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Nucleic acid programmed polymeric nanomaterials for biological communication

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NUCLEIC ACID PROGRAMMED POLYMERIC NANOMATERIALS FOR BIOLOGICAL COMMUNICATION

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy

in

Chemistry

by

Anthony Michael Rush

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2015
The dissertation of Anthony Michael Rush is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2015
I dedicate this thesis to my grandfather,

Thomas Edward Rush.

For introducing me to the power of the fianchetto.
“...technology is the real skin of our species.

*Humanity, correctly seen in the context of the last five hundred years,*

*is an extruder of technological material.*

*We take in matter that has a low degree of organization;*

*we put it through mental filters,*

*and we extrude jewelry, gospels, space shuttles.*

- Terence McKenna
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LIST OF ABBREVIATIONS

Å = Angstrom (10^{-10} m)
a.u. = arbitrary units
AFM = atomic force microscopy
AMA = ammonia:methylamine (1:1 v/v)
AMD = age-related macular degeneration
AMO = anti-miRNA oligonucleotide
AS = antisense
Ar = aryl
ATRP = atom transfer radical polymerization
B = bilayer
-b- = block, in block copolymer
br = broad
°C = degrees Celsius
C = cylinder
CHARMM = Chemistry at HARvard Molecular Mechanics
C_L = cylinder length
CMV = cytomegalovirus
CNT = carbon nanotube
CPG = controlled pore glass
CPP = cell penetrating peptide
CTA = chain transfer agent
Cy5, CY5 = cyanine 5 phosphoramidite
Δ = delta, change
δ = chemical shift
d = doublet, days, deuterated
dA/dT = derivative of absorbance with respect to temperature
DABCYL = 4-(4-dimethylaminophenyl) diazenylbenzoic acid
DC-Chol = 3β-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride
DCM = dichloromethane
DDAB = dimethyl dioctadecylammonium (bromide salt)
DIPEA = N,N-Diisopropylethylamine
DLS = dynamic light scattering
DMF = dimethylformamide
DMRIE = (1,2-dimyristoylpropyl-3-dimethyl-hydroxy ethyl ammonium bromide)
DMSO = dimethylsulfoxide
DMT = dimethoxytrityl
dn/dc = change in refractive index with respect to change in concentration
DNA = deoxyribonucleic acid
DNase = deoxyribonuclease
DNG = deoxynucleic guanidine
DOGS = 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl]
DOPE = dioleoylphosphatidylethanolamine
DOSPA = (+/-)-N,N-dimethyl-N-[2-(sperminecarboxamido)ethyl]-2,3-bis(dioleyloxy)-1-propaniminium pentahydrochloride
DOTAP = 1,2-dioleoyl-3-trimethylammonium-propane
DOTMA = 1,2-di-O-octadecenyl-3-trimethylammonium propane
DP = degree of polymerization
DPBS = Dulbecco’s phosphate buffered saline
DPA = DNA-polymer amphiphile

ds = double-stranded

EDTA = ethylenediaminetetraacetic acid

ELISA = enzyme-linked immunosorbent assay

ENA = ethylene bridged nucleic acid

EPR = enhanced permeation and retention effect

equiv = equivalents

ESI = electrospray ionization

eV = electron volts

EVE = ethyl vinyl ether

ExoIII = exonuclease III

F, FdT, FL = fluorescein-modified thymidine

FACS = fluorescence-activated cell sorting

FCD = fucoidan

FDA = food and drug administration

FISH = fluorescence in-situ hybridization

FPLC = fast protein liquid chromatography

FPN III = filipin III

FRET = Förster resonance energy transfer

FSC = forward scatter

g = grams

GAPDH = glyceraldehyde 3-phosphate dehydrogenase

h = hours

HATU = (1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate)
HCV = hepatitis C virus
HIV = human immunodeficiency virus
HNA = hexitol nucleic acid
HPA = 3-hydroxypicolinic acid
HPLC = high performance liquid chromatography
HPMA = N-(2-Hydroxypropyl)methacrylamide
HRMS = High Resolution Mass Spectrometry
Hz = Hertz (s⁻¹)
IFN = interferon
J = NMR coupling constant
K = degrees Kelvin, thousand
kcal = kilocalories
k_cat = turnover number
K_m = the substrate concentration at which the reaction rate is at half-maximum
λ_abs = absorbance wavelength
λ_em = emission wavelength
λ_ex = excitation wavelength
L = liters
LNA = locked nucleic acid
LPA = LNA-polymer amphiphile
LRMS = low resolution mass spectrometry
m = meter, multiplet
mRNA = messenger RNA
MALDI-TOF = matrix-assisted laser desorption ionization-time of flight
MALS = multi-angle light scattering
MβCD = methyl-β-cyclodextrin
MD = Molecular Dynamics
Me = methyl (CH₃)
MEM = minimum essential medium
MeOH = methanol (CH₃OH)
Mes = mesityl, 2,4,6–trimethylphenyl (2,4,6–Me₃C₆H₂)
min = minutes
miRNA = micro RNA
MLV = murine leukemia virus
MMP = matrix metalloproteinase
MMT = monomethoxytrityl
Mₘ = number average molecular weight
mol = moles
mon = monomer
MS = mass spectrometry
Mₘ = weight average molecular weight
MWCO = molecular weight cut-off
Nₐgg = aggregation number
NA = nucleic acid
Nb = norbornene
NHS = N-hydroxysuccinimide
NIPAM = N-isopropylacrylamide
NMP = nitroxide-mediated radical polymerization
NMR = Nuclear Magnetic Resonance
NP = nanoparticle
O.D. = outer diameter
ODN = oligodeoxynucleotide
OEG = oligoethylene glycol
Ph = phenyl (C₆H₅)
ppm = parts per million
π = pi
PAGE = polyacrylamide gel electrophoresis
PAMAM = poly(amidoamine)
PBS = phoshoate buffered saline
PCL = poly(ε-caprolactone)
PCR = polymerase chain reaction
PEG = polyethylene glycol
PEI = poly(ethyleneimine)
PLC = phospholipase C
PLL = poly(L-lysine)
PME = particle-mesh Ewald
PNA = peptide nucleic acid
POLY I = poly(inosinic acid)
PS = phosphorothioate
PTD = peptide transduction domain
PVP = poly(vinylpyrrolidone)
q = quartet
qPCR = quanitative PCR
R = organic group, alkyl group
RAFT = reversible addition-fragmentation chain-transfer
RES = reticuloendothelial system
RI = refractive index
RISC = RNA-induced silencing complex
RNA = ribonucleic acid
RNase = ribonuclease
ROP = ring-opening polymerization
ROMP = ring-opening methathesis polymerization
RP = reverse-phase
r.t. = room temperature
RT = reverse transcription
σ = sigma
s = singlet, seconds
S = sphere
SD = sphere diameter
SEC = size-exclusion chromatography
siRNA = small interfering RNA
SLS = static light scattering
SNA = spherical nucleic acid
ss = single-stranded
t = triplet
Tm = melting temperature
TD = DABCYL-modified thymidine
Tf = fluorescein-modified thymidine
T.A. = termination agent
T = temperature
TBE = tris boric acid EDTA
TCA = trichloroacetic acid
TEAA = triethylammonium acetate
TEM = transmission electron microscopy
Temp = temperature
TFA = trifluoroacetic acid
THAP = 2,4,6-trihydroxyacetophenone
THF = tetrahydrofuran
Tris, TRIS, = tris(hydroxymethyl)aminomethane
UV = ultraviolet
V = volts
VEGF = vascular endothelial growth factor
VMD = Visual Molecular Dynamics
WAX = weak anion exchange
X = halide
X-SCID = X-linked severe combined immunodeficiency
Y = Y-junction network
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PUBLICATIONS


ABSTRACT OF THE DISSERTATION

NUCLEIC ACID PROGRAMMED POLYMERIC NANOMATERIALS FOR BIOLOGICAL COMMUNICATION

by

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Doctor of Philosophy in Chemistry

University of California, San Diego, 2015

Professor Nathan C. Gianneschi, Chair

A number of nucleic acid-polymer conjugates were synthesized, resulting in amphiphilic polymer-nucleic acid conjugates with the capability to self-assemble into a range of discrete nanoscale architectures. These nanomaterials, termed DNA-polymer amphiphile nanoparticles (DPA NPs), were studied with respect to their enzymatic processing by both endo- and exonucleases and further deployed as antisense genetic regulatory elements in live cultured human cells.
DPA NPs were designed to act as substrates for both non sequence-specific exonucleases and a sequence-specific endonuclease. In all cases, nucleic acids arranged in the corona of spherical nanoparticles exhibited increased resistance to nucleolytic cleavage as compared to native single- or double-stranded analogues. For the exonucleases studied (Exonuclease III from E. Coli and phosphodiesterase I from Crotalus adamanteus), nanoparticle display retarded enzymatic processing by roughly a factor of five. For the endonuclease studied (Nt.CviPII), nanoparticle display prohibited virtually all enzyme activity on oligonucleotides within the nanoparticle shell.

To test the ability of these materials to regulate mRNA levels in live cultured human cells, LPA (LNA-polymer amphiphile) NPs were designed to be perfectly complementary to a 20-base region of mRNA encoding the anti-apoptosis protein survivin. In this study two key observations were made. The first observation is that packaging LNA into spherical micellar nanoparticles serves to dramatically enhance cellular uptake of LNA based on flow cytometry and fluorescence microscopy data. The second observation is that LPA NPs are capable of regulating mRNA levels by what is hypothesized to be activation of target mRNA for catalytic RNase H-mediated degradation.

These materials represent a unique class of DNA delivery system capable of rendering nucleic acids with natural backbone chemistry resistant to nuclease degradation and further serving to deliver DNA into cells to facilitate depletion of mRNA levels in a sequence-specific fashion. Notably, the use of detergents, charge-neutralizing, or DNA-sequestering components are not required for these materials to be effective in cells.
Chapter 1

Introduction and History

1.1 Introduction

To understand a given technology and to put it properly in context it is important to have an idea of what gave rise to that technology, what has fueled its propagation and success, and how it is connected to others. All research involving the use of synthetic or semi-synthetic DNA must pay homage to two key scientific revolutions beyond the discovery of DNA itself: the discovery and systematic exploitation of enzymes that can sequence-specifically manipulate strands of DNA\textsuperscript{1-4} and the development of automated DNA synthesis.\textsuperscript{5,6} These key advances have facilitated the development of genetic engineering, genome sequencing, and genetic therapy; all fields that have flourished in the age of genomics. Surfing these swells of discovery and innovation, DNA nanotechnology\textsuperscript{7} has emerged as a platform fueling the development of materials that are capable of interacting with exquisitely complex biological systems in programmable and unprecedented ways. The work to be presented in this thesis lies at the nexus of several scientific disciplines that have experienced and are currently experiencing strong and persistent interest. These disciplines, including synthetic DNA conjugates, DNA nanotechnology, and synthetic polymer chemistry, are integrally connected \textit{via} many common synapses. Therefore, they have each symbiotically propagated the success of the other. Scientific offspring include genetic interference and gene therapy, nucleic acid delivery, programmable nanomaterials, and polymer self-assembly.
The scientific goal, with respect to DNA-based nanomaterials, is to develop entities capable of communicating with cells, the basic constituents of life, in a programmable and predictable fashion. Such communication should serve both to instruct the cell to perform certain functions based on commands from the material and also to instruct the material to perform it’s own set of functions in response to cellular cues. It should be acknowledged that there are other informational polymers including peptides and oligosaccharides that can be used to program materials for biological interfacing.\(^8\,^9\) However, these approaches will not be discussed at length in this introduction as the focus will be on nucleic acids as the quintessential molecular programming element.

Chapter one is intended to provide insight into the key scientific advances that have fueled the development of DNA encoded nanomaterials and further to connect these sorts of materials to their applications in the realm of genetic interference and as entities capable of interfacing with complex biological systems in a programmable fashion.

### 1.2 The Birth of Synthetic DNA

Elucidation of the structure of the DNA double helix played an important role in bridging many scientific disciplines by illuminating the connection between the chemistry underlying genetics and the biological result of that chemistry.\(^10\,^12\) Predating this seminal work were many groundbreaking experiments that gradually led to the conclusion that there was indeed an informational and intelligent material actively programming living organisms. The early efforts of Mendel and Miescher identified a substance, present in life forms, encoding a message passed along to progeny in a systematic and statistically consistent fashion.\(^13\,^15\) Beadle, Tatum, and Lederburg would later posit that hereditary units, now termed “genes”, were each linked to the expression of a specific protein.\(^16\) A revolutionary set of experiments performed by Oswald Avery in 1944 deduced that bacteria transfer DNA to
each other in order to pass on important information conferring unique traits and behavior, including pathogenic ability.\textsuperscript{17} As scientists began to understand the importance of DNA, they naturally sought to determine the molecular underpinnings of this unique biopolymer. The chemical structure of DNA was first assigned by Levene based on estimations of elemental composition informed by combustion analysis, magnesium pyrophosphate precipitation, and Kjeldahl nitrogen estimation.\textsuperscript{18,19} In 1952, informed by the analysis of DNA extracted from sea urchins, Chargaff deduced that DNA was not composed of an equal amount of each of the four nucleotides (an assumption that had been held previously). Chargaff further concluded that it was in fact the ratio of guanine to cytosine and the ratio of adenine to thymine that were set at one-to-one.\textsuperscript{20,21} Subsequently, inspired by the work of Linus Pauling\textsuperscript{22} and utilizing the X-ray data of Maurice Wilkins and Rosalind Franklin,\textsuperscript{10,23} Watson and Crick put forth what came to be accepted as the proper physical and chemical structure of double helical anti-parallel B-form DNA.\textsuperscript{11} The groundwork had been laid for a revolution in science.

While the structure of the DNA double helix was slowly revealing itself to molecular biologists, chemists developed an urge to synthesize nucleic acids from the ground up. Motivated by the isolation of biochemically relevant small molecules and higher order biopolymers, Michelson and Todd pioneered the synthesis of nucleotides and their corresponding dimers in the 1940s and 50s.\textsuperscript{24-26} Oligonucleotide synthesis subsequently witnessed several key advances through the work of Khorana,\textsuperscript{27-29} Letsinger,\textsuperscript{30,31} and Caruthers\textsuperscript{32,33} until it reached such a state in the 1980s that it could be fully automated.\textsuperscript{6} Marvin Caruthers quickly teamed up with Leroy Hood, who became integral in the development of DNA sequencing technology and DNA microarrays, to cement the technology with Applied Biosystems (ABI) using automated DNA synthesizers to perform phosphoramidite chemistry that is still in use today.\textsuperscript{5}
The astounding efficiency of automated DNA synthesis has enabled the development of countless technologies that have all had profound impacts on human science. Synthetic DNA has facilitated extensive use of the polymerase chain reaction (PCR)\textsuperscript{3,4} for the amplification of nucleic acids for detection purposes and for the construction of synthetic genes - both powerful uses of combined synthetic DNA and naturally occurring enzymes. Further, synthetic DNA has fueled rapid genotyping via DNA microarray\textsuperscript{34} approaches and is intricately tied to the sequencing of entire genomes\textsuperscript{35,36}. The discovery and deployment of a multitude of enzymes capable of cutting, copying, and stitching together synthetic and natural DNA molecules\textsuperscript{1,2,37} laid the foundation for genetic engineering, recombinant protein engineering, and synthetic biology. These monumental technologies have eventually all come to rely heavily on synthetic DNA, and therefore have symbiotically encouraged its development and success.

### 1.3 Genetic Interference and the Development of Nucleic Acid Derivatives

As automated DNA synthesis and cloning technology became more reliable, researchers began to use synthetic DNA to induce and interfere with genetic expression, hence altering cellular behavior, function, and production via the use of properly designed genes and nucleic acid fragments.\textsuperscript{38,39} Scientists have had enormous success using semi-synthetic engineered plasmid DNA with respect to programming bacteria.\textsuperscript{40} This work is marked most impressively by the successful synthesis of entire genomes\textsuperscript{41} and the transfer of such genomes from one species of bacteria to another in order to transform that species into an entirely different species capable of self-replication.\textsuperscript{42,43} In eukaryotic cells, genetic engineering and manipulation is not so straightforward. This is largely due to the fact that
mammalian cells operate on chromosomal DNA instead of plasmid DNA and further do not internalize nucleic acids as readily as bacteria. Therefore, all sorts of tricks and tools have been established to facilitate nucleic acid delivery to eukaryotic cells – this will be a major subject of Chapter 1 of this thesis. In the context of this thesis, genetic interference will be discussed primarily in reference to its development in eukaryotic cell culture, tissue culture, and its application to human medicine.

The field of genetic interference encompasses all approaches to modulate cellular function at the genetic level by introducing either 1) genes to be directly transcribed into their protein products or 2) short fragments of nucleic acids to interfere with the process of transcription or translation. A common theme underlying the use of either mode of interference is that the nucleic acids intended to alter function must be protected from enzymatic degradation while being efficiently delivered to the proper location within the interior of a cell. It is also plausible to interfere with genetic information transfer by introducing or altering key components of the process such as transcription factors or polymerases, for example. However, these modes of interference will not be discussed in this introduction.

The introduction of genes intended for host transcription is usually achieved via viral delivery of plasmid DNA on the order of 5-150 kilobases in length. Upon delivery of a gene there can be two consequences. The first potential outcome is that the gene of interest is integrated into the chromatin of the host cell and subsequently propagated to daughter cells to achieve sustained expression. The second potential outcome is that the gene is delivered to the host cell, but remains as an extrachromosomal episome that will be expressed only as long as that episome remains intact and thus will not be copied to daughter cells. The type of virus used for gene delivery will determine whether or not that gene is incorporated into the host genome and therefore which potential result comes into fruition; this will be discussed in
more detail in section 1.4. Since the materials developed in the work described in this thesis act as antisense agents, the focus of this and remaining chapters will lie more heavily on short ODNs used for genetic interference, as this is significantly different from the delivery of entire genes. While gene delivery likely provides the most direct route to permanently influencing cell function, antisense technology provides an alternative means to study gene function in cell and animal models where gene knockout can have lethal results. For example, in work by Driver and coworkers,\(^45\) genetically engineered mouse E-cadherin embryos failed to properly form the blastocoele, thus resulting in failure at an early stage of embryogenesis. However, antisense ODNs, when administered in a later stage of development, were successfully employed to investigate a secondary role of E-cadherin.\(^45\) A further complication with gene delivery is that the insertion of gene fragments into existing chromatin can have severe negative consequences (for example, resulting in leukemia in patients undergoing gene therapy in the early X-SCID trials) that are difficult to predict.

Modulation of cellular function through the use of short fragments of nucleic acids (typically 20-30 bases) can be divided into pre- and post-transcriptional interference. A cartoon diagram summarizing post-transcriptional genetic interference using short oligonucleotides is provided in Figure 1.1. Pre-transcriptional interference is also termed “antigene” interference and involves the introduction of nucleic acid fragments capable of hybridizing directly to a gene, usually via triplex formation, thus inhibiting it’s transcription into RNA.\(^46\) Post-transcriptional interference is labeled as an antisense approach when nucleic acid fragments are used directly to modulate mRNA or pre-mRNA, most commonly via recruitment of RNase H. Alternatively, RNA interference is the term given to approaches involving the use of synthetic double-stranded RNA fragments for the regulation of mRNA via hijacking of a naturally occurring mRNA silencing pathway involving the RNA-induced silencing complex (RISC) and associated RNase Argonaute 2 (Ago2).\(^47,48\) Another major
difference between antisense and RNAi is that antisense oligonucleotides are effective in the
cell nucleus and therefore are able to degrade pre-mRNAs as well as mature mRNAs.\textsuperscript{48} It is
unclear which approach, antisense or RNAi, is superior in terms of gene regulating efficacy. Indeed, several studies have conflicting results and the efficacy of each construct is most
certainly dependent on the proper design and optimization of that construct.\textsuperscript{48,49} There also
exist approaches contrary to those that serve to reduce gene expression. Anti-miRNA
approaches are a unique type of genetic interference and thus are not typically categorized as
antisense or RNA interference and small activating RNAs (saRNAs) can be exploited to
directly enhance gene expression.\textsuperscript{50} It is clear that we will continue to discover new ways to
exploit natural pathways for regulating cellular information transfer as we learn more about
how these processes occur in living organisms.

Antisense interference involves hybridization of a synthetic fragment of DNA to a
target mRNA in the cytosol or cell nucleus. Once hybridized to a complementary sequence of
DNA, target mRNA is prevented from engaging in efficient translation typically \textit{via} one of
two pathways. The first, and major pathway involves RNase \textit{H}-mediated catalytic
degradation of mRNA engaged in an RNA-DNA heteroduplex (note that there are some
reports that other nucleases, namely RNase L, can play a role).\textsuperscript{51-54} The second mechanism by
which translation can be prohibited is through direct translational arrest, meaning that the
RNA-DNA heteroduplex formed does not allow proper ribosomal processing of the mRNA
transcript.\textsuperscript{55} RNase \textit{H}-mediated mRNA regulation is the major and preferred pathway due to
the fact that one antisense strand can bind and catalyze the destruction of multiple mRNAs.
Indeed, nanomolar (nM) doses are effective for \textit{in vitro} and \textit{in vivo} silencing effects.\textsuperscript{56}

RNA interference, which was not discovered until 20 years after antisense
interference,\textsuperscript{57} is achieved \textit{via} two major pathways – that of small interfering RNAs (siRNAs)
or that of microRNAs (miRNAs). Initially, guided by the discovery of endogenous gene
regulation mechanisms, researchers attempted to use long dsRNA to engage in siRNA interference. In this case, such strategies were not effective due to the activation of an antiviral interferon (IFN) response.\textsuperscript{58} Indeed, siRNA research exploded once key observations were made that short 21-22 base siRNA sequences that did not elicit the same IFN response could be used effectively.\textsuperscript{59,60} The use of synthetic siRNAs requires siRNA duplexes to enter the cellular cytosol and engage the RISC. The RISC then unwinds the double-stranded RNA and cleaves only one strand (the sense or passenger strand) leaving the antisense (or guide) strand intact to guide the RISC to a perfectly complementary mRNA for subsequent destruction. The RISC-guide strand complex then hybridizes and selectively degrades complementary mRNA in a catalytic fashion \textit{via} the RNase activity of associated Ago2.\textsuperscript{61,62} Unlike siRNAs, miRNAs exist as endogenous mRNA regulators. Endogenous miRNAs are imperfectly complementary stem-loop structures that require several levels of processing before reaching a mature state. The RNase DROSHA trims miRNAs to \textasciitilde70 base pair stem loop structures in the nucleus\textsuperscript{63} after which exportin 5 exports the pre-miRNA into the cytosol.\textsuperscript{64} Once in the cytosol, miRNAs share the same downstream processing as siRNAs. In the cytosol, DICER processes pre-miRNA into 20-25 base pair mature miRNAs while facilitating the activation of the RISC.\textsuperscript{65} It is at this point that synthetic double-stranded siRNAs can hijack the silencing pathway. After engaging the RISC, miRNAs are able to catalyze the degradation of target mRNA transcripts or translational repression depending on their degree of complementarity.\textsuperscript{66} Anti-miRNA oligonucleotides (AMOs) have been developed in order to silence the function of miRNAs that lead to disease states.\textsuperscript{54} It is hypothesized that this is achieved \textit{via} strong hybridization of nuclease resistant AMOs to miRNAs in order block miRNA processing \textit{via} unwinding or cleavage by Ago2 and hence prohibit loading into an active RISC.\textsuperscript{54,67} Given that AMOs are a fairly recently developed genetic interference technology, it is promising that there are AMOs currently in phase II
clinical trials. Miravirsen is an inhibitor of miR-122, a liver specific miRNA that the hepatitis C virus (HCV) requires for replication. Miravirsen is comprised of a 15 nucleotide sequence containing LNA and phosphorothioate modifications.

Further strategies towards genetic interference include the use of decoy DNA to inhibit transcription of a particular gene and the use of ribozymes and DNAzymes that simultaneously bind to and catalyze the degradation of complementary target mRNA sequences.

Figure 1.1. Genetic interference with short oligonucleotides. Note that antigene inhibition is not depicted in this graphic. Key references used to inform this graphic include Hoekstra and coworkers, Sah and coworkers, and Behlke and coworkers. It is also important to keep in mind that it is not entirely clear where anti-miRNA oligonucleotides enter in the processing scheme of miRNAs; it is hypothesized that one point of entry may be at the RISC processing stage. 1978 and 1998 mark the year that each independent interference pathway was discovered (note that anti-miRNAs were introduced later in 2003 and 2004). Red boxes indicate where synthetic oligonucleotides can hypothetically enter and interfere in the information transfer process.
1.3.1 Origins and History of Antisense

In the late 1970s researchers began to use short fragments of nucleic acids to interfere with genetic transcription and translation processes in cell-free yeast extracts. The idea was to use short pieces of DNA or RNA to hybridize either to mRNA or directly to the gene of interest in order to block translation or transcription of that gene and hence deplete levels of that particular gene’s protein product.\(^{78-80}\) Oligonucleotides used to block translation of a genetic transcript are termed “antisense” oligonucleotides, because they are complementary to “sense” DNA strands that are identical in sequence to mRNAs that translate into a particular protein product encoded by that gene. Paterson et al. and Inglis et al. independently demonstrated the first examples of antisense inhibition of mRNA translation using either DNA or RNA in 1977.\(^{81,82}\) In the work described by Inglis et al., avian influenza viral RNA was added to various viral mRNAs that had been synthesized inside of infected chick embryo fibroblast cells. Addition of the complementary genomic RNA was able to inhibit translation of viral mRNA in cell-free wheat germ extracts.\(^{82}\) In a similar fashion Paterson et al. used digested DNA plasmids to interfere with the translation of rabbit globin 9S mRNA in cell-free wheat germ extracts.\(^{81}\) In work marking the first use of completely synthetic antisense oligonucleotides, Zamecnik and Stephenson employed modified and unmodified short oligonucleotides to block translation of viral mRNA in cell-free wheat germ extracts.\(^{83}\) The DNA fragments used in these experiments were synthetic 13-base oligonucleotides that were presumably synthesized by hand using methods developed by Khorana and coworkers.\(^{84}\) Notably, each of these pioneering studies rely on cell-free extracts to demonstrate antisense inhibition of protein synthesis.

The second wave of antisense experiments came shortly after and were accomplished using engineered plasmids that encoded antisense RNAs complementary to mRNAs to be targeted for destruction.\(^{80}\) The difficulty with antisense RNA based on plasmid or gene
expression is that the control of this expression becomes an issue in that it is inconsistent and not addressable. More specifically, expression levels from plasmid DNA are difficult to control due to the fact that plasmid copy varies from cell to cell and ultimately plasmids rely on hijacking existing cellular machinery and hence are subject to stochastic fluctuations governed by cell state and environment. Further, in eukaryotic cells, plasmids are not always copied during cell division and thus are not propagated to daughter cells.85

Inspired by early successes, it was envisioned that genetic interference could have a significant impact on human health in curing diseases based on genetic abnormalities. Hence, experiments began to move into live cells and live organisms and therefore began to realize several significant barriers that were initially addressed via the use of nucleic acid derivatives.

