DNA Origami as a Drug Delivery Vehicle for *in vitro* and *in vivo* Applications

THESIS

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Abstract

DNA origami nanostructure technology allows for the precise control of size and structure formation using the building blocks of life. Here, DNA was not used as the blueprint for protein formation but as a delivery vehicle for chemotherapeutic drugs, such as the anthracycline antibiotic, daunorubicin. By itself, daunorubicin has limited pharmacokinetics and biodistribution profiles when applied in vivo. In addition, daunorubicin, like most small molecule drugs, is ineffective against cancer cells that have acquired multi-drug resistance (MDR). By delivering the chemotherapeutic using DNA origami allows the drug to travel through the endolysosomal pathway, bypassing MDR mechanisms. Here, we were able to overcome MDR mechanisms in a liquid tumor cell line using the “Trojan Horse” DNA origami nanostructure as a drug delivery vehicle.

Though promising, there are many barriers to pass before DNA origami nanostructures is a viable option for clinical use. This includes commercial level scale-up, target specificity and testing for immunogenicity and toxicity in vivo. Here, we discuss a method developed for the scale up of DNA origami production by 1500x standard volumetric reaction amounts. In addition, we were able to characterize a multitude of nanostructures for a more universal scaled process. Furthermore, we measured the effects that high concentrations of DNA origami nanostructures have in a mouse model. Lastly, since the binding and subsequent cellular internalization of DNA origami is non-specific, we were able to attached strategically located antibodies allowing for not only targeted drug specificity, but also blocking non-specific cell uptake. With these additions, the hope is that effective chemotherapeutics can be delivered to tumor sites while avoiding undesirable damage to healthy tissues in a clinical setting.
This work is dedicated to my wife, Jessica.
Acknowledgments

I would like to begin by thanking Dr. Castro for starting OhioMOD, Ohio State’s biomolecular design team; for without that opportunity, I would be miserable working in a factory right now. Also, to thank him for his continued support and occasionally letting me try things that no sane person would ever do, I’m not sure what that says about the two of us. In addition, I would like to thank the giants of the DNA origami field, Paul Rothemund and Shawn Douglas, and even though they do not know me, they have had a profound effect on my life.

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Fields of Study
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# Table of Contents

Abstract .......................................................................................................................... ii  
Acknowledgments ........................................................................................................ iv  
Vita ................................................................................................................................. vi  
Publications ................................................................................................................ vi  
Fields of Study ........................................................................................................... vi  
List of Tables .............................................................................................................. xiii  

Chapter 1 : An Introduction to Nanotechnology for Biological Uses ................................ 1  
  1.1 Introduction ........................................................................................................ 1  
  1.2 Background and Literature Review .................................................................... 1  
    1.2.1 DNA Structure .......................................................................................... 1  
    1.2.2 Nanotechnology Review ........................................................................ 3  

Chapter 2 Fabrication, Characterization, and Design of DNA Origami Nanostructures .. 10  
  2.1 Standard Processes for DNA Fabrication and Verification ................................. 10  
    2.1.1 CAD Design ............................................................................................ 10  
    2.1.2 Fabrication of DNA Origami Nanostructures ........................................... 12  
    2.1.3 Agarose Gel Electrophoresis ................................................................. 13  
    2.1.4 TEM of DNA Origami Nanostructures .................................................... 13  
  2.2 Methods and Protocols Developed ................................................................... 14  
    2.2.1 Rapid Fold .............................................................................................. 14  
    2.2.2 Stability of DNA Nanostructures ......................................................... 17  
    2.2.3 TEM Preparation of Cells ................................................................. 18  
    2.2.4 SEM Preparation of Cells ................................................................. 19  
  2.3 Structure Catalog ............................................................................................... 21  
    2.3.1 Trojan ‘Horse’ ...................................................................................... 21  
    2.3.2 DNA Hinge ............................................................................................ 23  
    2.3.3 Bennett Linkage .................................................................................... 25  
    2.3.5 Platform ............................................................................................... 27  
    2.3.4 Rothemund Rectangle .......................................................................... 29  
    2.3.6 18-Helix Bundle, Symmetrical Cross Section ....................................... 31  
  2.4 Design Considerations ....................................................................................... 33  

Chapter 3 : In Vitro Delivery of the Classic Chemotherapeutic Drug, Daunorubicin, to  
Circumvent Drug-Resistance ...................................................................................... 37  
  3.1 Design and construction of a DNA origami engineered rod-like Horse nanostructure drug delivery system ............................................................ 37  
  3.2 Daunorubicin intercalates into Horse DNA nanostructures ............................... 42  
  3.3 Daunorubicin-loaded Horse DNA nanostructures are internalized by HL-60/ADR cells ......................................................... 50  
  3.4 Visualization of Horse DNA nanostructure entry and lysosomal compartment localization in HL-60/ADR cells ......................................................... 55  
  3.5 Daunorubicin-loaded Horse DNA nanostructures circumvent drug resistance in HL-60/ADR cells .............................................................. 58  
  3.6 EXPERIMENTAL SECTION ...................................................................... 68
Chapter 4: Scaled Up Batch Production of DNA Origami Nanostructures

4.1 Introduction ............................................................................................................. 76
4.2 Characterizing DNA origami folding as a basis for scale up .................................... 79
  4.2.1 Critical Temperature ......................................................................................... 81
  4.2.2 Kinetics of Three Temperature Ramps ............................................................. 81
4.3 Scaling folding with a panel of DNA origami nanostructures .................................... 82
  4.3.1 DNA Origami Designs, goals of panel ............................................................... 83
  4.3.2 Mega Fold Scale Up ......................................................................................... 84
4.3 Thermal Annealing for Robust Self Assembly (TARSA) ............................................. 89
4.4 Further Alternative Methods .................................................................................. 92
4.5 Discussion ................................................................................................................ 93
4.6 Conclusions ............................................................................................................ 96
4.7 Materials and Methods ......................................................................................... 96

Chapter 5: Antibody Targeting Drug Delivery and in vivo Application of DNA nanotechnology

5.1 Introduction ............................................................................................................ 98
5.2 Antibody targeted DNA origami nanostructures ..................................................... 98
  5.2.1 Horse Redesign and Attachment of Antibody Modification .............................. 99
  5.2.2 Targeting DNA horse nanostructures to HL60, CD33+ cells ........................... 104
  5.2.3 Daunorubicin Loaded, Antibody Targeted DNA nanostructures ....................... 111
5.3 In vivo Pilot for DNA Origami Horse Nanostructure ............................................... 115
  5.3.1 Injection Method and Biodistribution ............................................................... 115
  5.3.2 Toxicity of DNA origami nanostructures in vivo ............................................. 119
  5.3.3 Immunogenicity of DNA origami nanostructures in vivo ................................ 121
5.4 Conclusions and Outlook ..................................................................................... 127

Works Cited .................................................................................................................. 128

Appendix A: Matlab Code for averaging of TEM images ............................................ 135
Appendix B: cadnano images ...................................................................................... 140
List of Figures

Figure 1.1. Watson-Crick Model, showing basic structure of double-helix DNA, adapted from (2) ................................................................. 2
Figure 1.2. Hydrogen Bonding Between Nucleotides, adapted from (2) .......... 3
Figure 1.3. Custom Shapes Using RNA Nanotechnology, adapted from (1) .... 5
Figure 1.4. RNA nanostructure delivery of siRNA, modified from (6) .......... 6
Figure 1.5. Seeman’s initial concept that formed a basis for DNA Nanotechnology, Rank 4 Nucleic Acid Junction, modified from (3) .......... 7
Figure 1.6. Early Example of the Power of Scaffolded DNA nanotechnology, modified from (5) ................................................................. 7
Figure 1.7. Examples of DNA origami increasing complexity, modified from (4) ................................................................. 8
Figure 2.1. Example of scaffold routing with internal crossover “seam” .......... 11
Figure 2.2. Design Concepts of DNA origami nanostructures, adapted from (7) on left and (9) on right ................................................................. 12
Figure 2.3. Representative agarose gel and TEM Image ........................................ 14
Figure 2.4. Agarose gel electrophoresis examples of rapid fold reactions .......... 15
Figure 2.5. TEM images of HL60 parental cell line, preparation using protocol listed ................................................................. 17
Figure 2.6. SEM images of HL60 parental cells, using protocol listed .......... 20
Figure 2.7. The DNA ‘Trojan horse’ nanostructure ........................................ 21
Figure 2.8. Introduction to the DNA hinge nanostructure ........................................ 23
Figure 2.9. Folding characterization of the hinge nanostructure ......................... 24
Figure 2.10. Introduction to the DNA Bennett Linkage nanostructure ................. 25
Figure 2.11. Folding characterization of the Bennett Linkage nanostructure ........ 26
Figure 2.12. Introduction to the platform nanostructure ........................................ 27
Figure 2.13. Folding characterization of the platform nanostructure .................. 28
Figure 2.14. Introduction to the Rothemund Rectangle ........................................ 29
Figure 2.15. Folding characterization of the rectangle nanostructure .................. 30
Figure 2.16. Introduction to the 18-helix bundle with a symmetrical cross section. 31
Figure 2.17. Folding characterization of the 18-helix bundle nanostructure .......... 32
Figure 2.18. Example of cadnano design, highlighting various sized segments .... 33
Figure 2.19. Graph of theoretical nucleotides that are bound versus temperature. 34
Figure 2.20. Histograms of DNA melting temperature of segments ................. 35
Figure 3.1. Design and construction of a DNA origami engineered rod-like Horse nanostructure drug delivery system ........................................ 38
Figure 3.2. Horse DNA nanostructures are stable in cell culture medium ............ 41
Figure 3.3. Loading of daunorubicin into Horse DNA nanostructures ............... 44
Figure 3.4. Daunorubicin effectively intercalates and releases from the Horse DNA nanostructure ................................................................. 45
Figure 3.5. Horse DNA nanostructures with varying base pair binding ratios of daunorubicin with a constant drug concentration of 0.1 µM were added to HL-
60/ADR cells for 24 hours followed by the CCK-8 assay to determine the percentage of viable cells. ................................................................. 46

**Figure 3.6.** Daunorubicin loaded Horse DNA nanostructures form aggregates at high daunorubicin concentrations. ................................................................. 47

**Figure 3.7.** Daunorubicin release profile from Horse DNA nanostructures. ........ 48

**Figure 3.8.** Daunorubicin-loaded Horse DNA nanostructures are internalized by HL-60/ADR cells. ......................................................................................... 49

**Figure 3.9.** Daunorubicin-loaded Horse DNA nanostructures are internalized by HL-60/ADR cells. ......................................................................................... 53

**Figure 3.10.** Horse DNA nanostructures enter intracellular lysosomal compartments in HL-60/ADR cells as shown via confocal microscopy. ................................. 54

**Figure 3.11.** Quantification of Cy3-Horse DNA nanostructure internalization. 55

**Figure 3.12.** Unloaded structures show limited to no toxicity on HL-60/ADR cells. 57

**Figure 3.13.** HL-60/ADR cells are drug resistant. ........................................... 58

**Figure 3.14.** Daunorubicin and daunorubicin loaded Horse DNA nanostructures induce cytotoxicity in HL-60 parental cells. .................................................. 60

**Figure 3.15.** Daunorubicin loaded double stranded (ds) M13mp18 has comparable effects on relative HL-60/ADR cell growth compared to free daunorubicin. ... 61

**Figure 3.16.** Daunorubicin-loaded Horse DNA nanostructures circumvent drug resistance in HL-60/ADR cells. ................................................................. 63

**Figure 3.17.** Daunorubicin loaded Horse DNA nanostructures reduce HL-60/ADR cellular proliferation. ................................................................. 65

**Figure 3.18.** Daunorubicin-loaded Horse DNA nanostructure drug delivery system proposed model. ................................................................................................. 66

**Figure 3.19.** Daunorubicin loaded Horse DNA nanostructures show limited effect in HL-60/ADR cell survival. ................................................................. 67

**Figure 4.1.** Folding characterization of the “horse” nanostructure. ..................... 80

**Figure 4.2.** Representative TEM images for the folding kinetics of the “horse” at different constant annealing temperatures. ..................................................... 83

**Figure 4.3.** Evolution of the Megafold. ............................................................ 85

**Figure 4.4.** Folding characterization of multiple structures using the Megafold method. ................................................................. 86

**Figure 4.5.** Verification of structure fabrication by Megafold method using TEM images. ................................................................................................. 87

**Figure 4.6.** Emission spectra of Cy3 oligo attached to “horse” nanostructure for Megafold and thermocycler folded structures. .............................................. 88

**Figure 4.7.** Summary of thermodynamic properties for the main panel of structures. ................................................................................................. 90

**Figure 4.8.** Folding characterization of the TARSA protocol of the original panel. 91

**Figure 4.9.** Folding characterization of the TARSA protocol of the expanded panel. ................................................................................................. 92

**Figure 4.10.** Trial and error approach to TARSA fold ....................................... 94

**Figure 4.11.** Experimental setup for the burner fold as depicted in Figure 4.3C. .... 95

**Figure 5.1.** DNA Horse nanostructures, with a single attachment point. .......... 99

**Figure 5.2.** Attachment Motif of Biotin/Streptavidin Antibody Conjugation. 100
Figure 5.3 Ease of Customizing DNA origami nanostructures................................. 102
Figure 5.4 Agarose Gel Electrophoresis of Antibody Conjugated Horse Nanostructures in 100% Fetal Bovine Serum for Various Time Points............ 103
Figure 5.5. CD33 targeted horse DNA origami nanostructures in human stromal and acute myeloid leukemia cells................................................................. 105
Figure 5.6. Coculture of HL60 and OSUCLL cell lines with Antibody Targeted and Streptavidin only DNA origami nanostructures........................................ 106
Figure 5.7. Colocalized Cy3 and Alexa 647 fluorophores on HL60 cells using targeted DNA origami nanostructures................................................................. 107
Figure 5.8. Antibody, streptavidin or ToPro-3 conjugated nanostructures incubated overnight in single cell populations................................................................. 109
Figure 5.9. A time course of antibody targeted DNA origami nanostructures..... 110
Figure 5.10. Antibody Targeted DNA origami nanostructure internalized through the endolysosomal pathway......................................................... 111
Figure 5.11. Overnight incubation of antibody targeted DNA origami nanostructures with daunorubicin and analyzed using a Cell Counting Kit-8 (CCK-8) (Sigma-Aldrich, St. Louis, MO)................................................................. 112
Figure 5.12. Antibody targeted, daunorubicin loaded DNA nanostructures effect on cell cocultures and drug internalization......................................................... 114
Figure 5.13. Horse DNA origami nanostructure stability in serum and plasma. ... 116
Figure 5.14. Intraperitoneal vs. Intravenous injection IVIS time course images... 117
Figure 5.15. Biodistribution of DNA origami nanostructures after injection by i.p. ......................................................................................................................... 118
Figure 5.16. Calendar for experimental plan of in vivo pilot project............... 119
Figure 5.17. Weight Analysis of DNA Origami Nanostructure in vivo........... 120
Figure 5.18. Organ Tissue Toxicity Evaluation of DNA Origami Nanostructure in vivo ............................................................................................................... 121
Figure 5.19. Ruling Out Organ Specific Accumulation of DNA origami nanostructure in vivo................................................................. 122
Figure 5.20. Cellular immunogenicity of DNA origami nanostructures in vivo.... 123
Figure 5.21. Molecular immunogenicity evaluation of DNA origami nanostructures in vivo................................................................. 125
Figure 5.22. Molecular immunogenicity evaluation of DNA origami nanostructures ex vivo............................................................................................................... 126
Figure B.1. Image of cadnano design for original “Trojan Horse” DNA origami nanostructure ........................................................................................................... 140
Figure B.2. Image of cadnano design for “Trojan Horse” DNA origami nanostructure with 36 ssDNA attachment site “overhangs” ........................................... 141
Figure B.3. Image of cadnano design for “Trojan Horse” DNA origami nanostructure with 44 ssDNA attachment site “overhangs” ........................................... 142
Figure B.4. Image of cadnano design for hinge DNA origami nanostructure.... 143
Figure B.5. Image of cadnano design for bennett linkage DNA origami nanostructure .................................................................................................................... 144
Figure B.6. Image of cadnano design for platform DNA origami nanostructure... 145
Figure B.7. Image of cadnano design for Rothemund’s Rectangle DNA origami nanostructure ................................................................. 146
Figure B.8. Image of cadnano design for 18-helix bundle with symmetrical cross section .................................................................................................................. 147
List of Tables

Table 3.1. Quantification of daunorubicin expulsion from Parental HL-60 and HL-60/ADR multidrug resistant cells. ............................................................... 51
Chapter 1: An Introduction to Nanotechnology for Biological Uses

1.1 Introduction

DNA is the design blueprint for all forms of life. This design is encoded in the form of a 4-base code and leads to the formation of RNA and proteins. Recently, DNA has also emerged as a highly promising building material for the creation of 2- and 3D nanostructures. These nearly unimaginably small structures, which are typically 50-400 nm in size, are smaller than the wavelength of visible light and over 17 million times smaller than the average human. Although they are so minute, DNA origami technology allows for precision down to ~1nm scale in their designs with possible design features including dynamic behavior, biologically-activated conformation changes, cavities for cargo, and detailed curvature (Alex, nanorobot, dietz curves). These features lend the engineer or inventor a multitude of options when delivering cancer treatments, such as small molecule drugs, targeting antibodies, or oligonucleotide therapies.

1.2 Background and Literature Review

1.2.1 DNA Structure

The Watson-Crick model of complementary double-stranded deoxyribonucleic acids (DNA) is well known and understood, shown in Figure 1.1(2). DNA consists of a combination of four molecules, adenine (A), cytosine (C), guanine (G), and thymine (T) that are combined by a phosphate bonds that make up a backbone; these molecules are known as nucleotides. When DNA is in its single stranded form (ssDNA) it is flexible and
each strand has a direction, typically denoted as going from the phosphate end group, known as the 5’ end, and the alcohol end group, known as the 3’ end. In addition, individual nucleotides will bind to their complementary nucleotide according to typical Watson-Crick base-pairing(2), in which C binds with G and T binds to A. The binding occurs by hydrogen bonding, where C binds to G using three hydrogen bonds and T binds to A with two (Figure 1.2). As a sequence of nucleotides, bound by the phosphate backbone, gets longer, the more specific and the higher affinity the binding site becomes. So in a case where there is a short strand, like CGTAG is bound to its anti-parallel complement CTACG it is a weaker bond than CGTATCGCA binding to its anti-parallel complement TGCGATACG; for this example, each sequence was written 5’ to 3’. Because of this specific binding, longer complementary strands allow for a stronger bond that remains intact up to higher temperatures (SantaLucia). In addition, when two pieces of single-stranded DNA (ssDNA) are bound together to become double-stranded DNA
(dsDNA) that takes the form of the well-known double helix, they become significantly more rigid.

