Brush Polymers for Nucleic Acid Delivery and Self Assembly

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Abstract of Dissertation

Difficult biopharmaceutical characteristics of oligonucleotides, such as poor enzymatic stability, rapid clearance by reticuloendothelial organs, unwanted stimulation of the immune system, and coagulopathies, limit their application as therapeutics. Many of these side effects are initiated via specific or non-specific interactions with proteins. In this thesis, we develop a non-cationic, polyethylene glycol (PEG) brush polymer/DNA conjugate that provides oligonucleotides with nanoscale steric selectivity: hybridization kinetics with complementary DNA remains nearly unaffected, but interactions with proteins are significantly retarded. The relative lengths of the brush side chain and the DNA strand are found to play a critical role in the degree of selectivity. We demonstrate that these unimolecular nanoparticles can enter cells and suppress gene expression without the need of a cationic polymer co-carrier. The PEG brushes also improve the in vivo biodistribution of oligonucleotide and suppress side effects induced by protein-nucleic acid interactions. Compared to traditional polycationic DNA carriers, our strategy is a radically new approach to addressing several long-lasting challenges in oligonucleotide therapeutics.

Beside the great potential in therapeutic applications, DNA is also envisioned as excellent molecular building blocks to construct a variety of nanomaterials. Herein, we investigate the use of DNA in the “polycondensation” of brush copolymers into much larger structures (in one or three dimensions), where the DNA serves as the functional groups. Triblock copolymer brushes are functionalized with nucleic acid sequences, which allow the polymers to connect head-to-tail and form supramolecular nanostructures. Two approaches were designed and implemented, using either a palindromic DNA attached to both ends of the polymer or two different DNA sequences attached regiospecifically. Given appropriate conditions, the DNA-brush conjugates self-assemble to form either nanoworms with length up to several microns or cross-linked networks.
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<tr>
<td>µ</td>
<td>micro</td>
</tr>
<tr>
<td>aPTT</td>
<td>activated partial thromboplastin time</td>
</tr>
<tr>
<td>calc</td>
<td>calculated</td>
</tr>
<tr>
<td>br</td>
<td>broad</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus retinitis</td>
</tr>
<tr>
<td>CPM</td>
<td>chlorpromazine</td>
</tr>
<tr>
<td>d</td>
<td>doublet</td>
</tr>
<tr>
<td>δ</td>
<td>chemical shift</td>
</tr>
<tr>
<td>Da</td>
<td>dalton</td>
</tr>
<tr>
<td>DBCO</td>
<td>dibenzocyclooctyne</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>D_h(n)</td>
<td>number averaged hydrodynamic diameter</td>
</tr>
<tr>
<td>DIPEA</td>
<td>N,N-diisopropylethylamine</td>
</tr>
<tr>
<td>DLS</td>
<td>dynamic light scattering</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
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<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
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<tr>
<td>DMT</td>
<td>dimethoxytrityl</td>
</tr>
<tr>
<td>DOTMA</td>
<td>1,2-di-O-octadecenyl-3-trimethylammonium propane</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double strand RNA</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>DX</td>
<td>double crossover</td>
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EDC  ethyl(dimethylaminopropyl) carbodiimide
ELISA  enzyme-linked immunosorbent assay
em  emission
EPR  enhanced permeation and retention
equiv  equivalent
EVE  ethyl vinyl ether
ex  excitation
FBS  fetal bovine serum
FDA  Food and Drug Administration
g  gram
GPC  gel permeation chromatography
h  hour(s)
HPLC  high performance liquid chromatography
Hz  Hertz
IFN  interferon
IL-6  interleukin 6
INF-β  interferon-β
IR  infrared
J  joule
k  kilo
L  liter
LiAlH₄  lithium aluminum hydride
LNA  locked nucleic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>m</td>
<td>milli</td>
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<tr>
<td>M</td>
<td>molar</td>
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<tr>
<td>MALDI-ToF MS</td>
<td>matrix-assisted laser desorption ionization-time of flight mass spectrometry</td>
</tr>
<tr>
<td>Me</td>
<td>methyl</td>
</tr>
<tr>
<td>MHz</td>
<td>megahertz</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
</tr>
<tr>
<td>$M_n$</td>
<td>number averaged molecular weight</td>
</tr>
<tr>
<td>mol</td>
<td>mole</td>
</tr>
<tr>
<td>mp</td>
<td>melting point</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>$M_w$</td>
<td>weight averaged molecular weight</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>MWCO</td>
<td>molecular weight cut off</td>
</tr>
<tr>
<td>MβCD</td>
<td>methyl-β-cyclodextrin</td>
</tr>
<tr>
<td>N-Br</td>
<td>norbornenyl bromide</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>N-NHS</td>
<td>norbornenyl hydroxysuccinimidy1 ester</td>
</tr>
<tr>
<td>N-PEG</td>
<td>norbornenyl polyethylene glycol</td>
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<tr>
<td>pacDNA</td>
<td>polymer-assisted compaction of DNA</td>
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<td>polyacrylamide gel electrophoresis</td>
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PAMAM       polyamidoamine
PBS         phosphate buffered saline
PBST        0.05% Tween-20 in phosphate buffered saline
PDI         polydispersity index
PEG         polyethylene glycol
PEI         polyethylene imine
PMSF        phenylmethanesulfonylfluoride
PNA         peptide nucleic acid
POPE        1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine
PT          prothrombin time
PVDF        polyvinylidene difluoride
QRT-PCR     quantitative real-time polymerase chain reaction
RISC        RNA-induced silencing complex
RNAi        RNA interference
ROMP        ring-opening metathesis polymerization
RP          reverse phase
rt          room temperature
scr         scrambled
sec         second
SELEX       systematic evolution of ligands by exponential enrichment
siRNA       small interfering RNA
ssRNA       single strand RNA
t_{1/2}      half-life
<table>
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<tr>
<td>TBE</td>
<td>Tris/Borate/EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>THPTA</td>
<td>tris(3-hydroxypropyltriazolylmethyl)amine</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TLR</td>
<td>toll like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor α</td>
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<tr>
<td>UV-Vis</td>
<td>ultraviolet-visible</td>
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Chapter 1

Introduction

1.1 Nucleic Acids as Biopharmaceuticals.

The development of small molecule drugs, either by rational design or high-throughput screening, has been proven exceedingly difficult, with the average developmental cost for a single drug being in the vicinity of 2.5 billion US dollars.\(^1\) Small molecule drugs that are of high specificity and high affinity for target, have proper pharmaceutical properties, and display minimal side effects are particularly difficult to come by.\(^2\) Therefore, research efforts have been extensively made to source therapeutic species directly from biological components, such as proteins, peptides, and antibodies. Those biopharmaceuticals are collectively termed biologics. Spanning from macromolecules to entirely living organs, biologics have shown improved selectivity for their disease target.\(^3\) The rapid development and commercial success of biologics are reflected in the fact that, during the last decade, one-third of Food and Drug Administration (FDA)-approved medicines are biologics.\(^4\) In addition, seven of the top eight drugs (by revenue) sold in the US in 2015 are biologics. Such success, however, is to a great extent achieved with antibodies alone. Both academia and industry are making significant efforts to push the boundary of biologic drugs to include nucleic acids.\(^5\)

The discovery of DNA double helical structure by Watson and Crick in 1953 laid the foundation of current research and therapeutic application of nucleic acids.\(^6\) Nucleic acid based therapy was conceptualized in the 1970s, when Zamecnik and Stephenson reported the inhibition of Rous sarcoma virus using a 13-mer oligonucleotide.\(^7\) Since then, the view of nucleic acids has
changed dramatically from being a genetic information storage device to potential therapeutics. The potential of the nucleic acid drug is unimaginable. It is a form of informational drug; a change in disease target in principle only requires a change in the DNA sequence itself. By separating the chemistry (dianophore) from the drug activity (pharmacophore), nucleic acids are poised to reduce drug development costs by at least an order of magnitude if not more.\textsuperscript{8} With the completion of the human genome project in 2003,\textsuperscript{9} gene therapy research has increased in intensity as more targetable biological pathways are identified. In addition, as rapid sequencing technologies are becoming available, nucleic acid-based therapies are among the few options for personalized therapy that have the potential to keep up with the pace of analytical advancement.\textsuperscript{10,11} So far, a variety of oligonucleotide-based treatment mechanisms have been explored, including antisense, RNA interference, and aptamer recognition.\textsuperscript{12-14}

The idea of antisense gene therapy is relatively straightforward. By introducing a synthetic antisense oligonucleotide, which can specifically hybridize with the complementary mRNA strand through Watson-Crick base pairing, the target mRNA is prevented from being translated to proteins, generally through two mechanisms.\textsuperscript{13,15} The first one is a catalytic process involving RNase H, in which RNase H specifically recognizes the DNA-RNA duplex and cleaves the mRNA. The other mechanism simply involves steric blocking of ribosomes, which prevents the mRNA from being translated. This is a stoichiometry mechanism; each mRNA requires an antisense strand to silence. A more efficient gene regulation mechanism using RNA interference (RNAi) pathway was proposed by Fire and Mello in 1998.\textsuperscript{16} They demonstrated effective gene regulation of homologue in \textit{C. elegans} using small double-stranded RNA. In the RNAi mechanism, the double strand small interfering RNA (siRNA) splits into the guide strand and the passenger strand after entering cells. The guide strand, which is complementary to target mRNA, is
incorporated into RNA-induced silencing complex (RISC), which can recognize and destroy mRNA by argonaute in the cytoplasm in a catalytic fashion.\(^{17}\) The two were awarded a Nobel prize in Physiology or Medicine in 2006 for their revolutionary discovery. Aptamers, on the other hand, do not involve hybridization to a complementary sequence within the cell. First discovered by Tuerk and Ellington in 1990,\(^ {18}\) the aptamer is a single-stranded oligonucleotide, which functions by folding and forming secondary structures that can bind with protein and small molecule targets, thereby modulating biological pathways. The identification of aptamer is achieved by an \textit{in vitro} assay named systematic evolution of ligands by exponential enrichment (SELEX).\(^ {19}\)

The current rate of output of oligonucleotide-based drugs is in stark contrast with their incredible promise: only three oligonucleotide-based drugs have reached the market. Vitravene was the first FDA-approved oligonucleotide drug (antisense, approved in 1998), which is used for the treatment of cytomegalovirus retinitis (CMV).\(^ {20}\) It was discontinued on 2006 due to the development of another highly active antiretroviral therapy, which significantly reduced the cases of CMV. An aptamer based drug for treatment of neovascular age-related macular degeneration, named Macugen, was approved by FDA in 2004.\(^ {21}\) A second antisense drug, Kynamro, was approved by FDA almost a decade later in 2013. Kynamro treats homozygous familial hypercholesterolemia by targeting the mRNA of apolipoprotein B-100, which is the major component of low-density lipoprotein.

The slow rate of translation for oligonucleotide-based drugs from bench to bedside reflects the many problems associated with the physiochemical and biological properties of oligonucleotides.\(^ {22,23}\) First, most nucleic acids (other than aptamers) need to enter the cytosol or the nucleus of the cell to act upon their target. The large size (at least 6-7 kDa for oligonucleotide sequences to be specific), the hydrophilicity (with a water/octanol log(p) in the negative range),
and the negatively charged phosphate backbone of the nucleic acid prevent it from passively entering cells, let alone accessing the cytosol or the nucleus.\textsuperscript{24} Endocytosis and subsequent endosomal escape is typically required for nucleic acid drugs.\textsuperscript{25} In addition to cell entry difficulties, nucleic acids also exhibit rapid enzymatic degradation both inside and outside the cells due to the presence of nucleases.\textsuperscript{26,27} The acidic and digestive environments of late endosomes and lysosomes can destroy nucleic acids rapidly. Furthermore, \textit{in vivo} experiments show that naked, unmodified nucleic acids have a half-life of several minutes in the blood stream owing to efficient hepatic capturing, which limits these systems to the treatment of liver-associated diseases.\textsuperscript{28} Furthermore, certain nucleic acid motifs can stimulate unwanted innate immune response.\textsuperscript{29} In particular, sequences containing unmethylated CG or GGGG motifs are capable of eliciting a strong immune response in B cells and dendritic cells,\textsuperscript{30} and are in fact used in clinical trials for cancer, allergies, and as a vaccine adjuvant.\textsuperscript{31} Unwanted immunostimulation can be a concern for designing oligonucleotide drugs. For example, the Phase I clinical trial for an siRNA drug (ApoB-SNALP), developed by Tekmira, was terminated because of the inflammatory response of the RNA.\textsuperscript{32} Other problems associated with oligonucleotide drugs include interference with coagulation pathway and toxicity of the cleaved nucleotide and nucleoside analogues (if non-natural sequences are used).\textsuperscript{33,34}

![Chemical modifications of nucleic acids](image)

\textbf{Figure 1-1.} Chemical modifications of nucleic acids.
Scientists have developed different methods to improve the biopharmaceutical properties of nucleic acids. Various chemical modifications have proved to enhance the stability, potency, binding affinity and reduce immunogenicity (Figure 1-1). The first-generation of modified oligonucleotide is the phosphorothioate, in which one of the non-bridging phosphate oxygens is replaced by a sulfur atom.\textsuperscript{35} The sulfurization dramatically reduces the action of endo- and exonucleases and increases the half-life against enzymes.\textsuperscript{36} Both FDA-approved antisense drugs, Vitravene and Kynamro, consist of phosphorothioates. However, phosphorothioate shows lowered binding affinity ($T_m$ -0.2 $^\circ$C/base), which may affect overall binding and the potency in gene regulation.\textsuperscript{37} The second-generation oligonucleotide is synthesized by 2'-alkylation.\textsuperscript{38} The 2'-O-methylated oligonucleotides also show improved stability against nuclease and significantly reduced immune system activation.\textsuperscript{39} The third-generation oligonucleotide, locked nucleic acid, (LNA) is synthesized by modifying with an extra bridge connecting the 2' oxygen and 4' carbon of natural nucleic acid.\textsuperscript{40} The locked ribose conformation enhances base stacking and backbone pre-organization. This significantly increases the hybridization properties, making it an efficient method of detecting mRNA \textit{in situ}.\textsuperscript{41} Another third-generation modification involves peptide nucleic acid (PNA), which comprises a peptide backbone. PNAs are not recognized by either nucleases or proteases, making them resistant to degradation.\textsuperscript{42} Despite significant progress, however, problems such as unwanted immune response, interference with coagulation pathways, ineffective cell uptake, and poor biodistribution still restrict the therapeutic application of oligonucleotides, as evidenced by the slow market adoption. These observations led us to believe that chemical modification alone is not sufficient to achieve satisfactory pharmaceutical properties.\textsuperscript{43}
Chemists have been working on an entirely different strategy to protect and transport nucleic acid to cells for over four decades. This strategy involves the use of various synthetic or biological gene delivery carriers, which are typically materials of a cationic nature, such as poly(ethylene imine), arginine and/or lysine-rich cell-penetrating peptides, quaternary ammonium-containing liposomes, etc (Figure 1-2).\textsuperscript{44} By far the most popular class of materials among these in clinical trials are lipid-based carriers.\textsuperscript{45} Lipid have been widely used as small molecule drug delivery vehicles in many commercial formulations.\textsuperscript{46} Cationic lipids, such as 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), can encapsulate or complex with anionic oligonucleotides to form

\textbf{Figure 1-2.} Structures of cationic materials for nucleic acid delivery.
lipid-oligo nanoparticles.\textsuperscript{47,48} Cationic lipid nanoparticles can have very high transfection efficacy \textit{in vitro}. For example, Lipofectamine 2000, which is a commercially available gene transfection agent, can effectively deliver DNA or RNA into the cytoplasm. However, efficacy is significantly reduced \textit{in vivo} at dosages where carrier toxicity is manageable.\textsuperscript{49} The phase III clinical trial of Allovecin-7, which is a lipid-DNA nanoparticle developed by Vical Inc. to treat advanced metastatic melanoma, was terminated due to the low efficacy.\textsuperscript{50}

Cationic polymers are another popular class of delivery vehicles due to high tunability and structure versatility.\textsuperscript{51} Cyclodextrin polymer is the first material for siRNA delivery in clinical trial for the treatment of cancer.\textsuperscript{52} Successful reduction of mRNA levels using cyclodextrin-siRNA nanoparticle was observed in translational experiments using monkeys. Many natural or synthetic cationic polymers, such as poly(ethylene imine), chitosan, polylysine, dendritic polyamidoamine (PAMAM), have been explored to form polyplexes with nucleic acids.\textsuperscript{53,54} The additional benefit of using cationic materials is their ability to disrupt the endosomal membrane of the cell, presumably \textit{via} a proton sponge effect, which increases release to cytosol and overall gene regulation efficacy.\textsuperscript{55} PEGylation of the nanoparticles is essential to enhance \textit{in vivo} biodistribution, since the cationic nature of the polyplex leads to rapid clearance.\textsuperscript{56-58} Despite significant effort, however, cationic polymers in general still face low delivery efficiency \textit{in vivo} and various degrees of cytotoxic and immunogenic reactions, resulting in less than 1\% of US gene therapy clinical trials using them.\textsuperscript{59}
Figure 1-3. (A) Structure anatomy of SNA nanostructure. (B) SNA shows successfully gene regulation of epidermal growth factor receptor (EGFR) in SCC12 cells. Modified with permission from reference 62.