1.3.2 DNA Derivatives as Antisense Agents

In the 1980s antisense DNA technology began to shift towards modified oligodeoxynucleotides (ODNs) to address issues of instability and poor cellular uptake. This section will focus on derivatives with enhanced nuclease resistance or cellular uptake. However, it is worth noting that nucleic acid derivatives possessing other advantages such as fluorescent nucleobases have also been successfully pursued.86 The seminal work of Miller and coworkers focused on using modified phosphotriesters, especially methylphosphonate DNA, as DNA derivatives due to enhance both resistance to nucleolytic degradation and cellular uptake.87-93 However, it was realized that methylphosphonate ODNs suffered several major setbacks in that they are highly susceptible to base catalyzed hydrolysis, exhibit limited solubility in aqueous media, and suffer decreased melting temperatures with respect to natural DNA duplexes resulting from steric hindrance in particular stereoisomers introduced by the existence of the phosphonate methyl group.94 Subsequently, Alpha-ODNs were developed as
alternatives that hybridize complementary DNA in a parallel fashion while maintaining standard duplex stability and conferring enhanced nuclease resistance. However, alpha-ODNs cost roughly thirty times more to synthesize than traditional beta-oligos and therefore have not been pursued as viable alternatives.\textsuperscript{95,96} Phosphorothioate (PS) ODNs were the first truly successful nucleic acid derivatives to be deployed as useful antisense tools due to their ease of synthesis and stability with respect to nuclease digestion.\textsuperscript{97} In fact, two out of the meager three FDA approved nucleic acid drugs contain phosphorothioate linkages (Fomivirsen and Mipomersen).\textsuperscript{98,99} Setbacks for phosphorothioate ODNs include a wide range of off-target effects due to their tendency to stick to proteins.\textsuperscript{100,101} In fact, they have been found to inhibit DNA polymerase and RNaseH.\textsuperscript{102} 2’-OMe and 2’-O-methoxyethyl RNA modifications have been introduced to conquer setbacks associated with PS-ODN drawbacks. These RNA analogues have high affinity for their targets, exhibit low toxicity, confer a high degree of nuclease resistance, exhibit low immunogenicity, and have long half-lives in tissue.\textsuperscript{103,104} However, 2’-modified RNAs have problems activating RNase H\textsuperscript{105} and therefore must rely on gapmer strategies wherein a region of nucleic acid amenable to RNase processing is flanked by regions of 2’-modified nucleotides.\textsuperscript{106,107} Note that most 2’-modifications, including locked nucleic acids (LNAs), result in the inability for the ODN to activate RNaseH.\textsuperscript{104,108,109} Similarly, morpholino DNA and peptide nucleic acids (PNAs) do not engage RNase H.\textsuperscript{110,111} LNA has also been developed as a bridged version of 2’-modified RNA with the capability of forming extremely stable duplexes with complementary DNA and RNA.\textsuperscript{112} Modifications to LNA chemistry resulting in so called alpha-L-LNA, LNA-T, and carbocyclic-ENA-T and carbocyclic-aza-ENA-T enhance antisense activity by increasing stability against nucleases and target duplex stability.\textsuperscript{113-115} For reviews on nucleic acid modifications for genetic interference see Sharma et al.\textsuperscript{104} and Kurreck.\textsuperscript{78}
Most ODNs currently in clinical trials contain backbones with some degree of modification. The most promising strategies take advantage of what are termed “gapmers” (also known as chimeras) in which a central DNA region is flanked by modified bases such as 2’-OMe so that the sequence retains its ability to recruit RNases for mRNA destruction while still maintaining a high resistance against nucleases. Promising structural modifications include various 2’-OH modifications (e.g., 2’-O-methyl), locked nucleic acids (LNAs), peptide nucleic acids (PNAs),116 hexitol nucleic acids (HNAs), and deoxynucleic guanidine (DNG).117,118 Figure 1.2 details the chemical structure of some of the most popular nucleic acid derivatives. Of course, several excellent reviews on nucleic acid derivatives and nucleic acids as therapeutics have been published.104,119-130

**Figure 1.2.** The chemical structures of some popular synthetic nucleic acid derivatives. Adapted and modified from Sharma et al.104
1.3.3 Nucleic Acid Conjugates

Conjugation of ODNs to other entities serves to add orthogonal functionalities like the ability to sense electrochemical reactions or penetrate through cell membranes. The development of ODNs covalently attached to other molecules began in the 1980s as DNA synthesis began to blossom. Major classes of nucleic acid conjugates include fluorophore conjugates for use in sequencing, molecular beacons, and fluorescence in situ hybridization (FISH). Peptide and protein nucleic acid conjugates were later developed to take advantage of the physical and functional properties of amino acid biopolymers. Specifically, cell-penetrating peptide (CPP)-ODNs and peptide transduction domain (PTD)-ODNs have been developed and serve to facilitate ODN uptake and cellular trafficking. Key examples in the literature include nucleic acids conjugated to an arginine and lysine-rich region of HIV-1 Tat protein and a nuclear localizing sequence. To harness the orthogonal functionalities of proteins, ODN-protein conjugates have been designed as artificial nucleases. Another class of biopolymer, namely carbohydrates, has also been used extensively for nucleic acid conjugation. Here, conjugates can be used to selectively target nucleic acids to glycoproteins including lectins. Carbohydrates have also been used to enhance cellular uptake and increasing stability against nuclease degradation. Another use for nucleic acid carbohydrate conjugates is in the preparation of microarrays. Lipophilic cholesterol conjugates have been used extensively for enhancing cell uptake of nucleic acids and also for the inhibition of viral replication. Additionally, cholesterol RNA conjugates have been used in vivo to silence miRNAs in mice. In terms of fundamental studies, Boxer and coworkers have used lipid nucleic acid conjugates to study vesicle fusion processes. Conjugation of nucleic acids to synthetic polymers has been demonstrated to enhance antisense activity, and also to enhance nuclease resistance and cellular uptake of nucleic acids. Finally, a great variety
of nucleic acid conjugates have been prepared as metal complex ODN conjugates, as artificial nucleases, for electron transport studies and electrochemical detection, as photoprobes, for metal-directed base pairing strategies, for the assembly of nanomaterials and as metal-nanoparticle ODN conjugates. The subject of ODN conjugates has been presented in a handful of reviews including reviews on conjugates in general, on fluorescent conjugates, on CPP conjugates, on nanoparticle conjugates, and on polymer conjugates.

1.3.4 Inadequacy of Naked Nucleic Acids for Genetic Interference

Although modified DNA and RNA oligonucleotides exhibit some level of success as therapeutics, they remain to suffer from several key pitfalls rooted in unfavorable pharmacokinetic and pharmacodynamic properties. Specifically, low bioavailability due to rapid renal excretion, off-target effects, ineffective cellular uptake, inability to pass the blood vessel endothelial wall and other tissue barriers, poor stability against nuclease degradation, RES clearance, and lack of target specificity all stunt the development of naked nucleic acid therapies. Furthermore, for the small fraction of administered dose that reaches the cell of interest, intact siRNA molecules need to efficiently escape from endosomal or lysosomal compartments in order to associate with the RISC and exert biological function. For all of these reasons, naked siRNAs are not entirely effective when administered systemically, and therefore are limited to administration in select areas of the body (e.g., eyes, lung, skin, mucus membranes, and local tumors). Certain types of nucleic acid conjugates are able to address some of these concerns and are discussed in section 1.4. Given the limitations of naked nucleic acids, new strategies have been undertaken to explore materials that can assist in prolonging oligonucleotide circulation while
also chaperoning nucleic acids into the appropriate subcellular regions with high efficiency.\textsuperscript{191}

\subsection*{1.4 Nanomaterials for Nucleic Acid Delivery}

Due to shortcomings associated with genetic interference using naked nucleic acids, nanoscale materials have been called upon in order to improve the efficiency of delivery for a variety of nucleic acid families. Materials for DNA delivery offer two key functions in that they protect nucleic acids from enzymatic degradation and they deliver negatively charged nucleic acids across cell membranes with high efficiency. Further, such materials offer unique attributes due to their physical properties including size, shape, and charge.

Most materials used for nucleic acid delivery have dimensions on the nanometer length-scale, hence they are trafficked in interesting ways \textit{in vivo}. For cancer therapies in particular, the EPR effect\textsuperscript{193} seems to play an important role in increasing delivery of nanomaterials to regions of inflammation or infection\textsuperscript{194} and tumors\textsuperscript{195,196} that are typically marked by highly fenestrated vasculature and poor lymphatic drainage. More specifically, the pore size of endothelial junctions in tumor neovasculature is between 100 nm and 780 nm, which is significantly larger than those in normal blood vessels.\textsuperscript{197,198} Nanoparticles with sizes of up to 200 nm in diameter tend to have a longer circulation time in the blood stream and are able to reach tumor masses at high concentrations through extravasation.\textsuperscript{199} It should be noted that the EPR effect remains a controversial theory given that supporting data are often acquired in model systems that do not always recapitulate or anticipate natural disease states. On the down side, RES clearance of nanoscale materials tends to hamper their efficacy leading to off-target accumulation in organs including the liver and spleen.\textsuperscript{200}

An advantage of material platforms for NA delivery comes as a result of their size and hence their ability to accommodate chemical complexity. Accordingly, materials can be
engineered to take advantage of biological stimuli in order to deliver payloads only when cued to do so.\textsuperscript{201} For example, in order to proliferate, cancer cells tend to have a high metabolic rate. Increased metabolism results in increased aerobic glycolysis thus resulting in high levels of lactate and a locally acidic environment.\textsuperscript{202} Indeed, pH-responsive materials can be taken advantage of in order to deliver ODNs specifically to environments with high acidity.\textsuperscript{203} Further, certain cancer cells express unique biomarkers, such as redox enzymes, glutathione, and matrix metalloproteinases (MMPs) - all stimuli that can be utilized to elicit specific responses in properly designed materials.\textsuperscript{204-206}

Materials used for nucleic acid delivery are typically either viral vectors (retroviruses, adenoviruses, adeno-associated viruses, baculovirus and vaccinia),\textsuperscript{44,207} protein complexes,\textsuperscript{208} or completely synthetic vectors (liposomes, cationic polymers, and DNA-nanotechnology based platforms).\textsuperscript{209-211} The key differences between materials for gene delivery, DNA oligo delivery, and RNA oligo delivery are guided by the requirements set for each different genetic interference element. Entire genes can be delivered for insertion into a genome or for transient expression. These genes are large fragments of DNA (5-150 kb)\textsuperscript{44} that need to be sequestered or condensed until they reach their desired location, usually the nucleus, at which point they must be released from the delivery vehicle. This modality sets guidelines in that the delivery vehicle must be large enough to house fairly long DNA strands. Additionally, these materials must reach and enter the nucleus of a cell in order to be properly integrated or transcribed.

RNA oligonucleotides, typically double-stranded siRNAs, are comprised of a guide and template strand and need to be sequestered or condensed until they are delivered into the cellular cytoplasm at which point they must be released to engage key protein machinery in order to take effect. DNA oligonucleotides to be delivered as antisense agents must also be sequestered for protection from enzymatic degradation and released into the cytoplasm or cell
nucleus for action. However, antisense DNA oligos need not be double-stranded and therefore enjoy a bit more freedom in their design in terms of material incorporation.

Other approaches to nucleic acid delivery that do not involve nanomaterials do indeed exist. Popular approaches involve electroporation, gene gun particle bombardment, microinjection, and alkali cation treatment coupled with heat shock. Recent approaches take advantage of new technologies, for example, microfluidic cell-squeezing has been used to efficiently deliver NAs to cells in a high throughput fashion. It is important to note that these methods are limited strictly to in vitro models and therefore do not offer direct therapeutic or diagnostic solutions for in vivo studies. Exceptions include electroporation and gene gun delivery, which can be used in vivo to assist in plasmid uptake in muscle fibers and neurons, for example.

1.4.1 Viral Vectors for Nucleic Acid Delivery

Viral vectors are thought of as premium candidates for nucleic acid delivery. This belief stems from the fact that viruses have naturally evolved for billions of years to achieve exactly this task. Further, using recombinant DNA technology, mutant viruses can be synthesized and tweaked in a very systematic fashion in order to create selectively replicating viruses, for example.

There are five main classes of viral vectors that can be categorized in two groups based on whether their genomes integrate into host cellular chromatin (oncoretroviruses and lentiviruses) or persist in the cell nucleus predominantly as extrachromosomal episomes (adeno-associated viruses, adenoviruses and herpes viruses). Non-integrating vectors can mediate persistent transgene expression only in non-proliferating cells. Integrating vectors are the tool of choice if stable genetic alteration needs to be maintained in dividing cells.
In light of problems associated with immune system activation and unpredictable insertion patterns, viruses have been engineered to be safer typically by limiting viral reproduction to certain environments only or by introducing so called “suicide genes” to signal self destruction in the case of oncogenesis. For example, selectively replicating mutant viruses have been engineered such that replication only occurs in tumor cells that provide a function that the mutant virus lacks due to deletion of that viral gene. Further, transgene expression can be restricted to particular cell types and switched on and off using tissue-specific and/or regulatable promoters.

Recombinant viral engineering is a very powerful strategy for assembling viruses with diverse and novel functionalities. Large libraries of viruses can be synthesized in order to screen for viruses that selectively target certain cell types. Through use of DNA family shuffling, recombinant envelope genes from six different strains of murine leukemia virus (MLV), produced a library of $1 \times 10^6$ MLV variants yielding an infectious clone with altered tropism for CHOK1 cells. In another approach, a library of rAAV clones with randomized peptide insertions in the viral capsid protein VP3 was generated in order to produce a viral variant with increased tropism for either the B-cell lymphocytic leukemia cell line Mec-1 or the human megakaryocytic cell line M07e.

Currently, the ‘Achilles heel’ of gene therapy using viral vectors is that host immunological defense systems are often activated against viral vectors at titers high enough to be effective therapies. Indeed, an infamous clinical trial resulted in death due to immune system activation after delivery of a high dose of adenovirus. Further, new transgene products resulting from proper integration of a delivered gene can often be recognized as foreign and therefore also evoke an immune response. These results underscore the complexity of viral delivery in that each individual presents a unique testing ground for a given virus.
Another potentially lethal complication resulting from viral gene delivery is insertional mutagenesis.\textsuperscript{231} This is despite the fact that the estimated risk of retroviral vector genomes integrated randomly into host chromatin and disrupting a cellular sequence connected with malignancy was predicted to be in the realm of 1 in 10 million insertions.\textsuperscript{230} This position on the rarity of insertional mutagenesis was reinforced by the fact that viral vector-induced cancer had not been observed in hundreds of patients that were treated with retroviral vectors in gene therapy trials. However, recent evidence from a number of separate studies has challenged beliefs about the low risks of using integrating retrovirus vectors for gene therapy.\textsuperscript{231,232} For example, the transplantation and expansion of a clone of retrovirally transduced bone marrow cells has been shown to induce leukemia in mice.\textsuperscript{233} In this instance, the development of cancer is thought to be a consequence of the transgene product and the fact that the retroviral integration event had disrupted the gene encoding a transcription factor implicated in the pathogenesis of acute myeloid leukaemia.\textsuperscript{234} Complications with random integrations were brought to the forefront in a clinical trial back in the year 2000. Despite viral gene therapy seemingly curing 3 of 11 patients, it was later discovered that 2 of the 11 patients treated during the successful severe combined immunodeficiency (X-SCID) trial\textsuperscript{231} had developed a leukemia-like disorder. It is hypothesized that the disorder is caused by retroviral vector genome integration in, or near, the oncogene LMO2.\textsuperscript{232} Indeed, it appears that retroviral integration is not random at all. It has been experimentally demonstrated \textit{via} the analysis of hundreds of human immunodeficiency virus (HIV) integration sites in cell culture that HIV is more likely to integrate into transcriptionally active genes than into non-coding regions of chromatin.\textsuperscript{235} Recent efforts focus on making viral vectors safer for delivery by mediating issues like unpredictable insertions. For example, site-specific integration machinery of bacteriophage $\phi$C31 has been exploited in non-viral delivery approaches to achieve the targeted integration of transgenes in mice and human cells.\textsuperscript{236-238}
1.4.2 Non-Viral Vectors for Nucleic Acid Delivery

There are two major classes of non-viral vectors for DNA delivery: condensing constructs and non-condensing constructs. Condensing materials are typically positively charged polymers that have the ability to condense DNA into aggregates of various size, shape, and zeta potential and thus facilitate passage of DNA across cell membranes. On the contrary, non-condensing materials such as lipids or polymers possess a neutral or negative charge and work by encapsulating nucleic acids or holding them in tact via hydrogen bonding in order to protect them from degradation and facilitate cell uptake.\textsuperscript{186} Outside of viral vectors, lipid-based transfection agents are by far the most utilized vectors for nucleic acid delivery, especially in cell culture. At the moment, popular transfection agents include the Lipofectamine®, DharmaFECT®, X-tremeGENE®, and FuGene® product lines. There are literally hundreds of reviews on materials for nucleic acid delivery, most of them focusing on condensing polyplex and lipoplex materials.\textsuperscript{119,211,239,240}

Condensing vectors include a range of polymers designed to bind DNA electrostatically and form aggregates capable of passing through cell membranes \textit{via} mechanisms that are not well understood.\textsuperscript{241,242} These vectors, often termed polyplexes (when polymers are the condensing agent) or lipoplexes (when lipid-like surfactant molecules are used as the condensing agent), tend to be very efficient at delivering NA payloads across cell membranes.\textsuperscript{243} Popular materials used as condensing vectors include cationic lipids such as DOTMA, DOTAP, DOSPA, DMRIE, DC-Chol, DOGS (Transfectam®), and DDAB or cationic polymers such as poly(ethyleneimine) (PEI), and poly(L-lysine) (PLL).\textsuperscript{147} Other condensing vectors include chitosans, cationic cyclodextrin derivatives, cationic fusogenic peptides (KALA),\textsuperscript{244} cationic nanogels (i.e. Pluronic®/PEI), dendrimers such as PAMAM, amine-functionalized gold nanoparticles, and solid lipid nanoparticles.\textsuperscript{147,245} Chemical
structures of some common commercially available lipid-based transfection agents are highlighted in Figure 1.3.

**Figure 1.3.** Chemical structures of some commercially available liposomal transfection reagents. Lipofectin® (Life Technologies) is a 1:1 (w/w) mixture of DOTMA and DOPE. Lipofectamine® (Life Technologies) is a 3:1 (w/w) mixture of DOSPA and DOPE.²⁴⁶

Many of these vectors can be PEGylated to enhance biological activity by stealthing the constructs and increasing circulation time.¹⁸⁶ However, cationic delivery vectors often suffer from severe setbacks in that the cationic polymer or lipid is typically toxic to the cell, positively charged complexes are rapidly cleared by the reticuloendothelial (RES) system, and intracellular unpacking of the nucleic acid constructs from charged complexes is typically inefficient.²⁴⁷

To avoid some of the setbacks incurred with the use of condensing nucleic acid delivery vectors, non-condensing vectors have been pursued wherein positively charged molecules and tight electrostatic binding of DNA can be avoided. There are a range of non-
condensing materials for gene delivery including gelatin, hydroxypropyl-methacrylate copolymers (HPMA), poly(ε-caprolactone) (PCL), poly(ethylene glycol) (PEG), poly(D,L-lactide-co-glycolide (PLG), poloxamer (Pluronic®), and poly(N-vinyl pyrrolidone) (PVP). The charge state of non-condensing systems dictates further advantages in that they are not typically engaged by the mononuclear phagocyte system for early clearance nor are they efficiently opsonized and sequestered via the innate immune response.

1.4.3 DNA Nanotechnology and DNA Delivery via Nanoparticle Display

Within the past 10 years, DNA nanotechnology has emerged as a paradigm enabling the synthesis of materials programmed by nucleic acids that serve not only as a platform for material synthesis with unprecedented precision, but also as a way of imparting novel properties on nucleic acids by virtue of three-dimensional arrangement. This field has been pioneered by people including Nadrian Seeman, Eric Winfree, Paul Rothemund, Chengde Mao, William Shih, and Peng Yin. DNA origami has ultimately led to the ability to create a multitude of three-dimensional shapes composed entirely of synthetic DNA that can be used to create functional patterns and structures with unprecedented precision. Expanding on structures made entirely of DNA, researchers like Hanadi Sleiman have built hybrid organic nucleic acid motifs that assemble into unique architectures that function as a result of the rigidity imposed by organic vertices and bridges. Indeed, origami-type structures have been assembled that facilitate cellular uptake of DNA due to the three-dimensional arrangement and packing of nucleic acids. It is interesting to note that structures made entirely of DNA have the opportunity to be assembled in vivo by cells and microorganisms.

The labs of Chad Mirkin and Paul Alivisatos discovered the power of functionalizing inorganic nanomaterials with DNA in order to direct and program patterns of assembly.
These experiments are the foundation for DNA nanotechnology based on hybrid structures in which materials are made only partially of DNA. A more in depth discussion on hybrid DNA nanotechnology can be found in Section 5.1.

Very recently, multiple groups have realized the power of arranging DNA into unique densely packed structures on the nanoscale. Several papers, including the work presented in this thesis, have collectively reached the conclusion that the three-dimensional arrangement of nucleic acids dictates the properties of those nucleic acids to a large and unexpected extent.\textsuperscript{158,260-262} Specifically, packing DNA into spherical arrangements either at the surface of gold nanoparticles or in the corona of spherical polymeric micellar nanoparticles greatly enhances cellular uptake of DNA as compared to that of free single stranded DNA.\textsuperscript{161,260} Likewise, as mentioned above, packing DNA into unique three-dimensional architectures using DNA origami also facilitates passage of these species across cellular membranes.\textsuperscript{257,258} Examples of materials capable of facilitating DNA uptake without charge-neutralizing lipids and polymers or viral packaging are rare and represent a new class of materials that will certainly be expanded upon in the near future. The benefit of structures composed of densely packed DNA at their exterior is that the DNA does not need to be released from a sequestered state in order to hybridize its target. Further, dense packing at the surface of a nanomaterial renders DNA nuclease resistant and therefore nucleic acids packaged in such a way do not require chemical modification. These benefits are of great consequence, and eliminate the need for toxic delivery vehicles entirely. A key difference between these types of materials and typical transfection agents is that these materials are engineered to have precise and well-defined geometries, with shape and nucleic acid density playing key roles in physical properties. One aspect to note is that DNA and especially certain DNA motifs\textsuperscript{263} are known to elicit immune responses.\textsuperscript{264-266} This can be viewed as a potential problem or as an aspect to take advantage of. For example, one could imagine
intentionally designing DNA nanostructures for immunotherapy. A further note is that large plasmid DNA may be difficult to incorporate at the surface of small nanoparticles. However, nanomaterials incorporating plasmid DNA in a well-controlled and precise fashion represent an interesting possibility and will surely manifest themselves as researchers focus on this as a fundamental materials science challenge.

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Chapter 2

Synthesis of DNA-Programmed Polymers for the Assembly of Nanostructures

2.1 Introduction

Nanomaterials have attracted tremendous interest over the past two decades due to their potential impact in a range of technologies including drug and gene delivery, advanced materials, biosensing, biodiagnostics, and electronics. Consequently, there is a great demand for the development of tools and methods for reliable and reproducible preparation of nanoscale particles, surface features and nanomaterials in general. Polymers have proven to be excellent candidates for the preparation of such materials, with the ability to facilitate patterning strategies and self-assemble into discrete architectures with unique properties at the desired length scale. Of particular interest is the preparation of nanoparticles generated utilizing controlled, living polymerization methods, which allow for the synthesis of well-defined polymers and block copolymers possessing a diverse array of chemical functionality and predictable molecular weights with low dispersity.

Significant progress towards synthesizing well-ordered nanomaterials using various living and controlled polymerization methods has been achieved. Atom transfer radical polymerization (ATRP), reversible addition-fragmentation chain transfer (RAFT) polymerization, nitroxide-mediated radical polymerization (NMP), ring-opening polymerization (ROP), and ring-opening metathesis polymerization (ROMP) are just a few of the living polymerization techniques utilized to generate nanoscale polymeric...
materials. Mild reaction conditions, high functional group tolerance, and a well-defined, stable, and tunable transition-metal based initiator\textsuperscript{22,23} render ring-opening metathesis polymerization (ROMP) particularly amenable for generating functionalized block copolymers in a living fashion with narrow molecular weight distributions. Furthermore, this method permits access to well-defined block copolymer architectures capable of self-assembly, generating nanoparticles with diverse structure and function.

The assembly of amphiphilic block copolymers to generate discrete nanoscale structures is primarily driven by the hydrophobic effect, with micelle size and shape governed by a set of basic principles rooted in surfactant phase separation behavior.\textsuperscript{24-32} Important parameters that control the size of micelles are the degree of polymerization of the polymer blocks, $N_A$ and $N_B$, and the Flory-Huggins interaction parameter $\chi$. The micellar structure is characterized by the core radius $R_c$, the overall radius $R_m$, and the distance $b$ between adjacent blocks at the core/shell-interface as shown in Figure 2.1. Distance between adjacent blocks, $b$, is often called grafting distance for comparisons to polymer brush models. The parameter $b^2$ is then the area per chain that correlates to the area per head group in the case of simple surfactant micelles. In the case of spherical micelles, the core radius $R_c$ and the area per chain $b^2$ are directly related to the number of polymers per micelle, i.e., the aggregation number $Z$, where $Z = 4\pi R_c^2 / b^2$.\textsuperscript{30}

Along with the hydrophobic effect, micelle formation is further guided by the volume ratio of the hydrophobic and hydrophilic polymer domains. Through these principles, it is possible to synthetically engineer soft matter to organize into various nanoscale morphologies that can be precisely tuned due to the chemical control provided by, in this particular discussion, a well-defined ROMP catalyst. A phase diagram of morphologies accessible from block copolymer surfactants is shown in Figure 2.2.
Figure 2.1. Important physical characteristics governing micelle architecture and morphology.

For simple surfactants the three-dimensional shape of the equilibrium aggregate is determined by the molecular packing parameter defined as $v_o/a_e l_o$, where $v_o$ is the surfactant tail volume, $a_e$ is the equilibrium area of the surfactant head and $l_o$ is the tail length. The connection between the molecular packing parameter and the aggregate shape is traditionally defined as: $0 \leq v_o/a_e l_o \leq \frac{1}{3}$ for sphere, $\frac{1}{3} \leq v_o/a_e l_o \leq \frac{1}{2}$ for cylinder, and $\frac{1}{2} \leq v_o/a_e l_o \leq 1$ for bilayer. However, slight deviations in this model have been presented when taking into account additional parameters concerning hydrophobic tail length.

Access to these various phases is possible via a number of synthetic ROMP-based approaches. These approaches can be divided into three distinct classes and involve grafting-through, grafting-to, or grafting-from ROMP polymer backbones in order to achieve amphiphilicity necessary to guide nanoparticle formation. Grafting-through employs sequential direct polymerization of monomer units yielding distinct hydrophobic and hydrophilic domains. Grafting-to ROMP polymer backbones is achieved in two ways: 1) through polymerization of conjugatable monomer units and subsequent conjugation or 2) through termination of polymerization with a conjugatable moiety. The major distinction between the two being that a conjugatable termination agent allows functionalization at exactly one location along the polymer backbone whereas polymerization of a conjugatable
monomer yields a statistical distribution of possible connection points. The grafting-from method involves the use of an orthogonal polymerization technique to either polymerize from a ROMP copolymer backbone or to provide a polymer scaffold capable of initiating ROMP. Here, the distinct domains of the resulting hybrid polymer are responsible for the self-assembly of the material. It should be noted that there are several other approaches to nanomaterials via ROMP including the synthesis of dispersed latex nanoparticles, hybrid nanoparticles achieved via scaffolded initiation of ROMP, and nanoparticles encapsulated in ROMP polymer matrices.

**Figure 2.2** Morphology diagram and representative electron micrographs of various phases accessible via ROMP-derived amphiphilic polymers. Shown are spherical micelles (S), cylindrical micelles (C), Y-junction networks (Y), and vesicular bilayer structures (B), scale bars are 100 nm for B, Y, and C and 50 nm for S.

The major drawback in materials made from purely synthetic and non-informational polymers is that there are limited ways to systematically address the materials post synthesis. One can use heat, pressure, light, and chemical reactions, for example, to manipulate materials made from polymers depending on how the polymers are engineered. However, these manipulations are non-specific and non-programmable. On the other hand, materials
encoded with informational polymers (i.e. nucleic acids) allow for programming of material
morphology and function and allow one to specifically address populations of materials based
on highly-specific molecular interactions.\textsuperscript{45} For this reason, programming polymers via the
covalent attachment of DNA provides an exceptional opportunity for the formation of
information rich functional materials taking advantage of structural and functional properties
from both the polymer scaffold and the DNA appendage.

DNA-polymer conjugates are of interest for building unique informational materials
while conferring new traits to nucleic acids. For example, nucleic acid-polymers conjugates
have been prepared as PCR primers,\textsuperscript{46} as hydrogels,\textsuperscript{47} for enhancing enzymatic resistance
through PEGylation,\textsuperscript{48} micellization,\textsuperscript{49} and nanoparticle formation,\textsuperscript{50} for purification of
biomolecules,\textsuperscript{51} for diagnostics and detection,\textsuperscript{52} and as novel antigen\textsuperscript{53} and antisense\textsuperscript{54}
agents.

\subsection*{2.2 Synthetic Routes for the Preparation of Nucleic Acid-Polymer
Conjugates}

Both solution and solid phase approaches have been successfully utilized for the
preparation of nucleic acid-polymer conjugates, each approach with its own set of concerns
and advantages. Specifically, solution phase conjugation is limited to conjugates wherein
both moieties are soluble in the same solvent. For traditional polyanionic nucleic acids this
means that the polymers must be soluble in water or in solvents composed of a certain
percentage of water. Recently, it has been proposed that surfactants or other charge
neutralizing agents offer a method for circumventing this limitation by solubilizing nucleic
acids\textsuperscript{55} in organic solvents for further functionalization.\textsuperscript{56,57} Note that DNA is soluble in
DMSO at reasonable concentrations (tens of micromolar), but must first be dissolved in
water, therefore, a small volume of water will be present in the final solution. DNA is only minimally soluble in 95% DMF (0.1 mg/mL plasmid DNA, roughly 200 nM), hence this solvent is considered unsuitable for conjugation reactions. For nucleic acid derivatives, such as PNA and methylphosphonamidite nucleic acids, there is a bit more flexibility in the solvent system, given that these derivatives are typically soluble in strong organic solvents like DMF. Solid phase conjugation represents a more enjoyable way to conjugate molecules onto DNA. In solid phase conjugation, moieties for conjugation can be dissolved in a variety of solvents (including DMF, acetonitrile, dichloromethane, and others) and washed over functionalized nucleic acids bound to a solid support. In this way, excess reagents that did not react with the DNA can be washed away before cleaving the conjugates from the solid support. The limitation in the solid phase approach to DNA conjugates is in the fact that anything conjugated to DNA must be able to withstand chemical conditions necessary to
release the conjugate from the solid support and also deprotect the nucleobases. Typically, these conditions are quite harsh, although gentler methods including UltraMILD protocols (http://www.glenresearch.com/Technical/TB_UltraMild_Deprotection.pdf) can be explored. Alas, there is no way to avoid subjecting the conjugate to basic conditions, at least with the current standard phosphoramidite synthesis approach.

