permission from references [38, 44].

2.1.6 Advantages and advancements of DNA origami for nucleic acid therapeutics

DNA origami NPs present a promising solution for the development of therapeutics because their strong control over the design of complex 2D and 3D structures enables us to design nanoparticles that can facilitate a therapeutic effect through structural modulation. Some of these structures include complex 2D shapes (e.g. stars, letters, and smiley faces), complex 3D structures (e.g. polyhedra, curved structures, amorphous bunny), and actuating 3D structures (e.g. boxes and propellers that open and close) [44, 49-51]. The design of DNA origami NPs also enables a wide-range of surface chemistries for the loading of molecules like drugs, peptides, nucleic acids and other molecules [52, 53]. Some of these surface chemistries include hybridization with ssDNA overhangs for the loading of biomolecules, DNA-PNA hybridization for the loading of semiconductor materials, like quantum dots, and covalent functionalization for the loading of molecules [54-58].

The field of structural DNA nanotechnology has made great strides to develop the advancement of DNA origami NPs for use as a new delivery platform, and more recently, as a new gene template for nucleic acid therapeutics. DNA origami NPs have demonstrated the facilitation of membrane transport and uptake without the use of transfection reagents; structural stability in cell lysates and in the cytosol of cells; the functionalization of nucleus-targeting peptides for intracellular targeting; and a higher biocompatibility over synthetic carriers like lipid nanoparticles (LNPs) because they can be degraded by physiological metabolic pathways and do not contain potentially immunogenic synthetic polymers like PEG [59-64]. Due to the versatility of their chemistry and design, DNA origami NPs have the potential to be multi-functionalized with different components, allowing us to customize the design of nanoparticles for use in specific disease applications. Some of the disease applications which have utilized the use of DNA origami NPs for the delivery of adjuvants for immunostimulation HIV, cancer and COVID-19 [58, 65-68].

2.1.7 Gene-encoding DNA origami NPs

In a regular DNA origami NPs, the molecule which is most commonly used as the ssDNA scaffold strand is the M13 bacteriophage genome (also known as M13 scaffold) because it is a naturally occurring, long, circular ssDNA molecule that is readily available in large quantities and relatively inexpensive compared to custom synthesized ssDNA. The M13 bacteriophage genome is the ssDNA molecule that Paul Rothemund used as a DNA origami scaffold in the first paper which he wrote to describe DNA origami method for assembling DNA NPs [44]. The size and complexity of the DNA origami NP which can be assembled is limited by the length of the scaffold strand that it utilizes, shown in **Figure 2.5b**. Because the cost of custom-synthesized ssDNA molecules increases exponentially based on length, the M13 scaffold

remains the most economical ssDNA molecule for use as a DNA origami scaffold based on cost per length. Almost all of the DNA origami NPs which have been created in this field utilize the M13 scaffold [49, 69, 70].

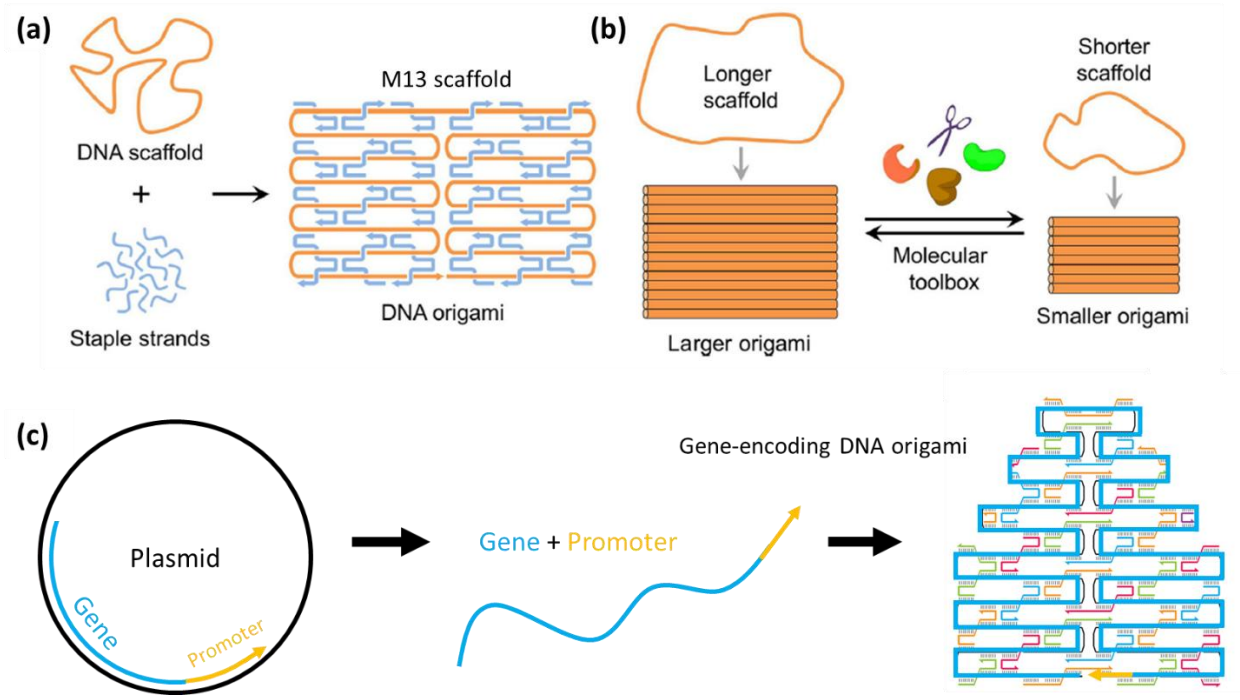

Figure 2.5. DNA origami scaffolds. (a) DNA origami made with M13 scaffold, (b) scaffold length and DNA origami size and (c) gene-encoding DNA origami. Modified and reprinted with permission from references [44, 69].

However, these new DNA origami NPs have replaced the traditional M13 bacteriophage genome with another scaffold molecule. In regular DNA origami, the M13 bacteriophage ssDNA molecule is primarily being used as a structural building platform, shown in **Figure 2.5a**. Therefore, it is not intended to be expressed. However, these new DNA origami NPs, known as gene-encoding DNA origami NPs, replace the use of the M13 bacteriophage ssDNA with another ssDNA that encodes a specific gene of interest and a promoter, which allows it to be expressed in biological systems, shown in **Figure 2.5c** [71-73]. Gene-encoding DNA origami NPs have utilized ssDNA scaffolds synthesized through various methods (bacteriophage

synthesis, aPCR, etc.) and have demonstrated expression in both bacterial and mammalian systems [70, 74].

The development of gene-encoding DNA origami NPs is exciting for the design of nucleic acid therapeutics because they incorporate the versatile structural and chemical properties of regular DNA origami NPs with the ability to be expressed as a gene template, so gene-encoding DNA origami NPs have the potential to be delivered and expressed inside of cells without the need for a carrier. Gene-encoding DNA origami NPs also have the potential to be functionalized for membrane transport, intracellular targeting and intra-nuclear delivery, which would remove the need for the use viral vectors for efficient DNA delivery. The strong control over the design of complex 2D and 3D structures of gene-encoding DNA origami NPs as gene templates allows us to structurally modulate their integration and expression efficiency in integrative gene therapies. DNA origami NPs have been demonstrated as a promising platform for the delivery of CRISPR/Cas for gene editing therapies, so it's possible that combining the use of gene-encoding DNA origami NP templates with CRISPR/Cas could be used to improve integration and gene editing efficiency [75-77]. However, almost nothing is known about the fundamental structure/function properties of DNA origami NPs for these types of applications (e.g. What is the most efficient way to present the promoter sequence on a gene-encoding DNA origami? Will functionalization of gene-encoding DNA origami NPs inhibit its expression?). In order to enable the development of safer, more effective and longer-lasting treatments for genetic diseases, this thesis establishes use of gene-encoding DNA origami NPs as a new and improved gene template and therapeutic delivery platform.

2.2 Coacervates

Another new material technology that has recently surfaced as a potential therapeutic delivery platform are coacervates. Coacervation is a form of liquid-liquid phase separation which occurs when dissolved components, such as proteins, polymers or colloids, interact in solution to form a coacervate phase, which has high concentrations of dissolved particles, and a supernatant phase, which is devoid of dissolved particles [78]. Coacervates can be formed through different types of molecular interactions including hydrophobic, pi-pi stacking, electrostatic and pi-cation interactions. There are two main types of coacervation—1) simple coacervation, which occurs between the same type of particles, and 2) complex coacervation (also known as associative coacervation), which occurs between different types of particles, shown in **Figure 2.6** [79]. The existence of coacervates in polymer science has been known for almost a century; however, they are currently being re-discovered in the field of biology due to recent findings that suggest how eukaryotic gene expression is regulated by the formation of coacervates in the cell which concentrate the relevant biomolecules in liquid-like micro-compartments [80, 81]. For this reason, they are also known as biomolecular condensates or membraneless organelles (MLOs) in the field of biology.

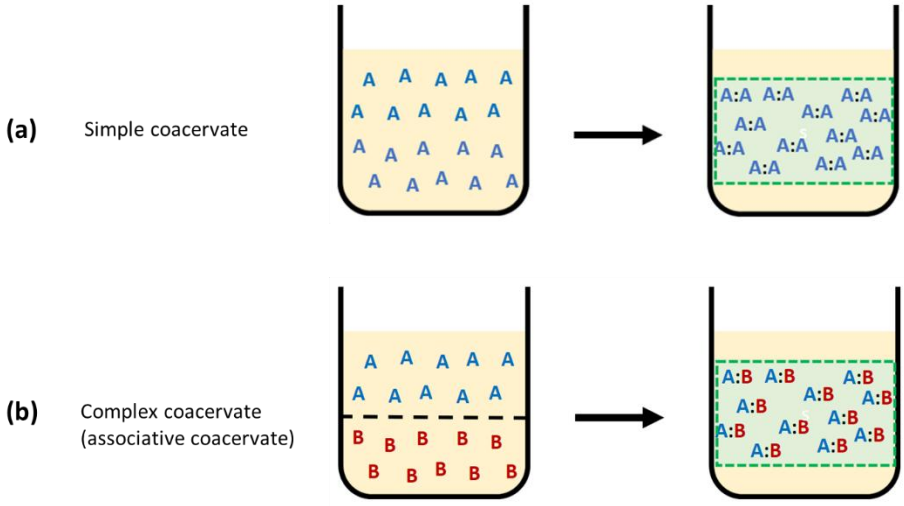

Figure 2.6. Types of coacervates. (a) Simple coacervates and (b) complex or associative coacervates. Modified and reprinted with permission from references [78].

2.2.1 Advantages of coacervates for nucleic acid therapeutics

Coacervate or MLO particles can be loaded with cargo such as drugs and nucleic acids for therapeutics, and they are advantageous as a therapeutic delivery platform due to their high loading efficiency, biocompatibility, membrane permeability and low cytotoxicity [82]. Coacervates are also relatively easy to fabricate and can be tuned for release using various mechanisms like changes in temperature and pH [83]. They have also demonstrated the ability to protect mRNA from degradation, which is favorable for nucleic acid delivery applications, and they have been utilized as a protein delivery platform for bone regeneration, tumor treatment and neurodegenerative disease treatment [84]. However, there is still much to learn about how to design and control these properties in de novo coacervates. Therefore, in order to enable the development of safer, more effective and longer-lasting treatments for genetic diseases, this thesis has explored the design of de novo coacervates in order to improve their loading efficiency, biocompatibility, release mechanisms, and establish their use as a nucleic acid delivery platform that can protect gene templates (such as plasmids, linear DNA, and DNA origami NPs) against exogenous nuclease degradation.

2.3 Cell-free transcription-translation systems as a platform for studying gene expression

Although gene-encoding DNA origami NPs and coacervates demonstrate a lot of promise to improve nucleic acid delivery technologies, one of the main challenges that remain in translating these technologies to the market is the cost and time required to produce enough sample to perform the required *in vitro* and *in vivo* experiments to assess the efficacy of gene expression. As known with therapeutic delivery experiments, the efficiency of membrane

transport into cells and retention in animal models strongly influences the minimum dosage required, sometimes requiring high concentrations of the delivered material, in order to observe a significant measurable effect [85]. Because of all the challenges with gene delivery that must first be addressed, it can be extremely difficult to decipher which specific component(s) of gene template design correlates with expression, highlighting the significant need for the development of platforms which can be used to more directly investigate the role of gene template design on gene expression.

Fortunately, cell-free gene expression, a technology which allows us to perform benchtop protein synthesis without the use of live cells, can help circumvent some of these issues. Cell-free transcription-translation (TXTL) systems are composed of cytoplasmic lysates, which are collected from cells which were stripped of membranes and genetic material, and then reconstituted in test tubes and supplemented with buffers to sustain proper functioning of transcription and translation [86]. Because cell-free TXTLs are simpler and more open systems than live cells, there is no need to address the challenge of membrane transport, allowing these systems to be extremely advantageous for focusing studies on gene expression, which can help us gain a better understanding of how the design of delivered genetic constructs might affect protein synthesis [87].

The specific pure cell-free TXTL system that was used in this thesis is the PURExpress In Vitro Protein Synthesis Kit, which was initially created by Y. Shimizu et al. as a platform for high throughput protein production [88-90]. Traditional *in vitro* protein expression systems are derived from purified cell lysates, which are difficult to optimize for high protein production due to the lack of control over the types and amounts of enzymes and other components in the system. However, the design of a pure cell-free TXTL system has greater control over the types

and amounts of individual components in the TXTL, allowing its components to be tuned to optimize specific properties, like protein production. The PURExpress In Vitro Protein Synthesis Kit used in this thesis has been optimized to yield the highest levels of protein production, so even very small amounts of added gene templates will result in a measurable amount of expressed protein.

This is advantageous because it allows cell-free TXTLs to be scaled down to small microliter reaction volumes which require only nanograms of DNA template material, as compared to cell or animal studies that might require micrograms or milligrams of material for measurable gene expression, avoiding the need to produce so much sample and enabling users to compare hundreds of design variations in parallel. Gene expression in cell-free TXTL systems have predominantly relied on the use of plasmids as the DNA template; a few papers that have studied the effect of the size of plasmids [91], as well as the stability of linear DNA [92, 93], on gene expression in *E. coli* cell-free TXTL systems. However, these studies have reported on the degradation of linear DNA templates by endogenous exonucleases, mainly RecBCD, present in cell-lysate based TXTLs [92-94], so to avoid exonuclease damage to linear DNA templates, here we will utilize the PURExpress cell-free extract system, a pure cell-free TXTL system reconstituted from purified *E. coli* components [88].

2.3.1 Prokaryotic v. Eukaryotic Gene Expression

Although the use of cell-free TXTL systems is advantageous for the study of gene template expression, it is important to recognize the limitations of this platform. The most important thing to keep in mind when using a cell-free TXTL system is the type of expression that the TXTL is modeled after. The PURExpress In Vitro Protein Synthesis Kit (used in this thesis) is derived from *E. coli* bacteria, which is a type of prokaryotic cell. Prokaryotic cells

typically form unicellular organisms, known as prokaryotes, and they do not contain a nucleus. Because prokaryotes are relatively simple organisms, their genome and expression system do not require as much complexity as eukaryotic cells. In fact, there are only two main components for prokaryotic gene expression—the sigma factor and bacterial RNA polymerase. The sigma factor is the bacterial transcription factor which recognizes and binds onto the promoter sequence of a gene for transcription, and RNA polymerase is the enzyme which transcribes DNA into mRNA. On the other hand, eukaryotic cells (i.e. mammalian cells) are much more complex because they are designed to form multicellular organisms. Because eukaryotic cell genomes are larger and more complex than prokaryotic cell genomes, they have a much more complex system for gene expression to ensure proper functioning and regulation. Eukaryotic gene expression involves not just one type of RNA polymerase, but three different types of RNA polymerase (RNA polymerase I, RNA polymerase II and RNA polymerase III) which are designed to transcribe different categories of genes. The initiation of RNA polymerase II it requires the assembly of the general transcription factors (TFIID, TFIIB, TFIIF, TFIIE and TFIIH), which recognize and bind onto different promoter sequences in the gene [11]. Because bacterial and mammalian gene expression have completely different transcription factors, they require different promoter sequences to initiate transcription, but in both expression systems it is important to ensure that the gene is designed to have the correct promoter sequence, ribosomal binding site and termination sequence.

When using cell-free TXTL platforms to study the expression of gene templates for nucleic acid therapeutics, it is important to keep in mind the differences between prokaryotic and eukaryotic gene expression At the moment, bacterial cell-free TXTL systems are more available, but mammalian cell-free TXTL systems, like HeLa cell-based TXTLs, have also been developed

and are now commercially available [95, 96]. For gene template expression studies, it would be good practice to confirm results in both bacterial and mammalian cell-free TXTL systems before scaling up to studying gene expression *in vivo*.

In order to enable the development of safer, more effective and longer-lasting treatments for genetic diseases, this thesis establishes the use of a pure cell-free TXTL system as a simple, high throughput platform to directly investigate the role of designed gene templates on expression without the use of live cells.

Chapter 3: Design and Characterization of a Gene-Encoding DNA Nanoparticle in a Cell-Free Transcription-Translation System

This chapter was originally published as Galvan AR, Green CM, Hooe SL, Oktay E, Thakur M, Díaz SA, Veneziano R, Medintz IL, Mathur D. "Design and Characterization of a Gene-Encoding DNA Nanoparticle in a Cell-Free Transcription-Translation System." *ACS Appl Nano Mater.* 2024 Jun 14;7(11):12891-12902. doi: 10.1021/acsanm.4c01456. Epub 2024 May 30. PMID: 39830902; PMCID: PMC11741557.

3.1 Introduction

Over the past two decades, great strides have been made within nucleic acids engineering in the form of DNA nanotechnology for biomedical applications along with the recent first in class COVID messenger RNA (mRNA) vaccine [97-102]. This success naturally suggests combining these two approaches into a single modality for future gene therapy and/or vaccine delivery [52, 65, 103, 104]. Gene-encoding DNA origami nanoparticles (NPs) may potentially allow the delivery of genetic material into cells without the need for synthetic nanocarriers like micelles and liposomes as is common now [105, 106]. The principle of DNA origami typically utilizes the canonical single-stranded DNA (ssDNA) M13 bacteriophage genome as a universal scaffold strand to which short synthetic staple strands bind and dictate the folding of the NP and the architecture of the final assembly [44, 48]. In a combined approach, the phage genome would be replaced by an ssDNA version of a gene encoding a desirable protein product to be delivered

to cells with the staple strands again determining the desired structure of the final DNA NP assembly.

Some preliminary reports toward this combined approach have already started to appear. For example, Kretzmann and colleagues demonstrated delivery and expression of the enhanced green fluorescent protein (EGFP)-encoding DNA NP in HEK293T cells using electroporation-based delivery [71]. They further enhanced the *in cellula* efficiency of gene expression by including multiple functional sequences such as a virus-inspired inverted-terminal repeat as an origin of replication from viral vectors into the scaffold along with confirming the co-transfection of multiple genes in stoichiometric ratios. Similarly, the Ding Lab utilized two complementary DNA strands of their desired functional gene as the DNA scaffold [72]. Following hybridization driven assembly, the encoded DNA origami NP exploited precisely organized lipids on its surface as a template for lipid growth to allow for efficient penetration through cell membranes during delivery and successful gene expression. By adding a further tumor targeting moiety, their p53 antitumor gene-encoded DNA origami NP yielded a pronounced upregulation of the p53 protein in tumor cells, confirming this as a potentially efficient tumor therapy. There have been some further examples in the same vein [73, 77, 107, 108]. Given the complexity inherent to both the structure of the DNA origami NP system along with the multiple steps involved in subsequent cellular targeting, transmembrane uptake, delivery, and intracellular expression without considering the additional endosomal sequestration and escape issues [109, 110], it can be extremely difficult to focus on assessing just gene expression itself and how this relates to the original DNA NP design. Thus, it is clearly desirable to have a preliminary and somewhat minimalist system to confirm and optimize the expression of the gene from the construct prior to dealing with any cellular uptake. Fortunately, cell-free

gene expression, a technology that allows us to perform benchtop protein synthesis without the use of live cells, can help circumvent some of these issues in the form of cell-free transcription−translation (TXTL) systems.

TXTL systems are typically composed of (bacterial) cytoplasmic lysates, which are collected from cells and stripped of membranes and genetic material, and then reconstituted in test tubes and supplemented with buffers to sustain proper functioning of the transcription and translation machinery [87, 88, 90, 94]. Some commercial TXTL kits such as the PURExpress *In Vitro* Protein Synthesis Kit consists of a reconstituted protein synthesis system where all components needed for *in vitro* transcription and translation are first purified following production in *E. coli* [111, 112]. Such kits contain a proprietary mix of optimized components with many of the extraneous materials found in cell lysate-based TXTL systems removed. Because cell-free TXTLs are obviously simpler and more open systems than living cells, there is no need to address the challenge of membrane transport, allowing these systems to be extremely advantageous for focusing studies on gene expression. This can help gain a better understanding of and correlate how the design of delivered genetic constructs and other related factors might affect protein synthesis. Further, cell-free TXTLs can be scaled down to small microliter reaction volumes, which require only nanograms of DNA template material, as compared to cell, tissue, or animal studies that might require micrograms or milligrams of material for measurable gene expression, thus avoiding the need to produce large quantities of test sample. Gene expressions in cell-free TXTL systems have predominantly relied on the use of plasmids as the DNA template; a few papers have also studied the effect of the size of plasmids as well as the stability of linear DNA on gene expression in *E. coli* cell-free TXTL systems [91-93].

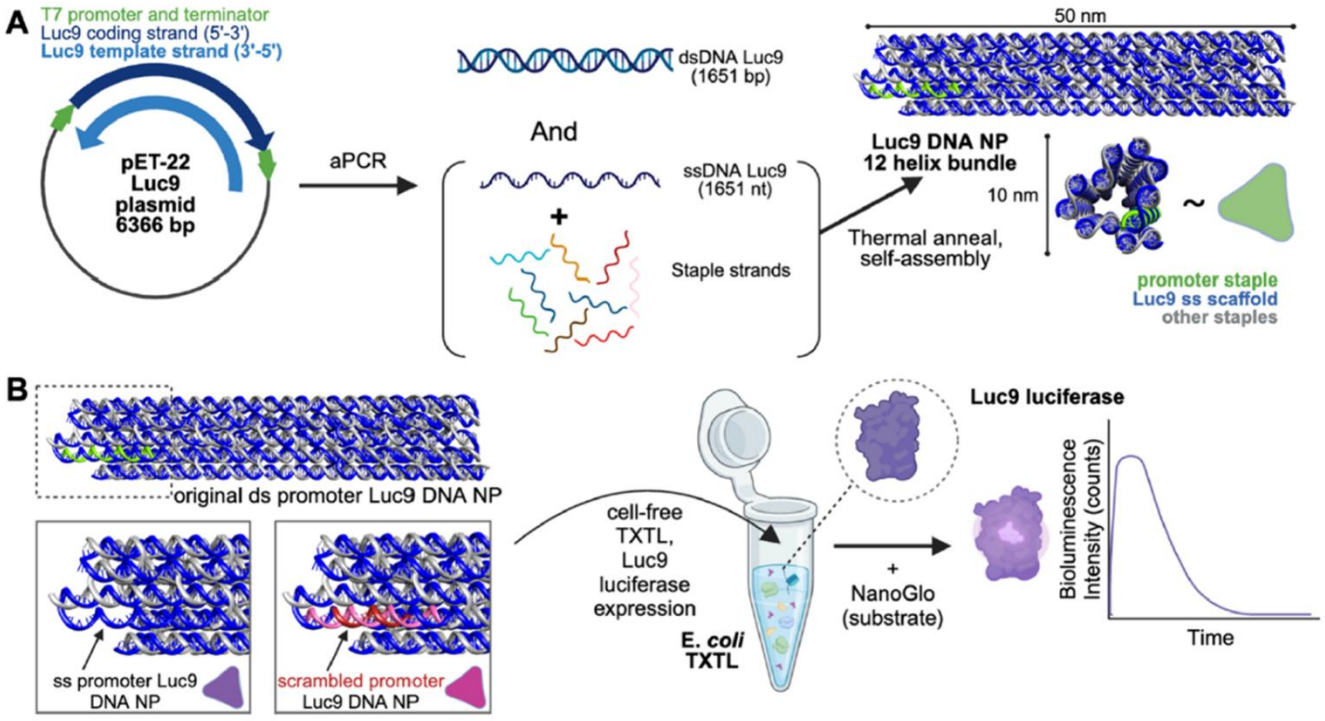

Figure 3.1. Schematic of the DNA NP design and gene expression in the cell-free TXTL system. (A) A DNA origami scaffold was obtained from a pET-22 plasmid encoding Luc9 gene with a T7 promoter via asymmetric PCR (aPCR). aPCR resulted in the Luc9 encoding ssDNA gene (scaffold) and a dsDNA Luc9 gene (byproduct). The ssDNA Luc9 scaffold was combined with staple strands and thermally annealed to assemble the final 12-helix bundle DNA NP, which underwent subsequent PEG purification. T7 promoter (green) is on the outer face of the DNA NP. (B) Three conformations of the Luc9 DNA NP were tested for luciferase protein expression in *E. coli* TXTL systems, namely, the original Luc9 DNA NP with its traditional dsDNA T7 promoter (represented in other figures as a green triangle), a construct missing the promoter staple, making a ssDNA promoter Luc9 DNA NP (purple triangle), and a version where the T7 promoter sequence was replaced by a scrambled sequence in the scaffold (pink triangle). Following TXTL, aliquots were tested for luciferase synthesis and bioluminescence by adding to NanoGlo solution. The bioluminescence assay served as a metric to determine DNA NP performance as a gene-encoded system for protein expression. Created with BioRender.com.

In this report, we apply a pure *E. coli* TXTL system in characterizing the expression of gene-encoded DNA NP systems. We determine the extent to which the nature of the promoter sequence upstream of the gene within a DNA NP affects protein expression in comparison to canonical plasmid and linearized single-stranded (ss) and double-stranded (ds) DNA versions of the gene; see the overview schematic in **Figure 3.1**. The gene for a previously optimized sea pansy (*Renilla reniformis*) luciferase is encoded into the scaffold strand of the DNA NP and

luciferase catalyzed bioluminescence serves as a quantifiable measure of DNA NP TXTL-enabled expression. We designed a 12-helix bundle DNA NP wherein the 1651-nucleotide long scaffold strand encoded a T7-expressing luciferase gene. Following confirmation of the DNA NP assembly, luciferase-based bioluminescence assays were used to characterize the relative protein expression level and function. Bioluminescence from protein expression in the canonical plasmid, linearized ds- and ssDNA gene versions, and alternate configurations of the T7 promoter site within the scaffold strand of the DNA NP revealed that protein expression via DNA NPs is programmable. Gene-encoded DNA NP with an ss promoter domain in its scaffold strand had markedly decreased protein expression compared to a DNA NP with an appropriate staple strand bound to the promoter scaffold region. The scrambled promoter sequence completely abrogates the protein expression. Fundamentally, the work formulates a methodology to characterize other gene-encoded DNA NPs in a facile, parallel, and cell-free manner.

3.2 Experimental methods

3.2.1 Materials

LB agar, LB broth, agarose, Tris-acetate ethylenediaminetetraacetic acid (TAE), Tris-borate-EDTA (TBE), Tris-HCl, $MgCl_2$, NaCl, and PEG-8000 were purchased from Sigma-Aldrich. Low melt agarose was purchased from IBI Scientific. Ethidium bromide and ampicillin were purchased from Fisher Scientific. GelRed was purchased from Biotium. ssDNA staples were purchased from Integrated DNA Technologies (IDT) at a final concentration of 100 μM in RNase-free water; primers and gene block (template for producing the scrambled promoter scaffold strand) were also purchased from IDT in lyophilized conditions and resuspended in RNase-free water (sequences for all can be found listed in the **Appendix**). Bacteriophage M13mp18 (M13) scaffold was purchased from Bayou Biolabs. Monarch Plasmid Miniprep Kit,

OneTaq Hot Start DNA Polymerase, deoxynucleoside triphosphates (dNTPs), OneTaq Standard Reaction Buffer, and PURExpress *In Vitro* Protein Synthesis Kit were purchased from New England Biolabs. The Zymoclean Gel DNA recovery kit was purchased from Zymo Research. NanoGlo Luciferase Assay Substrate was purchased from Promega. Black 384-well microtiter plates were purchased from Corning. MicroAmp Optical Adhesive Film was purchased from Thermo Fisher Scientific.