1.2.2 Nanotechnology Review

![Diagram of DNA structure](image)

*Figure 1.2. Hydrogen Bonding Between Nucleotides, adapted from (2)*

The concept of using nanotechnology in chemotherapeutic drug delivery was established several decades ago. Liposomes, which are made of a lipid bilayer that form a closed sphere were approved under the formulation of Doxil in 1995(10). Doxil itself, which contains doxorubicin as its active chemotherapeutics, also contains polyethylene glycol (PEG)–polymers that increase effectiveness(11). Some of the prime qualities that
PEGylated liposomes add, when compared to free drug, is an increase in terminal elimination pharmacokinetic half-life (from ~13 to ~90 hours), and improved biodistribution to tumor sites(10). Theory states that the size of the nanoparticles, between 100-400 nm, allows them to take advantage of the enhanced permeability and retention (EPR) effect(12). This permits nanoparticles to avoid filtration by the liver and kidneys increasing the half-life, while having enhanced permeability in the leaky vasculature associated with tumor tissue. In healthy tissues with tight junctions between endothelial cells, nanoparticles typically cannot easily escape circulation; though in tumor tissue the vasculature is more chaotic and ‘leaky’, allowing these particles to pass through the vessel walls more easily. The process of the EPR effect allows for a sort of passive targeting towards tumor locations(12). Since the approval of Doxil, there have been numerous liposome encapsulated drugs that have been successful for clinical use(13).

Abraxane is another FDA approved (2005) nanoparticle used for chemotherapeutic drug delivery(14). This nanoparticle is used for the treatment of metastatic breast cancer and contains a protein, albumin, linked to paclitaxel, a highly used chemotherapeutic. The attached protein allows for transendothelial transport to tumor sites and clinical studies have shown it nearly doubles the response rate of the therapeutic, increasing patient survival(15). In addition to Abraxane, there are many polymers, such as PLGA and chitosan, which have shown promise in the delivery of paclitaxel. The advantages include a programmed drug release due to the deterioration of many polymers to be predictable in physiological environments(16).

An additional technology that has shown promise in cancer detection and biological imaging is quantum dots (QDs)(17). Traditional fluorescent dyes can present troublesome
problems such as, low photostability and broad emission spectrums\((18)\). However, QDs contain unique properties that provide alternatives to traditional fluorophores, including narrow excitation, broad emission spectrum, and improved photostability\((19)\). Though light penetration into tissue is still an obstruction towards clinical use\((14)\).

Nanometal particles are another promising alternative tumor detection. Gold nanoparticles are versatile with the ability to either absorb or scatter light; providing a contrast agent to other forms of drug delivery\((20)\). Superparamagnetic iron oxide nanoparticles (SPIONS) provide similar contrast, but can be imaged and controlled using MRI technology\((21)\), which had FDA approval under the name of Feridex.

RNA nanotechnology has great potential for delivery of therapeutics, having unique polymeric qualities that can be advantageous for various applications (Figure 1.3). With 2’OMe modifications making RNA resistant to RNase degradation and RNA nanostructures having high stability otherwise allow for this technology to be a great tool.
in the delivery of more modern chemotherapeutics, such as therapeutics oligonucleotides(22). It has been shown to deliver siRNA antisense with a folate targeting agent in vivo with accumulation in the tumor site and gene knockdown (Figure 1.4)(6). Limiting this technology is its size (~10 nm), which is not effected by EPR, and its limited payload amount.

![Figure 1.4. RNA nanostructure delivery of siRNA, modified from (6)](image)

(top left) qRT–PCR with GADPH as endogenous control and by (bottom left) western blot assay with b-actin as endogenous control. (right) 3WJ-pRNA nanoparticles target FAþ tumour xenografts on systemic administration in nude mice. (upper right) whole body (lower right) organ imaging (Lv. liver; K. kidney; H. heart; L. lung; S. spleen; I. intestine; M. muscle; T. tumour) (6).
DNA nanotechnology was first proposed by Ned Seeman in 1982, with the intention of templating crystallization on a DNA scaffold to simplify crystallography based structure determination (3). He was able to use the complementary nature of dsDNA to design junctions of DNA (Figure 1.5). These junctions became the foundation of 3D structures, such as cubes (23) in 1991 and 2D DNA sheets in 1998 (24). In 2006, Paul Rothemund introduced the concept of scaffolded DNA origami,

Figure 1.5. Seeman’s initial concept that formed a basis for DNA Nanotechnology, Rank 4 Nucleic Acid Junction, modified from (3).

Figure 1.6. Early Example of the Power of Scaffolded DNA nanotechnology, modified from (5).
which allowed for larger, more diverse nanostructures (5) (Figure 1.6). Using the bacteriophage ssDNA m13mp18 genome as a template strand, Rothemund was able to use 150-200 custom oligonucleotides to make a variety of 2D nanostructures. In 2009, Shawn Douglas added a CAD program to the field, called cadnano, that allowed researchers to build complex 3D designs with relative ease (25, 26). Cadnano has remained an open source program (cadnano.org) and nanostructures have become increasingly complex (4, 27)(Figure 1.7).

DNA origami nanostructures have been utilized for the delivery of classic chemotherapeutics, such as anthracycline antibiotics, and have future potential to be used

![Figure 1.7. Examples of DNA origami increasing complexity, modified from (4)](image)

DNA origami machine components. A framework for designing DNA origami mechanisms and machines(8) starts with designing basic joints with motion in specified degrees of freedom.
as a vehicle of therapeutic oligonucleotides. Though specific studies and discussion will occur in their relevant chapters.
Chapter 2 Fabrication, Characterization, and Design of DNA Origami Nanostructures

I would like to acknowledge input from Christopher R. Lucas, Randy A. Patton, Joshua Johnson, Thomas Rudibaugh and Carlos E. Castro for contributions in establishing, optimizing and writing the methods and protocols outlined in this chapter.

This chapter summarizes basic information about the DNA origami nanostructure design, fabrication and verification processes. In addition, it contains characterization of structures that will appear in later chapters, as well as some analysis of factors that are important design considerations such as folding kinetics, annealing temperatures, stability of structures in physiological environments, which are aspects that the design parameters of nanostructures can affect.

2.1 Standard Processes for DNA Fabrication and Verification

This section contains the basic of protocols for DNA origami fabrication and verification. For a more comprehensive description, Castro et al. provides a detailed version of the entire process from design to verification(27).

2.1.1 CAD Design

The first step of the fabrication process for DNA origami is the design. Using cadnano, an open-sourced software, to design complex 3D structures is reasonably straight forward(25). There are two sets of components that make up a DNA origami nanostructure, the M13mp18 bacteriophage DNA scaffold and 150-200 chemically synthesized oligonucleotide staples. When starting a new design, each double helix is typically
envisioned as a cylindrical rod. The scaffold is a circular loop of single-stranded DNA. The scaffold most commonly used for scaffolded DNA origami is 7249 bases long and must be arranged in a continuous loop throughout the entire intended structure (Figure 2.1), which can be setup using either the honeycomb or square cross section lattice (26). To go from one helix to the next, the scaffold, or later staple, must “crossover” to the next helix, when double stranded this makes a holiday junction (Figure 2.2A).

Once the scaffold is routed into the desired structure, the staples must be incorporated. In most structures, nearly the entire scaffold is paired with a staple and junctions are typically placed at every possible location where the staple strand is pointing in the correct direction to make a cross-over to the neighboring helix. For a honeycomb lattice, this results in cross-overs every 21 bases, and for a square lattice it is every 32 (Figure 2.2B). Staples are typically designed make each one 40-60 bases long. Finally, cadnano facilitates the process of specifying a sequence for the scaffold, generating the staple sequences according to where they bind to the scaffold, and exporting a staple
sequence list for ordering. Full cadnano designs for the structures included in this thesis can be found in Appendix B.

2.1.2 Fabrication of DNA Origami Nanostructures
As described by Castro et al. a simple mixture of folding buffer (5 mM Tris, 1 mM EDTA, 5 mM NaCl), known as FOB), magnesium chloride (typically 10-24 mM), M13mp18 (20 nM), double distilled water (ddH$_2$O), and an even mixture of the staple list, which is at 10x the concentration of the M13mp18 scaffold, all concentrations listed are final(27). The

Figure 2.2. Design Concepts of DNA origami nanostructures, adapted from (7) on left and (9) on right
(Left) Representation of a holiday junction, a feature that allows DNA origami design to exist. (Right) DNA represented in ladder, filled-in, and cylinder formats, showing important angles for DNA origami design modified from .

2.1.2 Fabrication of DNA Origami Nanostructures
As described by Castro et al. a simple mixture of folding buffer (5 mM Tris, 1 mM EDTA, 5 mM NaCl), known as FOB), magnesium chloride (typically 10-24 mM), M13mp18 (20 nM), double distilled water (ddH$_2$O), and an even mixture of the staple list, which is at 10x the concentration of the M13mp18 scaffold, all concentrations listed are final(27). The
folding reaction is then subjected to a thermal annealing ramp in a thermocycler. The traditional ramp typically starts at 65°C for 15 minutes, then slowly drop the temperature over the course of 2.5 days to room temperature and then rapidly to 4°C. The results of the folding reaction may then be analyzed by gel electrophoresis and TEM or AFM imaging.

2.1.3 Agarose Gel Electrophoresis
Agarose gel electrophoresis is an effective analysis tool for DNA origami nanostructures. Structures, or unfolded scaffold, is typically diluted to 20 nM and mixed with a blue 6x gel loading dye (New England Biolabs, Ipswich, MA). Blue dye contains an amount of EDTA to chelate 10 mM of MgCl₂, if experiment involves low MgCl₂ concentrations, a custom orange or clear dye that does not contain EDTA can be used. Structure mixed with dye, 15 µl of structure and 3 µl of dye, can be loaded into the wells of an agarose gel and a voltage of 70 V is used to create migration driving force for 2-3 hours. Gels are generally made to include 1 µM of ethidium bromide, a DNA intercalator, to stain the DNA for fluorescence under UV light. Well folded structures usually migrate in the gel as tight bands that travel farther than the scaffold. An example of a gel with well folded structures is found in Figure 2.3A. Well-folded bands are excised from the gel and centrifuged at 16000 xg for 5 minutes in a Freeze 'N Squeeze DNA Gel Extraction Spin Column (Bio-Rad Laboratories, Hercules, CA).

2.1.4 TEM of DNA Origami Nanostructures
Proper folding and high-resolution structure evaluation can then be performed on gel-purified nanostructures via imaging by transmission electron microscopy (TEM). For DNA origami, typically the DNA is stained using 2% uranyl formate (UFo) and imaging can occur at up to 150,000x. Briefly, four µL of folded structures at 5-20 nM is incubated for
four minutes on a plasma cleaned copper TEM grid. Excess liquid is wicked off and briefly replaced with a 10 µL droplet of UFo which is also wicked away and a fresh 20 µL droplet of UFo is placed onto TEM grid for 40 sec. Again wick away excess liquid and allow grid to dry, the sample is now ready for imaging. A typical TEM image can be found in Figure 2.3B.

2.2 Methods and Protocols Developed

The following section contains protocols for experiments that are not contained in other chapters. These are some useful techniques for structure analysis and cell-structure interactions.

2.2.1 Rapid Fold

Standard DNA origami nanostructure annealing ramps generally begin with a high temperature, 65°C or above, and then the temperature is lowered 1-2 degrees every hour until the temperature reaches 25°C and then the temperature is dropped to 4°C(27). Depending on the laboratory and the structure, this process can be shortened by skipping...
temperatures or reducing the time spent at each individual temperature. Times vary wildly as different papers state times between 2 hours to 7 days.

In 2012, Sobczak et al. presented a novel way of approaching the annealing process (28). They monitored the progression of folding during self-assembly using qPCR to determine at what point of the annealing ramp folding of DNA nanostructures was actually occurring. It was found that most DNA origami nanostructures can fold relatively quickly (~5-100 min) at a single temperature. Since then other groups have studied the effects of cooperativity in the annealing process, which gives rise to this fast folding at a single temperature (29-31).

While using the qPCR to discover which temperature the structures begin to fold is a powerful tool, it was apparent that the method is highly challenging, and most laboratories do not contain the equipment required for qPCR. We took a different approach, which was to screen a range of constant annealing temperatures by folding a series of reactions using a temperature gradient across the heating block of a thermocycler. In this method, assembly reactions were heated to 65°C for 15 minutes, then lowered to the gradient for four hours so each well was at a different constant annealing temperature, and

*Figure 2.4. Agarose gel electrophoresis examples of rapid fold reactions*

(Left) Example of gel using four hour ramp with 20 degree gradient, “Trojan Horse” DNA nanostructure. (Right) Gel using four hour ramp with four degree gradient “zoomed in”, “Trojan Horse” DNA nanostructure.
finally all wells were rapidly cooled to 4°C. Based on this approach, we were able to find the temperatures that are suitable for constant temperature annealing.

Surprisingly, in many cases there was a large range of temperatures in which most structures will fold. The annealing temperatures for different DNA origami nanostructures can vary over a large range, necessitating the initial temperature gradient to find appropriate annealing temperatures to cover a large range. Therefore, to better quantify the upper limit of the annealing temperature, it became necessary to perform a series of gradients to focus in on the desired temperature. The process was standardized to begin with a 60-40°C gradient and then a four-degree gradient to increase resolution on the upper limit of appropriate annealing temperatures. Gel electrophoresis was performed to verify folding of the structures over the range of annealing temperatures (Figure 2.4).
2.2.2 Stability of DNA Nanostructures

![TEM images of HL60 parental cell line, preparation using protocol listed](image)

**Figure 2.5.** TEM images of HL60 parental cell line, preparation using protocol listed

Stability of nanostructures in conditions such as low salt, plasma, or serum is imperative to the success for *in vivo* applications. Using protocols developed by Stahl *et al.* for PEG precipitation of nanostructures, we were able to easily perform buffer exchanges for individual structures(32). Then structures able to be incubated in the desired condition, typically representing some physiological condition. If the buffer is water based, i.e. PBS, FOB (5 mM Tris, 1 mM EDTA, 5 mM NaCl), TBE (89 mM Tris, 89 mM Boric Acid, 2 mM EDTA), then standard gel electrophoresis procedures can be used. If the buffer contains a heavy amount of proteins or other debris, i.e. serum or plasma, the solution must again be PEG purified for an exchange back to a buffer that allows visualizing DNA origami structures on a gel. A few examples of this process are found in Chapter 5.
2.2.3 TEM Preparation of Cells

TEM preparation of cells was an attempt to view the details of the internalization of DNA nanostructures into cells. The following protocol is an adaptation of Murray et al’s work described in 1991. Though the preparation of cells was a success, there were no DNA origami structures found. Moving forward, this could likely be fixed by increasing the concentration of nanostructures in solution. Nevertheless, establishing the ability to image cells by TEM in our laboratory was an important step. The sample preparation begins by pelleting one million cells from a suspension cell line, in this case HL60, which is a human promyelocytic leukemia cell line. The whole process can be carried out in 1.5 mL polypropylene Eppendorf tubes. Extracellular proteins and other particles were separated from the cells by washing two times with PBS. Washing cells consists of centrifuging at 300 xg for 5 minutes to pellet cells, carefully removing the supernatant buffer, and adding new buffer; once cells are fixed, centrifuging is not required, just have care to not disturb the pellet while removing liquid. Cells are then resuspended in 0.1 M phosphate buffer with 2.5% gluteraldehyde and incubated two hours, this fixes the cell, stopping all biological processes and fixing the structure in place. Next, the fixed cells are rinsed with phosphate buffer three times, allowing the cells to sit for ten minutes each time. Then, 1 mL of 1% osmium tetroxide in cacodylate buffer is added and incubated for one hour.

Rinse cells with 0.1 M cacodylate buffer five times, allowing to incubate for 10 minutes between each wash step, followed by two washes with ddH₂O, incubating for 10 minutes between each wash. Add 2% uranyl formate for one hour, then rinse one time with ddH₂O followed by incubation for 10 minutes in ddH₂O. The next steps are a process
to remove all water from the cells by gradually exchanging the water for ethanol. Each step is one rinse per ratio, starting with 30% ethanol in water by volume. Then 50%, 70%, 90%, and then 100% ethanol in succession.

Now that the water is removed, the next step is to embed the cell pellet into a resin, though again it must be moved from ethanol to the resin in steps. The resin, LX-112 (Ladd Research, Williston, VT), should be prepared separately according to manufacturer specifications. Briefly, combine mix A (129g DDSA, 100g LX-112) and mix B (87g MNA, 100g LX-112) in a 60:40 ratio with .14 mL accelerator DMP-30. Use resin mixture in ethanol in a 1:2 mixture and mix on a rotator for two hours. Next, combine resin and ethanol in a 2:1 mixture and again mix on rotor for two hours. To suspend the pellet in 100% resin mixture, mix on rotor for 4-6 hours, carefully remove and replace with fresh resin and bake for 12 hours at 45°C overnight. Break open tubes with hammer and pull away extra plastic, bake again for 16-24 hours at 45°C. Superglue resin, with pellet facing outward, onto post and section into thin slices, using Leica EM UC6 and place onto TEM grid. If more contrast is needed, stain a second time with uranyl formate. The sample is now ready for viewing on a TEM, a sample image taken form a sample prepared using this process is shown in Figure 2.5 The process of fixing, staining, and embedding cells for TEM is complex, time consuming process that takes days to finish and though nanostructures were not found using the process, it could prove successful with an increase in nanostructure concentration.

2.2.4 SEM Preparation of Cells

Fixing and staining cells for scanning electron microscopy (SEM) can be performed in a single day. The following is an adaptation of Ohio State’s Campus Microscopy and
Imaging Facility’s protocol (http://www.cmif.osu.edu/). Begin by pipetting 100 µL of poly-L-lysine on 10 mm circular glass coverslip for one hour at room temperature. Rinse with PBS and place in a small petri dish. Place 300,000 cells in 20 µL PBS buffer onto coverslip and allow to rest for two hours before fixing cells in 0.1 M phosphate buffer with 2.5% glutaraldehyde. This fixing procedure should anchor cells onto the surface, so subsequent buffers can submerge coverslip. Rinse three times in phosphate buffer, no glutaraldehyde, incubating in buffer for five minutes each time. Incubate in 1% Osmium Tetroxide in phosphate buffer for one hour, followed by three rinses in phosphate buffer, holding for five minutes each time. Now, like the TEM prep, the sample will be slowly removed from water and exchanged into full ethanol, in the following mixtures, 50%, 70%, 80%, for 10 minutes each and then 95% two times. Now, using fresh 100% ethanol, make three buffer changes within 15 minutes. Next, the sample needs to be removed from ethanol and into HDMS. For 15 minutes each time use 2:1, 1:1 and then 1:2 ethanol to
HDMS. Then, replace the buffer two times in 100% HDMS with 15 minutes of incubation each time, then remove buffer and allow coverslip to dry overnight. Stick slide onto SEM post and use a light sputter coating. A sample SEM image of HL60 cells can be found in Figure 2.6.

2.3 Structure Catalog

![Diagram of DNA Trojan 'Horse' nanostructure]

*Figure 2.7. The DNA ‘Trojan horse’ nanostructure*

Top left is the cross section of the DNA ‘Horse’ nanostructure, with each rod representing a double helix. Bottom left is the side and isometric views of the ‘Horse’ nanostructure. Right image is a represent TEM image, scale bare is 100 nm.