Alongside the development of cationic materials and chemically modified DNA, other ideas, potentially revolutionary, were disseminated and tested. In 1996, a new class of nucleic acid delivery system termed spherical nucleic acid (SNA), which consists of oligonucleotide-functionalized gold nanoparticles, has emerged (Figure 1-3). The DNA densities on SNA can be very high (ca. 70% of theoretical maximum packing). Interestingly, despite being highly anionic, these particles are capable of entering a variety of cell lines in a large quantity and knocking down target genes without significant cytotoxic side effects associated with cationic agents. The sterics created by the high density does not prevent the oligonucleotides from binding with complementary strands when a sub-stoichiometry amount of DNA strands is added, but instead enhances the binding constant by two orders of magnitude. In a sterically congested spherical form, the SNAs are significantly more stable towards enzymatic degradation. Nonetheless, SNAs primarily accumulate in the liver after systemic injection, making them only appropriate for local or hepatic delivery. These data imply that one should look beyond the chemical and biological
traits of the nucleic acid and the co-carrier for gene regulation, to include a careful consideration of the nucleic acid’s immediate local environment.

*Therefore, a system that can improve nuclease stability, preserve target-binding capability, minimize off-target effects, and improve bio-distribution (e.g. passive tumor targeting) may prove to be the important missing link in achieving broad application of oligonucleotide-based therapies.*

In this thesis, we design and synthesize a new form of DNA-polymer conjugate (termed pacDNA: polymer assisted compaction of DNA) by covalently attaching oligonucleotides to the backbone of polyethylene glycol brush polymers. These single-molecule nanostructures have the potential to enhance the biopharmaceutical properties of almost all oligonucleotide drug candidates currently under investigation (Chapters 2-4). The innovation of pacDNA nanostructure consists of four aspects. (1) Using a biocompatible polymer for nucleic acid protection. This strategy represents a new route to nucleic acid protection. Instead of using cationic materials, a brush polymer architecture consisting of a well-established, FDA-approved, biocompatible polymer (polyethylene glycol, PEG) as the side chain is used to protect the nucleic acids via a novel mechanism of steric compaction instead of electrostatic complexation. No polycationic species is involved. (2) Unhindered accessibility to target nucleic acid strands. Because pacDNA does not rely upon polymer-DNA electrostatic interactions, the DNA remains hybridizable to target nucleic acid strands without shedding of the PEG side chains. Although the slower diffusion and steric of pacDNA may cause hybridization to become slower, our data demonstrate that the binding kinetics of pacDNA structures having 10-mer and 15-mer DNA strands at room temperature is indistinguishable from that of free DNA. Such unhindered accessibility to target strands was translated into higher in vitro and in vivo efficiencies. (3) Suppression of non-antisense side effects. Almost all cases of unwanted, non-antisense side effects are preceded by protein recognition of
the oligonucleotide, be it degradation, toll like receptor (TLR) activation and interferons IFN response, and inhibition of the coagulation cascade. The densely compacted structure of pacDNA circumvents the problems associated with protein binding, and minimizes related non-hybridization side effects. (4) Improved bio-distribution. Comprising pure PEG, pacDNA shows much enhanced blood circulation time compared with naked DNA in mice models. The size of pacDNA can also take advantage of the EPR effect for passive tumor targeting.

1.2 DNA as a Covalent Bond-equivalent for Nanoscale Assembly.

In nature, proteins non-covalently interact with each other to form quaternary structures in an extremely well defined fashion. For example, the tobacco mosaic virus consists of 2130 molecules of a coat protein connected in a helical manner with 16.3 proteins per helix, which gives it a rod-like appearance.\textsuperscript{65,66} Similarly, globular G-actin proteins polymerize in vitro to form thin, double helical linear fibers with a 6 nm diameter, which is highly regulated by accessory proteins.\textsuperscript{67} There has long been an interest in reproducing some of the programmability, directionality, and precise character of the supramolecular protein-protein interactions with synthetic materials.\textsuperscript{68,69}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure.png}
\caption{(A) Assembly of four-arm junction DNA structure through sticky ends. (B) Assembled cube-like and octahedron nanostructures from DNA. Modified from reference 118.}
\end{figure}
Chemists have achieved precise control over the molecular-level structures by building covalent bonds between atoms. However, the synthesis of highly complexed structure on the nanoscale is very much a nascent area due to the difficulty in forming limited-valency, orthogonal, and directional connections. DNA is a well-known biopolymer for storage of genetic information in biology. Beyond these properties, DNA is also an ideal candidate to build predictable structures owing to its precise recognition property.\textsuperscript{70} DNA hybridization is arguably the most well-defined, programmable, and predictable supramolecular coupling between two molecules than any other class of molecular interactions known to mankind, including protein-protein interactions.\textsuperscript{71} The DNA double helix itself also has a defined structure with a diameter of \~2 nm and a length of \~3.4 nm for a single helical turn.\textsuperscript{72} Using convenient DNA synthesis, one can create an essentially unlimited number of orthogonal connections.\textsuperscript{73} DNA nanotechnology emerges as a new field to construct controlled, well-defined nanostructures by using DNA as structural building blocks.\textsuperscript{74}

Seeman and co-workers pioneered the use of DNA to build nanostructures in 1980s.\textsuperscript{75} Inspired by the naturally occurring branched DNA structure, the Seeman group made the four-arm Holliday junctions with sticky ends, which guided the assembly of DNA into quadrilateral structures through DNA hybridization (Figure 1-4A).\textsuperscript{76} A range of structures with different topology were obtained by using the combination of branched DNA and sticky ends (Figure 1-4B).\textsuperscript{77,78} However, the high flexibility of single, long DNA duplex leads to poor control over the assembled structures and limits their potency to construct more complex structures. Being able to provide rigidity to DNA-DNA interaction, thus achieving directional DNA bonding, has become a major barrier to fabricate well-defined 2D and 3D structures. Since 1990s, two chemically and conceptually distinct pathways of using either multiple DNA strand crossover motifs or an inorganic nanoparticle template were developed to impart directionality of DNA hybridization.\textsuperscript{79}
Figure 1-5. Structures of DX and DX+J (junction) motifs (A) and their assembled 2D lattices (B). 3D lattice structures are constructed from three-point star tiles (C) or tensegrity tiles (D). Modified from reference 118.

The Seeman group designed and synthesized a double-crossover DNA motif, called DX tile, which comprised two crossover events in two DNA helixes (Figure 1-5A).\textsuperscript{80} The DX tile increased the structure rigidity of single DNA duplex and led to the formation of many well-defined 2D structures with controlled size and shape.\textsuperscript{81,82} For example, a series of DX tiles with proper designed sticky ends were synthesized to yield alternating AB or ABCD arrays (Figure 1-5B).\textsuperscript{83} Since then, a variety of DNA tiles with multiple crossover structures were investigated.\textsuperscript{84} The Mao group designed a three-point-star tile, which can be assembled into tetrahedron, dodecahedron, and buckyball structures in a concentration dependent-manner (Figure 1-5C).\textsuperscript{85} In 2009, the same group developed a 3D tensegrity tile, in which each edge (DNA helix) was not on
the same plane and pointed to different directions. Such tensegrity tiles were used to build a rhombohedral crystal, which showed a well-defined 3D conformation (Figure 1-5D).86

**Figure 1-6.** (A) A genomic DNA (black) is folded by short DNA staples (colored) to form arbitrary structures, such as star, smile face and triangle. (B) A 3D box DNA origami constructed from six DNA sheets. Adapted with permission from references 87 and 92.

In 2006, Rothemund developed a new method of constructing DNA nanostructure, called DNA origami. Instead of using tiles, a single, long DNA strand was folded into well-defined structure by using hundreds of short DNA strands as “staples” (Figure 1-6A).87 These staple strands can precisely hybridize to certain sections of the targeted DNA and determine the final structure. A series of complex 2D structures, such as triangle, star, smile face, were synthesized using DNA origami.88 Due to the high complexity of designing DNA staple sequences and
determining the hybridization sites, a computational software was developed to aid the precise control of DNA origami structures.\textsuperscript{89} Since 2009, scientists have successfully expanded the structure library of DNA origami from 2D to 3D.\textsuperscript{90,91} For example, a box structure with controlled lid-on and off was constructed by connecting six planar DNA origami sheets (Figure 1-6B).\textsuperscript{92} These origami structures have found applications in nanoparticle arrangement, sensors, proteomics and biomedicine.\textsuperscript{93-95} Even though origami is different from the tile-based method, the rigidity in DNA origami is also derived from multiple DNA hybridization events.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image.png}
\caption{Nanoparticle-DNA conjugates can be assembled to different crystal lattices with controlled structure parameters. Modified with permission from references 101 and 104.}
\end{figure}

Another approach of increasing the rigidity and therefore directionality of DNA-DNA interaction is by using inorganic nanoparticle as templates to immobilize and orient the DNA strands. By attaching DNA strands to the surface of inorganic particles with high density, the driving force of lattice formation is multiple DNA hybridization while the metallic core provides the necessary rigidity. This concept was first demonstrated in 1996 by the Mirkin group.\textsuperscript{96} The surface of gold nanoparticles was functionalized with thiol-modified DNA strands in a very high density, forming a spherical nucleic acid (SNA) structure. Then two kinds of SNA carrying complementary DNA strands were allowed to hybridize with each other to generate macroscopic network. Early attempts only resulted in structures with limited order due to the poor control of
the assembly process. After a decade of investigation on the fundamental properties of SNA structures and the interactions between SNA,\(^{97-99}\) the combination of annealing and weak DNA interactions between particles have been demonstrated to be the key steps to synthesize well-defined crystalline lattices.\(^{100,101}\) The idea of using short sticky ends in Seeman’s method was adopted in the design of SNA interactions. These sticky ends (usually 4 to 8 bases) enable the reorganization of SNA into a thermodynamically favored state, which is the lattice structure. Annealing can then drive the formation of thermodynamically stable, long-range ordered structures.\(^{102,103}\) By tweaking the parameters in the SNA building blocks (DNA length, atom element, particle size, DNA sequence, etc.), a large library of crystal structures with a diversity of compositions could be synthesized (Figure 1-7).\(^{101,104,105}\) For example, the inorganic nanoparticle could be replaced with silver, Fe\(_2\)O\(_3\), CdSe, and silica particles with different sizes and shapes (sphere, rod, triangle, disk).\(^{106-111}\) The nucleic acid shell can also be made by all forms of oligonucleotides, such as DNA, RNA or LNA.\(^{112-114}\) These studies have paved the way for building predictable lattices with controlled size, composition, and crystallographic symmetry.\(^{104}\) These materials have already found application in a number of fields spanning medical diagnostics and therapeutics, catalysis, energy, detection/sensing, etc.\(^{115-117}\)

The current two strategies of directional DNA assembly each have different limitations. The DNA tile method can be used to achieve a high level of structural diversity, e.g. arbitrary structures in 2D and 3D, but the chemical composition is limited to pure nucleic acid. On the other hand, the nanoparticle templating method opens up a great deal of compositional diversity, but structural diversity is limited to the repeating patterns of crystal lattices. A new methodology is needed to achieve both structural and material diversities, which is the ultimate goal of DNA nanotechnology as “the finest possible level of control over the spatial and temporal structure of
matter: Putting what you want where you want in the three dimensions, when you want it there. (Seeman, 2010)"

In this thesis, we test the possibility of using DNA-polymer conjugates as a new type of building blocks to achieve both structural and compositional diversity. DNA-polymer conjugates are an important class of materials, having both biological properties of DNA and the materials properties of the polymer. The polymer component can be highly diverse, ranging from biopolymers, bandgap polymers, to high performance polymers. Thus, such conjugates are useful in a broad range of applications, spanning drug delivery, gene therapy, energy harvesting, and detection/sensing. Polymers of various architectures (block, dendritic, star, hyperbranched, etc.) and compositions have been utilized in the synthesis of DNA conjugates. However, a strategy to program the ordered assembly of polymer molecules in a sequence-defined fashion is still very much in the embryonic stage. Even in the simplest form, directional assembly to form 1D structures (i.e. lines), represents a significant challenge, because sphere-like particles (such as SNA) uniformly interact across their surfaces, which leads to three-dimensional growth. Therefore, an opportunity exists to use DNA as a functional group equivalent to create a series of limited-valency macromonomers for topologically defined supramolecular polymerization. Because DNA is highly programmable, one can in principle create a very large number of “functional groups” that can work in one pot.

In Chapter 5, we design and synthesize bottle brush polymer-DNA conjugates to demonstrate programmable, reversible, and sequence-controlled supramolecular polymerization. The rigidity of DNA-DNA base pairing is achieved by the steric hindrance of densely packed brush polymer side chains, and we show the possibility of realizing DNA-programmed directional head-to-tail connection of bottlebrush polymers for the first time. The assembled polymer constructs
with precise connectivity of individual macromolecules have the potential to give rise to novel multiscale materials with unique electronic, optical, and magnetic properties that can be tuned by their dimensions and cooperativity. Such capability is expected to lead a rapid expansion of new materials with emergent properties otherwise difficult or impossible to obtain.
1.3 References.


(55) Benjaminsen, R. V.; Mattebjerg, M. A.; Henriksen, J. R.; Moghimi, S. M.; Andresen, T. L. The possible “proton sponge” effect of polyethylenimine (PEI) does not include change in lysosomal pH. *Mol. Ther.* **2013**, *21*, 149.


Chapter 2

Providing Oligonucleotides with Steric Selectivity by Brush Polymer-Assisted Compaction

This Chapter is based on a published paper with the same title.


Author contributions: X. L. and K. Z. conceived the study, designed the experiments, analyzed the data and wrote the manuscript. X. L. synthesized the materials and collected the in vitro data. H. T., K. S. and M. A. conducted the in vivo imaging experiment and analyzed data. F. J., X. T. and D. S. synthesized and purified some of the DNA strands. All authors edited the manuscript.
2.1 Introduction.