3.2.2 Scaffold production

The plasmid used in this study, pET-22 Luc9, expresses *Renilla luciferase* (*Renilla reniformis* EC No. 1.13.12.5) which was mutationally optimized by Rao et al.35 It has been used previously and encodes a T7 promoter, the Luc9 gene sequence, followed by a C-terminal (His)6 and a T7 terminator sequence (see **Figure 3.1A,B**) [73, 87]. *E. coli* TOP10 cells transformed with the Luc9 plasmid were cultured on LB agar plates containing 100 μg/mL ampicillin and grown overnight at 37°C. Afterward, a few colonies were picked and grown in 5 mL of LB medium supplemented with 100 μg/mL ampicillin overnight. The plasmids were extracted using the Monarch Plasmid Miniprep Kit according to manufacturer's instructions. Plasmid concentrations were measured using a Nano-Drop 2000 (Thermo Scientific), and samples were stored at −20°C until further use.

The Luc9-encoding scaffold strands were amplified using an aPCR protocol previously published by Veneziano et al [50]. Scaffold encoding the T7 promoter was amplified using the pET-22 Luc9 plasmid as the aPCR template, while the scaffold with the scrambled promoter was amplified from a gene-block dsDNA. The reaction mixture was as follows: 1 μM forward primer, 20 nM reverse primer, 0.2 μg/mL of plasmid (or 0.3 μg/mL Luc9 gene-block with scrambled promoter sequence), 0.2 mM of deoxynucleoside triphosphates (dNTPs), 1× OneTaq

Standard Reaction Buffer, and 1.25 U of OneTaq Hot Start DNA Polymerase enzyme. aPCR was performed in a Bio-Rad T100 thermal cycler using the following program: 94°C for 1 min for the initial denaturation followed by 35 cycles of 94°C for 20 s, 55°C for 30 s, and 68°C for 1 min per kilobase (kb) of the product to be amplified [92]. Samples were held at 4°C until the sample was retrieved from the thermal cycler. Formation of the ssDNA scaffold strands (and the dsDNA amplicon) was confirmed and simultaneously purified via gel extraction. Namely, aPCR products were loaded into a 1% low-melt agarose gel with 1× TAE buffer (pH 8.0) prestained with 0.5 µg/mL of ethidium bromide. Gel electrophoresis was run at 100 V/cm at room temperature for ~25 min. Gel images were taken (Azure c150 Gel Imaging Workstation) to confirm the presence of the ssDNA and dsDNA bands and validate the efficiency of the reaction (**Figure 3.2**). Both the ssDNA and dsDNA aPCR products were excised and purified from the gel using a Zymoclean Gel DNA recovery kit according to manufacturer's instructions. DNA concentrations were measured with a Nanodrop One (ThermoFisher) and calculated molecular weights of the ssDNA and dsDNA products, and the samples were stored at −20 °C until further use.

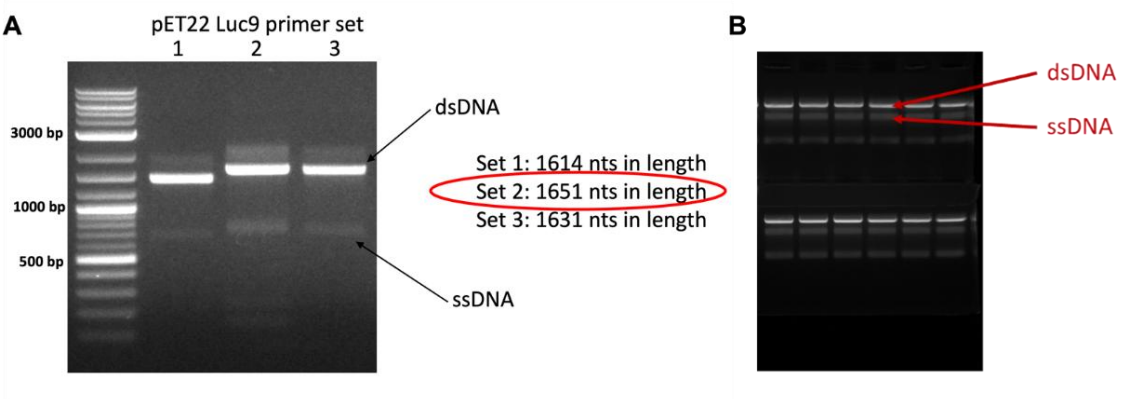

Figure 3.2: Agarose gel electrophoresis of aPCR ssDNA and dsDNA products. (A) 1% low-melt agarose gel electropherogram showing aPCR products synthesized using pET22 Luc9 plasmid as template and three different primer sets (lanes 1-3 indicating primer sets 1-3, respectively). Primer set 2 resulted in the highest yield of ssDNA (lane 2), which was used for the rest of the project. **(B)** 1% low-melt agarose gel electropherogram representing high-throughput amplification of ssDNA and dsDNA products from

pET22 Luc9 plasmid and primer set 2. From these lanes (which represent multiple lanes of the same sample type), bands representing ssDNA and dsDNA were excised and purified to yield the scaffold for the DNA NP and dsDNA control. The lower molecular weight band below the ssDNA band represents any excess primers.

3.2.3 DNA NP design, folding and purification

The Luc9-encoding 12-helix bundle DNA NP was designed using caDNAno (**Figure 3.3**) [113]; the scaffold sequence and list of ssDNA staple strands are found in the **Appendix**. Staple strands were added in 5-fold excess to the scaffold, and the mixture for a 50 µL volume was as follows: 40 nM scaffold, 12.5 mM $MgCl_2$, 0.5× TBE buffer (pH 8.4), and 200 nM staples. In a thermal cycler, the mixture was first annealed to 85°C and then held at 70°C for 10 min. Then, the mixture was cooled stepwise from 70 to 20°C over 8 h and held at 4°C until the sample was retrieved. The assembled DNA NPs were purified using PEG precipitation via the method described by Green et al [114]. Briefly, an equal volume of PEG precipitation solution (15% w/v PEG-8000, 5 mM Tris-HCl, 505 mM NaCl, and 12.5 mM $MgCl_2$) was added to the DNA NP solution; the tube was gently inverted 2−3 times and centrifuged (16000× rcf) for 25 min at 4°C. Afterward, the supernatant was collected (reserved for control experiments), and the DNA NP pellet was resuspended in 50 µL of 10 mM Tris-HCl pH 8.0, 12.5 mM $MgCl_2$. The DNA NP concentration was measured on the NanoDrop, and samples were stored at 4°C. As a negative control, a M13-scaffolded DNA snub cube was designed using DAEDALUS [50] and then assembled and purified using the above methods. For this structure, the M13 scaffold instead of the Luc9-encoding scaffold and a different set of staple strands were used (listed in the **Appendix**). All DNA template samples (**Figure 3.2**) were prepared at 16 nM using 10 mM Tris-HCl (pH 8.0) and 12.5 mM $MgCl_2$.

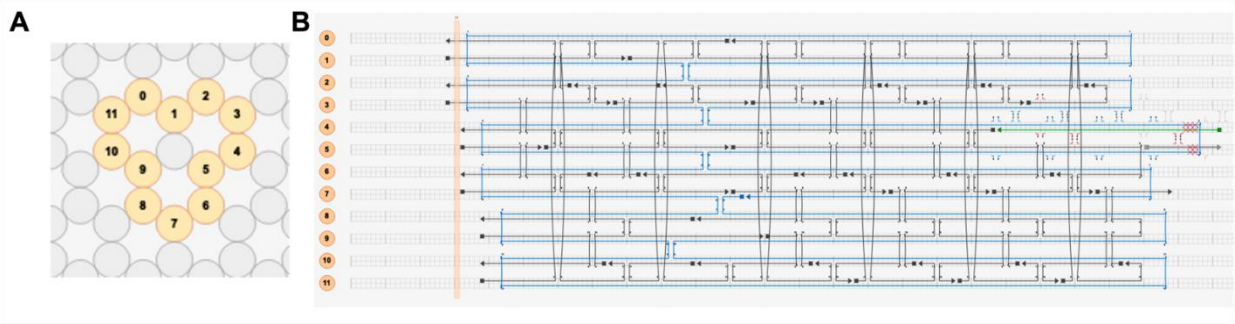

Figure 3.3: CaDNAno schematic of the Luc9 12 helix bundle DNA NP. (A) Helix cross section, **(B)** Expanded CaDNAno layout. The CaDNAno file will be posted on nanobase.org or can be provided upon request.

3.2.4 Agarose gel electrophoresis

1% (w/v) agarose gel in 0.5×TAE 8 mM $MgCl_2$ buffer was used to characterize the formation of the DNA NPs (**Figure 3.4**). No dye was added into the gel; 8 µL of each sample at 16 nM, 2 µL of 10× GelRed, and 10 µL of buffer were added into each well. Gel electrophoresis was run at 80 V/cm for 75 min at 4°C. Gel images were taken using a gel imager (BioRad).

3.2.5 Atomic force microscopy

Samples were prepared for atomic force microscopy (AFM) imaging by depositing 20 µL of sample at 4 nM on a freshly cleaved mica disk (1 cm) and allowed to adsorb for 5 min. The mica was rinsed using 0.5×TBE, 12.5 mM $MgCl_2$ buffer, and then placed on the AFM stage. Afterward, ~80 µL of buffer and ~7 µL of 100 mM $NiCl_2$ were added. Imaging was performed using a JPK Nanowizard 4 (JPK Instruments) on fast scan under the AC fast imaging mode (liquid) with a USC F0.3 similar to the methodology used previously [54, 67]. Images were acquired with these settings: Gain 600, Fast axis 1−2 µm, Pixels 1024 × 1024, and Line rate 6 Hz. AFM images were processed by using Gwyddion software.

3.2.6 Cell-free TXTL protein expression reaction

Expression of luciferase from the different Luc9 DNA templates was performed by using the commercially available PURExpress cell-free protein expression system. TXTL kit components were mixed according to the manufacturer-suggested ratios, but the total reaction volume was scaled down to 10 µL instead of the suggested 25 µL. In a 10 µL reaction, 1.9 nM DNA template (1.2 µL of DNA template at 16 nM was added), 4 µL of solution A, 2.8 µL of solution B, and 2 µL of water were added. The reaction was incubated at 37°C for 2 h and then transferred to the plate reader for measurements (see **Figure 3.4**).

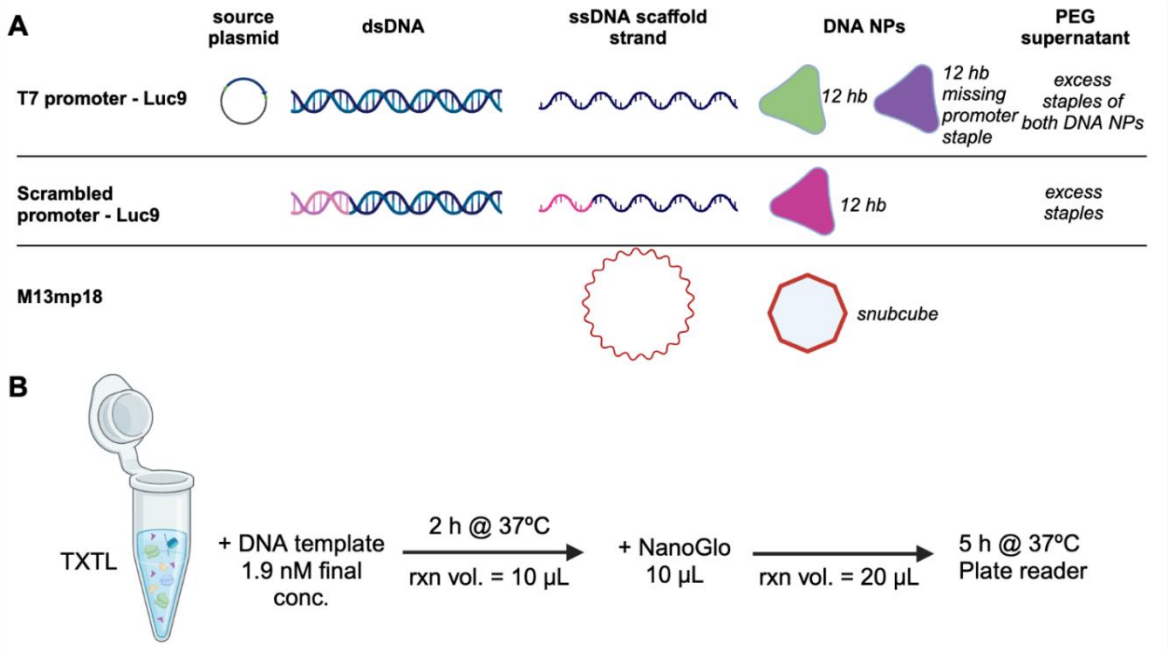

Figure 3.4: Diagram of (A) the 13 different DNA template types tested in TXTL for protein expression and (B) the experimental process. Created using BioRender.com.

3.2.7 Bioluminescence and analysis

Following protein expression, 10 µL of each reaction was pipetted into a black 384-well plate, and then 10 µL of NanoGlo Luciferase substrate in 1× PBS (1:100) was added to each well. The plate was sealed with MicroAmp optical adhesive film and placed into a Tecan Spark

multimode plate reader for bioluminescence measurements in a manner similar to that previously described [115, 116]. Measurements were collected at 460 nm wavelength (corresponding to the peak emission for the NanoGlo Furimazine substrate) for up to 5−6 h at 37°C with an integration time of 1000 ms for each sample. The data represented as bioluminescence intensity counts are arbitrary units of the instrument. The intensity counts are appropriate for a comparative study between different protein expression samples. The data were plotted using Origin Lab 2024 into curves as shown in this work. The curves shown are actual data and not fitted data by using any decay analysis.

3.3 Results

3.3.1 Design of the Luc9-encoded DNA NP

We sought a system that provided us with an easy assay to directly correlate protein synthesis from the gene-encoded DNA NP with some simple output metric. Following on the work of Kretzmann and colleagues, who monitored expression of fluorescent proteins for their gene encoding assembly, we selected a bioluminescent luciferase enzyme−substrate reporter system and direct monitoring of the light it would produce as our assay metric [71, 117]. This light generating enzyme was chosen as we wanted to avoid fluctuations in fluorescence due to fluorescent protein chromophore maturation rates along with the fact that the reactions would be taking place in a normal oxygenated atmosphere at standard pressure [117]. Moreover, since this is an enzymatic reaction, it inherently provides for a form of signal amplification over time. Luciferase has a very well-known, simple reaction mechanism that is only dependent on substrate presence and O_2 and leads to a bright luminescence intensity around 460 nm33 (see **Figure 3.5**). Therefore, we decided to encode *Renilla luciferase* (*Renilla reniformis* EC No. 1.13.12.5) protein into the scaffold strand of a DNA NP and measure its bioluminescence as a

function of DNA NP properties (**Figure 3.1A**). This version of the gene contains 9 mutations versus the original wild type that were introduced by Rao to optimize its activity and is therefore termed the Luc9 gene [118-121]. The Luc9 gene is inserted in the pET-22 vector and expressed from a T7 promoter (see **Appendix** for the complete plasmid sequence). Bioluminescence of Luc9 has been considerably studied and applied with DNA-based scaffolds previously, reinforcing it as a good prototypical protein for measuring gene expression [67, 70, 115, 116, 119]. Our choice of using Luc9 luciferase bioluminescence also took into consideration that the TXTL reactions themselves tend toward small reaction volumes (<10 μL) with low concentrations of reactants, and thus any product that was expressed would be difficult to detect directly against the background of proteins already present in the same mix using techniques such as Western blot and gel analysis.

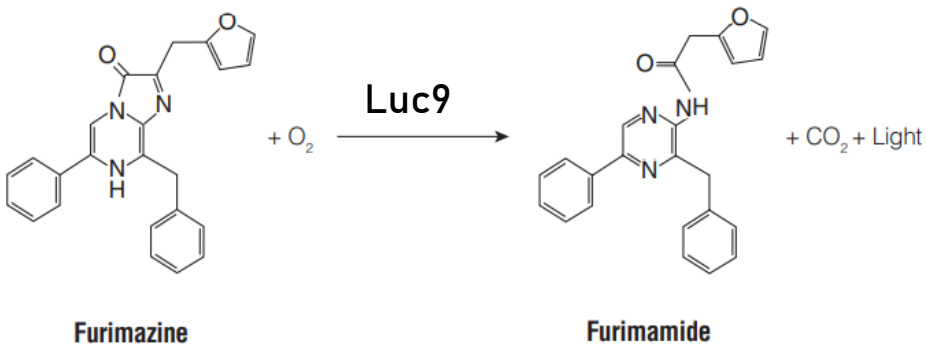

Figure 3.5. Chemical reaction of the Luc9 luciferase with furimazine to produce light. Chemical reaction adapted from the Nano-Glo® Luciferase Assay System Technical Manual (https://www.promega.com/resources/protocols/technical-manuals/101/nanoglo-luciferase-assay-system-protocol/ accessed 2.11.2024)

The synthesis of a scaffold strand that encodes the T7-controlled Luc9 gene was done using asymmetric polymerase chain reaction (aPCR) to amplify the gene-encoding subsequence from the pET-22 Luc9 plasmid (subsequently termed the plasmid) [50, 70]. aPCR allows the preferential amplification of one of the strands from a dsDNA template by using an asymmetric

ratio of forward and reverse primer strands. Previous studies have leveraged aPCR to amplify a gene encoded sequence for custom scaffold synthesis [108]. To this end, the promoter and gene encoding region of the plasmid was identified, and primers were designed in the corresponding sequence region (see **Appendix**). However, to identify which one of the two strands within the dsDNA plasmid should serve as the gene-encoding scaffold strand, we reverted to the fundamental principles of transcription of RNA from a dsDNA template. The two strands that constitute the plasmid are termed the coding strand (which is homologous to the resultant mRNA) and the template strand (which is the complement that is in fact read from during transcription to synthesize the mRNA) [11]. The *E. coli* RNA polymerase (RNAP) enzyme first identifies and binds to the promoter region, which would be T7 in this case, and then parses the template strand as the dsDNA strand is unwound, to produce mRNA. Therefore, for our studies, we prepared the scaffold encoding the ssDNA template strand and not the coding strand. The complementary sequence of the plasmid (which is the template strand) was used as the sequence to derive the forward and reverse primers accordingly. For the Luc9 plasmid (6366 base pairs (bp); **Figure 3.4**) [119], three different primer sets (listed in the **Appendix**) were initially tested in aPCR, and the one with the highest ssDNA yield according to agarose gel electrophoresisprimer set 2 was used to create all of the subsequent ssDNA scaffold (and dsDNA control samples) for this study (**Figure 3.4A**). The resultant amplified scaffold ultimately used was 1651 nucleotides (nt) long. aPCR performed on the plasmid yielded both the ssDNA Luc9 scaffold and dsDNA Luc9 products (**Figures 3.1 and 3.6 A,B**). Both ssDNA and dsDNA amplified products encoding Luc9 were recovered from subsequent agarose gel purification of the aPCR reactions and utilized in experiments for DNA NP synthesis as well as to serve as TXTL controls (**Figure 3.6B**).

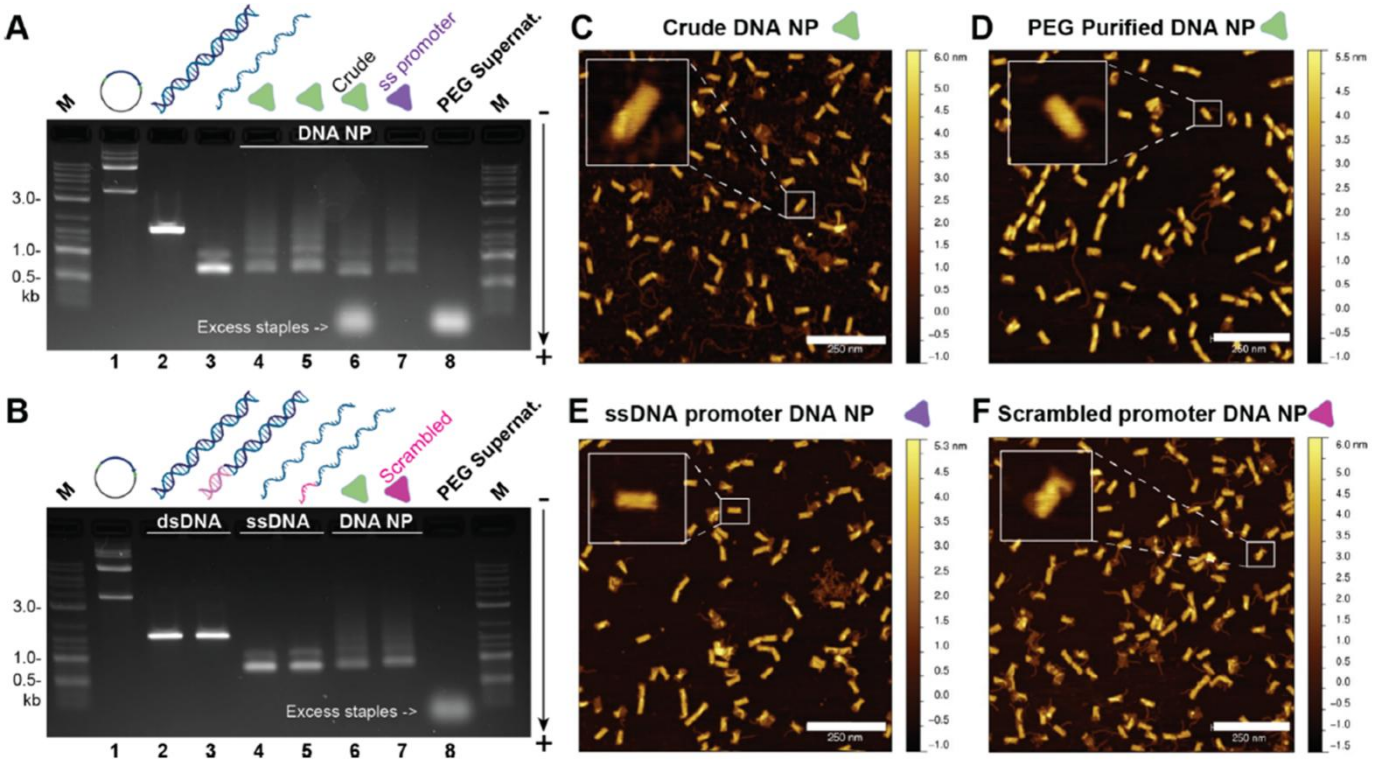

Figure 3.6. Characterization of the DNA NPs with different promoter variations. (A, B) 1% w/v agarose electrophoresis gels in 0.5× TAE 8 mM MgCl2 buffer. Gel contents comprised by the following lanes: (A) 1: pET-22 Luc9 plasmid; 2: dsDNA Luc9; 3: ssDNA Luc9; 4 and 5: PEGpurified Luc9 DNA NP; 6: crude Luc9 DNA NP; 7: PEG-purified Luc9 DNA NP with ssDNA promoter; 8: supernatant from PEG purification of DNA NP. Lanes 4 and 5 are experimental replicates of the PEG-purified original DNA NP. Lanes in (B) 1: pET-22 Luc9 plasmid; 2: dsDNA Luc9; 3: dsDNA Luc9 with scrambled promoter; 4: ssDNA Luc9; 5: ssDNA Luc9 with scrambled promoter; 6: PEG-purified Luc9 DNA NP; 7: PEGpurified Luc9 DNA NP with scrambled promoter; 8: PEG supernatant from DNA NP purification. (C−F) AFM micrographs of (C) crude DNA NP, (D) PEG-purified DNA NP, (E) PEG-purified DNA NP with ssDNA promoter, and (F) PEG-purified DNA NP with scrambled promoter. Scale bars in each panel: 250 nm.

Taking into consideration the desire for overall structural simplicity, assembly efficiency, and the need to confirm and characterize formation, a 12-helix DNA bundle with predicted dimensions of 50 × 10 × 10 nm3 was designed as the testbed DNA NP for this study (**Figure 3.1A and Figure 3.3**) [113]. The helix bundle architecture is frequently employed for biomedical applications and was recently shown to also serve well for gene expression of GFP and mCherry [71]. The NP was designed on CaDNAno using the canonical principles of DNA origami,

wherein the long ssDNA scaffold strand is routed in a rasterized format through a honeycomb lattice arrangement of double helices within the NP. The total length of the aPCRgenerated scaffold was 1651 nucleotides long, which was rasterized through the DNA NP to create the 12-helix bundle design. We maximized access to the promoter region for initiation of transcription by placing the promoter domain in the 12-helix bundle DNA NP on one of the extending helical ends (**Figure 3.1B**, green strand). The staple strand complementary to the promoter site on the scaffold was designed to be linear and without any crossover sites; another design feature that was hypothesized to promote transcription of the encoded gene. Previous studies have used a similar design feature [71]. The resultant DNA NP has 44 staples and, as explained above, one crossover-free linear staple strand complementary to the T7 promoter region on the scaffold– termed the "promoter staple" or staple C02 in **Table AS1**.

In addition to formulating a new approach to measuring gene-encoded DNA NP protein expression in this study, we wished to characterize changes in protein expression when the promoter sequence within the DNA NP is altered. Specifically, how does protein expression change when the promoter region is a single-stranded domain (or ss promoter) and when the conserved T7 sequence (5′-CTATAGTGAGTCGTATTA-3′ template strand sequence) is replaced with an arbitrary sequence (termed a "scrambled promoter"). **Figure 3.1B** shows the two variations relative to the DNA NP containing a dsDNA T7 promoter sequence. The ss promoter DNA NP variation (represented by the purple triangle icon in all the figures) is missing the C02 staple strand which binds to the T7 promoter region on the scaffold, leaving a single-stranded promoter segment on the scaffold. The "scrambled promoter" DNA NP (represented by the pink triangle icon in all the figures) encodes the same Luc9 gene but has an orthogonally different sequence where the T7 promoter would be. Consequently, the "scrambled promoter"

bearing scaffold strand is different in sequence from the pET22-Luc9 plasmid and was therefore produced using a synthetic "gene-block" template dsDNA commercially sourced. The arbitrary sequence to replace the T7 promoter sequence was chosen to be compatible with the requirements of gene-block synthesis while being orthogonal to the T7 promoter sequence. The ssDNA promoter NP analogue, therefore, was assembled using 43 instead of 44 staples (minus the C02 staple). The scrambled promoter analogue of the DNA NP was prepared using the common 43 staples, but a different scaffold strand and a different staple corresponding to the scrambled promoter sequence. **Figure 3.1** and **Table AS1** summarize the design and sequences required for building the primary and two derivative DNA NPs.

3.3.2 Assembly and initial characterization of the DNA NPs

All the DNA NPs were thermally annealed using published protocols [54, 58, 67, 70, 114]. Briefly, 5-fold excess staple strands were combined with 40 nM scaffold strands in the desired buffer solution (see **section 3.2 Experimental methods**). Thereafter, the DNA NPs were purified via PEG precipitation [122] and resuspended in Tris buffer (as suggested by the TXTL kit directions) supplemented with an optimal amount of MgCl2 for DNA NP stability in tandem to TXTL reaction efficiency [123]. The supernatant from the PEG-based purification of the DNA NPs was reserved for characterization of assembly and function (termed the PEG supernatant in **Figure 3.6**). We prepared a few other control samples; namely, an M13-scaffolded DNA wireframe snub cube NP served as a negative control for protein expression in TXTL [50]. The dsDNA byproduct synthesized in aPCR-based scaffold production (from the plasmid as well as scrambled promoter gene-block) was also tested in TXTL systems. Overall, we tested the original Luc9-encoding, ssDNA promoter, and scrambled promoter variations of the 12-helix bundle DNA NP, alongside an M13-encoding DNA snub cube (**Figure 3.7**), the precursor

scaffold-only, dsDNA Luc9 gene, and the pET22-Luc9 plasmid in subsequent experiments. **Figure 3.4** summarizes all of the samples that were tested as DNA templates in TXTL reactions.

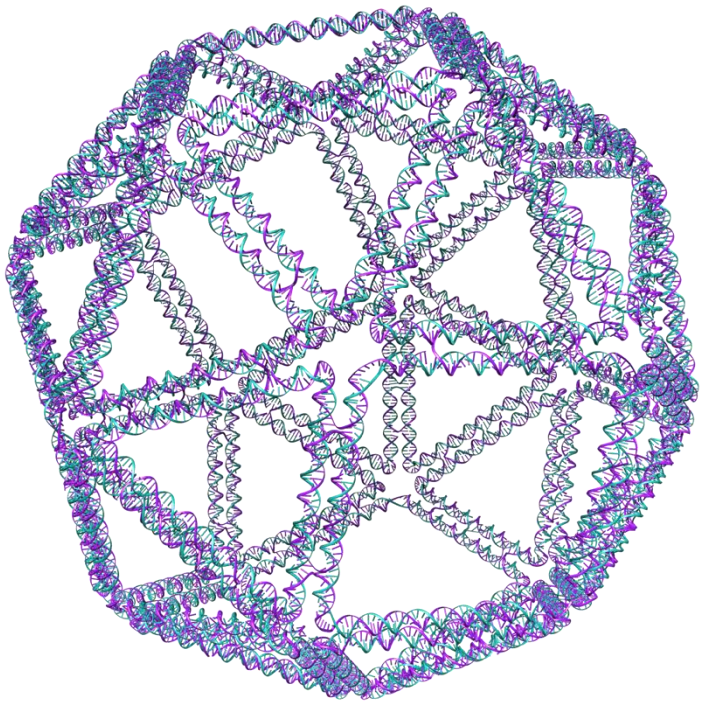

Figure 3.7: Molecular model of the M13-scaffolded DNA snubcube. Predicted diameter is 50 nm.