2.3.1 Trojan ‘Horse’

The DNA Trojan ‘horse’, as seen in Figure 2.7, is the primary structure we used as a drug delivery vehicle as discussed in Chapters 3 and 5 (33). It consists of 26 helices organized on a square lattice and containing four internal cavities (Fig. 2.7, top left). A number of design iterations on the ‘horse’ nanostructure have included ssDNA overhang attachment points at many locations on the surface or in internal cavities and in different quantities (1,
2, 4, 10, 36, 44 attachment sites). Cadnano designs for 2, 4, 10, 36, and 44 ssDNA attachment sites can be found in Appendix B.
2.3.2 DNA Hinge

The DNA hinge, presented in Figure 2.8, was introduced by Marras et al. as a dynamic structure and has well characterized mechanical properties (4, 8). It has also been used as a tool for probing nucleosome stability and structural dynamics (34). Each arm contains 18 helices organized in a six by three bundle cross section, designed using the square lattice. Figure 2.9 presents a characterization profile including rapid fold and annealing kinetics of the structure. The cadnano design can be found in Appendix B.
Figure 2.9. Folding characterization of the hinge nanostructure.
(A) Representative TEM images of nanostructures folded at different annealing temperatures and annealing times, including transitions from unfolded to folded. (B) Gel electrophoresis results for annealing time optimization at different annealing temperatures. (C) Gel electrophoresis results for annealing temperature optimization.
2.3.3 Bennett Linkage

The Bennett linkage nanostructure (Figure 2.10) consists of four links comprising 16 helices arranged in a 4x4 square lattice bundle. First introduced by Marras et al., it was presented as a dynamic structure that translated between a closed bundle configuration and an open frame configuration. The version used in Chapter 4 allows for free motion. Figure 2.11 presents a characterization profile including testing of different constant annealing temperatures and characterization of annealing kinetics of the Bennett linkage structure. The cadnano design can be found in Appendix B.

Figure 2.10. Introduction to the DNA Bennett Linkage nanostructure

Left images are different views of the Bennett Linkage nanostructure. Right image is a representative TEM image, scale bar is 100 nm.
Figure 2.11. Folding characterization of the Bennett Linkage nanostructure.
(A) Representative TEM images of nanostructures folded at different annealing temperatures and annealing times, including transitions from unfolded to folded. (B) Gel electrophoresis results for annealing time optimization at different annealing temperatures. (C) Gel electrophoresis results for annealing temperature optimization.
2.3.5 Platform

The platform nanostructure (Figure 2.12) consists of 40 helices, designed using the honeycomb lattice. The structure is designed to give a rigid, flat surface to template molecules in defined numbers and patterns. Figure 2.13 presents a characterization profile including rapid fold and annealing kinetics of the platform structure. The cadnano design can be found in Appendix B.
Figure 2.13. Folding characterization of the platform nanostructure

(A) Representative TEM images of nanostructures folded at different annealing temperatures and annealing times, including transitions from unfolded to folded. (B) Gel electrophoresis results for annealing time optimization at different annealing temperatures. (C) Gel electrophoresis results for annealing temperature optimization.
2.3.4 Rothemund Rectangle

The Rothemund Rectangle (Figure 2.14) was one of the first scaffolded DNA origami nanostructures introduced in 2006. It is made up of 25 double helices arranged side-by-side. It was chosen for the panel of structures in Chapter 4 due to its wide use in the field of DNA origami for templating molecules and nanoparticles. Because it is a relatively simple structure the Rothemund rectangle folds well across a wide variety of assembly conditions, but is only a single layer and not rigid making it difficult to visualize by TEM. Hence, we imaged the Rothemund rectangle via AFM. Characterization including rapid fold and annealing kinetics of the rectangle structure can be found in Figure 2.15. The cadnano design can be found in Appendix B.
Rothemund’s Rectangle

Figure 2.15. Folding characterization of the rectangle nanostructure.

(A) Representative AFM images of folded nanostructures folded at different annealing temperatures and annealing times, including transitions from unfolded to folded. (B) Gel electrophoresis results for annealing time optimization at different annealing temperatures. (C) Gel electrophoresis results for annealing temperature optimization.
2.3.6 18-Helix Bundle, Symmetrical Cross Section

The 18-helix bundle (Figure 2.16) consists of three six-helix bundles aligned in a symmetrical pattern. It is a rigid rod-like structure, chosen because of its similarity to the ‘horse’ nanostructure, but is designed using the honeycombed lattice. Figure 2.16 presents a characterization profile including rapid fold and annealing kinetics of the 18-helix bundle nanostructure and the cadnano design can be found in Appendix B.

*Figure 2.16. Introduction to the 18-helix bundle with a symmetrical cross section.*

Top left is the cross section of the 18-helix bundle nanostructure, with each rod representing a double helix. Bottom left is the side and isometric views of the 18-helix bundle nanostructure. Right image is a represent TEM image, scale bare is 100 nm.
Figure 2.17. Folding characterization of the 18-helix bundle nanostructure

(A) Representative AFM images of folded nanostructures folded at different annealing temperatures and annealing times, including transitions from unfolded to folded. (B) Gel electrophoresis results for annealing time optimization at different annealing temperatures. (C) Gel electrophoresis results for annealing temperature optimization.
2.4 Design Considerations

Using an in-house MATLAB program to analyze the nanostructure designs from cadnano allows for some insight on which factors can effect DNA origami formation. Recent studies have shown that the order in which a structure will fold is determined by segments of DNA with higher melting temperatures (30, 31). A segment is defined as continuous binding of DNA before a staple or scaffold crossover occurs (Figure 2.18).

We analyzed the melting temperatures of these segments using SantaLucia et al.’s nearest neighbor method (35). Due to cooperativity in the annealing process, only a small percentage of DNA oligonucleotide segments need to bind in order for a structure to fold. The catalog of characterized structures gives us a unique opportunity to correlate

Figure 2.18. Example of cadnano design, highlighting various sized segments

Section of the cadnano design of the 18-helix bundle. Light blue section is the M13mp18 scaffold, and the green are the staple oligonucleotides. Dark blue highlighting segments of different lengths and ending at either a staple crossover or strand break. Note: Not all segments are highlighted.
nanostructure formation with cadnano design. **Figure 2.19** presents the information gathered from MATLAB as the percent of DNA nucleotides that are double stranded. This plot does not consider any cooperativity that may be occurring. The graphs show only the data points for which the structure actually folds using the rapid fold gels that are in section 2.3.

A few points of interest can be gleaned from this data. The first is that structures can anneal even when only 5 to 20 percent of their bases are stably bound according to their segment melting temperature. The Bennett Linkage presents an interesting case, as the curve matches the best performing structure (Rothemund’s Rectangle), as it has over 20% of its bases bound before it folds. This is likely because its design is separated into four distinct regions. Another way to assess the data is placing the segments into a
35

Figure 2.20. Histograms of DNA melting temperature of segments

Histogram by melting temperature (Figure 2.20). By doing this, clear shifts become apparent between the structures that are designed using the square lattice and those that are designed with the honeycomb. With the honeycomb lattice, crossovers occur every 21 bases between each set of helices, which for a compact structure makes most segments
either seven or 14 bases long. However, when designing with the square lattice
crossovers occur every 32 bases, which allows for most segments to be either eight or 16
bases long. These extra few bases drive the overall melting temperatures of the segments
and allow for more thermodynamically stable structures. This analysis is limited because
of the small number of designs available that have been fully characterized.
Chapter 3 : In Vitro Delivery of the Classic Chemotherapeutic Drug, Daunorubicin, to Circumvent Drug-Resistance

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“Daunorubicin-Loaded DNA Origami Nanostructures Circumvent Drug-Resistance Mechanisms in a Leukemia Model”

The focus of this chapter is in vitro drug delivery using the classic chemotherapeutic daunorubicin. The purpose of the study was to circumvent multi drug resistant (MDR) mechanisms in a liquid tumor model. This was intended to be a first step towards clinical application of the Trojan “horse” DNA origami nanostructure as a delivery vehicle for therapeutic agents.

3.1 Design and construction of a DNA origami engineered rod-like Horse nanostructure drug delivery system

One previous report in an adherent cell line revealed that a doxorubicin-loaded DNA nanostructure engineered via DNA origami outperformed free doxorubicin in the ability to induce cell death in doxorubicin-resistant MCF-7 adenocarcinoma cells.\(^{(36)}\)

Furthermore, the effect was shown to be shape-dependent as rod-shaped DNA nanostructures loaded with doxorubicin outperformed triangular-shaped nanostructures.\(^{(36)}\)

In addition, a number of prior studies have demonstrated efficient uptake of nanoparticles 100 nm and smaller, as summarized by Shang et al.\(^{(37)}\) Together, these findings provide
strong rationale to explore the ability of a rod-shaped DNA nanostructures in the ~10-100 nm size range to serve as a drug delivery device for not only the treatment of solid tumors but also hematologic malignancies.
Here we expand on this work to characterize the function of DNA origami nanostructures for circumventing drug resistance in delivering daunorubicin at clinically relevant concentrations to leukemia cells, specifically an acute promyelocytic leukemia (APL) model. We designed the scaffolded DNA origami rod-like Horse nanostructure (~92.5 x ~13.2 x ~11 nm) using the DNA design software cadnano.\(^{(25)}\) While nanoparticle uptake in HL-60 cells has not been quantified, previous research has shown suspension cells effectively uptake particles in the range of ~100 nm.\(^{(37-40)}\) The structure contains four internal cavities in order to maximize the surface area accessible to solution. The larger cross-section also allows for increased mechanical stiffness, and the cavities yield larger thermal stability since it reduces the number of cross-overs per helix thereby increasing the length of segments (\textbf{Figure 3.1A}). The full cadnano design is provided in \textbf{Appendix B}. To ensure proper molecular self-assembly and purify well-folded structures, self-assembled nanostructures were subjected to agarose gel electrophoresis. Figure 3.1B shows an image of a gel including (from left to right) a 1kb DNA ladder (L), the 7249 M13mp18 scaffold starting material (S), the Horse DNA nanostructure (1), the Horse nanostructure containing 10 (2) and 36 (3) overhangs, a Yoyo-1-labeled Horse structure (4), and a daunorubicin-loaded Horse DNA nanostructure (5). We defined a normalized loading parameter, the base pair binding ratio, as the ratio between the concentration of daunorubicin- (drug) loaded into Horse DNA nanostructures to the total concentration of DNA base pairs in solution (sample calculation in \textbf{Equation 3-1}). The last lane of the gel shows structures loaded with a BPBR of 0.42. A low bp binding ratio was used for gel electrophoresis since highly
loaded Horse nanostructures (bp binding ratios ≥ 1) migrated in the reverse direction because of the positive charge of the daunorubicin. In all Horse DNA nanostructure reactions, sharp bands were present that ran further than the 7249 M13mp18 scaffold control indicative of well-folded structures (Figure 3.1B). Leading bands were excised and visualized via transmission electron microscopy (TEM) and atomic force microscopy (AFM) revealing well-folded Horse DNA nanostructures (Figure 3.1C). The nanostructures exhibited a banded pattern in TEM images, with the dark bands along the length corresponding to the inner cavities of the Horse geometry. Daunorubicin-loaded Horse structures at a bp binding ratio of 1.2 (TEM) and 0.8 (AFM) were visualized 24 hours post loading (Figure 3.1D) and revealed drug-loading caused a twisting and fraying effect on the Horse nanostructures consistent with previous studies,\(^{26, 41}\), which is due to the local structural changes imposed by drug intercalation. With the intention of applying Horse DNA nanostructures in cell culture conditions, we tested the stability in RPMI 1640 cell culture medium supplemented with 20% FBS at various time points at 37°C over a 24-hr period followed by analysis via agarose gel electrophoresis (Figure 3.1E). At each time point the Horse DNA nanostructures remained intact both in the presence (Figure 3.1E) or absence (Figure 3.2) of 20% FBS heat inactivated at 56°C. Interestingly, a recent study showed that both a 6-helix nanotube and a 24-helix nanorod structures were digested by serum nucleases in a time-dependent manner in standard RPMI cell culture medium supplemented with 10% FBS and heat inactivated at 56°C.\(^{42}\) The same study showed this effect could be reversed by heat inactivation of serum at 75°C or via nuclease competitive

\[
\frac{\text{Loaded Drug Concentration (µM)}}{\text{Structure Concentration (nM)}} \times \frac{1}{7.249} = \text{Basepair Binding Ratio (BPBR)}
\]
binding using actin, and the same group demonstrated membrane encapsulation can improve \textit{in vivo} stability.\textsuperscript{(43)} Our results suggest that structure design may be another viable route to obtain enhanced stability in cell culture conditions. The enhanced stability of the Horse structure may be due to the square lattice design with internal cavities, which allow for lower total cross-over density equating to higher local thermodynamic stability. Furthermore, square lattice structures contain internal stresses and denser packing of helices on the outer layer,\textsuperscript{(44)} which might inhibit nuclease recognition and degradation.

Although it is often not taken into consideration, ease of fabrication is also an essential characteristic for scalability toward pre-clinical development for a novel biomedical nanodevice. Therefore, we aimed to demonstrate straightforward and rapid assembly of the Horse nanostructures following a previous report that demonstrated assembly of DNA nanostructures on the timescale of $\sim$5-60 minutes via thermal annealing at constant temperature.\textsuperscript{(28)} To address this, Horse DNA nanostructure folding reactions were subjected to thermal annealing at 52°C utilizing a procedure described previously\textsuperscript{(28)} for various time points over a 2 hr time course followed by evaluation via agarose gel electrophoresis (Figure 3.1F). Pronounced well-folded structure bands were observed.
already after ~5 min demonstrating an ability to manufacture Horse DNA nanostructures in a robust manner over a short time scale. Interestingly Sobczak et al.\(^{(28)}\) demonstrated folding of 2D (single-layer) structures within 5 minutes, while compact 3D (multi-layer) structures took ~30-60 minutes, suggesting that in addition to enhanced stability the Horse nanostructure design is remarkably fast folding for a compact 3D nanostructure. This may also be due to the high local thermodynamic stability enabled by the square lattice and internal cavities. Taken together, these findings confirm the construction of well-folded Horse DNA nanostructures that can be easily and quickly fabricated and are capable of drug loading, albeit with slightly altered internal structure. Furthermore, Horse DNA nanostructures are stable in cell culture conditions containing 20% FBS providing strong support for its use as a novel drug delivery device.

### 3.2 Daunorubicin intercalates into Horse DNA nanostructures

While previous studies of DNA nanostructure mediated delivery employed doxorubicin,\(^{(36, 41, 45)}\) we chose to study delivery of daunorubicin due to its widespread use in the clinic for treatment against adult and pediatric acute leukemia.\(^{(46-50)}\) To optimize drug loading, daunorubicin was mixed with Horse DNA nanostructures at varying conditions. The concentration of daunorubicin loaded into DNA nanostructures was determined from the concentration difference between free daunorubicin in solution prior to and post Horse nanostructure addition as determined by absorbance measurements at 480 nm, the absorption wavelength of daunorubicin.\(^{(51)}\) The procedure for loading and concentration measurements is depicted schematically in the inset of Figure 3.3A. Daunorubicin at a concentration of 250 µM was incubated with 10 nM Horse DNA nanostructures at varying temperatures and for a range of times. Base pair binding ratios were comparable as a
function of temperature (data not shown) and drug loading increased up to ~30 min of incubation and then remained steady for up to several days (Figure S3.3). In addition, varying concentrations of Horse nanostructures (5-240 nM) were mixed with 500 µM daunorubicin, and in separate experiments varying
Figure 3.3. Loading of daunorubicin into Horse DNA nanostructures.

(A) We quantified drug loading in terms of the basepair binding ratio, which defines the ratio of drug molecules per dsDNA base-pair in the structure, and the fraction of drug loaded, which defines the ratio of the drug loaded into the structure divided by the total amount of drug initially in solution. (B) Effect of incubation time for daunorubicin loading into Horse DNA nanostructure. 30 nM of Horse nanostructures were added to a 500 µM daunorubicin solution for varying time points (30min—2weeks) followed by absorbance determination at 480 nm, the absorbance wavelength of daunorubicin. The BPBR and fraction of loaded daunorubicin is plotted as a function of time. The fraction of drug loaded was also quantified for (C) varying DNA origami structure concentration incubated with 500 µM daunorubicin and (D) varying daunorubicin concentrations incubated with 20 nM nanostructure. Note that (C) and (D) are the same results as in Figure 2 quantified in terms of fraction of drug loaded. (E) Agarose gel electrophoresis to assess electrophoretic mobility (left to right; L = DNA ladder, S = 7249 M13mp18 scaffold, 1 = free daunorubicin (125 µM), 2 = unloaded Horse DNA nanostructure (20 nM), 3 = daunorubicin-loaded Horse nanostructure (20 nM, BPBR of 0.5), 4 = daunorubicin-loaded Horse nanostructure (20 nM, BPBR of 0.7), and 5 = daunorubicin-loaded Horse nanostructure (20 nM, BPBR of 2.5). Daunorubicin fluorescence was determined via Typhoon fluorescence imaging at 473 nm (top), total DNA was visualized via post staining the gel with ethidium bromide (middle), overlay of fluorescence and ethidium bromide (bottom). The reverse migration of the drug is consistent with the positive charge of daunorubicin. For BPBR < 1.0, the electric field strips the drug from the nanostructures without significantly affecting mobility of either drug or nanostructure. However, for overloaded nanostructures (BPBR=2.5) the concentration of nanostructures in the leading band is reduced and a pronounced smear of drug runs shorter in the reverse direction. This is consistent with aggregations of structure and suggests the drug is not as easily stripped from the structure in the case of overloading, which may be a result of aggregation or some distinct mechanism of interaction, perhaps electrostatic in nature.
concentrations of daunorubicin (62.5-2500 µM) were mixed with 20 nM Horse nanostructures, all for 24-hour incubation times. The bp binding ratio depended inversely on DNA origami concentration (Figure 3.4A) and linearly on the daunorubicin concentration (Figure 3.4B) present in the loading reaction. The efficiency of daunorubicin loading (i.e. fraction of drug loaded into nanostructures from solution) remained in the

Figure 3.4. Daunorubicin effectively intercalates and releases from the Horse DNA nanostructure.

A) Varying concentrations of Horse DNA nanostructures (5–240 nM) were added to 500 µM daunorubicin, and the amount of loaded daunorubicin was determined colorimetrically by absorbance. The inset illustrates the process of drug loading including mixing, incubation, centrifugation, and measuring the daunorubicin concentration left in the supernatant. The data are presented in triplicate as mean bp binding ratio ± SD (SD was smaller than the marker size for larger concentrations). B) Varying concentrations of daunorubicin (62.5–2500 µM) were added to Horse nanostructures at 20 nM for 24 h followed by free daunorubicin removal. Linear regression statistical analysis was applied to the data set. The data are presented as normalized mean relative intensity ± SD in triplicate and represent three independent experiments.
range of 40-70% as a function of time or varying daunorubicin in solution, but did increase up to ~90% with increasing DNA origami in solution (Figure 3.3C) suggesting efficiency of drug loading may be controlled by the amount of DNA origami nanostructures in solution. Interestingly, incubating low concentrations of DNA origami with high daunorubicin concentrations (≥ 500 nM) led to bp binding ratios above 1.0 (Figure 3.4B). This was likely due to the ability of daunorubicin to bind in the minor groove of DNA and electrostatic interactions mediated by the positively charged daunorubicin molecules due to protonated amines physically wrapping around the poly-anionic backbone of the Horse DNA nanostructure helices, as previously described for anthracycline binding to DNA. Gel electrophoresis analysis of daunorubicin loaded Horse nanostructures revealed that overloading (base pair binding ratios above 1.0) impacted electrophoretic...
mobility (Figure 3.3E), while base pair binding ratios <1.0 had little impact on migration of nanostructures. While increased drug loading initially seemed desirable, bp binding ratios much larger than 1 resulted in reduced efficacy (Figure 3.5). The fluorescence properties of daunorubicin allowed direct imaging of daunorubicin-loaded nanostructures, which revealed large aggregates of structures loaded with bp binding ratios much greater than 1, effectively reducing the concentration of structure in solution (Figure 3.6B). These large aggregates, which might prove toxic or immunogenic in vivo, were not observed at lower bp binding ratios (≤1.0) (Figure 3.6A).