In this work, nucleic acid-polymer conjugates have been prepared such that the polymer, synthesized via ROMP, serves as a hydrophobic domain while the nucleic acid serves as a hydrophilic domain. In a selective solvent, that is, a solvent suitable to dissolve only one domain of the conjugate, the amphiphilic nature of the conjugates drives assembly into a range of nanoscale materials. DNA-polymer conjugates were prepared in one of two ways: either by conjugating a 5’ amino-modified nucleic acid sequence to a ROMP polymer containing an active ester moiety (a graft-from approach) or by directly polymerizing a peptide nucleic acid (PNA) sequence derivatized with a norbornene moiety (a graft through approach). Note that nucleic acid-containing polymers prepared via direct polymerization are not true conjugates, as no conjugation between the polymer and nucleic acid elements is necessary. Nevertheless, in context of this thesis, the term nucleic acid-polymer conjugate will serve to describe nucleic acid-polymer hybrids prepared via both graft to and graft through approaches. Polymerization of a hydrophobic monomer followed polymerization of a monomer suitable for conjugation (i.e. an $N$-hydroxysuccinimide-modified norbornene) provides a block copolymer scaffold with multiple sites for nucleic acid conjugation. Alternatively, polymerization of a hydrophobic monomer followed by termination with a carboxylic acid functionalized chain transfer agent (CTA) provides a polymer with exactly one site for nucleic acid conjugation. For work described in this thesis, all graft-to approaches for the preparation of DNA-polymer conjugates are carried out via reacting solubilized polymer with oligonucleotides still bound to a solid support. This method is used due to the
disparate solubilities of the polymer and oligonucleotides. Solution phase conjugation approaches are certainly amenable to the preparation of other conjugates. Recently, methods have been developed to conjugate nucleic acids to hydrophobic moieties in organic solvents using surfactant-based approaches.\textsuperscript{56,57}

Of the three approaches to amphiphilic nucleic acid-polymer conjugates, block copolymers containing multiple NHS-modified norbornene monomers provide the least desirable route. This is due to the fact that, in this case, conjugation of amino-functionalized nucleic acids results in nucleic acid-polymer conjugates with various degrees of functionalization and multiple substitution patterns. Therefore, conjugation of amino-modified nucleic acids to polymer termini functionalized with carboxylic acid moieties is preferable in that the resulting conjugate is well defined and reproducible. Direct polymerization of PNA-functionalized norbornene monomers resulting in amphiphilic block copolymers is also desirable in that each block copolymer will have incorporated the same number (plus or minus a standard deviation) of nucleic acid elements, with no need for post-polymerization conjugation. Thus, direct polymerization provides a quick route to reproducible nucleic acid-polymer hybrids. The three approaches used for preparing nucleic acid-polymer conjugates are described in Figure 2.3 and demonstrated in a variety of settings in work prepared by the author.\textsuperscript{50,54,60,61}

Characterization of nucleic acid-polymer conjugates and their components is necessary at multiple stages to ensure high fidelity materials. Both the polymer and nucleic acid, appropriately functionalized with reactive chemical moieties, must be characterized prior to conjugation to ensure that the conjugation reaction will connect two species of known composition and purity. Obviously, starting materials (i.e. monomers for polymer synthesis and phosphoramidites and monomers for nucleic acid synthesis) must be of high purity before synthesis of the respective macromolecules. Polymers prepared \textit{via} ROMP must be
monitored in order to ensure complete monomer conversion and complete termination and analyzed to determine the molecular weight of the resulting polymer. Monomer conversion and termination efficiency can be tracked via \( ^1 \)H NMR, owing to key resonances at the olefinic norbornyl positions and at the ruthenium-based alkylidene position respectively. Polymer molecular weight is best-determined using size exclusion chromatography coupled to a multi-angle light scattering detector (SEC-MALS). Light scattering analysis, unlike gel permeation chromatography on its own, is not prone to error in molecular weight determination associated with variations in polymer confirmation that affect retention time.\(^{62}\)

Amino-modified nucleic acid sequences must be released from solid support, purified, and analyzed by MALDI-TOF in order to confirm molecular weight associated with a given sequence and all incorporated modifications including the crucial amino modifier. This analysis should be done for a small fraction of the solid support, as polymer conjugation is to be performed with nucleic acids still bound to the support. The final analysis that can be conducted before polymer conjugation is release of the monomethoxytrityl (MMT) protecting group from the 5'-amino modified oligonucleotide bound to the solid support. Cleavage of this protecting group can be performed on the DNA synthesizer, and the resulting wash collected for analysis by measuring absorbance of the solution at 472 nm. This absorbance can be compared to the dimethoxytrityl (DMT) cation release from the previous coupling in order to determine the efficiency of amino labeling (see Glen Research “5'-MMT-AMINO-MODIFIERS”, http://www.glenresearch.com/Technical/TB_MMT_Amino_Modifiers.pdf). Alternatively, the cleavage wash can be assessed qualitatively, as the solution (roughly 10 mL of MMT cation in 3% trichloroacetic acid (TCA)/dichloromethane (DCM)) should be a dark yellow if the 5’-amino modifier has been efficiently incorporated and removed. After conjugation, an assessment of the efficiency of the reaction can be carried out via gel electrophoresis. Alternatively, conjugates can be directly analyzed via mass spectrometry.\(^{63,64}\)
however, no success has been realized in this approach with any of the polymer conjugates prepared in this work. This is presumably due to the lack of a solvent suitable to dissolve both the polymer and nucleic acid domains. Despite the lack of success of the author, there most likely exist conditions in which such materials can be effectively analyzed via MS.

2.3 Self-Assembly of DNA-Polymer Conjugates and Amphiphiles

Nucleic acid-polymer conjugates in this work are either formed on solid support or during ROMP. If the conjugates are formed on solid support then their assembly into nanomaterials occurs at the instant that they are released from the solid support into a given solution. At this point, the morphology of these materials can be manipulated via dramatically switching solvent systems. For example, switching solvent from pure aqueous phase to 50% THF in water or altering the length of DNA at the nanomaterial shell via enzymatic processing both have effects on DPA morphology. If the conjugates are formed during ROMP, as is the case for directly polymerized PNA block copolymers, assemblies are encouraged to form by dialyzing the nucleic acid-polymer hybrids from a non-selective solvent (i.e. DMSO or DMF) into a selective solvent (i.e. water). In this case, it is theorized that due to the fact that peptide nucleic acids are less hydrophilic than traditional phosphate-bearing nucleic acids, hybrid polymers composed of PNA are fully soluble in organic solvents such as DMF and DMSO. Therefore, these block copolymers can be transitioned from the organic phase into the aqueous phase in order to promote phase separation and assembly into discrete architectures.

Nucleic acid-polymer conjugates synthesized on solid support are rapidly released into solution via base catalyzed ester hydrolysis. Using an equal mixture of concentrated ammonium hydroxide and 40% aqueous methylamine (AMA), the conjugates are released into solution within minutes. It is hypothesized that DPA nanoparticles form spontaneously
upon release of the conjugate into solution. Based on the experience of the author, conjugates of various oligonucleotide length (ca. 20 to 40 bases) bound to polymers on the order of 20 monomer repeats in length always assemble into spherical nanoparticles with diameters of roughly 20 nm. Similar materials have been prepared at least 25 times with no deviation from a spherical morphology. Figure 2.4 shows several examples of nanoparticles prepared on separate occasions and with different oligonucleotide sequences. Each of these DPA NPs were prepared as described in section 3.7.6. These results highlight the reproducibility of this synthetic approach towards well-defined DNA-programmed nanomaterials. This reproducibility has been key in demonstrating several unique features of these materials such as enhanced stability against enzymatic degradation and the ability to enter live mammalian cells and silence mRNA transcripts.

Figure 2.4. A criterion collection of DPA nanoparticles prepared for various uses. All scale bars are 50 nm. Underlined bases are LNA, T_f and T_D denote fluorescein and DABCYL modified thymidine.
Things get more interesting when the aforementioned spherical nanoparticles are manipulated post formulation. Upon dialyzing DPA NPs from 10 mM Tris pH 8.5 into a solution of 50% THF in water, some very wonderful nanostructures are formed. It is theorized that the THF acts to swell the polymer core\textsuperscript{65} of the materials while simultaneously dehydrating the nucleic acid shell.\textsuperscript{59,66} Very complex assemblies are formed as the materials search for a local thermodynamic minimum (Figure 2.5).

\textbf{Figure 2.5.} Materials formed via dehydration and swelling of DPA nanoparticles with THF.

The most interesting structures formed are shown in Figure 2.6. These structures can be thought of as segmented micelles or lamellar droplet-type phases.\textsuperscript{67}
Figure 2.6. Segmented micelles formed from dehydration and swelling of DPA nanoparticles via dialysis into 50% aqueous THF.

Similar assemblies have been reported in the literature but remain quite rare. Wooley and Pochan describe similar structures as resulting from the one-dimensional packing of disc-like micelles. Craig Hawker’s group has reported what they term axially stacked lamellae structures and dispersed droplets by controlling phase separation behavior of polystyrene-b-poly(2-vinylpyridine) block copolymers with functional surfactants. To the author’s knowledge, there have been no reports in the literature of these structures assembled from DNA-conjugated polymers. Unfortunately, so far, these structures are extremely difficult to reproduce. Even when recapitulating the experiment under identical conditions, the structures have not been revisited. There are two conclusions that can be drawn based on this evidence – 1) the structures are metastable and exist only for a limited window in time and 2) the parameters governing structure formation are not under appropriate control. To elaborate on
the second point, very subtle changes in the rate of influx of the THF/water mixture (controlled by stirring, osmotic pressure, and dialysis membrane integrity) and the rate of THF evaporation from the dialysis unit (controlled by sealing or not sealing the cap of the mini dialysis unit - Thermo Pierce Slide-A-Lyzer MINI Dialysis Device, 20K MWCO, 0.1mL Product #69590) may play an important role in structure formation. Further, the volume of solution placed inside the dialysis unit plays an important role in that the influx of THF will have a more dramatic impact on the solution composition when a smaller volume of solution is placed in the dialysis unit prior to the onset of dialysis. At the time of writing, this work is unpublished. However, it is the author’s intention to reproduce the phase-separated worms and study their behavior with respect to DNA hybridization and biological interfacing.

Hybrid PNA-polymer nanostructures are formed via a different route than DPA NPs. In this case, the amphiphilic polymer-peptide nucleic acid hybrids are formed in solution during the polymerization process as depicted in Figure 2.7.
Figure 2.7. Synthesis and characterization of a poly-(oligonucleotide). A) Peptide nucleic acid (PNA) modified norbornene monomer (PNA-Nb) polymerized using ROMP initiator (IMesH$_2$)(C$_5$H$_5$N)$_2$(Cl)$_2$Ru=CHPh (‘Ru’) to form poly-PNA homopolymers, I, and poly-PNA block copolymers, II. B) Representative percent conversion for I determined by the disappearance of the olefin signal associated with PNA-Nb in $^1$H NMR. C) Representative SEC-MALS for III. $M_n = 28,270$ indicating a degree of polymerization of 6.

As the norbornyl PNA monomer has a signature $^1$H NMR resonance associated with the norbornyl olefin protons, the percent incorporation of PNA monomer into a polymeric species can be tracked via NMR. Tracking of percent conversion via NMR is depicted in Figure 2.18, while percent conversions for various homo- and block copolymers are given in Table 2.1. To encourage assembly of PNA-based block copolymers in nanostructures, one must transition the resulting hybrids into a selective solvent, such as water. Upon transition in
water via dialysis, the polymers assemble into a range of structures shown in Figures 2.8 and 2.9.

**Figure 2.8.** Polymerization of norbornyl PNA monomers into homopolymer and block copolymer architectures via ROMP. Poly-PNA amphiphiles were dialyzed from DMSO into H$_2$O to generate nanoparticles. A) Dynamic light scattering (DLS) data indicating a hydrodynamic radius of 25 nm. B) $T_m$ of PNA-NP with a complimentary DNA sequence was found to be 58.1°C. C) Negative-stained TEM of PNA-NP provided evidence of spherical 20 nm diameter nanoparticles. Atomistic models of D) III, and E) PNA-NP. II is shown in a conformation present within PNA-NP.
Figure 2.9. Antisense PNA-based nanostructures from poly(PNA) ROMP copolymers.

It is believed that the PNA occupies the surface region of these nanoparticles, since they are able to hybridize complementary strands of DNA. A molecular model of the nanoparticles shown in Figure 2.8 has been generated, corroborating this assumption. Rendering the PNA sequences more hydrophilic via the incorporation of a sulfated cyanine 5.5 moiety seems to encourage the formation of different structures, more amenable to solubilization in water. PNA block copolymer structures benefiting from the incorporation of a sulfated cyanine 5.5 are depicted in Figure 2.9. Surely the structures formed are highly variable depending on the rate of dialysis, concentration of polymer, block size of each polymer domain, and a host of other factors. Indeed, most self-assembled structures are under kinetic control and therefore can be encouraged to occupy many different phases. Information including SEC-MALS traces and synthetic procedures for some PNA copolymers is not included in this section. For more information see James et al. 61

2.4 Methods

Note: Methods on the preparation of DPA NPs can be found in Section 3.7.
Figure 2.10. Norbornyl monomers for the incorporation into PNA block copolymers.

General Methods for PNA Polymer Preparation. All reagents were purchased from commercial sources and used without further purification unless otherwise indicated. N-phenyl-cis-5-norbornene-exo-dicarboximide (1),70 2-(2,5,8,11-tetraoxatridecan-13-yl)-3a,4,7,7a-tetrahydro-1H-4,7-methanoisoindole-1,3(2H)-dione (2),71 N-benzyl-2-(1,3-dioxo-1,3,3a,4,7,7a-hexahydro-2H-4,7-epoxyisoindol-2-yl)-N,N-dimethylethan-1-aminium (3),72 and N-(glycine)-cis-5-norbornene-exo-dicarboximide (4)73 were all prepared as described. All PNA sequences were made using NovaPEG Rink Amide resin, purchased from EMD Millipore, with a loading of 0.49 mmol/g and a swelling volume of 8.8 mL/g in CH$_2$Cl$_2$. The synthesis of all PNA oligomers used Fmoc/Bhoc protected monomers (Fmoc-A(Bhoc)-aeg-OH, Fmoc-C(Bhoc)-aeg-OH, Fmoc-G(Bhoc)-aeg-OH, and Fmoc-T-aeg-OH) purchased from Panagene. PNA sequences were manually synthesized in house using standard solid-phase peptide synthesis conditions. RP-HPLC analysis of PNAs was performed on a Hitachi-Elite LaChrom L-2130 pump with a step-wise gradient. Detection was at 260 nm using an in-line UV-Vis detector (Hitachi-Elite LaChrom L-2420). For analysis, an analytical scale Phenomenex Jupiter 4µm Proteo 90Å column (150 x 4.60 mm) was utilized. For purification, a semi-preparative Phenomenex Jupiter 4µm Proteo 90Å column (250 x 10.0 mm) was utilized. Mass spectra were obtained at the UCSD Chemistry and Biochemistry Molecular Mass Spectrometry Facility. Low-resolution mass spectra were obtained using a Thermo LCQdeca mass spectrometer. Concentrations of oligonucleotides and peptide nucleic acids
were determined using a Thermo Scientific NanoDrop 2000c spectrophotometer. Modified 2nd Generation Grubbs’ Ruthenium initiator (IMesH2)(C5H5N)2(Cl)2Ru=CHPh was prepared as described by Sanford et al.5 Sealed ampules of (CD3)2NCOD (DMF-d7) used in polymerization reactions was purchased from Cambridge Isotope Laboratories Inc. and was distilled and degassed with 3 freeze-pump-thaw cycles prior to use. 1H (400 MHz) NMR spectra were recorded on a Varian Mercury Plus spectrometer. Chemical shifts (1H) are reported in δ (ppm) relative to the DMF-d7 residual proton peaks (8.03, 2.92, and 2.75 ppm). Polymer molecular weight and dispersity were determined via size-exclusion chromatography (Phenomenex Phenogel 5µm 10, 1K-75K, 300 x 7.80 mm in series with a Phenomex Phenogel 5µm 10, 10K-1000K, 300 x 7.80 mm (mobile phase: 0.05 M LiBr in DMF)) using a Hitachi-Elite LaChrom L-2130 pump equipped with a DAWN HELEOS multi-angle light scattering (MALD) detector (Wyatt Technology) and a refractive index detector (Hitachi L-2490) normalized to a 30,000 g/mol polystyrene standard. The dn/dc values used were 0.179. Hydrodynamic diameter (Dh) of nanoparticles was measured via DLS using a DynaPro NanoStar (Wyatt Technology). TEM samples were deposited on carbon/formvar-coated copper grids (Ted Pella Inc.), stained with 1% w/w uranyl acetate, and imaged using a Tecnai G2 Sphera operating at an accelerating voltage of 200 kV. Complementary and non-complementary DNA sequences were purchased from Integrated DNA Technologies (purified by HPLC, confirmed by ESI-MS). DNA melting temperature analysis was conducted using a Cary Series 100 UV-Vis spectrophotometer equipped with a Cary temperature controller.
Figure 2.11. Synthesis of PNA norbornyl monomers amenable to polymerization via ROMP.

**PNA Norbornyl Monomer Synthesis.** All PNA sequences were manually synthesized. All reactions and washes of the resin were performed in a fritted glass peptide synthesis vessel, with the exception of the cleavage and deprotection of the oligomer from the resin, which was done in a polypropylene Poly-Prep Chromatography Column, purchased from Bio-Rad Laboratories. Unless otherwise stated the following standard protocol was used:

1) Swelling of the NovaPEG Rink Amide resin in $\text{CH}_2\text{Cl}_2$ for 2 hours. Deprotection of the resin is not required as it is sold without protecting groups.

2) Resin is washed with a steady flow of DMF (30 seconds) followed by a steady flow of with $\text{CH}_2\text{Cl}_2$ (30 seconds).

3) Activation of PNA monomer (5 equivalents with respect to total active sites on the resin) occurred by addition of 4.5 equivalents $\text{N},\text{N},\text{N},\text{N}$-Tetramethyl-$\text{O}$-(7-azabenzotriazol-1-yl)uronium hexafluorophosphate (HATU) (slightly less equivalents were used to ensure total activation of monomer with HATU and to prevent occurrence of unreacted HATU from reacting with resin amine or unprotected amines in the growing PNA sequence. Unprotected amines can form a guanidine moiety with HATU that blocks further elongation) and 10 equiv. of diisopropylethylamine (DIPEA) in DMF. The final concentration of the monomer
was 0.2M in DMF. Monomer activation was allowed to proceed for 10 minutes before being added to the resin.

4) A steady stream of N\textsubscript{2} (g) was bubbled through the peptide synthesis vessel while coupling occurred. Coupling time was 60 minutes.

5) Upon completion of coupling, the activating solution was vacuumed off the resin, and the resin was washed with a steady stream of DMF for 30 seconds (3 times), followed by CH\textsubscript{2}Cl\textsubscript{2} for 30 seconds (3 times). No capping steps were necessary for the PNA sequences chosen.

6) Deprotection of the Fmoc was done using a solution of 20% piperidine in DMF for 5 minutes.

7) Steps 2-6 were repeated until chain length was complete.

8) Following the removal of the final Fmoc group, and subsequent washings of the resin with DMF and CH\textsubscript{2}Cl\textsubscript{2}, the HATU-activated carboxylic acid-substituted norbornene (1) was added and a steady stream of N\textsubscript{2} (g) bubbled through the vessel for 60 minutes (the carboxylic acid-substituted norbornene was activated using the same protocol used for the PNA monomers in step 3).

9) Upon completion of coupling, the activating solution was vacuumed off the resin, and the resin was washed with a steady stream of DMF for 30 seconds (3 times), followed by CH\textsubscript{2}Cl\textsubscript{2} for 30 seconds (3 times).

10) Step 8 was repeated (carboxylic acid-substituted norbornene (1) coupling to the resin).

11) Upon completion of coupling, the activating solution was vacuumed off the resin, and the resin was washed with a steady stream of DMF for 30 seconds (3 times), followed by CH\textsubscript{2}Cl\textsubscript{2} for 30 seconds (3 times).

12) The resin was dried under vacuum for several hours.

13) The removal the Bhoc protecting groups and cleavage from the resin was accomplished by treatment with a solution of TFA:m-cresol (80:20) for 90 minutes. The cleavage was
performed in a polypropylene Poly-Prep Chromatography Column. After cleavage, the TFA:cresol solution was separated from the resin by centrifugation. The TFA:cresol solution was then evaporated until near dryness by applying a stream of air to the solution for several hours.

14) The crude PNA-norbornene oligomer crashed out of the TFA:cresol solution upon addition of 5 equivalents of diethyl ether with respect to the TFA:m-cresol solution, yielding an off-white powdery solid.

15) Reverse-phase preparatory HPLC was used to purify all sequences, and masses were confirmed by MALDI-TOF.

**HPLC Purification of PNA Sequences.** RP-HPLC analysis of PNA was performed using 0.1% TFA/H2O as solvent A, and 0.1% TFA/CH3CN as solvent B. Gradient: 0% solvent B in 2 min, 0% to 5% solvent B in 3 min, 5% to 20% solvent B in 10 min, and 20% to 100% solvent B in 20 min.
Figure 2.12. HPLC and ESI-MS of purified PNA norbornyl monomers.

PNA Homopolymer Synthesis via ROMP (Figure 2.7). PNA-Nb monomer, CGAGTCATT-T-Nb, was polymerized via ROMP using Grubbs’ modified 2nd generation catalyst (IMesH$_2$)(C$_5$H$_3$N)$_2$ClRu=CHR in a glove box. The PNA-Nb monomer (3mg, 1 µmol) in a J-Young NMR tube was dissolved in 250 µL of anhydrous and degassed DMF-d$_7$. The tube was removed from the glove box and a $^1$H NMR spectrum (t = 0) was taken. The tube was returned to the glove box and the catalyst (0.2 µmol or 0.1 µmol) was added to the reaction solution. $^1$H NMR spectra were recorded at the indicated time points until consumption of the olefin. It was observed that as the PNA monomer olefin disappeared, the corresponding DMF-d$_7$ solutions became cloudy, and that the polyolefin peaks typically seen at ~5.5 ppm were absent, indicating that the resultant polymer had limited to no solubility in
DMF. In addition, the homopolymers were insoluble in H₂O, MeOH, and DCM solutions and had limited solubility in a solution of 0.05 M LiBr in DMF. Upon consumption of the olefin, the tube was returned to the glove box and termination agent ethyl vinyl ether (100 µL, excess) was added to the reaction mixture, and the mixture was allowed to sit at room temperature for 20 minutes. The crude polymer was precipitated from cold methanol and analyzed by SEC-MALS.

**Figure 2.13.** Polymerization of PNA monomer to a degree of ten monitored via ¹H NMR. Integrations are based on five equivalents of PNA olefin at t=0. DMF residual proton, (CH₃)₂NC(O)H, integration is then held constant for the remaining time points.
Figure 2.14. Polymerization of PNA monomer to a degree of ten monitored via $^1$H NMR. Integrations are based on ten equivalents of PNA olefin at t=0. DMF residual proton, $(\text{CH}_3)_2\text{NC(O)H}$, integration is then held constant for the remaining time points.

Figure 2.15. SEC-MALS chromatogram for PNA homopolymer I. The Mn was determined to be 13,790 with a PDI of 1.388, giving a degree of polymerization of 5 by LS, as opposed to 10 by $^1$H NMR. This discrepancy can be attributed to error in the assignment of the dn/dc. The dn/dc used to calculate the $M_n$ was 0.179, the known dn/dc for polystyrene in DMF. In addition, a large LS peak can be seen at 16 minutes. This peak corresponded to a Mn of $7.3 \times 10^6$ and indicated polymer aggregation in DMF. SEC-MALS for III could not be obtained due to polymer insolubility in DMF.
Figure 2.16. General scheme for the preparation of PNA block copolymers using Grubb’s 2nd generation modified catalyst. A small aliquot of block 1 was terminated using III (ethyl vinyl ether) for SEC-MALS analysis before adding PNA. This provided an accurate $M_n$ and degree of polymerization for the first block. After polymerization of the PNA block, the complete block copolymer was again analyzed using SEC-MALS.

Synthetic procedure for ROMP of II (Figures 2.7 and 2.8). N-phenyl-cis-5-norbornene-exo-dicarboximide1 (1) in a J-Young NMR tube (5 mg, 0.02 mmol) was dissolved in 250 µL anhydrous and degassed DMF-d$_7$ in a glove box. Catalyst (IMesH$_2$)(C$_5$H$_5$N)$_2$Cl$_2$Ru=CHPh (0.411 mg, 0.56 µmol) was added and the tube was removed from the glove box and $^1$H NMR spectra were recorded until complete consumption of olefin. After olefin consumption, the tube was returned to the glove box and PNA-Nb (8.2 mg, 2.8 µmol, 5 equivalents w.r.t. catalyst) was added in 100 µL anhydrous and degassed DMF-d$_7$. The tube was removed from the glove box and $^1$H NMR spectra were recorded at the indicated time points. Upon consumption of the olefin, the tube was returned to the glove box and termination agent ethyl vinyl ether (100 µL, excess) was added to the reaction mixture, and the mixture was allowed to sit at room temperature for 20 minutes. The crude polymer was precipitated from cold methanol and analyzed by SEC-MALS.
Figure 2.17. $^1$H NMR timescale for PNA block copolymer II. To a live catalyst on the end of a polyphenyl was added PNA (5 eq w.r.t. catalyst). The timescale shown is after 17 hours of reaction, at which point the polymer was terminated. The integrals shown are based on the amount of added phenyl-functionalized norbornene (35 eq. w.r.t catalyst, 5 protons/phenyl plus 5 protons of phenyl alkylidene for a total of 180 protons) and the amount of added PNA-Nb (5 eq w.r.t. catalyst).

ROMP conditions for PNA block copolymers VII-X (Figure 2.18). N-phenyl-cis-5-norbornene-exo-dicarboximide (1) (3.47 mg, 13.7 µmol), N-benzyl-2-(1,3-dioxo-1,3,3a,4,7,7a-hexahydro-2H-4,7-epoxyisoindol-2-yl)-N,N-dimethylethan -1-amininium (3) (4.47 mg, 13.7 µmol), and 2-(2,5,8,11-tetraoxatridecan-13-yl)-3a,4,7,7a-tetrahydro-1H-4,7-methanooisoindole-1,3(2H)-dione (2) (4.8 mg, 13.7 µmol) in 85.5 µL DMF-d$_7$ were polymerized by mixing with 0.274 mg (0.38 µmol) of catalyst (14.5 µL of a 0.026 M soln. in DMF-d$_7$) for a total volume of 100 µL DMF-d$_7$. After complete polymerization, 10 µl of this reaction (0.038 µmol w.r.t. catalyst) was taken out and added to 1 mg (0.34 µmol) of PNA norbornyl monomer (this reaction described below). The remaining polymerized first block reaction mixture was quenched with excess ethyl vinyl ether, precipitated and analyzed by SEC-MALS.
For incorporation of PNA norbornyl monomer as the second block in a phenyl-PNA block copolymer, 4.2 mg (1.4 µmol) PNA monomer was dissolved with heating in 25 µL DMSO-\(d_6\) to yield a stock solution of PNA monomer at a concentration of 57.5 mM. 5.95 µL of this PNA stock solution (1mg, 0.34 µmol) was added to 10 µL of phenyl homopolymer solution with live ruthenium catalyst (as described above) for a total reaction volume of 15.95 µL. After 12 hours at r.t., all four reactions were diluted to 90 µL total volume with DMF-\(d_7\) and added to a 3mm O.D. NMR tube via a heat-pulled glass pipette in order to provide enough volume for NMR analysis while keeping the concentration at a maximum.