Nucleic acids and derivatives have been envisioned as biopharmaceutical agents in many forms of therapies, including oncolytic virotherapy,\(^1\) suicide gene therapy,\(^2\) anti-angiogenesis,\(^3\) therapeutic vaccines,\(^4\) and RNA interference/antisense gene silencing therapies.\(^5\) However, unlike antibodies, nucleic acids are not directly part of the natural biological defense system. Thus, utilizing them as therapeutics faces an uphill battle against stability and delivery issues,\(^6\) and many sequence- and/or chemical structure-specific, non-hybridization activities, such as stimulation of the immune system and coagulopathies.\(^7\)

For oligonucleotides, while nuclease stability can be improved by chemical modification of the phosphodiester backbone, e.g. phosphorothioates,\(^8\) LNAs,\(^9\) PNA,\(^10\) morpholinos,\(^11\) and ribose 2’ O-alkyl modifications,\(^12\) etc., problems such as immune system stimulation and delivery to target site still plague development. Cationic materials (e.g. polymers, peptides, nanoparticles, liposomes, etc.) have been designed to form polyplexes with nucleic acids to assist cell entry, endosomal release, and co-delivery of a drug, etc.\(^13\) However, these materials remain largely limited to in vitro applications, because their benefits \textit{in vivo} are oftentimes overshadowed by carrier-induced side effects.\(^14\)

Recently, a class of nucleic acid nanostructure consisting of densely arranged oligonucleotides (termed spherical nucleic acids, SNA) has emerged.\(^15\) Despite the steric created by the high density arrangement, SNAs remain hybridizable with complementary strands.\(^16\) Furthermore, SNAs show increased stability against enzymatic degradation, and can enter cells and regulate cellular gene expression without using a polycationic carrier.\(^17\)
Inspired by the structure of the SNA, we have designed and synthesized a novel form of brush polymer-DNA conjugate, termed pacDNA (polymer-assisted compaction of DNA). We hypothesize that that the “compaction” of DNA by high density side chains of brush polymers can provide the DNA with selective accessibility, favoring a complementary DNA strand to species with larger cross-section dimensions such as proteins (Scheme 2-1). The majority of unwanted, non-hybridization side effects are preceded by protein recognition of the oligonucleotide, be it degradation, toll-like receptor activation and interferon response, and inhibition of the coagulation cascade. In principle, the steric selectivity resulting from the densely compacted structure of pacDNA can circumvent many of the side effects associated with protein binding. Given that the mechanism of DNA shielding is by steric hindrance (as opposed to electrostatic polyplexation), polymer compositions not typically considered for DNA protection can now be utilized. For a

**Scheme 2-1.** Mechanism for the steric selectivity of pacDNA.
proof of concept, we choose a common biocompatible, low fouling polymer, poly(ethylene glycol) (PEG), as the side chains of the pacDNA.\textsuperscript{18}

![Scheme 2. Schematics for pacDNA synthesis.]

At least two parameters must be established to provide the desired binding selectivity and biological functionalities. First, the relative lengths of the PEG and the DNA must be such that the DNA can receive sufficient polymer coverage.\textsuperscript{19} Second, the PEG side chains must be dense enough to create steric congestion, requiring the brush to have sufficiently high degrees of polymerization along the backbone. Owing to recent advances of ring-opening metathesis polymerization (ROMP) and bioconjugation chemistries, control over these parameters can be easily accomplished.\textsuperscript{20}

\textbf{2.2 Results and Discussion}

To systematically probe the relationship between the structural parameters of the pacDNA and its steric selectivity, a library of six pacDNA structures has been synthesized by conjugating two DNA strands (10 or 15 bases; DNA-1: 5'-NH\textsubscript{2}-CCC AGC CCT C-F-3' and DNA-2: 5'-NH\textsubscript{2}-CCC AGC CTT CCA GCT-F-3') with three brush polymers (brushes a-c: side chain PEG \(M_n = 2, 3, 5\) kDa, respectively; PDI < 1.05). The brushes are synthesized via sequential ROMP of norbornenyl hydroxysuccinimidylic ester (N-NHS) and norbornenyl PEG (N-PEG), to yield a diblock architecture (pN-NHS\textsubscript{2,3}-b-pN-PEG\textsubscript{32-38}, Scheme 2-2 and Table 2-1). The short first block
containing NHS esters is incorporated for subsequent coupling with amine-modified DNA strands. Gel permeation chromatography (GPC) shows narrow molecular weight distribution for all brush polymers (Figure 2-1). Infrared spectroscopy shows characteristic vibrations of the NHS groups at 1739 cm$^{-1}$, 1780 cm$^{-1}$, and 1807 cm$^{-1}$ (Figure 2-2), confirming their successful incorporation into the brushes.$^{18}$ To quantify the number of reactive NHS esters available for coupling, polymers and an excess amounts of fluorescein 5-thiosemicarbazide are allowed to react overnight in dimethylformamide (DMF). After removing the unreacted fluorescein by dialysis, optical absorbance was measured and compared to a standard curve to calculate the number of NHS groups per polymer. For brushes a-c, there are 2.1, 2.0 and 2.4 NHS esters, respectively.

Table 2-1. GPC analyses for the brush polymers used.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Composition</th>
<th>M$_n$ (kDa)</th>
<th>M$_w$ (kDa)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brush-a</td>
<td>pN-NHS$<em>2$-b-pN-PEG(2k)$</em>{38}$</td>
<td>76.3</td>
<td>88.8</td>
<td>1.18</td>
</tr>
<tr>
<td>Brush-b</td>
<td>pN-NHS$<em>2$-b-pN-PEG(3k)$</em>{32}$</td>
<td>96.2</td>
<td>106.2</td>
<td>1.10</td>
</tr>
<tr>
<td>Brush-c</td>
<td>pN-NHS$<em>2$-b-pN-PEG(5k)$</em>{35}$</td>
<td>174.3</td>
<td>196.4</td>
<td>1.13</td>
</tr>
<tr>
<td>Brush-d</td>
<td>pN-NHS$<em>2$-b-pN-PEG(10k)$</em>{30}$</td>
<td>310.9</td>
<td>350.0</td>
<td>1.13</td>
</tr>
</tbody>
</table>

Figure 2-1. DMF GPC chromatograms of brushes a-d (orange, red, blue, and black, respectively).
Figure 2-2. Overlaid IR spectra of brush-b and its constituent monomers, showing retention of the NHS ester carbonyl vibrations in the brush polymer.

For conjugation to the brushes, the DNA strands are designed to have a 5' amine group. A fluorescein tag is also incorporated at the 3' to facilitate tracking and quantification. The conjugation is carried out in pH 8.0 bicarbonate buffer at 0 °C using an excess of DNA, and the products are purified by aqueous GPC equipped with a photodiode array detector. The conjugates have a much larger molecular weight compared with free DNA, and thus the two components have baseline separation (Figure 2-3). Agarose gel electrophoresis and GPC chromatograms for purified pacDNA show no residual free DNA (Figures 2-4 and 2-5). Quantification of the amount of DNA strands per brush by peak integration indicates that there are 1-2 strands for each pacDNA (Table 2-2), which is consistent with the numbers of reactive NHS ester groups. Dynamic light scattering (DLS) shows that pacDNAs have number-average hydrodynamic diameters between 25±5 nm and 32±8 nm, with narrow size distributions (PDI < 0.1, Table 2-2 and Figure 2-6A). Transmission
electron microscopy (TEM) shows a spherical morphology for all pacDNAs with a dry-state diameter in the range of 27±4 to 31±5 nm (Figure 2-6B). The spherical morphology is not surprising, because the brush polymers have relatively long side chains and short backbone length, making them structurally analogous to star polymers.²²

**Figure 2-3.** Aqueous GPC traces for brushes a-c (top row) and their reaction mixtures with DNA-1 to form pacDNAs (bottom row). Because the total amounts of DNA and brush polymer are known, the number of DNA strands per pacDNA can be calculated from the relative peak integrations of the pacDNA and free DNA in the 488 nm channel (fluorescein).

**Figure 2-4.** Aqueous GPC chromatograms of purified pacDNAs compared with free DNAs (black and red lines) and free brushes (dashed lines).
Figure 2-5. Agarose gel (1%) electrophoresis of fluorescein-labeled free DNAs and pacDNAs (fluorescein filters applied), showing that free DNA is absent from their pacDNA conjugates.

Figure 2-6. (A) Number-average size distributions of pacDNAs, as determined by DLS. Numeric diameter values are tabulated in Table 2-2. (B) TEM images of selected pacDNAs (a1, b1 and c1). Scale bar is 200 nm.
Table 2-2. Number of DNA strands per pacDNA.

<table>
<thead>
<tr>
<th>pacDNA</th>
<th>a1</th>
<th>b1</th>
<th>c1</th>
<th>a2</th>
<th>b2</th>
<th>c2</th>
</tr>
</thead>
<tbody>
<tr>
<td># of DNA/pacDNA</td>
<td>2.3</td>
<td>1.4</td>
<td>1.5</td>
<td>1.5</td>
<td>1.4</td>
<td>1.9</td>
</tr>
<tr>
<td>$D_{h(o)}$ (nm)</td>
<td>25±5</td>
<td>30±7</td>
<td>32±8</td>
<td>25±5</td>
<td>33±8</td>
<td>34±8</td>
</tr>
</tbody>
</table>

To examine whether the brush component inhibits DNA hybridization, we adopted a fluorescence quenching assay,\textsuperscript{23} in which a quencher (dabcyl)-linked complementary DNA strand is added to fluorescein-tagged pacDNA. The rate at which fluorescence decreases is an indicator of the kinetics for duplex formation (Figure 2-7A). All pacDNAs are mixed with 2 equiv. of complementary dabcyl-DNA in phosphate buffered saline at room temperature. A dummy strand (DNA-7) that is unable to form a duplex with the pacDNA is used as a control. Fluorescence is measured immediately upon mixing and every 3 sec for 60 min. Strikingly, all pacDNAs hybridize immediately with their respective antisense dabcyl-DNA strands (Figure 2-7B), with little to no difference in the kinetics between the pacDNA and the free DNA. When the dummy dabcyl-DNA control is used in the presence of free DNA or pacDNA, fluorescence signals remain constant, ruling out non-specific binding. There are, however, differences in the hybridization kinetics between DNA-1 and DNA-2, and between the pacDNAs containing them. This observation is likely because DNA-2 can form a hairpin structure (calc $T_m = 34.1$ °C). The intramolecular secondary structure stabilizes single-strand conformation and increases the energy barrier for intermolecular hybridization, which then slows the hybridization kinetics.\textsuperscript{24} The thermodynamics of the duplexes are not significantly changed, as manifested by the nearly identical melting transitions for pacDNA and free DNA (0.1–1.8 °C, 0.5 M NaCl, Figure 2-9 and Table 2-3).
Figure 2-7. (A) Schematics of assays for determining DNA hybridization and nuclease degradation kinetics. (B) Hybridization kinetics for pacDNA vs free DNA. (C) Nuclease degradation kinetics for pacDNA vs free DNA.
**Figure 2-8.** Half-lives (A and C) and initial rates (B and D) for the enzymatic degradation of free DNA and pacDNA by DNase I. Numeric values are listed in Table 2-4.

**Figure 2-9.** Melting transitions of free DNA duplexes and pacDNAs. Numeric $T_m$ values are presented in Table 2-3.

**Table 2-3.** Duplex melting temperature of free DNAs and pacDNAs.

<table>
<thead>
<tr>
<th>Name</th>
<th>DNA-1</th>
<th>pacDNA-a1</th>
<th>pacDNA-b1</th>
<th>pacDNA-c1</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_m$ (°C)</td>
<td>64.3±0.3</td>
<td>63.3±0.2</td>
<td>63.2±0.2</td>
<td>62.5±0.2</td>
</tr>
<tr>
<td>Name</td>
<td>DNA-2</td>
<td>pacDNA-a2</td>
<td>pacDNA-b2</td>
<td>pacDNA-c2</td>
</tr>
<tr>
<td>$T_m$ (°C)</td>
<td>64.3±0.3</td>
<td>64.3±0.3</td>
<td>64.3±0.3</td>
<td>64.3±0.3</td>
</tr>
</tbody>
</table>
To test if pacDNAs are able to sterically inhibit proteins from accessing their DNA component, we utilize DNase I as a model protein to act on fluorescein-pacDNAs that are pre-hybridized with dabcy1-DNA. The fluorescence of the pacDNAs are quenched when hybridized. When DNase I is introduced, the duplexes are degraded, and the fluorophores are released, leading to an increase of fluorescence (Figure 2-7A). While free DNA-1 and DNA-2 duplexes are both degraded rapidly, with half-lives of 9.3±4.2 min and 7.3±1.7 min, respectively, all pacDNA conjugates show enhanced stability against the enzyme, as evidenced in prolonged half-lives and reduced initial rates (Figures 2-7C and 2-8). The best among these is pacDNA-c1, which has the longest PEG side chain (5 kDa) and the shorter DNA component, showing ca. 14.5x longer half-life and ca. 0.09x of the initial enzymatic activity. In contrast, pacDNA-a2, having the shortest PEG side chains (2 kDa) and the longer DNA-2, shows only ca. 2.4x increase in half-life and 0.47x of the initial degradation rate.

We anticipate that, for oligonucleotides considered for therapeutic purposes (typically 13-25 mers) the PEG side chain MW needs to be adjusted accordingly for optimal selectivity. Therefore, we synthesized and tested pacDNA-d2, which has a PEG side chain MW of 10k Da. As predicted, pacDNA-d2 shows significantly enhanced protection compared with pacDNA-c2, with 21x longer half-life and 0.07x initial degradation rate compared with those of free DNA, but its binding kinetics with complementary strands remains nearly unaffected. These results indicate that, provided with appropriate design parameters, the pacDNA can achieve substantial selectivity for DNA hybridization vs. protein recognition. Such selectivity is possible on the molecular level. First, the DNA is ca. 18-22 ångströms wide, while proteins are generally 3-10 nm in hydrodynamic diameter, giving complementary DNA a kinetic advantage for access. Second, upon hybridization, the dsDNA does not occupy additional space relative to ssDNA; the hydrodynamic
volume that it occupies does not change significantly.\textsuperscript{27} In contrast, in order for a protein to access the ssDNA confined within the dense side chains, steric congestion would have to increase. Both kinetics and thermodynamics favor DNA hybridization rather than binding with a protein.

Figure 2-10. Blood clotting time of free DNA, brush-c, and pacDNAs vs DNA concentration in (A) aPTT and (B) PT assay.

To test the inhibition of protein association in a more complex biological environment, we examined the anticoagulation properties of pacDNA versus free DNA in human plasma. Oligonucleotide sequences exhibit nonspecific and specific interactions with serum proteins, including thrombin, resulting in the prolongation of activated partial thromboplastin time (aPTT) and prothrombin time (PT).\textsuperscript{28} This unwanted interaction of oligonucleotides with blood components remains a problem for intravenous gene targeting; effective control of thrombin activity and coagulation cascade is beneficial in therapeutic applications.\textsuperscript{29} To test if pacDNA can restrict the access of pro/thrombin, an identified thrombin-binding aptamer (DNA-3) is used to form pacDNAs (a3, b3, and c3),\textsuperscript{7c} and aPTT and PT assays are carried out. Although free DNA-3 shows a marked anticoagulation behavior, doubling and tripling the coagulation times in the aPTT and PT assay at 4000 nM, respectively, all pacDNAs exhibit only slight increases in clotting times in both assays compared to those of free brush-c (Figure 2-10). The data clearly demonstrates that
pacDNA is able to inhibit the propensity of DNA to bind with serum proteins and to mask the anticoagulation effect of the DNA.