The three variations of the Luc9-encoded DNA NP, namely, one with the original dsDNA T7 promoter, one with ssDNA T7 promoter, and one with a scrambled promoter, assembled with high efficiency, as characterized with agarose gel electrophoresis and atomic force microscopy (AFM) (**Figure 3.6**). Assembly of the original DNA NP and the ssDNA promoter DNA NP was initially confirmed via agarose gel electrophoresis (**Figure 3.6A**). All of the lanes that contain DNA NP samples (lanes 4−7; **Figure 3.6A**) have a distinct band just under 1.0 kb, which corresponds to around the same location where the ssDNA Luc9 scaffold (lane 3) had migrated. However, the ssDNA scaffold and DNA NP lanes also have higher molecular weight bands present, which most likely correspond to a subspecies of interacting or cross-linked

NP concatemers. Under these conditions, the addition of the staple strands does not appear to significantly change the migration rate of the formed NP vice to that of the ssDNA scaffold. This arises since the 3D DNA NP does not migrate in the gel based solely on the mass-to-charge ratio of a simple dsDNA that is not supercoiled, but because its migration rate is now far more complex due to its origami architecture. More stringent gel methodologies, such as those based on polyacrylamide, are required when separating closely related species of DNA origami [124, 125]. The band for the ssDNA promoter DNA NP (lane 7) migrates in a manner identical to that of the original Luc9 DNA NP as expected, also indicating successful formation. All the PEG-purified versions of the DNA NP (**Figure 3.6A** lanes 4, 5, and 7) have marginally slower migration than the crude DNA NP (lane 6) which could be attributed to the excess Na+ present in the purified DNA NP samples post PEG precipitation. Alterations in band migration due to different salt conditions have been known to occur in DNA NPs. **Figures 3.6C and 3.6D** show representative AFM images collected from samples of the crude and PEG-purified DNA NPs, respectively. A small degree of end-to-end chaining can be observed in the DNA NP (even though the DNA NP is designed to contain ssDNA poly-T extensions and scaffold loops along the helix ends to prevent this; see **Figure 3.3**). However, this was not a cause for concern because the TXTL reactions (explained below) were performed at 37°C, a temperature at which nonspecific chaining is known to be reduced. The presence of excess staple strands in the crude sample manifests as visible background noise (**Figure 3.6C**) compared to purified DNA NP in its three promoter variations (**Figures 3.6D−F**), but these are not distinct due to their small size and the resolution limits of the instrument. The presence of these excess staple strands is also visualized as the lower molecular weight band in lane 6 (crude original DNA NP) and lane 8 (supernatant recovered post PEG-based purification of the DNA NP) on the gel shown in **Figure**

3.6A. Nevertheless, formation of the 12-helix bundle DNA NPs with dimensions reflecting approximately what was expected from the original design criteria serves as confirmation of structural assembly. The AFM image in **Figure 3.6E** also shows that the ssDNA promoter Luc9 DNA NP is identical with that of the parent structure above it.

The scrambled promoter DNA NP was similarly characterized by gel separation, as shown in **Figure 3.6B**. As mentioned above, the scrambled promoter variation of the DNA NP required a separate gene block template to synthesize the scaffold strand (lane 5, **Figure 3.6B**) and dsDNA control (lane 3, **Figure 3.6B**). The new scaffold in lane 5 and the original scaffold in lane 4 (synthesized via aPCR from the pET-22 Luc9 plasmid shown in lane 1) appear to migrate at the same rate, confirming equivalent lengths, despite the difference in sequence at the promoter site. Similarly, following PEG purification, there does not seem to be a difference in the formation and migration rate of the original DNA NP (lane 6) and the scrambled promoter DNA NP (lane 7). Successful formation of the scrambled promoter DNA NP is further validated by the appearance of well-formed 12-helix bundles, as visualized by AFM (**Figure 3.6F**). Overall, this data confirms formation and PEG purification of the desired DNA NPs with each of the different scaffold strands containing correct or scrambled promoter species and with or without a promoter staple. Formation, purification, and characterization of the DNA snubcube control was performed in a similar manner (data not shown).

3.3.3 Measuring Luc9 expression from the DNA NPs in bacterial cell-free TXTL reactions

Some previous studies have reported the degradation of linear DNA templates by endogenous exonucleases, mainly RecBCD, present in cell lysate-based TXTLs. To avoid exonuclease damage to linear DNA templates, here we utilize the PURExpress cell-free extract system, a cell-free TXTL reconstituted from selected purified *E. coli* components and provided

as a set of preoptimized reaction mixes [112]. The comparative protein expression assay that was implemented to evaluate gene expression consisted of adding equal concentrations and volumes of a given DNA transcription substrate (DNA NP or a control DNA) to the TXTL reaction mixture for a fixed amount of time. Following the protein expression incubation, an aliquot was removed from this reaction mixture and added to the NanoGlo luciferase substrate in buffer, and relative bioluminescence was assayed in a Tecan plate reader using the luminescence mode thereafter. Alternatively, in some optimization experiments, the NanoGlo substrate was added directly to the expression reaction. The averaged relative intensities collected over time from these measurements were then compared to each other and used as a metric of gene expression efficiency, as delineated in **Figure 3.8**.

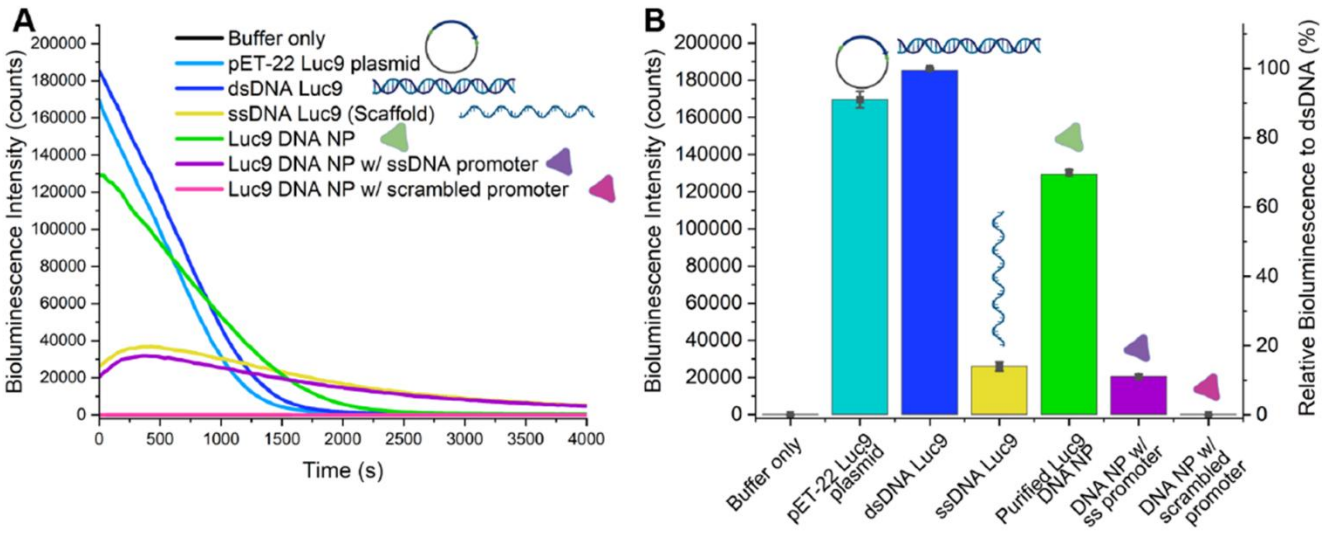

Figure 3.8. Bioluminescence intensity collected from different Luc9 DNA templates in the TXTL system over time. (A) Time-resolved bioluminescence intensity from the three DNA NP variants and the plasmid, dsDNA, and ssDNA templates. (B) Bioluminescence intensity from the same samples at t = 0 s and represented relative to the bioluminescence from dsDNA TXTL (navy colored bar). Error bars represent the standard deviation of the mean from 3 to 6 independently assembled and assayed samples.

Prior to DNA NP-related TXTL experiments, the individual parts of each of the expression and assay components underwent empirical optimization with a focus specifically toward monitoring output from the expression of Luc9 from the pET22-Luc9 plasmid template via enzymatically generated bioluminescence. We first determined whether the luciferase substrate could be added simultaneously to the TXTL reaction to measure bioluminescence as protein expression took place. We observed that adding the Luc9 substrateNanoGlo alongside the DNA plasmid into the TXTL reaction resulted in poor bioluminescence (**Figures 3.9 and 3.10**). Therefore, two coupled components were required that functioned stepwise to evaluate gene expression from the plasmid (and DNA NPs), namely, (i) the cell-free TXTL system where the luciferase protein is first produced following transcription of the encoded scaffold within the DNA NP (or any of the other DNA transcription substrates), and (ii) the luciferase enzymesubstrate reporter system where the expressed enzyme's activity is assayed after 1 h. This coupled format was implemented in contrast to having the luciferase substrate jointly present within the TXTL reactions as that resulted in lower to no bioluminescence signal being generated because the compounds present in the substrate buffer presumably inhibited protein synthesis if added too soon (**Figure 3.9**). We next optimized the concentration of a series of simple DNA transcription substrates or templates (the pET-22 Luc9 plasmid, dsDNA, ssDNA Luc9 genes, and negative controls) to ensure use of a minimum amount of template but with production of a high enough signal of subsequent bioluminescent intensity; see the representative data in **Figures 3.10−3.14**. For the 10 μL total TXTL reaction volume utilized here, the concentration of the DNA template used was optimized to 1.9 nM (**Figure 3.10**). We observed that enough protein was synthesized for sufficient bioluminescence signal when the cell-free TXTL reaction was incubated for 2 h at 37°C prior to adding to the luciferase substrate with 1 h being a working

minimum (**Figure 3.9**). For the substrate reporter system, we optimized the type of substrate used by testing several different luciferase substrate analogues (coelenterazine versus NanoGlo; **Figure 3.11**), the type of aqueous conditions used (PBS, water, mini-prep elution buffer, or vendor-supplied NanoGlo buffer; **Figure 3.12**), and the ratio of substrate concentration relative to buffer (0.5:50 up to 10:50; **Figures 3.13 and 3.14**) in order to obtain the highest signal of bioluminescence intensity. The observed higher luminescence from NanoGlo in comparison to coelenterazine validates what has been shown previously [126]. We observed that the NanoGlo substrate dissolved in 1× PBS and a 0.5:50 substrate:buffer ratio consistently produced the highest signal and used these parameters for all of the subsequent experiments performed.

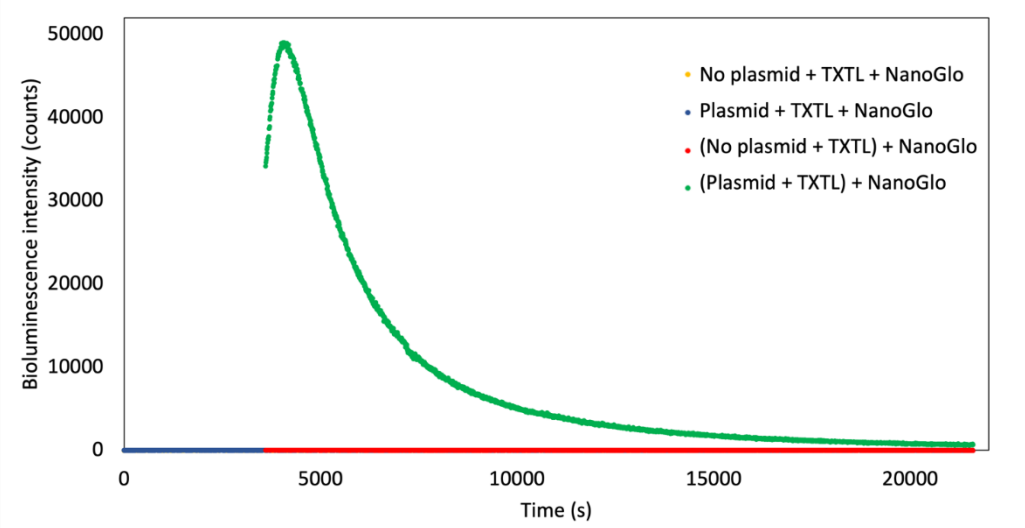

Figure 3.9: Cell-free TXTL reaction versus time. This representative optimization result shows that the reaction needs to be incubated for at least one hour to have enough protein synthesis for sufficient luminescent signal before adding the luciferase substrate NanoGlo. Samples with "no plasmid" indicate that water was added in place of the DNA template (pET22 Luc9 plasmid). Parenthesis, such as (plasmid+TXTL) indicates that the NanoGlo was added after allowing the TXTL reaction to proceed for 1 hour. Plasmid/no plasmid + TXTL + NanoGlo represents the simultaneous addition of substrate without 1 hour incubation. TXTL reaction volume: 25 µL. DNA template concentration in each TXTL reaction: 0.6 nM. Upon adding the substrate (25 µL) the total reaction volume becomes 50 µL. Substrate to buffer volume ratio is 1:50. NanoGlo buffer is used to prepare the substrate solution. Reactions were measured in the plate reader at 37°C for 6 hrs (either 1 h incubation + 5 hr measurement or 0 h incubation + 6 hr measurement).

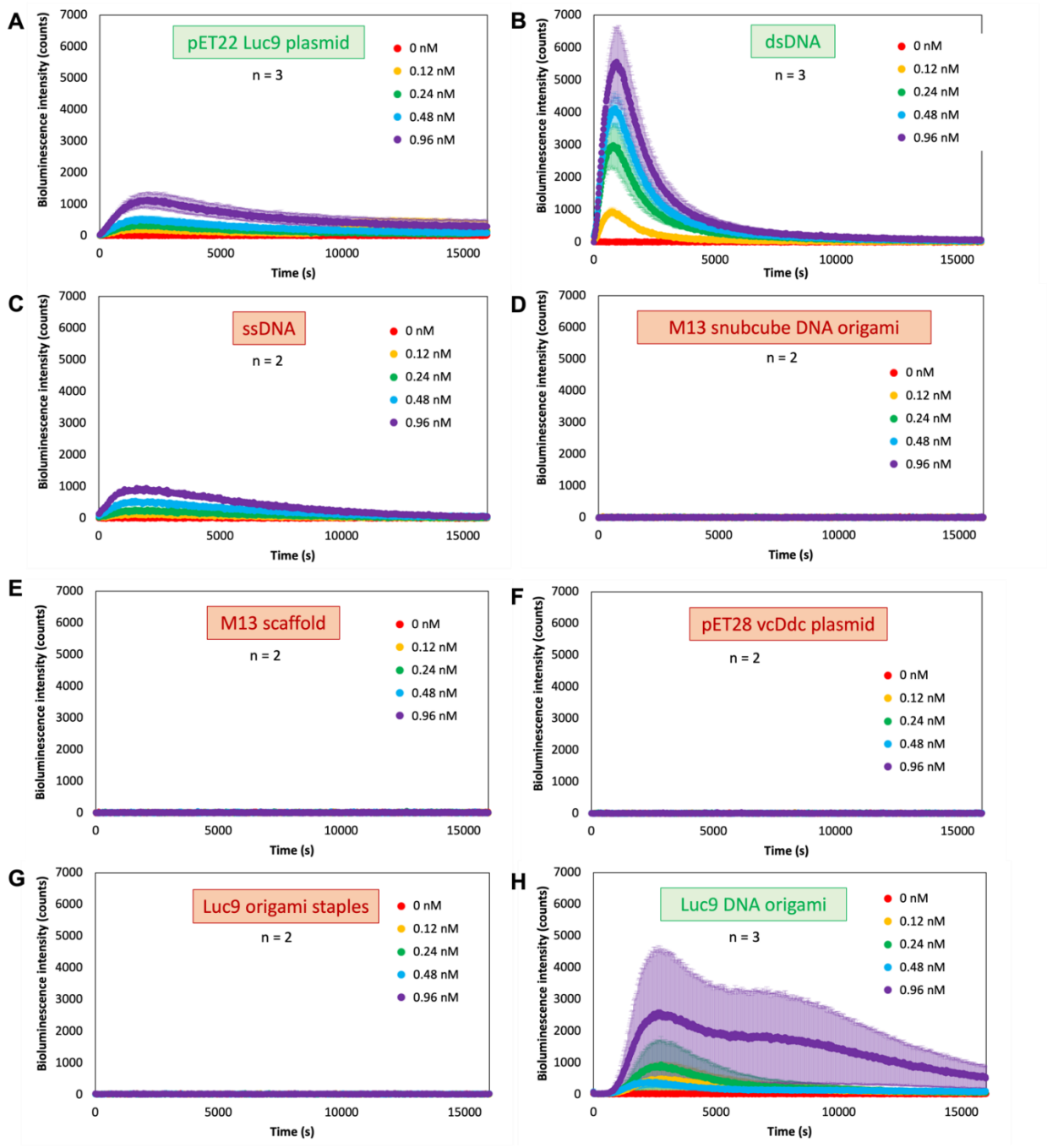

Figure 3.10. Testing of different concentrations of DNA templates in cell-free TXTL. (A) Parent pET22 plasmid encoding Luc9 gene. **(B)** dsDNA aPCR product. **(C)** ssDNA aPCR product which serves as the scaffold for the DNA NP. **(D)** M13 scaffolded DNA snubcube. **€** M13 only. **(F)** pET28 plasmid encoding a diaminobutyrate decarboxylase serving as an initial negative control. **(G)** DNA NP staples only (no scaffold present). **(H)** DNA NP encoding Luc9. Luciferase substrate was added immediately after combining the DNA template and TXTL mix. DNA concentration in TXTL reaction was tuned from 0-1.9 nM. Final DNA template concentration in each reaction 0 – 0.96 nM (since substrate was added immediately). Total reaction volume: 20 µL. Substrate to buffer volume ratio is 0.5:50. Buffer is 1× PBS. Reactions was measured in the plate reader at 37°C for 4 hrs.

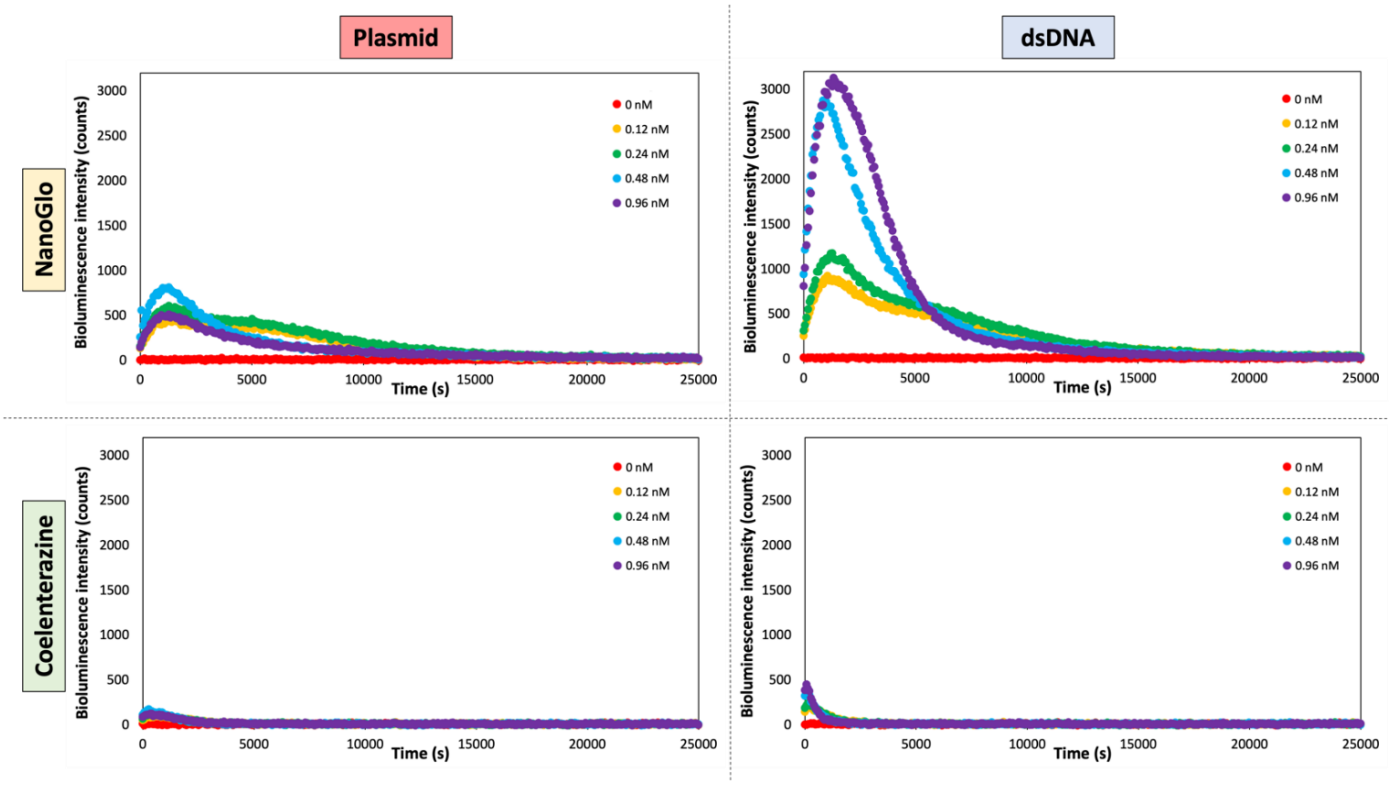

Figure 3.11. Bioluminescence intensity testing with different reporter substrates. This representative result tested two substrate types, namely – NanoGlo and coelenterazine. Two DNA template types were used for protein luciferase synthesis – the parent pET22 Luc9 plasmid and the aPCR-amplified dsDNA product. TXTL reaction volume: 10 µL. No pre-incubation was performed prior to adding the substrate in these experiments. After adding 10 µL of substrate solution, the total reaction volume: 20 µL. DNA template concentration in each reaction: 0 – 0.96 nM (since substrate was added immediately). Substrate to buffer volume ratio is 0.5:50. Buffer is 1× PBS. Reactions were measured in the plate reader at 37°C for 7 hrs.

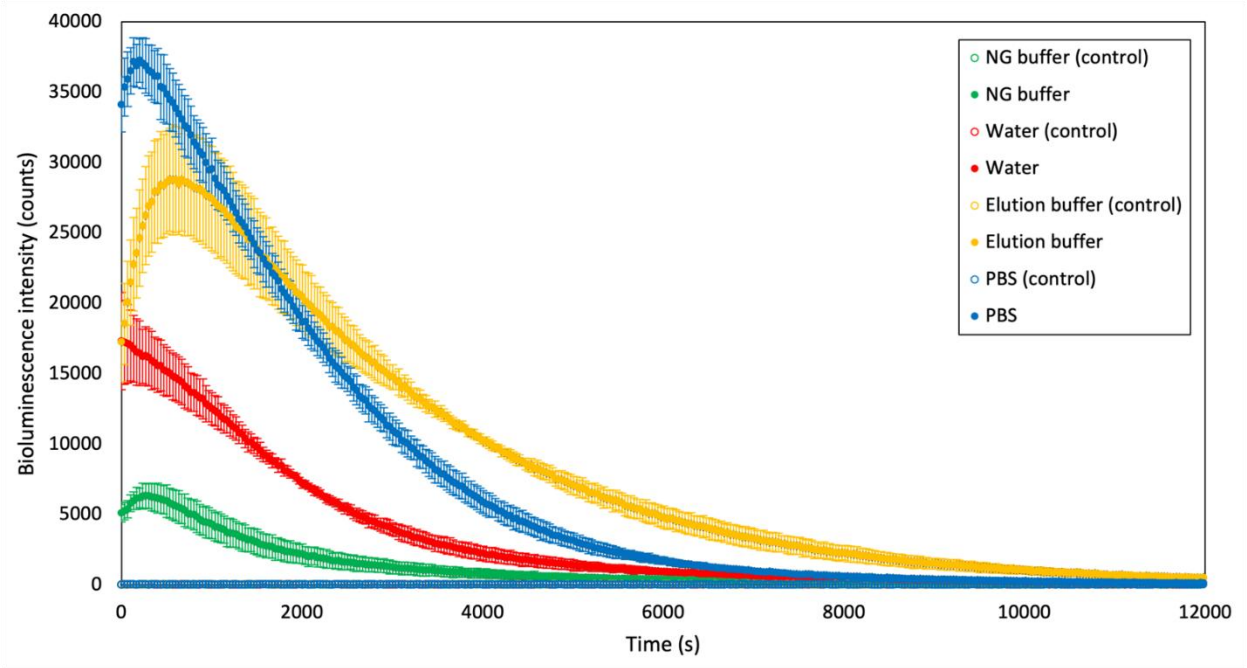

Figure 3.12. Testing bioluminescence intensity with different substrate buffers. TXTL reactions with different types of buffer solutions at 1:50 substrate to buffer ratio. We learned that NanoGlo solution could alter TXTL performance, and it was important to identify the optimum aqueous environment to prepare the substrate solution. The parent pET22 Luc9 plasmid was combined with TXTL at 37°C for 1 hr. After incubation, NanoGlo (NG) was added. Different types of buffer were tested to prepare the NanoGlo solution. Error bars represent the standard deviation of the mean for each sample (n=3). A negative control representing no DNA plasmid was tested and plotted in each case. The buffer provided with the NanoGlo substrate is in indicated in green. TXTL reaction volume: 10 µL. DNA template concentration in each TXTL reaction: 0.6 nM. After adding 10 µL substrate solution, the total reaction volume: 20 µL. Reactions were measured in the plate reader at 37°C for 5 hrs.

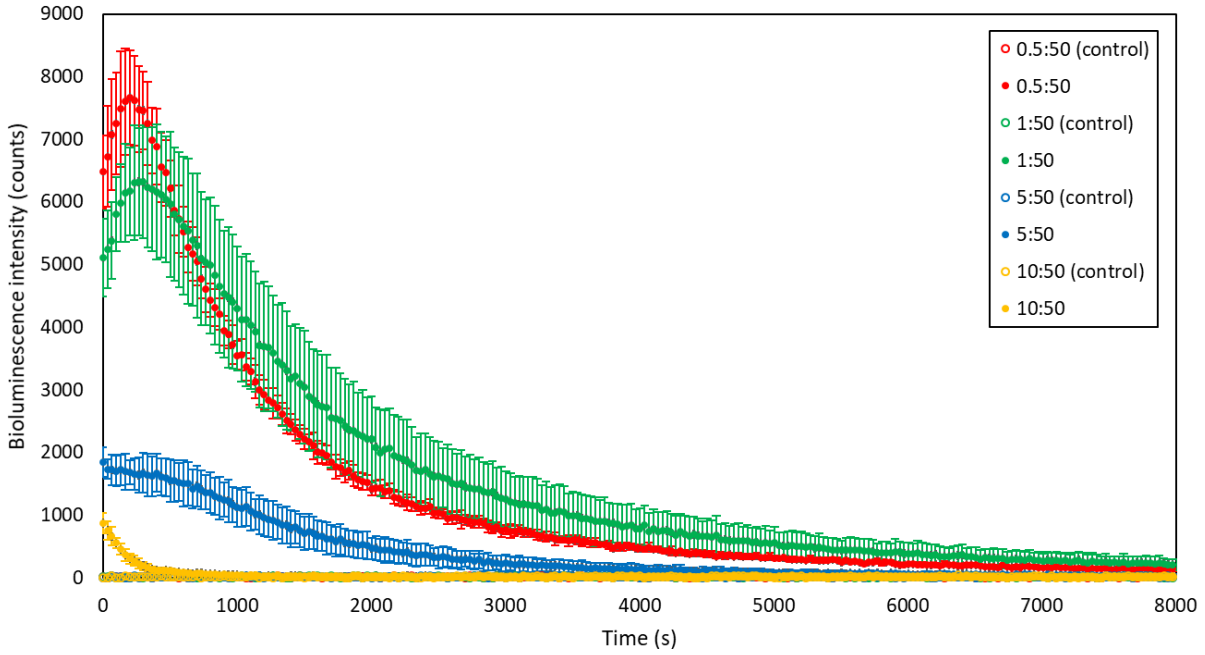

Figure 3.13. Optimizing the NanoGlo substrate to buffer ratio for the TXTL reaction. We wanted to test the extent to which substrate amount in the reaction could be tuned. The parent pET22 Luc9 plasmid was combined with the TXTL solution and incubated at 37°C for 1 hr. After incubation, NanoGlo was added (in vendor-supplied buffer). Error bars represent the standard deviation of the mean for each sample (n=3). A negative control with no DNA plasmid was tested for each variable. The TXTL reaction kit suggested substrate to buffer ratio at 1:50 (indicated in green). NanoGlo solution was prepared using vendor supplied buffer. TXTL reaction volume: 10 µL. DNA template concentration in each TXTL reaction: 0.6 nM. After adding 10 µL substrate the total reaction volume: 20 µL. Substrate to buffer volume ratio is 1:50. Buffer is NanoGlo buffer. Reactions were measured in the plate reader at 37°C for 5 hrs.