**Table 2.1. Polymers and Copolymers of PNA with Monomers Shown in Figure 2.11.**

<table>
<thead>
<tr>
<th>polymer</th>
<th>mon(_1)^a</th>
<th>mon(_2)^c</th>
<th>m(^d)</th>
<th>n(^d)</th>
<th>% con.(^e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>PNA-Nb (10:1)(^b)</td>
<td>—</td>
<td>10</td>
<td>—</td>
<td>99</td>
</tr>
<tr>
<td>II</td>
<td>1 (35:1)</td>
<td>PNA-Nb (5:1)</td>
<td>35</td>
<td>5</td>
<td>97</td>
</tr>
<tr>
<td>III</td>
<td>PNA-Nb (5:1)</td>
<td>—</td>
<td>5</td>
<td>—</td>
<td>97</td>
</tr>
<tr>
<td>IV</td>
<td>1 (30:1)</td>
<td>PNA-Nb (7.5:1)</td>
<td>30</td>
<td>5</td>
<td>65</td>
</tr>
<tr>
<td>V</td>
<td>1 (30:1)</td>
<td>PNA-Nb (7.5:1)</td>
<td>30</td>
<td>5</td>
<td>65</td>
</tr>
<tr>
<td>VI</td>
<td>1 (30:1)</td>
<td>PNA-Nb (7.5:1)</td>
<td>30</td>
<td>6</td>
<td>79</td>
</tr>
<tr>
<td>VII</td>
<td>1 (36:1)</td>
<td>PNA-Nb (9:1)</td>
<td>35</td>
<td>8</td>
<td>88</td>
</tr>
<tr>
<td>VIII</td>
<td>1 (36:1)</td>
<td>PNA-Nb (18:1)</td>
<td>35</td>
<td>16</td>
<td>87</td>
</tr>
<tr>
<td>IX</td>
<td>2 (36:1)</td>
<td>PNA-Nb (9:1)</td>
<td>33</td>
<td>7</td>
<td>74</td>
</tr>
<tr>
<td>X</td>
<td>3 (36:1)</td>
<td>PNA-Nb (9:1)</td>
<td>41</td>
<td>5</td>
<td>56</td>
</tr>
</tbody>
</table>

\(^a\)Indicates identity of monomer polymerized first (degree of polymerization, DP = m). \(^b\)Ratios shown indicate monomer to initiator ratio, or intended DP. \(^c\)Indicates identity of monomer polymerized second (DP = n). \(^d\)Observed degree of polymerization of mon\(_1\) (m), or mon\(_2\) (n). \(^e\)Percent conversion of PNA-Nb determined by \(^1\)H NMR.
Figure 2.18. Synthesis of various PNA block copolymers with conversion of PNA monomer monitored by $^1$H NMR. Roman numeral labels correspond to Table 2.1.

**Molecular Dynamics (MD) Simulations.** A single PNA-polynorbornyl unimer was initially built in fully extended conformation with GaussView software. 60 identical unimers, first relaxed in vacuum for 0.3 ns at the temperature of 300 K, were spherically distributed in space, with PNA ends oriented towards the outside. The resulting PNA-NP of 60 unimers was then immersed in a cubic (TIP3P) water box with solvate plugin in VMD; water molecules present within a 65 Å radius of PNA-NP center were deleted. The resulting unit cell with solvated 60-monomer PNA-NP contained 2,122,554 atoms.

MD simulations of solvated PNA-NP were performed with NAMD2 software, where the molecules were described using the CHARMM force field. The parameters for the unimer units (PNA, norbornyl) were obtained by analogy to molecules already parameterized in the CHARMM force field, using the ParamChem Server. All simulations were performed in NpT ensemble using periodic boundary conditions, at a constant temperature.
T= 300 K, a Langevin constant $\gamma_{\text{Lang}} = 0.001 \text{ ps}^{-1}$ (to ensure fast dynamics), and at a constant pressure $p= 1.01325 \text{ bar}$. The particle-mesh Ewald (PME) method\textsuperscript{80} was used for evaluation of long-range Coulombic interactions. The time step was set to 1.0 fs, and long range interactions were evaluated every 1 (van der Waals) and 2 time steps (Coulombic).

In the prepared system, water was minimized for 10 ps around the fixed PNA-NP, then for additional 8 ps around the constrained PNA-NP. The whole system was then heated to the temperature of 300 K and equilibrated at this temperature for 16 ns without constraints. See James et al. for more information.\textsuperscript{61}

**Partial Atomic Charges.** CHARMM parameters for all atoms were prepared by analogy to known molecules, using the ParamChem Server. The obtained partial charges were slightly modified to ensure that the unimer had no net charge. Below are the partial atomic charges for each PNA base, a unit of the PNA peptide chain, and a unit of the hydrophobic chain. See James et al. for more information.\textsuperscript{61}

## 2.5 Acknowledgements

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2.6 References


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Chapter 3

Enzymatic Processing of Nanoparticles

Assembled From DNA-Polymer Amphiphiles

3.1 Introduction

Nucleic acids are unique, informational molecules with exceptional potential in the preparation of complex nanostructured materials\(^1\) with utility as potent and specific therapeutic agents \textit{in vivo} and as powerful investigative tools \textit{in vitro}.\(^2\)-\(^4\) Despite this promise, unmodified nucleic acids are inherently susceptible to enzymatic degradation in biological milieu, limiting their practical utility in detection and as therapeutics in real-world applications.\(^5\),\(^6\) To mitigate these issues, considerable effort has been applied to the generation of DNA analogues capable of resisting attack.\(^7\),\(^8\) Unfortunately, in biological settings, these synthetic analogues exhibit unpredictable off-target effects\(^9\) and preclude advantageous interactions with key cellular machinery necessary for antisense interference.\(^10\),\(^11\) Therefore, two nanotechnology-based approaches for packaging nucleic acids for resistance have been pursued whereby the base identity is preserved along with backbone chemistry: (1) utilizing gold nanoparticles as templates for arranging oligonucleotides as a spherical brush,\(^12\) (2) packing nucleic acids as DNA origami.\(^13\),\(^14\) Importantly, both approaches use well-defined nanostructures to arrange nucleic acids in a given pattern. Herein, we describe a third route that avoids two key limitations imposed by these current approaches: (1) the need for preparing nucleic-acid-intensive DNA origami structures that, by necessity, consist of large portions of double-stranded DNA, susceptible to structural breakdown\(^15\) and unavailable for
subsequent hybridization; and (2) the need for a metal nanoparticle template that precludes
the incorporation of a chemically diverse core. Our strategy is based on the hypothesis that
single-stranded oligonucleotides, consisting of natural nucleotide structures, may be made
resistant to nuclease attack by densely packing them as organic polymeric micellar
nanoparticles. The design is predicated on the idea that steric hindrance through dense
packaging limits the accessibility of DNA to selective and nonselective nucleases. We sought
to demonstrate this approach to resistant nucleic acids by utilizing a polymerization strategy
that is known to be highly functional group tolerant, would allow efficient end-terminus
functionalization, and provides polymers of low polydispersity. Nucleic acids were packed
as DNA–polymer amphiphiles (DPAs) into micellar nanoparticles consisting of a high-
density ssDNA corona with a hydrophobic organic polymer core. We demonstrate that this
morphology allows free access to additional complementary DNA strands while preventing
and/or inhibiting the activity of various types of nucleases.

Note that there are a few other examples in the literature that report rendering nucleic
acids resistant to enzymatic degradation via tethering to polymer scaffolds. In one instance,
nucleic acids are rendered resistant upon condensation of DNA-poly(NIPAM) conjugates
induced by heat. Other examples describe stability conferred by PEGylation alone, PEGylation and micellization, or siRNA duplexes flanked by polymer modifications on
either end of the duplex.

It is posited that exonucleases, especially 3’ exonucleases, are responsible for nucleic
acid degradation in cell culture medium (see Uhlmann et al. beginning on page 565). In
these instances it is important to keep in mind that the rate of degradation depends on the
culture medium and cell line used for the experiment. On the contrary, it is believed that
endonucleases are the major culprits when it comes to nucleic acid degradation in cellulo.
3.2 Preparation of DPA Nanoparticles for Enzymatic Processing

DPAs were prepared via conjugation of a hydrophobic polymer (prepared via ring-opening metathesis polymerization), terminally modified with a carboxylic acid moiety, to a 5’-amino-modified oligonucleotide on solid support (Figure 3.1). The resulting DNA–polymer conjugate was separated from unmodified polymer by thoroughly rinsing the support. Subsequent cleavage and dialysis gave a mixture of spherical micellar nanoparticles and free, non-conjugated single-stranded DNA (ssDNA, Figure 3.1a, lane 1). The particles were separated from ssDNA via size-exclusion chromatography (SEC-FPLC) to give purified material (Figure 3.1a, lane 3). This procedure was utilized in the preparation of two micellar nanoparticles, P1 and P2, both exhibiting low dispersity with diameters on the order of 20 nm, as determined by TEM (Figure 3.1c) and dynamic light scattering (DLS, see Figure 3.5). Static light scattering (SLS) was utilized to confirm aggregation numbers (N_{agg}) on the order of ~200 DNA strands per particle (see Figure 3.5). Therefore, DNA is at exceptionally high densities of approximately 0.2 DNA strands/nm² on the surface of the micelles.
Figure 3.1. Preparation of DNA–polymer amphiphiles (DPAs) and assembly of micelles. Synthesis: (i) a hydrophobic polymer, terminally modified with a carboxylic acid moiety was mixed with a coupling agent and reacted with a 5′-amino-modified oligonucleotide on solid support (controlled pore glass, CPG); (ii) deprotection and cleavage of the resulting DNA–polymer conjugate from solid support; (iii) dialysis of cleaved DPA into deionized water to form a mixture of micelles and free, non-conjugated nucleic acid. T_F and T_D correspond to fluorescein- and DABCYL-modified thymidine phosphoramidites. (a) PAGE analysis. Lane 1: Crude material post micelle (P1) formation showing conjugate (top band) and free ssDNA (lower band). Lane 2: HPLC purified sample of ssDNA-1. Lane 3: Purified P1, isolated via size-exclusion chromatography (SEC). (b) SEC trace of purified P1 (λ_{abs} = 260 nm). (c) Transmission electron micrograph of P1. See Figure 3.9 for P2 data.
Figure 3.2. Carboxylic acid-terminated polymer synthesis via ROMP.

Figure 3.3. $^1$H NMR overlay showing complete termination of ROMP polymer with termination agent 2 (x-axis = $\delta$ in ppm). The resonance of the alkylidene proton in the polymeric species is at 17.7 ppm. Upon termination with compound 2, an alkylidene proton resonance corresponding to the metathesis product appears at 18.4 ppm. At 20 minutes, the resonance corresponding to the polymeric species is absent, indicating completion of the polymer termination reaction. At this point ethyl vinyl ether is added to quench the catalyst.
**Figure 3.4.** Polymer $I_{20}$ SEC-MALS chromatogram (LS = light scattering Rayleigh ratio, RI = refractive index difference, UV = UV absorbance at 280 nm, $M_w$ = weight averaged molecular weight).

**Figure 3.5.** Light scattering data for DPA nanoparticles P1 and P2. a) DLS histogram for P1 showing aggregates with a hydrodynamic diameter of 20 nm (10 mM Tris pH 8.5, 25 °C, mass weighted intensity signal). b) DLS histogram for P2 showing aggregates with a hydrodynamic diameter of 20 nm (10 mM Tris pH 8.5, 25 °C, mass weighted intensity signal). c) SLS/DLS analysis of P1 versus BSA protein standard, $M_w = 4,000,000$ g/mol, $N_{agg} = 295$ DPA/nanoparticle (DPA $M_w = 5221$ g/mol (polymer) + 8295 g/mol (oligo) = 13516 g/mol; 4,000,000/13516 = 295, 295/(4*3.14*(10^2)) = 0.23 DNA/nm²). d) DLS histogram of P1 in Exo III reaction buffer at 37 °C showing stability against aggregation. Potassium acetate was added in order to prevent aggregation due to MgCl₂ in NE Buffer 1. e) DLS histogram of P1 in Nt.CviPII reaction buffer at 37 °C showing stability against aggregation.
Figure 3.6. DNA sequences and chemical modifications. The recognition site for Nt.CviPII is indicted in red and the nick site is indicated by an asterisk.

Figure 3.7. HPLC purification and MALDI-TOF analysis of ssDNA-1 and ssDNA-2. a) HPLC chromatogram of purified ssDNA-1 (λ_{abs} = 290 nm, gradient: 36-60% B in 50 minutes). b) HPLC chromatogram of purified ssDNA-2 (λ_{abs} = 290 nm, gradient: 36-60% B in 50 minutes). c) MALDI-TOF mass spectrum of ssDNA-1, m/z = obs: 8295.45; theo: 8292.07 (calibration was performed externally). d) MALDI-TOF mass spectrum of ssDNA-2, m/z = obs: 8295.36; theo: 8292.07 (calibration was performed internally, left-to-right std. m/z = obs: 3646.21; theo: 3646.4, obs: 6118.48; theo: 6118.0, obs: 9191.64; theo: 9192.0).
The two particles (P1 and P2) were engineered to incorporate ssDNA sequences (ssDNA-1 and ssDNA-2) as substrates for a selective endonuclease and also a pair of indiscriminate exonucleases. ssDNA-1 and ssDNA-2 differ only in the location of dye and quencher labels. ssDNA-1 consists of a DABCYL modifier located toward the 3′-terminus and a fluorescein modifier 13 bases away toward the 5′-terminus. By contrast, ssDNA-2 has the reverse arrangement with a fluorescein modifier toward the 3′-terminus. This pair of sequences was designed to detect nuclease activity via a FRET assay in which the enzyme-triggered release of a DABCYL-modified (ssDNA-1) or fluorescein-modified (ssDNA-2) fragment from the oligonucleotide sequence results in an increase in fluorescence signal (vide infra). Additionally, to serve as independent controls, ssDNA-1 and ssDNA-2 were purified as non-polymer conjugated oligonucleotides.

![Figure 3.8. SEC-FPLC purification of DPA nanoparticles P1 and P2.](image-url)
3.3 Endonuclease Activity on DPA Nanoparticles

To examine how DPA nanoparticles respond to sequence-selective endonucleases, we incorporated a substrate for nicking endonuclease Nt.CviPII (5'...CCA...3'; see Figure 3.6) between fluorescein- and DABCYL-labeled thymidine moieties of the oligonucleotide. Nt.CviPII is a nicking endonuclease that recognizes double-stranded DNA (dsDNA) and introduces a single-strand break on the 5' side of the recognition site (5'...*CCX...3', X = A, G, or T). The system was designed such that nucleolytic cleavage occurs on the sequence of the DPA nanoparticle or ssDNA analogue while leaving the complementary sequence of the duplex fully intact. We reasoned that this design would facilitate a catalytic degradation of both the nanoparticle and ssDNA in response to small quantities of complementary DNA in the presence of the enzyme. Specifically, we programmed the nick site between bases 10 and
11 of the 20 base pair duplex such that the melting temperature ($T_m$) of the nicked product would drop to approximately half that of the full 20 base pair duplex (from ~60 to 30 °C). Through subsequent, thermodynamically favorable strand invasion, intact ssDNA or nanoparticle DNA would then be allowed to hybridize to its complement in order to recycle the target.

To monitor Nt.CviPll activity, we employed two complementary analytical techniques: a fluorescence assay and an assessment of DNA $T_m$ with and without enzyme treatment (Figure 3.10). The first method involved a fluorescence dequenching experiment wherein the particles or ssDNA sequences were allowed to hybridize to complementary DNA and subsequently introduced to the endonuclease. Fluorescein fluorescence was monitored over time in order to assess the activity of the enzyme. In this case, an increase in fluorescein fluorescence corresponded to a nick in the oligonucleotide sequence and a dissociation of the quencher- and fluorophore-labeled fragments. Indeed, after hybridization to complementary DNA, the labeled ssDNA sequence was readily destroyed in the presence of the nicking endonuclease (Figure 3.10b,c). On the contrary, the DPA nanoparticles showed virtually no activity via fluorescence under identical conditions (Figure 3.10a,c). Notably, this observation was independent of the dye and quencher arrangement in the nanoparticle substrates. This is a critical observation because for P1 there is the possibility that neighboring, fully intact strands within the particle shell may quench the fluorescein-labeled fragment, whereas this is not possible for P2, as the fluorescein-labeled fragment should be free in
Figure 3.10. Endonuclease resistance of DPA nanoparticles. (a) Scheme depicting DPA nanoparticle (P2) resistance to nicking endonuclease (Nt.CviPII) and consequently intact, quenched duplex DNA at the particle surface. (b) Scheme depicting dsDNA degradation by Nt.CviPII and consequently a decrease in duplex melting temperature and increase in fluorescein fluorescence. (c) Nt.CviPII activity over time, monitored via fluorescein fluorescence dequenching (λ_{ex} = 485 nm, λ_{em} = 535 nm). (d) Thermal denaturation analysis with and without Nt.CviPII treatment for P1 and ssDNA-1; λ_{abs} = 260 nm. Sample subjected to enzyme for 100 min at 37 °C. ssDNA-1 + complement: T_m = 63.9 °C; ssDNA-1 + Nt.CviPII + complement: T_m = 58.3 °C; P1 + complement: T_m = 58.8 °C; P1 + Nt.CviPII + complement: T_m = 58.3 °C. (e) Thermal denaturation analysis with and without Nt.CviPII treatment for P2 and ssDNA-2; λ_{abs} = 260 nm. Sample subjected to enzyme for 100 min at 37 °C. ssDNA-2 + complement: T_m = 63.9 °C; ssDNA-2 + Nt.CviPII + complement: T_m = 58.3 °C; P2 + complement: T_m = 56.9 °C; P2 + Nt.CviPII + complement: T_m = 55.3 °C. See Figure 3.11 for derivative plots of melting temperatures. Complement: 5’-TATTATATCTTTAGACACTGACTGGACATGACTCT-3’.
solution following nicking ($T_m \sim 37 \, ^\circ C$). Alternatively, we reasoned that perhaps the lack of fluorescence increase for both $P_1$ and $P_2$ could be due to the possibility that a nicked sequence on the particle may not dissociate into solution due to the density of DNA in close proximity to the cleaved product. To rule out these possibilities, we analyzed the $T_m$ of both single-stranded and nanoparticle-based systems following nuclease treatment (Figure 3.10d,e). This analysis confirms that the activity of the endonuclease on the ssDNA–complement duplex is accompanied by a significant decrease in the $T_m$ of the duplex ($\Delta = -26.1 \, ^\circ C$ for ssDNA-1 and ssDNA-2), consistent with complete nicking of the oligonucleotide. By contrast, the $T_m$ of the nanoparticle–complement duplex remains consistent ($\Delta = -0.5 \, ^\circ C$ for $P_1$, $-1.6 \, ^\circ C$ for $P_2$) after nuclease treatment, thus indicating the presence of an intact 20-base oligonucleotide shell on the DPA nanoparticle.

![Figure 3.11](image-url). Derivative plots of thermal denaturation analysis with and without Nt.CviPII treatment for ssDNA-1 and $P_1$ (left), and ssDNA-2 and $P_2$ (right).

We note that unlike previously reported DNA-functionalized gold nanoparticle systems, we do not see an enhanced $T_m$ on the initial DPA nanoparticles, which would be
indicative of cooperative hybridization of complementary DNA. Rather, we observe a slight depression in the $T_m$ (Figure 3.10d,e), an observation consistent with steric hindrance at the interface between DNA and the hydrophobic polymer. This type of effect has been noted by others with respect to unusual DNA hybridization characteristics at interfaces.\textsuperscript{33}

### 3.4 Exonuclease Activity Against DPA Nanoparticles

Given that DPA nanoparticles exhibit a high level of resistance against a sequence-specific nicking endonuclease, we were interested in determining how they would respond as substrates to a nonspecific 3′-exonuclease (Figure 3.12). Exonuclease III (Exo III, from Escherichia coli) is reported to catalyze the stepwise removal of mononucleotides from the 3′-hydroxyl termini of duplex DNA with preferred substrates being blunt or recessed 3′-termini.\textsuperscript{34} However, in our hands, the enzyme exhibits indiscriminate activity on both ssDNA and dsDNA substrates (Figure 3.13).
Figure 3.12. Exonuclease resistance of DPA nanoparticles. (a) Scheme depicting DPA nanoparticle resistance to exonuclease III and, consequently, intact DNA at the particle surface available for hybridization with complementary ssDNA. (b) Scheme depicting ssDNA being degraded by Exo III and, consequently, no intact DNA available for hybridization with complementary ssDNA. (c) Exonuclease III activity over time monitored by fluorescein fluorescence dequenching ($\lambda_{\text{ex}} = 485$ nm, $\lambda_{\text{em}} = 535$ nm). (d) Thermal denaturation analysis with and without Exo III treatment for P1 and ssDNA-1; $\lambda_{\text{abs}} = 260$ nm. Samples subjected to enzyme for 60 min at 37 °C. P1 + complement: $T_m = 58.8$ °C; P1 + Exo III + complement: $T_m = 55.8$ °C. (e) Thermal denaturation analysis with and without Exo III treatment for P2 and ssDNA-2; $\lambda_{\text{abs}} = 260$ nm. Samples subjected to enzyme for 60 min at 37 °C. P2 + complement: $T_m = 56.9$ °C; P2 + Exo III + complement: $T_m = 53.9$ °C. See Figure 3.15 for derivative plots of melting temperatures. Complement: 5′-TATTATATCTTTAGACACTGACTGGACATGACTCT-3′.
**Figure 3.13** Indiscriminate Exo III activity on ss (green) and ds DNA (red, grey, orange) substrates. Concentration refers to the amount of complementary, unlabeled DNA added.

Therefore, we analyzed the activity of Exo III against ssDNA and corresponding DPA nanoparticles in the absence of any additional complementary DNA. Exo III activity against ssDNA and DPA nanoparticles was monitored *via* fluorescein fluorescence dequenching over time. Upon initial observation, P1 appears to be highly resistant to Exo III digestion, while P2 appears to be degraded at a substantially higher rate. Indeed, a detailed kinetic analysis of P2 with respect to Exo III reveals that it is a substrate, albeit a significantly poorer one than ssDNA-2 with a 3-fold difference in the magnitude of the second-order rate constant and a greater than 4-fold difference in initial rates (Figure 3.12c and Table 3.1). However, these kinetic data, derived from fluorescence measurements (see Figure 3.17), reveal only that P2 is indeed a substrate with respect to removal of the first few bases at the 3′-terminus, that is, at the location where fluorescein is liberated and hence detectable. Therefore, we reasoned that the apparent discrepancy between Exo III activity on P1 and P2 (as monitored by fluorescence) is most likely due to the fact that, for P2, the fluorescein-labeled nucleotide is located only one base from the 3′-hydroxyl terminus. Therefore, liberation of the
fluorescent product into solution (i.e., detection of fluorescence) only requires the removal of two bases. In the case of \textbf{P1}, the liberation of a DABCYL-labeled nucleotide does not have the same effect. Here, we conclude that the fluorescein-labeled nucleotide is not liberated into solution but remains in an environment surrounded by DABCYL quencher molecules still present on intact, neighboring DNA strands, as well as neighboring deoxyguanosine bases, which are also known to quench fluorescein fluorescence.\textsuperscript{35} Therefore, the discrepancy between \textbf{P1} and \textbf{P2} response to Exo III is consistent with the nuclease digesting or “shaving” away a limited fraction of 3′-terminal bases.

To confirm observations and conclusions drawn from fluorescence kinetic studies (Table 3.1) and to determine the extent of digestion, a hybridization study via DNA duplex melting analyses was required. Briefly, DPA nanoparticles or ssDNA analogues were allowed to react with Exo III for 1 h before deactivating the enzyme with EDTA and heat. Following enzyme deactivation, an equimolar quantity of complementary DNA was allowed to hybridize to the nanoparticle or ssDNA. Thermal denaturation analysis reveals the absence of a melting transition in the case of both ssDNA strands, indicating complete degradation following enzyme treatment (Figure 3.12d, e, Figure 3.15, and Figure 3.16). By contrast, in the case of enzyme-treated DPA nanoparticles, \textbf{P1} and \textbf{P2}, we observe a sharp melting transition of the particle duplex indicative of an intact DPA nanoparticle. However, we do observe a slight decrease in the particle duplex Tm ($\Delta = -3$ °C in each case, Figure 3.12d, e and Figure 3.15), consistent with fluorescence evidence suggesting that the enzyme digests several bases of the nanoparticle nucleic acid shell at the outer edge and is subsequently sterically hindered, thus preventing complete digestion. Indeed, the data following this partial digestion are consistent with a duplex on the order of approximately 18 base pairs compared to 20 base pairs for the full-length sequence without enzymatic treatment. Encouraged by our results demonstrating DPA nanoparticle resistance to Exo III, we aimed to determine whether
a nuclease routinely used for complete digestion of synthetic oligonucleotides would yield similar results. In addition, we sought to answer whether DPA nanoparticles serve to protect DNA against general exonuclease digestion and not just specifically Exo III digestion. Therefore, we subjected the single-stranded particles and corresponding ssDNA analogues to snake-venom phosphodiesterase (phosphodiesterase I from *Crotalus adamanteus*), an enzyme known for its aggressive 3’-exonuclease activity and routinely utilized for complete digestion of synthetic oligonucleotides (Figure 3.14).

![Figure 3.14](image)

**Figure 3.14.** SVP activity over time monitored by fluorescein fluorescence dequenching $(\lambda_{ex} = 485 \text{ nm}, \lambda_{em} = 535 \text{ nm})$.

Indeed, on the basis of fluorescence dequenching experiments identical to those for Exo III activity analysis, the DPA nanoparticles exhibit exceptional resistance consistent with observations made utilizing Exo III (Figure 3.14). Although the relative initial rates differ between SVP and Exo III depending on substrate, it is clear that the trends in activity are consistent between the two nucleases; that is, **P2** is resistant compared to ssDNA-2, and **P1** is resistant compared to ssDNA-1.
**Figure 3.15.** Derivative plots of thermal denaturation analysis with and without ExoIII treatment for ssDNA-1 and P1 (left), and ssDNA-2 and P2 (right).

**Figure 3.16.** DNA melting temperature analysis with and without ExoIII treatment. For substrate plus Exo III, the experiment was conducted as described in the text. Specifically, the data marked by symbols is the same data presented in Figure 3.12. Here, this data is compared to the absorbance spectra for ssDNA or DPA NP on their own added to the absorbance spectra for the complementary DNA on it’s own (solid lines without symbols). For substrate control melting curves, the substrate (ssDNA or DPA-nanoparticle) was treated in an identical fashion to those in the experiments except no enzyme or complementary DNA was added before conducting melting temperature analysis. This comparison highlights the fact that ssDNA-1 and ssDNA-2, after digestion with Exo III, are unable to form a significant duplex with their complement and therefore exhibit thermal denaturation curves nearly identical to the sum of the melting curves for the ssDNA and complement on their own.
Figure 3.17. Exo III kinetic analysis against ssDNA-2 and P2. a) Exo III activity, shown as fluorescence de-quenching over time for ssDNA-2 and P2, plotted as concentration of liberated fluorescein dT. b) Initial rate data for Exo III vs. ssDNA-2 at varying concentrations of ssDNA-2. c) Initial rate data for Exo III vs. P2 at varying concentrations of P2. d) Lineweaver-Burk plot for Exo III activity on ssDNA-2. e) Lineweaver-Burk plot for Exo III activity on P2. f) Fluorescein dT fluorescence calibration curve showing a linear increase in fluorescence with increasing concentration of the free phosphoramidite (red). Shown in black is an analogous calibration curve for a DPA-nanoparticle identical to P1 but not containing a DABCYL quencher moiety. The drastic difference in slopes for the two fluorescent systems is indicative of the unique environment of the fluorescein molecule on the nanoparticle. g) Bradford assay calibration curve (black) constructed using BSA standard and dye reagent (Bio-Rad #500-0002). Exo III sample is plotted in red, indicative of a concentration of 55 µg/mL.
Table 3.1. Exonuclease III Kinetics on ssDNA-2 and P2 Substrates.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Initial Rate (x 10^9 M/s)</th>
<th>V_{max} (x10^7 M/s)</th>
<th>K_{m} (x 10^{-6} M)</th>
<th>k_{cat}</th>
<th>k_{cat}/K_{m} (x 10^7/M*s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exo III</td>
<td>ssDNA-2</td>
<td>18.6</td>
<td>50</td>
<td>10.6</td>
<td>1.42</td>
<td>1.3</td>
</tr>
<tr>
<td>Exo III</td>
<td>P2</td>
<td>4.4</td>
<td>12.5</td>
<td>7.0</td>
<td>0.36</td>
<td>0.5</td>
</tr>
</tbody>
</table>

3.5 Nanomaterial Morphology Switching via Enzymatic Activation

Self-assembled amphiphiles arrange themselves into various shapes and aggregation states that are dependent on a range of variables including solvent composition, ionic strength, temperature, and volume ratio of each domain in the amphiphile constituent. The tuning of parameters such as heat or solvent composition can be readily achieved by an experimenter. However, such a dramatic change is certain to affect all entities in the system of interest. This may have negative consequences, for example, if one desires to change the shape of a material inside of a cell without affecting the entire cell. Specific manipulation of a material in a given environment requires chemical tools that serve to recognize and process such a material with high fidelity. Enzymes are prime candidates for such tools, as they are experts at carrying out chemical modifications in a highly specific and catalytic fashion. Functioning as nature’s workhorses, enzymes provide inspiration to materials scientists through their elaborate demonstrations of material construction and deconstruction at ambient temperature and pressure.\textsuperscript{38-40} It can therefore be reasoned that enzymes should be capable of manipulating entirely synthetic materials to yield dramatic changes in material properties including nanoscale architecture. Further, these manipulations can be carried out in complex biological environments, thus serving to interface synthetic nanomaterials with living systems en route to the development of novel drug delivery systems and hybrid semi-synthetic cellular systems.
Processing materials using enzymes is not a new concept; indeed it is this very idea that gave rise to molecular cloning.\textsuperscript{41} However, using enzymes to process entirely synthetic nanomaterials is a fairly recent paradigm.\textsuperscript{17,42-53} In this work, we have synthesized DPA nanoparticles that serve as enzyme substrates in efforts to probe how nuclease and DNAzyme processing affects DPA aggregation state and morphology.