Retention of drug in the nanostructure prior to cell uptake is a critical aspect of drug delivery function. Here we evaluated the in vitro retention of daunorubicin in drug-loaded

Figure 3.6. Daunorubicin loaded Horse DNA nanostructures form aggregates at high daunorubicin concentrations.

(A) To visualize daunorubicin bound Horse DNA nanostructures, 20 nM of Horse DNA nanostructures were added to 0.5 µM daunorubicin for 24 hours. Upon free daunorubicin removal and re-suspension in PBS with 10 mM MgCl\(_2\) added, structures were visualized via TIRF imaging with a 488 nm excitation laser and 100x objective. Structures are at a concentration of ~750 pM and the bp binding ratio is 1. (B) To visualize daunorubicin bound Horse DNA nanostructures at a high base pair binding ratio, 20 nM of Horse DNA nanostructures were added to 2 µM daunorubicin for 24 hours. Upon free daunorubicin removal and re-suspension in PBS with 10 mM MgCl\(_2\) added, structures were visualized via TIRF imaging with a 488 nm excitation laser and 100x objective. Structures are at a concentration of ~750pM and the base pair binding ratio is 4.5. (C) A proposed model for base pair binding ratios > 1 involves minor groove binding of daunorubicin to DNA in addition to intercalation, which ultimately drives aggregation.
Horse nanostructures under experimental conditions. Incubation for varying times up to 24 hours in either storage buffer (PBS, 10 mM MgCl₂) or culture media (clear RPMI 1640, 20% FBS) led to release of approximately 30% or 50% of loaded daunorubicin, respectively (Figure 3.7A). In both cases drug release occurred in a time-dependent manner up to 6 hours followed by a gradual increase with approximately 31% and 50% of the drug released over 24 hours in storage buffer and culture media, respectively.
Interestingly, removing free drug from solution via a series of washes (i.e. centrifugation, removal of supernatant, and resuspension) triggers additional drug release, and under these conditions, low pH and the presence of serum proteins enhance drug release (Figure 3.7B). Therefore, in our experiments, a significant fraction of drug should remain loaded in the nanostructures at the point of cellular entry, and upon cellular uptake, low pH and interactions with enzymes or proteins may trigger additional release of daunorubicin. These

Figure 3.8. Daunorubicin-loaded Horse DNA nanostructures are internalized by HL-60/ADR cells.

A) Either free daunorubicin or daunorubicin-loaded Horse DNA nanostructures (1 Å~ 10−6 m daunorubicin) were introduced to HL-60/ADR cells for 24 h. The level of intracellular daunorubicin fluorescence was determined via flow cytometry. Cells were considered viable using Near IR LIVE/DEAD viability cell stain. A representative histogram overlay is shown from three independent experiments. B) Either free daunorubicin or daunorubicin-loaded Horse DNA nanostructures (1Å~10−6 mdaunorubicin) were introduced to HL-60/ADR or HL-60 parental cells for 3 h followed by a PBS wash and subject to live cell fluorescence imaging via total internal reflection (TIRF) (488 nm excitation) at 10 min intervals for approximately 3 h. Scale bars are 10 µm. C) Time traces of the level of intracellular daunorubicin were measured from at least 50 individual cells where the data are presented as the mean relative fluorescent unit (RFU) ± SEM where data and images represent two independent experiments.
results can also inform future \textit{in vivo} studies of drug pharmacokinetics using DNA origami delivery as the technology progresses toward clinical development.

\subsection*{3.3 Daunorubicin-loaded Horse DNA nanostructures are internalized by HL-60/ADR cells}

The generation of drug resistance in MDR models of APL is mediated through the increased expression of the MDR1 and MRP1 protein efflux pumps in the plasma membrane that effectively export small molecules to sustain cell survival and growth.\cite{54}

Since a primary mechanism for entry of free anthracycline drugs is passive diffusion across the cell membrane,\cite{55} they are susceptible to efflux-pump mediated expulsion.\cite{54} To characterize the ability of the DNA origami Horse nanostructures to circumvent efflux pumps, we used flow cytometry and fluorescence imaging to directly quantify the level of daunorubicin internalization when presented to cells either free in solution or via Horse DNA nanostructures. Parental HL-60 and drug-resistant HL-60/ADR cells were cultured in the presence of either free daunorubicin or loaded Horse DNA nanostructures for 24 hours. The level of daunorubicin present in cells was measured via flow cytometry. An increase in daunorubicin fluorescence was evident when delivered via Horse DNA nanostructures relative to free daunorubicin and untreated controls at 24 hours, illustrated by a shift to the right of the histogram in Figure 3A. In order to monitor intracellular daunorubicin in real time, parental HL-60 and HL-60/ADR cells were cultured in the presence of free daunorubicin or loaded Horse DNA nanostructures for three hours followed by a PBS wash. The level of fluorescent daunorubicin present in the cells was then monitored in an imaging chamber under physiological conditions (5\% \text{CO}_2, 37\textdegree C in a humidified environment). Quantification of
Daunorubicin fluorescence present in individual cells revealed approximately 2.9-fold increased levels of daunorubicin in HL-60 cells relative to HL-60/ADR cells (Figures 3.8B and 3.8C, and Table 3.1), which is expected due to the elevated expression and activity of protein efflux pumps on the HL-60/ADR cell surface. While a steady decline in daunorubicin fluorescence was evident among HL-60 cells over time, HL-60/ADR cells exhibited a slightly faster decrease until 1.5 hours followed by sustained fluorescence (Figure 3.8C). Delivering daunorubicin via Horse nanostructures resulted in ~1.4-fold more fluorescence retained in both HL-60 parental and HL-60/ADR cells relative to free daunorubicin (Figure 3.8B and 3.8C, and Table 3.1). Some difference is already present at time zero of the measurement, which suggests that nanostructure-
mediated delivery leads to more drug initially entering the cell. Interestingly, the rate of expulsion of daunorubicin (i.e. slopes in Figure 3.8) is similar for the nanostructure delivery and free delivery cases. It is possible that some amount of daunorubicin leaks
out of structures during or after cellular internalization and is expelled at a similar rate to
Nevertheless, approximately 40% increases in intracellular daunorubicin fluorescence persist in both HL-60 and HL-60/ADR (Table 3.1) when the drug is delivered via Horse DNA nanostructures compared to free daunorubicin. This is likely due to a combination of more drug initially entering the cell and some fraction of the drug remaining sequestered in structures taken up by cells long enough to prevent

Figure 3.10. Horse DNA nanostructures enter intracellular lysosomal compartments in HL-60/ADR cells as shown via confocal microscopy.

(A) Alexa647-labeled Horse DNA nanostructures were added to HL-60/ADR cells that were pretreated with Lysotracker Green and imaged after 1 hour via confocal microscopy. Left column represents merged image of 488 nm and 640 nm channels, center column represents 488 nm channel, and right column represents 640 nm channel. (B) HL-60/ADR cells were treated with Lysotracker green for 1 hour followed by confocal imaging with 488 and 640 nm excitation. Three representative images are shown from at least two independent experiments.

the free daunorubicin. Nevertheless, approximately 40% increases in intracellular daunorubicin fluorescence persist in both HL-60 and HL-60/ADR (Table 3.1) when the drug is delivered via Horse DNA nanostructures compared to free daunorubicin. This is likely due to a combination of more drug initially entering the cell and some fraction of the drug remaining sequestered in structures taken up by cells long enough to prevent
expulsion. These findings confirm that drug loaded Horse DNA nanostructures are not only internalized by HL-60 and HL-60/ADR cells, but they also allow for larger amounts of intracellular daunorubicin implicating potential for drug resistance circumvention.

3.4 Visualization of Horse DNA nanostructure entry and lysosomal compartment localization in HL-60/ADR cells

To further explore the mechanism of drug delivery, we visualized nanostructure uptake and intracellular localization via fluorescence microscopy. Previous work has demonstrated...
DNA nanostructures can be uptaken via endocytic pathways\(^{(56, 57)}\) and a recent study revealed the presence of doxorubicin loaded DNA nanostructures within lysosomal compartments and proposed lysosomal acidification of DNA nanostructures as a potential mechanism driving drug release\(^{(36)}\). Therefore, we hypothesized that upon spontaneous uptake, daunorubicin loaded Horse DNA nanostructures similarly localized to intracellular lysosomal compartments of HL-60/ADR cells to be degraded followed by daunorubicin release. In order to test this hypothesis, Cy3-labeled Horse DNA nanostructures were added to cells labeled with Lysotracker Green (Life Technologies) and monitored using epifluorescence and bright field DIC time lapse imaging from zero to 18 hours after introduction of nanoparticles. Labeled structures consistently entered HL-60/ADR cells in a time-dependent manner (\textbf{Figure 3.9A}) and localized to lysosomal compartments as shown via epifluorescence and confirmed by confocal microscopy using Alexa647-labeled Horse DNA nanostructures using (\textbf{Figure 3.9A and 3.9B, Figure 3.10A}). As an important control, we verified that Lysotracker Green failed to fluoresce in the red channel (640 nm excitation), confirming that fluorescence in this channel is from Alexa647 labeled nanostructures (\textbf{Figure 3.10B}). In addition, the level of Cy3-Horse DNA nanostructure internalization measured at the single cell level revealed a time-dependent increase in Cy3-Horse signal that appeared to reach steady state around 5 hours (\textbf{Figure 3.11}). Based on a previous study that quantified the number of DNA nanostructures uptaken by KB-3–1 cells to be in the range of tens of DNA origami nanostructures per cell at lower concentrations of nanostructure\(^{(58)}\), here we likely have at least that many Horse nanostructures internalized and likely much more per HL-60/ADR cell. It was confirmed that internalization of unloaded Horse structures failed to induce cell death of HL-60/ADR cells.
DIC and epifluorescence microscopy was used to image HL-60/ADR cells after adding 750 pM of Cy-3 labeled unloaded Horse DNA nanostructures. Cells uptake the unloaded Horse DNA nanostructures and expel them over the time course of approximately 10-15 hours and ultimately recover a healthy round morphology. Cells were monitored from zero to 18 hours with DIC and fluorescence images (561 nm excitation) collected every 10 min. Overlays of DIC and 561nm excitation images (yellow) are shown at several time points.

(Figure 3.12), even though they were effectively internalized. Loaded Horse structures were observed to induce cell death as monitored via bright field (DIC) and epifluorescence time lapsed imaging.

Together, these findings suggest a distinct mechanism of delivery where daunorubicin-loaded Horse DNA nanostructures enter HL-60/ADR cells through the endolysosomal pathway, which may effectively localize the drug in vesicular structures where it can
bypass resistance efflux pumps such as MDR1. In addition, daunorubicin-loaded Horse DNA nanostructures provide a high local dose of drug, which may exceed the local capacity of efflux pumps, or these pumps may simply not be able to access drug that is sequestered in the nanostructure. These characteristics of DNA nanostructure-mediated delivery result in an increase in total number of daunorubicin molecules in the cell to cause cell death or disrupt replication. The DNA nanostructures eventually localized to acidic compartments, where the low pH, or enzymatic degradation, could induce drug release to ultimately cause cell death or impair cellular growth.

3.5 Daunorubicin-loaded Horse DNA nanostructures circumvent drug resistance in HL-60/ADR cells

Since DNA origami nanostructures are taken up by cells and improve drug retention, we hypothesized that the Horse nanostructures could effectively circumvent drug resistance in the HL-60/ADR acute myeloid leukemia tumor model, which over-expresses MDR1, using
the drug daunorubicin, which is structurally similar to doxorubicin but more widely used to treat adult and pediatric acute leukemia.\textsuperscript{(46, 49)} We first confirmed drug resistance of HL-60/ADR cells by challenging them with doxorubicin and daunorubicin concentrations in the expected range of drug resistance (0-2.0 µM) and evaluated efficacy using the CCK-8 assay, quantified in terms of the relative number of viable cells, which is the total number of viable cells in each treatment normalized to the untreated controls. The relative number of viable HL-60/ADR cells remained at approximately 100% at 24 hours (time point chosen to reflect the elimination half-life of daunorubicin\textsuperscript{(62)}) post daunorubicin addition (Figure 3.13) and decreased only slightly with increasing concentrations of daunorubicin (Figure 3.14). Compared to HL-60/ADR cells, daunorubicin at similar concentrations was
significant more effective against parental HL-60 cells at the clinically relevant 1-2 µM range\(^{46, 47, 63}\) (Figure 3.14).

To identify optimal drug loading conditions, Horse DNA nanostructures with varying daunorubicin bp binding ratios were added to HL-60/ADR cells for 24 hours followed by evaluation of efficacy using the CCK-8 assay. While bp binding ratios of 0.46, 0.85, and 1.0 (structure concentration was adjusted to achieve total of 0.1 µM daunorubicin) all induced comparable reductions in the relative number of viable cells, a bp binding ratio of 2.1 was not as effective (Figure 3.5B) likely due structure aggregation caused by overloading with daunorubicin (Figure 3.6B). We therefore chose to perform functional
experiments using a bp binding ratio of ~1.0 or slightly less. For all experiments, the loaded Horse DNA nanostructure concentration was chosen accordingly to achieve target daunorubicin concentrations, and the same nanostructure concentration was then used for unloaded Horse nanostructure controls.

To determine whether Horse DNA nanostructures circumvented resistance mechanisms in HL-60/ADR cells, a range of clinically relevant doses (0.1-1.0 µM) of either free daunorubicin, or daunorubicin loaded in Horse DNA nanostructures were added to HL-60/ADR cells. The relative number of viable cells was determined 24 hours post drug
addition via the CCK-8 assay and combined DIC/fluorescence microscopy using the dead
Figure 3.16. Daunorubicin-loaded Horse DNA nanostructures circumvent drug resistance in HL-60/ADR cells.

A) Varying concentrations of free daunorubicin (0.1 × 10⁻⁶, 0.25 × 10⁻⁶, and 1 × 10⁻⁶ M), unloaded Horse nanostructures, or daunorubicin-loaded Horse DNA nanostructures (bp binding ratio of ≈0.9) were added to HL-60/ADR cells and cell viability was evaluated after 24 h using the CCK-8 assay. Statistical differences are shown between groups. *, p < 0.05 (0.1 × 10⁻⁶ M, p < 0.05, 95% CI: 0.3840 to 37.52; 0.25 × 10⁻⁶ M, p < 0.05, 95% CI: 0.9718 to 37.36; 1.0 × 10⁻⁶ M, p < 0.05, 95% CI: 1.8613 to 38.19). B) Varying concentrations (0.1 × 10⁻⁶, 0.25 × 10⁻⁶, and 1 × 10⁻⁶ M) of free daunorubicin or daunorubicin-loaded Horse nanostructures were added to HL-60/ADR cells for 24 h and the number of viable cells and relative density (indicated by n = number of viable cells) was evaluated using fluorescence microscopy using the viability dye, Sytox Red, with 561 nm laser excitation. The image shows a representative DIC image overlaid with the Sytox Red fluorescence image. Statistical differences are shown between groups. *, p < 0.05 (0.1 × 10⁻⁶ M, p = 0.0423, 95% CI: 0.9487 to 38.35; 0.25 × 10⁻⁶ M, p = 0.0104, 95% CI: 9.9797 to 41.09; 1.0 × 10⁻⁶ M, p = 0.0230, 95% CI: 3.057 to 28.60). C) Either free daunorubicin or daunorubicin-loaded Horse nanostructures (1 × 10⁻⁶ M daunorubicin) were added to HL-60/ADR cells for the first 24 h over a 4 day time course while the cells were washed with PBS at 24 and 72 h post-initial treatment. The relative number of viable cells was determined via counting beads via flow cytometry. The mean number of viable cells ± SEM is shown at 24, 48, 72, and 96 h post-initial nanostructure addition. Statistical differences are shown between groups. *, p < 0.05 (24 h, p = 0.0151, 95% CI: 3.144 to 19.89; 48 h, p = 0.0492, 95% CI: 0.1682 to 60.32). D) Either free daunorubicin or daunorubicin-loaded Horse nanostructures (1 × 10⁻⁶ M daunorubicin) were added to HL-60/ADR cells over a four day time course while the cells were washed with PBS at 24 and 72 h post-initial treatment. The near IR live/dead viability stain and the Violet V450 proliferation dye was added to evaluate proliferation via flow cytometry. Cells negative for near IR LIVE/DEAD viability stain were gated and considered viable. Proliferation was evaluated via V450 proliferation dye fluorescence represented by a representative histogram overlay at 96 h from three independent experiments. The quantitative data in A, B, and C were normalized to cells only controls and are presented in triplicate as the mean relative% (or relative number, C) viable cells ± SEM and represent three independent experiments.

cell indicator Sytox Red. Both CCK-8 and fluorescence imaging revealed that delivering
daunorubicin via the Horse DNA nanostructures produced significant reductions in the relative number of viable HL-60/ADR cells relative to free daunorubicin (Figures 3.16A and B). Importantly, a daunorubicin-loaded plasmid M13mp18 DNA allowed for comparable levels of relative growth of HL-60/ADR cells compared to free daunorubicin (Figure 3.15). Furthermore, a four-day time course revealed significant reductions in the number of viable HL-60/ADR cells at 24 and 48 hours and marked reductions at 72 and 96 hours were evident when daunorubicin was delivered via loaded Horse DNA nanostructures relative to free daunorubicin as evaluated via flow cytometry (Figure 3.16C). These results were confirmed by fluorescence imaging, which similarly showed a significant reduction in the relative number of viable HL-60/ADR cells at 24 hours and reductions at 48, 72, and 96 hours (Figure 3.17). These findings are consistent with previous work demonstrating that delivering anthracyclines (previous studies used doxorubicin) via DNA origami nanostructures produces enhanced efficacy relative to free drug\(^{41}\) and can circumvent drug resistance\(^{36}\) in MCF-7 adenocarcinoma cells. Importantly, direct imaging of HL-60/ADR cells revealed that while the number of viable cells was reduced, the percentage of viable cells within the given treatment was comparable between free daunorubicin and daunorubicin-loaded Horse nanostructure treatments (Figure 3.20A), suggesting that delivery via Horse DNA nanostructures may not enhance the direct cytotoxicity of daunorubicin. This was confirmed via flow cytometry (Figure 3.20B). Therefore, cellular survival did not appear to be the primary mechanism responsible for enhanced efficacy with Horse DNA nanostructure delivery. This is not unexpected since the primary mechanism of action of daunorubicin is to impair cellular growth.\(^{64-66}\) Therefore, we hypothesized that loaded Horse DNA nanostructures affected
cellular proliferation. To test this, HL-60/ADR cells were cultured in the presence of either a continuous treatment of free daunorubicin or daunorubicin loaded Horse DNA nanostructures for four days where cell growth was evaluated by flow cytometry. A shift in violet proliferation fluorescence, indicative of reduced proliferation, was evident in the presence of daunorubicin loaded Horse DNA nanostructures relative to free daunorubicin (Figure 3.16D), suggesting that the improved drug retention induced by DNA nanostructure delivery indeed enhances the ability of daunorubicin to disrupt proliferation.