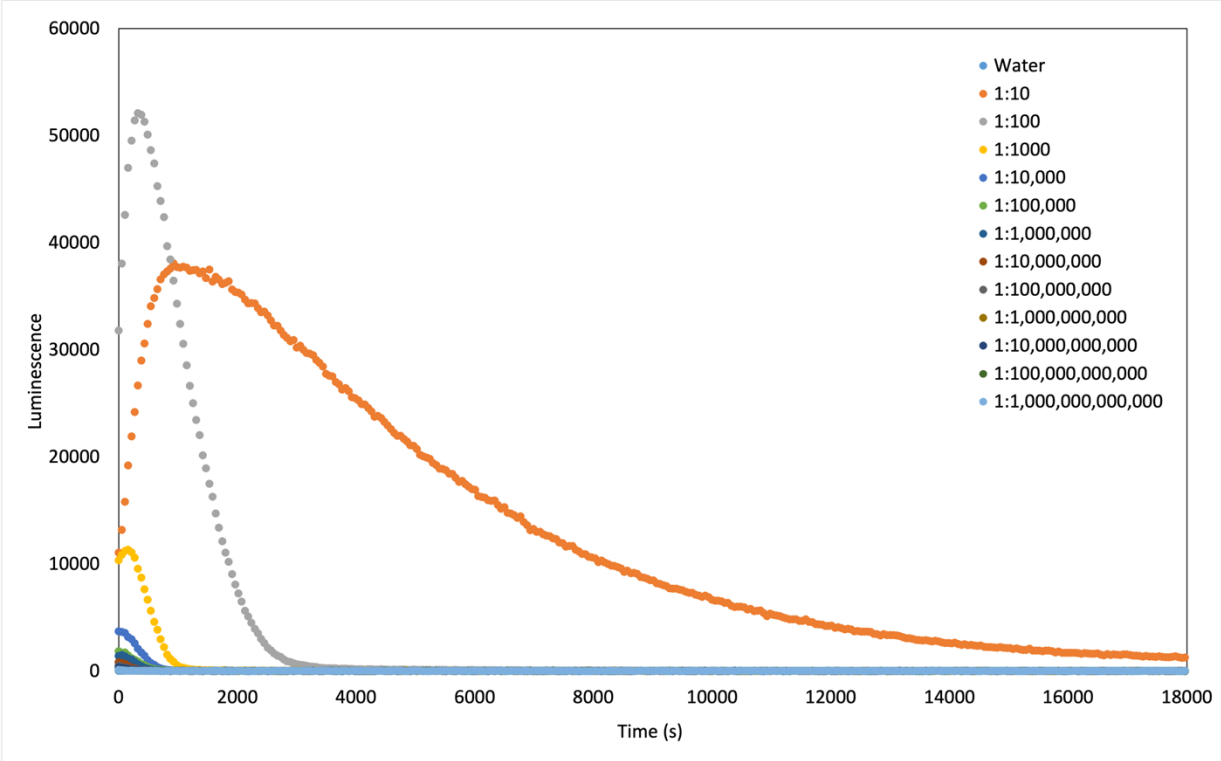

Figure 3.14. Testing luminescence at further diluted substrate concentrations. Following up on results shown in Figure S9, we wanted to test if more dilute substrate solutions (represented as substrate:buffer ratio in the legend) altered bioluminescence intensity. Parent pET22 Luc9 plasmid was combined with TXTL reaction and incubated at 37°C for 1 hr. After incubation, NanoGlo substrate was added at different ratios to 1× PBS. A negative control with no NanoGlo substrate (water only) was tested. No negative control for the DNA plasmid. No positive control for the kit recommended substrate:buffer ratio (which would be 1:50 NanoGlo buffer). TXTL reaction volume: 10 µL. DNA template concentration in each TXTL reaction: 0.6 nM. Ater adding 10 µL of each of the diluted substrate solutions to the TXTL solution, the total reaction volume: 20 µL. Buffer is 1× PBS. Reactions were measured in the plate reader at 37°C for 5 hrs.

As described above, we were interested in testing protein expression from the Luc9-encoding DNA NPs while we were focused on evaluating the effect of promoter variations within the DNA NP on the overall protein expression. To that end, the three DNA NP variations were compared, namely, the original dsDNA T7 promoter encoding Luc9 DNA NP, a DNA NP with a ssDNA promoter (missing the C02 staple), and a DNA NP with a scrambled sequence in place of the T7 promoter (synthesized from a different gene-block template). These choices were made to provide information on how the design of a DNA NP's starting promoter sequence

might influence transcription initiation in a cell-free TXTL reaction. Also evaluated in this format were a plethora of controls that included buffer only, M13 scaffold, the original pET-22 Luc9 source plasmid, aPCR-amplified dsDNA and ssDNA Luc9 products (of which the ssDNA product serves as scaffold for the DNA NP), crude and PEG purified NPs and corresponding supernatant, and a M13-scaffolded DNA origami snub cube. All nucleic acids (or DNA precursors) were added to the TXTL mixture at the same overall molar concentration (1.9 nM). The DNA precursors with TXTL reaction mix were allowed to incubate at 37°C for 2 h to allow for sufficient *in vitro* protein expression. Thereafter, an aliquot was added to luciferase substrate NanoGlo (see **section 3.2 Experimental methods**), and the samples were immediately moved to a Tecan plate reader for luminescence measurements over the next 5 h at 37°C. The DNA template concentration after the addition of luciferase substrate was estimated to be 0.96 nM.

Luciferase expression was successfully achieved from the TXTL reaction of gene-encoded DNA NP. As reflected in the representative data of **Figure 3.8A**, the maximum luminescence is collected at t = 0 for most samples, with luminescence decreasing over time as a function of substrate consumption. Fortuitously, for some of the reactions that did not display maximum luminescence, an initial rise to a peak around 300−500 s is seen followed by a steady decrease. This indicates that for those reactions where small amounts of luciferase were produced during the 2 h preincubation, additional time was required for the enzyme to reach an apparent steady state of turnover or light production, i.e., V_{MAX}. This also reflected that the ratio of substrate to luciferase present was appropriate for this type of experimental format. High amounts of enzyme present did not consume all the substrate too rapidly and potentially cause the assay signal to be missed in the time required to set up the experiment and start collecting data. Lastly, since the Luc9 gene in dsDNA form (that was recovered along with the ssDNA

scaffold from aPCR) consistently yielded the highest initial luminescence in experiments as expected (navy curve in **Figure 3.8A**), it was used as a comparator in **Figure 3.8B** with its value normalized to 100% for reporting relative luminescence (see **Table AS3** for luminescence values). **Figure 3.15** represents the bioluminescence intensity for a number of control experiments performed to support results shown in **Figure 3.8**.

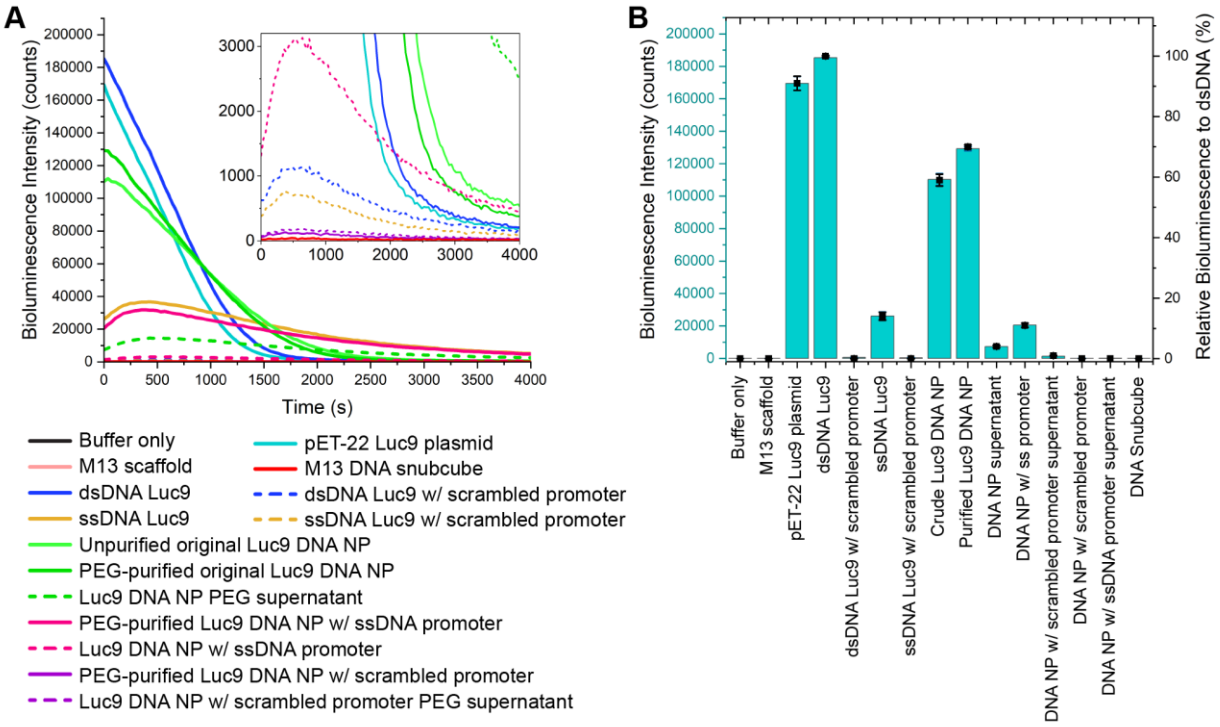

Figure 3.15: Complete time-resolved luminescence assay of Luc9 DNA NPs with control samples. (A) Bioluminescence intensity over time for different controls as well as DNA NP samples shown in Figure 3A. **(B)** The maximum (or peak) luminescence was measured at t = 0 s. Each sample luminescence was represented as a fraction % of the luminescence of the dsDNA Luc9 sample. Table S3 lists the luminescence values that were used to calculate the relative intensity (%).

The top 3 most efficient producers of enzymatic luminescence in decreasing order are the dsDNA encoding Luc9 (100%), the source pET-22 Luc9 plasmid (91%), and the PEG-purified original DNA NP (70%). Naturally, the original crude DNA NP without PEG purification also demonstrated high luminescence albeit lower than the purified version (59%; plotted in **Figure 3.15**). This is likely because the excess staple strands in the crude version affect transcription

processivity and delay activity of the TXTL. The pET-22 plasmid and the dsDNA Luc9 both express with somewhat similar efficiency, although the dsDNA consistently yielded the highest luminescent signal. DNA NP (crude and purified) samples were less efficient than the dsDNA Luc9 template, perhaps due to the complex routing of the gene through the DNA NP architecture, which might take more time to parse during the transcription process, but this data clearly indicates that it is possible to express gene-encoded DNA NP templates in cell-free TXTLs with good efficiency.

The ssDNA promoter variant of the DNA NP also demonstrated luminescence (11%), which was comparable to that of the ssDNA Luc9 (scaffold only; 14%) control (purple and yellow samples, respectively, in **Figure 3.8**). Surprisingly, this shows that the ssDNA Luc9 template also expresses in the cellfree TXTL albeit at a low level, even though it is ssDNA in form. This is perhaps due to the closer proximity and high concentration of biomolecules for transcription and translation present in the system, which make it possible for the system to engage in some low baseline initial transcription activity even though the scaffold is not in dsDNA form. That the ssDNA promoter containing Luc9 DNA NP expresses similarly to the ssDNA Luc9 template is unsurprising because they both have promoters in ssDNA form. The low luminescence of the ssDNA promoter Luc9 DNA NP confirms that the protein expression being observed from the purified original Luc9 DNA NP is due to well-formed DNA NPs and not because of any residual ssDNA or dsDNA scaffold that could be present in that sample. These results are followed by the ssDNA Luc9 scaffold itself (14%), the PEG-purified ssDNA promoter Luc9 DNA NP (11%), and the PEG supernatant from the Luc9 DNA NP purification (4%). We speculate that the small amount of activity for this last sample is ascribed to some

DNA NP or partial-to-fully ssDNA Luc9 scaffold that remained unprecipitated and/or was not removed during purification.

The DNA NP variant with a scrambled promoter sequence showed <0.5% expression, which served to validate the correct formation of the T7 promoter-bearing DNA NP discussed above. Surprisingly, the dsDNA and ssDNA templates with the scrambled promoter showed nonzero albeit trivial levels of luminescence as compared to the T7 dsDNA template (**Figure 3.15**). Also, all three samples are close to 0% relative activity at the start of the measurement, confirming the minimal expression levels. This result also serves to again confirm the key role that the dsDNA gene promoter sequence plays in these systems. Lastly, negative controls such as the buffer-only sample (containing no DNA template), the M13 scaffold (nonspecific template), and the M13-scaffolded DNA snub cube (nonspecific DNA NP template) showed 0% expression.

3.4 Discussion and conclusions

In this preliminary proof-of-concept report, we demonstrate that an appropriately assembled gene-encoded DNA origami NP can be expressed in bacterially derived TXTL systems. For this, we appropriate the gene for an optimized Luc9 luciferase enzyme, which allows us to indirectly assay subsequent protein expression and compare relative levels using a simple bioluminescence-based assay on a commercial plate reader, the latter of which could also enable high throughput assays. This format was utilized as visualizing the small amount of protein expressed in the reaction against a background of many other proteins was not readily achievable or quantifiable. An optical response from enzymatic bioluminescence provides time-resolved data that can often elude RNA transcription assays alone [108]. We further probe the system to confirm some initial assumptions made about the nature of the promoter sequence and

the critical role that it plays toward efficient expression of the gene it is appended to despite the latter being folded into a programmable origami configuration; namely, we seek to confirm that the promoter sequence is required and that it must be present in a dsDNA configuration regardless of how the DNA is presented to the reaction system.

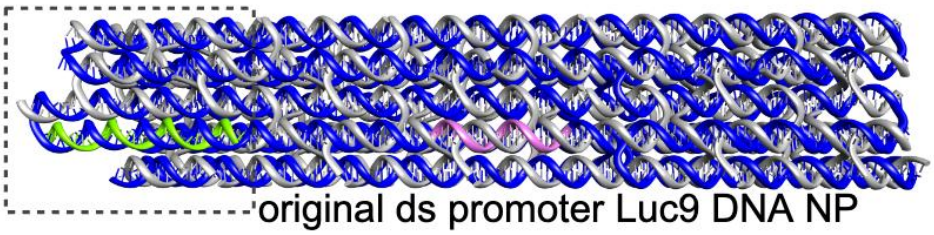

Figure 3.16: Schematic of the DNA NP showing the promoter staple in green and the ribosome binding site (RBS) in pink on the scaffold strand.

We began by routing the T7 promoter sequence on the outer section of the 12-helix bundle at a slight overhang so that it could be clearly recognized and bound by the transcription machinery present in the reaction during transcription. The ribosome binding site (RBS)—relevant later during the mRNA translation stage—is also on the outer face of the DNA NP, though its role may not have any direct impact during transcription (**Figure 3.16**). As expected, the most efficient gene expression was demonstrated from constructs displaying the T7 promoter in a dsDNA configuration regardless of whether the gene was provided simply as the plasmid, the linear dsDNA product from the aPCR reactions, or the fully folded DNA NP. More interesting was the fact that the ssDNA scaffold along with the DNA NP displaying the ssDNA promoter sequence assayed with around 10−15% of the relative efficiency of the dsDNA comparator. Although somewhat rare in biology, promoter sequences that function in the ssDNA configuration have been described [127]. However, the general consensus is that most promoter sequences, and especially those in prokaryotes, are meant to function in a dsDNA configuration [11]. In this case, we ascribe this result to more of a “leakage” type of a phenomena, which is

facilitated by the fact that the reaction mixture contains large concentrations of primarily just the transcription and translation machinery and, moreover, has been extensively optimized to undertake these specific types of reactions. That the samples with the scrambled promoter sequence display <1% of the activity of those with the correct promoter supports this. Clearly some promoter recognition and transcription initiation are being undertaken with the scaffold in the ssDNA conformation, and this is significantly reduced when the promoter sequence is scrambled beyond recognition. Moreover, the low luminescent output of the ssDNA promoter DNA NP also suggests a preference of the RNAP for the template strand to the coding strand of the gene-encoded precursor plasmid. The low level of expression from ssDNA constructs need not be deemed a liability as it may represent another way to control gene expression levels in these type of systems beyond just the gross DNA NP concentration added to the reaction mix. It is also interesting to speculate whether addition of just the cognate C02 staple strand in excess to a TXTL reaction with a ssDNA promoter DNA NP already present in it could rapidly upregulate its protein expression. Here, the added staple strand could perhaps form the promoter into the correct dsDNA configuration in situ in the reaction in real time as a form of an instant ON switch.

Looking forward, we foresee the use of TXTL reactions and gene encoding DNA NPs in this manner as a mechanism to initially confirm the ability of a construct to engage in gene expression and also perhaps test different structural design variations and how they may affect the protein expression efficiency. Several parameters are yet to be tested and optimized, such as accessibility to important sequences during transcription, compactness, stability, and density of crossover sites within the DNA NP. In this study, the location of the promoter was chosen to be present at a marginally extending helix end within the bundle with the intent to minimize steric

hindrance for enzyme binding during transcription. The helix bundle architecture rather than a wireframe polyhedral shape was motivated by the fact that wireframe structure have shown to sterically hinder nuclease attack [128], possibly making transcription enzyme binding more challenging, too. By strategically routing the gene-encoded scaffold strand and leveraging staple strands for installing a Förster resonance energy transfer reporter system on the DNA NP [123], one could apply luminescence and fluorescence in tandem to learn the nuances of DNA NP stability and functionality. In fact, measuring protein expression efficiency from a DNA NP could serve as a much-needed analytical tool to determine nucleotide-level structural integrity of DNA NPs under physicochemical stressors [125]. Use of DNA binding protein fusions may also allow the NPs to bring proteins attached to it into the TXTL reaction or eventually even a cell in a piggyback-like manner to complement any requirements not present or to focus activity to just a given target [129]. In a manner mechanistically similar to what has been shown previously, the addition of hard NP scaffolds to the TXTL reactions such that they cross-link the proteins present into dense nanoclusters could perhaps help speed up both the reaction rate and its efficiency by allowing the enzymatic components to also engage in enzymatic channeling processes [112, 130, 131].

Of course, this approach is not limited to bacterial TXTL reactions and should be similarly applicable to eukaryotic TXTL systems although appropriate codon biasing and optimization requirements will have to implemented [132-134]. One could optimize protein expression from gene-encoded DNA NPs to inform *in vivo* gene therapy experimentation by including other stressors in the TXTL system, such as degrading enzymes, serum, and digestive cell organelles. Even though the protein expression levels from the gene-encoded DNA NP were lower than the dsDNA gene segment, DNA NPs have proven value in their superior transfection

efficiency, targeting, and stability compared to linear duplexes for therapeutic applications in general [135, 136]. In light of current limitations of gene delivery from viral and lipid NP formulations, namely, gene-length loading size cutoff, stringent cold-chain storage requirements, and toxicity [137, 138], the field anticipates that DNA NP-based gene therapies could offer potentially higher loading, targeting, and modular capabilities [65]. DNA NPs have higher resistance to degradation compared to plasmids, and strategies such as ligating the ends can enhance stability further [128]. The success of several DNA NP-based vaccine therapies where the NP architecture is leveraged to spatially organize epitopes is highly encouraging [139]; the scaffold strand used in these DNA NPs so far is bacteriophage-based, but it can be programmed for protein expression to add a gene therapy functionality as well. Naturally, such application of gene-encoded DNA NPs would require concomitant ways to produce the ss custom scaffold strand in desirable quantities. While producing custom plasmids in large quantities is an established process, scaling up of the synthesis of gene-encoded DNA NPs is rapidly advancing as well due to leading efforts to formulate enzymatic and phage-based techniques of producing custom scaffold strands [49, 140]. Traditionally gel extraction is used to acquire purified ssDNA for custom scaffold strands, but new analytical techniques are emerging that reduce material loss and improve DNA NP production efficiency [141]. And the assembly process of DNA NPs lends itself directly to scale up in that the reactions can be done in a massively parallel format. Our approach will certainly help with potential future downstream applications of these DNA NP materials, whether they are for gene delivery and designer therapies or even for point of care protein production of diagnostics and active biotherapeutic/drug molecules [142].

Chapter 4: Peptide Coacervates can Protect Sequestered Oligonucleotides from Nucleases and Release them for Transcription and Translation

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4.1 Introduction

Coacervates, also known as membraneless organelles/compartments or biomolecular condensates, are liquid-liquid phase separations where the coacervate phase is highly concentrated in biomolecules as compared to the surrounding continuous aqueous phase. Biomolecular condensates (the most common term when they are naturally occurring within cells) gained initial interest due to their observed role in cellular process regulations, such as chromatin packing [143]. It is also postulated that they support catalytic cascades by transiently bringing substrate and enzymes together in highly packed environments [144, 145]. The recent focus on coacervates (the more common term when seen from a materials perspective) arises from our capability to design *de novo* coacervates from biopolymers and exploit them for their unique properties [146]. For example, the self-assembly and membraneless nature of the coacervates make them optimal cell delivery vehicles [147]. Coacervates can be grouped depending on how they are formed, the principal groups being simple coacervates (composed of a single type of polymer) and associative coacervates (composed of oppositely charged macromolecules) [146].

Being composed of biopolymers and lacking highly apolar membranes suggests that coacervates might be a biocompatible cellular delivery mechanism [79, 148]. Work from the Keating groups demonstrated that coacervates, particularly associative coacervates, are very efficient at sequestering oligonucleotides within the phase separated domain [144, 149]. They also showed that while short-range secondary structure is maintained, longer range structure is often lost [150]. Our own work demonstrated that this sequestration within coacervates can be exploited to enhance nucleotide biosensing [151]. Building further, the Miserez lab recently showed how tailoring the peptide design can modify the delivery capability [152]. Importantly, in their work they showed that coacervate delivery of oligonucleotides (i.e. siRNA, CRISPR/Cas) to cells outperformed commercial strategies [153].

One important aspect that requires further investigation is: what is the accessibility of the nucleotides within the coacervates? Within cells it appears that coacervates, through their colocalization of enzymes and nucleotides, are important in transcribing and translating nucleotides [154]; suggesting that enzymes can bind nucleotides within coacervates. The question arises: if this is true for transcriptases, would this also hold for nucleases? Free oligonucleotides can be degraded by nucleases both within the cytosol as well as in the extracellular environment [155], but would coacervates provide a protection mechanism. Particularly, if considering coacervates as delivery vehicles for cells, it would be useful to know whether nucleotides might be degraded before delivery. It is not sufficient to merely sequester the nucleotides, it would be important to understand if the nucleotides, e.g. a gene or siRNA, could be released and retain its intended functionality.

Here we study associative and simple phase separated coacervates to understand their differing capability to sequester, protect, and then subsequently release nucleotides so that

enzymes (e.g. transcriptases) could interact efficiently with them. We initially studied them in vitro utilizing dye-labeled DNA Förster resonance energy transfer (FRET) based reporters to detect degradation of the DNA through exposure to nucleases [156]. We observed that within our associative model (polyhistidine peptides and ATP) the DNA was protected until we caused the coacervate to dissociate with an external stimuli resulting in DNA release and cleavage (**Figure 4.1**). In our simple coacervate (based on a repeat HGLGY sequence peptide that has been previously reported) [157] the DNA could be protected, though to a much lesser extent than in the associative coacervates. The general stability of the HGLGY coacervate also did not allow for external stimuli responsive results. With this initial evidence, we proceeded to expand the work with the use of a luciferase gene and a cell-free transcription-translation (TXTL) model system to demonstrate that a functional gene can be sequestered into an associative coacervate, protected from enzymatic degradation, then released to create the desired protein product.

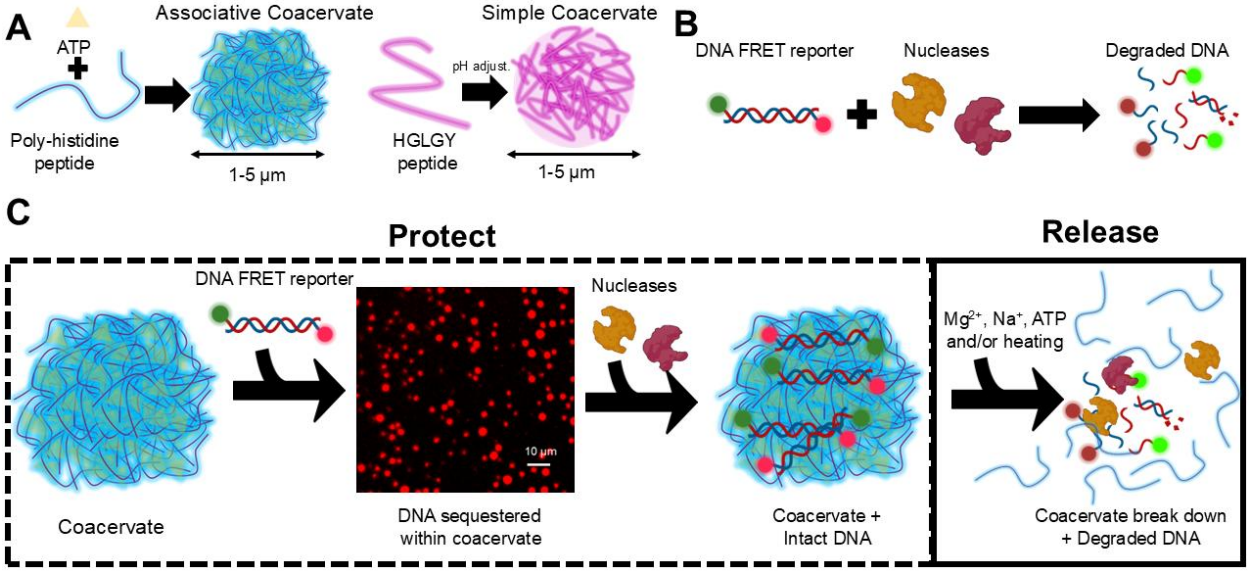

Figure 4.1. Schematic of in vitro coacervate capability to protect and release oligonucleotides. A) Formation of associative and simple coacervates. B) DNA stability assay based on FRET. C) Coacervates capable of sequestering DNA and protecting them from nuclease degredation. Upon perturbation of the coacervate through an external stimuli, i.e. change in ionic strength, or temperature increase, the DNA is released and can be acted upon by the enzymes. Sections of the figure created in https://BioRender.com.

4.2 Materials and methods

Peptides (trifluoroacetic acid removed by commercial vendor) and DNA were purchased from Bio-Synthesis, Inc. and IDT, respectively. ATP (CAS: 34369-07-8) and salts for buffers purchased from Sigma Aldrich and used as is.

4.2.1 Sample preparation

Associative coacervates were formed by creating stock solutions of H_9, H_{12}, ATP, and the required DNA in H_2O, along with the buffer solution (10 mM TRIS, 15 mM KCl, 0.5 mM $MgCl_2$, pH 7.4). The H_x was added to the buffer along with the DNA in an Eppendorf tube and then ATP was added subsequently and mixed through pipetting. Simple coacervates were formed by creating stock solutions of the HGLGY at 10 mM in 10 mM acetic acid buffer (pH 3.2) the required DNA was then added to the peptide and the solution was titrated with NaOH 0.1 M until a pH of 7.0 ± 0.2 was achieved.

4.2.2 Fluorescence spectra

Spectra were measured at 20 °C using a TECAN Spark plate reader exciting from above on a 384-microwell plate. An excitation wavelength of 520 nm was used to excite the sample and the fluorescence emission was measured from 540–725 nm with 5 nm steps when collecting the entire spectra, or the Cy3 and Cy5 peaks were followed at 570 and 670 nm, respectively.

4.2.3 Nucleotide protection experiments

The solution containing either buffer or coacervate along with the DNA was added to a fluorescence 384-microwell plate and the samples were allowed to stabilize at 25°C and baseline Cy5/Cy3 fluorescence ratio was obtained. Time traces were then obtained with excitation at 520 nm and the fluorescence emission measured from 540–720 nm with 5 nm steps when collecting the entire spectra, or the Cy3 and Cy5 peaks were followed at 570 and 670 nm. Using

a multipipetter the respective, water/buffer/nuclease was added to each well and the fluorescence time trace was restarted immediately after addition.