**Figure 2-11.** (A) Near-IR imaging of live mice over 24 h (Cy5.5 channel). (B) *Ex vivo* imaging of tissues from numbered mice (T-tumor, H-heart, Lg-lung, K-kidney, S-spleen, Lv-liver). (C) Dual-channel imaging of organs from mice treated with dual-labeled pacDNA (polymer: Cy5.5, DNA: Cy3).
Finally, because the physical size of the brush can be tuned by controlling the degree of polymerization and side-chain length, it is possible for pacDNA to take advantage of the enhanced permeation and retention effect (EPR) for passive cancer targeting. Being able to target cancer via EPR would necessitate sufficient blood circulation times, which in turn require appropriate pacDNA size (10-100 nm) and low opsonization of the pacDNA surface. PacDNA-c1 and –a2 are used to study in vivo bio-distribution, because these two structures provide a contrast in protein shielding capabilities. To enable in vivo imaging, a near-infra red tag (Cy5.5) is incorporated into the pacDNA by consuming a small amount of the brush NHS ester groups (< 10% by mol). The DNA is modified with a Cy3 tag to allow for independent tracking. For animal model, xenograft mice with orthotopically implanted mouse breast cancer cells (4T1) in the right mammary fat pad are used. In addition to pacDNAs, free dye, free DNA, and brush polymers are used as controls. For pacDNA-c1 and its parent polymer (brush-c), the nanostructures appear gradually on the surface of the mice after 2 h and persists for 24 h (after which mice are sacrificed), suggesting good blood circulation (Figure 2-11A). Significant tumor uptake is also evident in images obtained after 8 h, which is confirmed by ex vivo imaging of tissues at 24 h (Figure 2-11B). It can be seen that pacDNA-c1 show higher liver uptake than its polymer brush counterpart, suggesting that the DNA is not completely shielded. Images of both Cy3 and Cy5.5 channels show that the signals from the DNA and the polymer components are colocalized in the tumor, which is indicative that the DNA is successfully delivered to the tumor (Figure 2-11C).
Figure 2-12. A) Near-IR imaging over 24 h (Cy5.5 channel) of live mice injected in the tail vein with free Cy5.5, brush-a, and pacDNA-a2. B) Ex vivo imaging of tissues from numbered mice (T-tumor, H-heart, Lg-lung, K-kidney, S-spleen, Lv-liver).

In contrast, lacking the shielding effect from the brush polymer, free DNA is rapidly cleared by the liver. On the other hand, pacDNA-a2 shows primarily hepatic uptake and minimal tumor accumulation, while its parent polymer (brush-a) shows moderate levels of tumor uptake but is
largely cleared by the kidney (Figure 2-12). One interpretation of these results is that pacDNA-a2 does not have sufficient shielding of the DNA, and the exposed DNA leads to the recognition and capture by liver endothelial cells.\textsuperscript{31} For the parent polymer brush-a, which has a $M_n$ of only 76.3 kDa, renal clearance \textit{via} glomerular filtration is possible.\textsuperscript{32} These data are consistent with the fluorescence-based protein accessibility analyses (\textit{vide supra}), and suggest that, when designed appropriately, the pacDNA can be a viable platform for systemic oligonucleotide delivery.

\textbf{2.3 Conclusion.}

A novel form of brush polymer-DNA nanostructure has been developed. The densely packed side chains of the brush shield the DNA from proteins but allow unhindered DNA hybridization to take place. These structures stand apart from polycationic carrier based approaches because the mode of NA protection is based on steric compaction instead of polyplexation, allowing noncharged polymers to be used. The pacDNA is expected to be minimally immunostimulative because the recognition of possible pathogen-associated patterns in the DNA sequence (e.g., CpG) by pattern-recognition receptors is similarly sterically hindered. We anticipate that pacDNA will exhibit significantly better biopharmaceutical characteristics compared to naked or polyplexed DNA. These data also imply that one should look beyond the chemical and biological properties of the NA and the cocarrier for oligonucleotide-based therapies to include a careful consideration of the NA’s immediate local environment.
2.4 Materials and Experimental Procedures.

Phosphoramidites and supplies for DNA synthesis were purchased from Glen Research Co. ω-amine terminated poly(ethylene glycol) methyl ether (M₄=2 kDa, PDI=1.05) was purchased from Polymer Source Inc., Montreal, Canada. All other materials were purchased from Sigma-Aldrich Co., VWR International LLC., or Fisher Scientific Inc., and used without further purification unless otherwise indicated. DLS data were acquired from a MALVERN Zetasizer Nano-ZSP. MALDI-ToF measurements were carried out on a Bruker Microflex LT mass spectrometer (Bruker Daltonics Inc., MA, USA). Ultraviolet-visible spectroscopy (UV-Vis) data were obtained on a Cary 4000 UV-Vis spectrophotometer (Varian Inc., CA, USA). Fluorescence spectroscopy was measured on a Cary Eclipse fluorescence spectrophotometer. ¹H and ¹³C NMR spectra were recorded on a Varian 400 MHz spectrometer (Varian Inc., CA, USA). Chemical shifts (δ) were reported in ppm. Infrared (IR) spectra were obtained on a Bruker Tensor FT-IR spectrometer (Bruker Corporation). Gel permeation chromatography (GPC) measurements were performed on a TOSOH EcoSEC HLC-8320GPC system equipped with an RI and an UV-Vis detector and with a flow rate of 0.5 mL/min. N, N-Dimethylformamide (DMF) with 0.2 M LiBr was used as the eluent. The GPC was calibrated based on polystyrene standards (706 kDa, 96.4 kDa, 5970 Da, 500 Da). Gel electrophoresis was performed using 1% agarose gel in 0.5x Tris/Borate/EDTA (TBE) buffer with a running voltage of 100 V. Gel images were acquired on an Alpha Innotech Fluorochem Q imager. For TEM analysis, samples were deposited on carbon-coated copper grids for 5 min before being carefully wicked away by filter paper. The grids were then stained by pipetting 10 μL of 1.5% uranyl acetate directly onto the grid. The stain was allowed to stay for 3 min before being wicked away. All TEM samples were imaged on a JEOL JEM 1010 electron microscope utilizing an accelerating voltage of 80 kV.
Oligonucleotide synthesis

Oligonucleotides were synthesized on a Model 391 DNA synthesizer (Applied Biosystems, Inc., Foster City, CA) using standard solid-phase phosphoramidite methodology. Oligonucleotides were purified by reverse-phase high performance liquid chromatography (HPLC, Waters Corporation) equipped with a C-18 column (Thermo Scientific Inc., 3 μm, 250 mm × 4 mm). All DNA strands were cleaved from the CPG support by using aqueous ammonium hydroxide (28-30% NH₃ basis) at 55 ºC for 17 h. The dimethoxytrityl (DMT) protecting group was removed by treatment with 20% acetic acid in H₂O for 1 h, followed by extraction with ethyl acetate for three times. The successful syntheses of DNA sequences were verified by MALDI-TOF MS.

Table 2-4. Oligonucleotide sequences used in this study.

<table>
<thead>
<tr>
<th>Name of Strand</th>
<th>Application</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA-1</td>
<td>Couples with p(N-NHS)₂-b-p(N-PEG)ₙ</td>
<td>5'-NH₂-CCC AGC CCT C - fluorescein-3'</td>
</tr>
<tr>
<td>DNA-2</td>
<td>Couples with p(N-NHS)₂-b-p(N-PEG)ₙ</td>
<td>5'-NH₂-CCC AGC CTT CCA GCT-fluorescein-3'</td>
</tr>
<tr>
<td>DNA-3</td>
<td>Couples with p(N-NHS)₂-b-p(N-PEG)ₙ</td>
<td>5'- NH₂- GGT TGG TGT GGT TGG -3'</td>
</tr>
<tr>
<td>DNA-4</td>
<td>Thrombin binding aptamer</td>
<td>5'-GGT TGG TGT GGT TGG-3'</td>
</tr>
<tr>
<td>DNA-5</td>
<td>Antisense strand of DNA-1</td>
<td>5'-Dabcyl-GAG GGC TGG G-fluorescein-3'</td>
</tr>
<tr>
<td>DNA-6</td>
<td>Antisense strand of DNA-2</td>
<td>5'-Dabcyl-AGC TGG AAG GCT GGG-3'</td>
</tr>
<tr>
<td>DNA-7</td>
<td>Dummy dabcyl DNA</td>
<td>5'-NH₂-TTT ACT-dabcyl AAC CTT TCC GTC GCA GCT AAA-3'</td>
</tr>
<tr>
<td>DNA-8</td>
<td>Cy3-modified strand for in vivo imaging</td>
<td>5'-NH₂-CCC AGC CCT C –Cy₃-3'</td>
</tr>
<tr>
<td>DNA-9</td>
<td>Cy5.5-modified strand for in vivo imaging</td>
<td>5'-Cy5.5-CCC AGC CCT C-3'</td>
</tr>
</tbody>
</table>
Figure 2-13. RP-HPLC and MALDI-ToF MS data for all DNA strands used in this study. The asterisk-labeled peaks, which represent DMT-linked target strands, were collected and deprotected by using 20% acetic acid. MALDI-ToF MS spectra were obtained using purified strands.

General method for synthesizing brush copolymers

Modified 2nd generation Grubbs’ catalyst was synthesized based on a published protocol.33 The catalyst (0.02 M) was dissolved in DCM in a 5 mL Schlenk flask. All the monomers (0.04 M) were dissolved in DCM in two different 5 mL Schlenk flasks. All reagents were separately
degassed by 3x freeze-pump-thaw cycles. The flask containing N-NHS (5 equiv.) was cooled to -20 °C by using ice-salt bath, to which a solution containing modified Grubbs’ catalyst (1 equiv.) was added via a microsyringe. The reaction mixture was stirred for 30 min (TLC shows complete consumption of the N-NHS), before the second monomer, N-PEG (40 equiv.), was added. The reaction mixture was further stirred for 2 h. At the end of the reaction, several drops of ethyl vinyl ether (EVE) were added to the mixture and the reaction was stirred overnight. The mixture was then concentrated and precipitated into ethyl ether 3x and the precipitant was dried in vacuo.

**Quantitation of the available NHS groups in brush polymers**

In a round bottom flask, a brush polymer (67 nmol) was dissolved in 2.0 mL DMF, to which fluorescein 5-thiosemicarbazide (1.43 mg, 3.4 µmol) and DIPEA (0.44 mg, 3.4 µmol) were added. The reaction mixture was allowed to stir at room temperature overnight, before being dialyzed against NaCl solution (0.15 M) using dialysis tubing (MWCO 6-8 kDa) for 48 h. The concentration of fluorescein in the polymer solution was determined by UV-Vis spectroscopy of the brush polymer solution and comparison with a standard curve (Figure 2-14). The number of fluorescein molecules per polymer was calculated based on the known polymer concentration. Approximately 2.1, 2.0, and 2.4 fluorescein tags were attached to brushes a-c, respectively.
Figure 2-14. Determination of the numbers of NHS ester groups per brush polymer by UV-Vis absorbance.

**General method for synthesizing pacDNA**

Brush polymers were dissolved in anhydrous DMSO (ca. 100 µL) to give a final concentration of 1.0 mM. Amine-modified DNA (15 nmol) was dissolved in 10 µL aqueous buffer containing NaHCO₃ (50 mM) and NaCl (1.0 M). The polymer solution (5 µL) was added to the DNA solution, and the mixture was shaken gently overnight at 0 °C on an Eppendorf Thermomixer. The reaction mixture was then dialyzed against Nanopure™ water using a MINI dialysis unit (MWCO 3500 Thermo Fisher) for desalting. The dialysate was further subjected to aqueous GPC purification. The fractions containing the conjugate was collected and dialyzed against Nanopure™ water to remove NaNO₃. The final solution was lyophilized to yield a light-green powder.

**General method for the synthesis of dual-labeled pacDNA for in vivo imaging**

Brush polymers and Cy5.5 hydrazide were dissolved in anhydrous DMSO in separate
flasks to give stock solutions with a final concentration of 1 mM. DNA-1’ was dissolved in an aqueous buffer containing NaHCO₃ (50 mM) and NaCl (1 M) with a concentration of 1 mM. Then, DNA-1’ (200 µL), the brush solution (100 µL), and the Cy5.5 solution (20 µL) were rapidly mixed in a microcentrifuge tube and shaken gently at 0 ºC overnight on an Eppendorf Thermomixer. For the brush-c polymer control (no DNA), only the polymer and Cy5.5 stock solutions were mixed. The reaction mixture was then dialyzed first against 0.5 M NaCl solution, and then against Nanopure™ water, using MINI dialysis units (MWCO 3500 Da) to remove residue Cy5.5 and DMSO. The aqueous solution was further purified by aqueous GPC. The fraction containing the conjugate was collected and desalted by dialysis. The final solution was lyophilized to yield a purple powder. UV-Vis spectroscopy indicated that there are 0.2 Cy5.5 and 1.8 DNA-1’ per brush-c (Figure 2-15)

![UV-Vis spectra of dual labeled pacDNA-c1 (DNA: Cy3, brush: Cy5.5).](image)

**Figure 2-15.** UV-Vis spectra of dual labeled pacDNA-c1 (DNA: Cy3, brush: Cy5.5).

**Hybridization kinetics assay**

All free DNA and pacDNA were each dissolved in microcentrifuge tubes in PBS buffer
(pH = 7.4) to give final concentrations of 100 nM. Each solution (1 mL) was transferred to a fluorescence cuvette, to which a complementary dabcyl-DNA strand or a dummy DNA strand (2 equiv.) was added via 1 µL of PBS solution and thoroughly mixed. The fluorescence of the mixture (ex = 490 nm, em = 520 nm) was monitored before the mixing every 3 sec using a Cary Eclipse fluorescence spectrometer, and the monitoring was continued for 60 min after the mixing. The endpoint is determined by adding a large excess of complementary dabcyl-DNA to the mixture followed by incubation for an extended period of time (> 1 h). The kinetics plots are normalized to the end points determined for each sample.

**Nuclease degradation kinetics assay**

All free DNA and pacDNA (1 µM) were each mixed with their respective complementary dabcyl-labeled DNA (2 µM) in PBS buffer. The mixtures were heated to 80 °C and allowed to cool slowly to room temperature in a thermally insulated container during a period of 10 h. The mixture was then diluted to 100 nM in assay buffer (10 mM Tris, 2.5 mM MgCl₂, and 0.5 mM CaCl₂, pH = 7.5), and 1 mL of the mixture was transferred to a fluorescence cuvette which was mounted on a fluorimeter. DNase I (Sigma-Aldrich) was then added and rapidly mixed to give a final concentration of 0.1 unit/mL. The fluorescence of the samples (ex = 490 nm, em = 520 nm) was measured immediately and every 3 sec for 6 h. The endpoint was determined by adding a large excess of DNase I (ca. 2 units/mL) to the mixture, and the fluorescence was monitored until no additional increase was observed. The kinetics plots were normalized to the endpoints determined for each sample, and all experiments were performed in triplicates.

**dsDNA melting transitions**

Free DNA and pacDNAs (1000 nM) were mixed with 2 equiv. of complementary dabcyl-
labeled DNA in 0.5 M NaCl solution. The mixtures were heated to 80 °C and allowed to cool slowly to room temperature in a thermally insulated container during a period of 10 h. The samples were then added into a 96 well plate. The melting curves were measured on a qPCR instrument (CFX96 Touch™ Real-Time PCR Detection System, Biorad, USA). The temperature increases from 30 °C to 90 °C at a rate of 0.2 °C/min. All experiments were performed in triplicates.

**Activated partial thromboplastin time (aPTT) assay and prothrombin time (PT) assays**

A model BFT-2 coagulometer (Siemens, USA) was used to run both activated partial thromboplastin time (aPTT) assay and prothrombin time (PT) assay to determine the clotting times for each sample. For the aPTT assay, 50 μL of normal human plasma was incubated with 50 μL TriniClot aPTT S (Trinity BioTech, Ireland) at 37 °C for 5 min before adding the controls/samples. The mixture was incubated at 37 °C for another 5 min, and CaCl₂ (50 μL) was added to initiate the assay. For the PT assay, 50 μL of normal human plasma was incubated with controls/samples at 37 °C for 5 min. TriniClot PT Excel reagent (Trinity BioTech, Ireland) (100 μL) was added to initiate the reaction. All the tests presented here were conducted at the same final concentration of DNA. The times until clot formation after the addition of CaCl₂ or TriniClot PT Excel reagent were automatically recorded by the coagulometer. All experiments were performed in triplicates.