Figure 3.17. Daunorubicin loaded Horse DNA nanostructures reduce HL-60/ADR cellular proliferation.

HL-60/ADR cells were cultured with either daunorubicin loaded or free daunorubicin (1.0 µM) for 24, 48, 72, and 96 hours. Sytox Red Dead Cell Stain epifluorescence and bright field microscopy were used for cell counts. The normalized numbers of viable cells are shown with respect to buffer only controls in comparison to the % of viable cells and represent 1 (72 hrs) or 3 (24, 48, 96 hrs) independent experiments. Statistical differences are shown between groups. *p < 0.05 (24 hrs, p = 0.0230; 95% CI: 3.057 to 28.60).

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Where free drug delivered in solution enters cells via passive diffusion (Figure 3.18A),
delivery via Horse nanostructures facilitates drug uptake via endocytosis (Figure 3.18B),
which leads to larger amounts of drug entering and ultimately staying in the cell thereby
improving efficacy. The results presented here along with other studies\(^{36, 41, 45}\) support
DNA origami nanostructures as an attractive option for drug delivery, and specifically as
a promising approach to circumvent efflux-pump mediated drug resistance. In addition,
our results and previous work have shown that DNA origami nanostructures themselves do
not induce cytotoxic or significant immunogenic effects \textit{in vitro}\(^{67, 68}\) or \textit{in vivo}.
\(^{45}\)
Furthermore, recent findings suggest that nuclease activity and immune recognition \textit{in vivo}
is reduced when either siRNAs are modified with 2′-OMe groups\(^{45, 69, 70}\) or when DNA
nanostructures are enveloped with a PEGylated lipid bilayer,\(^{43}\) and here we demonstrated
A structure design that provided good stability in cell culture conditions over at least 24 hours. These results suggest combined strategies of structure design and modification can yield sufficient structural stability. With respect to other delivery vehicles, the DNA origami approach allows unprecedented control over nanoscale geometry and precise functionalization with nucleotides (i.e. CpG sequences\(^{67}\) or RNA\(^{71}\)), a variety of proteins,\(^{72}\) inorganic nanomaterials,\(^{73, 74}\) small drug molecules,\(^{36, 41}\) and combinations thereof, which could provide opportunities to optimize nanostructure design and functionalization for cellular uptake, localization, tunable drug release,\(^{45, 75}\) and multifunctional drug delivery. It also offers the advantage of easy loading, which is not typical

\(\text{Figure 3.19. Daunorubicin loaded Horse DNA nanostructures show limited effect in HL-60/ADR cell survival.}\)

(A) HL-60/ADR cells were cultured with either daunorubicin loaded or free daunorubicin (0.1 µM, 0.25 µM, and 1.0 µM) for 24 hours. Sytox Red Dead Cell Stain epifluorescence and bright field microscopy were used for cell counts. The normalized number (gray outlined bars and left y-axis from Fig. 5B for comparison) and raw percentage of viable cells (black outlined bars and right y-axis) is shown with respect to buffer only controls and represents three independent experiments.

(B) HL-60/ADR cell survival assessed via flow cytometry (Near IR-/Annexin V-) population at 24 hours post treatment with either free daunorubicin or loaded Horse DNA nanostructures. The data are presented as the mean % Viable HL-60/ADR cells ± SEM and represent three independent experiments. Statistical differences are shown between groups. *\(p < 0.05\).
of many other nanoparticle delivery systems. While combination therapies can also be achieved with other nanoparticles, the DNA origami approach enables exquisite control over the absolute quantifiable number and location of modifications, which has recently been shown to impact cellular responses.\(^{76}\) While significant work remains to be done to enable clinical applications of DNA origami nanostructures, such as understanding distribution, pharmacokinetics, and toxicity limits \textit{in vivo}, this proof-of-concept work presented here gives strong merit to further optimization of DNA origami nanostructures as a drug delivery vehicle, and demonstrates that DNA origami nanostructures can aid in addressing drug resistance mechanisms in disseminated diseases such as acute myeloid leukemia, for which the outcome is still poor and for which no therapy has been approved in the past 30 years. Lastly, our specific DNA origami structure design enabled good stability in cell culture and rapid fabrication, which could be key advantages for a wide range of applications and gives fundamental insight into design principles for DNA origami-based nanostructures for cellular, \textit{in vivo}, or clinical use.

3.6 EXPERIMENTAL SECTION

Cell lines. The HL-60 human acute promyelocytic leukemia cell line, previously described,\(^ {77, 78}\) was obtained from ATCC (Manassas, VA). HL-60/ADR doxorubicin-resistant cells were described previously\(^ {79-81}\) and kindly provided by Dr. Kapil Bhalla (Houston Methodist Research Institute, Houston TX). HL-60/ADR cells were challenged with doxorubicin to confirm resistance. All cells were cultured in complete RPMI 1640 (CellGro, Manassas, VA) with 20% fetal bovine serum (FBS) (Atlas Biologicals, Ft. Collins, CO), 100 U/ml penicillin (Gibco, Life Technologies, Grand Island, NY), 100
µg/ml streptomycin (Gibco) and 2 mM L-glutamine (Gibco) in a humidified atmosphere containing 5% CO₂ at 37°C.

**Horse nanostructure design and fabrication via scaffolded DNA origami.** Scaffolded DNA origami allows for the generation of precise nanostructures in high yield through molecular self-assembly using ~150-200 short (30-50 base) oligonucleotide strands referred to as “staples.” The staples are designed to be piecewise complementary to the scaffold to drive folding into specific two- and three-dimensional nanoscale geometries. The Horse nanostructure (~92.5 x ~13.2 x ~11 nm) was designed using the computer-aided design software caDNAno. Staple sequences output from caDNAno were ordered from a commercial vendor (Eurofins Genomics, Huntsville, AL), and the scaffold was produced in our lab as previously described. Structures were made via molecular self-assembly following protocols established in Castro et al. Briefly, a M13mp18 ssDNA scaffold was placed in solution at 100 nM in a buffer containing 5 mM Tris, 5 mM NaCl, 1 mM EDTA, 20 mM MgCl₂, and 152 oligonucleotide staple strands each in a 10-fold molar excess to scaffold. The mixture underwent a 17-hour thermal ramp including heating to 65°C followed by slow cooling to 25°C to facilitate molecular self-assembly driven by complementary binding of staples. Rapid fold thermal annealing ramps were held at 52°C following protocols previously described. To confirm and purify well-folded nanostructures, folding reaction products were subjected to 2% agarose gel electrophoresis (0.5X TBE) in the presence of 11 mM MgCl₂ followed by excision of bands containing well-folded structures. DNA origami nanostructures were extracted from the excised agarose bands via centrifugation with “freeze ‘N squeeze” purification tubes (BioRAD, Hercules, CA) and visualized via transmission electron microscopy. Alternatively, in
order to concentrate Horse DNA nanostructures, unpurified structures were placed in equivolume amounts of 15% PEG 8000 (Sigma Aldrich, St. Louis, MO) and centrifuged for 20 minutes at 20,000 x g to remove excess staple DNA strands.\(^{(32)}\) Purified structures were resuspended in 1X PBS (Gibco, Life Technologies) in the presence of 10 mM MgCl\(_2\). PEG precipitations were performed two times to eliminate all excess staples in solution. Horse nanostructure concentrations, characterized by UV absorbance measurements on a Nanodrop 2000 (Thermo Scientific, Waltham, MA), were typically ~20 nM after resuspension. To test Horse DNA nanostructure stability in complete RPMI 1640 (described above) in the presence or absence of 20% FBS, Horse DNA nanostructure pellets were resuspended in 200 µl of medium and allowed to incubate in a humidified atmosphere containing 5% CO\(_2\) at 37° C for 3, 6, 9, 12, 15, and 24 hrs and evaluated via agarose gel electrophoresis.

**Transmission Electron Microscopy (TEM).** TEM grids were prepared as described in Castro et al.\(^{(27)}\) Briefly, a 3 µl droplet of purified structures was pipetted onto a copper TEM grid coated with carbon and formvar (Electron Microscopy Sciences, Hatfield, PA) and incubated for three minutes. The solution was then wicked away using filter paper and a 15 µl droplet of 2% Uranyl Formate negative stain was applied to the grid and immediately wicked off using filter paper, followed by application of a 20 µl droplet of 2% Uranyl Formate, which was incubated for 40 seconds prior to wicking off the stain solution and allowing the grids to dry for at least 30 minutes prior to imaging. Horse DNA nanostructures were visualized on a Tecnai G2 BioTWIN transmission electron microscope (FEI, Hillsboro, OR) at an electron acceleration voltage of 80 kV.

**Atomic Force Microscopy**
AFM images were collected on a Bruker AXS Dimension Icon Atomic Force Microscope in ScanAsyst mode (Bruker Corporation, Billerica, MA). A 5 µl drop of annealed unloaded or daunorubicin-loaded Horse DNA nanostructure was deposited onto the surface of freshly cleaved mica and allowed to incubate for 2 min. The samples were then washed with 1 ml ddH₂O and dried with compressed nitrogen. Images were collected using a ScanAsyst-Air silicon nitride cantilever with a measured spring constant of 0.79 N/m and nominal tip radius of 2 nm (Bruker Corporation, Billerica, MA).

**Nanostructure Daunorubicin Binding.** Daunorubicin HCl (10 mM) (Santa Cruz Biotechnology, Dallas, TX) was resuspended in 1X PBS with 10 mM MgCl₂ and added in equimolar amounts to varying concentrations of Horse DNA origami nanostructures. After 24 hours incubation with daunorubicin, the nanostructures were centrifuged at 16,000 x g for 25 minutes yielding a visible red-orange precipitated pellet. The absorbance of the supernatant was measured at 480 nm via a plate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA) to determine the concentration of remaining daunorubicin, from a known standard curve. The concentration of drug loaded into the DNA origami structures was taken as the difference between the original concentration of daunorubicin and supernatant post centrifugation. We defined the loading of daunorubicin into DNA nanostructures as the base pair binding ratio as described previously (Equation 3-1). The interaction between daunorubicin and Horse nanostructures was also evaluated by gel electrophoresis. In this experiment, daunorubicin fluorescence was visualized on a Typhoon FLA 9500 imaging system (General Electric, Fairfield, CT) at 473 nm followed by an ethidium bromide post stain to visualize Horse DNA nanostructures.

**Daunorubicin release from Horse DNA nanostructures**
Daunorubicin-loaded Horse DNA nanostructures (40 nM, 300 µM daunorubicin) were incubated with clear RPMI 1640, 20% FBS, pH 7 and PBS, pH 7, 10 mM MgCl₂ and incubated at 37°C for 1, 3, 6, and 24 hrs followed by centrifugation (16,000 x g, 10 min, room temperature). In addition, daunorubicin-loaded Horse DNA nanostructures (40 nM, 1 mM daunorubicin) were incubated with various buffers (PBS, pH 7, 10 mM MgCl₂; PBS, pH 4; 10 mM MgCl₂; PBS pH 7; clear RPMI 1640, no FBS, pH 7; clear RPMI 1640, 20% FBS, pH 7) through a series of four centrifugation (16,000 x g, 10 min, room temperature) and resuspension steps at 1 hr increments at 37°C each time resuspending in 200 µl of fresh buffer to remove drug that had already been released into solution. Supernatants were collected after each centrifugation and daunorubicin absorbance was measured at 480 nm in a Sub-microCell 100 µl cuvette (ThermoElectron, Madison, WI) via a Nanodrop 2000 (Thermo Fisher Scientific).

**Confocal Microscopy.** HL-60/ADR cells were incubated with 400 nM Lysotracker Green (Life Technologies) for 60 minutes. Cells were centrifuged and re-suspended in 1.5 ml of imaging buffer containing 750 pM Alexa647 Horse DNA origami structures and seeded on 35-mm No. 1.5 glass bottom dishes (MatTek, Ashland, MA). After 60 minutes incubation at 37°C, samples were moved to a live-cell imaging chamber at 37°C of a TI-E inverted research microscope (Nikon Instruments Inc., Melville, NY) controlled by Nikon Elements software and equipped with spinning disk confocal unit (Yokogawa Electric, Tokyo, Japan), 100x objective lens (Plan Apochromat Lambda, NA 1.45, Nikon) and EMCCD camera (iXon DU897 Ultra, Andor Technology, South Windsor, CT). 3D confocal stacks were captured with a step size of 0.5 µm along the z-axis.
**Fluorescence Microscopy.** Fluorescence microscopy was performed to determine cell viability using the Sytox Red Dead Cell Stain (1:100) (Life Technologies). 24 hours after addition of drug loaded nanostructures, cells were washed and resuspended in imaging buffer (clear RPMI 1640 (Gibco) + 2% penicillin/streptomycin/L-Glutamine). Cells were then imaged on a Nikon TiE microscope (Belmont, CA) under Differential Interference Contrast (DIC) imaging to view cell morphology, and fluorescence imaging with an excitation wavelength of 640 nm to assess presence of the Sytox Red impermeant dye in individual cells. The number of viable cells was counted, and the data are presented as % viable cells relative to controls without the addition of any drug. In addition, Horse nanostructures were labeled with either YOYO-1 intercalating dye (Life Technologies), or Cy3-labeled oligonucleotides (Eurofins Genomics) for fluorescence imaging experiments. YOYO-1 was added to Horse nanostructures at a ratio of 10 base pairs to one YOYO-1 particle and incubated for 6 hours at 37° C followed by centrifugation and removal of excess YOYO-1 in solution and resuspension in PBS with 10 mM MgCl₂ buffer. Cy3 oligonucleotides were attached via ssDNA overhangs present on the Horse nanostructure in the initial folding process, treating the labeled oligonucleotides as an additional staple. Fluorescently labeled nanostructures were employed in experiments to image Horse nanostructures in solution (YOYO-1) or to visualize cellular uptake and localization (Cy3). Lysotracker Green (Life Technologies) was employed according to manufacturer’s instructions to visualize lysosomal compartments in intracellular localization experiments. In all cases, Horse (drug loaded and unloaded) nanostructures were added to cells at a concentration of 250-750 pM and images were collected using an ANDOR EMCCD camera on an automated Nikon TiE microscope under DIC imaging and fluorescence
imaging with 488 nm (daunorubicin, lysotracker green, and YOYO-1) or 561 nm (Cy3) laser excitation.

**In Vitro Cell Viability Assay.** To evaluate cell viability, the formazan dye cell assay Cell Counting Kit-8 (CCK-8) (Sigma Aldrich, St. Louis, MO) was performed according to manufacturer’s instructions. Briefly, 5 x 10^4 cells/ml were resuspended in a 96-well plate in complete RPMI 1640 and incubated at 37° and 5% CO_2 for 24 hours. Cells were treated with varying concentrations of free daunorubicin (0.1-1.0 µM), daunorubicin-loaded Horse (0.1-1.0 µM drug concentration corresponding to 15-150 pM of DNA nanostructures), and corresponding concentrations of unloaded and loaded double stranded M13mp18 plasmid DNA (Bayou Biolabs, Metairie, LA) and unloaded Horse (15-150 pM), and then placed in wells in quadruplicate and incubated for 24 hours. CCK-8 (10 µl) was added into each well and incubated for 2 hours. Absorbance was measured at 450 nm (SpectraMax M2, Molecular Devices). Raw absorbance values were normalized and presented as % of buffer only controls.

**Flow Cytometry.** Flow cytometry was performed on a Gallios Flow Cytometer (Beckman Coulter, Brea, CA) to evaluate cell viability and apoptosis as previously described using Near IR LIVE/DEAD viability stain and Alexa647- and Pacific Blue-conjugated Annexin V (Life Technologies). Near IR LIVE/DEAD/Annexin V double negative cells were considered viable. Proliferation was evaluated with the Violet V450 nm Proliferation Dye gated on (Near IR LIVE/DEAD− cells) and the number of cells was measured via counting beads (BD Biosciences, San Jose, CA). Intracellular daunorubicin fluorescence was also determined.
**Statistical Analysis.** Statistical analysis was conducted using GraphPad Prism software (GraphPad Software, La Jolla, CA). Linear regression statistical analysis was applied to data representing Basepair Binding Ratio as a function of DNA origami solution concentration and daunorubicin solution concentration. Data from multiple treatment groups were analyzed using a one-way ANOVA to determine whether an overall statistical change existed. $p$ represents a parameter that determines statistical significance and is typically set at a 95% significance level. Certain $p$ values were calculated using a Bonferroni post hoc analysis. A two-sided Students $t$ test was used to determine whether a statistically significant difference existed between two datasets where a $p$ value $\leq 0.05$ indicated statistically significant results. Exact $p$-values (two-sided Students $t$ test) and 95% CIs are reported in the figure legends.
Chapter 4: Scaled Up Batch Production of DNA Origami Nanostructures

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**Scaling DNA origami nanofabrication for the clinic and the classroom**

Manuscript in preparation for publication

4.1 Introduction

An inherent issue with a bottom-up design approach to nanotechnology is the inability to manufacture a large amount product, while maintaining precision and control over the process. The molecular self-assembly process known as scaffolded DNA origami (5, 27, 44, 85) is a powerful nanostructure fabrication method that allows for intricate control over structure shape and size (86, 87). These nanostructures are often constructed with bundle components (5, 26, 44, 85), and recent research has expanded to a range of lattice-free architectures (88) enabling the design of nanoscale shapes with nearly arbitrary geometric complexity (85, 89) and controllable dynamic behavior (8, 82). The ability to design complex DNA origami nanostructures and incorporate numerous functionalities such as proteins (34, 76, 90, 91) and nanoparticles (92-96) has led to several highly promising applications including use *in vitro* and *in vivo* as a carrier for chemotherapeutics (33, 36, 41, 45), controlled release containers (97, 98), nanomechanical devices and measurement tools (8, 34, 82, 99-101), and templates for chemical (102) and enzymatic reactions (103) and functional ligand attachment (43, 104, 105). Despite these promising applications,
development and implementation for commercial and clinical applications, is hindered by the current small scales of fabrication. Furthermore, the need for expensive thermal annealing equipment, usually a polymerase chain reaction (PCR) machine, currently limits broader use in research and education. With the myriad uses that DNA origami presents, the need for a scaled up assembly is critical in order to produce material at industrially and therapeutically relevant production scales with efficient and economical fabrication methods and equipment.