4.2.4 Nucleotide release experiments.

The experiment was realized as stated above; once it was confirmed that the coacervate was stable and that the buffer sample was being degraded (anywhere between 500 and 3000 s) then the perturbation was applied. If the perturbation included increase in ionic strength then a multipipetter was utilized to add the perturbation to all wells simultaneously. If increased temperature was the perturbation then the heating function of the TECAN Spark was utilized. The time trace were restarted immediately after addition.

4.2.5 Microscopy

Imaging was performed using a Nikon A1 HD25 with a ×60 objective lens (oil immersion). Samples (10 μL) were imaged on polyethylene functionalized glass slide-coverslips [151]. Functionalized slides were used for fluorescence imaging as it allows the condensates to adopt more spherical shapes. 532 nm excitation laser source was used with emission determined either through band-pass filters (Cy3 or Cy5 emission) or 10 nm windows for spectral determinations.

4.2.6 Cell-free TXTL protein expression reaction

Expression of luciferase from the different Luc9 DNA templates was performed by using the commercially available PURExpress cell-free protein expression system. TXTL kit components were mixed according to the manufacturer-suggested ratios, but the total reaction volume was scaled down to 10 μL instead of the suggested 25 μL. In a 10 μL reaction, 1.9 nM DNA template (1.2 μL of DNA template at 16 nM was added), 4 μL of solution A, 2.8 μL of

solution B, and 2 μL of water were added. The reaction was incubated at 37°C for 2 h and then transferred to the plate reader for measurements.

4.2.7 Bioluminescence and analysis

Following protein expression, 10 μL of each reaction was pipetted into a black 384-well plate, and then 10 μL of NanoGlo Luciferase substrate in 1× PBS (1:100) was added to each well. The plate was sealed with MicroAmp optical adhesive film and placed into a Tecan Spark multimode plate reader for bioluminescence measurements in a manner similar to that previously described. Measurements were collected at 460 nm wavelength (corresponding to the peak emission for the NanoGlo Furimazine substrate) for up to 5–6 h at 37 °C with an integration time of 1000 ms for each sample. The intensity counts are appropriate for a comparative study between different protein expression samples.

4.3 Results

The peptide systems utilized to create the coacervates studied in this work were either based on the associative model of a positively charged peptide (a poly-histidine peptide of varying length, H_x) along with a negatively charged voluminous counterion for which we chose ATP [158]. Or the second alternative of a simple coacervate based on proteins with prion-like domains [159] on which previous work has been published [157]. The complete sequences of the peptides are available in **Table 4.1**.

Name	Sequence[a]	Mw [g/mol]	pI
H_9	(NH_2)WHHHHHHHHH(COOH)	1438.5	8.2
H_{12}	(NH_2)WHHHHHHHHHHHH(COOH)	1849.9	8.2
HGLGY	(NH_2)HGLGYGLGHGLGYGLGHGLGYG(COOH)	2112.3	7.7

a) A tryptophan (W) was added to the poly-histidine sequences to allow for simple spectral quantification.

Table 4.1. Coacervate peptide properties.

We note that the experimental results of the H_9 and H_{12} peptides are very similar. Therefore, though the precise experimental details are provided in each case, for purposes of the discussion of the results whether the longer or shorter peptide was utilized is not important. In work not presented here, we have observed similar coacervate formation for any polyhistidine with lengths going from 6 to 20 amino acids (we were unable to form coacervates with peptides shorter than 5 amino acids). As will be discussed below, the important distinction arises from the associative and simple coacervate distinction. The detailed protocols for coacervate creation are available in **section 4.2 Materials and methods**. We note that results are based on multiple experimental repeats with controls run with each experiment; the presented figures are representative results.

4.3.1 Nuclease Protection

As an initial test of the inherent capability of coacervates to protect nucleotides, a FRET based reporter was developed from dye-labeled complementary single stranded (ss)DNAs, referred to as EcoCy3 and EcoCy5. When the reporter forms a double stranded (ds)DNA, it presents a Cy3 donor fluorophore and a Cy5 acceptor fluorophore within 5.5 nm of each other which is well within the Förster distance of the dye pair (5.5 ± 0.3 nm in water, 6.8 ± 0.3 nm in the associative coacervates, and 4.8 ± 0.4 in the simple coacervates) [151]. We also utilized a dual labeled ssDNA, again using Cy3 and Cy5 as the FRET pair separated by 9 nucleotides, to allow for testing of further nucleases that might modify ssDNA but not dsDNA. As has been shown in the literature, DNA will sequester in coacervates efficiently [151] and in **Figure 4.2** we can see the spectra for the stable dsDNA form as well as a cleaved, or separated, form of the DNA reporter. We note that due to differences in fluorescence QY within the coacervate environment, and even the structural difference that DNA undergoes in the particular

surroundings, that the spectra will have unique starting and end points whether they are in buffer or coacervate (**Figure 4.2A**). See **Table 4.2** for DNA sequences and properties.

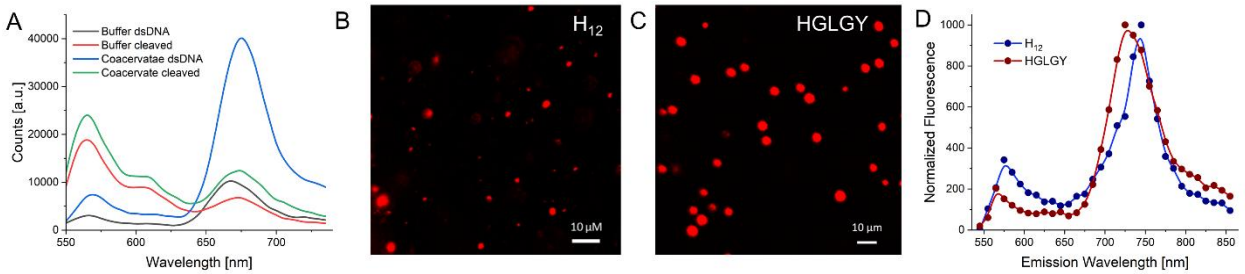

Figure 4.2. Coacervates loaded with DNA. A) Ensemble fluorescence spectra of 250 nM dsDNA reporter in associative coacervate (H_{12}) or in buffer solution. Excitation 530 nm. The dsDNA samples were whole, while the cleaved samples consisted of dsDNA that had been previously cleaved. B) Confocal microscopy image of dsDNA reporter in associative coacervate (H_{12}). 532 nm excitation and 740-750 nm detection. C) Confocal microscopy image of dsDNA reporter in simple coacervate (HGLGY). 532 nm excitation and 730-740 nm detection. D) Spectra of associative coacervate (H_{12}) or simple coacervate (HGLGY) containing the dsDNA FRET reporter after 532 nm excitation and using 10 nm detection bins.

Name	Sequence 5′→ 3′	DNA nucleotide length	Melting temp [°C]
EcoCy3	TCGTT**GAATTC**TTGCT(Cy3)	16	48
EcoCy5	AGCAA**GAATTC**AACGA(Cy5)	16	48
ssDNA FRET	GGATCAGAG(Cy3)TGGACG ACA(Cy5)TGACGTAGGTCC	30	-

The bold sections in EcoCy3 and EcoCy5 are the target sequence for EcoRI nuclease. Melting temperature based on nearest neighbor estimates and taking into account the buffer conditions.

Table 4.2. DNA sequences used for coacervate experiments.

The associative coacervate experiments consisted of creating parallel preparations of the DNA reporter in a buffer (10 mM TRIS, 15 mM KCl, 0.5 mM $MgCl_2$, pH 7.4) where all solutions had mM concentrations of the counterion ATP, but only the ‘coacervate’ samples contained the polypeptide required to form the coacervate. Concentrations did vary but were generally in the range of 1-2 mM for ATP and 0.4-1.0 mM (400 µM being the most common concentration) for the polypeptide and 250-400 nM of the dsDNA or ssDNA FRET reporter. Structural conformation and DNA co-localization was confirmed by scattering signals seen in the

absorbance spectra and fluorescence confocal microscopy (see **Figure 4.2B** and **Figure 4.3**). Based on our confocal images and previous DNA-coacervate characterization, we can predict that greater than 99% of the DNA was sequestered within the associative coacervate [151]. Kinetic characterization of DNA sequestration (**Figure 4.4**) resulted in estimates of ~20 min for DNA to fully sequester within associative coacervates, while the simple HGLGY based coacervates would take 15 min to sequester as ssDNA, but approximately an hour to form dsDNA if they were added individually.

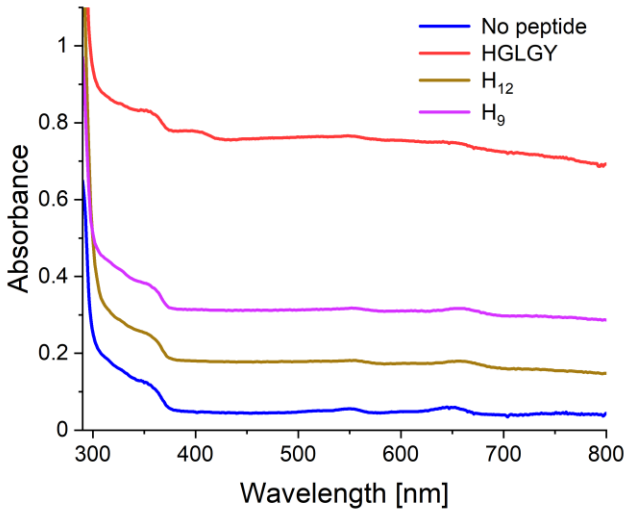

Figure 4.3. Absorbance spectra of the different systems at 60 min. The increase in baseline absorbance of the coacervate samples arises from the scattering. The Cy3 and Cy5 absorbance can also be observed in all the samples.

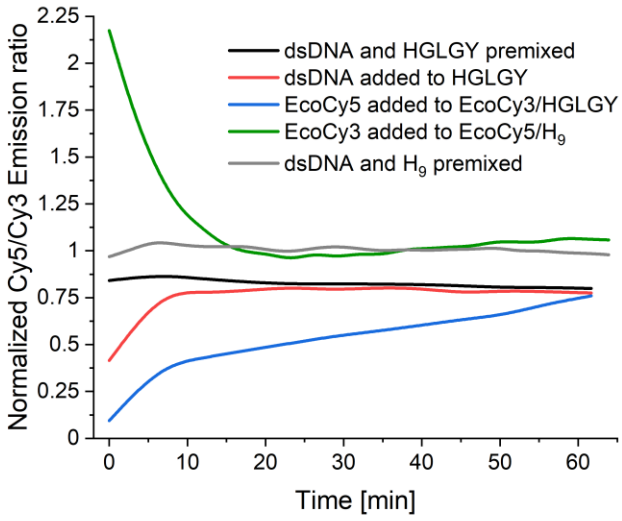

Figure 4.4. Kinetics of DNA uptake into coacervates. Coacervates samples were prepared using the EcoCy3 and EcoCy5 strands (Table 2 main text), dsDNA means the strands were preformed and either added to the coacervate previously (premixed, black and grey lines) or at time = 0 (red line). We also tested whether complementary strands could enter the coacervates and form the dsDNA conformation. It was observed that while both coacervates took about ~15-20 min to uptake the strands (including the preformed dsDNA) in the case of the associative coacervates the formation of the dsDNA was almost immediate (green line), while in the simple HGLGY coacervate (blue line) it took ~60 min for the dsDNA to form.

Working with a Tecan plate-reader and 384 well plates, we ran both experimental and independent replicates of the experiment. After initial characterization and reaching stability at 20-25°C on the plate we could spike the individual wells with varying nucleases (presented in **Table 4.3**), as well as buffer and/or water as controls, and follow the respective changes in fluorescence emission ratio of the Cy3-Cy5 donor-acceptor FRET pair. The chosen enzymes were meant to cover a range of sizes, charge, substrate, and hydrolytic mechanisms, with the intent of discerning if particular guide rules could be understood. If there was enzyme activity, the Cy5 divided by Cy3 emission ratio would decrease. **Figure 4.5** shows representative data of how the buffer samples were quickly degraded while the coacervate samples were stable.

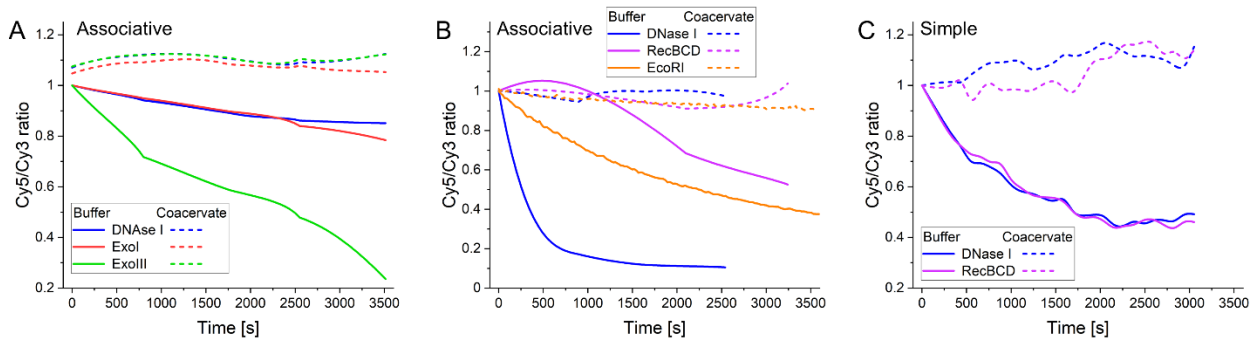

Figure 4.5. Coacervate protection capacity. Cy5/Cy3 fluorescence emission ratio was followed over time and normalized to the buffer addition samples. Enzyme was added at t = 0. A) Buffer (solid) compared to associative coacervate (dashed, H_{12}) with ssDNA FRET reporter and either DNase I (10 u/ml), ExoI (200 u/ml), or ExoIII (1000 u/ml). B) Buffer (solid) compared to associative coacervate (dashed, H_{12}) with dsDNA FRET reporter and either DNase I (10 u/ml), RecBCD (500 u/ml), or EcoRI (1500 u/ml). C) Buffer (solid) compared to simple coacervate (dashed, HGLGY) with dsDNA FRET reporter and either DNase I (5 u/ml) or RecBCD (500 u/ml).

Nuclease	**Source** [a]	**Mw [kDa] / pI** [b]	**Described recognition/action site**
Exo I	*E. coli*	55.7 / 6.2	Linear ssDNA 3′ → 5′
Exo III	*E. coli*	31.0 / 5.8	Linear ssDNA 3′ → 5′, linear dsDNA
Exo V (RecBCD)	Recombinant combination of the three subunits of *E. coli* Exonuclease V	270.3 / 6.8	Linear and circular ssDNA, linear dsDNA
DNAse I	Recombinant b*ovine pancreatic*	39 / 5.2	Linear and circular ssDNA, linear dsDNA
EcoRI	*E. coli*	31 / 7.8	Specific cleavage site in dsDNA

a) All enzymes purchased from New England Biolabs and used as received.
b) Values not available from vendor calculated by https://web.expasy.org/compute_pi/

Table 4.3. Nucleases tested for DNA degradation.

For the simple HGLGY based coacervates, the experimental approach was slightly different. The coacervate formation method required initial dissolution of the peptide in a 10 mM acetic acid (pH 3.0) based buffer and subsequent titration of the buffer with NaOH 0.1 M until a pH near 7.0 was reached and coacervate formation was observed [157]. The peptide concentration required for coacervate formation was higher, ranging from 1.0 to 2.5 mM. The

DNA reporter, at a final concentration of 35 nM, was then added to the coacervate to allow for sequestration (see **Figure 4.2C,D**). The nuclease experiment was realized in a similar manner, save for the lower DNA concentration, as that described for the associative coacervate experiments. The amount of DNA that could be protected was ~2 orders of magnitude less than for the associative coacervates if we consider DNA/peptide ratios.

As can be seen from the results in **Figure 4.5** both the associative and simple coacervates are capable of protecting the DNA reporters from enzymatic degradation, i.e. nucleases. We do have evidence that the effect is not boundless as large excesses of DNAse I (100-times greater concentration than generally recommended for DNA elimination) are capable of showing activity in the buffer and the coacervate systems (see **Figure 4.6**) and there are limits on the amount of DNA that can be sequestered, particularly in the simple coacervates (see **Figure 4.7**).

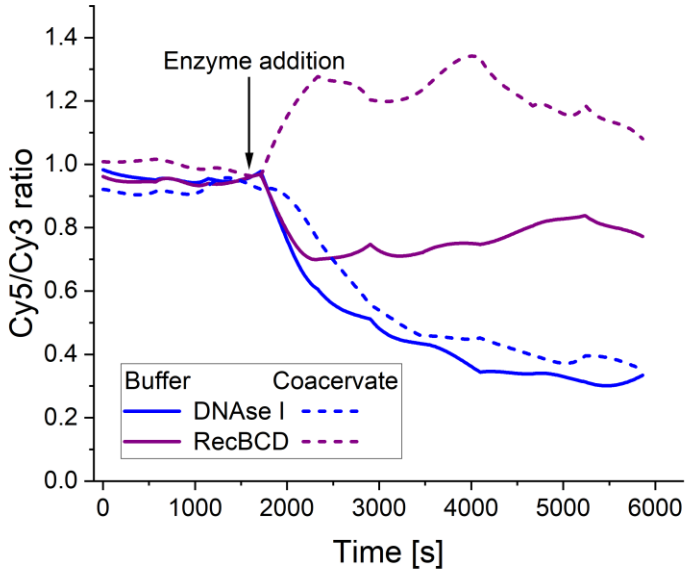

Figure 4.6. Coacervate protection capability. Cy5/Cy3 fluorescence emission ratio was followed over time. Buffer (solid) compared to associate coacervate (dashed, H_{12}) with dsDNA FRET reporter and either DNase I (200 u/ml), RecBCD (250 u/ml). At ~1500 s, the enzymes were added to each well. As can be observed the coacervate does protect against RecBCD, which is at the normal concentration levels, while the DNAse I, which is at 20-fold excess the normal concentration used, does result in FRET reporter degradation.

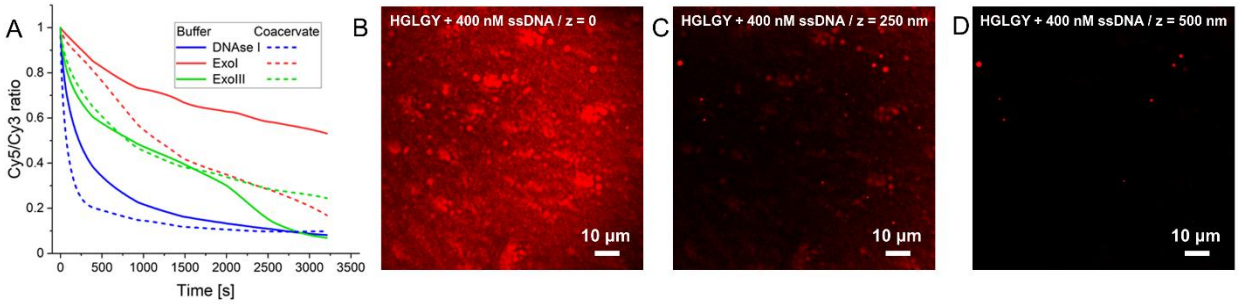

Figure 4.7. Coacervate release for nuclease activity observation. A) Cy5/Cy3 fluorescence emission ratio was followed over time. Buffer solution (solids) and simple coacervate (dashed, HGLGY) with 400 nM ssDNA FRET reporter. Enzymes were added at time = 0, 50 u/ml of DNAse I, 4000 u/ml ExoI enzyme, or 4000 ExoIII. The amount of DNA added was more than the coacervate could sequester, and as such, the coacervate was unable to protect it from degradation. B-D) Fluorescence confocal microscopy images of HGLGY coacervates with 400 nM ssDNA FRET reporter. Images are part of z-stack, with the glass coverslip as z = 0. It can be observed in part B and C that excess DNA is found bound to the coverslip, i.e. would be found in continuous phase of the solution. The coacervates themselves (as seen in part C and D) also show the presence of the sequestered ssDNA.

4.3.2 DNA release from coacervates

For applications, such as cellular delivery, it is necessary that the DNA be protected by the nuclease but then be released from the delivery vehicle, in this case the coacervate. Of course, the dynamic nature of the coacervates signifies that cargo does enter and leave the continuous and coacervate phase, but this process is not inherently controllable [153]. Approaches have utilized thiol reduction [152, 160], changes in pH [161], host-guest interactions [162], as well as light irradiation [163, 164], to control coacervate formation and dissolution. Here, we show that changes in the ionic strength of the buffer along with increases to physiological relevant temperatures can dissociate the coacervate and result in DNA release.

Experiments were realized in line with the approach mentioned above; after demonstrating protection from nucleases the samples were perturbed and the ratio of the Cy5/Cy3 emission was followed. The perturbation could be addition of concentrated monovalent or divalent salt solutions and/or heating of the solution. The equivalent perturbation was applied to control samples where the DNA was in aqueous buffer solutions and control samples where

no enzymes were present were run in all cases to assure that spectral changes were due to nuclease activity and not environmental changes.

As can be seen in **Figure 4.8**, upon perturbation of the coacervate the DNA is released from the protective environment in which it had been sequestered, allowing the nucleases to degrade the FRET pair. Interestingly, this does not appear to be a binary effect, as can be seen in Figure 4A,C where addition of Na^+ (171 mM, concentrated PBS buffer) only results in partial degradation of the DNA FRET reporter, while addition of Mg^{2+} results in full degradation. Though, of course the controlled release is not only ion dependent, but also concentration dependent as seen in **Figure 4B,C**. Similarly, heating only (no Mg^{2+}), has a lower level of cleavage than 20 mM Mg^{2+} and lower than the combined perturbation. We note that the temperature increase was generally from 25°C to 37°C (at most 42°C, which was the limit of the plate reader). At these temperatures the coacervate is destabilized, but it is not completely dissolved into a continuous phase [165]. Increasing ATP concentration up to 4 mM was also tested (See **Figure 4.9**) and it was observed that considerable perturbation was obtained, resulting in approximately 52% of the DNA being cleaved.

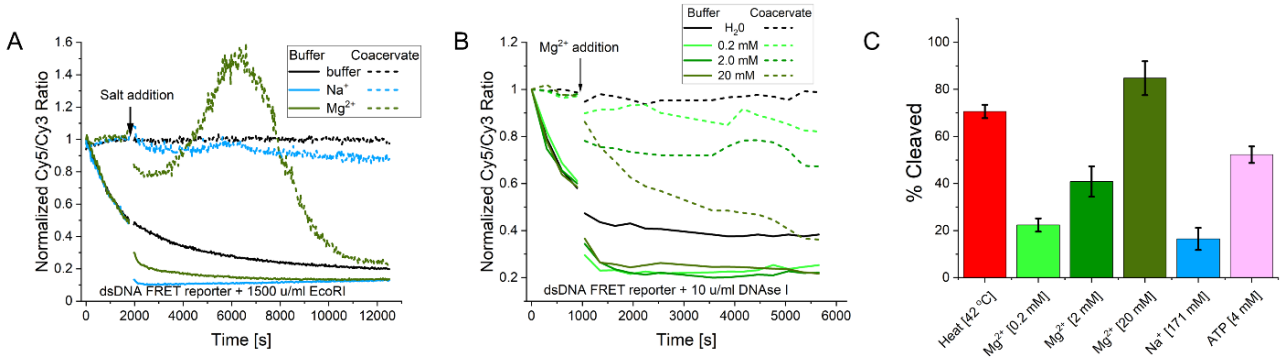

Figure 4.8. Coacervate release for nuclease activity observation. Cy5/Cy3 fluorescence emission ratio was followed over time. A) Buffer solution (solids) and associative coacervate (dashed, H_9) with dsDNA FRET reporter and 1500 u/ml of EcoRI enzyme. At ~2000 s, either 147 mM Na^+ or 20 mM Mg^{2+} was added. Buffer addition was realized as a negative control. B) Buffer solution (solids) and associative coacervate (dashed, H_9) with dsDNA FRET reporter and 10 u/ml of DNase I enzyme. At ~1000 s, varying concentrations of Mg^{2+} were added. C) Percentage of FRET reporter that was cleaved from the coacervate

samples at end-point of experiment as a function of perturbation applied in the presence of 10 u/ml of DNAse I. The buffer solution was assumed to have 100% cleaved DNA.

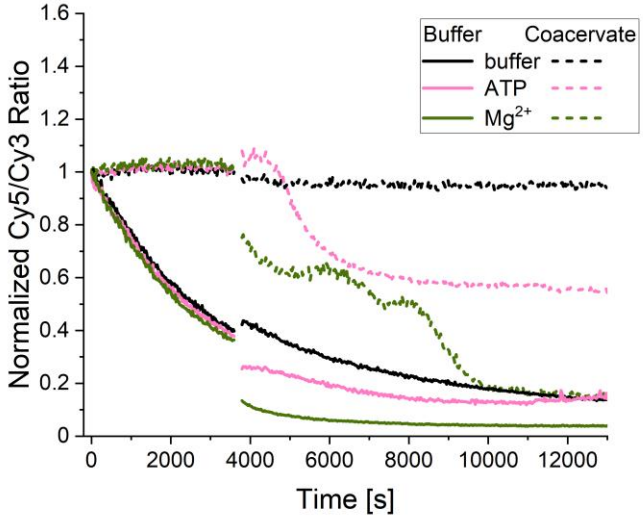

Figure 4.9. Coacervate release for nuclease activity observation. Cy5/Cy3 fluorescence emission ratio was followed over time. Buffer solution (solids) and associative coacervate (dashed, H_9) with dsDNA FRET reporter and 1500 u/ml of EcoRI enzyme. At ~3000 s, either 4 mM ATP or 20 mM Mg2+ was added.

The distinct shape that the Mg^{2+} curve shows in **Figure 4.8A**, and seen to a lesser extent in **Figure 4.6**, we adjudicate to the reconfiguration of the system and its subsequent effects on the FRET due to changes in QY and Cy5-Cy3 distance. A reasonable interpretation would suggest that initially the coacervate is degraded by Mg^{2+}, lowering viscosity and dropping dye QYs [151] resulting in the drop in Cy5/Cy3 ratio. The additional Mg^{2+} in solution then results in tighter DNA binding increasing the FRET signal, until eventually the EcoRI begins to degrade the dsDNA causing the Cy5/Cy3 signal to drop once more. In the case of **Figure 4.8B** the DNase I may be acting so quickly that the initial increase is never observed. The results in **Figure 4.8** are representative of the associative coacervate results. Similar results were observed using the ssDNA and the other enzymes (see **Figure 4.10**).

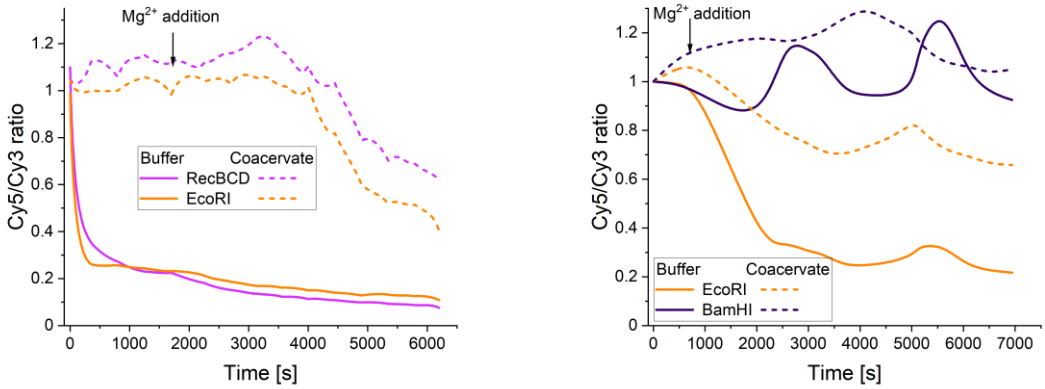

Figure 4.10. Coacervate release for nuclease activity observation. Cy5/Cy3 fluorescence emission ratio was followed over time. Buffer solution (solids) and associative coacervate (dashed, H_{12}) with dsDNA FRET reporter. (Left) 1200 u/ml of EcoRI or 100 u/ml RecBCD enzyme. At ~1800 s, 20 mM Mg^{2+} was added. (Right) 150 u/ml of EcoRI or 150 u/ml BamHI enzyme, which is a negative control as the dsDNA FRET reporter does not have the BamHI cleavage sequence. At ~900 s, 20 mM Mg^{2+} was added.

We present these results as a qualitative description of the system. The DNA FRET reporters are protected from all the tested nucleases and upon a perturbation that weakens the electrostatics that drive coacervation, the DNA is cleaved. The DNA release appears to correlate with the degree of coacervate disruption.