**Mouse model and in vivo imaging**

Nude mice were orthotopically implanted with mouse breast cancer cells (4T1) in the right mammary fat pad. Animals were divided into six groups: pacDNAs, brush polymers, free dye, free DNA, with each group having two to three animals. All samples dispersed in PBS buffer were injected intravenously via tail vein on day 21 post-tumor implantation with equivalent dose of Cy5.5. The mice were fed with a special diet 5 days before the imaging study to eliminate any
interference of fluorescence from the food. *In vivo* images were acquired at different time points 1-24 h post-injection in an IVIS instrument (PerkinElmer Inc., USA). The animals were sacrificed at 24 h post-injection and perfused with PBS to remove blood from all organs. Clean organs were used for *ex vivo* imaging of tissues (Cy3 and Cy5.5 channels).
2.5 References.


Chapter 3

Effective Antisense Gene Regulation via Non-cationic, Polyethylene Glycol Brushes

This Chapter is based on a published paper with the same title.


Author contributions: X. L. and K. Z. conceived the study, designed the experiments, analyzed the data and wrote the manuscript. X. L. synthesized the materials and collected the in vitro data. F. J. and X. T. synthesized and purified some of the DNA strands. D. W., X. C. and J. Z. helped on cell culture and confocal laser scanning microscopy. All authors edited the manuscript.
3.1 Introduction.

Oligonucleotide-based gene therapy holds tremendous promise for treating a variety of disorders with a genetic basis, including cancers, neurological diseases, and metabolic conditions.\(^1\) However, since its conceptualization in the 1970s,\(^2\) there have only been a relatively small number of commercial successes (e.g. Vitravene, Macugen, and Kynamra),\(^3\) despite powerful advancement in the understanding of the underlying biology.\(^4\) This contrast exemplifies the difficulties in transforming nucleic acids to drugs: poor accumulation at target sites, unwanted innate and adaptive immune responses, nuclease degradation, coagulopathy, poor cellular uptake, and overall low biochemical efficacy.\(^5\)

In Chapter 2, we have developed a novel form of polymer-DNA conjugate, termed polymer-assisted-compaction of DNA (pacDNA), which consists of oligonucleotide (1-3 strands) covalently attached to the backbone of a sterically congested brush polymer with polyethylene glycol (PEG) side chains.\(^12\) By carefully designing the relative lengths of the DNA strands and the PEG side chains, we have shown that the pacDNAs can achieve >20-fold increase in half-life for DNase I, while hybridization with complementary strands remains kinetically unaffected. Therefore, we contemplate that it is possible for the pacDNA to endure the endosome/lysosome environment, and thus enter the cytosol through normal endosomal processing pathways\(^13\) and regulate gene expression with minimal perturbation to the cell. In contrast, cationic species often cause cell membrane/endosome perforation, leading to toxicity.\(^14\) The use of PEG for oligonucleotide delivery can also improve the biopharmaceutical properties of the oligonucleotide by suppressing unwanted, non-antisense interactions with various proteins.\(^15\) Furthermore, factors previously recognized as important for co-carrier systems such as nucleic acid dissociation from
complex and proton buffering capacity do not apply to the pacDNA, thereby simplifying carrier design.\textsuperscript{11}

![Chemical structure](image)

**Scheme 3-1.** Structures of pacDNA and Y-shaped PEG-DNA conjugate.

### 3.2 Results and Discussion.

To test our hypothesis, we have designed an antisense pacDNA having 10 kDa PEG side chains that targets the human epidermal growth factor receptor 2 (Her2) mRNA (pacDNA\textsubscript{10k}, Scheme 3-1). Her2 is an important biomarker for many cancers including several types of breast and ovarian cancers,\textsuperscript{16} and antisense control of the Her2 gene has been previously demonstrated.\textsuperscript{17}

For controls, we use an improper pacDNA with overly short side chains (5 kDa), and a Y-shaped PEG-DNA conjugate (γPEG-DNA). The pac-DNA\textsubscript{5k} is incapable of effectively protecting the
embedded DNA against enzymatic degradation (*vide infra*). The γPEG is routinely used to form bioconjugates and is found in commercial oligonucleotide drug formulations (Macugen). However, because of the low density of the PEG chains (2 chains), adequate enzymatic protection to the DNA is not anticipated.

**Table 3-1.** GPC analyses for the brush polymers used.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Composition</th>
<th>$M_n$ (kDa)</th>
<th>$M_w$ (kDa)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brush$_{5k}$</td>
<td>pN-azide$<em>5$-b-pN-PEG(5k)$</em>{35}$</td>
<td>178.8</td>
<td>197.2</td>
<td>1.10</td>
</tr>
<tr>
<td>Brush$_{10k}$</td>
<td>pN-azide$<em>5$-b-pN-PEG(10k)$</em>{28}$</td>
<td>285.5</td>
<td>329.1</td>
<td>1.15</td>
</tr>
</tbody>
</table>

**Figure 3-1.** DMF GPC chromatograms of brush$_{10k}$ and brush$_{5k}$.

**Figure 3-2.** IR spectra of Br-brush$_{10k}$ (black) and azide-brush$_{10k}$ (red).
Previously, the pacDNA structure was synthesized by coupling amine-modified DNA to diblock brush polymer containing N-hydroxyl succininimide (NHS) groups in an aqueous bicarbonate buffer. The reaction efficiency is affected by the hydrolysis of NHS groups, requiring the use of large excesses (>20:1 mol:mol) of the DNA. In this chapter, cyclooctyne-mediated copper-free click chemistry\textsuperscript{19} replaces the amidation reaction, resulting in near-quantitative yields. To achieve the coupling, the Her2 antisense DNA strand is modified with 5' dibenzocyclooctyne (DBCO) group (sequence: 5' DBCO CTC CAT GGT GCT CAC TTT 3'), while the brush polymer bears the azide groups. The brush polymers are synthesized \textit{via} sequential ring opening metathesis polymerization (ROMP) of norbornenyl bromide (N-Br) and norbornenyl PEG (N-PEG, \( \text{M}_n=5 \) or 10 kDa, PDI<1.05), followed by azide substitution of the bromide.\textsuperscript{20} The resulting brush is of a diblock structure, with the first, oligomeric block (ca. 5 repeating units) serving as a reactive region for DNA conjugation, and the second, longer block (ca. 30 repeating units) creating the brush architecture and the steric congestion needed to protect the DNA. Dimethylformamide gel permeation chromatography (DMF GPC) shows narrow molecular weight distribution (PDI<1.15) for the brush polymers (Table 3-1 and Figure 3-1; polystyrene-equivalent MW is shown), and the successful incorporation of the azide group is verified by infrared spectroscopy, which shows characteristic vibration of the azido group at 2029 cm\(^{-1}\) (Figure 3-2).\textsuperscript{21}
Figure 3-3. Agarose gel (1%) electrophoresis of fluorescein-labeled pacDNA\textsubscript{10k}, pacDNA\textsubscript{5k}, γPEG-DNA, and free DNA.

Figure 3-4. Aqueous GPC traces for pacDNA\textsubscript{5k}, pacDNA\textsubscript{10k}, and γPEG-DNA. The γPEG-DNA was purified on three tandem Ultrahydrogel 500 columns to achieve baseline separation. Other samples only require one column. Because the total amounts of DNA and brush polymer are known, the number of DNA strands per pacDNA can be calculated from the peak integrations for the pacDNA and the free DNA.
Figure 3-5. (A) Aqueous GPC chromatograms and (B) zeta potential measurements of free DNA, ϒPEG-DNA, pacDNA5k, and pac-DNA10k. (C-F) Number-average hydrodynamic diameter distributions for pacDNA5k (C) and pacDNA10k (D), and corresponding TEM images (E-F, images are negatively stained with uranyl acetate).

Coupling of the DBCO-modified DNA strand to the brush polymers and the ϒPEG is achieved by incubation in 2 M NaCl solution at 40 °C for 48 h (3:1 alkyne:azide mol:mol). The elevated salt concentration is required to achieve high DNA loading by screening the charge between DNA strands. Purified conjugates are free of unconjugated DNA as shown by aqueous GPC and agarose gel electrophoresis (Figures 3-3 and 3-5A). The numbers of DNA strands per brush was determined by peak integration of the GPC chromatograms recorded at 488 nm to be 5.7 and 4.9 for pacDNA10k and pacDNA5k, respectively (Figure 3-4). The pacDNAs exhibit a spherical morphology, with a dry-state diameter of 18.2±2.5 for pacDNA5k and 21.9±3.1 nm for
pacDNA\textsubscript{10k}, as evidenced by transmission electron microscopy (TEM) (Figures 3-5E and F). These measurements are consistent with dynamic light scattering analysis, showing number-average hydrodynamic diameters of 17.0±4.2 nm and 23.7±5.9 nm for the 5k and 10k pacDNAs, respectively (Figure 3-5C and D). Zeta potential measurements indicate that pacDNAs and the γPEG-DNA have significantly reduced negative surface charge (from -9.8 mV to -20.3 mV) compared with free DNA in Nanopure water (-47.4 mV, Figure 3-5B), which is expected from the dilution of surface charge for the conjugates.

![Figure 3-6](image)

**Figure 3-6.** (A) Schematics of DNA hybridization and DNase I degradation assays. (B-C) Hybridization and degradation kinetics of pacDNAs, γPEG-DNA, and free DNA.

**Table 3-2.** Half-lives of free DNA and conjugates (1 μM DNA) in the presence of 0.1 unit/mL DNase I.

<table>
<thead>
<tr>
<th>Name</th>
<th>Free DNA</th>
<th>γPEG-DNA</th>
<th>pacDNA\textsubscript{5k}</th>
<th>pacDNA\textsubscript{10k}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Half-lives (min)</td>
<td>6.0±1.3</td>
<td>8.2±2.1</td>
<td>13.2±1.0</td>
<td>141.9±21.4</td>
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</tbody>
</table>
We next compare the ability of the DNA conjugates to hybridize with complementary strands and resist nuclease degradation. Hybridization is monitored by a fluorescence quenching assay, where a quencher (dabcyl)-modified complementary strand is added to fluorescein-labeled conjugates. The rate of fluorescence decrease is a direct indicator of the hybridization kinetics (Figure 3-6A). Remarkably, both pacDNAs and the γPEG-DNA hybridize rapidly with complementary DNA ($t_{1/2} < 10$ s), with negligible difference compared with free DNA (Figure 3-6B). When a dummy (non-complementary) dabcyl-DNA strand is added, fluorescence intensities are not affected, ruling out non-hybridization interactions. In order to probe the extent of nuclease protection, DNase I is added to fluorescein-labeled DNA conjugates that are pre-hybridized to dabcyl-modified complementary strands. Upon DNase I action, the fluorophore is released, which leads to an increase of fluorescence (Figure 3-6A). The pacDNA$_{10k}$ exhibits significantly extended half-life ($t_{1/2}$) of ~141.9 min compared with free DNA, which is degraded rapidly with a $t_{1/2}$ of ~6.0 min (Figure 3-6C and Table 3-2). On the other hand, pacDNA$_{5k}$ has very limited protective power, showing a $t_{1/2}$ of ~13.2 min. This is not surprising because the fluorophore is located at the periphery (3’) of the DNA; once the DNA extends beyond the PEG shell of the brush, the exposed portion should experience a rapid drop-off in steric protection. Similarly, the γPEG barely lend any protection to conjugated DNA, with a $t_{1/2}$ of ~8.2 min, despite having twice the length of the side chains of pacDNA$_{10k}$. These results suggest that both the brush side chain length and steric congestion created by a densely grafted architecture are critical to providing oligonucleotides with steric selectivity.
Figure 3-7. Confocal fluorescence microscopy images of SKOV3 cells incubated with 100 nM of free Cy3-DNA (A) or Cy-3 pac-DNA_{10k} (B). Cell nuclei were stained with DAPI (blue). Scale bar is 20 µm. (C) Flow cytometry measurements of cells treated with 100 nM Cy3-labeled samples. (D) MTT cytotoxicity assay for SKOV3 cells.
Figure 3-8. Confocal fluorescence microscopy images of mouse 4T1 cells incubated with 100 nM free Cy3-DNA (top row) and Cy3-pacDNA_{10k} (bottom row, scale bar is 20 µm).

In order for the pacDNA to serve as an antisense agent, it needs to efficiently enter cells. We evaluated the cell uptake efficiency in SKOV3, a human ovarian cancer line. To enable tracking, conjugates are labeled at the DNA component with the fluorophore Cy3. Cells are incubated with the conjugates and free DNA for 6 h, followed by flow cytometry analysis. Interestingly, cell uptake appears to be a function of DNA accessibility; the better hidden the DNA, the greater the cell uptake (Figure 3-7C). This is an advantageous phenomenon because DNA accessibility is inversely correlated with nuclease stability. Confocal microscopy confirms the cell up-take of the pacDNA. As shown in the Figures 3-7A, B and 3-8, free DNA-treated cells produce very small amount of fluorescence signals, while the same concentration of DNA, when packaged into pacDNA_{10k}, results in much stronger fluorescence signals under identical imaging settings. Quantification using cell lysates shows that there are ca. $7.7 \times 10^5$ pacDNA_{10k} particles/cell when
the cells are incubated with an equivalent of 1 µM of DNA for 6 h (Figure 3-9). While the pacDNA clearly improves DNA uptake, the extent of uptake is still much below that of the SNA, for which the number of particles/cell often exceeds $10^6$ when similar DNA concentrations (~10 nM SNA) are used.\textsuperscript{22}

**Figure 3-9.** Quantification of the cell uptake of pacDNA and free DNA in SKOV3 cells. Cells were treated with 1 µM equivalent of DNA for 6 h.

**Figure 3-10.** Comparison of DNase I degradation kinetics for pacDNA\textsubscript{10k} containing a scrambled sequence and pacDNA\textsubscript{10k} containing a Her2 antisense sequence.
Having demonstrated that the pacDNA has improved cell uptake, we next studied its antisense activity towards Her2 in SKOV3 cells, which is a Her2-overexpressing cell line. For positive controls, Lipofectamine, an effective cationic liposomal transfection agent, and SNAs with 13 nm gold nanoparticle cores bearing identical antisense strands, are used. PacDNA\textsubscript{10k} containing a scrambled sequence (scr-pacDNA\textsubscript{10k}) and brush polymers devoid of DNA strands are used as negative controls. We verified that scr-pacDNA\textsubscript{10k} exhibits similar levels of cell uptake and resistance to DNase I as the pacDNA\textsubscript{10k} bearing the antisense sequence (Figures 3-10 and 3-11). SKOV3 cells were treated with samples and controls at varying concentrations (10-1000 nM DNA) for 20 h, followed by culturing for another 48 h in fresh media. The total cellular protein for each sample is harvested and analyzed by western blot. It is found that the Her2 levels are significantly reduced by pacDNA\textsubscript{10k}, SNA, and Lipofectamine-complexed DNA (Figure 3-12). Strikingly, even at a low concentration (10 nM), the pacDNA\textsubscript{10k} was able to reduce Her2 expression to only 5% of untreated, as determined by band densitometry analysis, while scrambled pacDNA\textsubscript{10k} does not reduce Her2 expression. On the other hand, pacDNA\textsubscript{5k}, γPEG-DNA, and free DNA show no Her2 expression reduction compared with untreated cells.
Figure 3-12. Western blot analysis of pacDNAs and controls. Her2 expression reduction was observed for pacDNA10k, Lipofectamine, and SNA, while pacDNA5k and γPEG-DNA did not show significant antisense activity.

Figure 3-13. MTT cytotoxicity assay for free DNA, brush10k, pacDNA10k, and Lipofectamine2000-complexed DNA in mouse 4T1 cells.

These data corroborate our hypothesis that DNA stability plays an important role in non-cationic gene regulation; only adequately protected oligonucleotides are able to withstand the digestive endosomal processing and perform down-stream action. Unprotected nucleic acids are efficiently cleaved and deactivated by the cells. Because pacDNA consists of non-toxic
components (PEG and DNA), we anticipate its cytotoxicity to be minimum. Indeed, MTT cytotoxicity assays for SKOV3 and 4T1 cells show essentially no cytotoxicity at 4000 ng of DNA, the highest concentration tested (Figure 3-7D and 3-13), while Lipofectamine results in significant cell death (>50%) above 400 ng of DNA.