Typical DNA origami fabrication methods, referred to hereafter as “folding”, yield 1-20nM of structure in 50-100 ul volumes, which equates to ~0.2-5 µg, in batch reactions that take anywhere from tens of hours up to several days. While this is often sufficient for research use, the vast majority of commercial applications would require at least milligram quantities. For example, when considering in vivo research or clinical applications, .2-1 mg, or 10-50 mg/kg per mouse, is typically required for a single experiment or test in a mouse model, which corresponds to hundreds of typical folding batches for a single experiment. Clearly the ability to fabricate DNA origami nanostructures at significantly larger scales could facilitate their commercial, clinical, and larger scale research applications, and eliminating the need for expensive PCR equipment could facilitate new research and broader use of DNA origami at various levels of research and education.

The scaffolded DNA origami self-assembly process involves binding of 150-200 custom-designed oligonucleotide strands, known as “staples,” to a long (~7000-8000 bases) ssDNA scaffold strand usually derived from the M13mp18 bacteriophage genome to form 2D- and 3D-nanostructures (5, 25-27). Traditionally, the folding is performed in a thermal annealing ramp starting at high temperatures (~65-80°C) and slowly cooling
through a range of annealing temperatures (~30-60°C) at intervals of 1-4 hr/°C, and finally cooling to room temperature or 4 °C. Recent studies of the self-assembly process have revealed staple binding is a cooperative process (29, 106). While traditional folding is performed via slow thermal annealing recent publications have taken advantage of this cooperativity to anneal structures in a guided or piecemeal manner (30, 31). By controlling and even visualizing the folding pathway, researchers have allowed for greater understanding of the folding process which allows for better control of structure formation(107). Therefore, it is now understood that much of structure formation occurs at a single temperature and most DNA origami nanostructures can self-assemble in ~5-100 minutes at a single temperature(28).

Here, we present a one-pot batch folding reaction on a scale 1500x the conventional process and the methods needed to apply this technique to other designed DNA origami structures. We further present methods to optimize this technique to other DNA origami structures as well as robust folding protocols that work for a wide variety of structures. Our fabrication optimization process also reveals new fundamental insight into the kinetics of the DNA origami self-assembly process. In particular, using modified folding protocols initially developed by Sobczak et. al. (28) we show that annealing can occur over a range of constant temperatures. We further show that assembly can proceed on the timescale of a few minutes, and kinetics of folding may vary at different annealing temperatures, which is not always correlated to the final yield. Furthermore, the methods established here can help streamline paths to commercial and clinical applications of DNA origami and broaden use in research and teaching laboratories.
4.2 Characterizing DNA origami folding as a basis for scale up

The DNA origami “Trojan Horse” nanostructure (~13nm x 15nm x 93nm), was previously introduced as a chemotherapy delivery vehicle, a structure diagram and transmission electron microscope images can be found in Figure 4.1A & 4.1B. The “Horse” structure was presented with beneficial qualities such as speed of folding and
stability in cell culture media with fetal bovine serum (33). Here, we further characterize this structure in order to add additional understanding of the speed and scalability of its self-assembly process.
4.2.1 Critical Temperature

Sobczak et. al. have previously shown that most DNA origami nanostructures fold at a single temperature, as part of a three-stage temperature ramp, instead of gradually cooling a reaction from 95°C to 20°C, which is the process originally laid out by Rothemund in 2006(28). Using a systematic trial-and-error approach for finding the critical temperature provided a streamlined and less experimentally sensitive method. In addition, it was desirable to eliminate the need for expensive, real-time PCR equipment, which most laboratories without a focus on gene expression or nucleic acid biochemistry would not have. Using the typical solution conditions for self-assembly described by Castro et. al. (27), we developed a strategy to screen annealing temperatures to identify the temperature that structures begin to fold. All ramps began with a standard 65°C melting period for 10 minutes. Then a temperature gradient is established across the thermocycler heating block from 60°C to 40°C for four hours. This allows efficient testing of a range of constant annealing temperatures. Finally, all reactions are then rapidly cooled to 4°C for 10 minutes. A standard agarose gel is run and analyzed to determine at which temperatures of the gradient that the structure begins to form bands of folded structure, afterwards a smaller gradient, typically four degrees was used to increase resolution of what we deem the “critical temperature” or temperature which allows the structure to fold (Figure 4.1C). Surprisingly, structures were able to fold at not only a single critical temperature, but a range of critical temperatures.

4.2.2 Kinetics of Three Temperature Ramps

Using the 60°C to 40°C gradient as a coarse guide for temperatures at which the structure fold, three temperatures were chosen to explore the speed at which folding occurs
and whether the kinetics would vary at different possible annealing temperatures. Thermal ramps underwent a standard melting of 65°C for 15 minutes, and then were set at one of three critical temperatures (high, middle or low) in the range of previously determined appropriate annealing temperatures. This temperature was held for varying amounts of time ranging from 1 to 30 minutes. As usual, reactions were rapidly cooled to 4°C for 10 minutes after the annealing phase. Structures were run on agarose gels for each temperature (Figures 4.1D) and TEM images (Figure 4.1E) analyzed to determine effect of time on the folding process. Representative TEM images for each condition can be found in Figure 4.2. Finding a the critical temperature in which a structure will efficiently fold allows for a condensed process that can shorten folding times to as little as a total of 20 minutes.

4.3 Scaling folding with a panel of DNA origami nanostructures

When nanostructures are used for biological process, like drug delivery, it is often necessary to use greater amounts of structures at higher concentrations than what results from typical DNA origami folding reactions. As it was found that the “Horse”
nanostructure folded quickly (<15 min), at all tested annealing temperatures, and with minimal secondary structure the first attempt for a scaled up production of horse was performed by attempting to insulate the solution, therefore slow down cooling, after heating it to 65°C (Figure 4.3A). Though successful, it was quickly apparent with the limited ability to easily confirm this process’ success for other structures, before scaling up, it was not an effective method for folding many structures at a larger scale. Therefore, more control was desired which was established using water baths.

4.3.1 DNA Origami Designs, goals of panel

To create the comprehensive panel for scaled-up production of DNA origami nanostructures, we employed a combination of structures that have presented both ease and
challenges in their ability to assemble and complexity of design. An 18-helix bundle with a symmetrical cross-section (~14 nm x 12 nm x 140 nm), similar to the “Horse”, provides a simple structure that is relatively easily folded, except the design uses the honeycomb lattice cross-section, which is more widely used. The platform structure (~68 nm x 30 nm x 6 nm) poses a challenge due to the likelihood of high quantity of secondary or undesirable structure, based on prior experience. Rothemund’s 2D rectangle is used due to its popularity in the field. The last two structures, introduced by Marras et. al. represent nanosized mechanisms, providing more complex structures previously studied for their dynamic properties (8) (Figure 4.4A),

4.3.2 Mega Fold Scale Up

As mentioned above, the “Horse” was formed using an insulated foam cooler with small fragments of Styrofoam surrounding a 50 mL Erlinmeyer flask with a rubber stopper and thermometer combination as depicted in Figure 4.3A. The folding reaction contained 2 mL of 10x folding buffer (50 mM Tris, 50 mM NaCl, 10mM EDTA), 2 mL of 200 mM
MgCl$_2$, 4 mL of ddH$_2$O, 4 mL of oligonucleotides at 500 nM, and 8 mL of 100 nM M13mp18 viral DNA with the ratios of parts being the same as described by Castro et. al. but now contains a total of 20 mL. This original method provided limited ability to consistently fold a variety of structures, due to inadequate insulation and temperature control. Though the band for the scaled-up fold lagged slightly behind the 20-hour folding time control, no visible differences between nanostructure formation were viewed in the averaged TEM images of the 2.5 day thermocycler (TC) and Mega Fold (MF) folds (Figure 4.3 Evolution of the Megafold).

(A) In the initial design, structure components were heated to above 65°C on a heating plate, then placed in an insulated chamber to cool slowly to room temperature before being stored at 4°C. (B) In the second iteration, structure components were melted as before but then placed in an electronic water bath set to the optimized annealing temperature for 4 hours before cooling the solution in an ice bath. (C) The alternative schematic replaces the hot plate and electronic water bath with the more economical water heated with a propane burner.
The images in Figure 4.5 are an average of more than 100 individual structure images that are combined by a custom MATLAB program that can be found in Appendix A. Though successful for the “horse” structure, it was feared that this original method provides limited ability to consistently fold a variety of structures, due to inadequate insulation and temperature control.

The subsequent method provides a more universal solution to scale up folding with a little extra preparation. Using the information gathered from the critical temperature gradients and subsequent gels, an optimal single folding temperature, which is typically
just below the critical temperature, was determined qualitatively by band. Characterization for each of the structures from the panel can be found in **Appendix B**. As before, a 50mL Erlenmeyer flask with a rubber stopper, thermometer combination was filled with the proper ratios of buffer, magnesium chloride, staples and scaffold. As depicted in **Figure 4.3B**, the flask was quickly heated on a hotplate and a temperature between 65°C and 70°C was maintained for 15 minutes while occasionally stirring and venting bottle. The stopper is necessary so not to lose excess liquid due to evaporation, but it does become necessary to vent the flask every ~3 minutes during this high temperature phase. Then, the flask was moved to a water bath that was set to the optimal folding temperature determined by the gradient folding analysis. This temperature was maintained between 3-4 hours, while occasionally swirling flask, though ~1 hours is enough for many structures (data not shown). After incubation time, the flask was then placed in a cool water bath until the liquid reaches 4°C, or approximately 5 minutes depending on volume. Flask ventilation is critical during cooling to prevent stopper lodging in the flask. The optimal folding

*Figure 4.5 Verification of structure fabrication by Megafold method using TEM images.*

On left is a representative image of the “horse” structure folding using Megafold method in Figure 4.3A. On the top right shows an average image of the “horse” nanostructure using the Megafold method in Figure 4.3A (n>100), and bottom right is an average image of the “horse” nanostructure using the standard 2.5-day fold in a thermocycler (n>100).
temperatures used for the “Horse”, hinge, bennett linkage, platform, rectangle, and symmetrical 18-helix were, 55°C, 52°C, 53°C, 48°C, 58°C and 52°C respectively. Structures were then analyzed by agarose gel electrophoresis and TEM (Figure 4.4B and 4.4C). In addition, to confirm the quality of folding, two DNA strands with Cy3 fluorophores were added to the “Horse” structure design and folded by both a standard 2.5-day annealing ramp and the more efficient of the Mega Fold methods. The fluorescent emission intensity was measured on a Horiba Fluoromax 4 fluorimeter and compared in Figure 4.6, reactions and measurements were performed in duplicate. Furthermore, dual dry-bath tabletop incubators were used to fold an additional 10 structures in 1 mL amounts in 1.5 mL polypropylene tubes. This shows that this method is not limited to the 10-100 mL scale or to the equipment described.

Figure 4.6. Emission spectra of Cy3 oligo attached to “horse” nanostructure for Megafold and thermocycler folded structures.

Blue spectrum are nanostructures folded for 2.5-day using thermocycler. Red spectrum are nanostructures folded by Megafold method in Figure 4.3B. Structures concentrations are normalized at 20 nM.
Taken together, these findings suggest that once establishing information as the critical temperature and optimal folding temperatures, that these structures and likely all DNA origami nanostructures can be folded on a much larger scale than previously demonstrated. Once the proper initial data is gathered for an individual structure, this approach provides a reliable method that forms structures comparable to their smaller scaled, 2.5-day equivalent.

4.3 Thermal Annealing for Robust Self Assembly (TARSA)

The investigation and characterization of the panel of structures, enabled the possibility to exploit our results to design a single short annealing ramp (< 1 hour) that could be performed in dual water baths that would ideally work all structures. Figure 4.7 shows our panel of six structures with the vertical bars representing the annealing
temperature ranges at which the individual structures fold. Based on these results, a set of annealing temperatures were chosen from the structure panel results to create a multi-temperature ramp. The temperatures 58°C, 51°C, 46°C, 37°C, were chosen to test folding of an extended panel of structures, with the goal to fold all of the structures while maintaining reasonably folding quality (i.e. fast running narrow bands) for as many of the structures as possible. The panel of structures was also expanded to assure the effectiveness of the TARSA protocol. Ultimately, the ramp contained times of 10 minutes, 45 seconds, 4 minutes, 15 minutes, 10 minutes and 5 minutes for 65°C, 58°C, 51°C, 46°C, 37°C, 4°C respectively as shown in Figure 4.8 for the initial panel and Figure 4.9 for the extended. These structures were all formed simultaneously, 200µL liquid volumes were
placed in .5 mL Eppendorf tubes and reactions were performed in dual water baths. However, during the shorter timed steps it was necessary to add ice in order to facilitate cooling. Though likely not as effective heat transfer as when reactions are performed in a thermocycler, structures still formed reasonably well. Structures were then analyzed by agarose gel electrophoresis and TEM (Figures 4.8B, 4.8C, 4.9B, 4.9C). A trial-and-error approach was needed for proper folding of certain structures and additional ramps and corresponding gels can be found in Figure 4.10. If equipment or time is limited for analysis of a newly designed structures, this TARSA ramp can be confidently used to provide reasonable quality folded structures for a wide range of DNA origami design.

Figure 4.8. Folding characterization of the TARSA protocol of the original panel.

(A) 3D models showing diversity of structures tested. (B) Electrophoresis gel comparing structures folded using the standard 2.5 day procedure in the thermocycler (2.5) and structures folded using the DRUMPF protocol in water baths. (C) TEM images showing representative images of well folded structures using the standard protocol (top) and the DRUMPF protocol (bottom).
4.4 Further Alternative Methods

In the spirit of the study and in order to limit the equipment required for DNA origami, further simplified fabrication methods were developed. Using the equipment, such as tabletop burners and ring stands, we were able to build an apparatus based on equipment contained even in almost all High School Chemistry laboratories in order to fold DNA origami nanostructures (Figure 4.11). Two 1L beakers were situated on burner stands of different heights with Bunsen burners located underneath. It was possible to keep the temperatures of the baths well within the high (65-70°C) and critical temperature range of the “horse” nanostructure for the 30 minutes necessary for the fold to easily occur.
Structures were then analyzed by agarose gel electrophoresis and TEM to confirm proper folding (data not shown).

4.5 Discussion

In order for the DNA origami nanostructure fabrication method to be elevated for use as a delivery vehicle for treatments for cancer in a clinical setting, the requirement for structure formation on a larger scale is imperative. Before addressing the clinical possibilities, \textit{in vivo} studies must be conducted for pharmacokinetics, bio-distribution, and therapeutic effectiveness. As a quick estimate for the scale of structures needed, we present
a standard 15 mouse pharmacokinetics study, using doxorubicin as a controlled, known standard. Using a doxorubicin injection concentration of 10 mg/kg with the molar ratio of doxorubicin to DNA nanostructure at 15:2, the study would require approximately 35.5 mL of folded DNA at the standard 20 nM. This would effectively be 700 folding reactions at the traditional 50 µL scale. This example would be a small, simple study that only uses one structure and limited experimental samples and animal model size. If the study was expanded to a full study with multiple structures and a full biodistribution panel, it is not

**Figure 4.10. Trial and error approach to TARSA fold**

Five gels in top left use the following structures in order (not accounting for the one with a red ‘x’); Horse, Hinge, BL, LPP, 18hb-S, 6hb, D18, F18, Scissor-jack, Otter, Block O. Each of the ‘UF’ gels have the corresponding ramp shown in the table directly to the gel’s right. After it became apparent that most structures can fold at these temperatures, there were two exceptions, the scissor jack and block ‘O’ structures. There were three tests done on only these two structures shown in the bottom gels.
difficult to image a full liter of folded structures would be needed. These numbers would increase quickly if looking at a larger animal model or human trials.

Creating DNA nanostructures for diagnostics (108), enzymatic reactions (103) or as measurement tools will eventually require greater amounts if they are ever to be used for commercial or industrial means. Here we assembled four distinct DNA nanostructures in volumes as large as 50 mL, as well as developed an all-purpose method of DNA origami formation that could be done with confidence using most structures. These methods can be implemented once more large-scale uses are needed.

The main limiting factor in folding DNA origami on a larger scale is, of course, cost. These costs are associated with both the staples and scaffold, but in the future can be mitigated by already established processes. Bacteriophage creation relies on the growth of competent E. coli cells, which has many options for high throughput growth including
change of growth media and extraction methods (109, 110). Though not used in this study, in-house staple creation can easily occur, once methods are established, using chip-set inkjet printing and has been used in both DNA origami and gene synthesis (86, 111-114).

4.6 Conclusions
Here, we were able to show that DNA origami can be folded in a large one-pot folding reaction on the scale of 500x that is typically done. We were also able to show that the rapid folding techniques shown by Sobczak et. al. can be used not only for folding but for the information gathered by the process, which could be expanded on in later studies (28). Currently the need for larger-scale formation of DNA origami is needed only in limited situations, but for DNA origami to succeed in biologic applications, this scale up will quickly become necessary. There will be alternative methods of folding on larger scales, from the building of large-scaled thermocyclers to continuous flow reactors. This study demonstrates that volume is not a limiting factor in whether or not DNA origami will be used in these large-scale applications.

4.7 Materials and Methods
Nanostructure design and assembly via scaffolded DNA origami. All structures were designed using the computer-aided design software cadnano (85) and thermocycler-folded structures were formed using the process described by Castro et. al. (27). Two of the structures, the hinge and the Bennett linkage were previously presented by Marras et al (8). All designs can be found in Appendix B. Staple sequences output from cadnano were ordered from a commercial vendor (Eurofins Genomics, Huntsville, AL), and several versions of the m13mp18 scaffold was either produced in our lab as previously described (27, 82) or purchased (Guild Biosciences Inc, Dublin, OH). Briefly, A M13mp18 ssDNA
scaffold was placed in solution at 100 nM in a buffer containing 5 mM Tris, 5 mM NaCl, 1 mM EDTA, 20 mM MgCl₂, and oligonucleotide staple strands, each in a 10-fold molar excess to scaffold. The mixture underwent a 65 hour thermal ramp including heating to 65°C followed by slow cooling to 25°C to facilitate molecular self-assembly driven by complementary binding of staples.

**Agarose gel electrophoresis.** For conformation of purity, folding reaction products were subjected to 2% agarose gel electrophoresis (0.5X TBE) in the presence of 11 mM MgCl₂ followed by removal of bands containing well-folded structures (27). DNA origami nanostructures were extracted from the excised agarose bands via centrifugation with “freeze ‘N squeeze” purification tubes (BioRAD, Hercules, CA) and visualized via transmission electron microscopy (27).

**Transmission Electron Microscopy (TEM).** Copper TEM grids coated with carbon and formvar (Electron Microscopy Sciences, Hatfield, PA) were prepared as described in Castro et al. (27). A 3 µl droplet of purified structures was placed onto a grid and incubated for four minutes. The solution was then wicked away using filter paper, the grid was then washed with 10 µl droplet of 2% Uranyl Formate and immediately wicked off using filter paper, followed by a 40 second application of a 20 µl droplet of 2% Uranyl Formate. DNA nanostructures were visualized on a Tecnai G2 BioTWIN transmission electron microscope (FEI, Hillsboro, OR) at an electron acceleration voltage of 80 kV.
Chapter 5: Antibody Targeting Drug Delivery and *in vivo* Application of DNA nanotechnology

I would like to acknowledge input from Christopher R. Lucas, Amjad Akif Chowdhury, Laura M. Heyeck, and Carlos E. Castro for execution, protocols, and writing in this chapter.