The first set of experiments was designed to investigate the effect of DNAzyme processing on DPA nanoparticle architecture. Here DNA polymer conjugates were prepared by conjugation of a 5'-amino modified DNA sequence to an NHS-labeled norbornyl block copolymer prepared via ROMP as depicted in Figure 3.18.

\textbf{Figure 3.18.} Synthesis of DPA nanoparticles via post polymerization conjugation of amino-modified DNA to an NHS-derivatized norbornyl block copolymer backbone.

Resulting DPA nanoparticles were spherical in morphology with diameters of approximately 25 nm. The nanoparticles were designed such that the oligonucleotides constituting the hydrophilic shell served as substrates for a synthetically evolved DNAzyme.\textsuperscript{54} In this
instance, the oligonucleotide contains an RNA base flanked by regions of 7 and 10 bases of DNA on either side. These 7 and 10 base regions are perfectly complementary to the “arms” of the DNAzyme such that the DNAzyme will hybridize to these regions and subsequently catalyze hydrolysis of the phosphate backbone at the RNA site of the oligonucleotide in the shell of the nanoparticle. Such a cleavage event serves to eliminate 10 bases of DNA along with a small oligoethylene glycol (OEG) moiety from the nanoparticle shell. It is hypothesized that removal of multiple DNA-OEG fragments from a single spherical DPA NP destabilizes the structure such that it needs to repack itself to optimize its surface curvature. Indeed, processing of the DPA NPs by the DNAzyme catalyst results in a population wide shift in material morphology from spheres to cylinders over a time period of hours to days as shown in Figures 3.19 and 3.20.

Figure 3.19. Assembly of DNA-brush copolymers into micelles with spherical or cylindrical morphologies. Amphiphile structures are represented as cones for each respective morphology, with the hydrophobic domain highlighted in red. TEM images of a) 25 nm spherical micelles assembled from initial DNA-brush copolymers; b) cylindrical morphology formed following DNAzyme addition to spheres; c) spherical micelles (green) formed after addition of \( \text{In}_1 \) to cylinders.
Figure 3.20. DNA-directed size and phase change over time. Conditions: particles (0.14 gL$^{-1}$), Tris (20 mM, pH 7.4), MgCl$_2$ (50 mM). DNAzyme (5 nM) was mixed with particles at t=0 min. a) t=0 min, b) 2 min, c) 2 h, d) 1 day, e) 2 days. f) Particle size as a function of time following DNAzyme addition. Blue curve: average cylinder length (C$_L$) measured by TEM. Green curve: Dh value of largest aggregates measured by DLS. Red curve: Sphere diameter (S$_D$) measured by TEM. DLS data was taken at the time points shown following DNAzyme addition. Data points for C$_L$ and S$_D$ are averages of multiple measurements made from TEM images, with error bars indicating standard deviations.

Subsequent work has shown that the rate of DNAzyme processing at the surface of DPA NPs is increased with respect to the processing of single-stranded analogues.$^{55}$ Therefore, the hypothesis is that although cleavage of the oligonucleotide shell of each DNA NP is rapid (minutes to hours), the rearrangement of the material from spheres to cylinders is slower and requires days to complete. It is an interesting observation that, in this case, DNAzymes are more active on oligonucleotide substrates arranged at the surface of a nanomaterial as compared to those freely dispersed in solution. On the contrary, as described in sections 3.3 and 3.4, naturally occurring enzymes appear to follow the opposite trend in that their activity is hampered on oligonucleotide substrates arranged at the surface of spherical nanoparticles.$^{56}$

It must be noted that comparing these two studies is not completely rigorous in that the DPA NPs differ in the chemistry of their nucleic acid polymer conjugates. Furthermore, it has been demonstrated that enzyme activity on nanomaterial surfaces is dependent on the enzyme in question. Indeed, some naturally occurring enzymes exhibit enhanced activity at nanomaterials surfaces.$^{57}$
As demonstrated in section 3.4, exonuclease activity (initial rate) at the surface of DPA NPs is retarded approximately four-fold with respect to that of single-stranded analogues. Further, exonuclease III digestion seems to taper off as the enzyme approaches the core of the DPA NP. Despite slow kinetics and incomplete digestion, we hypothesized that exonuclease activity at the periphery of DPA NPs might be enough to destabilize the nanoparticles and induce a morphological shift in a similar fashion to DNAzyme-catalyzed morphological switching as described above. Indeed, after only 30 minutes of incubation, exonuclease III digestion of DPA NP substrates induces aggregation of the spherical nanoparticles into large networks as shown in Figure 3.21.

**Figure 3.21.** Exonuclease III processing of DPA nanoparticles results in a population-wide shift in assembly aggregation state and morphology.
Figure 3.22. Control experiments demonstrating the importance of enzymatic processing for initiating a morphological change in DPA micelles. Each experiment is carried out under identical conditions with the initial and final materials imaged under identical conditions. Treatment in each case, either with or without enzyme or with denatured enzyme, is carried out for 30 minutes at 37 °C.

Importantly, as depicted in Figure 3.22, control experiments at 37 °C indicate that a non-denatured enzyme is necessary to impart this phase transformation. In these results, as well as the results above obtained using DNAzymes, it is theorized that new morphologies resulting from enzymatic manipulation are a result of the aggregation of spherical DPA NPs that have been truncated and destabilized in certain regions. Due to the fact that the glass transition temperature of phenyl norbornyl polymers (ca. 20 monomers in length) is above 100 °C, it is unlikely that physical rearrangement of DPA chains is occurring at room temperature or even at elevated temperatures in water. Indeed, heating at 90 °C for two hours does not induce aggregation or morphology transitions in DPA NPs. This is in contrast to work involving shape-shifting DNA-lipid based materials that are capable of physical re-equilibration even at
At the time of writing, this work is unpublished and a further investigation into nuclease-driven DPA NP phase transformations is underway.

3.6 Conclusion

In summary, we have described a novel approach for rendering DNA resistant to two key classes of nuclease that are otherwise capable of rapidly degrading substrates in a sequence-selective or nonselective fashion. We propose that steric hindrance due to dense packing is the simplest explanation for the observation that the endonuclease has undetectable activity on P1 and P2, whereas 3'-nucleases show some activity but only at the outer few bases. Further, we have demonstrated that enzymatic processing of DPA NPs results in dramatic population wide shifts in material morphology. These manipulations can be programmed to be sequence specific and performed by synthetic enzymes whose activity is enhanced at the surface of DPA NPs or they can be non-sequence specific and performed by general exonucleases whose activity is hampered at the surface of DPA NPs.

Inspiration for these investigations is drawn from the increasing interest in novel approaches for packaging and delivering nucleic acids for in vivo applications. This interest has led to an array of materials designed to facilitate potent and selective communication with important cellular machinery. Our approach is predicated on the idea that a key requirement for any enabling technology of this type is a well-defined nucleic-acid-based material that maintains the integrity of the base sequence in nuclease-rich environments. The utilization of DPA nanoparticles for targeted delivery of intact hybridization competent nucleic acids in vitro and in vivo is currently underway in our laboratories. This approach, together with other well-defined DNA-based nanomaterials, constitutes a concerted effort to move away from amorphous, poorly defined, multi-component, and cytotoxic transfection agents. Finally, we note that this approach is likely
general in terms of particle core chemistry, as other polymerization strategies are amenable to the incorporation of DNA and potentially the preparation of resistant micellar particle systems.\textsuperscript{18,33,71}

3.7 Methods

3.7.1 Instruments and Reagents

All reagents were purchased from commercial sources and used without further purification. (IMesH\textsubscript{2})(C\textsubscript{5}H\textsubscript{5}N\textsubscript{2})(Cl)\textsubscript{2}Ru=CHPh was prepared as described by Sanford \textit{et al.}\textsuperscript{72}

DNA synthesis was carried out on an ABI 394 DNA/RNA synthesizer utilizing standard phosphoramidite chemistry. DNA synthesis reagents and custom phosphoramidites were purchased from Glen Research Corporation. CPG support columns and standard phosphoramidites were purchased from Azco Biotech Inc. Nucleases Nt.CviPII and Exonuclease III were purchased from New England Biolabs. Phosphodiesterase I from \textit{Crotalus adamanteus} was purchased from USB Corporation as a lyophilized powder. All deuterated solvents were purchased from Cambridge Isotope Laboratories Inc. \textsuperscript{1}H (400 MHz) and \textsuperscript{13}C (100 MHz) NMR spectra were recorded on a Varian Mercury Plus spectrometer. Chemical shifts (\textsuperscript{1}H) are reported in \(\delta\) (ppm) relative to the CDCl\textsubscript{3} residual proton peak (7.27 ppm). \textsuperscript{13}C chemical shifts are reported in \(\delta\) (ppm) relative to the CDCl\textsubscript{3} carbon peak (77.00 ppm). Mass spectra were obtained at the UCSD Chemistry and Biochemistry Molecular Mass Spectrometry Facility. Low-resolution mass spectra were obtained using a Thermo LCQdeca mass spectrometer and high-resolution mass spectra were obtained using an Agilent 6230 Accurate Mass time of flight mass spectrometer. Polymer molecular weight and dispersity were determined \textit{via} size-exclusion chromatography (Phenomenex Phenogel 5u 10, 1K-75K, 300 x 7.80 mm in series with a Phenomenex Phenogel 5u 10, 10K-1000K, 300 x 7.80 mm (mobile phase: 0.05 M LiBr in DMF)) using a Hitachi-Elite LaChrom L-2130 pump equipped
with a DAWN HELEOS multi-angle light scattering (MALS) detector (Wyatt Technology) and a refractive index detector (Hitachi L-2490) normalized to a 30,000 g/mol polystyrene standard. Hydrodynamic diameter \( (D_h) \) of DPA nanoparticles was measured via DLS using a DynaPro NanoStar (Wyatt Technology).

DPA nanoparticle molecular weight was determined via batch mode SLS using a DAWN HELEOS MALS detector. Concentrations of oligonucleotides, DPA nanoparticles, and fluorescein phosphoramidite standards were determined using a Thermo Scientific NanoDrop 2000c spectrophotometer. HPLC analysis and purification of oligonucleotides was accomplished utilizing a Phenomenex Clarity 5u Oligo-RP (150 x 4.60 mm) or Clarity 10u Oligo-WAX (150 x 4.60 mm) column and a Hitachi-Elite LaChrom L-2130 pump equipped with a UV-Vis detector (Hitachi-Elite LaChrom L-2420). Oligonucleotide molecular weights were determined by mass spectrometry performed on a Bruker Daltronic Biflex IV MALDI-TOF instrument using a combination of 2’, 4’, 6’- trihydroxyacetophenone monohydrate (THAP) and 3-hydroxypicolinic acid (3-HPA) as matrices and a three-point calibration standard (Oligonucleotide Calibration Standard #206200, Bruker). Denaturing polyacrylamide gel electrophoresis was performed using a Bio Rad Criterion Mini-PROTEAN Tetra cell and precast TBE-Urea gels. Size-exclusion FPLC was accomplished using a HiPrep 26/60 Sephacryl S-200 High Resolution-packed size-exclusion column (mobile phase: 10 mM Tris, 0.5 mM EDTA pH 8.3) and an Akta purifier (Pharmacia Biotech) equipped with a P-900 pump and a UV-900 UV-Vis multi-wavelength detector. TEM samples were deposited on carbon/formvar-coated copper grids (Ted Pella Inc.), stained with 1% w/w uranyl acetate, and imaged using a Technai G2 Sphera operating at an accelerating voltage of 200 kV. Fluorescence data were acquired using a Perkin Elmer HTS 7000 Plus Bio Assay Reader (excitation filter: IB14326, B126002, EX-A, emission filter: IB21654, B126103, EM-A). DNA melting temperature analysis was conducted using a
Beckman Coulter DU 640 spectrophotometer equipped with a high performance temperature controller. Enzyme kinetics were calculated using Lineweaver-Burk plot analysis with enzyme concentrations determined via a standard Bradford assay.

3.7.2 Monomer, Termination Agent, and Polymer Synthesis

*Synthesis of (N-Benzyl)-5-norborne-exo-2,3-dicarboximide (1).* See Figure 3.2 for chemical structure. Compound 1 was prepared according to a modification of a previously reported procedure. To a stirred solution of N-benzylamine (2.85 g, 26.6 mmol) in dry toluene (125 mL) were added 5-norbornene-exo-2,3-dicarboxylic anhydride (4.10 g, 25.0 mmol) and triethylamine (3.83 mL, 27.5 mmol). The reaction was heated to reflux overnight under an atmosphere of N$_2$. The reaction was cooled to room temperature and washed with 10% HCl (3 × 50 mL) and brine (2 × 50 mL). The aqueous layers were combined and extracted with ethyl acetate (60 mL). The combined organic layers were dried with MgSO$_4$, filtered, and concentrated to dryness, yielding a pale yellow solid that was then recrystallized from ethyl acetate/hexanes to give 1 (4.98 g, 79%) as white crystals. $^1$H NMR (CDCl$_3$): δ (ppm) 1.07 (d, 1H, CH$_2$, J = 9.6 Hz), 1.42 (d, 1H, CH$_2$, J = 9.6 Hz), 2.69 (s, 2H, 2 × CH), 3.26 (s, 2H, 2 × CH), 4.61 (s, 2H, CH$_2$), 6.28 (s, 2H, CH═CH), 7.25–7.40 (m, 5H, Ar). $^{13}$C NMR (CDCl$_3$): δ (ppm) 42.18, 42.28, 45.13, 47.62, 127.74, 128.48, 135.76, 137.76, 177.48. LRMS (CI), 253.99 [M + H]$^+$, HRMS, theor: 254.1176 [M + H]$^+$, found: 254.1175 [M + H]$^+$.

*Synthesis of (Z)-4,4′-(But-2-ene-1,4-diylbis(oxy)) Dibenzoic Acid (2).* See Figure 3.2 for chemical structure. To a stirred solution of ethyl 4-hydroxybenzoate (5.5 g, 33.1 mmol) in 100 mL of dry DMF was added potassium carbonate (7.28 g, 52.7 mmol). To this stirred suspension was added cis-1,4-dichlorobutene (2.0 g, 16 mmol). The solution turned brown within minutes, and the reaction was allowed to stir under an atmosphere of N$_2$ at 90 $^\circ$C overnight. The mixture was then cooled to room temperature, filtered, and concentrated to
dryness. The resulting solid was dissolved in ethyl acetate and washed three times with H₂O. The organic layer was dried over magnesium sulfate and concentrated to dryness to yield solid white crystalline needles. This solid was recrystallized from ether to yield the pure diester (2.18 g, 35%). ¹H NMR (CDCl₃): δ (ppm) 1.38 (t, 6H, 2 × CH₃), 4.35 (q, 4H, 2 × CH₂), 4.74 (d, 4H, 2 × CH₂), 5.96 (t, 2H, CH═CH), 6.92 (d, 4H, 4 × ArH), 8.0 (d, 4H, 4 × ArH). The diester (2.18 g, 5.66 mmol) was dissolved in 95% ethanol, and potassium hydroxide was added (12.0 g, 215 mmol). The reaction was heated to reflux for 5 h, cooled to room temperature, diluted with an equal volume of H₂O, and acidified with HCl to form a white precipitate. The precipitate was filtered off to yield 2 as an orange-tan solid (1.78 g, 100%). ¹H NMR (DMSO-d₆, residual 1H = 2.50 ppm): δ (ppm) 3.38 (br s, 2H, 2 × COOH), 4.80 (d, 4H, 2 × CH₂), 5.89 (t, 2H, CH═CH), 7.03 (d, 4H, 4 × ArH), 7.87 (d, 4H, 4 × ArH). ¹³C NMR (DMSO-d₆, residual 13C = 39.51 ppm): δ (ppm) 64.11, 114.50, 123.18, 128.33, 131.34, 161.72, 166.98. LRMS, 327.03 [M – H]⁻, HRMS, theor: 327.0874 [M – H]⁻, obs: 327.0877 [M – H]⁻.

Synthesis of Polymer (1₂₀-2). See Figure 3.2 for chemical structure. Monomer 1 (870 mg, 3.4 mmol) was dissolved in 5 mL of CDCl₃ and cooled to −78 °C. Ruthenium catalyst (IMesH₂(C5H5N)₂(Cl)₂Ru═CHPh (124 mg, 0.17 mmol) was added as a powder, followed by 1 mL additional CDCl₃ to solubilize the catalyst. The reaction was then allowed to warm to room temperature and stir under N₂ for 35 min (NMR confirms the absence of the original olefin resonance from monomer 1 at 6.28 ppm, and the presence of broad cis- and trans-polymer backbone olefin resonances at 5.45 and 5.71 ppm). At this point, 200 µL of the reaction mixture was removed and quenched with an excess of ethyl vinyl ether to provide a homopolymer for molecular weight determination (SEC-MALS: Mₙ = 5221 g/mol, PDI = 1.075, Figure 3.4). Termination agent 2 (111 mg, 0.34 mmol) was dissolved in 2.0 mL of DMF-d₇, added to the reaction mixture, and the mixture was allowed to stir at
room temperature for 20 min. The ruthenium alkylidene proton resonance was monitored in order to track the completion of the polymer termination event (Figure 3.3). Once termination was determined to be complete, excess ethyl vinyl ether was added to quench the ruthenium catalyst. The crude polymer was precipitated from cold methanol and further purified by column chromatography in order to eliminate any traces of unreacted termination agent. The crude precipitated polymer was dry loaded onto a silica column, the column was washed with 200 mL of CH₂Cl₂, and the polymer was eluted with 3% methanol in CH₂Cl₂ to yield a glassy yellow-brown solid as the pure polymer (905 mg, 97%, Rₚ = 0.56).

3.7.3 DNA Synthesis

Oligonucleotides ssDNA-1 and ssDNA-2 were synthesized in-house using automated phosphoramidite chemistry and saccharin 1-methylimidazole as an activator. Standard 2'-cyanoethyl protected phosphoramidites include (N-Bz) dA, (N-dmf) dG, (N-acetyl) dC, and T. Oligonucleotides were synthesized on a 1.0 µmol scale using columns packed with 1000 Å CPG beads. A 5’-amino modifier was incorporated into each synthetic oligonucleotide through use of 5’-amino modifier C12 phosphoramidite (Glen Research). In the case of ssDNA-1 and ssDNA-2, the 5’-amino terminus was acetylated on solid support using the automated synthesizer. The MMT group was removed by treatment with 3% trichloroacetic acid in CH₂Cl₂ for 2 min (until the yellow color due to the MMT⁺ cation was no longer visible in the eluting deblock solution) followed by a standard capping cycle to acylate the free amine with acetic anhydride. Fluorescein and DABCYL labels were incorporated into the oligonucleotides via use of fluorescein dT and DABCYL dT phosphoramidites (Glen Research). Oligos were cleaved from solid support and deprotected by treatment with AMA (concentrated NH₄OH/40% methylamine, 1:1 v/v) at 55 °C for 20 min, purified by HPLC, and characterized by MALDI-TOF MS. Complement DNA was purchased from Integrated
DNA Technologies (purified by HPLC, confirmed by ESI-MS). Detailed sequences and enzyme recognition/cleavage sites are shown in Figure 3.6.

3.7.4 HPLC Purification of Oligonucleotides

See Figure 3.7 for corresponding chromatograms. Synthetic oligonucleotides ssDNA-1 and ssDNA-2 were purified via reverse-phase HPLC using a binary gradient as indicated on each chromatogram in Figure 3.7 (solvent A: 10% methanol in 50 mM triethylammonium acetate (TEAA) pH 7.1; solvent B: methanol). For ssDNA-2, weak anion exchange (WAX) HPLC was also necessary to purify the oligonucleotide. A quaternary gradient was used for WAX HPLC analyses and purification (solvent A: nanopure H2O; solvent B: methanol; solvent C: 100 mM tris(hydroxymethyl)aminomethane (Tris) pH 8.0; solvent D: 2 M NaCl). Oligonucleotides were desalted post-WAX HPLC purification using Sep-Pak Plus C18 environmental cartridges.

3.7.5 MALDI-TOF MS of Oligonucleotides

See Figure 3.7 for corresponding spectra. A MALDI target plate was spotted with 1 µL of matrix solution A for each sample to be analyzed and allowed 20 min to dry completely (matrix A was prepared as follows: dissolve 50 mg of 3-Hydroxypicolinic acid in 500 µL of HPLC grade acetonitrile/nanopure water 1:1 v/v and mix 454 µL this solution with 45 µL of 100 mg/mL diamonium hydrogen citrate in nanopure water). Oligonucleotide samples were prepared for MALDI-TOF MS analysis using Zip-Tip C18 pipette tips. Oligos were loaded onto the C18 tips from concentrated stock solutions (ca. 50–100 µM) and eluted with matrix solution B (matrix solution B was prepared as follows: dissolve 50 mg of 2’, 4’, 6’ Trihydroxyacetophenone monohydrate (THAP) in 500 µL of HPLC grade acetonitrile, assist dissolution by sonication, and centrifuge the resulting solution to pellet any solid remaining, mix 250 µL of the supernatant with 250 µL of 23 mg/mL diamonium hydrogen citrate in
nanopure H2O). One microliter of the oligonucleotide in matrix solution B was mixed with 1 µL of oligonucleotide calibration standard (Bruker) dissolved in nanopure H2O. One µL of this solution was then spotted onto the MALDI plate on top of crystallized matrix A. The samples were allowed to dry for 15–30 min before analyzing via MALDI-TOF MS.

### 3.7.6 DNA–Polymer Amphiphile Synthesis and Micellar Nanoparticle Formation

To a solution of polymer (150 mg, 27.8 µmol) dissolved in 250 µL of DMF were added N,N-diisopropylethylamine (48 µL, 280 µmol) and HATU (10.6 mg, 28 µmol). The solution was vortexed for 10 min at room temperature in order to activate the polymer carboxylic acid terminus. At this point, 5′-amino-modified DNA on CPG solid support (ca. 1 µmol, MMT deprotected) was added. The mixture was allowed to vortex at room temperature overnight. The CPG beads were filtered away from the solution using an empty synthesis column and then washed with DMF (2 × 20 mL) and CHCl3 (2 × 20 mL). The DNA–polymer amphiphile (DPA) was cleaved from solid support via treatment with AMA at 65 °C for 30 min. The CPG beads were filtered off using glass wool and subsequently washed consecutively with H2O (2.0 mL), DMSO (2.0 mL), formamide (2.0 mL), H2O (3.0 mL), and DMSO (1.0 mL). To form micellar nanoparticles, this solution of DPA was transferred to 3500 MWCO snakeskin dialysis tubing (Thermo Scientific), and 2.0 mL of H2O, used to wash the filtrate container, was added. The resulting solution was dialyzed against 2.0 L of nanopure H2O overnight. This dialyzed solution was then concentrated to 3.0 mL via speed vac evaporation. The resulting crude DPA nanoparticle/ssDNA mixture was analyzed by denaturing PAGE and agarose gel electrophoresis to confirm the presence of DPA conjugates and free ssDNA. It is important to note that low molecular weight ssDNA impurities (≤ 8295 g/mol) remained present despite extensive dialysis attempts (20k MWCO Slide-a-lyzer dialysis cassette). Therefore, the crude mixture was purified via SEC FPLC (mobile phase: 10
mM Tris, 0.5 mM EDTA pH 8.3, flow rate = 2 mL/min, $\lambda_{abs} = 260$ nm). The DPA nanoparticles (P1/P2) elute at ca. 50 min (Figures 3.1 and 3.8). Crude P1/P2 samples were purified using HiLoad 16/60 Superdex 200 prep grade SEC media and exhibit a retention time differing from that of pure P1/P2 as subsequent purifications and reinjections of pure material were performed using HiPrep 26/60 Sephacryl S-200 high-resolution SEC media.

3.7.7 Gel Electrophoresis

Denaturing PAGE was accomplished using Bio-Rad Criterion 15% TBE-urea precast gels (#345-0091) and loading 200 ng of DNA per lane for each sample to be analyzed. In the case of crude conjugate, 400 ng of DNA was loaded per lane. Samples were prepared to load by mixing 1:1 (v/v) with TBE-urea sample buffer (#161-0768, Bio-Rad) and heating to 90 °C for 2 min followed by rapid cooling on ice. The gels were run in 1× Tris/boric acid/EDTA (TBE) buffer pH 8.4 at 200 V for 70 min, stained with ethidi bromide (200 ng/L) for 30 min, and visualized using a Bio-Rad Fluor-S MultImager.

3.7.8 DNA Concentration Determination

Nucleic acid concentrations were determined by UV absorbance at 260 nm using a quartz cuvette (Fisher #14-385-928A, path length = 10 mm). An extinction coefficient of 294 554.58 L/mol·cm was used for ssDNA-1, ssDNA-2, P1, and P2. This coefficient was calculated as the extinction coefficient of the entire sequence without the two thymine-modified bases (226 654.58 L/mol·cm, OligoCalc) plus the extinction coefficients for each dye-labeled base at 260 nm (38 800 L/mol·cm for fluorescein dT and 29 100 L/mol·cm for DABCYL dT, Glen Research). Due to the fact that P1 and P2 contain additional aromatic groups capable of absorbing UV radiation, a slight correction factor was introduced. This correction factor was calculated as the ratio of absorbance of ssDNA-1 or ssDNA-2 at 492 nm versus 260 nm ($A_{260}/A_{492}$). This correction factor was multiplied by P1 or P2 absorbance
at 492 nm in order to calculate what the absorbance at 260 nm would be if the system behaved as the standard ssDNA analogues. This corrected absorbance at 260 nm was then averaged with the actual DPA nanoparticle absorbance at 260 nm and used to determine nucleic acid concentration. For example, for P1, \( A_{260} = 0.168 \) (0.57 \( \mu \)M) and \( A_{260} \) corrected = 0.130 (0.44 \( \mu \)M). Therefore, \( A_{260} \) average = 0.149 (0.50 \( \mu \)M).

### 3.7.9 Transmission Electron Microscopy

Copper grids (Formvar/carbon-coated, 400 mesh copper, Ted Pella #01754) were prepared by glow discharging the surface at 20 mA for 1.5 min followed by treatment with 3.5 \( \mu \)L of 250 mM MgCl\(_2\) in order to prepare the surface for DPA nanoparticle adhesion. The MgCl\(_2\) solution was wicked away with filter paper, and 3.5 \( \mu \)L of DPA nanoparticle (ca. 50 \( \mu \)M DNA in 10 mM Tris pH 8.5) solution was deposited on the grid surface. This solution was allowed to sit for 5 min before being washed away with 4 drops of glass distilled H\(_2\)O and subsequent staining with 3 drops of 1% w/w uranyl acetate. The stain was allowed to sit for 30 s before wicking away with filter paper. All grid treatments and sample depositions were on the dark/shiny/glossy Formvar-coated face of the grid (this side face up during glow discharge). Samples were then imaged via TEM.

### 3.7.10 Fluorescence Measurements

Each experiment was measured in triplicate and plotted as a normalized average (i.e., time point zero was set to zero fluorescence) with standard deviation plotted as error bars. Sigmoidal fits were performed for each data set. Fluorescein fluorescence dequenching was monitored over time using a Perkin Elmer HTS 7000 PLUS Bio Assay Reader and a 96-well plate reader and a 96-well plate (Corning, flat bottom nonbinding surface #29110009). Time points were collected in 15 s intervals, integrating three flashes per measurement. Identical gain and filter settings were used in every case. For measuring Nt.CviPII activity, the
following conditions were used in each experiment: 5 µM ssDNA or DPA nanoparticle, 300 nM ssDNA complement, 25 mM NaCl, 1× NE Buffer 4 (New England Biolabs), 10 mM Tris pH 8.5, and 5 units of Nt.CviPII (New England Biolabs, 100 units in 20 µL was diluted to 100 µL with 80 µL of diluent A, 5 µL of this solution was used per reaction) all in 50 µL total volume. NE Buffer 4 and enzyme were mixed and added to each well. All other components were mixed and added to each enzyme/buffer-containing well simultaneously using a multichannel pipettor. The plate reader was set to 37 °C for the duration of the 100 min experiment. For measuring Exo III activity, the following conditions were used in each experiment: 5 µM ssDNA or DPA nanoparticle, 25 mM NaCl, 50 mM potassium acetate, 1× NE Buffer 1 (New England Biolabs), 10 mM Tris pH 8.5, and 10 units of Exo III (New England Biolabs, 5000 units in 50 µL was diluted to 500 µL with 450 µL of diluent A, 1 µL of this solution was used per reaction) all in 50 µL total volume. NE Buffer 1 and enzyme were mixed and added to each well. All other components were mixed and added to each enzyme/buffer-containing well simultaneously using a multichannel pipettor. The plate reader was set to 37 °C for the duration of the 60 min experiment. For measuring SVP activity, the following conditions were used in each experiment: 5 µM ssDNA or DPA nanoparticle, 25 mM NaCl, 50 mM potassium acetate, 12.5 mM MgCl₂, 10 mM Tris pH 8.5, and 0.6 units of SVP (1.58 mg of lyophilized SVP powder (63 units/mg) was dissolved in 1.58 mL of buffer containing 100 mM Tris-HCl, pH 8.9, 110 mM NaCl, 15 mM MgCl₂, and 50% glycerol, 10 µL of this solution was used per reaction) all in 50 µL total volume. MgCl₂ and enzyme were mixed and added to each well. All other components were mixed and added to each enzyme/buffer-containing well simultaneously using a multichannel pipettor. The plate reader was set to 37 °C for the duration of the 60 min experiment.
3.7.11 DNA Melting Temperature Analysis

Melting temperature analyses were performed by heating each sample from 25 °C (5 min equilibration time) to 70 °C using a temperature gradient of 1 °C/min. Tm's were calculated as first derivatives of the curve. Each strand was at a concentration of 0.83 µM. For melting analysis after Nt.CviPII treatment, the reaction mixture was heated to 70 °C for 20 min in order to denature the enzyme. The mixture was then cooled to room temperature, and 228.1 µL of 10 mM Tris pH 8.5 was added, followed by 12.5 µL of 2 M NaCl and 9.4 µL of 24.8 µM complementary DNA in 10 mM Tris pH 8.5. Final concentrations of each strand are 0.83 µM, and final NaCl concentration is 87.5 mM all in a total volume of 300 µL. At this point, the sample was heated at 90 °C for 5 min and then allowed to cool to room temperature over a period of 2 h. The sample was refrigerated at 8 °C for 15 min and subsequently analyzed. For Tm analysis after Exo III treatment, after the reaction was complete (60 min), 10 µL of 0.5 M EDTA was added to inhibit the enzyme. The reaction was heated at 70 °C for 20 min and allowed to cool to room temperature. Then, 217.5 µL of 10 mM Tris followed by 12.5 µL of 2 M NaCl and 10 µL of 24.8 µM complementary DNA was added. At this point, the sample was treated identical to those in the case of Nt.CviPII.