3.3 Conclusion.

In summary, our data suggest that efficient cell uptake and enhanced oligonucleotide stability is a successful combination for non-cationic gene regulation; facilitated endosomal release by a membrane-disrupting agent is not required. The pacDNA have desired characteristics to make it an ideal non-cationic oligonucleotide delivery platform, thanks to the densely arranged side chains of the brush and the biocompatibility of PEG. Because of its ability to shield DNA from proteins and bypass serum opsonization, the pacDNA has the potential to be applied in vivo for many oligonucleotide-based applications.
3.4 Materials and Experimental Procedures.

Synthesis of monomers

**Compound 1.** In a round bottom flask, 2.0 g (20.6 mmol) of maleimide and 1.54 g (22.6 mmol) of furan were dissolved in 20 mL ethyl acetate. The solution was refluxed for 4 h, and a white solid, 1, precipitated from the reaction mixture during the course of the reaction. The solids were isolated by filtration, washed with ethyl ether, and dried under vacuum.

$^1$H-NMR (400 MHz, CDCl$_3$): δ 8.14 (s, 1H, -CNHC-), 6.52 (s, 2H, CH=CH), 5.31 (s, 2H, CHOCH), 2.99 (s, 2H, CH-CH); $^{13}$C-NMR (400 MHz, CDCl$_3$): δ 176.2, 136.8, 81.2, 48.9.

**Compound 2.** 2.59 g (12 mmol) of 1, 4-dibromobutane, 2.07 g of K$_2$CO$_3$ (15 mmol), and 5 mL DMF were added to a round bottom flask, to which a solution of 1 (0.5 g, 3 mmol) in 5 mL DMF was added dropwise over a period of 30 min with stirring. The reaction mixture was allowed to stir overnight at room temperature. Silica gel chromatography (3:1 v:v hexane:EtOAc) was used to purify 2. Upon drying under vacuum, 2 appears as a white solid.

$^1$H-NMR (400 MHz, CDCl$_3$): δ 6.51 (s, 2H, CH=CH), 5.26 (s, 2H, CHOCH), 3.51 (t, 2H, NCH$_2$), 3.41 (t, 2H, CH$_2$Br), 2.84 (s, 2H, CH-CH), 1.84-1.71 (m, 4H, CH$_2$-CH$_2$); $^{13}$C-NMR (400 MHz, CDCl$_3$): δ 176.5, 136.8, 81.2, 47.6, 38.1, 33.1, 29.8, 26.4.
General method for synthesizing brush copolymers

Modified 2nd generation Grubbs’ catalyst (0.02 M) was dissolved in DCM in a 5 mL Schlenk flask. The monomers (0.04 M) were each dissolved in DCM in 5 mL Schlenk flasks. All reagents were separately degassed by 3x freeze-pump-thaw cycles. The flask containing norbornenyl bromide (N-Br, 5 equiv.) was cooled to -20 ºC by using an ice-salt bath, and the stock solution containing modified Grubbs’ catalyst (1 equiv.) was added via a microsyringe. The reaction mixture was stirred for 30 min (TLC shows complete consumption of the N-Br), before the second monomer, N-PEG (30 equiv.), was added. The reaction mixture was further stirred for 6 h. At the end of the reaction, several drops of ethyl vinyl ether (EVE) were added to the mixture and the reaction was stirred overnight. The mixture was then concentrated and precipitated into ethyl ether 3x and the precipitant was dried in vacuo. The polymer was then allowed to react with excess NaN₃ in DMF overnight at room temperature, and was dialyzed against Nanopure™ water for 24 h. Thereafter, the polymer was injected into an aqueous GPC column, and the fractions containing the polymer were collected, combined, concentrated, and was further desalted by a NAP-10 column. Infrared spectroscopy showed successful incorporation of azide groups.

Quantitation of the azide groups in brush polymers

In a 1.5 mL microcentrifuge tube, brush polymer (5 nmol) was dissolved in 200 µL Nanopure™ water, to which fluorescein-alkyne (Lumiprobe, 0.207 mg, 500 nmol), copper(II) sulfate pentahydrate (CuSO₄·5H₂O, 500 nmol, 5 µL 100 mM aqueous solution), Tris(3-hydroxypropyltriazolymethyl)amine (THPTA, 600 nmol, 6 µL 100 mM aqueous solution), and sodium ascorbate (2.5 µmol) were added. The reaction mixture was shaken on an Eppendorf shaker at room temperature overnight, before being dialyzed against a NaCl solution (0.15 M) using
dialysis tubing with a MWCO of 6-8 kDa for 48 h. The UV-Vis absorption of the polymer solution at 491 nm was measured and compared with a standard curve. The number of fluorescein molecules per polymer was calculated based on the known polymer concentration. Approximately 5.5 and 5.8 fluorescein tags were attached to brush$_{5k}$ and brush$_{10k}$, respectively (Figure 3-14).

![Figure 3-14. Standard curve generated using free fluorescein in 0.15 M NaCl solution for the quantification of available azide groups on azide-functionalized brushes.](image)

**General method for pacDNA synthesis**

Azide-brush polymers were dissolved in aqueous NaCl solution (2 M) to give a final concentration of 0.1 mM. DBCO-modified DNA (60 nmol) was dissolved in 200 µL aqueous NaCl solution (2 M). The polymer solution (40 µL) was added to the DNA solution, and the mixture was shaken gently for 48 h at 40 °C on an Eppendorf Thermomixer. The reaction mixture was then dialyzed against Nanopure™ water using a MINI dialysis unit (MWCO 3500 Thermo Fisher) for desalting. The dialysate was further subjected to aqueous GPC purification. The fraction containing the conjugate was collected and dialyzed against Nanopure™ water to remove NaNO$_3$. 

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The final solution was lyophilized to yield a white powder (or green/red powders for fluorescein and Cy3-labeled conjugates).

**Synthesis of γPEG-DNA**

Y-shaped PEG NHS ester (80 mg, 2 µmol), 3-azido-1-propanamine (N₃-amine, 0.16 mg, 1.6 µmol), and N, N-diisopropylethylamine (0.65 mg, 5 µmol) were added to a 25 mL round bottom flask. The reaction was vigorously stirred for 30 min. TLC shows complete consumption of N₃-amine. Then, another 0.16 mg (1.6 µmol) of N₃-amine was added and the reaction mixture was allowed to stir for 1 h before being precipitated into diethyl ether 3x. The product was dried under vacuum and further purified by a NAP-10 column to remove impurities. The final solution was lyophilized to give a white powder. To synthesize γPEG-DNA conjugate, 2 mg (50 nmol) of Y-shaped PEG azide was combined with 60 nmol DBCO-modified DNA strands in 200 µL Nanopure™ water. The reaction mixture was shaken at 40 °C for 48 h and purified by aqueous GPC. To achieve baseline separation between conjugate and free DNA, three Waters Ultrahydrogel™ 500 columns were used in tandem combination. After purification, the conjugate was desalted by a NAP-10 column and was lyophilized to yield a white powder (or green/red powders for fluorescein and Cy3-labeled conjugates).

**Hybridization kinetics assay**
Free DNA, γPEG-DNA, and pacDNA (all fluorescein-labeled) were each dissolved in microcentrifuge tubes in PBS buffer (pH = 7.4) to give final concentrations of 100 nM. Each solution (1 mL) was transferred to a fluorescence cuvette, to which a complementary dabcyl-DNA strand or a non-complementary strand (2 equiv.) was added via 1 µL of PBS solution. The fluorescence of the mixture (ex = 490 nm, em = 520 nm) was monitored before mixing and every 3 sec thereafter for 60 min using a Cary Eclipse fluorescence spectrometer. The endpoint is determined by adding a large excess of complementary dabcyl-DNA to the mixture followed by incubation for an extended period of time (> 1 h). The kinetics plots are normalized to the endpoint determined for each sample.

Nuclease degradation kinetics assay

Free DNA, γPEG-DNA and pacDNA (1 µM, all fluorescein-labeled) were each mixed with their respective complementary dabcyl-labeled DNA (2 µM) in PBS buffer. The mixtures were heated to 80 °C and allowed to cool slowly to room temperature in a thermally insulated container during a period of 10 h. The mixtures were then diluted to 100 nM in assay buffer (10 mM Tris, 2.5 mM MgCl₂, and 0.5 mM CaCl₂, pH = 7.5), and 1 mL of each mixture was transferred to a fluorescence cuvette which was mounted on a fluorimeter. DNase I (Sigma-Aldrich) was then added and rapidly mixed to give a final concentration of 0.1 unit/mL. The fluorescence of the samples (ex = 490 nm, em = 520 nm) was measured immediately and every 3 secs for 6 h. The endpoint was determined by adding a large excess of DNase I (ca. 2 units/mL) to the mixture, and the fluorescence was monitored until no additional increase was observed. The kinetics plots were normalized to the endpoints determined for each sample, and all experiments were performed in triplicates.
Synthesis of anti-Her2 SNA

In a typical synthesis, 60 nmol of thiol modified antisense DNA strands were treated with 100 µL of 100 mM dithiothreitol (DTT) in 50 mM pH 8.0 phosphate buffer for 0.5 h and desalted using a NAP-10 column. These purified strands were then added to 15 mL of gold nanoparticles (13 nm, 10 nM, synthesized via a literature method\textsuperscript{23}) pre-mixed with 15 µL of 10% TWEEN 20. The mixture was then heated to 50 °C in an Eppendorf Thermomixer for 2 h. Over 10 h, the particles were brought to an elevated salt concentration (0.5 M) by adding aliquots of 5 M NaCl. The particles were shaken for an additional 48 h at 50 °C on an incubator shaker. Particles were then purified by centrifugation and resuspended in Nanopure\textsuperscript{TM} water. This process was repeated 3x. After the final centrifugation, SNA particles were resuspended in 15 mL of PBS buffer containing 0.01% TWEEN 20.

Cell culture

Human SKOV3 and mouse 4T1 cells were grown in DMEM medium with 10 % heat inactivated fetal bovine serum, 1% antibiotics, 1% L-glutamine, and were maintained at 37 °C in 5 % CO\textsubscript{2}.

Confocal fluorescence microscopy

To study the cellular uptake of pacDNA, 4T1 or SKOV3 cells were seeded at a density of 1.0×10\textsuperscript{5} cells/well in 24-well glass bottom plates and were cultured overnight at 37 °C and 5% CO\textsubscript{2}. Serum-free DMEM containing Cy3-labeled free DNA or Cy3-labeled pacDNA\textsubscript{10k} at equal doses of DNA (100 nM) were added to each well, followed by incubation for 6 h at 37 °C. The cells were then gently washed with PBS 3x, fixed with a 4% formaldehyde solution for 15 min, and stained with 10 µM DAPI for 3 min. The cells were imaged on an LSM-700 confocal laser
scanning microscope (Carl Zeiss Ltd., Cambridge, UK) at excitation wavelengths of 408 nm (DAPI) and 543 nm (Cy3). Imaging settings were identical for free DNA- and pacDNA-treated cells.

**Flow cytometry**

SKOV3 cells were seeded at a density of 2.0×10^5 cells/well in a 6-well plate and were cultured overnight at 37 °C and 5% CO₂. Serum-free DMEM medium containing Cy3-labeled free DNA, YPEG-DNA, pacDNA_{5k}, and pacDNA_{10k} (100 nM equivalent of DNA) were added to each well, followed by further incubation for 6 h. The cells were washed with PBS 3x, harvested by trypsinization, and transferred into fluorescence activated cell sorter tubes. All samples were analyzed by flow cytometry (FACS Calibur, BD Bioscience, San Jose, CA) to determine the extent of cellular internalization.

**Quantification of cell uptake**

SKOV3 cells were seeded at a density of 2.0×10^5 cells/well in a 24-well plate and were cultured overnight. Serum-free DMEM medium containing fluorescein-labeled free DNA and pacDNA_{10k} (1 µM equivalent of DNA) were added to each well, followed by further incubation for 6 h. The cells were washed with PBS 3x, harvested by trypsinization, and were counted using a TC20™ automated cell counter (Bio-Rad, MA, USA). The cells were then centrifuged, lysed with 100 µL RIPA Cell Lysis Buffer, and transferred to a 96-well plate. The fluorescence of each cell lysate was determined by a microplate reader (Biotek Synergy HT, BioTek Instruments, Inc. VT, USA) and was compared with a fluorescein standard curve (Figure 3-15, created by serial dilution of fluorescein-labeled free DNA in RIPA buffer). The number of DNA strands per cell
was calculated based on Equation 1. There are ca. 7.7±1.0×10^5 particles in pacDNA10k-treated samples and ca. 5.1±0.16×10^4 DNA strands in free DNA-treated samples, respectively.

\[
\text{pacDNA/cell} = \frac{\text{Concentration of DNA} \times \text{Volume of lysate} \times \text{Avogadro's Constant}}{\text{Number of cells per well} \times \text{Number of DNA strands per pacDNA}} \quad \text{(Equation 1)}
\]

**Figure 3-15.** Standard curve generated using free fluorescein-labeled DNA in RIPA buffer for the quantification of cell uptake.

**MTT assay**

The cytotoxicity of pacDNA was evaluated with the MTT assay. Briefly, SKOV3 or 4T1 cells were seeded in a 96-well plate in 100 μL medium and cultured for 24 h. The cells were then treated with free DNA, free brush10k, and pacDNA_{10k} at varying concentrations of total DNA (0.1, 0.25, 0.5, 1 and 4 μg). Lipofectamine 2000 (Invitrogen) was used as a positive control using conditions suggested by the manufacturer. Cells treated with PBS were used as a negative control. After 48 h, 3-[4,5-dimethylthiazol-2-yl]-3,5-diphenyltetrazolium bromide (MTT dye, final
concentration of 0.5 mg/mL) was added. The cells were incubated for 4 h and the absorbance was measured at 570 nm using a microplate reader (Biotek Synergy HT).

**Western blotting**

SKOV3 cells were plated in 6-well plates at a density of 2.0×10⁵ cell per well and cultured overnight. Cells were incubated with free DNA, free brush10k, γPEG-DNA, pacDNA5k, scrambled pacDNA10k, pacDNA10k (10 nM, 100 nM, 1000 nM), and Lipofectamine-complexed DNA in opti-MEM. The incubation concentration of all samples except pacDNA10k was 100 nM (DNA concentration). After 20 h, the medium was replaced with fresh, full growth medium and cells were cultured for another 48 h. Whole cell lysates were prepared in 100 μL of RIPA Cell Lysis Buffer with 1 mM phenylmethanesulfonylfluoride (PMSF, Cell Signaling Technology) according to the manufacturer’s suggested protocol. Protein concentrations were determined using a BCA Protein Assay Kit (Pierce). Equal amounts (5 μg) of protein samples were fractionated by 4-20% SDS-PAGE and transferred to PVDF membrane, and were analyzed by western blotting with Her2 and GAPDH antibodies (Invitrogen) using an ECL Western Blotting Substrate (Pierce).
Table 3-3. Oligonucleotide sequences used in this study.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Her2 antisense</td>
<td>5'-DBCO-TTT CTC CAT GGT GCT CAC-3'</td>
</tr>
<tr>
<td>Fluorescein-labeled Her2</td>
<td>5'-DBCO-TTT CTC CAT GGT GCT CAC-fluorescien-3'</td>
</tr>
<tr>
<td>Cy3-labeled Her2</td>
<td>5'-DBCO-TTT CTC CAT GGT GCT CAC-Cy3-3'</td>
</tr>
<tr>
<td>Dabcyl-labeled Her2</td>
<td>5'-Dabcyl-GTG AGC ACC ATG GAG-3'</td>
</tr>
<tr>
<td>Scrambled antisense</td>
<td>5'-DBCO-TTT TAA CTC TGA CAT GAT GTC-3'</td>
</tr>
<tr>
<td>Fluorescein-labeled scrambled</td>
<td>5'-DBCO-TTT TAA CTC TGA CAT GAT GTC-fluorescien-3'</td>
</tr>
<tr>
<td>Cy3-labeled scrambled</td>
<td>5'-DBCO-TTT TAA CTC TGA CAT GAT GTC-Cy3-3'</td>
</tr>
<tr>
<td>Dabcyl-labeled scrambled</td>
<td>5'-Dabcyl-GAC ATC ATG TCA GAG TTA-3'</td>
</tr>
<tr>
<td>Her2 antisense for SNA</td>
<td>5'-CTC CAT GGT GCT CAC T_{(10)}-SH-3'</td>
</tr>
</tbody>
</table>
3.5 References.