Release from HGLGY coacervates was more complex to detect, as neither ionic concentration increases nor heating to 42°C, were sufficient to observe notable DNA release. Attempts at pH changes were unsuccessful as control experiments showed that the enzymes were not functional at the new pH. Based on the literature results [153], one would expect HGLGY to eventually release the nucleotide cargo, perhaps through a dilution mechanism or merely through passive exchange. We were unable to observe this in a controllable *in vitro* manner.

4.3.3 Protection and release of a gene sequence

To this point the experiments were realized on a short oligonucleotide (16 and 30 nucleobases for dsDNA and ssDNA, respectively), which would be useful for siRNA, for example. Yet, for most biological applications it is more pertinent to understand whether this

same approach is compatible with the protection and release of genetic information [166]. The clear distinctions include the need to sequester-protect-release a larger nucleotide moiety (~2-orders of magnitude larger) as well as requiring that the genetic information component of the nucleotide be maintained. We chose to use the gene for *Renilla luciferase* (*Renilla reniformis* EC No. 1.13.12.5) which encodes for the bioluminescent enzyme Luc9 (optimized mutant of the wildtype luciferase enzyme original produced by the Rao lab) [120, 121]. Addition of the Luc9 substrate, Nano-Glo^{TM} (Promega), created a bioluminescent signal that was correlated to the amount of enzyme produced, providing a simple assay to determine protection-release of a gene [167].

As the focus of our work was in studying the capability of the coacervate to sequester and release the genetic information, we chose to use a cell-free transcription–translation (TXTL) system. The chosen TXTL system, PURExpress *In Vitro* Protein Synthesis Kit (New England Biolabs), is composed of optimized components from *E. coli* cell lysates [111]. TXTL systems have been shown as efficient high-throughput approaches that can avoid the delivery/membrane transport variable. Additionally TXTL systems benefit from microliter reaction volumes, requiring much less material (DNA, peptides, etc.) for quantitative gene expression [167]. These facts made the TXTL systems optimal for our proof-of-concept use. Details on how the dsDNA containing the Luc9 gene and T7 promoter, 1651 nucleotides long, was obtained from the pET-22 Luc9 plasmid are available in our previous publication [167]. The Luc9 gene sequence and additional information is available in the **Appendix**.

We first wished to observe whether the Luc9 could be expressed from DNA with the TXTL system in the presence of the coacervates. The dsDNA gene was added at a final concentration of 15.6 nM along with 5 mM ATP; coacervate samples included either 500 μM H_9

peptide or 1.9 mM of HGLGY. We note that the TXTL system has endogenous ATP, this additional ATP should not affect coacervate formation as we are already working at a high concentration. The DNA and coacervate were allowed to interact for 90 min at 25°C. A fraction of this was then added to the TXTL system in accordance with the kit recommendations (final dsDNA concentration was 1.9 nM) and the temperature was increased to 37°C for two hours. Then 10 µL of the TXTL solution was combined with 10 µL of Nano-Glo substrate in 384-well plates and bioluminescence measurements were collected at 460 nm wavelength for up to 5–6 h at 37°C. The majority of the bioluminescence was observed within the first hour of Nano-Glo addition. The intensity values provide a comparative expression read-out between different conditions. It can be observed in **Figure 4.11B, C** that adding the coacervate does not appear to inhibit gene expression.

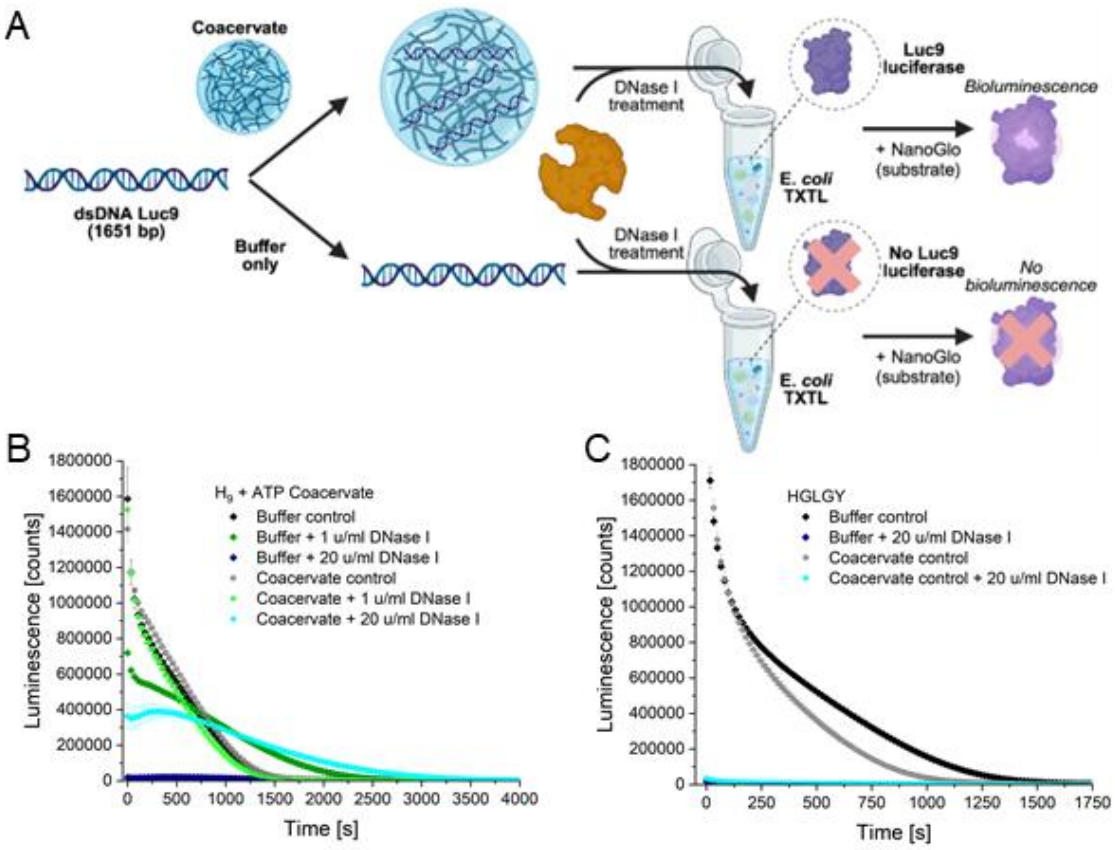

Figure 4.11. Coacervate protection and release of dsDNA gene sequence. A) Representative schematic of coacervate protect and release experiment in TXTL system. Created in biorender.com. B) Luminescence of samples that contained dsDNA gene for Luc9 with and without associative coacervate (H_9). The samples were challenged with 20 or 1 u/ml of DNase I or just buffer (positive control). C) Luminescence of samples that contained dsDNA gene for Luc9 with and without simple coacervate (HGLGY). The samples were challenged with 20 u/ml of DNase I or just buffer (positive control).

In line with the *in vitro* protection assays we proceeded to challenge our samples with exogenous nucleases to see if the coacervates could improve the gene expression. The nuclease was added after an initial DNA-Coacervate sequestration stage (30 min) and was allowed to act on the samples during the 90 min at 25°C incubation. Initially we wished to deactivate the enzymes after the incubation by heating the samples to 85°C. We observed high levels of degradation of the dsDNA when heating with the associative coacervate, this was independent of whether the enzymes were present or not (See **Figure 4.12**). This confirms other reports that poly-histidine peptides are capable of cleaving nucleotide bonds [168, 169], which in our case goes from insignificant at RT to considerable at 85°C. In our second approach, we transferred the incubated samples, nucleases and all, into the TXTL system. **Figure 4.11B** has representative results (all experiments are run in triplicate, as well as independent repeats), it can be observed that the associative coacervate are capable of protecting the Luc9 gene and allowing considerable expression as measured by the bioluminescence. Addition of 1 u/ml of DNAse I resulted in almost no decrease in bioluminescence, while 20 u/ml DNAse I had ~33% of the bioluminescence at the initial time point. Conversely, the sample without coacervate had the gene almost completely degraded resulting in no expression for 20 u/ml DNAse I, and ~50% less signal for 1 u/ml DNAse I. Similar experiments were realized with RecBCD as the nuclease (see **Figure 4.13**) and though some protective power was observed (~20% for 100 u/ml RecBCD), it was lower and more erratic than that seen for DNAse I.

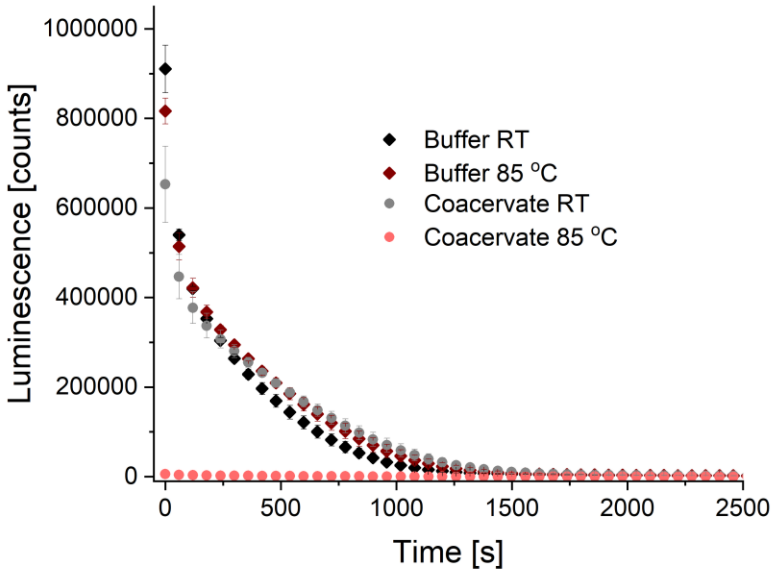

Figure 4.12. Bioluminescence as a function of time from Luc9 formed off addition of the 15 nM dsDNA Luc9 gene that had been previously incubated in Buffer or Coacervate (H_{12}) solution at either RT or 85 °C for 1 hour before combining with TXTL system. It can be observed that the gene was degraded upon heated incubation with the coacervate and no protein was expressed, incubation at RT with the coacervate or at 85 °C in buffer did not degrade the DNA.

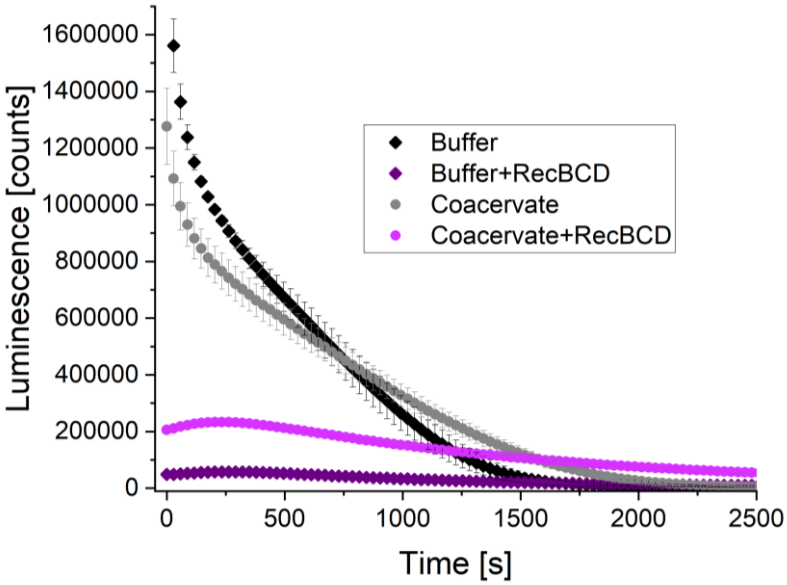

Figure 4.13. Bioluminescence as a function of time from Luc9 formed off addition of the 15 nM dsDNA Luc9 gene that had been previously incubated in Buffer or Coacervate (H_{12}) and exposed to 100 u/ml of RecBCD nuclease for one hour before combining with TXTL system. It can be observed that the coacervate sample had nearly 5-fold more protein production than the buffer system. In general it was observed that the associative coacervates provided better relative protection against DNAse I than RecBCD.

The same experiment with the HGLGY simple coacervate showed that the enzyme was expressed in the presence of the coacervate. Yet the coacervate provided no protective power when challenged by DNAse I or RecBCD, in fact in some experiments it appeared to have been degraded to an even greater extent in the presence of coacervates. We hypothesize that due to the much larger size of the dsDNA gene that the sequestration within the HGLGY coacervates is very limited in comparison to the short DNA strands we utilized to test the system in vitro. This is also consistent with the lower amount of DNA that could be protected by the HGLGY coacervates. It is important to remember that a single cleavage in the gene could result in a completely non-functional enzyme, so it is imperative that the entire dsDNA gene be protected.

4.4 Discussion

The experimental results demonstrate that the associative coacervates are capable of sequestering and protecting DNA from nucleases, and that by perturbing the coacervate through ionic strength or heat, the nucleotides can be released. For simple coacervates it appeared that small amounts of short DNA could also be sequestered and protected, but these were also not efficiently released and longer genes did not access protection at all in these materials. The key reason for the distinction between the two coacervates appears to be their mechanism of formation; while the associative coacervate is charge driven, the simple coacervate is largely driven by hydrophobicity.

The HGLGY peptide simple coacervate formation is well represented by the "sticker-and-spacer" model, where the sticker amino acids (H and Y) drive the intra- and intermolecular interactions while the GLG acts as the spacer [157]. Furthermore, the GLG spacer provided intermediate hydrophobicity and steric bulk, in turn reducing interfacial water structuring at the coacervate-continuous phase interface required for formation of liquid condensates [170]. As is

apparent, there is no strong electrostatic force driving HGLGY coacervate interaction with the highly charged oligonucleotides. It is likely that some combination of π-π and hydrogen bonding are the principal sequestration mechanism [157]. This results in a weak interaction and the limited protection capability of the coacervate; able to only protect relatively short and small amounts of DNA (tens of nM oligo concentration vs mM peptide).

In contrast the associative coacervate is largely driven by electrostatics and the anionic complexation of the counterion that can be either ATP or the chemically similar oligonucleotides [171]. We have seen in other positively charged peptides, e.g. polyarginine, that ssDNA are capable of forming associative coacervates without other counterions. This permits much stronger sequestration within the coacervates (hundreds of nM of DNA vs μM peptide). As examples of the strong interactions that occur, Ohno et al. observed that addition of positively charged polypeptides, in their case polylysine, disrupted DNA complementarity through electrostatic bridging [172]. Ponnuswamy and colleagues also demonstrated that DNA nanoparticles could be coated with polylysine to avoid nuclease degradation [173]. The Keating lab similarly showed that polyamine based associative coacervates can partition RNA ~10,000 fold over the dilute continuous phase [149]. This suggests that our polyhistidine peptides are functioning in a very similar manner. We hypothesize that it is these strong interactions that limit accessibility of the DNA to the nuclease binding/active site. Using the ExoIII nuclease as an example, the active site of the nuclease is at the bottom of a long groove between two β-sheets, where the oligonucleotide is cleaved by a single metal-ion aided nucleophilic attack [174]. This metal ion active site is a negatively charged cavity within an extended positively charged (to aid DNA-binding) facet. The transition of the DNA through this long groove would therefore be easily inhibited by the presence of the polyhistidine. DNase I and RNase H have very similar

endonuclease mechanisms [175]. When focusing on the binding/recognition sites of the enzyme, we find that there are two loops that bind with the major and minor grooves of the dsDNA, that interact with about 1700 $Å^2$ of the DNA surface [174], which could be inhibited by the presence of a polyhistidine peptide that was strongly bound to the DNA. There is some evidence that binding, but not translation into the active site may be occurring in some of the enzymes. For example, in **Figure 4.11A and C**, we observe an increase of the Cy5/Cy3 ratio when the enzyme is added. This could be because the binding of the enzyme is either changing the dyes local environment and modifying the dye QY or the structure of the FRET reporter causing the dyes to be slightly closer and showing enhanced FRET.

While many enzymes have been shown to colocalize within coacervates [176-178], a hypothesis arose that charge-based enzyme exclusion was part of the protection mechanism. Yet, if we observe the properties of the enzymes in **Table 4.3**, we see that they present pI both below and above the buffer pH. Similarly, the enzymes range from 31 to 270 kDa in size, suggesting that enzyme size is not the determinant factor. Since all enzymes were similarly inhibited of their nuclease activity by the associative coacervates it stands to reason that it had more to do with the interaction with the substrate, as opposed to a competitive inhibitor in line with GamS (RecBCD inhibitor) [179]. As noted above, there is also some evidence that the addition of the enzyme is resulting in changes in FRET signals, which suggests the enzyme is capable of entering the coacervate.

The final consideration is whether the coacervates were functioning as metal chelators, specifically of Mg^{2+}, and therefore inhibiting the enzyme's activity through ion sequestration. While polyhistidines are well known to strongly bind to divalent cations, the strongest binding occurs with larger ions (e.g. Ni^{2+}, Co^{2+}, Cu^{2+}, and Zn^{2+}) with Mg^{2+} binding at a lower affinity

[114]. Frankel et al, also showed that in associative coacervates containing nucleotides, Mg^{2+} will be concentrated in the coacervate phase [149]. Furthermore, our baseline buffer contained 0.5 mM $MgCl_2$ with the peptide being at 400 μM concentration, assuming each peptide could coordinate 2-3 Mg^{2+} that would still leave more than 0.35 mM Mg^{2+} in solution.

Considering the experimental evidence, we hypothesize that the inhibition mechanism arises from the strong electrostatic interaction that occurs when nucleotides are sequestered into the coacervates. This binding inhibits access of the DNA to the recognition and active sites of the nucleases and/or TXTL system through a combination of steric and charge inhibition. Disruption of the coacervate, through heating, change in ionic strength, and/or dilution shields the DNA from the interaction with the peptides and allows it to interact with the enzymes.

4.5 Conclusion

We have shown that oligonucleotides sequestered within coacervates are protected from nuclease degradation. The mechanism of protection is likely peptide-oligonucleotide binding, and as such the method of coacervation (i.e. associative vs simple) modifies the level of protection. Associative coacervates, based on their strong electrostatic interactions, are capable of greater oligonucleotide binding and as such are more efficient at sequestering oligonucleotides from interaction with enzymes as compared to the simple HGLGY coacervate we tested. Of greater interest, this sequestration was reversible, with the oligonucleotide being released from the coacervate upon heating or increase in ionic strength. We were able to demonstrate this with nuclease degradation of short oligonucleotide FRET reporters and then transition the same approach to the increased production of a Luc9 enzyme in a TXTL system through protection-release from an associative coacervate when nucleases were present.

As shown recently by the Miserez lab, there is considerable interest in coacervates as cellular delivery vehicles [153]. Polyhistidine systems have also been used previously for cellular delivery as they may provide an endosomal escape mechanism through swelling [180, 181]. Therefore, knowing a designed coacervate's capability to protect and release oligonucleotides cargos such as plasmids or mRNA in a simple high-throughput approach, such as using TXTL systems, could maximize their application by allowing for broad materials surveys. Additionally, the amount of material required is much smaller, allowing only promising coacervates to transition to cellular and eventually in vivo experiments. As it stands, it appears that associative coacervates present an advantage for protection/release of oligonucleotides. Especially as we have shown that DNAse I degradation is well controlled by the coacervates and it is the most common nuclease in eukaryotic systems [182]. Yet associative coacervates may not be compatible with cellular delivery due to dilution limitations. Further investigation into peptides that may coacervate and present the strong sequestration and protection of oligonucleotides, stability upon dilution, and simple release mechanism is still required.

A system that may be more amenable to using the currently designed associative coacervates are cell-free detection approaches (which mirror TXTL systems). These systems can sense an analyte and transform that into protein production, often times fluorescent or bioluminescent enzymes, that provide a readout [123, 183]. These systems could benefit from coacervates, resulting in increased production through stabilization of intermediates, i.e. DNA and RNA, as well as through substrate-enzyme colocalization [112].

Chapter 5: Summary and Contributions to Science

5.1 Summary

Nucleic acid therapeutics are extremely powerful biomedical technologies that have the potential to save millions of lives worldwide. However, at present, there are only about 37 nucleic acid therapeutics which are approved by the FDA, as compared to more than 10,000 approved pharmaco-chemical drugs, highlighting the immense potential to develop these therapeutics in terms of their application space and complexity. Although the most widely known nucleic acid therapeutics are COVID vaccines, these therapeutics were initially developed with the promise to provide solutions for genetic diseases. Currently, the standard treatment for most genetic diseases only work to delay disease progression and/or to minimize the effects of the disease, which are temporary and short-term. The most direct way to prevent genetic disease is to edit or replace the genetic mutations inside of the chromosome, highlighting the significant need to develop technologies which can be used for gene replacement and editing in order to provide more permanent, long-term solutions for genetic diseases. While current technologies have made great strides in addressing challenges involved with cellular stability and nucleic acid delivery, enabling the approval of nucleic acid therapeutics for genetic disease treatment, about half of these therapeutics are designed to be administered *ex vivo* and therefore limited in their range of cellular targets. Also, almost all of these therapeutics utilize live viral vectors which pose a high safety risk to infect cells with unedited viral genome. Therefore, in order to enable the development of safer, more effective and longer-lasting nucleic acid therapeutics for genetic diseases, this thesis has established the use of gene-encoding DNA origami nanoparticles (NPs) as a new and improved gene template and potential therapeutic delivery platform (Chapter 3); established the use of *de novo* peptide coacervates as a nucleic acid delivery platform that can

protect gene templates (such as plasmids, linear DNA and DNA origami) against exogenous nuclease degradation (Chapter 4); and established the use of a pure cell-free transcription-translation (TXTL) system for benchtop studies and used this to evaluate the design and expression of gene-encoding DNA origami NPs and coacervates loaded with DNA (Chapter 3 and 4).

5.1.1 Establishing the expression of gene-encoding DNA origami in bacterial cell-free TXTLs

In Chapter 3, I designed a 12-helix bundle DNA NP wherein the 1651-nucleotide long scaffold strand encoded a T7-expressing luciferase gene (Luc9), which allowed us to indirectly assay subsequent protein expression and compare relative levels using a simple bioluminescence-based assay on a commercial plate reader, enabling the design of high throughput assays for gene-encoding DNA origami NPs. I applied a pure E. coli TXTL system in characterizing the expression of gene-encoded DNA NP systems, and demonstrate that an appropriately assembled gene-encoded DNA origami NP can be expressed in bacterially derived TXTL systems. I probed the system to confirm some initial assumptions made about the nature of the promoter sequence and the critical role that it plays toward efficient expression of the gene and determined the extent to which the nature of the promoter sequence upstream of the gene within a DNA NP affects protein expression in comparison to canonical plasmid and linearized single-stranded (ss) and double-stranded (ds) DNA versions of the gene. I confirmed that the promoter sequence is required and that it must be present in a dsDNA configuration regardless of how the DNA is presented to the reaction system. I also confirmed that promoter recognition and transcription initiation is undertaken with the scaffold in the ssDNA conformation, and this is significantly reduced when the promoter sequence is scrambled beyond recognition. The work completed in Chapter 3 establishes the expression of gene-encoding DNA origami in bacterial

cell-free TXTLs, providing us with a platform to directly investigate the role of DNA template structure on gene expression without the use of live cells and improve the structural design of DNA templates, which can be advantageous for the development of gene-editing and gene-replacement therapies.

5.1.2 Establishing the use of peptide coacervates to protect nucleotides from nuclease degradation in bacterial cell-free TXTLs

In Chapter 4, I demonstrated that coacervates can protect sequestered oligonucleotides from nuclease degradation. I achieved this by studying the ability of simple and associative coacervates to sequester, protect and release nucleotides *in vitro* by utilizing dye-labeled DNA Förster resonance energy transfer (FRET) based reporters to detect degradation of the DNA through exposure to nucleases. I demonstrated that nucleotides can be sequestered into, protected from nucleases, and released from an associative coacervate model (polyhistidine peptides and ATP) through an external stimuli (e.g. perturbation of the coacervate through ionic strength or heat). I also demonstrated that small amounts of short nucleotides can be sequestered into, protected from nucleases, and released from a simple coacervate model (based on a repeat HGLGY sequence peptide), though to a much lesser extent than in the associative coacervates. Longer genes were not able to be protected from nuclease degradation in simple coacervates and were not efficiently released. With this initial evidence, I expanded the work with the use of a luciferase gene and a cell-free transcription-translation (TXTL) model system and demonstrated that a functional gene can be sequestered into an associative coacervate, protected from enzymatic degradation, and then released to create the desired protein product. The work completed in Chapter 4 establishes the use of peptide coacervates as a nucleic acid delivery platform that can protect against nuclease degradation as well as the use of bacterial cell-free

TXTLs as a platform to evaluate the nuclease degradation of DNA templates, enabling us with the tools to expand on the technology available for the delivery and protection of exogenous nucleic acids.

5.2 Scientific contributions

5.2.1 Publications

Galvan AR, Tiboni IM, Mathur D, Green CM, He Y, Ulijn RV, Medintz IL, and Díaz SA. "Peptide Coacervates can Protect Sequestered Nucleotides from Nucleases and Release them for Transcription and Translation". *Biomacromolecules* (2025): 26 (9), 5767-5777. (doi: 10.1021/acs.biomac.5c00600).

Meares AA, Ansteatt SR, Díaz SA, Kim YC, Cunningham PD, Thomas SG, Segal VM, **Galvan AR**, Pascual G, Roy SK, Patten L, Yurke B, Knowlton WB, Lee J, Medintz IL, and Melinger JS. "Control of Cy5 Dimer Geometry Using Variable-Length Linkers to DNA Scaffolds." *Journal of the American Chemical Society* (2025): 147 (32), 28651-28664. (doi: 10.1021/jacs.5c00128).

Rad LM, Hughes KR, Wheeler SN, Decker JT, Orbach SM, **Galvan A**, Thornhill J, Griffin KV, Turkistani H, Urie RR, Irani DN, Shea LD, and Morris AH. Engineered immunological niche directs therapeutic development in models of progressive multiple sclerosis. *Proceedings of the National Academy of Sciences* (2025): 122(7):e2409852122. (doi: 10.1073/pnas.2409852122).

Galvan AR, Green CM, Hooe SL, Oktay E, Thakur M, Díaz SA, Veneziano R, Medintz IL, and Mathur D. "Design and Characterization of a Gene-Encoding DNA Nanoparticle in a Cell-Free Transcription–Translation System." *ACS Applied Nano Materials* (2024): 7 (11), 12891-12902. (doi: 10.1021/acsanm.4c01456).

Mathur D, **Galvan AR**, Green CM, Liu K, Medintz IL. "Uptake and stability of DNA nanostructures in cells: a cross-sectional overview of the current state of the art." *Nanoscale 15.6* (2023): 2516-2528. (doi: 10.1039/D2NR05868E).

5.2.2 Conference presentations

Green CM, **Galvan AR**, Segal VM, Breger JC, Tiboni IM, Lysne DP, Ulijn RV, Medintz IL, and Díaz SA*. Oral presentation: "Peptide Based Liquid-Liquid Coacervates for Biosensing, Degradation Resistance, and as Biofoundries." *Biotechnology for Defense Symposium*. Falls Church, VA. February 2025.

Lysne D, Green CM, Sementa D, Melinger JS, Tiboni IM, **Galvan AR**, Medintz IL, Ulijn R, and Díaz SA*. Poster presentation: "DNA sequestration into peptide liquid-liquid coacervates: Applications in biosensing, degradation defence, and computing." *The 30th International Conference on DNA Computing and Molecular Programming (DNA 30)*. Baltimore, MD. September 2024.

Mathur D*, **Galvan AR**, Green CM, Hooe SL, Oktay E, Thakur M, Diaz SA, Veneziano R, and Medintz IL. Poster presentation: "Cell free protein expression from a gene-encoded DNA nanoparticle." *The 30th International Conference on DNA Computing and Molecular Programming (DNA 30)*. Baltimore, MD. September 2024.

Galvan AR*, Green CM, Hooe SL, Oktay E, Thakur M, Díaz SA, Veneziano R, Medintz IL, and Mathur D. Poster presentation: “Gene-encoding DNA nanostructure expression in a cell-free TXTL system”. *Mid-Atlantic DNA Nanotechnology Symposium (MaDNAno)*. Manassass, VA. May 2024.

Galvan AR*, Mathur D, Green CM, Medintz IL. Poster presentation: “DNA origami immunotherapies in biomedicine”. *Mid-Atlantic DNA Nanotechnology Symposium (MaDNAno).* Rockville, MD. May 2022.

5.2.3. Patents

Díaz SA, Medintz IL, Green CM, Tiboni I, and **Galvan AR**. “Sequestration and enzymatic degradation protection of nucleotides within peptide-based coacervates coupled to a subsequent activatable release mechanism.” Navy Case # 212489. Filed December 2024. Provisional application filed.

Meares AA, Melinger JS, Medintz IL, Díaz SA, Kim YC, Cunningham PD, Thomas SG, Segal VM, and **Galvan AR**. “Controlled Linker Length Modulation of DNA Scaffolded Dye Aggregate Provides For Subsequent H- or J-Aggregate Behavior.” Navy Case # 212008. Filed February 2024. Provisional application filed.