5.1 Introduction

As shown in previous chapters, DNA origami nanostructures are a promising and versatile drug delivery vehicle. Up to this point, the manuscript has focused on *in vitro* work and scale up, in order to make DNA origami technology a viable option in the future for clinical use. Though promising, the Horse nanostructure loaded with the anthracycline daunorubicin relies on non-specific uptake into cells in a dish. Here, we will focus on structure modification and pilot studies of *in vivo* delivery of DNA nanostructures.

5.2 Antibody targeted DNA origami nanostructures

Antibodies have been attached to many nanoparticles with different levels of success (115-119). Although polymers and liposomes are the most researched fields in nanotechnology drug delivery, both lack the versatility and precise control of DNA origami nanostructures. The programmed self-assembly of DNA technology allows for precise placement of any modification that can be attached to an oligonucleotide. This includes biotin, which is highly utilized for *in vitro* work, or functional groups such as
amines or thiols, which allow for covalent attachment. Here we exploit the ability to functionalize DNA nanostructures for the goal of attachment of antibodies to a nanostructure is to increase specificity to a certain cell type, in this case a CD33+ AML cell line, HL60. A commercially biotinylated monoclonal anti-CD33 antibody was purchased (Abcam, Cambridge, MA) and attached via a biotin, streptavidin interaction, which attachment scheme used for most experiments in this chapter. Anti-CD20 antibodies, commercially known as Obinutuzumab and kindly provided by Dr. John Byrd’s CLL Experimental Therapeutics Laboratory at The Ohio State University Comprehensive Cancer Center, were also attached using a commercially available kit (Solulink, San Diego, California) (Figure 5.1).

5.2.1 Horse Redesign and Attachment of Antibody Modification

In Chapter 3, Figure 3.1B, the DNA horse nanostructure was modified to contain ssDNA ‘overhangs’, which serve as attachment sites for complementary oligonucleotide conjugated with fluorescent molecules, such as Alexa647 and Cy3. These modifications are commercially available through a multitude of DNA synthesis companies, such as IDT, Eurofins Genomics, and Sigma-Aldrich. For this study, a minimal redesign of the

Figure 5.1. DNA Horse nanostructures, with a single attachment point.

Conjugated with streptavidin (left), biotinylated anti-CD33 Antibodies (center), and by covalent bound anti-CD20 antibodies (right).
Horse was required because of a desire for antibody conjugation to occur at specific locales on the structure, in this case the ends. A total of six oligonucleotides, out of 150 total, were replaced to add four overhangs as attachment points leaving the rest of the structure identical to the initial design. The ssDNA overhangs have two sets of sequences, both with a 21-nucleotide sequence, to allow for two distinct binding sites. This allows two different molecules to be simply attached to the structure, allowing for added flexibility of the structure. The location of the overhangs is important, as we hypothesize that rod-like structures are internalized through endocytosis by tip-dependent response as it was modelled in a previous study (120). Having the antibodies attached to the ends of the structure hopefully allows an improvement on the current method of internalization and not acting counterproductive to the natural cellular uptake of the structures.

Briefly, the process of antibody conjugation is as follows. DNA origami nanostuctures with ssDNA attachment sites are incubated with 10x excess attachment
sites of biotinylated oligonucleotides that are complementary to the ssDNA sites and incubated for 1 hr at 37°C. Structures are then precipitated with PEG purification protocol, a 1:1 volumetric mixture of nanostructures to PEG mixture (15% PEG 8000, 5 mM Tris, 1 mM EDTA, 500 mM NaCl) as described in Chapter 2, resuspended in PBS with 2.5 mM MgCl₂ and PEG purified a second time, then resuspending the pellet again. Attachment and purification is optimized using higher concentrations of DNA nanostructures (>100 nM) at volumes >100 µL. Streptavidin is then mixed with biotinylated nanostructures at an excess of 500:1 and incubated at 37°C for 1 hr. Structures are then PEG purified two times, each time resuspending in PBS with 2.5 mM MgCl₂ added. Now that the excess streptavidin is removed from solution, biotinylated antibodies are now incubated at 10x the attachment sites for 2 hrs at 37°C. Nanostructures are again PEG purified 2x, this time using a PEG mixture with lower NaCl (15% PEG 8000, 5 mM Tris, 1 mM EDTA, 50 mM NaCl). Resuspend nanostructures in PBS with 2.5 mM MgCl₂ and antibody-conjugated nanostructures are
are ready for use. For covalent conjugation of antibodies, amine conjugated oligonucleotides are linked to antibody using an Antibody-Oligonucleotide All-in-One Conjugation Kit (Solulink, San Diego, CA), following instructions provided. Antibody conjugated oligonucleotides are then incubated at 10x concentration of nanostructures for 2 hrs at 37°C. Nanostructures are PEG purified 2x, using the PEG mixture with lower

\textit{Figure 5.3 Ease of Customizing DNA origami nanostructures.}

(A) TEM images of either unloaded or daunorubicin-loaded Horse DNA origami nanostructures functionalized with none, 1, or 2 targeting (biotin-anti-CD33) antibodies. (B) TEM images of Horse DNA origami nanostructures functionalized with PEG (2kDa), 2′-OMe, or PEI with molecular structures shown. (C) All molecular modifications were confirmed via agarose gel electrophoresis (EtBr stain) where a shift was present upon each molecular modification relative to the unmodified Horse DNA origami nanostructure control.
NaCl (15% PEG 8000, 5 mM Tris, 1 mM EDTA, 50 mM NaCl). Resuspend nanostructures in PBS with 2.5 mM MgCl₂ and antibody-conjugated nanostructures are ready for use.

**Figure 5.3A** shows examples of successful conjugation of CD33 antibodies with and without the addition of daunorubicin loading using the methods described in Chapter 3. Attachment stability is a concern for both *in vitro* and *in vivo* experiments; therefore,

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**Figure 5.4** Agarose Gel Electrophoresis of Antibody Conjugated Horse Nanostructures in 100% Fetal Bovine Serum for Various Time Points.

Top gel is EtBr stain with UV excitation of DNA nanostructures, with structures beginning to breakdown between four and six hours with near complete at 24. Bottom is 640 nm excitation and red, high pass emission filter indicating streptavidin with Alexa 647 fluorophores remain attached to structure to at least eight hours. The control is horse DNA origami nanostructure with 2- Alexa647 conjugated streptavidin molecules in PBS with 2.5 mM MgCl₂.

we incubated structures in 100% fetal bovine serum (FBS) to resolve the greater of the fears (**Figure 5.4**). Structure begin to degrade in between four and six hours and are still
lightly present after 24 hours and it appears that as long as the structure in formed in solution the Alexa647 conjugated streptavidin remained attached to the structures. The control on this gel also shows successful removal of excess streptavidin and free streptavidin-biotinylated antibody conjugates from solution.

**5.2.2 Targeting DNA horse nanostructures to HL60, CD33+ cells**

To test the targeting capabilities of the antibody-conjugated horse nanostructure, we first used a cell culture of GFP-labeled HS-5 stromal and HL60 cell lines, both kindly provided by Dr. John Byrd’s Laboratory. HS-5 GFP stromal cells are not considered to
be CD33+, and hence are a good complementary cell to HL60, which are CD33+, both being naturally located in the bone marrow. Cells were cultured together and 1 nM origami structure, with CD33 antibody attached by a streptavidin conjugated with Alexa 647 fluorophores was incubated for 30 minutes, 1 hr, 3 hr, and 16 hr (Figure 5.5). The structures used for this experiment have two CD33 antibodies attached to each end and

**Figure 5.5. CD33 targeted horse DNA origami nanostructures in human stromal and acute myeloid leukemia cells.**

GFP+/CD33- HS-5 human stromal cells (green) are cocultured with CD33+ HL60 cells and incubated with horse DNA nanostructures (red) with two CD33 antibodies attached via streptavidin, biotin linkage. Structures were incubated for the times listed on the left. Representative images of 15 locations and three experiments.
two ssDNA overhangs with nothing attached. In each of the time points there is no apparent colocalization of the Alexa 647 that is attached to the structure and the GFP+/CD33- HS-5 cells. By 30 minutes, the structures appear to attach to the cell membrane of the HL60s further developing a thicker ring at 1 hour, a possible indication that internalization has begun, or more structures have bound to the surface. At the three-hour time point, further internalization of the nanostructures occurs. After an overnight incubation of 16 hours, structures seem to be fully internalized in only the HL60, CD33+ cells (Figure 5.5). By performing these experiments on a fluorescent microscope, it is difficult to maintain focus on both the adherent HS-5 and the suspended HL60 cell lines at the same time, though there was never apparent colocalization of DNA origami structures with the stromal cells.

Using CellTracker Green (Thermo Fisher, Waltham, MA) to stain a Chronic Lymphocytic Leukemia (CLL) cell line, OSUCLL, again kindly provided by Dr. Byrd’s Laboratory, similar experiments were performed using two suspension cell lines. In Figure 5.6, we show that after 16 hours incubation the Alexa 647 signal does not overlap

**Figure 5.6.** Coculture of HL60 and OSUCLL cell lines with Antibody Targeted and Streptavidin only DNA origami nanostructures.
with the green stained OSUCLL; again the antibody conjugated structures are only internalized by the CD33+ cell line. Interestingly, when the structure only has the streptavidin with Alexa 647 attached to it with no antibody, the structures are not internalized by either cell line (Figure 5.6), suggesting the addition of the streptavidin might deter non-specific binding and uptake into cells.

While these results are promising, since the fluorescent label is located on the streptavidin molecule, it is possible that free streptavidin antibody conjugates are binding to cells. Figure 5.4 shows that excess streptavidin is purified out during the functionalization process, however, purifying excess antibodies out is challenging to verify. It is possible that streptavidin antibody conjugates could fall off structures or streptavidin could fall off and bind to antibodies in solution that were not purified out. To

![Image](image_url)

**Figure 5.7. Colocalized Cy3 and Alexa 647 fluorophores on HL60 cells using targeted DNA origami nanostructures.**

CD33 conjugated using streptavidin with Alexa 647 (red) attached to DNA origami nanostructures that are labelled with Cy3 (green). Matlab script was used to remove bleed over from Alexa 647 into 561 excitation channel. Structures were incubated for 30 minutes before imaging and yellow indicates that both antibody remains attached to structure.
rule out this possibility, we performed imaging experiments to simultaneously image streptavidin and directly labeled DNA nanostructures. For this experiment, Horse nanostructures that contained two CD33 antibodies attached via Alexa647 labeled streptavidin and two Cy3 fluorophores directly to the Horse nanostructures. After 1 hour of structures incubated with CD33+ HL60 cells, it was shown that the signal for the nanostructure, Cy3 fluorophore, and antibody, Alexa 647 fluorophore, were colocalized on the cell membrane (Figure 5.7). There is some signal bleed over from the Alexa 647 into the Cy3, which was removed using a custom Matlab script, kindly provided by Randy Patton and modified and used by Laura Heyeck. Nanostructures with and without Cy3 were added to target cells and 647 nm and 561 nm intensity signals were measured for both. A ratio of 561nm/647nm was taken from the structures without Cy3. The 647 nm intensity of the nanostructures with Cy3 was multiplied with this ratio and the corresponding 561 nm value was subtracted from total 561 nm intensity on the image. These results confirm that antibody labeled Horse nanostructures indeed bind to cells via antibody-mediated interactions. Interestingly, we found that streptavidin labeled Horse nanostructures did not bind to cells like our previously unlabeled Horse nanostructures. Based on this result, we
hypothesized that modifying the ends of the structures blocks the typical binding and uptake mechanism of unlabeled DNA nanostructures.

To test whether non-specific uptake into cells was blocked using the current design, further experiments were performed. Targeted and streptavidin only structures were incubated with only OSUCLL or HS-5 cells overnight, with no internalization shown in either case (Figure 5.8), though using the original horse design with an
intercalating dye, TO-PRO-3 (Thermo Fisher, Waltham, MA) allowed for cell internalization in both cell lines. A small amount of structures did appear to bind to either cell surfaces or the extra cellular matrix in the HS-5 cell line. This binding was qualitatively distinct from cell uptake, which could be due to aggregation or some
binding to extracellular materials. Further experiments are required to confirm whether this is consistent and would be important to be aware of in translating to *in vivo* applications. Similar experiments were performed using HL60 cells, with internalization occurring in both the targeted and non-targeted structures, with a more robust appearance of structures in the targeted cells (Figure 5.9).

### 5.2.3 Daunorubicin Loaded, Antibody Targeted DNA nanostructures

As stated in Chapter 3, one of the main advantages of using DNA origami for drug delivery is its ability to overcome multi-drug resistance (MDR) cellular mechanisms. Our proposed mechanism for bypassing MDR is that structures internalize through the endolysosomal pathway. To verify that attaching antibodies does not affect the mechanism of cellular uptake, HL60 cells were stained with Lysotracker Green (Life Technologies, Grand Island, NY). After 16 hours incubation, colocalization of targeted structures and lysosomal compartments was prevalent in the culture (Figure 5.10), indicating that structures are still endocytosed.

![Figure 5.10. Antibody Targeted DNA origami nanostructure internalized through the endolysosomal pathway.](image)
Since antibody attachment improves specificity to targeted cells, at least in the case of CD33, efficacy experiments of delivering a payload with antibody targeted Horse nanostructures were performed. Again, we used the intercalating chemotherapy drug, daunorubicin, as our payload (Figure 5.2A). Drugs were loaded to a base pair binding ratio of 0.7-1 with methods described in Chapter 3, after antibodies were already conjugated. Initially, a CCK-8 cell viability assay was performed on both HL60 Parental and HL60-ADR cell lines after a 16 hour incubation of loaded targeted and non-targeted
structures at .1 μM, or equivalent structure concentration, of daunorubicin (Figure 5.11).
The results being similar to the ones found in Chapter 3, with significance (P-value < .05, student’s t-test) found in the HL60-ADR cell line, between drug alone and loaded structures. Although using the targeted nanostructures did not improve efficacy of the structures on a targeted cell type in vitro, it appeared to still bypass MDR resistance. Though there was not significance in the parental cell line, trends for improved efficacy were still observed (Figure 5.11). Structure with antibody attachment only, or no daunorubicin loaded, showed no reduction of viable cells in either cell line.

To assess ability of antibody-labeled Horse nanostructures to deliver drugs to a specific cell type among multiple types of cells, OSUCLL and HL60 cell lines were tested in co-culture and analyzed by fluorescent microscopy. Daunorubicin, which fluoresces easily with an excitation laser of 488 nm, can be quantified by region of interest analysis using Nikon Elements Software. After 3 hours incubation with the daunorubicin-loaded and antibody-conjugated nanostructures there was a marked increase in daunorubicin internalization HL60 cells, but not OSUCLLs. Because of the wide emission spectrum of daunorubicin, CellTracker Deep Red (Thermo Fisher) was used to stain the OSUCLL cell line. The fluorescence intensity of the daunorubicin
indicates that twice the amount of daunorubicin was internalized using the targeted system at the 3 hr time point. Also, the relative cell number using the targeted structures was markedly decreased using the targeted, loaded structures (Figure 5.12); this was done by a manual count of live and dead cells, with dead cells being marked with Live/Dead Blue-UV excitation stain (Thermo Fisher) and a morphology test and

Figure 5.12. Antibody targeted, daunorubicin loaded DNA nanostructures effect on cell cocultures and drug internalization.

(Top Left) Daunorubicin fluorescence average intensity for region of interest over HL60 vs. OSUCLL cells in either targeted nanostructures or free daunorubicin incubated for 3 hrs, n>30 for each data set. (Top Right) Relative cell numbers, normalized by cells only control, in HL60 and OSUCLL coculture when incubated with targeted nanostructures or free daunorubicin for 3 hrs, one experiment. (Bottom) Representative image of data on top left.
normalizing it to a PBS treated controls. These results are promising, but this experiment will need to be repeated to verify results, with more time points to assure efficacy.

5.3 *In vivo* Pilot for DNA Origami Horse Nanostructure

There have been two previous *in vivo* studies published in the field of DNA origami nanostructures (43, 45). One study, Zhang *et al.*, loaded doxorubicin into different shaped origami structures and, though limited, showed promising results. The other, Perrault *et al.*, was more comprehensive, doing pharmacokinetics and biodistribution, and, after struggling with stability issues, encapsulated their complex structure with an anchored liposome and polyethylene glycol modifications to increase stability. Here, in a pilot study, we focus on the DNA origami horse nanostructure itself. The aim of this study was to confirm the tolerability of the Horse nanostructure testing whether the unloaded structure causes systemic or local toxicity or immunogenicity. Prior to performing in vivo pilot tests, we performed more extensive characterization of the stability in physiological buffers.

5.3.1 Injection Method and Biodistribution

Each of the previous *in vivo* studies were single injection using intravenous (i.v.) route of delivery. With Perrault *et al.* doing a single injection at approximately 50 nM (1.2 mg/kg) and Zhang *et al.* using about 3.33 nM (.08 mg/kg) injections every 3\textsuperscript{rd} day for 12 days. The advantage of using intercalating drugs, such as doxorubicin or daunorubicin, is that the load of drug molecules per DNA nanostructure is very high (7000+), when comparing it to overhang or site loading (<150 attachments). This
allowed Zhang et al. to use a very low concentration of origami nanostructures, but if we were looking to deliver therapeutic oligonucleotides, it would be necessary to use a much greater concentration of nanostructures. With this in mind, we performed a simple, 24-hour single i.v. injection experiment with five, ICR mice with 100 µL at 500 nM (12 mg/kg). No apparent toxicity was seen 24 hours postinjection.

![Figure 5.13. Horse DNA origami nanostructure stability in serum and plasma.](image)

(A) To address Horse DNA origami structural stability in serum, Horse DNA origami nanostructures were resuspended in various percentages of FBS for 24 hrs followed by PEG precipitation and analysis via agarose gel electrophoresis. (B) To evaluate Horse DNA origami structural stability as a function of time streptavidin with Alexa 647- Horse DNA origami nanostructures were resuspended in 100% FBS and incubated at 37 deg C for various times followed by PEG precipitation and analysis via fluorescent gel electrophoresis. Fluorescent bands were imaged by a Typhoon Imaging System (General Electric) and post-stained with EtBr (total DNA stain). (C) To monitor Horse DNA origami structural stability in complete plasma, Horse DNA origami nanostructures were resuspended in 100% Human or Mouse plasma and incubated at 37 deg C for 1, 3, 6, and 24 hrs followed by PEG precipitation and analysis via agarose gel electrophoresis.
Perrault *et al.* used a complex, octahedron structures that they presented in two studies\((42, 43)\). This structure was unstable in cell culture media with 10% serum with added Mg\(^{2+}\). On the other hand, we showed in Chapter 3 that the horse nanostructure is stable in 20% serum and no added Mg\(^{2+}\). Additionally, Figure 5.13 shows that structures

*Figure 5.14. Intraperitoneal vs. Intravenous injection IVIS time course images.*

To evaluate biodistribution as functions of injection route and time, ICR mice (female, 6 weeks old) were injected either i.p. \((n=3)\) or i.v. \((n=3)\) with Cy5.5-labeled Horse DNA origami nanostructures \((500nM, 100\mu l \text{ injections})\) and subjected to live animal imaging fluorescence imaging (IVIS) directly following injection, 6 hrs post injection, and 24 hrs post injection. A representative mouse injected i.p. \((\text{left})\) or i.v. \((\text{right})\) is shown.
are stable in 100% human and mouse plasma for over six hours, and 100% fetal bovine serum for over 8 hours without modifications.