(21) Singh, K. S.; Svitlyk, V.; Mozharivskyj, Y. Mono and dinuclear areneruthenium (ii) triazoles by 1, 3-dipolar cycloadditions to a coordinated azide in ruthenium (ii) compounds. *Dalton Trans.* **2011**, *40*, 1020.

Chapter 4

Modulating the Cellular Immune Response of Oligonucleotides by Brush Polymer-assisted Compaction

This Chapter is based on a manuscript that has been submitted for publication.

Cao. X; † Lu, X; † Jia, F.; Wang, D.; Tan, X.; Corley, M.; Chen, X.; Zhang, K. “Reducing the Inflammatory Response of Oligonucleotide by Brush Polymer-assisted Compaction” Submitted. (†Equal first author)

Author contributions: X. C., X. L. and K. Z. conceived the study, designed the experiments, analyzed the data and wrote the manuscript. X. L. synthesized and characterized all the materials. X. C. performed all the PCR and ELISA experiments. D. W. helped on cell culture and confocal microscopy experiments. M. C. and C. X. helped on flow cytometry and ELISA experiments. J. F. and X. T. synthesized and purified some of the DNA strands. All authors edited the manuscript.
4.1 Introduction.

Nucleic acids (NA) hold great promise for treating a variety of genetic disorders,\textsuperscript{1} infectious diseases,\textsuperscript{2,3} and cancer.\textsuperscript{4,5} However, development of NA therapeutics is greatly hindered by the poor biopharmaceutical properties of the NA (e.g. rapid degradation, hepatic/renal clearance, extreme hydrophilicity) as well as specific or non-specific, non-hybridization side effects, including coagulopathy and unwanted activation of the immune system.\textsuperscript{6-9} In particular, RNA sequences containing 5'-GUCCUCAA-3' or 5'-UGUGU-3',\textsuperscript{10,11} and DNA sequences containing unmethylated cytosine-phosphate-guanosine (CpG) motifs are able to elicit strong innate immune responses even at low concentrations,\textsuperscript{12,13} and are in fact used in clinical trials as a vaccine adjuvant.\textsuperscript{14} Immune system activation is mediated by several toll-like receptors (TLRs), including TLR7/8, TLR3, and TLR9, which are implicated in the recognition of single stranded RNA (ssRNA), double strand RNA (dsRNA), and DNA, respectively.\textsuperscript{15,16} Unwanted activation can be a difficulty for biopharmaceutical development, as patients may develop local swelling and symptoms resembling influenza infection (e.g. headache, fever, myalgia, and nausea).\textsuperscript{17} For example, the Phase I clinical trial of anti-ApoB siRNA, which was developed by Tekmira Pharmaceuticals, was terminated due to the potential of immune system stimulation by the RNA.\textsuperscript{18} Many antisense DNA candidates, such as GEM231 of Idera, Inc. (antisense inhibitor of the oncoprotein Bcl-2), contain at least one CpG motif, which may eventually limit their success.\textsuperscript{19} While 2’ O-methylation mitigates the risk associated with CpG motifs, methylated CpG-containing sequences have oftentimes been found to be non-inert and still carry immunostimulatory properties.\textsuperscript{20} In addition to the immunogenicity of the NA, many widely used gene transfection materials in clinical trials, such as the adenovirus and cationic polymers, also elicit strong immune responses and cause inflammation-associated symptoms.\textsuperscript{21-25} Therefore, an
ideal NA carrier system should not only mask the immunogenicity of the NA payload but also be non-immunostimulative itself.

**Scheme 4-1.** Synthetic scheme of pacDNAs and γPEG-DNA conjugate.

We have developed a new class of PEGylated DNA, termed pacDNA (polymer-assisted compaction of DNA), which uses brush polymers consisting of many PEG side chains as the PEGylating agent (as opposed to linear or slightly branched PEG, e.g. Y-shaped PEG).\(^{26}\) It is discovered that the pacDNA can be made to have an intermediate PEG density, such that the formation of dsDNA with the pacDNA is not inhibited, but protein access to the embedded DNA is significantly retarded. This selectivity results from the sterically congested PEG environment, which creates a significant access barrier for bulky species such as proteins, but allows the slender complementary DNA strand to penetrate freely. We have shown that the steric congestion can increase the nuclease half-life of pacDNA by 30-fold when compared with naked DNA.\(^{27}\) The same principle also provides the pacDNA the ability to effectively block DNA-thrombin interactions, which reduces DNA-induced blood coagulopathy. In contrast, the DNA within the brush polymer exhibits nearly identical hybridization kinetics and free energy as naked DNA. Furthermore, the pacDNA has the added benefit of being able to enter cells and regulate cellular
gene expression without an additional helper system,\textsuperscript{28} and can persist in blood circulation thanks to the stealth property of the polymer. These results have positioned the pacDNA as an attractive material for designing antisense therapeutics. Nonetheless, it is still unclear whether the pacDNA exhibit similar immunogenicity challenges as free antisense strands containing unmethylated CpG motifs. We hypothesize that the pacDNA architecture provides a general selectivity that can bypass many if not all side effects associated with DNA-protein interactions. Thus, by sterically blocking TLRs from recognizing embedded foreign nucleic acid, the pacDNA should offer a new mechanism to diminish the immunogenicity of TLR-agonist sequences. This mechanism may also be combined with methylation to further reduce the immunogenic properties of certain nucleic acid strands.

4.2 Results and Discussion

\textbf{Synthesis and characterization of PEG-DNA conjugates.} To test our hypothesis, we designed and synthesized three PEG-DNA conjugates, all of which contain the same 20-mer CpG-rich DNA sequence: TCC ATG ACG TTC CTG ACG TT.\textsuperscript{13} The sequence is modified at the 5$'$ with a dibenzocyclooctyne (DBCO) functionality for conjugation to the PEG component, and a Cy3 fluorophore at the 3$'$ for fluorescence tracking and quantification. The PEG components used to modify the DNA sequence include a brush with 10 kDa PEG side chains (pacDNA\textsubscript{10k}), a brush with 5 kDa PEG side chains (pacDNA\textsubscript{5k}), and a Y-shaped, 40 kDa PEG (YPPEG-DNA, each arm 20 kDa). The different PEG structures are expected to have varying levels of protection over the embedded DNA strands (vide infra). Both brushes are of a diblock architecture, with a very short first block (~5 repeat units) containing azido groups for coupling with DNA, followed by a relatively long (28 and 35 repeat units) block bearing the PEG side chains, which create the necessary steric congestion. These diblock brush polymers were synthesized by sequential ring-
opening metathesis polymerization (ROMP) of norbornenyl bromide (N-Br) and norbornenyl PEG (N-PEG) monomers (Scheme 4-1).\textsuperscript{29} Nucleophilic substitution by sodium azide in dimethylformamide gives two conjugation-ready brush polymers: p(N-azide)$_5$-$b$-p(N-PEG$_{5k}$)$_{35}$ and p(N-azide)$_5$-$b$-p(N-PEG$_{10k}$)$_{28}$. Azide-modified Y-shaped PEG was synthesized by reacting a PEG-NHS ester (JenKem, USA) with 3-azido-1-propanamine. Conjugation is achieved by reacting DBCO-modified DNA with the respective polymers in a high salt (2 M NaCl) aqueous solution at 40 °C for 48 h. After purification using aqueous gel permeation chromatography (GPC), pacDNA$_{5k}$ and pacDNA$_{10k}$ were obtained with ~5.1 and ~5.3 DNA strands on each brush, respectively, and $\gamma$PEG-DNA has ~1 DNA strand attached, as determined by GPC peak integrations (546 nm channel, Figure 4-1). Aqueous GPC and agarose gel electrophoresis of purified samples (Figures 4-2A and B) show successful synthesis of the PEG-DNA conjugates. Transmission electron microscopy (TEM) show that the pacDNA$_{5k}$ and the pacDNA$_{10k}$ have a spherical morphology with a dry-state diameter of 18.8±2.4 nm and 23.7±2.9 nm, respectively (Figure 4-2D), consistent with the hydrodynamic diameter measurements ($D_{h(n)}$ of 15.7±4.5 nm and 25.2±7.7 nm), as determined by dynamic light scattering (DLS, Figure 4-2C).

![Figure 4-1](image_url)

**Figure 4-1.** Aqueous GPC traces of reaction mixtures containing pacDNA$_{5k}$, pacDNA$_{10k}$, and $\gamma$PEG-DNA (546 nm channel) prior to their purification. The peak at 32 min represents excess, unconjugated DNA. The number of DNA strands per conjugate was calculated from peak integrations of conjugates and excess free DNA.
Figure 4-2. (A-B) Aqueous GPC and agarose gel electrophoresis analyses of pacDNAs and γPEG-DNA conjugate. (C-D) Number-average hydrodynamic size distribution and TEM images (negatively stained) of pacDNA\textsubscript{5k} and pacDNA\textsubscript{10k} (scale bar is 100 nm).

**Determination of the extent of steric protection.** Stimulation of innate immune system is directly correlated with DNA accessibility by the TLR9, which is located in early endosomes.\textsuperscript{30} Therefore, it is important to determine the extent of steric shielding of the DNA by the different PEGylation strategies. A model protein system, bovine pancreatic DNase I,\textsuperscript{31,32} is used to probe the three conjugates, because the rate of DNase I cleavage of dsDNA is inversely correlated with the accessibility of the DNA. To measure DNase I activity, Cy3-labeled PEG-DNA conjugates are first pre-hybridized with quencher (dabcyl)-modified complementary strands. Upon introduction of DNase I, the duplexes are cleaved, leading to the separation of fluorophore from the quencher and an increase of fluorescence (Figure 4-3A). It is found that pacDNA\textsubscript{10k} has the highest resistance to DNase I degradation, with an extended half-life (t\textsubscript{1/2}) of 5-fold longer than that of naked DNA (Figure 4-3B). Having much lower PEG density, pacDNA\textsubscript{5k} only slightly increases the nuclease t\textsubscript{1/2} (1.2x longer), while the Y-shaped 40 kDa PEG is unable to offer noticeable shielding (t\textsubscript{1/2} for γPEG-DNA is 1.08x of naked DNA). These observations are consistent with our hypothesis that
the brush architecture with long, densely grafted side chains can best block large species from accessing the embedded DNA. Since DNase I is a relatively small protein (30.1 kDa) compared with TLR9 (115.8 kDa), we anticipate the steric blocking effect to be more pronounced for TLR9.

![Figure 4-3](image)

**Figure 4-3.** (A) Schematics for nuclease stability assay. (B). Degradation kinetics of free DNA, γPEG-DNA, and pacDNAs by DNase I.

![Scheme 4-2](image)

**Scheme 4-2.** (A) Mechanism of how pacDNA exhibits reduced innate immune system activation in RAW 264.7 cells.
Measurement of innate immune response in RAW 264.7 cells. To systematically investigate the inflammatory response of the PEG-DNA conjugates, we used the murine macrophage cell line RAW 264.7, which plays an important role in non-specific immunity by recognizing foreign pathogens through C-type lectin-like domains.\textsuperscript{33,34} The initiation of innate immune response is indicated by the production of several characteristic inflammatory cytokines (Scheme 4-2), including interferon-β (INF-β), tumor necrosis factor α (TNF-α), and interleukin 6 (IL-6).\textsuperscript{35} The degree of immune system activation can therefore be determined by comparing the level of these pro-inflammatory cytokines.\textsuperscript{36} We treated RAW 264.7 cells with samples containing equal concentration of the DNA (1.0 µM), and quantified the mRNA and protein levels of INF-β and TNF-α by using quantitative real-time polymerase chain reaction (QRT-PCR) and commercial enzyme-linked immunosorbent assay (ELISA) kits. As shown in Figures 4-4B and C, all three PEG-DNA conjugates exhibit significantly reduced of INF-β and TNF-α in mRNA levels compared with Lipofectamine-complexed DNA. The same trend was also observed in protein levels (Figures 4-4 C-E). Of note, Lipofectamine itself induces a robust INF-β mRNA response, while free PEG (both brush and Y-shaped) does not lead to noticeable changes in mRNA and protein levels of all measured cytokines, suggesting that the PEG-based materials are inherently biocompatible.\textsuperscript{37} The combined results support our hypothesis that the pacDNA architecture can mitigate unwanted innate immune system activation by shielding its DNA component from TLRs.
Figure 4-4. Upon treatment, the relative mRNA levels of INF-β (A) and TNF-α (B) is measured by QRT-PCR. All results are normalized to GAPDH mRNA expression. The relative protein levels for INF-β, TNF-α, and IL-6 are determined by ELISA and shown in plots (C), (D), and (E) as mean ± SD, respectively.

Interestingly, although γPEG-DNA exhibits the least shielding against enzymatic cleavage, it does not induce production of both TNF-α and INF-β more than pacDNAs. This unusual trend contradicts our hypothesis that the brush architecture is more effective at lowering TLR recognition. We attribute this observation to the different levels of cellular uptake for the conjugates, since TLR recognition is an intra-endosomal event. Indeed, flow cytometry shows that the cell uptake for pacDNA_{10k} and the pacDNA_{5k} are 9.6x and 4.0x more than γPEG-DNA, respectively. (Figures 4-5B and 4-6) Confocal laser scanning microscopy images also show much brighter Cy3 fluorescence for pacDNAs vs. γPEG-DNA under identical imaging settings (Figure
Normalizing cytokine production against cellular uptake reveals a trend that is predicted by the DNase I accessibility assay (*vide supra*): more steric protection leads to greater suppression of the TLR9 agonist’s activity (Figure 4-6). However, the mathematical normalization is only appropriate when cytokine level has a linear response to cellular uptake. To more accurately study the relationship between the steric congestion and immune system activation, we adjusted the incubation concentration of γPEG-DNA, pacDNA5k, and pacDNA10k to achieve nearly identical cellular uptake within the same timeframe (Figure 4-5C; concentrations used: 0.105 µM pacDNA10k, 0.248 µM pacDNA5k, and 1.0 µM γPEG-DNA). It is found that both pacDNAs induce dramatically reduced levels of INF-β and TNF-α mRNA production compared with the γPEG-DNA. The same trend is also observed in protein levels (Figure 4-5D). The difference between pacDNA10k and pacDNA5k is negligible in this case, likely due to the small amount of delivered DNA, which is insufficient to induce a measurably different response. At higher concentrations (1 µM), pacDNA10k elicits lower cytokine production than pacDNA5k, despite higher cellular uptake (Figures 4-4A-E). Collectively, these data indicate that the pacDNA structure is effective in mitigating unwanted activation by immunogenic oligonucleotide sequences.
**Figure 4-5.** Confocal laser scanning microscopy images (A) and flow cytometry measurements (B) of RAW 264.7 cells treated with 1 µM, Cy3-labeled γPEG-DNA, and pacDNAs (green). The nuclei are stained by Hoechst 33342 (Blue). (C) Flow cytometry measurements of RAW 264.7 cells, showing roughly equal uptake of all samples (cells were treated with 0.105 µM pacDNA_{10k}, 0.248 µM pacDNA_{5k}, and 1 µM γPEG-DNA). (D) Relative mRNA and protein levels of INF-β and TNF-α following 4 h incubation at uptake-adjusted concentrations. Data shown are mean ± SD (**p<0.01, ***p<0.001).
Figure 4-6. (A) Relative amount of cell uptake of pacDNA$_{5k}$, pacDNA$_{10k}$, and γPEG-DNA, as determined by flow cytometry. (B-F) Cytokine production in mRNA and protein levels (numerically normalized to cell uptake) for INF-β (B and D), TNF-α (C and E), and IL-6 (F).