Chapter 6: Future Work and Outlook

6.1 Future work

6.1.1 Gene-encoding DNA origami nanoparticles and cell-free TXTLs

Looking forward, I foresee the use of TXTL reactions and gene encoding DNA NPs in this manner as a mechanism to initially confirm the ability of a construct to engage in gene expression and also perhaps test different structural design variations and how they may affect the protein expression efficiency. Several parameters are yet to be tested and optimized, such as accessibility to important sequences during transcription, compactness, stability, and density of crossover sites within the DNA NP. In Chapter 3, the location of the promoter was chosen to be present at a marginally extending helix end within the bundle with the intent to minimize steric hindrance for enzyme binding during transcription. The helix bundle architecture rather than a wireframe polyhedral shape was motivated by the fact that wireframe structure have shown to sterically hinder nuclease attack [128], possibly making transcription enzyme binding more challenging, too. By strategically routing the gene-encoded scaffold strand and leveraging staple strands for installing a Förster resonance energy transfer reporter system on the DNA NP [123], one could apply luminescence and fluorescence in tandem to learn the nuances of DNA NP stability and functionality. In fact, measuring protein expression efficiency from a DNA NP could serve as a much-needed analytical tool to determine nucleotide-level structural integrity of DNA NPs under physicochemical stressors [125]. Use of DNA binding protein fusions may also allow the NPs to bring proteins attached to it into the TXTL reaction or eventually even a cell in a piggyback-like manner to complement any requirements not present or to focus activity to just a given target [129]. In a manner mechanistically similar to what has been shown previously, the addition of hard NP scaffolds to the TXTL reactions such that they cross-link the proteins

present into dense nanoclusters could perhaps help speed up both the reaction rate and its efficiency by allowing the enzymatic components to also engage in enzymatic channeling processes [112, 130, 131].

Of course, this approach is not limited to bacterial TXTL reactions and should be similarly applicable to eukaryotic TXTL systems although appropriate codon biasing and optimization requirements will have to implemented [132-134]. One could optimize protein expression from gene-encoded DNA NPs to inform *in vivo* gene therapy experimentation by including other stressors in the TXTL system, such as degrading enzymes, serum, and digestive cell organelles. Even though the protein expression levels from the gene-encoded DNA NP were lower than the dsDNA gene segment, DNA NPs have proven value in their superior transfection efficiency, targeting, and stability compared to linear duplexes for therapeutic applications in general [135, 136]. In light of current limitations of gene delivery from viral and lipid NP formulations, namely, gene-length loading size cutoff, stringent cold-chain storage requirements, and toxicity [137, 138], the field anticipates that DNA NP-based gene therapies could offer potentially higher loading, targeting, and modular capabilities [65]. DNA NPs have higher resistance to degradation compared to plasmids, and strategies such as ligating the ends can enhance stability further [128]. The success of several DNA NP-based vaccine therapies where the NP architecture is leveraged to spatially organize epitopes is highly encouraging [139]; the scaffold strand used in these DNA NPs so far is bacteriophage-based, but it can be programmed for protein expression to add a gene therapy functionality as well. Naturally, such application of gene-encoded DNA NPs would require concomitant ways to produce the ss custom scaffold strand in desirable quantities. While producing custom plasmids in large quantities is an established process, scaling up of the synthesis of gene-encoded DNA NPs is rapidly advancing

as well due to leading efforts to formulate enzymatic and phage-based techniques of producing custom scaffold strands [49, 140]. Traditionally gel extraction is used to acquire purified ssDNA for custom scaffold strands, but new analytical techniques are emerging that reduce material loss and improve DNA NP production efficiency [141]. And the assembly process of DNA NPs lends itself directly to scale up in that the reactions can be done in a massively parallel format. Our approach described in Chapter 3 will certainly help with potential future downstream applications of these DNA NP materials, whether they are for gene delivery and designer therapies or even for point of care protein production of diagnostics and active biotherapeutic/drug molecules [142].

6.1.2 Coacervates and cell-free TXTLs

As shown recently by the Miserez lab, there is considerable interest in coacervates as cellular delivery vehicles [153]. Polyhistidine systems have also been used previously for cellular delivery as they may provide an endosomal escape mechanism through swelling [180, 181]. Therefore, knowing a designed coacervate's capability to protect and release oligonucleotides cargos such as plasmids or mRNA in a simple high-throughput approach, such as using TXTL systems, could maximize their application by allowing for broad materials surveys. Additionally, the amount of material required is much smaller, allowing only promising coacervates to transition to cellular and eventually in vivo experiments. As it stands, it appears that associative coacervates present an advantage for protection/release of oligonucleotides. Especially as we have shown that DNAse I degradation is well controlled by the coacervates and it is the most common nuclease in eukaryotic systems [182]. Yet associative coacervates may not be compatible with cellular delivery due to dilution limitations. Further investigation into

peptides that may coacervate and present the strong sequestration and protection of oligonucleotides, stability upon dilution, and simple release mechanism is still required.

A system that may be more amenable to using the currently designed associative coacervates are cell-free detection approaches (which mirror TXTL systems). These systems can sense an analyte and transform that into protein production, often times fluorescent or bioluminescent enzymes, that provide a readout [123, 183]. These systems could benefit from coacervates, resulting in increased production through stabilization of intermediates, i.e. DNA and RNA, as well as through substrate-enzyme colocalization [112].

6.1.3 Design requirements for nucleic acid therapeutics

As described by J.A. Kulkarni et al., the design requirements for nucleic acid therapeutics for gene editing and replacement include—membrane transport, endosomal escape, cytosolic stability, intracellular targeting, RNA translation, intranuclear transport and DNA transcription—and of all the fundamental technologies which have enabled the FDA approval of nucleic acid therapeutics, only adeno-associated viral vectors have demonstrated the ability to perform all these properties [1]. However, the use of viral vectors for delivery pose a high safety risk to infect cells with unedited viral genome and potentially cause the development of other diseases. Therefore, it is important to develop new non-viral therapeutics which can be safely administered for gene editing and replacement in vivo.

6.1.4 Limitations of DNA origami NPs and coacervates

Adding to the body of literature to push the use of DNA origami NPs for use in nucleic acid therapeutics, including the demonstration of membrane transport by L. Liang et al. and M.M.C. Bastings et al. [60, 61]; the demonstration of cytosolic stability by Q. Mei et al. and D. Mathur et al. [63, 155]; and the demonstration of intracellular targeting by L. Liang et al. and

M.A. Zanta et al. [61, 64], in Chapter 3, we demonstrated the DNA transcription of gene-encoding DNA origami NPs. Although we have not demonstrated all of these properties in our gene-encoding DNA origami NP, we expect that our NP will be able to exhibit the same properties of these DNA origami NPs because we believe that these properties are primarily based on the structure of DNA origami NPs and not the gene sequence encoded into them. In the future, we recommend the characterization of these properties in gene-encoding DNA origami to enable their translation for use as nucleic acid therapeutics.

As compared to the study of DNA origami NPs for use in nucleic acid therapeutics, the study of coacervates for use in nucleic acid therapeutics are relatively new. In Chapter 4, we demonstrated that coacervates can improve the cytosolic stability of gene templates against nuclease degradation and still be used for DNA transcription. However, there is much work that needs to be done. One of the biggest limitations of this technology is its sensitivity to different environments. We do not believe that it would be able to successfully deliver nucleic acids to cells if administered intravenously due to its high sensitivity to temperature and pH, as well as its lack of ability to encapsulate cargo. However, we can utilize what we've learned from our studies and use polyhistidine peptides to help prevent nuclease degradation in conjunction with other delivery technologies, like lipid nanoparticles, to create polyhistidine functionalized lipid nanoparticles, which would help increase cytosolic stability.

The pharmacokinetics of DNA origami NPs has been noted in different disease systems, including cancer, HIV and COVID, so we believe that it can target and be uptaken by cells without the use of a carrier [58, 66, 67, 85]. However, one of the biggest challenges for translating this technology as a therapeutic is the manufacturing of gene-encoding ssDNA scaffolds. At the moment, there are not many high throughput, non-viral production methods for

scaffolds [125]. In the future, we recommend improving current methods to increase scaffold production efficiency to have high enough concentrations of DNA origami NPs for clinical studies.

6.2 Outlook

Our work displays the importance of taking into account the design of the promoter sequence in gene-encoding DNA origami NPs and the critical role that it plays towards efficient expression of the gene, as well as an initial understanding in the differences of how simple and associative coacervates are able to sequester and release nucleotides and protect them from nuclease degradation. In this dissertation we discover important links between the structural design of gene-encoding DNA origami NPs and gene expression efficiency, as well as the type of coacervate peptide material and nuclease degradation protection efficiency. These aspects of gene templates and nucleic acid delivery vehicles have not yet been studied, and the field will benefit from further exploration of the structural design of gene templates and the mechanisms in which peptide coacervates can protect nucleotides from nuclease degradation.

Appendix: Supplementary information

A. DNA, plasmid, scaffold, primer, and staple sequences.

A.1 pET-22 Luc9 plasmid sequence (5' → 3').

Complement of this sequence forms the template for scaffold strand.

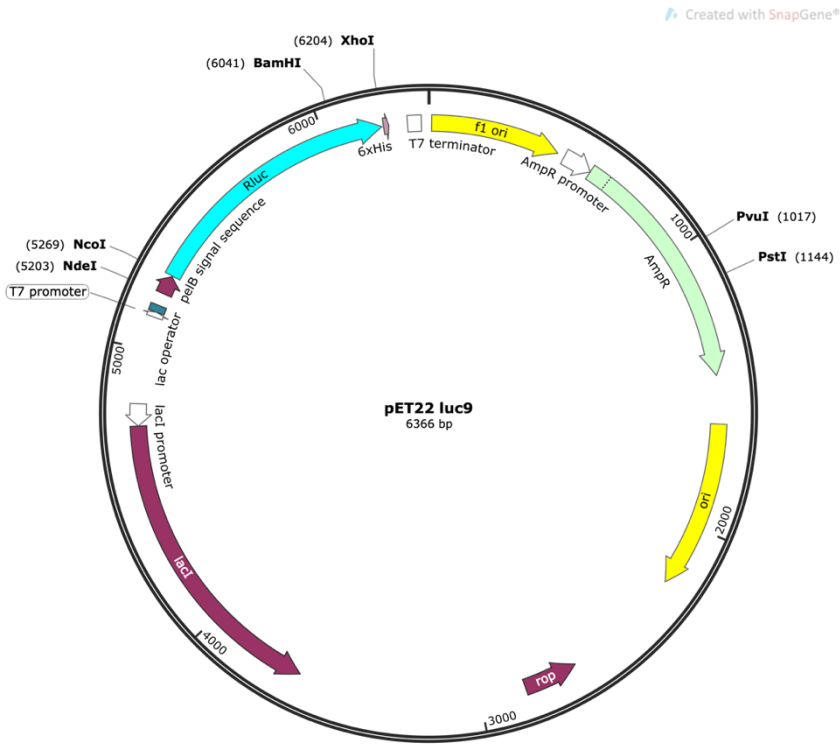

Figure A.1: Map of the pET-22 Luc9 plasmid

Legend: pET22 plasmid; Luc9 gene; T7 promoter

5′-
TGGCGAATGGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACAC
TTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAA
GCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGG
TGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATA
GTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGATTTTGCCG
ATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAACAAAATATTAACGTTTAC
AATTTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGT
ATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTC
CGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAA
AGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTT
TTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGAC
GCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAA
GCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACT

TACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTT
GATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGCAGCAATGGCAAC
AACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGG
ATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAG
CGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGG
GAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGT
CAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATC
CTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGAT
CAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGG
TGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAAT
ACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCT
AATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGG
ATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTG
AGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGG
CAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTC
GCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCG
GCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGA
TAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCG
AGGAAGCGGAAGAGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCACACCGCATATATGGTGCA
CTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGTATACACTCCGCTATCGCTACGTGACTGGGTCATGG
CTGCGCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAA
GCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTCATCACCGAAACGCGCGAGGCAGCTGCGGTA
AAGCTCATCAGCGTGGTCGTGAAGCGATTCACAGATGTCTGCCTGTTCATCCGCGTCCAGCTCGTTGAGTTTCTCCA
GAAGCGTTAATGTCTGGCTTCTGATAAAGCGGGCCATGTTAAGGGCGGTTTTTTCCTGTTTGGTCACTGATGCCTCC
GTGTAAGGGGGATTTCTGTTCATGGGGGTAATGATACCGATGAAACGAGAGAGGATGCTCACGATACGGGTTACTGA
TGATGAACATGCCCGGTTACTGGAACGTTGTGAGGGTAAACAACTGGCGGTATGGATGCGGCGGGACCAGAGAAAAA
TCACTCAGGGTCAATGCCAGCGCTTCGTTAATACAGATGTAGGTGTTCCACAGGGTAGCCAGCAGCATCCTGCGATG
CAGATCCGGAACATAATGGTGCAGGGCGCTGACTTCCGCGTTTCCAGACTTTACGAAACACGGAAACCGAAGACCAT
TCATGTTGTTGCTCAGGTCGCAGACGTTTTGCAGCAGCAGTCGCTTCACGTTCGCTCGCGTATCGGTGATTCATTCT
GCTAACCAGTAAGGCAACCCCGCCAGCCTAGCCGGGTCCTCAACGACAGGAGCACGATCATGCGCACCCGTGGGGCC
GCCATGCCGGCGATAATGGCCTGCTTCTCGCCGAAACGTTTGGTGGCGGGACCAGTGACGAAGGCTTGAGCGAGGGC
GTGCAAGATTCCGAATACCGCAAGCGACAGGCCGATCATCGTCGCGCTCCAGCGAAAGCGGTCCTCGCCGAAAATGA
CCCAGAGCGCTGCCGGCACCTGTCCTACGAGTTGCATGATAAAGAAGACAGTCATAAGTGCGGCGACGATAGTCATG
CCCCGCGCCCACCGGAAGGAGCTGACTGGGTTGAAGGCTCTCAAGGGCATCGGTCGAGATCCCGGTGCCTAATGAGT
GAGCTAACTTACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAAT
GAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCCAGGGTGGTTTTTCTTTTCACCAGTGAGACGGG
CAACAGCTGATTGCCCTTCACCGCCTGGCCCTGAGAGAGTTGCAGCAAGCGGTCCACGCTGGTTTGCCCCAGCAGGC
GAAAATCCTGTTTGATGGTGGTTAACGGCGGGATATAACATGAGCTGTCTTCGGTATCGTCGTATCCCACTACCGAG
ATATCCGCACCAACGCGCAGCCCGGACTCGGTAATGGCGCGCATTGCGCCCAGCGCCATCTGATCGTTGGCAACCAG
CATCGCAGTGGGAACGATGCCCTCATTCAGCATTTGCATGGTTTGTTGAAAACCGGACATGGCACTCCAGTCGCCTT
CCCGTTCCGCTATCGGCTGAATTTGATTGCGAGTGAGATATTTATGCCAGCCAGCCAGACGCAGACGCGCCGAGACA
GAACTTAATGGGCCCGCTAACAGCGCGATTTGCTGGTGACCCAATGCGACCAGATGCTCCACGCCCAGTCGCGTACC
GTCTTCATGGGAGAAAATAATACTGTTGATGGGTGTCTGGTCAGAGACATCAAGAAATAACGCCGGAACATTAGTGC
AGGCAGCTTCCACAGCAATGGCATCCTGGTCATCCAGCGGATAGTTAATGATCAGCCCACTGACGCGTTGCGCGAGA
AGATTGTGCACCGCCGCTTTACAGGCTTCGACGCCGCTTCGTTCTACCATCGACACCACCACGCTGGCACCCAGTTG
ATCGGCGCGAGATTTAATCGCCGCGACAATTTGCGACGGCGCGTGCAGGGCCAGACTGGAGGTGGCAACGCCAATCA
GCAACGACTGTTTGCCCGCCAGTTGTTGTGCCACGCGGTTGGGAATGTAATTCAGCTCCGCCATCGCCGCTTCCACT
TTTTCCCGCGTTTTCGCAGAAACGTGGCTGGCCTGGTTCACCACGCGGGAAACGGTCTGATAAGAGACACCGGCATA
CTCTGCGACATCGTATAACGTTACTGGTTTCACATTCACCACCCTGAATTGACTCTCTTCCGGGCGCTATCATGCCA
TACCGCGAAAGGTTTTGCGCCATTCGATGGTGTCCGGGATCTCGACGCTCTCCCTTATGCGACTCCTGCATTAGGAA
GCAGCCCAGTAGTAGGTTGAGGCCGTTGAGCACCGCCGCCGCAAGGAATGGTGCATGCAAGGAGATGGCGCCCAACA
GTCCCCCGGCCACGGGGCCTGCCACCATACCCACGCCGAAACAAGCGCTCATGAGCCCGAAGTGGCGAGCCCGATCT
TCCCCATCGGTGATGTCGGCGATATAGGCGCCAGCAACCGCACCTGTGGCGCCGGTGATGCCGGCCACGATGCGTCC
GGCGTAGAGGATCGAGATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTC
CCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGAAATACCTGCTGCCGACCGCTGCTGCTGG
TCTGCTGCTCCTCGCTGCCCAGCCGGCGATGGCCATGGCTTCGAAAGTTTATGATCCAGAACAAAGGAAACGGATGA

TAACTGGTCCGCAGTGGTGGGCCAGATGTAAACAAATGAATGTTCTTGATTCATTTATTAATTATTATGATTCAGAA
AAACATGCAGAAAATGCTGTTATTTTTTTACATGGTAACGCGaccTCTTCTTATTTATGGCGACATGTTGTGCCACA
TATTGAGCCAGTAGCGCGGTGTATTATACCAGATCTTATTGGTATGGGCAAATCAGGCAAATCTGGTAATGGTTCTT
ATAGGTTACTTGATCATTACAAATATCTTACTGCATGGTTTGAACTTCTTAATTTACCAAAGAAGATCATTTTTGTC
GGCCATGATTGGGGTGCTgcgTTGGCATTTCATTATgcgTATGAGCATCAAGATcgtATCAAAGCAATAGTTCACat
gGAAAGTGTAGTAGATGTGATTGAATCATGGGATGAATGGCCTGATATTGAAGAAGATATTGCGTTGATCAAATCTG
AAGAAGGAGAAAAAATGGTTTTGGAGAATAACTTCTTCGTGGAAACCgtgTTGCCATCAAAAATCATGAGAAAGTTA
GAACCAGAAGAATTTGCAGCATATCTTGAACCATTCAAAGAGAAAGGTGAAGTTCGTCGTCCAACATTATCATGGCC
TCGTGAAATCCCGTTAGTAAAAGGTGGTAAACCTGACGTTGTACAAATTGTTAGGAATTATAATGCTTATCTACGTG
CAAGTGATGATTTACCAAAActgTTTATTGAATCGGATCCAGGATTCTTTTCCAATGCTATTGTTGAAGGCGCCAAG
AAGTTTCCTAATACTGAATTTGTCAAAGTAAAAGGTCTTCATTTTctgCAAGAAGATGCACCTGATGAAATGGGAAA
ATATATCAAATCGTTCGTTGAGCGAGTTCTCAAAAATGAACAActcgaGCACCACCACCACCACCACTGAGATCCGG
CTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCC
TCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATATCCGGAT – 3′

A.2 *Luc9 DNA sequence and primers (5′ → 3′)* highlighted on the pET22 Luc9 plasmid subsequence.

Single stranded DNA amplified based on these primers was used as the scaffold strand for the 12 helix bundle DNA NP.

Legend: Luc9 gene sequence; T7 promoter; ***RBS site***; Primer set 1; Primer set 2; Primer set 3

5′: GCTTAATGCGCCGCTACA
3′: CATACCCACGCCGAAACA

5′: AGGGAAGAAAGCGAAAGGAG
3′: CGACTCCTGCATTAGGAAGC

5′: TTTAGAGCTTGACGGGGAAA
3′: AATGGTGCATGCAAGGAGAT
5′–
…CCCTAATCAAGTTTTTTGGGGTCGAGGTGCCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGG
GAAAGCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTG
CGCGTAACCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCCCATTCGCCAATCCGGATATAGTTCCTCCTTTCAGC
AAAAAACCCCTCAAGACCCGTTTAGAGGCCCCAAGGGGTTATGCTAGTTATTGCTCAGCGGTGGCAGCAGCCAACTCAGCTTCCTT
TCGGGCTTTGTTAGCAGCCGGATCTCAGTGGTGGTGGTGGTGGTGCtcgagTTGTTCATTTTTGAGAACTCGCTCAACGAACGATT
TGATATATTTTCCCATTTCATCAGGTGCATCTTCTTGcagAAAATGAAGACCTTTTACTTTGACAAATTCAGTATTAGGAAACTTC
TTGGCGCCTTCAACAATAGCATTGGAAAAGAATCCTGGATCCGATTCAATAAAcagTTTTGGTAAATCATCACTTGCACGTAGATA
AGCATTATAATTCCTAACAATTTGTACAACGTCAGGTTTACCACCTTTTACTAACGGGATTTCACGAGGCCATGATAATGTTGGAC
GACGAACTTCACCTTTCTCTTTGAATGGTTCAAGATATGCTGCAAATTCTTCTGGTTCTAACTTTCTCATGATTTTTGATGGCAAc
acGGTTTCCACGAAGAAGTTATTCTCCAAAACCATTTTTTCTCCTTCTTCAGATTTGATCAACGCAATATCTTCTTCAATATCAGG
CCATTCATCCCATGATTCAATCACATCTACTACACTTTCcatGTGAACTATTGCTTTGATacgATCTTGATGCTCATAcgcATAAT
GAAATGCCAAcgcAGCACCCCAATCATGGCCGACAAAAATGATCTTCTTTGGTAAATTAAGAAGTTCAAACCATGCAGTAAGATAT
TTGTAATGATCAAGTAACCTATAAGAACCATTACCAGATTTGCCTGATTTGCCCATACCAATAAGATCTGGTATAATACACCGCGC
TACTGGCTCAATATGTGGCACAACATGTCGCCATAAATAAGAAGAggtCGCGTTACCATGTAAAAAAATAACAGCATTTTCTGCAT
GTTTTTCTGAATCATAATAATTAATAAATGAATCAAGAACATTCATTTGTTTACATCTGGCCCACCACTGCGGACCAGTTATCATC
CGTTTCCTTTGTTCTGGATCATAAACTTTCGAAGCCATGGCCATCGCCGGCTGGGCAGCGAGGAGCAGCAGACCAGCAGCAGCGGT
CGGCAGCAGGTATTTCATATGTATA***TCTCCTTCTTAAAGTTAAACAAA***ATTATTTCTAGAGGGGAATTGTTATCCGCTCACAATTC
CCCTATAGTGAGTCGTATTAATTTCGCGGGATCGAGATCTCGATCCTCTACGCCGGACGCATCGTGGCCGGCATCACCGGCGCCAC
AGGTGCGGTTGCTGGCGCCTATATCGCCGACATCACCGATGGGGAAGATCGGGCTCGCCACTTCGGGCTCATGAGCGCTTGTTTCG
GCGTGGGTATGGTGGCAGGCCCCGTGGCCGGGGGACTGTTGGGCGCCATCTCCTTGCATGCACCATTCCTTGCGGCGGCGGTGCTC
AACGGCCTCAACCTACTACTGGGCTGCTTCCTAATGCAGGAGTCGCAT…–3′

A.3 *Luc9 DNA "scaffold"/gene block designed with scrambled promoter (5' → 3').*

Single stranded DNA amplified based on this template was used as the scaffold strand for the 12 helix bundle DNA NP with scrambled promoter.

Legend: Gene block sequence; current primers used (same as primer set 2); Scrambled promoter sequence

5′ –
AGGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTAACCACCACACCCGCCGCGCTTA
ATGCGCCGCTACAGGGCGCGTCCCATTCGCCAATCCGGATATAGTTCCTCCTTTCAGCAAAAAACCCCTCAAGACCCGTTTAGAGG
CCCCAAGGGGTTATGCTAGTTATTGCTCAGCGGTGGCAGCAGCCAACTCAGCTTCCTTTCGGGCTTTGTTAGCAGCCGGATCTCAG
TGGTGGTGGTGGTGGTGCtcgagTTGTTCATTTTTGAGAACTCGCTCAACGAACGATTTGATATATTTTCCCATTTCATCAGGTGC
ATCTTCTTGcagAAAATGAAGACCTTTTACTTTGACAAATTCAGTATTAGGAAACTTCTTGGCGCCTTCAACAATAGCATTGGAAA
AGAATCCTGGATCCGATTCAATAAAcagTTTTGGTAAATCATCACTTGCACGTAGATAAGCATTATAATTCCTAACAATTTGTACA
ACGTCAGGTTTACCACCTTTTACTAACGGGATTTCACGAGGCCATGATAATGTTGGACGACGAACTTCACCTTTCTCTTTGAATGG
TTCAAGATATGCTGCAAATTCTTCTGGTTCTAACTTTCTCATGATTTTTGATGGCAAcacGGTTTCCACGAAGAAGTTATTCTCCA
AAACCATTTTTTCTCCTTCTTCAGATTTGATCAACGCAATATCTTCTTCAATATCAGGCCATTCATCCCATGATTCAATCACATCT
ACTACACTTTCcatGTGAACTATTGCTTTGATacgATCTTGATGCTCATAcgcATAATGAAATGCCAAcgcAGCACCCCAATCATG
GCCGACAAAAATGATCTTCTTTGGTAAATTAAGAAGTTCAAACCATGCAGTAAGATATTTGTAATGATCAAGTAACCTATAAGAAC
CATTACCAGATTTGCCTGATTTGCCCATACCAATAAGATCTGGTATAATACACCGCGCTACTGGCTCAATATGTGGCACAACATGT
CGCCATAAATAAGAAGAggtCGCGTTACCATGTAAAAAAATAACAGCATTTTCTGCATGTTTTTCTGAATCATAATAATTAATAAA
TGAATCAAGAACATTCATTTGTTTACATCTGGCCCACCACTGCGGACCAGTTATCATCCGTTTCCTTTGTTCTGGATCATAAACTT
TCGAAGCCATGGCCATCGCCGGCTGGGCAGCGAGGAGCAGCAGACCAGCAGCAGCGGTCGGCAGCAGGTATTTCATATGTATATCT
CCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGGGAATTGTTATCCGCTCACAATTCCCTAGATTTCTAGACGATGTATTTCGCG
GGATCGAGATCTCGATCCTCTACGCCGGACGCATCGTGGCCGGCATCACCGGCGCCACAGGTGCGGTTGCTGGCGCCTATATCGCC
GACATCACCGATGGGGAAGATCGGGCTCGCCACTTCGGGCTCATGAGCGCTTGTTTCGGCGTGGGTATGGTGGCAGGCCCCGTGGC
CGGGGGACTGTTGGGCGCCATCTCCTTGCATGCACCATTCCTTGCGGCGGCGGTGCTCAACGGCCTCAACCTACTACTGGGCTGCT
TCCTAATGCAGGAGTCG – 3′

Table AS1. List of Luc9 DNA 12 helix bundle NP staples. C02 promoter staple was used for assembling the original Luc9 DNA NP while the scrambled promoter staple was used for the scrambled promoter Luc9 DNA NP. In the ss promoter DNA NP, neither was included. Tm (°C) = melting temperature.