To expand on the original \textit{in vivo} pilot, the horse nanostructure had another minimal redesign to add three Cy5.5 fluorescent located in three of the internal cavities. The structure was folded and verified by gel electrophoresis as seen in Figure 5.3C.

With the desire to inject a series of mice every other day for ten days, it became necessary to test if intraperitoneal (i.p) is distributed in a similar way to i.v. Three 6-week old ICR mice were injected by i.v. and three were i.p. and then were imaged using an IVIS fluorescent imaging, right after injection, then again at six hours and 24 hours (Figure 5.14). Immediately after injecting most i.v. mice showed a distribution in the blood stream, where the nanostructures were distributed in the i.p. mice by six hours.

\textit{Figure 5.15. Biodistribution of DNA origami nanostructures after injection by i.p.}

(A) To assess DNA origami biodistribution \textit{in vivo}, ICR mice (female, 6 weeks old) were injected (i.p.) with either i) 1X PBS + 1mM MgCl$_2$ (n=1), ii) LPS (Sigma) (10µg, n=1), iii) CpG-ODN 2395 (Invivogen) (Class C, 10µg, n=1), or iv) Cy5.5-labeled Horse DNA origami nanostructures (500nM, n=5); (100µl) followed by live animal fluorescence imaging (IVIS imaging system) 3 hours post i.p. injection. Three representative DNA origami treated mice are shown.
This was satisfactory to use i.p. injections for the larger pilot study. Five more 6-week old ICR mice were injected with 100 µL at 500 nM, in addition to one injected with PBS with 1 mM MgCl₂ and two inflammation causing positive controls. Although there was some autofluorescence, likely due to mild inflammation, which made the choice of using Cy5.5 less than ideal, IVIS imaging showed full circulation of structures throughout the body (Figure 5.15). All experiments were performed in cooperation with Dr. Amy Johnson’s group at Ohio State’s Comprehensive Cancer Center.

5.3.2 Toxicity of DNA origami nanostructures in vivo

The experimental plan for the larger in vivo pilot can be found in Figure 5.16, which included injections every other day and strategic blood draws to test for inflammation cytokines and blood counts. For the two positive control mice, one was
given 10 µg LPS (Sigma Aldrich) and the other 10 µg of CPG-ODN 2395 (Invivogen).

After five i.p. injections each of their respective treatments over the course of eight days, all eight mice survived. The five mice that were injected with horse DNA nanostructures maintained a weight above their starting point (Figure 5.17). After day eight, all the animals were sacrificed and their organs and tissues collected. Three of the mice, two treated with DNA origami and the PBS control, had a complete necropsy pathology evaluation performed by the Comparative Pathology and Mouse Phenotyping Shared Resource at Ohio State. The pathologist reported no significant findings; samples of the H&E stained kidney and liver tissues are found in Figure 5.18. In addition, tissues of the mice that did not have a necropsy performed had their organs imaged to rule out
accumulation of nanostructures in any tissues over the course of the study. Figure 5.19 shows the major organs, with the only single located in the stomach, which again is likely autofluorescence caused by inflammation (121).

5.3.3 Immunogenicity of DNA origami nanostructures in vivo

A key factor in the use of DNA origami as a chemotherapeutic delivery system is whether or not DNA nanostructures cause an immune response. One potential immune response could come from toll-like receptor (TLR9) response caused by the m13mp18 bacteriophage DNA that is used a scaffold in DNA origami design and construction.

Figure 5.18. Organ Tissue Toxicity Evaluation of DNA Origami Nanostructure in vivo.

On day 8 post the 5 repeated dosing regimen, three mice (1 PBS control)- and (2 Horse DNA origami)-treated were subjected to complete necropsy evaluation. H&E stained slides of the liver (top) and kidney (bottom) sections of the animals are shown.

A key factor in the use of DNA origami as a chemotherapeutic delivery system is whether or not DNA nanostructures cause an immune response. One potential immune response could come from toll-like receptor (TLR9) response caused by the m13mp18 bacteriophage DNA that is used a scaffold in DNA origami design and construction.
TLR9 is involved in the innate detection of foreign material and activated in response of DNA (122). Another potential source of immunogenicity is the endotoxins that can be leftover from the m13mp18 production process, which is grown using E.coli bacterial cells. Therefore, the scaffold used for the in vivo pilot was filtered through an endotoxin removal kit (Qiagen, Valencia, CA) during growth process. In addition, we selected positive controls for these experiments including TLR9 inducing CPG-oligodeoxynucleotide named ODN 2395 (Invivogen) and endotoxin response using LPS (Sigma Aldrich), as well as a negative control of a PBS injection. Each were given by i.p injection in doses of .5 mg/kg at the same time points as the origami nanostructures.

Whole blood was collected by submandibular bleed at 24 hours and day 8 after the first injection from all mice for complete blood counts to be performed (Figure 5.19. Ruling Out Organ Specific Accumulation of DNA origami nanostructure in vivo).

In order to determine organ specific accumulation of DNA origami nanostructures, at the conclusion of the 8 day experiment previously described, treated mice (1 LPS, 1 CpG-ODN, and 3 Cy5.5-labeled Horse DNA origami nanostructures; the remaining animals were subject to complete necropsy) animals were euthanized, organs were harvested and subjected to fluorescent imaging (IVIS). Animals were anesthetized via isoflurane via live animal imaging with the following specifications (Cy5.5 channel, 680 nm excitation, 710 emission, 1 minute exposure).
In addition, cardiac blood was collected from each animal and flow cytometry was performed for T-Cell and B-Cell counts and respective cell activations (Figure 5.20).
The complete blood counts shows average counts in the normal ranges for white blood cells, red blood cells, hemoglobin, and platelets; with no other significant findings found. In addition, the T-cell panel shows that the DNA origami T-cell total count and activation levels were in normal ranges. However, the B-cell panel indicates a high number and activation of B-cells.

To further analyze immune response, the levels of key immunogenic cytokine factors including IFN-γ, TNF-α, IL-6, IL-1β, IL-12p70, and an anti-inflammatory, IL-10, were measured in the plasma by Luminex Technology using a Multiplex system (Milliplex, Darmstadt, Germany) (Figure 5.21). Samples were collected by submandibular bleed into EDTA lined tubes and samples were centrifuged and plasma removed from top layer for the experiment before initial injections and then at 24, 48, 72 hours, and 7 and 8 days after first injection. The results show an increased cytokine response, especially in IL-6 and IL-12 but not one that matches either of the positive controls. This likely indicates that the DNA origami cytokine response is not caused by either a reaction to endotoxins or TLR9 activation, but rather an independent response.

The main advantage of using DNA origami nanostructures for drug delivery is the ability to modify the design with ease and precision. Although having an immune response does not rule out DNA origami nanostructures for clinical applications, there are modification that can be done to the structures to dampen any inherent response. Three modifications were used to dampen an immune response for ex vivo experiments in total splenocytes (Figure 5.3B). 2 kDa amine reactive Methoxy-PEG succinic acid NHS (NanoCS, New York, NY) were attached to amine conjugated oligonucleotides (IDT, San Jose, CA) and attached in PBS buffer at pH 7.4 using a simple reduction reaction and
then purified using a dialysis capsule (Thermo Fisher) and then attached to 44 complementary ssDNA located around the horse nanostructure. 2’OMe RNA (IDT) is commercially available and again attached to the 44 sites. Last is Polyethylenamine (PEI), which is positively charged and attaches to the DNA nanostructure through electrostatic interactions and are incubated at a 1:1 base pair to PEI ratio.

To evaluate the level of molecular immunogenicity and potential inflammatory response to DNA origami nanostructures in vivo, ICR mice (female, 6 weeks old) were injected (i.p.) with either i) 1X PBS + 1mM MgCl₂ (n=1), ii) LPS (Sigma) (10µg, n=1), iii) CpG-ODN 2395 (Invivogen) (Class C, 10µg, n=1), or iv) Cy5.5-labeled Horse DNA origami nanostructures (500nM, n=5); (100µl) under a repeat dosing regimen every 48 hrs over an 8 day time course. Whole blood was collected in EDTA-treated tubes (sub-mandibular) on day -3, day 1, day 2, day 3, day 7, and day 8 and plasma was isolated. The levels of key immunogenic soluble cytokine factors including (IFN-γ, TNF-α, IL-6, IL-1β, IL-12p70, and anti-inflammatory (IL-10) were measured in the plasma by Luminex Technology (Milliplex). Plasma samples were measured in duplicate and are expressed as the mean cytokine concentration (ng/ml) ± SEM.

Figure 5.21. Molecular immunogenicity evaluation of DNA origami nanostructures in vivo.

To evaluate the level of molecular immunogenicity and potential inflammatory response to DNA origami nanostructures in vivo, ICR mice (female, 6 weeks old) were injected (i.p.) with either i) 1X PBS + 1mM MgCl₂ (n=1), ii) LPS (Sigma) (10µg, n=1), iii) CpG-ODN 2395 (Invivogen) (Class C, 10µg, n=1), or iv) Cy5.5-labeled Horse DNA origami nanostructures (500nM, n=5); (100µl) under a repeat dosing regimen every 48 hrs over an 8 day time course. Whole blood was collected in EDTA-treated tubes (sub-mandibular) on day -3, day 1, day 2, day 3, day 7, and day 8 and plasma was isolated. The levels of key immunogenic soluble cytokine factors including (IFN-γ, TNF-α, IL-6, IL-1β, IL-12p70, and anti-inflammatory (IL-10) were measured in the plasma by Luminex Technology (Milliplex). Plasma samples were measured in duplicate and are expressed as the mean cytokine concentration (ng/ml) ± SEM.

then purified using a dialysis capsule (Thermo Fisher) and then attached to 44 complementary ssDNA located around the horse nanostructure. 2’OMe RNA (IDT) is commercially available and again attached to the 44 sites. Last is Polyethylenamine (PEI), which is positively charged and attaches to the DNA nanostructure through electrostatic interactions and are incubated at a 1:1 base pair to PEI ratio. These
modifications were verified using gel electrophoresis (Figure 5.3C) and visualized using TEM imaging (Figure 5.3B). With the extra oligonucleotides attached, Figure 5.3C shows a band shift in the lanes with PEG and 2’OMe attachments, with some of the PEG molecule attachments not running well through the gel. The PEI, with its positive charge
causes the structure band to smear; which is indicative of the more uneven binding of the process.

To measure these modifications effect on immune response \textit{ex vivo}, total splenocytes were taken from 6-8 week old C57/BL6 mice and cultured with horse DNA origami nanostructures alone and with modifications at 50 nM. ELISA assays for IL-6 and IL-12p70 (R&D Systems, Minneapolis, MN) were evaluated and showed a marked decrease in activity for both cytokines for all modifications (\textbf{Figure 5.22}).

\textbf{5.4 Conclusions and Outlook}

Although in earlier chapters, we discussed the promise of DNA origami as a drug delivery vehicle, this chapter focused on the future of drug delivery for DNA origami nanostructures. The potential of targeted delivery with a minimal immune response allows for a more wide spread use of DNA nanostructures in clinical application. A non-specific immune response could even be employed as an advantage in whole gene delivery or even vaccine development. The pilot study will need to be expanded upon for both an increased sample size and different positive controls.

In addition, for the near future, therapeutic oligonucleotides can be delivered using DNA origami nanostructures which are not limited to just a few attachment sites, allowing for a high density payload. With the possibility of a combination of therapies that can be employed, using less specific, but more powerful drugs and targeted with antibodies could lead to an increased result.
Works Cited


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90. F. Li et al., A DNAzyme-mediated logic gate for programming molecular capture and release on DNA origami. Chem Commun (Camb) 52, 8369-8372 (2016).
Appendix A: Matlab Code for averaging of TEM images

The following contains sections of code from related MATLAB scripts written by Dr. Carlos Castro and Joshua Johnson

% Halley Structure Averages
% Averaging individual structures from a TEM image
clc, clear all, close all

file_name = uigetfile('.tif');
Pic = imread(file_name); %Load image to select structures
double = im2double(Pic);
flip_q = questdlg('Would you like to have an option to flip?','','No','Yes','Yes');

% M = inputdlg('How many structures would you like to input?');
% N = str2double(M{1}); %turn into a scaler number

rect1 = [69.7500 133.6600 146.5100 31.5100];

%%Selecting Structure

doubledouble = im2double(double);
double2 = padarray(doubledouble,[200 200],'replicate','both');

figure(1);
rect = [15.5100 1.5100 450.98 400.98];%can change default box size (last two numbers) for fig 1
imshow(double2)
hold on
xlabel('Select All Structures and Press Enter or Double Click Last One','FontSize',20,'Fontweight','bold')
[xpos1,ypos1] = getpts(1);

%%for simple selection
% gel_crop_rect = imrect(gca, rect);
% pause
% rect = gel_crop_rect.getPosition;
% xpos = rect(1);
% ypos = rect(2);

xpos = xpos1 - rect(3)/2;
ypos = ypos1 - rect(4)/2;
box_width = rect(3); % set above
box_height = rect(4); % set above
[N, XX] = size(xpos);
hold off
NN = num2str(N);

for i=1:N

double2 = padarray(doubledouble,[200 200], 'replicate', 'both');
crop_im1 = double2(ypos(i):(ypos(i)+box_height),xpos(i):(xpos(i)+box_width));

rectx(i) = rect(1)+rect(3)/2; % for previous spot
recty(i) = rect(2)+rect(4)/2; % for previous spot
image_norm_2 = crop_im1;
image_norm = padarray(image_norm_2,[100 100], 'replicate', 'both');

% Rotating image

theta_deg = -5:0.2:5;
col_num_zero = 1000*ones(size(theta_deg));
max_col_sum = zeros(size(theta_deg));

ii = num2str(i);
figure(2)
imshow(image_norm,[0 max(max(image_norm))])
title([ii ' of ' NN])
xlabel('Set line for rotation, Press Any Key','FontSize',20,'Fontweight','bold')
rot_line = imline(gca);
pause
rot_pos = rot_line.getPosition;
rot_x1 = rot_pos(1,1);
rot_x2 = rot_pos(2,1);
rot_y1 = rot_pos(1,2);
rot_y2 = rot_pos(2,2);
theta_rot = 180/pi*atan((rot_y2-rot_y1)/(rot_x2-rot_x1));

Pic_rot = imrotate(image_norm,theta_rot, 'bicubic');
[r1, c1] = size(Pic_rot);

h1 = figure(3);
set(gcf,'Position',[500 25 600 600])
imshow(Pic_rot)

if strcmp(flip_q,'Yes')
    flip_q2 = questdlg('Do we need to flip?','''',''No','Yes','Yes');
    if strcmp(flip_q2,'Yes')
        k=0;
    136
while k==0
    flip_q3 = questdlg('Flip this image?', '', 'x-axis', 'y-axis', 'both', 'x-axis');
    k=0;
    if strcmp(flip_q3, 'y-axis')
        flip_image = fliplr(Pic_rot);
        Pic_rot = flip_image;
        imshow(Pic_rot)
    end
    if strcmp(flip_q3, 'x-axis')
        flip_image = fliplr(Pic_rot);
        Pic_rot = flip_image;
        flip_image = imrotate(Pic_rot, 180, 'bilinear');
        Pic_rot = flip_image;
        imshow(Pic_rot)
    end
    if strcmp(flip_q3, 'both')
        flip_image = imrotate(Pic_rot, 180, 'bilinear');
        Pic_rot = flip_image;
        imshow(Pic_rot)
    end
    flip_q4 = questdlg('Is it good?', '', 'yes', 'no', 'yes');
    if strcmp(flip_q4, 'yes')
        k=1;
    end
end
end

title([ii ' of ' NN])
xlabel('Change Box to Surround Structure, Press Any Key', 'FontSize', 20, 'Fontweight', 'bold')

gel_crop_rect = imrect(gca, rect1);
pause
rect1 = gel_crop_rect.getPosition;
rect2x = rect1(1);
rect2y = rect1(2);
rect2w = rect1(3);
rect2h = rect1(4);

% xpos1 = rect1(1)-35;  % adjust (by half of change) if adjusting below
% ypos1 = rect1(2)-90;  % adjust (by half of change) if adjusting below
% box_width1 = 170.51;  %
% box_height1 = 170.51;  % hinge control
% xpos1 = rect1(1)-75;  % adjust (by half of change) if adjusting below
% ypos1 = rect1(2)-120;  % adjust (by half of change) if adjusting below
below
% box_width1 = 350.51; %horse sized(85kx for turned)
% box_height1 = 300.51; %horse sized

box_width1 = 355.51; %horse sized - need large box to rotate
box_height1 = 300.51; %horse sized (115kx)
for_horse_x = (box_width1 - rect2w)/2;
for_horse_y = (box_height1 - rect2h)/2;
xpos1 = rect1(1)-for_horse_x; %auto-centered (x)
ypos1 = rect1(2)-for_horse_y; %auto-centered (y)

crop_im1 =
Pic_rot(ypos1:(ypos1+box_height1),xpos1:(xpos1+box_width1));
image_add = crop_im1;

if i==1
    tot_image = image_add;
else
    tot_image = tot_image + image_add;
end

close (2)
close (3)
end

%%%If you have existing image
take_data = questdlg('Would you like to add to a previous file?', '', 'No', 'Yes', 'Yes');
if strcmp(take_data,'Yes')
go_get = uigetfile('.mat'); %Get file name
previous = load(go_get); %Load addition image for add
add_to = previous.tot_image;
M2 = inputdlg('How many structures were in previous file?'); %number of images averaged previously
    tot_image = tot_image + add_to;
    N2 = str2double(M2{1}); %turn into a scaler number
    N = N+N2;
end

NN = num2str(N);
NNN = ['N is ' NN];
%%%saving as data
save_data = tot_image;
take_data = questdlg('Would you like to save this file?', '', 'No', 'Yes', 'Yes');
if strcmp(take_data,'Yes')
    save_data = uiputfile('.mat', NNN); % save images to add later
    save(save_data, 'tot_image');
end
%%%displaying for image save
meanImage = tot_image / N;
figure(3)
contrast_image = adapthisteq(meanImage);
imshow(contrast_image)
Figure B.1. Image of cadnano design for original “Trojan Horse” DNA origami nanostructure
Figure B.2. Image of cadnano design for “Trojan Horse” DNA origami nanostructure with 36 ssDNA attachment site “overhangs”
Figure B.3. Image of cadnano design for “Trojan Horse” DNA origami nanostructure with 44 ssDNA attachment site “overhangs”
Figure B.4. Image of cadnano design for hinge DNA origami nanostructure
Figure B.5. Image of cadnano design for bennett linkage DNA origami nanostructure
Figure B.6. Image of cadnano design for platform DNA origami nanostructure
Figure B.7. Image of cadnano design for Rothemund’s Rectangle DNA origami nanostructure
Figure B.8. Image of cadnano design for 18-helix bundle with symmetrical cross section