3.7.12 Morphology Switching of DPA Micelles (Figures 3.18-3.20)

Synthesis of copolymer (138-b-218) for conjugation. To a stirred solution of 1 (0.400 g, 1.58 mmol) in dry CH2Cl2 (2 mL) cooled to −78°C was added a solution of the catalyst ((IMesH2)(C5H5N)2Cl)2Ru=CHPh) (41.0 mg, 0.00564 mmol) in dry CH2Cl2 (1 mL) also cooled to −78 °C. After 5 min the cold bath was removed and the reaction was left to stir under nitrogen while warming to room temperature. After 40 min a 0.25 mL aliquot was removed and quenched with ethyl vinyl ether. After 25 min the polymer was precipitated by addition to cold MeOH to give the homopolymer as an off white solid. To the remaining
reaction mixture a solution of 2 (0.164 g, 0.517 mmol), in dry CH₂Cl₂ (1 mL) was added. The mixture was left to stir under N₂ for 40 min followed by quenching with ethyl vinyl ether (0.1 ml). After 25 min the solution was concentrated to approx. 1/3 the original volume then precipitated by addition to cold MeOH to give the copolymer as an off white solid. ¹H NMR of the polymer confirms the absence of monomer (no olefin peak at 6.30 ppm) and the presence of broad trans and cis olefin peaks of the polymer backbone at 5.73 and 5.50 ppm respectively. SEC-MALS: homopolymer 1: Mₘ = 9814, Mₘ/Mₙ = 1.019, degree of polymerization for 1 = 38. Copolymer of 1-b-2: Mₘ = 15380, Mₘ/Mₙ = 1.023, degree of polymerization for 2 = 18.

**Figure 3.23.** SEC-MALS traces of homopolymer 1ₚ₈ (blue) (Mₘ = 9814) and copolymer 1ₚ₈-b-2₁₈ (red) (Mₘ = 15,380) (0.5 mL/min CHCl₃).

**DNA conjugation.** 0.05 µmol of copolymer was dissolved in 0.25 mL of chloroform, followed by addition of 1 equivalent of N,N-Diisopropylethylamine (DIPEA) and 0.2 µmol of HBTU to which 0.1 mL of acetonitrile was added. Freshly synthesized CPG-support-bound-DNA was removed from the capsule and placed in a 1.5 mL eppendorf tube to which was added the copolymer mixture described above. This reaction was shaken at room temperature
overnight, deprotected in ammonium hydroxide/ethanol (3:1 ratio) for 4 hrs, and volume reduced in vacuo to approx. 50 µL.

**Figure 3.24.** SEC-MALS traces of copolymer 1\textsubscript{37}-b-2\textsubscript{22} (red) (M\textsubscript{w} = 15380) and DNA-copolymer 1\textsubscript{38}-b-(COOH-DNA)\textsubscript{18} (black) (1.0 mL/min DMF).

**Spherical micelle formation.** To the DNA-brush copolymer in 50 µL of water was added 1 mL of Tris buffered water (20 mM, pH 7.4). This solution was then transferred to a 10,000 MWCO dialysis tubing. Buffer was changed three times, one time per day.
**Phase Transition Studies.** Spherical micelles at 1 µM with DNAzyme (5 nM) were used in studies of phase transitions. The experiments were run in Tris/MgCl₂ (20 mM, pH 7.4, MgCl₂, 50 mM) solutions at room temperature. TEM grids were prepared at time points as indicated in Figure 3.20. 4 µL of sample was spotted on the grid for 1 min, and washed with water (8 drops), then stained with 1 % uranyl acetate solution in water (3 drops) then wicked away with filter paper. DLS samples were also taken at the same time points, from
the same solutions. Cylinder lengths (C\textsubscript{L}) were determined as the average of multiple TEM images at given time points.

### 3.8 Acknowledgements

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### 3.9 References


Chapter 4

Intracellular mRNA Regulation with Self-Assembled Locked Nucleic Acid-Polymer Nanoparticles

4.1 Introduction

Modulation of intracellular RNA abundance provides an exceptional opportunity to study and influence gene function and cellular behavior. In order to systematically exploit this opportunity, the delivery of nucleic acids to relevant biological compartments has been extensively investigated for the past 50 years.\textsuperscript{1,2} Despite exhaustive efforts, nucleic acid therapies have realized limited success in the clinics. This shortcoming is largely due to insufficient biostability of naked nucleic acids, off-target effects of modified nucleic acids, and ultimately the inability to deliver naked nucleic acids across phospholipid membranes.\textsuperscript{3,4} The success of nucleic acid-based therapies relies on the ability to rationally design well-defined and stable materials capable of overcoming these barriers. Multi-component, vector-facilitated nucleic acid delivery has emerged as a powerful tool in the past decade due to convenience, effectiveness, and the ability to adapt materials for \textit{in vivo} experimentation.\textsuperscript{5-7} However, progress in multi-component nucleic acid delivery using viral vectors, lipoplex formulations, or cationic transfection agents has been hindered by numerous setbacks including toxicity, immunogenicity, DNA release, and nucleic acid instability.\textsuperscript{8,9} More recently, single-component nucleic acid based materials have been
developed as well-defined alternatives to multi-component DNA delivery systems.\textsuperscript{10-12} Spherical nucleic acids (SNAs)\textsuperscript{13} represent a unique class of stable\textsuperscript{14} DNA delivery vehicles that display nucleic acids at the surface of the nanomaterial, hence eliminating the need to release nucleic acids from a condensed or sequestered state. Materials capable of regulating mRNA abundance \textit{in cellulo} without the need for the incorporation of a cellular internalizing component have only been demonstrated using metal-templated SNAs.\textsuperscript{15,16} Despite the success of gold-core SNAs, the requirement for metal templation imposes certain constraints and limitations on the resulting SNAs including oligo attachment chemistry, chemical diversity of the core itself, and maximum nucleic acid density achievable in the nanoparticle shell.\textsuperscript{17} Furthermore, in order to avoid toxicity associated with gold nanoparticle accumulation,\textsuperscript{18,19} the template must be chemically dissolved once the material has been synthesized.\textsuperscript{20} In the interest of developing multifunctional and nontoxic oligonucleotide delivery agents with novel properties, it is necessary to develop new strategies toward accessing and expanding upon this unique class of materials. In this work we demonstrate that efficient cellular uptake and potent mRNA regulation can be achieved with a new class of spherical nucleic acid, namely LNA-polymer amphiphile (LPA) nanoparticles.

\section*{4.2 LPA Nanoparticle Preparation}

LPA nanoparticles are discrete assemblies of a well-defined polymer-LNA conjugate prepared \textit{via} solid-phase coupling of a carboxylic acid terminated norbornyl polymer with an amine-modified LNA oligonucleotide on controlled pore glass (CPG) beads. After conjugation, LPAs are cleaved from the solid support with an aqueous base to yield well-defined spherical polymeric micellar nanoparticles (Figure 4.1) LPA nanoparticle formation
in aqueous solution is driven by the hydrophobic effect,\textsuperscript{21} hence the micelles are composed of a hydrophobic polynorbornyl core with each polymer covalently bound through an amide linkage to one solvated hydrophilic oligonucleotide in the shell. This chemistry is important in that it drives the dense packing of negatively charged, self-repulsive nucleic acids in the micelle corona via energetically favorable solvent exclusion governed by the hydrophobic

Figure 4.1. LPA composition and characterization by electron microscopy. LPA nanoparticles as they are released from solid support and dispersed into aqueous solution. The resulting nanoparticles are roughly 20 nm in diameter as evidenced by negative stain TEM and DLS. LPA nanoparticles consist of a hydrophobic polynorbornyl core and a fluorescently labeled hydrophilic LNA shell designed to be complementary (antisense) or non complementary (nonsense) to a 20-base region of mRNA responsible for synthesizing the protein survivin. LNA bases are indicated in orange, “Pol” indicates the norbornene polymer conjugated to the 5′ end of the LNA sequence, F and FdT represent fluorescein-modified thymidine, and Cy5 represents an incorporated cyanine 5 phosphoramidite.
polymer core. It is noteworthy to mention that nucleic acid density achieved in the nanoparticle shell is exceptionally high as evidenced in previous work from our laboratory demonstrating that analogous DNA-based nanoparticles can render DNA resistant to degradation by both endo- and exonucleases.\textsuperscript{22} LPA nanoparticles average 20 nm in diameter as evidenced by transmission electron microscopy (TEM) and dynamic light scattering (DLS; see Figures 4.1-4.4). These materials are the first example of a non-templated and purely organic single-component nanoparticle to demonstrate efficient cellular uptake and subsequent mRNA regulation \textit{via} antisense activity in live cells.

To examine the efficiency of LPA nanoparticle cellular uptake and subsequent interaction with intracellular mRNA, three different LPA nanoparticles were designed and synthesized (Figures 4.1 and 4.16). The first micelle, termed antisense fluorescein-labeled LPA (\textbf{AS-FL-LPA}) nanoparticle, contains a fluorescently labeled LNA sequence complementary to a 20-base region located in the second exon of survivin mRNA in HeLa cells. As a control, a second micelle was synthesized, termed nonsense fluorescein-labeled LPA (\textbf{NS-FL-LPA}) nanoparticle, in which the nucleotide sequence was scrambled. We anticipated that comparison of the activity of these two distinct materials would facilitate determination of the sequence-specific nature of LPA nanoparticle mediated survivin mRNA regulation. A third micelle, termed antisense cyanine 5-labeled LPA (\textbf{AS-CY5-LPA}) nanoparticle, was designed to interrogate the influence of the incorporated dye on LPA nanoparticle uptake in HeLa cells.\textsuperscript{23-25}
Figure 4.2. Antisense fluorescein (AS FL) LPA nanoparticle characterization. Negative stain (1% w/w uranyl acetate) TEM micrographs are at 29,000x (A), 80,000x (B), and 150,000x (C) magnification. FPLC (D) was carried out using 50 mM Tris pH 8.5 at a flow rate of 1.8 mL/min while monitoring absorbance at 260 nm. DLS data is reported as the reweighted intensity by mass % (E), the raw intensity % (F), and the correlation function (G).
Figure 4.3. Antisense CY5 (AS CY5) LPA nanoparticle characterization. Negative stain (1% w/w uranyl acetate) TEM micrographs are at 29,000x (A), 80,000x (B), and 150,000x (C) magnification. FPLC (D) was carried out using 50 mM Tris pH 8.5 at a flow rate of 1.8 mL/min while monitoring absorbance at 260 nm. DLS data is reported as the reweighted intensity by mass % (E), the raw intensity % (F), and the correlation function (G).
Figure 4.4. Nonsense fluorescein (NS FL) LPA nanoparticle characterization. Negative stain (1% w/w uranyl acetate) TEM micrographs are at 29,000x (A), 80,000x (B), and 150,000x (C) magnification. FPLC (D) was carried out using 50 mM Tris pH 8.5 at a flow rate of 1.8 mL/min while monitoring absorbance at 260 nm. DLS data is reported as the reweighted intensity by mass % (E), the raw intensity % (F), and the correlation function (G).
4.3 Cellular Internalization of LPA Nanoparticles

As an initial experiment, AS-FL-LPA nanoparticles and the corresponding naked single-stranded fluorescein-labeled LNA sequence were incubated with HeLa cells to investigate the extent of uptake of each species measured by fluorescence-activated cell sorting (FACS, Figure 4.5). After incubation with 5 nM AS-FL-LPA nanoparticle or 1 µM (the equivalent concentration with respect to LNA and fluorescein dye) naked fluorescein-labeled ssLNA analogue for 2 h, FACS analysis reveals an approximately 10-fold increase in population-wide fluorescence at 533 nm for those cells treated with LPA nanoparticles as compared to those treated with the ssLNA analogue (Figures 4.5A and 4.19). Uptake for Cy5-labeled LPA nanoparticles shows a similar trend (Figures 4.5B and 4.20). However, in contrast to fluorescein-labeled ssLNA, there is observable association of the naked Cy5-labeled ssLNA analogue with HeLa cells. As the LNA nucleobase sequence is identical to that of the fluorescein-labeled ssLNA, this effect may be a result of cyanine 5 incorporation, as certain dye molecules are known to associate with cell membranes. Nevertheless, HeLa cells incubated with 5 nM AS-CY5-LPA nanoparticles demonstrate a ca. 10-fold increase in fluorescence per cell at 675 nm as compared to those cells treated with the Cy5-labeled ssLNA analogue. These results underscore the importance of the three-dimensional arrangement of oligonucleotides in facilitating cellular association. Indeed, it has been recently demonstrated that other varieties of nanostructured DNA-based materials undergo cellular uptake more efficiently than single-stranded analogues.
Figure 4.5. Uptake of dye-labeled LPA nanoparticles in HeLa cells. (A) FACS distributions showing the intensity of fluorescence among HeLa cells treated with the antisense fluorescein-labeled LNA-polymer amphiphile (AS-FL-LPA) after a 2 h incubation in the presence of nanoparticles. Data are gated on 2500 total events, $\lambda_{ex} = 488$ nm, $\lambda_{em} = 533 \pm 15$ nm; see Figure 4.19 for details. (B) FACS data showing fluorescent cell population due to antisense Cy5-labeled (AS-CY5-LPA) micelle uptake in HeLa cells after incubation for 2 h. Data are gated on 2500 total events, $\lambda_{ex} = 640$ nm, $\lambda_{em} = 675 \pm 12.5$ nm; see Figure 4.20 for details. (C–E) Confocal fluorescence microscopy of AS-FL-LPA-treated HeLa cells (C, $\lambda_{ex} = 488$ nm), untreated HeLa cells (D, $\lambda_{ex} = 488$ nm, 635 nm), and antisense Cy5 LPA nanoparticle-treated HeLa cells (E, $\lambda_{ex} = 635$ nm) showing material uptake and distribution in a single z-slice. Time points are indicated on each panel; scale bars are 50 µm. See Figures 4.21 and 4.22 for z-stack images.
Figure 4.6. Confocal fluorescence microscopy Z-stack imaging for fixed HeLa cells incubated with CY5-AS-LPA micelles for 3.5 hours ($\lambda_{ex} = 635$ nm, colored red). Nuclei are stained with NucBlue ($\lambda_{ex} = 405$ nm, colored blue). Z-spacing is 0.5 $\mu$m.
Having established that LPA nanoparticles associate with HeLa cells by FACS, we performed live-cell z-stack confocal fluorescence microscopy to determine the extent of nanoparticle internalization as well as intracellular distribution (Figures 4.5C-E, 4.6, 4.21, and 4.22). Live HeLa cells were imaged after LPA nanoparticle incubation in order to discern the distribution of LPA nanoparticles relative to cellular boundaries without the introduction of artifacts associated with cell fixation. Based on confocal fluorescence images it is apparent that LPA nanoparticles are distributed diffusely throughout the cell body in some cells (ca. 70% of cells, as determined by visual inspection of the imaged area) after 10 min of incubation and in almost all imaged cells (ca. 98% of cells, as determined by visual inspection of the imaged area) after 2 h of incubation with LPA nanoparticles. This, along with FACS data, suggests that micelles are rapidly taken up into HeLa cells and effectively distributed in the cytosol.

Figure 4.7. FACS data showing HEK-293 uptake of antisense fluorescein-labeled LPA nanoparticles after 10 minute and 2 hour incubations. Histograms were constructed from gated and ungated data on a total of 5000 event counts and the x-axis is plotted as FL-1 Area ($\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 533 \pm 15 \text{ nm}$).
4.4 RNA Regulation with LPA Nanoparticles

RNA plays a central role in regulating and propagating genetic information; hence numerous efforts have demonstrated the utility of manipulating the expression of disease-causing genes via interference with various RNAs. To determine if LPA nanoparticles are able to modulate intracellular mRNA levels, we designed nanoparticles capable of base-pairing in an antisense fashion specifically with a 20-base region of survivin mRNA, an RNA associated with proliferation of HeLa and other cancerous cells.

![Figure 4.8. LPA nanoparticle uptake and survivin mRNA depletion.](image)

(A) FACS data showing similar uptake for both antisense and nonsense LPA nanoparticles after incubation with HeLa cells for 2 h. $\lambda_{ex} = 488$ nm, $\lambda_{em} = 533 \pm 15$ nm; see Figure 4.18 for more details. (B) RT-PCR results showing sequence-selective survivin mRNA knockdown due to treatment with antisense LPA nanoparticles (AS-FL-LPA). See Tables 4.1 and 4.6 for more information.
Table 4.1. RT-qPCR statistical analysis for antisense versus nonsense LPA micelle treatments.

<table>
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<th>antisense</th>
<th>untreated</th>
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<td>0.0164</td>
<td>0.0652</td>
</tr>
<tr>
<td>SD</td>
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<td>0.0201</td>
</tr>
<tr>
<td>SEM</td>
<td>0.0027</td>
<td>0.0082</td>
</tr>
<tr>
<td>N</td>
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<td>6</td>
</tr>
<tr>
<td>two-tailed P value</td>
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<td></td>
</tr>
<tr>
<td>conclusion</td>
<td>very statistically significant</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>nonsense</th>
<th>untreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.0572</td>
<td>0.0652</td>
</tr>
<tr>
<td>SD</td>
<td>0.0037</td>
<td>0.0201</td>
</tr>
<tr>
<td>SEM</td>
<td>0.0021</td>
<td>0.0082</td>
</tr>
<tr>
<td>N</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>two-tailed P value</td>
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<td></td>
</tr>
<tr>
<td>conclusion</td>
<td>not statistically significant</td>
<td></td>
</tr>
</tbody>
</table>

Here, we have used an LNA-DNA chimera that encourages strong hybridization between the LPA NP and target mRNA and also provides a region of unmodified DNA-RNA duplex to facilitate RNase H mediated degradation of target RNA. After treatment with antisense (AS-FL-LPA) or nonsense (NS-FL-LPA) micelles, total HeLa RNA was harvested and analyzed for relative abundance of survivin mRNA (Figure 4.8). Treatment with antisense LPA nanoparticles significantly depleted survivin mRNA levels relative to endogenous GAPDH mRNA transcripts, suggesting efficient, sequence-specific regulation of mRNA levels (Figure 4.8B). Treatment with nonsense LPA nanoparticles showed no significant effect on survivin mRNA levels when compared to levels in untreated cells. To our knowledge, other than gold-templated SNAs, there has been only one other example in the literature of a DNA-based nanomaterial demonstrating gene-specific interactions without the need for an auxiliary uptake-enhancing component.

Note that other antisense ODN-polymer conjugates have been developed that rely on nucleic acid complexation with a secondary cationic delivery-enhancing reagents in order to be effective. Delivery of antisense DNA-PEG conjugates with DNA sequestered in the
material core by PEI complexation demonstrates anti-proliferative effects on tumor cell growth in cell culture and also tumor growth suppression. In this example, there is no analysis of mRNA levels post-antisense treatment. Another study using the same materials demonstrates anti-tumor proliferation activity in mice models. Indeed, these materials in can be labeled with folic acid moieties to improve tumor targeting. Delivery of antisense DNA-PEG complexes with the fusogenic KALA peptide shows anti-proliferative activity in cell culture. Again, in this instance, no analysis of mRNA levels after antisense treatment is conducted. Antisense ODN-PEG conjugates complexed with branched-PEI have been shown to enhance antisense activity compared to ODN-PEG conjugates alone. In this study, the incorporation of an S-S linkage between the ODN and PEG also increased antisense activity. Additionally, two other publications prepare conjugates as “antisense agents” but neither not show any antisense activity. Rather, these publications suggest that the materials could be used as antisense agents or have only demonstrated antisense-like behavior via in vitro RNA hybridization studies.

4.5 Uptake Mechanism, Uptake Kinetics, and Cytotoxicity of LPA Nanoparticles

Given that cellular uptake and internalization of LPA nanoparticles appears to be rapid and efficient, we investigated the kinetics, cytotoxicity, and potential mechanism for LPA nanoparticle uptake (Figure 4.9). Previous reports concerning spherical nucleic acids indicate rapid cellular internalization that is dependent on Type A scavenger receptors and cholesterol-dependent caveolae-mediated endocytosis. LPA nanoparticles exhibit rapid uptake within 10 min of introduction to adherent cells across five different cell lines including human embryonic kidney cells (Figures 4.9A, 4.7, 4.23, 4.24, and Table 4.3). LPA
nanoparticle association with the cell appears to reach a maximum between 30 and 60 min after incubation for each of the four cancerous cell lines studied. Furthermore, LPA nanoparticle uptake in HeLa cells appears to be dependent on cholesterol, as treatment with methyl-β-cyclodextrin significantly decreases association of LPA nanoparticles (Figure 4.9B). In our hands, treatment with other pharmacological inhibitors or disruptors of the aforementioned endocytotic pathways did not have a significant effect on cellular association in HeLa cells (Figures 4.10 and 4.11 and Tables 4.2 and 4.5).

**Figure 4.9.** AS-FL-LPA Nanoparticle uptake kinetics, dependence on cholesterol, and cytotoxicity. (A) Compiled FACS data showing LPA nanoparticle uptake over time in various cell lines. Data are gated on a minimum of 2500 events, $\lambda_{ex} = 488$ nm, $\lambda_{em} = 533 \pm 15$ nm; see Figures 4.23 and 4.24 and Table 4.3 for more information. (B) Compiled FACS data showing a decrease in LPA nanoparticle uptake after HeLa cells were treated with methyl-β-cyclodextrin $\lambda_{ex} = 488$ nm, $\lambda_{em} = 533 \pm 15$ nm; see Figures 4.10 and 4.11 and Tables 4.2 and 4.5 for more information. (C) Compiled FACS data showing relative cytotoxicity for 5 nM and 10 nM LPA nanoparticle treatments versus treatment with 0.25% Triton-X 100. Cytotoxicity was assessed via cell-associated propidium iodide fluorescence, $\lambda_{ex} = 488$ nm, $\lambda_{em}$ filter = 670 nm LP. See Figure 4.12 and Table 4.4 for more information.
Figure 4.10. FACS data showing HeLa uptake of fluorescein antisense LPA (FL-AS-LPA) micelles after treatment with pharmacological inhibitors of various pathways of endocytosis. FACS histograms (left) display only untreated, LPA micelle-treated, and HeLa cells treated with methyl-β-cyclodextrin and LPA micelle. Treatment of HeLa cells with other known inhibitors of endocytosis did not show a significant difference in LPA micelle uptake as compared to incubation of HeLa cells with LPA micelles only. Untreated cells were not incubated with inhibitor or LPA micelle in order to serve as a reference for minimum cell-associated fluorescence. HeLa cells were incubated with inhibitor for 30 minutes at 37 °C in 5% CO₂ followed by 5 nM LPA micelle for 1 hour at 37 °C in 5% CO₂. FACS histograms are displayed as ungated (top) and gated (bottom) data (see Figure 4.11) and are based on 2500 total event counts for each experimental condition with the x-axis plotted as FL-1 Area ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 533 +/- 15$ nm).
Figure 4.11. FACS scattering analysis of HeLa cells after treatment with endocytosis inhibitors and/or fluorescein antisense LPA (FL-AS-LPA) micelles. Gates for relevant experiments are drawn as pink polygons and the percentage of the total cell population (2500 events total) within the gate is displayed.
Table 4.2. Pharmacological inhibitors and experimental concentrations for HeLa treatment.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Stock</th>
<th>Experimental</th>
<th>Volume (µl) in 250 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>MβCD</td>
<td>250 mg/ml in H₂O</td>
<td>12.5 µg/ml</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>2 mg/ml in DMSO diluted to 0.28 mg/ml with DPBS</td>
<td>2.5 µg/ml</td>
<td>2.23</td>
</tr>
<tr>
<td>Filipin III</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospholipase C</td>
<td>5 units/ml in DPBS</td>
<td>0.5 units/ml</td>
<td>25.0</td>
</tr>
<tr>
<td>Sodium Azide</td>
<td>1 M in H₂O</td>
<td>50 mM</td>
<td>12.5</td>
</tr>
<tr>
<td>Polyinosinic acid</td>
<td>0.83 mg/ml in H₂O:DPBS, 10:2</td>
<td>50 µg/ml</td>
<td>15.0</td>
</tr>
<tr>
<td>Fucoidan</td>
<td>1 mg/ml in DPBS</td>
<td>50 µg/ml</td>
<td>12.5</td>
</tr>
</tbody>
</table>

Furthermore, LPA nanoparticles show no appreciable cytotoxicity in HeLa cells when analyzing membrane integrity after 1 h of incubation using propidium iodide as a probe (Figures 4.9C and 4.12 and Table 4.4).

Figure 4.12. FACS cytotoxicity analysis measured via fluorescence of propidium iodide incorporated into compromised HeLa cells. HeLa cells were incubated with 5 nM LPA NP or 10 nM LPA NP for 1 hour at 37 °C in 5% CO₂ or 0.25% Triton-X 100 for 5 minutes. FACS data displays 5000 event counts for each experimental condition. The cells contained within the gated area (pink polygon) are denoted as a percent of the total event count. The histogram data is not gated (compromised cells do not fall within the same scattering region) and displays FL-3 Area as the x-axis (λex = 488 nm, λem filter = 670 nm LP).
4.6 Conclusion

The development of hybrid nucleic acid-based materials capable of facilitating potent and specific interactions in complex biological milieu hinges upon the ability to create well-defined elements with predictable attributes and diverse composition. The straightforward synthesis, high-density display of covalently bound nucleic acids, and the potential for chemical diversification make LPA nanoparticles ideal candidates in forming the basis of next generation smart biomaterials capable of recognizing and responding to particular gene expression features.

4.7 Methods

4.7.1 Instruments and Reagents

All reagents were purchased from commercial sources and used without further purification. (IMesH$_2$)(C$_5$H$_5$N)$_2$(Cl)$_2$Ru=CHPh was prepared as described by Sanford et al.$^{41}$ DNA synthesis was carried out on an ABI 394 DNA/RNA synthesizer utilizing standard phosphoramidite chemistry. DNA synthesis reagents and custom phosphoramidites were purchased from Glen Research Corporation. CPG support columns and standard phosphoramidites were purchased from Azco Biotech Inc. LNA phosphoramidites were purchased from Exiqon Inc. DNA/RNA oligos that were not synthesized were purchased from Integrated DNA Technologies. All cell lines were purchased from ATCC (HeLa CCL-2, MCF7 HTB-22, A549 CCL-185, HT1080 CCL-121, HEK-293 CRL-1573). All deuterated solvents were purchased from Cambridge Isotope Laboratories Inc. $^1$H (400 MHz) and $^{13}$C (100 MHz) NMR spectra were recorded on a Varian Mercury Plus spectrometer. Chemical shifts ($^1$H) are reported in δ (ppm) relative to the CDCl$_3$ residual proton peak (7.27 ppm). $^{13}$C chemical shifts are reported in δ (ppm) relative to the CDCl$_3$ carbon peak (77.00 ppm). Mass
spectra were obtained at the UCSD Chemistry and Biochemistry Molecular Mass Spectrometry Facility. Low-resolution mass spectra were obtained using a Thermo LCQdeca mass spectrometer and high-resolution mass spectra were obtained using an Agilent 6230 Accurate Mass time of flight mass spectrometer. Polymer molecular weight and dispersity were determined via size-exclusion chromatography (Phenomenex Phenogel 5u 10, 1K-75K, 300 x 7.80 mm in series with a Phenomex Phenogel 5u 10, 10K-1000K, 300 x 7.80 mm (mobile phase: 0.05 M LiBr in DMF)) using a Hitachi-Elite LaChrom L-2130 pump equipped with a DAWN HELEOS multi-angle light scattering (MALS) detector (Wyatt Technology) and a refractive index detector (Hitachi L-2490) normalized to a 30,000 g/mol polystyrene standard. Hydrodynamic diameter (D_h) of DPA nanoparticles was measured via DLS using a DynaPro NanoStar (Wyatt Technology). Concentrations of oligonucleotides and DPA nanoparticles were determined using a Thermo Scientific NanoDrop 2000c spectrophotometer. HPLC analysis and purification of oligonucleotides was accomplished utilizing a Phenomenex Clarity 5u Oligo-RP (150 x 4.60 mm) or Clarity 10u Oligo-WAX (150 x 4.60 mm) column and a Hitachi-Elite LaChrom L-2130 pump equipped with a UV-Vis detector (Hitachi-Elite LaChrom L-2420). Oligonucleotide molecular weights were determined by mass spectrometry performed on a Bruker Daltronics Biflex IV MALDI-TOF instrument using a combination of 2’, 4’, 6’-trihydroxyacetophenone monohydrate (THAP) and 3-hydroxypicolinic acid (3-HPA) as matrices and a three-point calibration standard composed of three purchased oligonucleotides. Denaturing polyacrylamide gel electrophoresis was performed using a Bio Rad Criterion Mini-PROTEAN Tetra cell and precast TBE-Urea gels. Size-exclusion FPLC was accomplished using a HiPrep 26/60 Sephacryl S-200 High Resolution-packed size-exclusion column (mobile phase: 50 mM Tris, pH 8.5) and an Äktapurifier (Pharmacia Biotech) equipped with a P-900 pump and a UV-900 UV-Vis multi-wavelength detector. TEM samples were deposited on carbon/formvar-coated
copper grids (Ted Pella Inc.), stained with 1% w/w uranyl acetate, and imaged using a Technai G2 Sphera operating at an accelerating voltage of 200 kV.