Name	Sequence	T_m (°C)
A01	GTACTTGCCAGCGCCCTCGGGTCTTGAGGGGCTAACAAAGCCCGATCTT	73.1
A02	CGCTCCCCTCTAGAAATAACCGCACCTGTGGACGCCGAAACAAGCAGTA	71.1
A03	CCGACGCGCAGCGTGACCTGAAAGGAGGAACACCACCACTGAGATTGCA	73.1
A04	CATATCTGGTAATGGTTGCTGTTATTTTTTTCAAATGAATGTTCTCCGC	63.4
A05	TTTTTCAAAGAGAAAGGTGAGCATATCTAAC	55.8
A06	AGATCTCGATTTTT	35.2
B01	TTTTTTTGGAGAATTGAACCATTTTT	51.4
B02	ATTTGCAAGTTCGTCTGACGTTGTACAAATTGTTTTT	61
B03	AGTGGCGTCCTGCAGCTCCTTTCGCTTTCTAATCC	67.6
B04	TTTTATCATTTTTGTCGGCCATGCAAAGCAATAGTTCACATGTTTT	64
B05	GCCTGCGTCCGGCGTAGAGGATCGGCGCCCAACAGTCCGGTG	75.9
B06	CCTCGACAGCCCGATCTTCATGAGAAAGATGGCCTCGTGATC	68.4
C01	GATCATTTCAGAAAAACATGCTTTATTAATTATTATGATGAAATAC	57.1
C02 (promoter staple)	TTTTCCCGCGAAATTAATACGACTCACTATAGGGGAATTGTGA	64.2
scrambled promoter staple	TTTTCCCGCGAAATACATCGTCTAGAAATCTAGGGAATTGTGA	64.1
C03	GATGATCAAATCTGAAGTTCGTGGAAACCGTCAGAAGA	63.1
C04	CGGTGATGGAGATATACATAAGAAGATATCAAAAATCCCCAT	61.1
C05	AAAACATGGTCAGGCAATTTCTGCAAGAAGACCGG	64.7
C06	ACCTATATCCGGTGGTTCCGCCGCAAGGAATCCCG	69.8
D01	TTCAAGGAGAGATGTGATTGAATCATCG	56.8
D02	CTGCTGCCGATGATTCAAGAAAATCTTATAGGTCAAAGTAAAAGGAAGG	64.2
D03	CATGGCTTCGAAAGTTTACAA	51.4
D04	TTTTTAGGAATTATAATGCTTATTATTGAATCGGATCCAGGATTTT	59.9
D05	ATGGACGCGCCGCATTAGCAAGGAGATTTT	65.4
D06	CCTTGGTATGGGCAAATAACGCGACCTCTTCCAGTGGTGGGCCAGTCCT	72.2
E01	CCGTTATTTAATCTTATGATGAAATGGGAAACACC	58
E02	CTGAGATTTACCAACGCCAAGTCTTACTGCATGGTTGCTGCG	67.5
E03	AACGTTGCCATTGCGTTGAATGGCCTGATATTGTATGC	65.6
E04	CGTTAGTAAAAGGTCAAGTGATGCAATAACTAGCATAACCCC	62.9
E05	GTATGAGCATCAAGATGG	47.4
F01	TTTTGAAAGTGTAGTAAAAAATGGTTTTT	51.9
F02	ATCGTGTATTATACCAGTGGCGACATGTTGTAAACGGATGATAACTGGC	66.3
F03	TTGGCATTTCATATACAAATAAAGTTTCCTAATACGCCACCG	62.2
F04	TGCTTTAACTTTAAGAAGTCGGCGATATAGGAGCCCGA	64.7
F05	GTTCTACGTGGGTAAACCGTCCAACATTATCTTAG	60.9

G01	TTTTTTCTTTTCCAATTCTTAATTTACCAAAGAAGTTTT	56.6
G02	AAGCTCTAAAAGCGCCCTTAGGAAGCAGCCCGCTC	69.1
G03	CCCCGCCGGTTAACAATTGCCCAGCCGGCGATGGT	73.6
G04	ATGCGCCAGCAATTTTGTGCTGGTCTGCTGCATGT	69
G05	TTGGGGCCTGAGTTGGCTGCTTGAATTTGTTACTT	65.6
H01	CATAGCGCGGCGGGTGTGGATTGGCGAATGGAACAACTCGAGCACATAT	71.7
H02	GCGGAGATGCCGGCCACGAACGGGGCCTGCCACAGCA	77.2
H03	CTGTTTTTTGCGCTACAGGTTGAGGCCGTTGCATA	66
H04	TATATTGGGGTTGAACTGCTATTGTTGAAGGAACT	60.7
H05	AGGGCCACATTAGCGCGAAATCGTTCGTTGAAAAA	65.7

Table AS2. List of M13 DNA origami snubcube staples. Tm (°C) = melting temperature.

Sequence Name	Sequence	T_m (˚C)
Snub-cube_1_6232_V	GTT GAA AGG AAT TTT TTT GAG GAA GGT AGA ACC TAC CTT TTT ATA TCA AAA T	62
Snub-cube_12_1762_V	CCG GAA GCA AAT TTT TCT CCA ACA GGA TAC TGC GGA ATT TTT TCG TCA TAA A	66.2
Snub-cube_30_615_V	TTG GGA AGG GCT TTT TGA TCG GTG CGG GGG ATG TGC TTT TTT GCA AGG CGA T	72.6
Snub-cube_45_3424_V	TTG CTC AGT ACT TTT TCA GGC GGA TAC CGG AAT AGG TTT TTT GTA TCA CCG T	67.5
Snub-cube_2_6127_V	GAC TTT ACA AAT TTT TCA ATT CGA CAT TAA TTT TAA ATT TTT AGT TTG AGT ACC AGA AGG AGC TTT TTG GAA TTA TCA	65.5
Snub-cube_10_1915_V	AGT TCA GAA AAT TTT TCG AGA ATG ACC TAT TAT AGT CTT TTT AGA AGC AAA GCC GAA AGA CTT TTT TTC AAA TAT CGC	66.8
Snub-cube_27_1395_V	ATA AAG CCT CAT TTT TGA GCA TAA AGC CTG TAA TAC TTT TTT TTT GCG GGA GTT TTA GAA CCC TTT TTT CAT ATA TTT	67.2
Snub-cube_44_1189_V	TAT TCA ACC GTT TTT TTC TAG CTG ATG AGA TCT ACA ATT TTT AGG CTA TCA GAA TCG ATG AAC TTT TTG GTA ATC GTA	66.9
Snub-cube_2_44_V	TTA TAA ATC AAT TTT TAA GAA TAG CCT AGT AAA TTG GTT TTT GCT TGA GAT G	60.7
Snub-cube_15_200_V	GCG CCA GGG TGT TTT TGT TTT TCT TTT TCA CCG CCT GTT TTT GCC CTG AGA G	69.9
Snub-cube_33_773_V	TTG GTG TAG ATT TTT TGG GCG CAT CGG ACA GTA TCG GTT TTT CCT CAG GAA G	68.6
Snub-cube_50_2434_V	AAG AGT AAT CTT TTT TTG ACA AGA ACT ATG CGT TAT ATT TTT CAA ATT CTT A	60.5
Snub-cube_4_5817_V	CAT CGG GAG AAT TTT TAC AAT AAC GGC AAA ATC GCG CTT TTT AGA GGC GAA TAT GAT GAA ACA TTT TTA ACA TCA AGA	68.2
Snub-cube_13_1658_V	ATG CTG TAG CTT TTT TCA ACA TGT TTT TTC ATT CCA TTT TTT ATA ACA GTT GAC GAC GTT GTA TTT TTA AAC GAC GGC	67.8
Snub-cube_28_1553_V	TTG ACC ATT AGT TTT TAT ACA TTT CGA GAT TCA AAA GTT TTT GGT GAG AAA GGC TCA TTT TTT TTT TTA ACC AAT AGG	66.4
Snub-cube_48_3318_V	CCG CCA CCC TCT TTT TAG AAC CGC CAA AGA ACT GGC ATT TTT TGA TTA AGA CAA ATA CAT ACA TTT TTT AAA GGT GGC	69.1
Snub-cube_4_5974_V	TCA TCA ATA TAT TTT TAT CCT GAT TGC TAA TAG ATT ATT TTT GAG CCG TCA A	61.5
Snub-cube_17_3738_V	GAG CCG CCG CCT TTT TAG CAT TGA CAA TAT TCA CAA ATT TTT CAA ATA AAT C	64.6
Snub-cube_35_4103_V	GAC GGA AAT TAT TTT TTT CAT TAA AGT TTG GGA ATT ATT TTT GAG CCA GCA A	63
Snub-cube_52_305_V	ATT AAT TGC GTT TTT TTG CGC TCA CTA GCT GCA TTA ATT TTT TGA ATC GGC C	66.3
Snub-cube_5_5612_V	AAT AAC CTT GCT TTT TTT CTG TAA ATA TAA GAA TAA ATT TTT CAC CGG AAT CAG CTG CTC ATT TTT TTC AGT GAA TAA	66.4
Snub-cube_15_458_V	TCT AGA GGA TCT TTT TCC CGG GTA CCG TTT CCT GTG TTT TTT GAA ATT GTT ACG GAA GCA TAA TTT TTA GTG TAA AGC	68.5
Snub-cube_31_667_V	CTG GTG CCG GAT TTT TAA CCA GGC AAT CCT GTA GCC ATT TTT GCT TTC ATC AGG ATT CTC CGT TTT TTG GGA ACA AAC	70.7
Snub-cube_50_2225_V	AAA ATC TAC GTT TTT TTA ATA AAA CGA AGA TTC ATC ATT TTT GTT GAG ATT TAT AAC GCC AAA TTT TTA GGA ATT ACG	65.3
Snub-cube_6_5664_V	TTT GAA TTA CCT TTT TTT TTT TAA TGG CGT AGA TTT TTT TTT CAG GTT TAA C	61.1
Snub-cube_21_4939_V	ATT CCA AGA ACT TTT TGG GTA TTA AAA TTT TCG AGC CTT TTT AGT AAT AAG A	62
Snub-cube_36_4155_V	CCA AAG ACA AAT TTT TAG GGC GAC ATG GAA CCA GAG CTT TTT CAC CAC CGG A	69
Snub-cube_56_1501_V	TCA TTT GGG GCT TTT TGC GAG CTG AAA TTA ACA TCC ATT TTT ATA AAT CAT A	64
Snub-cube_6_5507_V	ATT TAT CAA AAT TTT TTC ATA GGT CTG TTG GGT TAT ATT TTT TAA CTA TAT GAA AGA ACG CGA TTT TTG AAA ACT TTT	65.3

Snub-cube_19_5092_V	TCT GTC CAG ACT TTT TGA CGA CAA TAC TGT TTA TCA ATT TTT CAA TAG ATA ATA ATT TAC GAG TTT TTC ATG TAG AAA	65.6
Snub-cube_34_3529_V	ATA AAC AGT TAT TTT TAT GCC CCC TGA AGT ATT AAG ATT TTT GGC TGA GAC TAA TAA GTT TAT TTT TTT TTG TCA CAA	66.1
Snub-cube_52_3632_V	TGA ATT TAC CGT TTT TTT CCA GTA AGT GTA CTG GTA ATT TTT TAA GTT TTA ACC GCC ACC CTC TTT TTA GAG CCA CCA	68.7
Snub-cube_8_5354_V	GAC CTA AAT TTT TTT TAA TGG TTT GAC TTG AAA ACA TTT TTT AGC GAT AGC T	62.1
Snub-cube_24_2590_V	GCC TGA TAA ATT TTT TTG TGT CGA AAC GAG GCG CAG ATT TTT CGG TCA ATC A	67.1
Snub-cube_38_4625_V	TCC AAA TAA GAT TTT TAA CGA TTT TTT ACA GAG AGA ATT TTT TAA CAT AAA A	59.4
Snub-cube_58_2954_V	GAG GTG AAT TTT TTT TCT TAA ACA GCT GCA CCC AGC TTT TTT ACA ATT TTA T	63.8
Snub-cube_8_5197_V	TAA TTG AGA ATT TTT TCG CCA TAT TTC CGT TTT TAT TTT TTT TTC ATC GTA GGG ACA GAT GAA TTT TTC GGT GTA CAG	67
Snub-cube_22_4835_V	CAG ATA TAG AAT TTT TGG CTT ATC CGC TCC CGA CTT GTT TTT CGG GAG GTT TCA ACC ATC GCC TTT TTC ACG CAT AAC	70.1
Snub-cube_39_4677_V	CCA GAG CCT AAT TTT TTT TGC CAG TTA CAA AGT TAC CTT TTT AGA AGG AAA CTA GCA AGC CCA TTT TTA TAG GAA CCC	68.3
Snub-cube_53_3999_V	CCG GAA ACG TCT TTT TAC CAA TGA AAA GAA TCA AGT TTT TTT TGC CTT TAG CCG GTC ATA GCC TTT TTC CCT TAT TAG	68.5
Snub-cube_10_2072_V	CTC GTT TAC CAT TTT TGA CGA CGA TAC CTT ATG CGA TTT TTT TTT AAG AAC T	63.7
Snub-cube_27_2748_V	CAT GAG GAA GTT TTT TTT CCA TTA AAC CAA CCT AAA ATT TTT CGA AAG AGG C	63.7
Snub-cube_42_3112_V	ATG GGA TTT TGT TTT TCT AAA CAA CTG AAC AAC TAA ATT TTT GGA ATT GCG A	63.7
Snub-cube_60_1032_V	TTG ATA ATC AGT TTT TAA AAG CCC CAA TTG TAA ACG TTT TTT TAA TAT TTT G	60.9
Snub-cube_9_2020_V	GAA GTT TTG CCT TTT TAG AGG GGG TAT TTG ATA AGA GTT TTT GTC ATT TTT GCC AGC AGG CGA TTT TTA AAT CCT GTT	68.8
Snub-cube_25_2642_V	CCC AGC GAT TAT TTT TTA CCA AGC GCG TTA AAG GCC GTT TTT CTT TTG CGG GCG GAA CGA GGG TTT TTT AGC AAC GGC	72.3
Snub-cube_40_3006_V	AAA AAG GCT CCT TTT TAA AAG GAG CCA CAA ACT ACA ATT TTT CGC CTG TAG CAC GAT CTA AAG TTT TTT TTT GTC GTC	68.8
Snub-cube_54_4519_V	GAA CAC CCT GAT TTT TAC AAA GTC AGC CCA CAA GAA TTT TTT TGA GTT AAG CGC AAT AGC TAT TTT TTC TTA CCG AAG	68.1
Snub-cube_1_2338_E	AGA ACG AGC GAG ATA GGG TTG AGT GTT GTT C	62.6
Snub-cube_5_5878_E	AAG AAA TTG AAA CAG TAC ATA AAT CAA TAT A	50.9
Snub-cube_9_2286_E	GTG AAT TAA AAA CCA AAA TAG CGA GAG GCT T	58.1
Snub-cube_13_1666_E	TGA ATA TAA GTT GCA GCA AGC GGT CCA CGC T	64.7
Snub-cube_17_3901_E	GGC ATT TTG TCA GAC TGT AGC GCG CCA GAA C	65.7
Snub-cube_21_4790_E	TAG CGA ACG TAT TCT AAG AAC GCG CTG TCT T	62.3
Snub-cube_1_6240_E	AAT CAA CAG GCT TGC CCT GAC GAG AAA CAC C	65.1
Snub-cube_5_5620_E	TGT GAG TGT ATT TGC ACG TAA AAC AGA AAT A	57.2
Snub-cube_9_2028_E	TTG CAA AAG TTT AAT TTC AAC TTT AAT CAT T	52.7
Snub-cube_13_103_E	GGT TTG CCC GGA TGG CTT AGA GCT TAA TTG C	64.3
Snub-cube_17_3746_E	CAC CAC CAC CCT CAG AGC CGC CAT TTT CAT C	66.9
Snub-cube_21_4947_E	TCC TTA TCC CAA TCA ATA ATC GGA GGC GTT T	61.1
Snub-cube_2_6135_E	AAG TAT TAT AGA TAA TAC ATT TGA AAT CGG C	52.5
Snub-cube_6_5515_E	AAT AGT GAT AGA TTA AGA CGC TGA CAT TTA A	54.7
Snub-cube_10_1923_E	CTT TAA ACT ATT CAT TGA ATC CCC AAC ACT A	55.6

Snub-cube_14_155_E	GAT TGC CCT CAC CAG TGA GAC GGG TTT GCC C	68.3
Snub-cube_18_3954_E	GTA GCG ACC CAT CGA TAG CAG CAC CCT GAG C	67.4
Snub-cube_22_4843_E	AAG CAA ATT AAG GGA ACC GAA CTG ACC AAC T	61.7
Snub-cube_2_52_E	AAA ATC CCT GAT GGT GGT TCC GAG GAT TTA G	61.5
Snub-cube_6_5672_E	CAA TTT CAA AAC AAA ATT AAT TAG AAG AGT C	51.5
Snub-cube_10_2080_E	TCA TAA CCA GGC ATA GTA AGA GCC TCA AAT G	59.7
Snub-cube_14_6084_E	GAA CGT TAA CTC GTA TTA AAT CCC AAC AGC T	58.7
Snub-cube_18_5723_E	AAA AGA AGT ATT CAT TTC AAT TAC GTA ATC A	52.7
Snub-cube_22_2493_E	TTG AAA GAG AAT CAT TAC CGC GCC CAA TAG C	62.1
Snub-cube_3_6188_E	ACT AAC AAT TTG GAT TAT ACT TCT GAA TAA T	52
Snub-cube_7_5568_E	TTA GAA TCA ATA CCG ACC GTG TGA TAA ATA A	56.2
Snub-cube_11_1976_E	AGC GTC CAT CAG GAT TAG AGA GTA CCT TTA A	59.9
Snub-cube_15_466_E	AGG TCG ACA ACG CGC GGG GAG AGG CGG TTT G	72.2
Snub-cube_19_5100_E	AAA GTA ATG AAT ATA AAG TAC CGA ATC CAA T	53.6
Snub-cube_23_2545_E	AGC CGG AAT CCG CGA CCT GCT CCA ATT ATT A	65.6
Snub-cube_3_5930_E	GGA AGG GTT TAT CTA AAA TAT CTT TAG GAG C	55.4
Snub-cube_7_5310_E	GGC GTT AAC GTC GCT ATT AAT TAA TTT TCC C	58.3
Snub-cube_11_1718_E	TTG CTC CTA TAG TAA AAT GTT TAG ACT GGA T	55.9
Snub-cube_15_208_E	CGT ATT GGC AGT GCC AAG CTT GCA TGC CTG C	68
Snub-cube_19_5413_E	CGC AAG ACT AAA TGC TGA TGC AAC AAA AGG T	61.4
Snub-cube_23_2182_E	CAG GTA GAA ACT AAC GGA ACA ACT GTT ACT T	58.3
Snub-cube_4_5825_E	ACC TTT TAG TCA GAT GAA TAT ACA TCA GAT G	54.8
Snub-cube_8_5205_E	GTA GGG CTC CAG TAT AAA GCC AAC TTA ATT T	58.5
Snub-cube_12_1613_E	TCT GGA AGT AAA TAT GCA ACT AAA TCA AAG C	56.1
Snub-cube_16_3693_E	TGG CCT TGG GAG GTT GAG GCA GGT TTG AAT A	65.9
Snub-cube_20_5153_E	GCA GAG GCC CAA GTA CCG CAC TCA TCG AGA A	67.2
Snub-cube_24_2858_E	TGC AGG GAG AAA CAA AGT ACA ACG GAG ATT T	61.5
Snub-cube_4_5982_E	ATG GCA ATT CAT ATT CCT GAT TAG TAA CAG T	56.5
Snub-cube_8_5362_E	CAT CTT CTT CAA ATA TAT TTT AGG CTC AAC A	54.3
Snub-cube_12_1770_E	GAA CCA GAG TTT TAA TTC GAG CTG TAC GGT G	60.5
Snub-cube_16_5774_E	CCA AGT TAA TTC GCC TGA TTG CTC AGA CGA T	61.8
Snub-cube_20_4895_E	CAA GCA AGA ACA ACG CCA ACA TGT AAT TTA G	58.8
Snub-cube_24_2598_E	GTA TCA TCC GAT ATA TTC GGT CGC TGA GGC T	62
Snub-cube_25_2650_E	CTT TGA CCA AAA GAA TAC ACT AAA AAC TAA T	53.3
Snub-cube_29_570_E	GGC GAA AGG GCC TCT TCG CTA TTA ATC ATG G	63.6
Snub-cube_33_3484_E	AAA CAT GAC CTA TTT CGG AAC CTA ATG GGA T	59.5
Snub-cube_37_4580_E	GCA GCC TTT GTT TAA CGT CAA AAA TAA TGC A	59
Snub-cube_41_3067_E	ATA GAA AGT TCA ACA GTT TCA GCG CAA AAA C	58.5
Snub-cube_45_3432_E	GCG GGG TTA ACA TAT AAA AGA AAC GCA AAG A	59.4
Snub-cube_25_2131_E	GCA GAT ACA GGA ATA CCA CAT TCA CAC TCA T	59.8

Snub-cube_29_415_E	TCA TAG CTG AGC TCG AAT TCG TAC GCC AGC T	65.1
Snub-cube_33_781_E	AGG TCA CGG GCG GAT TGA CCG TAT TAT TCT G	64.2
Snub-cube_37_5049_E	GAA CGC GCA ACA ACA TGT TCA GCT GAA AAT A	61.5
Snub-cube_41_1352_E	ATT ATG ACC TAA ATC GGT TGT ACG AGT GAG A	58.7
Snub-cube_45_4214_E	CAC CAC GGC CTC AAG AGA AGG ATT AGG ATT A	62.3
Snub-cube_26_2703_E	CGA AGG CAC GGG TAA AAT ACG TAA AGG TCT T	61.6
Snub-cube_30_883_E	TCT GGC CTA GCG CCA TTC GCC ATT CAG GCT G	69.4
Snub-cube_34_3537_E	GTG CCC GTA CCG CCT CCC TCA GAG CCG CCA C	73.7
Snub-cube_38_4371_E	ATA GCC GAA CAA AAT AAA CAG CCA TAT TAT T	55.8
Snub-cube_42_1091_E	AAC AAG AGG TCA TTG CCT GAG AGT AAA TGA A	59.7
Snub-cube_46_4266_E	AAC GTA GAT CCT TAT TAC GCA GTA AGA AAC A	57.6
Snub-cube_26_1872_E	TAC CCT GAC ATA AAT CAA AAA TCT GCC ACT A	58
Snub-cube_30_623_E	CGC AAC TGA ACG CCA TCA AAA ATA ATT CGC G	62.2
Snub-cube_34_3797_E	CCT CAG AAC GGG GTC AGT GCC TTG AGT AAC A	65.5
Snub-cube_38_4633_E	TAT CCC AAC CCT TTT TAA GAA AAG TAA GCA G	57.3
Snub-cube_42_3120_E	TTT TCT GTT TTC CAG ACG TTA GTC TGG AGC A	61.2
Snub-cube_46_4423_E	ATG AAA TAC CAA TAA TAA GAG CAT GTT AGC A	55.3
Snub-cube_27_1403_E	ATT AAG CAC AGG CAA GGC AAA GAA GAC TAA A	60.3
Snub-cube_31_675_E	CAC CGC TTA TCG CAC TCC AGC CAG ACA CAA C	66.6
Snub-cube_35_3849_E	AAA TCA CCT CAA CCG ATT GAG GGA GGG AAG G	64.3
Snub-cube_39_4685_E	GCG TCT TTC CTG AAT CTT ACC AAC AAT AAT A	57.1
Snub-cube_43_1144_E	ATT TTT GAA AAT TAA TGC CGG AGA CGC AAG G	60.1
Snub-cube_47_4319_E	ATA CCC AAC CCT CAG AGC CAC CAC CCT CAT T	66.8
Snub-cube_27_2756_E	GAC TTT TTT ACA GAG GCT TTG AGT TAG CAA A	57.8
Snub-cube_31_364_E	ATA CGA GCT CCG CTC ACA ATT CCC TTT CCG G	65.9
Snub-cube_35_4111_E	TAA ATA TTC GTT TGC CAT CTT TTC ATA ATC A	54.4
Snub-cube_39_4998_E	TCC CAT CCG TCC TGA ACA AGA AAG CTA ACG A	63.7
Snub-cube_43_1301_E	ATA AAA ATA AGC CTT TAT TTC AAG GGT AGC T	55.5
Snub-cube_47_3274_E	TTC AGG GAC GAG GAA ACG CAA TAA TAA CGG A	62.5
Snub-cube_28_1561_E	GAT TTA GTT AAG TTG GGT AAC GCC AGG GTT T	60.4
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Snub-cube_36_4474_E	AGA GAT AAA GGG TAA TTG AGC GCT TAT GGT T	59.4
Snub-cube_40_3014_E	TCT CCA AAA TAA TAA TTT TTT CAC AGC GAA A	54.9
Snub-cube_44_1197_E	CAA TAT GAT TAA AAT TCG CAT TAA ATT TTT G	50.8
Snub-cube_48_3326_E	CCT CAG AAA CTC AGG AGG TTT AGT CTC ATA G	59.1
Snub-cube_28_518_E	TCC CAG TCA TTC CCA ATT CTG CGA ACG AGT A	63.8
Snub-cube_32_3589_E	TAC AGG AGC GTC ATA CAT GGC TTA GTT TGA G	61
Snub-cube_36_4163_E	TAC CAG CGT CAA TAG AAA ATT CAA ATA TCA G	55.7
Snub-cube_40_2807_E	GAC AGC ATA TCG TCA CCC TCA GCG TTG AAA A	63.2
Snub-cube_44_935_E	TTA AAT CAG CCG GAG ACA GTC AAA TCA CCA T	61.3

Snub-cube_48_3171_E	TTA GCG TAA TTC CAC AGA CAG CCA CCG CCA C	66.2
Snub-cube_49_5258_E	TAG TAT CAC GGA TAT TCA TTA CCC AAA TCA A	56
Snub-cube_52_3640_E	GCA GTC TCC TCA TTA AAG CCA GAA AGT GAG C	62.1
Snub-cube_55_1456_E	GTA GTA GCA AGG TGG CAT CAA TTC AGA TTA A	58.5
Snub-cube_58_3222_E	TCA CCA GTT TTA ATT GTA TCG GTT TAT CAG C	57.4
Snub-cube_49_2390_E	CGT AAC AAA TAA TTA CTA GAA AAA GCC TGT T	54.6
Snub-cube_52_313_E	TAA CTC ACC TGG GGT GCC TAA TGT GGA AAG C	64.6
Snub-cube_55_1821_E	GAG GAA GCC GGA TTG CAT CAA AAT ACT AAT A	58.6
Snub-cube_58_2962_E	TTG CTT TCA TGT ACC GTA ACA CTG AGT TTC G	60.4
Snub-cube_50_2233_E	TGG GAA GAG GCT CAT TAT ACC AGT TGG CTG A	63.6
Snub-cube_53_4007_E	TAG CAA GGA ATC ACC AGT AGC ACC TAA CCT C	62.2
Snub-cube_56_1249_E	GTA GGT AAC AAA TGG TCA ATA ACC TGT TTA G	55.9
Snub-cube_59_987_E	ATA TTT AAA AAA CAG GAA GAT TGT GAG TAA C	53.1
Snub-cube_50_2442_E	CCT TCA TCA CCA GGC GCA TAG GCC AGG ACG T	69
Snub-cube_53_5464_E	CGG CTT AGG AGA GAC TAC CTT TTA TTA CCA T	59
Snub-cube_56_1509_E	CTA TAT TTT AAA TGC AAT GCC TGA GTA ATG T	54.7
Snub-cube_59_832_E	AAC CCG TCA CAT TAA ATG TGA GCA TAA GCA A	60.5
Snub-cube_51_260_E	GTC GTG CCG CCC GCT TTC CAG TCG GGA ACA A	71
Snub-cube_54_4527_E	AAT TAA CTA CAG GGA AGC GCA TTA CAC CGA C	61.6
Snub-cube_57_4738_E	TGC TAT TTT TGA TAC CGA TAG TTG CGC CGA C	61.9
Snub-cube_60_3379_E	AGT ATA GCA GTG CCG TCG AGA GGG TCA TAT G	63.4
Snub-cube_51_6033_E	AGA AAC CAA CAT TAT CAT TTT GCG GAA ACC T	59.3
Snub-cube_54_4060_E	TTG AGC CAG TGA ATT ATC ACC GTG ACG GGA G	64.3
Snub-cube_57_2910_E	AAT GAC AAT GAA GCC TTA AAT CAA GAT TAG T	55.6
Snub-cube_60_1040_E	TAC CCC GGA AAC TAG CAT GTC AAT TGA TAT A	59

Table AS3: Average bioluminescence values at t = 0s for each sample.

S.D. represents standard deviation about the mean of n = 3-6 replicates. Bolded dsDNA Luc9 sample was used to normalize the values to 100%.

Sample	Luminescence (counts) **	S.D.
Buffer only	7.5	4.5
ssDNA M13 scaffold	8.5	4.5
pET-22 Luc9 plasmid	169483	4331
dsDNA Luc9	**185319.5**	**414.5**
dsDNA* (scrambled promoter)	626	71
ssDNA Luc9	26168.5	2457.5
ssDNA* (scrambled promoter)	383	55
Unpurified DNA NP	110248.5	3653.5
Original DNA NP	129193.5	1192.5
PEG-supernatant	7355	751
ssDNA promoter DNA NP	20517.5	1292.5
ssDNA promoter NP PEG-sup.	1316	169
DNA NP* (scrambled promoter)	58	19
Scrambled promoter NP PEG-sup.	74	0
M13 DNA snubcube	30.5	3.5

Notes: NP – nanoparticle; PEG buffer - 15% w/v PEG-800 5 mM Tris-HCl, 505 mM NaCl, 12.5 mM $MgCl_2$;

*Indicates presence of scrambled promoter.

**Values derived from at least 3 to 6 separate independently-assembled experimental replicates.

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Tables 1.1-1.4

Between hope and reality: treatment of genetic diseases through nucleic acid-based drugs

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Publication: Communications Biology
Publisher: Springer Nature
Date: Apr 23, 2024

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Last updated October 2022

Figure 2.4 and Figure 2.5

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