4.7.2 Monomer, Termination Agent, and Polymer Synthesis

![Chemical Diagram]

**Figure 4.13.** Synthesis of carboxylic acid-terminated phenyl-norbornene polymer 1\textsubscript{18}-2 via ROMP.

**Figure 4.14.** Polymer termination with termination agent 2 (T.A.) monitored via \textsuperscript{1}H NMR.
Synthesis of (N-Benzyl)-5-norborne-exo-2,3-dicarboximide (1). To a stirred solution of N-benzylamine (2.85 g, 26.6 mmol) in dry toluene (125 mL) was added 5-norbornene-exo-2,3-dicarboxylic anhydride (4.10 g, 25.0 mmol) and triethylamine (3.83 mL, 27.5 mmol). The reaction was heated to reflux overnight under an atmosphere of N₂. The reaction was cooled to room temperature and washed with 10% HCl (3 x 50 mL) and brine (2 x 50 mL). The aqueous layers were combined and extracted with ethyl acetate (60 mL). The combined organic layers were dried with MgSO₄, filtered and concentrated to dryness yielding a pale yellow solid that was then recrystallized from ethyl acetate/hexanes to give 1 (4.98 g, 79%) as white crystals. ¹H NMR (CDCl₃): δ (ppm) 1.07 (d, 1H, CH₂, J=9.6 Hz), 1.42 (d, 1H, CH₂, J=9.6 Hz), 2.69 (s, 2H, 2 x CH₂), 3.26 (s, 2H, 2 x CH₂), 4.61 (s, 2H, CH₂), 6.28 (s, 2H, CH=CH), 7.25-7.40 (m, 5H, ArH). ¹³C NMR (CDCl₃): δ (ppm) 42.18, 42.28, 45.13, 47.62, 127.74, 128.48, 135.76, 137.76, 177.48. LRMS (Cl), 253.99 [M+H]+. HRMS, theo: 254.1176 [M+H]+, found: 254.1175 [M+H]+.

Synthesis of (Z)-4,4′-(but-2-ene-1,4-diylbis(oxy)) dibenzoic acid (2). To a stirred solution of ethyl 4-hydroxybenzoate (5.5 g, 33.1 mmol) in 100 mL dry DMF was added potassium carbonate (7.28 g, 52.7 mmol). To this stirred suspension was added cis-1,4-dichlorobutene (2.0 g, 16 mmol). The solution turned brown within minutes and the reaction was allowed to stir under an atmosphere of N₂ at 90 °C overnight. The mixture was then cooled to room temperature, filtered, and concentrated to dryness. The resulting solid was dissolved in ethyl acetate and washed three times with H₂O. The organic layer was dried over MgSO₄ and concentrated to dryness to yield solid white crystalline needles. This solid was recrystallized from ether to yield the pure diester (2.18 g, 35%). ¹H NMR (CDCl₃): δ (ppm) 1.38 (t, 6H, 2 x CH₃), 4.35 (q, 4H, 2 x CH₂), 4.74 (d, 4H, 2 x CH₂), 5.96 (t, 2H, CH=CH), 6.92 (d, 4H, 4 x ArH), 8.0 (d, 4H, 4 x ArH). The diester (2.18 g, 5.66 mmol) was dissolved in 95% ethanol and potassium hydroxide was added (12.0 g, 215 mmol). The reaction was
heated to reflux for 5 hours, cooled to room temperature, diluted with an equal volume of 
H₂O and acidified with HCl to form a white precipitate. The precipitate was filtered off to 
yield 2 as an orange-tan solid (1.78 g, 100%). ¹H NMR (DMSO-d₆, residual ¹H = 2.50 ppm): 
δ (ppm) 3.38 (s broad, 2H, 2 x COO⁻), 4.80 (d, 4H, 2 x CH₂), 5.89 (t, 2H, CH=CH), 7.03 (d, 
4H, 4 x ArH), 7.87 (d, 4H, 4 x ArH). ¹³C NMR (DMSO-d₆, residual ¹³C = 39.51 ppm): δ 
(ppm) 64.11, 114.50, 123.18, 128.33, 131.34, 161.72, 166.98. LRMS, 327.03 [M-H]⁺, HRMS, 

Synthesis of Polymer (1₁₈-2). Monomer 1 (870 mg, 3.4 mmol) was dissolved in 5 
ml CDCl₃ and cooled to −78 °C. Ruthenium catalyst (IMesH₂(C₅H₅N)₂(Cl)₂Ru=CHPh (124 
mg, 0.17 mmol) was added as a powder, followed by 1 mL additional CDCl₃ to solubilize the 
catalyst. The reaction was then allowed to warm to room temperature and stir under N₂ for 35 
minutes (NMR confirms the absence of the original olefin resonance from monomer 1 at 6.28 
ppm, and the presence of broad cis and trans polymer backbone olefin resonances at 5.45 and 
5.71 ppm). At this point, 200 µL of the reaction mixture was removed and quenched with an 
excess of ethyl vinyl ether to provide a homopolymer for molecular weight determination 
(SEC-MALS: Mₙ = 4575 g/mol, PDI = 1.001, Figure 4.15). Chain transfer agent 2 (111 mg, 
0.34 mmol) was dissolved in 2.0 mL DMF-d₇, added to the reaction mixture, and the mixture 
was allowed to stir at room temperature for 20 minutes. The ruthenium alkylidene proton 
resonance was monitored in order to track end labeling of the polymer (Figure 4.14). Once 
the reaction was determined to be complete, excess ethyl vinyl ether was added to quench the 
ruthenium catalyst. The crude polymer was precipitated from cold methanol and further 
purified by column chromatography in order to eliminate any traces of unreacted chain 
transfer agent 2. The crude precipitated polymer was dry loaded onto a silica column, the 
column was washed with 200 mL CH₂Cl₂, and the polymer was eluted with 3% methanol in 
CH₂Cl₂ to yield a glassy yellow-brown solid as the pure polymer (905 mg, 97%, rᵣ = 0.56).
Alternatively, the polymer can be precipitated from cold basic methanol (0.1% triethylamine) three times in order to yield pure polymer free of excess chain transfer agent.

![SEC-MALS chromatogram for polymer](image)

**Figure 4.15.** SEC-MALS chromatogram for polymer I\textsubscript{18} (LS = light scattering Rayleigh ratio, RI = refractive index difference, UV = UV absorbance at 280 nm, M\textsubscript{w} = polymer molecular weight).

### 4.7.3 LNA Synthesis

Oligonucleotides were synthesized in house using automated phosphoramidite chemistry (ABI 394 DNA/RNA Synthesizer) with 4,5-Dicyanoimidazole (Glen Research cat. #30-3150-52) as the activator and 0.02 M iodine in tetrahydrofuran/pyridine/water (Glen Research cat. #40-4330-52) as the oxidizer. Standard 2-cyanoethyl protected phosphoramidites include dA (N-Bz), dG (N-dmf), (N-acetyl) dC, and T. LNA phosphoramidites were vacuum dried using a Schlenk line for 24 hours prior to dissolving at 0.07 M in anhydrous acetonitrile or 25% tetrahydrofuran in acetonitrile v/v (for LNA mC only). Coupling times of 35 s (dC and T), 100 s (dA and dG), and 120 s (LNA amidites) were used. Oxidation time for LNA phosphoramidites was increased to 45 s as compared to 15 s for standard phosphoramidites. Oligonucleotides were synthesized on a 1.0 µmol scale using
columns packed with 1000 Å CPG beads (both AZCO universal support, cat. #19-8051-10, and Glen Research Universal Support III 40 µmol/g, cat. #G308237). No significant differences between the two support types were noticed. However, a pore size of at least 1000 Å was found to be critical for polymer conjugation on solid support. A 5’-amino modifier was incorporated into each synthetic oligonucleotide through use of 5’-amino modifier C12 phosphoramidite (Glen Research).

![Chemical Structures](image)

**Fluorescein anti-survivin ssLNA and LPA (FL-AS-ssLNA and FL-AS-LPA)**

![Chemical Structures](image)

**Cyanine 5 anti-survivin ssLNA and LPA (CY5-AS-ssLNA and CY5-AS-LPA)**

![Chemical Structures](image)

**Fluorescein nonsense LPA (FL-NS-LPA)**

![Chemical Structures](image)

**survivin mRNA target sequence hybridized to FL-AS-LPA**

![Chemical Structures](image)

**Figure 4.16.** LNA and RNA sequence information, modifications, and chemical structure. T_F denotes fluorescein dT phosphoramidite and CY5 denotes cyanine 5 phosphoramidite. Underlined positions indicate LNA bases. LNA C is incorporated as the 5'-methyl cytosine analogue as sold by Exiqon.
FL-AS-ssLNA and CY5-AS-ssLNA 5'-amino termini were acetylated on solid support using the automated synthesizer as follows: the MMT group was removed by treatment with 3% trichloroacetic acid in CH$_2$Cl$_2$ for three minutes (until the yellow color due to the MMT$^+$ cation was no longer visible in the eluting deblock solution) followed by a standard capping cycle to acetylate the free amine with acetic anhydride. Fluorescein and CY5 labels were incorporated into the oligonucleotides via use of Fluorescein dT and Cyanine 5 phosphoramidites (Glen Research) using coupling times of 10 minutes and 3 minutes respectively. Oligos were cleaved from solid support by treatment with ~150 µl 2.0 M ammonia in methanol for 1 hour at room temperature (recommended for universal support III chemistry) and further deprotected by addition of ~1.5 ml concentrated ammonium hydroxide at room temperature for 24 hours (to avoid degradation of CY5), purified by HPLC, and characterized by MALDI-TOF MS. Survivin RNA oligo was purchased from Integrated DNA Technologies (purified by HPLC, confirmed by ESI-MS). Detailed sequences are shown in Figure 4.16.

4.7.4 HPLC Purification of Oligonucleotides

Synthetic LNA oligonucleotides were purified via reverse-phase HPLC using a binary gradient (buffer A: 10% methanol, 90% 50 mM triethyl ammonium acetate (TEAA) pH 7.1; buffer B: 100% methanol.). For FL-AS-ssLNA, weak anion-exchange (WAX) HPLC was also necessary to purify the oligonucleotide. A quaternary gradient was used for WAX HPLC analyses and purification (Solvent A: nanopure H$_2$O, solvent B: methanol, solvent C: 100 mM Tris(hydroxymethyl)aminomethane (Tris) pH 8.0, solvent D: 2 M NaCl). FL-AS-ssLNA was desalted post WAX HPLC purification using a Sep-Pak Plus C18 Environmental Cartridge.
Figure 4.17. HPLC chromatograms and corresponding MALDI-TOF MS spectra for synthesized LNA sequences. HPLC was carried out using a linear binary gradient from 0-100% Buffer B in 60 minutes while recording UV absorbance at 260 nm (Buffer A: 10% methanol, 90% 50 mM triethyl ammonium acetate (TEAA) pH 7.1; Buffer B: 100% methanol).

4.7.5 MALDI-TOF MS of Oligonucleotides

A MALDI target plate was spotted with 1 µL of matrix solution A for each sample to be analyzed and allowed 20 minutes to dry completely. Matrix solution A was prepared by dissolving 50 mg of 3-HPA in 1000 µL of HPLC grade acetonitrile/Nanopure H₂O (1:1 v/v). Subsequently, 454 µL of this solution was mixed with 45 µL of 100 mg/ml diammonium hydrogen citrate in Nanopure H₂O. Oligonucleotide samples were prepared for MALDI-TOF MS analysis using Zip-Tip C18 pipette tips. Oligos were loaded onto the C18 tips from concentrated stock solutions (ca. 50-100 µM), desalted (protocol: Sample Preparation of Oligonucleotides Prior to MALDI-TOF MS Using ZipTipC18 and ZipTip μ-C18 Pipette Tips), and eluted with matrix solution B. Briefly, desalting was achieved by washing/wetting the Zip-Tip with 50% acetonitrile, equilibrating with 0.1 M TEAA, loading the oligo, washing with 0.1 M TEAA, washing with H₂O, and finally eluting the bound oligo with ~ 3.0 µl of matrix solution B. Matrix solution B was prepared by dissolving 50 mg of THAP in 500 µL of HPLC grade acetonitrile. Dissolution was assisted by sonication and the resulting
solution was centrifuged to pellet any remaining solid. Subsequently, 250 µL of this supernatant was mixed with 250 µL of 23 mg/mL diammonium hydrogen citrate in Nanopure H2O. 1 µL of this solution was then spotted onto the MALDI plate on top of crystallized matrix A. The samples were allowed to dry for 15-30 minutes before analyzing via MALDI-TOF MS. External three-point calibration was achieved using a mixture of three oligonucleotides purchased from Integrated DNA Technologies. Aliquots of 25 pmol 5’-TCTTATACCTTAAT-3’ (3898.6 g/mol), 125 pmol 5’-CACTGACTGGACATGACTCT-3’ (6077.0 g/mol), and 700 pmol 5-AAGATCGATCACTGACTGGAC-3’ (11644.6 g/mol) were all dissolved together in 100 µl nanopure H2O and spotted (1 µl) on top of crystallized matrix A (1 µl spot) for MALDI-TOF MS analysis in order to create an external calibration reference.

4.7.6 LNA-Polymer Amphiphile Synthesis and Micellar Nanoparticle Formation

To a solution of polymer (118-2) (50 mg, 10.9 µmol) dissolved in 150 µL DMF was added N,N-diisopropylethylamine (5 µL, 29.1 µmol) and HATU (3.42 mg, 9 µmol). The solution was vortexed for 10 minutes at room temperature in order to activate the polymer carboxylic acid terminus. At this point, 5’-amino modified LNA on CPG solid support (ca. 0.5-1.0 µmol, MMT deprotected by washing with 3% TCA in DCM for 3 minutes) was added. 100 µl DMF was added to wash CPG beads to the bottom of the microcentrifuge tube and immerse the beads in solution entirely. The mixture was allowed to vortex at room temperature for 2 hours. At this point, the CPG beads were centrifuged and washed with DMF three times followed by a final rinse with DCM. The beads were dried using a SpeedVac vacuum concentrator and subsequently a second coupling of 50 mg polymer for 2 hours was carried out under conditions identical to those described above. The CPG beads were then filtered away from the solution using an empty synthesis column and then washed.
with DMF (2 x 20 ml) and CHCl₃ (2 x 20 ml). The LNA-polymer amphiphile (LPA) was cleaved from solid support via treatment with concentrated ammonium hydroxide for 24 hours at room temperature. The CPG beads were away from the solution using glass wool and subsequently washed consecutively with H₂O (2.0 ml), DMSO (2.0 ml), Formamide (2.0 ml), H₂O (3.0 ml), and DMSO (1.0 ml). This solution was transferred to 3,500 MWCO snakeskin dialysis tubing (Thermo Scientific) and 2.0 mL H₂O, used to wash the filtrate container, was added. The resulting solution was dialyzed against 2.0 L of Nanopure H₂O overnight. This dialyzed solution was then concentrated to ~3.0 ml via rotary evaporation (water bath temperature set to 55 °C). The resulting crude LPA-nanoparticle/ssLNA mixture was analyzed by denaturing PAGE and agarose gel electrophoresis to confirm the presence of LPA conjugates and free ssLNA. It is important to note that low molecular weight ssLNA impurities (≤10,000 g/mol) remain present despite extensive attempts to dialyze away these species using 20,000 MWCO slide-a-lyzer dialysis cassettes or mini dialysis units (Thermo cat. #PI87736, #PI69590). Therefore, the crude mixture was purified via SEC FPLC (mobile phase: 50 mM Tris pH 8.5, flow rate: 1.8 ml/min, λₑₑₑ = 260, 492, 646 nm). The LPA-nanoparticles elute at ca. 55 minutes while unconjugated ssLNA impurities elute at ca. 85 minutes. All crude LPA micelles were purified using HiPrep 26/60 Sephacryl S-200 High Resolution SEC media. The pure LPA fraction (~15 ml) was then concentrated to ~0.5-1.0 ml via rotary evaporation (water bath temperature set to 55 °C) and dialyzed (20,000 MWCO slide-a-lyzer mini dialysis unit, Thermo Cat. #PI69590) against 10 mM Tris pH 8.5 overnight. The pure LPA micelles in 10 mM Tris pH 8.5 were then filtered through a 13 mm 0.45 µm pore size PTFE syringe filter (GE Whatman cat. #6784-1304).

**Transmission Electron Microscopy.** Copper grids (formvar/carbon-coated, 400 mesh copper, Ted Pella # 01754) were prepared by glow discharging the surface at 20 mA for 1.5 minutes followed by treatment with 3.5 µL 250 mM CaCl₂ in order to prepare the surface
for DPA nanoparticle adhesion. The CaCl₂ solution was wicked away with filter paper, the grid allowed to dry for 1 minute, and 3.5 µL of LPA nanoparticle (ca. 50 µM DNA in 10 mM Tris pH 8.5) solution was deposited on the grid surface. This solution was allowed to sit for 10 seconds before being washed away with 20 drops of glass distilled H₂O and subsequent staining with 3 drops of 1% w/w uranyl acetate. The stain was allowed to sit for 10 seconds before wicking away with filter paper. All grid treatments and sample depositions were on the dark/shiny/glossy formvar-coated face of the grid (this side face up during glow discharge). Samples were then imaged via TEM.

**Dynamic Light Scattering.** All samples were filtered through a 0.45 µm PTFE filter (Whatman #6784-1304) prior to DLS analysis using a DynaPro NanoStar (Wyatt Technology) and Dynamics version 7 software. A total of ten 4.0 s acquisitions were averaged for each reported measurement. Analyses are reported as both the reweighted intensity by mass % and the raw intensity (see Figures 4.2-4.4).

### 4.7.7 Cellular Internalization of LPA NPs Analyzed by Flow Cytometry

All FACS analyses were carried out using an Accuri C6 flow cytometer set to the default “3 blue 1 red” configuration with standard optics. Medium fluidics (35 µl/min, 16 µm core size) were used with no compensation set for any channel. All cells were grown in Dulbecco’s Modified Eagle Medium (DMEM + 4.5 g/L glucose, - L-Glutamine, - Sodium Pyruvate, Gibco Life Tech. #11960-044) with fetal bovine serum (FBS, Omega Scientific, Cat. # FB-02) added to 10%, and antibiotics (100 x Penicillin-Streptomycin, Corning Cellgro, #30-002-CI), non-essential amino acids (100x MEM-NEAA, Gibco Life Tech. #11140-050), sodium pyruvate (100x, Gibco Life Tech. #11360-070) and glutamine (Glutamax 100x, Gibco Life Tech. #35050-061) all added to a final concentration of 1x. All cells were treated similarly for all FACS analyses, unless otherwise noted. In each experiment cells were grown
to 80-100% confluency before splitting and plating 15-24 hours prior to treatment and subsequent FACS analysis. All incubations were carried out in Opti-MEM reduced serum media (Gibco Life Tech. #31985-070). Cells were plated in plastic 24-well tissue culture plates (24-well, flat bottom, 1.93 cm² growth area, Genesee Scientific Olympus Plastics #25-107) at 20,000 cells/well with 500 µl DMEM (as used for growth) per well. After treatment, cells were washed three times with Dulbecco’s Phosphate Buffered Saline (DPBS, without Calcium or Magnesium, Corning Cellgro #21-031-CM) and trypsinized with 250 µl 0.25% trypsin (diluted from 10x stock with DPBS, 2.5% trypsin, Gibco Life Tech. #15090-046) for 10 minutes at 37 °C and 5% CO₂. Following trypsinization, 500 µl DMEM (as used for growth) was added and the cells were aspirated and dispensed three times before harvesting in 1.5 mL microcentrifuge tubes (CNT-1.5F, DNase/RNase free, Bio Pioneer Inc.). Cells were then pelleted by centrifugation at 300 g for five minutes. Subsequently, the media was aspirated and the pellet was resuspended in 100 µl cold DPBS and put on ice until FACS analysis (analysis carried out within one hour of resuspension). Cells were never fixed at any point for any experiment. Cells were vortexed gently immediately prior to FACS analysis.

**Antisense versus nonsense LPA NP uptake.** HeLa cells were plated at 20,000 cells/well in a 24-well plastic tissue culture plate ca. 24 hours prior to incubation with 5 nM antisense fluorescein (AS-FL-LPA) or nonsense fluorescein (NS-FL-LPA) LPA micelles in 250 µl Opti-MEM for 2 hours at 37 °C and 5% CO₂. Cells were then washed three times with DPBS and harvested via trypsinization and subsequently pelleted and resuspended in cold DPBS. A total of 2500 event counts were collected and gated to include only events in the population that gave rise to significant scattering (>2M FSC). These cells comprised 32-45% of the total population analyzed for each experiment. Fluorescence for each event was recorded using an excitation wavelength of 488 nm and an emission filter of 533 ± 15 nm.
Data is plotted as overlaid histograms displaying relative event count versus fluorescence per event.

**Figure 4.18.** FACS data showing HeLa uptake of both antisense and nonsense fluorescein-labeled LPA nanoparticles. HeLa cells were incubated with 5 nM LPA micelle for 2 hours at 37 °C in 5% CO₂. FACS data displays 2500 event counts for each experimental condition. The cells contained within the gated area (pink polygon) are denoted as a percent of the total event count. The histogram data is gated to each corresponding scatter plot and displays FL-1 Area as the x-axis (λ_ex = 488 nm, λ_em = 533 ± 15 nm).

**LPA NP versus ssLNA uptake.** HeLa cells were plated at 20,000 cells/well in a 24-well plastic tissue culture plate ca. 24 hours prior to incubation with 5 nM antisense LPA micelles or 1 µM ssLNA (equivalent concentration with respect to LNA and fluorophore) analogue in 250 µl Opti-MEM for 10 minutes or 2 hours at 37 °C and 5% CO₂. Cells were then washed three times with DPBS, harvested via trypsinization, and subsequently pelleted and resuspended in cold DPBS. Cells harvested at 10 minutes were put on ice until 2 hour
treatment was finished, hence FACS analysis was carried out at the same time for both time points. No significant reduction in LPA micelle-cell association was noticed for cell samples stored on ice for less than 4 hours. A total of 2500 event counts were collected and gated to include only events in the population that gave rise to significant scattering (>2M FSC). Ungated histograms are also displayed for each experiment. These cells comprised 52-86% of the total population analyzed for each experiment. Fluorescence for each event was recorded using an excitation wavelength of 488 nm and an emission filter of 533 ± 15 nm (FL-1, fluorescein-labeled materials) or an excitation wavelength of 640 nm and an excitation filter of 675 ± 12.5 nm (FL-4, cyanine 5-labeled materials). Data is plotted as overlaid histograms displaying relative event count versus fluorescence per event.
Fluorescein Antisense ssLNA vs LPA Micelle

Figure 4.19. FACS data showing the difference in HeLa uptake between fluorescein-labeled LPA nanoparticles and the corresponding fluorescein-labeled ssLNA at both 10 minutes (top) and 2 hours (bottom). HeLa cells were incubated with 5 nM LPA micelle for the denoted time at 37 °C in 5% CO₂. FACS data displays 2500 event counts for each experimental condition. The cells contained within the gated area (pink polygon) are denoted as a percent of the total event count. The histogram data on the left is gated to each corresponding scatter plot and displays FL-1 Area as the x-axis ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 533 \pm 15$ nm).
**Cy5 Antisense ssLNA vs LPA Micelle**

**Figure 4.20.** FACS data showing the difference in HeLa uptake between Cy5-labeled LPA nanoparticles and the corresponding Cy5-labeled ssLNA at both 10 minutes (top) and 2 hours (bottom). HeLa cells were incubated with 5 nM LPA micelle for the denoted time at 37 °C in 5% CO₂. FACS data displays 2500 event counts for each experimental condition. The cells contained within the gated area (pink polygon) are denoted as a percent of the total event count. The histogram data on the left is gated to each corresponding scatter plot and displays FL-4 Area as the x-axis ($\lambda_{ex} = 640$ nm, $\lambda_{em} = 675 \pm 12.5$ nm).
**LPA micelle uptake in human embryonic kidney cells.** HEK 293 cells were plated at 20,000 cells/well in a 24-well plastic tissue culture plate ca. 24 hours prior to incubation with 5 nM antisense fluorescein LPA (AS-FL-LPA) micelles in 250 µl Opti-MEM for 10 minutes or 2 hours. At the end of each time point, 250 µl of 0.25 % trypsin was added to each well and the cells were incubated for 10 minutes at 37 °C 5% CO₂. After three aspirate/dispense cycles, the cells were collected in a 1.5 ml microcentrifuge tube and centrifuged at 300 g for 5 minutes. The supernatant was aspirated and the cells resuspended in 1.0 ml DPBS. The cells were pelleted again at 300 g, the supernatant removed, and the pellet resuspended in 100 µl cold DPBS for FACS analysis. A total of 5000 event counts were collected and gated to include only events in the population that gave rise to significant scattering (>2M FSC). These cells comprised 17-26% of the total population analyzed for each experiment. Fluorescence for each event was recorded using an excitation wavelength of 488 nm and an emission filter of 533 ± 15 nm. Data is plotted as overlaid histograms displaying relative event count versus fluorescence per event.

**4.7.8 Cellular Internalization of LPA NPs Analyzed via Live Cell Laser Scanning**

**Confocal Fluorescence Microscopy**

HeLa cells were plated at 20,000 cells/well in a 24-well glass-bottom tissue culture plate (No. 1.5, γ-irradiated, MatTek Corporation cat. #P24G-1.5-10-F) ca. 24 hours prior to incubation with 10 nM antisense fluorescein (AS-FL-LPA) or 10 nM antisense cyanine 5 (AS-CY5-LPA) micelles in 250 µl Opti-MEM for 5 minutes or 2 hours at 37 °C and 5% CO₂. Glass bottom wells were coated with human fibronectin (BD Biosciences cat. #354008) and stored at 8 °C the evening before plating the cells. Fibronectin coating was achieved by first treating each well with 250 µl of 1 M HCl for 15 minutes. Following this treatment, the wells were washed three times with DPBS and two times with ultrapure H₂O. Fibronectin
solution (250 µl, diluted to 50 µg/ml with DPBS) was added to each well and incubated for 1 hour before aspirating and washing three times with DPBS.

Figure 4.21. Confocal fluorescence microscopy Z-stack montage for live HeLa cells incubated with FL-AS-LPA micelles for 2 hours ($\lambda_{ex}= 488$ nm). The distance between each image in the z-axis is 1.25 µm.
**Figure 4.22.** Confocal fluorescence microscopy Z-stack montage for live HeLa cells incubated with CY5-AS-LPA micelles for 2 hours ($\lambda_{ex} = 635$ nm). The distance between each image in the z-axis is 1.25 µm.
At each time point, the Opti-MEM media was removed and replaced with warm DPBS. The cells were then imaged via confocal fluorescence microscopy in an incubation chamber at 37 °C and 5% CO₂. For figures 4.21 and 4.22, imaging was accomplished using a 20x air objective at 2x optical zoom. For single-slice images (Figure 4.5), scan size was set to 800 x 800 pixels with a scan speed of 10 µs/pixel. For Z-stack imaging, slice thickness was 1.25 µm with a scan size of 512 x 512 pixels and a scan speed of 10 µs/pixel. Cell imaging for fluorescein fluorescence was accomplished using a 488 nm laser and the following settings for all images acquired: 488 laser set to 30% power, HV=458, Gain=2, Offset=7. Cell imaging for cyanine 5 fluorescence was accomplished using a 635 nm laser and the following settings for all images acquired: 635 laser set to 50% power, HV=361, Gain=2, Offset=6. Identical contrast, brightness, and intensity settings were applied to each fluorescent channel for all images post acquisition. To image a high-resolution z-profile of cells treated with LPA nanoparticles with an added nuclear stain, a separate experiment was conducted (Figure 4.6). In this case, HeLa cells were plated exactly as described for the previous experiment. Treatment with 5 nM AS-CY5-LPA for 3.5 h was then carried out, followed by fixation with paraformaldehyde, permeabilization with Triton X-100, and staining according to standard procedures. Imaging was accomplished using a 60x oil objective at 2x optical zoom. Scan size was set to 640 x 640 pixels at a scan speed of 10 µs/pixel and a slice thickness of 0.5 µm. For cyanine 5 imaging, a 635 nm laser was used with the following settings: 3% laser power, HV = 484, gain = 4, offset = 6. For NucBlue imaging, a 405 nm laser was used with the following settings: 32% laser power, HV = 355, gain = 3, offset = 6.

4.7.9 LPA NP Uptake Kinetics Across Multiple Cell Lines

HeLa, A549, HT1080, and MCF7 cells were plated at 20,000 cells/well in a 24-well plastic tissue culture plate ca. 24 hours prior to incubation with 5 nM antisense fluorescein
LPA (AS-FL-LPA) micelles in 250 µl Opti-MEM for 10 minutes, 30 minutes, 1 hour, or 4 hours at 37 °C and 5% CO₂.

Figure 4.23. FL-AS-LPA micelle uptake kinetics for four different cell lines as analyzed via FACS. Histograms were constructed from gated data (see Figure S16) on a total of 5000 event counts. The x-axis is plotted as FL-1 Area (λ<sub>ex</sub> = 488 nm, λ<sub>em</sub> = 533 ± 15 nm).

Table 4.3. Tabulated data for LPA micelle uptake kinetics in four different cell lines.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>10 min</th>
<th>30 min</th>
<th>1 hr</th>
<th>4 hr</th>
<th>untreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>7.50</td>
<td>6.10</td>
<td>5.76</td>
<td>5.39</td>
<td>0.80</td>
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<td>HT1080</td>
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<td>14.5</td>
<td>14.6</td>
<td>11.4</td>
<td>0.83</td>
</tr>
<tr>
<td>A549</td>
<td>5.02</td>
<td>6.72</td>
<td>6.59</td>
<td>6.81</td>
<td>0.67</td>
</tr>
<tr>
<td>MCF7</td>
<td>20.6</td>
<td>17.2</td>
<td>16.3</td>
<td>13.6</td>
<td>1.26</td>
</tr>
</tbody>
</table>

*values reported as mean FL-1 area of gated population divided by 1x10⁴

The data for the 4 hr incubations are not reported in the main text, as triplicate measurement were only carried out for the first three time points. At the end of each time point, the cells were washed three times with DPBS, harvested via trypsinization, and subsequently pelleted.
and resuspended in cold DPBS and put on ice. All cells were analyzed via FACS ca. 90 minutes after the start of the experiment.

Figure 4.24. Representative FACS scatter plots showing percentage (5000 events total) of cells falling within the gated region for each experiment. Each gate was drawn to accommodate the population of events scattering significantly in the forward direction for the untreated cells.

No significant reduction in LPA micelle-cell association was noticed for cell samples stored on ice for less than 4 hours. A total of 5000 event counts were collected and gated to include only events in the population that gave rise to significant scattering (>2M FSC). These cells comprised 44-88% of the total population analyzed for each experiment. Fluorescence for each event was recorded using an excitation wavelength of 488 nm and an emission filter of 533 ± 15 nm. Data is plotted as overlaid histograms displaying relative event count versus fluorescence per event.
4.7.10 Cytotoxicity of LPA NPs

HeLa cells were plated at 20,000 cells/well in a 24-well plastic tissue culture plate ca. 24 hours prior to incubation with 5 nM antisense fluorescein LPA (AS-FL-LPA) micelles or 10 nM antisense fluorescein LPA (AS-FL-LPA) micelles in 250 µl Opti-MEM for 1 hour at 37 °C and 5% CO₂. Cells treated with 0.25% Triton-X 100 in 250 µl DMEM were incubated for 5 minutes at room temperature. The shorter incubation time in this instance was necessary due to nearly complete cell lysis after 1 hour of incubation (thus precluding FACS analysis).

After incubation, cells were then washed three times with DPBS, harvested via trypsinization, and subsequently centrifuged and resuspended in 100 µl of DPBS. To this cell suspension was added 10 µl of a 200 µg/ml solution of propidium iodide in DPBS. The cells were then incubated at room temperature for 5 minutes before putting on ice. Cells were incubated on ice for 15 minutes prior to FACS analysis.

Table 4.4. Tabulated data for LPA micelle toxicity studies by propidium iodide fluorescence.

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>5 nM Micelle</th>
<th>10 nM Micelle</th>
<th>Triton-X</th>
</tr>
</thead>
<tbody>
<tr>
<td>repeat 1</td>
<td>1.07</td>
<td>1.05</td>
<td>1.53</td>
<td>57.2</td>
</tr>
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<td></td>
<td>1.33</td>
<td>1.17</td>
<td>1.13</td>
<td>59.4</td>
</tr>
<tr>
<td>repeat 2</td>
<td>2.09</td>
<td>1.11</td>
<td>1.07</td>
<td>52.4</td>
</tr>
<tr>
<td></td>
<td>1.08</td>
<td>4.73</td>
<td>1.25</td>
<td>58.0</td>
</tr>
<tr>
<td>repeat 3</td>
<td>1.07</td>
<td>2.89</td>
<td>1.94</td>
<td>49.7</td>
</tr>
<tr>
<td></td>
<td>0.87</td>
<td>1.86</td>
<td>1.67</td>
<td>58.5</td>
</tr>
<tr>
<td>Average</td>
<td>1.25</td>
<td>2.14</td>
<td>1.43</td>
<td>55.9</td>
</tr>
<tr>
<td>Std. Dev.</td>
<td>0.44</td>
<td>1.45</td>
<td>0.34</td>
<td>3.89</td>
</tr>
</tbody>
</table>

*values reported as mean FL-3 area divided by 1.0 x 10⁵

A total of 5000 event counts were collected for each sample. Gating for the population of cells with significant scattering signal in the regions associated with healthy HeLa cells excluded greater than 95% of the cell population treated with Triton-X 100. Therefore, histograms were constructed for ungated cell populations in order to assess the fluorescence due to propidium iodide for each event. Fluorescence for each event was recorded using an
excitation wavelength of 488 nm and a 670 nm LP emission filter. Data is plotted as overlaid histograms displaying relative event count versus fluorescence per event.

4.7.11 Pharmacological Inhibition of LPA NP Uptake

HeLa cells were plated at 20,000 cells/well in a 24-well plastic tissue culture plate ca. 24 hours prior to incubation for 30 minutes with a given pharmacological inhibitor in 211 µl Opti-MEM at 37 °C and 5% CO₂ in order to compare results to previous literature. Following incubation with inhibitor, 39 µl of 32 nM antisense fluorescein LPA (AS-FL-LPA) micelle was added to each well to achieve a final concentration of 5 nM LPA micelle. The cells were then incubated for 1 hour at 37 °C and 5% CO₂. After incubation, cells were then washed three times with DPBS, harvested via trypsinization, and subsequently centrifuged and resuspended in 100 µl of DPBS. A total of 2500 event counts were collected for each sample. As methyl-β-cyclodextrin was the only inhibitor to show a significant effect on LPA micelle-cell association, this data was compared directly to the experiments not involving any inhibitor treatment in both gated and ungated FACS analyses. In the instance of gated populations, these cells comprised 25-59% of the total population analyzed for each experiment. Fluorescence for each event was recorded using an excitation wavelength of 488 nm and an emission filter of 533 ± 15 nm. Data is plotted as both as overlaid histograms displaying relative event count versus fluorescence per event and as bar graphs displaying average fluorescence per event (mean FL-1 A, gated and ungated) in each experiment (error bars are the result of running the experiment three times with three separate passages of the cells, see Figures 4.10 and 4.11). Experimental and stock concentrations of pharmacological inhibitors are shown in Table 4.2.
Tabulated data for pharmacological inhibition of endocytosis effect on LPA micelle uptake in HeLa cells.

### Mean Ungated Fluorescein Fluorescence/Cell* via FACS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>exp 1</th>
<th>exp 2</th>
<th>exp 3</th>
<th>average</th>
<th>std. dev.</th>
</tr>
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<tr>
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<td>MBCD</td>
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<td>FCD</td>
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<td>11.7</td>
<td>12.7</td>
<td>2.14</td>
</tr>
<tr>
<td>POLY I</td>
<td>13.4</td>
<td>13.3</td>
<td>9.00</td>
<td>11.9</td>
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<td>NaN3</td>
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<td>12.5</td>
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<td>1.94</td>
</tr>
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<td>12.8</td>
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<td>7.53</td>
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<tr>
<td>PLC</td>
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<tr>
<td>Untreated</td>
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<td>7.31</td>
<td>7.83</td>
<td>0.33</td>
</tr>
</tbody>
</table>

*values reported as mean FL-1 area of ungated population divided by 1x10^3

### Mean Gated Fluorescein Fluorescence/Cell* via FACS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>exp 1</th>
<th>exp 2</th>
<th>exp 3</th>
<th>average</th>
<th>std. dev.</th>
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</thead>
<tbody>
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</tbody>
</table>

*values reported as mean FL-1 area of gated population divided by 1x10^3

### 4.7.12 RNA Regulation with LPA NPs Analyzed by RT-qPCR

HeLa cells were treated with 5 nM LPA micelle for three consecutive days as follows:

*Day 0, morning:* HeLas were grown in DMEM in a T75 flask to 60-80% confluency. Media was aspirated, cells were washed with PBS and dissociated with trypsin (TrypLE Express, Life Technologies #12605010) for 5 minutes. DMEM with FBS was added to inactivate trypsin and the cells were aspirated and dispensed gently to break up any aggregates. 20,000 cells were plated in each well of a 24-well tissue culture plate.

*Day 1:* Micelles were diluted in Opti-MEM in a 1.5mL microcentrifuge tube to 5 nM immediately before use. For each LPA experiment (3 wells for fluorescein antisense micelle, 3 wells for fluorescein nonsense micelle), 39 μL of LPA Micelle (concentration = 32 nM
LPA micelle) was added to 211 μL of Opti-MEM. For the untreated control (6 wells), 39 μL molecular-grade water was added to 211 μl of Opti-MEM. The media was aspirated from the cells gently followed by the gentle addition of micelle suspension. Beginning at 3:00 pm, the cells were incubated with LPA micelles for four hours at 37 °C and 5% CO₂. After four hours the media with LPA micelles was aspirated and replaced with DMEM growth media.

**Day 2:** Cells were incubated with 5 nM LPA micelle from 6:00-10:00 pm. After four hours the media with LPA micelles was aspirated and replaced with DMEM growth media.

**Day 3:** Cells were incubated with 5 nM LPA micelle from 6:00-10:00 pm. At 10:00 pm 400 μl DMEM was added to each well without aspiration of Opti-MEM micelle solution.

**Day 4:** Cellular RNA was harvested with RNAeasy Mini Kit (Qiagen cat. #74-104) at 5:00 pm.

**RNA harvest, reverse transcription, and qPCR.** On Day 4, HeLa RNA was harvested from each experimental well using Qiagen’s RNAeasy mini kit following the associated protocol. The cells were lysed directly in each well using 350 μl RLT buffer and the lysate collected by vigorous aspirate and dispense cycles. The lysate was then homogenized using QIAshredder spin columns and RNA was harvested using RNeasy spin columns. Remaining DNA was digested using TURBO DNase and a TURBO DNA-free kit (Life Technologies #AM1907M) and following the associate protocol. Subsequently, the total RNA concentration was determined using a NanoDrop 2000c spectrophotometer. Reverse transcription of cellular RNA into cDNA was achieved using Superscript III reverse transcriptase and Superscript III First-Strand Synthesis System (Invitrogen #18080-051). Reverse transcription was carried for each experimental well (3 experimental wells for antisense micelle treatment, 3 experimental wells for nonsense micelle treatment, and 6 experimental wells for untreated HeLa cells) using oligo dT primers, 1 μg of RNA per reaction, and digesting RNA with RNase H after the reaction was complete. Quantification of
GAPDH and survivin transcripts in 1 µl of resulting cDNA for each experiment was achieved by qPCR using Fast SYBR Green Master Mix (Life Technologies #4385612) and measuring threshold cycle in the presence of survivin primers\textsuperscript{16} relative to cycle threshold in the presence of glyceraldehyde 3-phosphate dehydrogenase primers (GAPDH, Primer Bank ID 83641890b1) for each experimental condition in triplicate.

**Table 4.6.** RT-qPCR cycle threshold (CT) data for qPCR of survivin cDNA normalized to endogenous glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA.

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<th>CT (with GAPDH Primers)</th>
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<td>repeat 2</td>
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<td>20.86</td>
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<tr>
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<table>
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<th>Survivin-GAPDH</th>
<th>2^-{survivin-GAPDH}</th>
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Data analysis was carried out using GraphPad Software $t$ test calculator (http://www.graphpad.com/quickcalcs/ttest1/?Format=SEM) by entering the mean, standard
error of the mean (SEM), and number of repeats (N) and using the unpaired $t$ test. Survivin forward: 5'-ATG GGT GCC CCG ACG TTG, Survivin reverse: 5'-AGA GGC CTC AAT CCA TGG, GAPDH forward: 5'-AAG GTG AAG GTC GGA GTC AAC, GAPDH reverse: 5'-GGG GTC ATT GAT GGC AAC AAT A. All primers were purchased from Integrated DNA Technologies with standard desalting used as the sole purification method.

4.8 Acknowledgements

Chapter 4 is adapted from Anthony M. Rush, David A. Nelles, Angela P. Blum, Sarah A. Barnhill, Erick T. Tatro, Gene W. Yeo, and Nathan C. Gianneschi. “Intracellular mRNA Regulation with Self-Assembled Locked Nucleic Acid Polymer Nanoparticles.” J. Am. Chem. Soc., 2014, 136 (21), 7615-7618. Copyright 2014 American Chemical Society. Permission to use copyrighted images and data in the manuscript was also obtained from David A. Nelles, Angela P. Blum, Sarah A. Barnhill, Erick T. Tatro, Gene W. Yeo, and Nathan C. Gianneschi. The dissertation author is the first author of this manuscript.

4.9 References


Chapter 5

Conclusion - Programming Materials for The Future

5.1 Applications for Materials Encoded with DNA

This section is intended as a brief summary on some of the applications of DNA nanotechnology and will be split into those based on DNA origami and those based on hybrid DNA nanomaterials (i.e. conjugates). Excellent reviews on the applications of both nanostructures comprised solely of DNA\textsuperscript{1,2} and hybrid DNA nanomaterials\textsuperscript{3-5} have been published in the literature. Therefore the reader should refer to those reviews for a more extensive discussion.

Nanostructures composed entirely of DNA are traditionally assembled \textit{via} a tile-based method or an origami-like approach. Tile-based approaches typically produce one and two-dimensional repeating DNA lattices wherein junctions and crossover motifs have been engineered between many DNA sequences to program ordered overlap and assembly.\textsuperscript{6,7} DNA origami typically involves the folding of one long single-stranded piece of DNA, often “stapled” at multiple points with smaller DNA pieces to hold the structure in place.\textsuperscript{8,9} Structures assembled using the DNA origami approach tend to exhibit greater control over precise size and shape and offer great utility in the preparation of three-dimensional structures and large patterns. Due to molecularly precise architectures and sequence-specific addressability, DNA origami structures have been useful in studies that necessitate such spatial rigor including the elucidation of protein structure and function\textsuperscript{10,11} or the design of
fluorescent barcodes\textsuperscript{12} that can be used to label nanoscale structures and features for super resolution fluorescence microscopy visualization.\textsuperscript{13} Further, the DNA origami-guided preparation of carbon nanotubes (CNTs), metallic nanomaterials, and extracellular matrices with precise and tunable dimensions allow researchers to study phenomena that are otherwise difficult to address. For example precise organization and alignment of CNTs,\textsuperscript{14} distant dependent optical properties of plasmonic nanostructures,\textsuperscript{15} and cellular cytoskeletal arrangement and shape\textsuperscript{16} have all been studied \textit{via} the use of DNA origami as an assembly guide. Taking advantage of DNA-protein interactions, researchers have designed DNA nanostructures that can guide biosynthesis of metabolites\textsuperscript{17} or increase hydrogen production\textsuperscript{18} in bacteria \textit{via} protein scaffolding. In other work involving DNA aptamer-protein interactions, nanostructures have been engineered as drug delivery systems that release cargo in response to levels of various proteins in a logically guided fashion.\textsuperscript{19} Modifying enzyme activity based on folding DNA into shapes of various curvature and steric accessibility has also been demonstrated.\textsuperscript{20} In one example, DNA origami was used to induce different levels of tension in double helical DNA in order to regulate DNA methylation by EcoRI methyltransferase.\textsuperscript{21} Structures assembled purely from nucleic acids have even been used \textit{in vivo} to demonstrate gene knockdown \textit{via} RNA interference.\textsuperscript{22} Finally, perhaps the most exciting aspect of functional DNA origami nanostructures is that they can be synthesized \textit{in vivo} by genetically encoding the proper sequence or sequences.\textsuperscript{23,24}

DNA nanomaterials composed entirely of DNA rely on base-pairing interactions for assembly and structural integrity and therefore suffer a few consequences as a result. Although DNA nanostructures have been determined to be somewhat resistant to enzymatic degradation,\textsuperscript{20} nucleases do break down these structures over time.\textsuperscript{25} Further, pH and ionic strength effect structural integrity, often leading to disassembly or aggregation.\textsuperscript{25} The last
Hybrid DNA nanostructures benefit not only from the programmability provided by DNA but also from the properties of the other component including increased structural stability, unique optical properties, unique structural arrangement, increased lipophilicity, and unique electrochemical properties. DNA conjugates began to be reported in the mid 1980s but it wasn’t until the 1990s that researchers began to take advantage of these conjugates for the formulation of true nanomaterials. Initially, most DNA-based nanomaterials were being used to organize other materials like proteins and inorganic nanoclusters.\textsuperscript{26,27} Pioneered by Niemeyer,\textsuperscript{28,29} Tour,\textsuperscript{30} Park,\textsuperscript{31} and Mirkin,\textsuperscript{32} DNA conjugates began to be realized as interesting materials in their own right. Another early and elegant example, demonstrated by Smith et al., involved the use of sequence-specific DNA-protein interactions to guide assembly of nanomaterials.\textsuperscript{33} Other early work includes studies on assembly kinetics of DNA-porphyrin conjugates.\textsuperscript{34} Arguably the most renowned work came out of the labs of Chad Mirkin and Paul Alivisatos, who independently demonstrated that DNA could be covalently bound to gold nanoparticles in order to direct nanoparticle assembly.\textsuperscript{35,36}

As it turns out, nanostructures built partially from DNA make excellent DNA detectors. Almost 20 years ago, Mirkin and coworkers discovered that DNA decorated gold nanoparticles could be used to detect DNA in a colorimetric fashion at concentrations reported down to 250 fM of genomic DNA without PCR amplification or the need for expensive equipment.\textsuperscript{37-39} More recently, this assay was coupled to silver enhancement and indirect target amplification in order to push DNA detection down to 500 zM using a scanometric approach.\textsuperscript{40} A related platform used in DNA analysis for detection of multiple pathogens simultaneously takes advantage of magnetic nanoparticles functionalized with DNA.\textsuperscript{41} In truly remarkable work, bio-bar-code assays relying on oligonucleotide
functionalized metallic nanoparticles have been developed that push detection of proteins beyond that of the traditional ELISA approach and achieve nucleic acid detection rivaling that of PCR.\textsuperscript{3,40,42} Microcantilever approaches to DNA detection have been developed with the capability to detect DNA down to 0.05 nM.\textsuperscript{43} In this approach, gold cantilevers functionalized with nucleic acids bind to target sequences in solution and subsequently perturb silver nucleation on the cantilever. This perturbation manifests itself as a change in the cantilever resonance frequency and therefore can be readily detected. Indeed, other detection strategies relying on DNA hybridization have been achieved using nanomechanical cantilevers.\textsuperscript{44,45} Surface plasmon resonance spectroscopy has also been used to detect the hybridization of DNA sequences labeled with gold nanoparticles.\textsuperscript{46} Pushing towards nucleic acid detection in real-time, gold core spherical nucleic acids have been developed as one of the only ways to detect RNA in living cells.\textsuperscript{47} Other detection platforms based on hybrid DNA materials rely on perturbations in electrochemistry at the surface of DNA-coated electrodes.\textsuperscript{48-54} Using fluorescence-based readouts, DNA-decorated surfaces in the form of DNA microarrays have lent an enormous helping hand in genotyping and gene expression analysis.\textsuperscript{55-57} Beyond detection, DNA nanoparticles are being used in DNA sequencing and related analyses, exemplified in Illumina’s TruSeq\textsuperscript{®} Nano technology.

Of course, materials incorporating DNA have also fueled research in the realms of antisense and siRNA interference; these materials have been discussed in section 1.4. Hybrid materials wherein DNA is covalently linked to a second moiety such as a polymer or metallic nanoparticle are less common when it comes to genetic interference applications. However, these sorts of materials are being developed with increasing pace and participation and will be strong candidates for advanced therapeutics and diagnostics. Indeed, DNA-decorated gold nanoparticles, termed spherical nucleic acids, have been successfully deployed as potent
antisense agents\textsuperscript{47} and siRNA platforms that show promise as DNA delivery systems capable of crossing the blood brain barrier\textsuperscript{58} and penetrating through the epidermis.\textsuperscript{59}

Outside of biotechnology, hybrid DNA nanomaterials have been used to direct crystallization of programmable nanoparticle “atom equivalents” in efforts towards unprecedented precision in building macroscopic materials.\textsuperscript{60,61} These materials provide unique fundamental insights into the crystallization process in general. Towards the synthesis of nanoscale electric circuits, surfaces decorated with DNA have been used to prepare nanoscale silver wires capable of conducting electricity.\textsuperscript{62}

5.2 The State of Nucleic Acid Therapeutics

There are currently three FDA approved nucleic acid drugs. The first of these therapeutics to be licensed, Fomivirsen (also known as Vitravene\textsuperscript{TM}), was licensed by the FDA in 1998 as an antiviral antisense agent for combating retinal inflammation caused by cytomegalovirus (CMV) in immunocompromised individuals. Fomivirsen is a 21 base nucleic acid comprised entirely of phosphorothioate linkages. The drug is introduced \textit{via} intravitreal injection at a concentration of 6.6 mg/mL and functions by inhibiting translation of a key mRNA associated with CMV proliferation.\textsuperscript{63} Due to the success of anti-HIV drugs, especially protease inhibitors, there has been a steady decline in opportunistic infections in individuals infected with HIV; therefore, Isis Pharmaceuticals decided to discontinue Fomivirsen.

In 2004, Pegaptanib (also known as Macugen\textsuperscript{TM}) was approved by the FDA for the treatment of wet age-related macular degeneration (AMD). Pegaptanib is a 29 base PEGylated RNA aptamer with 2’-fluoro and 2’-O-Methyl modifications incorporated to enhance stability against enzymatic degradation. Here, the PEG moieties serve to increase circulation time of the drug. The RNA aptamer binds the 165 isoform of vascular endothelial
growth factor (VEGF), a protein involved in angiogenesis and increased permeability of blood vessels. Both of these processes are responsible for vision loss associated with neovascular wet AMD. Pegaptanib is administered via intravitreal injection at a dose of 0.3 mg once every 6 weeks. As of 2011, market sales for Pegaptanib have declined as Ranibizumab (a monoclonal antibody for the treatment of wet AMD) sales have risen.

In 2013, Mipomersen (also known as Kynamro™) was FDA approved for the treatment of homozygous familial hypercholesterolemia. Mipomersen is a 20 base antisense oligonucleotide that targets mRNA coding for apolipoprotein B; a protein believed to be involved in the formation of plaques in blood vessels that cause vascular disease and can eventually lead to heart disease. Mipomersen is composed entirely of phosphorothioate linkages and flanked on both the 3’ and 5’ ends with 5 bases of 2’-O-methoxyethyl RNA. Treatment with Mipomersen involves weekly intravenous injections.

As of 2012, 1843 clinical gene therapy trials had been conducted in 31 countries. Of these trials, 64.4% targeted cancer and roughly 67% used viral vectors for DNA delivery. Outside of viral delivery, naked DNA was used in 18.3% of all trials while lipofection was used in 5.9% of all trials. Roughly 100 trials are approved each year and it is estimated that approximately 300 gene therapy trials are ongoing in a given year.

Infamous clinical trials back in 1999 and 2000 have scarred our outlook on the use of viral vectors in gene therapy. In 1999, Jessie Gelsinger died from an immune response resulting from a viral based gene therapy treatment aiming to cure him of ornithine transcarbamylase deficiency, an X-linked genetic disease. Interestingly, a patient given a similar dose (3.6 versus $3.8 \times 10^{13}$ viral particles) in the same trial did not exhibit an immune response, thus highlighting the fact the each individual patient will indeed respond differently to a given therapy. In 2000, three children were cured of X-linked severe combined immunodeficiency (X-SCID), a fatal immunodeficiency disorder, following gene therapy
Unfortunately, two years later, the treatment led to the development of a leukemia-like disorder in two of the 11 patients that underwent the therapy. Since then, several other gene therapy clinical trials aimed at curing X-SCID were conducted with a total of 18 of 20 individuals reported to be alive with better immune systems. However, five of those 20 patients have since developed leukemia due to insertional mutagenesis from the viral vector. More recently, researchers have developed self-inactivating retroviruses that are less prone to mutagenesis. These vectors have been successfully deployed in clinical trials for the treatment of X-SCID. Here, of the nine patients enrolled in the trial, eight remained alive for the duration of the trials while seven of those eight were reported to regain immune function and have remained healthy after roughly three years. Note that in previous trials leukemia had a latency period of 2.0 to 5.5 years. It was reported that the single patient death was the result of a preexisting infection and that death occurred four months after the gene therapy treatment, reportedly before immune system function could be restored.

In 2011, James Wilson wrote an excellent perspective on the history and promise of gene therapy. Here, Wilson assures that technology backing viral-based gene therapy will meet the challenge as second-generation delivery vectors are developed. This sentiment is backed by Kay and coworkers, who offer a wonderful review and perspective on the topic. Wilson continues that the ultimate concern here will be one of commercialization and public acceptance of the controversial therapy. He argues that engagement of the biotech industry in gene therapy is necessary and will be difficult - stating that putting a price on a cure is a challenging task. On the positive side, it seems that the FDA is optimistic about the future of genetic therapy. One thing is clear: new materials that facilitate the delivery of nucleic acids into cells in vivo are in high demand.
5.3 Conclusion and Perspective

The power of materials programmed with DNA is twofold. First, with DNA as the guide, the precision with which synthetic nanoscale materials can be architected is currently unparalleled. Structures built using DNA as a template or guide are able to facilitate precision patterning and positioning of functional molecular features. These patterns can be used for fundamental studies and practical applications as described in section 5.1. The second outstanding attribute of materials incorporating DNA is that they are inherently capable of interfacing with biological systems in a synthetically programmable manner. For example, they provide a means to communicate with cells in ways that are impossible to achieve with small molecules or materials lacking true information. Indeed, although in their infancy, materials programmed with DNA have realized use in biodiagnostics and show early promise for in vivo settings. Despite major clinical setbacks suffered near the turn of the millennium, many believe that nucleic acid therapies will begin to impact the general population within the next ten or twenty years.

The next frontier in nucleic acid-programmed materials will be the development of robust and orthogonal stimuli-responsive platforms. Materials able to respond to specific environmental cues by emitting a strong detectable signal or instruct cells to perform a given function in response to patterns of cellular information will have a significant impact on how biological systems are studied and how biotechnologies are implemented in the future. For example, a major barrier to the development of successful stem cell therapies remains the inability to track the fate of implanted stem cells. Without knowledge of how, when, and where stem cells differentiate in vivo, the iterative development of such a technology will remain a daunting task. Materials designed to detect and respond to RNA expression patterns in vivo, for example, would provide invaluable information in such a setting.
Inspiring work in developing materials that lie at the interface of the synthetic and the natural world is currently underway. By designing materials to incorporate elements of nature’s own programming language, researchers are able influence and interact with life via communication at the molecular level.\textsuperscript{58,79-83} Concurrently, we are learning how to use nature to build advanced multi-component materials that we cannot build on our own.\textsuperscript{84,85} This feedback loop between living organisms and the material world they exist in sets the scene for a profoundly interesting and fruitful era in science. The ability to harness cellular machinery that can conduct specific functions reliant on programmable molecular information will aid in the interfacing of materials and biology.\textsuperscript{86-89} Progress in this arena hinges upon our ability to monitor material-cell interactions non-invasively in real-time at high spatial and temporal resolution in a multiplexed fashion. Further, proteomics- and genomics-based approaches offer large amounts of valuable information when it comes to studying dynamic living systems. These approaches will become commonplace in studying how materials interface with and influence cells, tissues, organisms, and ecosystems.

5.4 References