Intracellular trafficking of pacDNA. Our working hypothesis is that the pacDNA can bypass immune system activation by sterically blocking protein access to the DNA component. In order for this proposed mechanism to be correct, we need to verify that the pacDNA indeed enter the cells via endosomal compartments (and therefore can potentially be recognized by TLRs). Therefore, we investigated the cell uptake mechanism and intracellular trafficking pathway of the pacDNA in RAW 264.7 cells.$^{38}$ Using chemical blockers, rottlerin, methyl-β-cyclodextrin (MβCD), and chlorpromazine (CPM),$^{39}$ we investigated whether micropinocytosis, caveolae-mediated endocytosis, clathrin-mediated endocytosis, or their combination was primarily responsible for the cell uptake. As shown in Figures 4-7, the amount of internalized pacDNA decreases markedly after treatment with MβCD and CPM, suggesting significant contributions
from both caveolae- and clathrin-mediated pathways. The lack of response to rottlerin rules out substantial uptake by micropinocytosis.

**Figure 4-7.** Flow cytometry analysis of cellular uptake of pacDNA5k(left) and pacDNA10k (right) in RAW 264.7 cells pretreated with chemical blockers. Data are shown as mean ± SD (**p<0.001).

To further follow the intracellular trafficking of the pacDNA, cells were incubated with pacDNA10k at different time points, and immunostaining was used to determine particle colocalization with early endosomes, late endosomes, and lysosomes. Confocal laser scanning microscopy images show that the pacDNA10k enters cells via vesicle-like compartments (Figure 4-8), which colocalize with early endosomes. The extent of early endosome colocalization decreases as a function of time, suggesting particle trafficking into downstream locations (Figure 4-9). Colocalization with late endosome was found to increase during 1-4 h, and stays relatively constant thereafter. Remarkably, no apparent colocalization between pacDNA10k and lysosome was seen during the entire observation period (1-12 h). This phenomenon may be used to explain the high antisense activity of the pacDNA: by avoiding the harsh digestive environment in the lysosome, the pacDNA can potentially remain active for a longer period of time.
Figure 4-8. Time-resolved confocal images of RAW 264.7 cells incubated with Cy3-pacDNA\textsubscript{10k} (green). Scale bars are 10 µm. Early endosomes, late endosomes, and lysosomes are immunostained by anti-EEA1, anti-Rab-9, and anti-LAMP-1 antibodies, respectively (red). The nuclei are stained by Hoechst 33342 (blue). Arrows show sites of colocalization between pacDNA and early endosomes.
Manders colocalization analysis. The percentage of colocalization was calculated according to Manders colocalization coefficient (M1).\textsuperscript{41} M2 information is provided in Figure 4-11.

4.3 Conclusion.

In conclusion, we have demonstrated that the pacDNA nanostructure can effectively reduce the innate immune response of molecular DNA by sterically blocking TLRs from interacting with the DNA component. Our data indicate that the pacDNAs with higher steric congestion leads to greater cellular uptake and lower activation of the innate immune system. This observation allows us to design new polymer brushes to modulate the innate immune response by varying the PEG density of the brush. Taken together, these results suggest that the brush-type PEG is a superior alternative to linear or slightly branched PEG for creating non-immunogenic, oligonucleotide-based biopharmaceuticals.
4.4 Materials and Experimental Procedures.

Nuclease degradation kinetics assay. Cy3-labeled free DNA, γPEG-DNA, and pacDNAs (100 nM DNA concentration) were each mixed with complementary dabcyl-labeled DNA (200 nM) in assay buffer (10 mM Tris, 2.5 mM MgCl₂, and 0.5 mM CaCl₂, pH = 7.5). The mixtures were heated to 80 °C and allowed to cool slowly to room temperature in a thermally insulated container during a period of 10 h. Each mixture (100 μL) was transferred to a 96-well optical bottom plate (Fisher Scientific Inc.). DNase I (Sigma-Aldrich) was then added with a multichannel pipette and rapidly mixed to give a final concentration of 0.1 unit/mL. The fluorescence of the samples (ex = 540±25 nm, em = 590±35 nm) was measured immediately and every 30 secs for 3 h in a Synergy Neo2 microplate reader (BioTek Instruments Inc.). The endpoint was determined when no additional increase of fluorescence was observed. The kinetics plots were normalized to the endpoints determined for each sample, and all experiments were performed in triplicates.

Quantification of cytokine excretion by QRT-PCR and ELISA. RAW 264.7 cells were plated at a density of 2.0×10⁵ cells per well in 24-well plates and cultured overnight. Cells were incubated with free DNA, brush₁₀k, brush₅k, γPEG, γPEG-DNA, pacDNA₅k, pacDNA₁₀k, and Lipofectamine-complexed DNA (1 μM DNA concentration) in serum-free DMEM for 4 h. A two-step QRT-PCR method was used to quantify cytokine mRNA levels. After incubation, the total RNA was extracted using the Trizol reagent following manufacture-suggested protocols. The RNA concentration was determined using a NanoDrop™ 2000 spectrophotometer (Thermo Scientific). Total RNA (0.2 OD) was reverse transcribed using Superscript III at 42 °C for 30 min and 85 °C for 5 min. The obtained cDNA was amplified with SsoAdvanced™ Universal SYBR Green Super Mix on a Bio-Rad CFX96 Touch™ System. The results were normalized to GAPDH expression. The primer sequences used were: TNF-α forward, 5′-CAT CTT CTC AAA ATT CGA GTG ACA
A-3', TNF-α reverse, 5'-TGG GAG TAG ACA AGG TAC AAC CC-3', IFN-β forward, 5'-CCA TCC AAG AGA TGC TCC AG-3', IFN-β reverse, 5'-GTG GAG AGC AGT TGA GGA CA-3', GAPDH forward, 5'-TGC ACC ACC AAC TGT TTA GC-3', and GAPDH reverse, 5'-GCC ATG GAC TGT GGT CAT GAG-3'. To determine the cytokine protein levels, the supernatant of cells was collected after 4 h of incubation. Commercially available ELISA kits were used following the manufacture-suggested protocols. All QRT-PCR and ELISA experiments were performed in triplicates and the results were averaged.

**Cellular uptake and intracellular trafficking.** To study the cellular uptake of γPEG-DNA and pacDNAs, RAW 264.7 cells were seeded at a density of 1.0×10^5 cells/well in a 24-well glass-bottom plate and were cultured overnight at 37 °C and with 5% CO₂. Serum-free DMEM containing Cy3-labeled pacDNA_{5k}, pacDNA_{10k}, and γPEG-DNA (1 μM DNA concentration) were added to each well, followed by incubation for 4 h at 37 °C. The cells were then gently washed with PBS 3x, fixed with 4% formaldehyde solution for 15 min, and stained with 10 μM Hoechst 33342 for 5 min. Cells were imaged at excitation wavelengths of 350 nm (Hoechst 33342) and 543 nm (Cy3) on an LSM-700 confocal laser scanning microscope (Carl Zeiss Ltd., Cambridge, UK). Imaging settings were kept identical for all samples. To study the contribution of different endocytotic pathways, cells were pre-incubated with rottlerin (1-3 μM), methyl-β-cyclodextrin (MβCD, 2.5 or 7.5 mg/mL), or chloropromazine (CPM, 1 or 5 μg/mL) for 2 h before addition of samples. The cells were then washed with PBS 3x, harvested by trypsinization, and transferred into fluorescence-activated cell sorter tubes. All samples were analyzed by flow cytometry (FACS Calibur, BD Bioscience, San Jose, CA) to determine the extent of cellular internalization. All measurements were performed in triplicates and the results were averaged.
To investigate the intracellular trafficking of pacDNA, RAW 264.7 cells were seeded at a density of $1.0 \times 10^5$ cells/well in 24-well glass-bottom plates and were cultured overnight at 37 °C and with 5% CO$_2$. After incubation with Cy3-labeled pacDNA$_{10k}$ (1 μM DNA concentration) for different durations of time (15 min-12 h), cells were gently washed with PBS 3x, fixed with 4% formaldehyde solution for 15 min, and incubated with 0.1% Triton-100 in PBS for 10 min, which increases the permeability of cell membrane. Cells were then blocked with 2% BSA in PBS for 1 h and incubated with anti-EEA1, anti-Rab9, or anti-LAMP1 primary antibodies at 4 °C overnight. Thereafter, cells were washed with 0.05% Tween-20 in PBS (PBST) 3x and incubated with Alexa Fluor 488-labeled secondary antibody for 1 h at room temperature. Following incubation, cells were washed with PBST 3x and stained with Hoechst 33342 for 5 min. Imaging was carried out on a confocal microscope using excitation wavelengths of 350 nm (Hoechst 33342), 488nm (EEA-1, RAB-9, LAMP1), and 543 nm (Cy3-labeled samples). To quantify the colocalization between fluorescence signals of pacDNA and cellular compartments, Zen-2009 digital imaging software was used to calculate the Manders colocalization coefficient according to Equation 1, where G represents green pixel signals (DNA) and R represents red pixel signals (early endosome, late endosome, and lysosome). The threshold value was kept identical for all images analyzed. The reported data is averaged from multiple cells (Figures 4-10 and 4-11).

$$M1 = \frac{\sum_i G_i,\text{coloc}}{\sum_i G_i}, \quad M2 = \frac{\sum_i R_i,\text{coloc}}{\sum_i R_i} \quad (\text{Equation 1})$$

Table 4-1. DNA sequences used in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CpG DNA</td>
<td>5'-DBCO-TCC ATG ACG TTC CTG ACG TT-Cy3-3'</td>
</tr>
<tr>
<td>CpG complementary DNA</td>
<td>5'-Dabcyl-AAC GTC AGG AAC GTC ATG GA-3'</td>
</tr>
</tbody>
</table>
Figure 4-10. Time-resolved confocal images of RAW 264.7 cells incubated with Cy3-pacDNA_{10k} (green). Scale bars are 20 μm. Early endosomes, late endosomes, and lysosomes are immunostained by anti-EEA1, anti-Rab-9, and anti-LAMP-1 antibodies, respectively (red). The nuclei are stained by Hoechst 33342 (blue). Arrows show sites of colocalization between pacDNA and early endosomes.
Figure 4-11. Manders colocalization analysis. The percentage of colocalization was calculated according to Manders colocalization coefficient (M2), which is the ratio between the number of red pixels that are colocalized with green pixels (samples) and the number of overall red pixels (cellular compartments).
4.5 References.


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Chapter 5

Polycondensation of Polymer Brushes via DNA Hybridization

This Chapter is based on a published paper with the same title.


Author contributions: X. L. and K. Z. conceived the study, designed the experiments, analyzed the data and wrote the manuscript. X. L. synthesized and characterized all the materials. E. W., F. J. and X. T. synthesized and purified some of the DNA strands and polymer-DNA conjugates. All authors edited the manuscript.
5.1 Introduction.

DNA has been recently explored as a structure component of a variety of nanomaterials, owing to its tailorability and programmability.\textsuperscript{1-5} For instance, origami structures of arbitrary shapes consisting of pure DNA have been created using multiple bespoke DNA sequences.\textsuperscript{6-10} DNA has also been used to mediate the assembly of various inorganic nanoparticles to form novel, complex crystals for which analogous atomic structures are oftentimes absent in nature.\textsuperscript{11-13} DNA-polymer conjugates are another important class of materials, having both biological properties of the DNA and the architectural and physiochemical properties of the polymer.\textsuperscript{14-17} These properties have rendered such conjugates useful in a broad range of applications, spanning drug delivery,\textsuperscript{18-21} gene therapy,\textsuperscript{22-24} and detection/sensing.\textsuperscript{25,26} Herein, we explore the use of DNA in the “polycondensation” of brush copolymers into much larger structures (in one or three dimensions), where the DNA serves as the functional group equivalent in step-growth polymerization.\textsuperscript{27}

The self-assembly strategy involves the synthesis of triblock bottle brush polymers as “macromonomers”,\textsuperscript{28-30} which can be selectively functionalized by amine- or thiol-modified oligonucleotides at the regions near the α- and ω-ends of the brush’s linear backbone. Given appropriate conditions, the hybridization between the nucleic acid strands should allow the monomers to self-assemble head-to-tail, connecting them either linearly or with branching, to form higher order assemblies (Scheme 1).

To create these assemblies, several design parameters must be established. First, the polymer backbone should be rigid enough such that it is energetically unfavorable for monomers to cyclize. This hurdle can be overcome by synthesizing a brush copolymer with sufficient side-chain length, which acts as a barrier to prevent intramolecular DNA hybridization, allowing the
multivalent conjugate to behave as a divalent structure. Second, there must be at least one DNA strand per block, but not too many as to open up space near the termini to allow more than one brush to form a connection, which will be important in the formation of linear structures where limiting the degree of branching is important. This will require the synthesis of oligomeric blocks having no more than a few repeating units for DNA conjugation. Recent advances in ring-opening metathesis polymerization (ROMP) have paved the way for the synthesis of the required building blocks.\textsuperscript{31,32}

\textbf{Scheme 5-1.} Synthesis of the hairpin DNA-polymer conjugate and formation of worm-like nanostructures \textit{via} DNA hybridization.
5.2 Results and Discussion.

Our first approach is to utilize a palindromic hairpin DNA sequence, which undergoes a hairpin-to-self-dimer transition when the temperature is raised above the $T_m$ of the hairpin structure. We designed and synthesized an amine-modified DNA sequence (DNA-1, 5'-NH$_2$-TTT TTA ATC CGT AGC GCT AGC CAT T-F-3') for polymer conjugation. A fluorescein tag was incorporated at the 3' to enable tracking and quantification of the resulting DNA-polymer conjugate. The calculated Gibbs free energy for the melting of the hairpin (-3.2 kJ/mole, $T_m$=36.2 °C) is higher than that of the self-dimer (-62.8 kJ/mole, $T_m$=57.0 °C), which allows us to control the polycondensation process by changing the temperature.

![Figure 5-1](image.png)

**Figure 5-1.** (A) MALDI-ToF MS spectra of p(N-NHS)$_5$-b-p(N-PEG)$_{35}$-b-p(N-NHS)$_8$ (polymer 1, blue) and p(N-NHS)$_{10}$-b-p(N-PEG)$_{29}$-b-p(N-MI)$_{10}$ (polymer 2, black). (B) GPC chromatograms of polymer 1 ($M_n$=70.0 kDa, $M_w$=80.7 kDa, PDI=1.15) and polymer 2 ($M_n$=45.9 kDa, $M_w$=52.8 kDa, PDI=1.15).

In order for this DNA sequence to control the assembly of the brush units, it must be conjugated to both chain termini of the brush. Towards this end, we synthesized norbornenyl N-
hydroxysuccinimidyl ester (N-NHS) as a reactive monomer, which was sequentially copolymerized by ROMP using a modified 2nd-generation Grubbs’ catalyst with norbornenyl poly(ethylene glycol) (Mₙ=2 kDa, PDI=1.05, N-PEG) to yield a triblock copolymer, p(N-NHS)₅-b-p(N-PEG)₃₅-b-p(N-NHS)₈ (Scheme 5-1). Due to the short backbone length, the polymer can alternatively be viewed as a star polymer. The NHS groups enable the coupling with amine-modified DNA strands, while the PEG side chains provide water solubility. Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-ToF MS) indicates a polymer Mₙ of 80.4 kDa and a PDI of 1.15 (Figure 5-1A, GPC data see Figure 5-1B). The successful incorporation of the N-NHS groups is confirmed by the stretching vibration modes for NHS carbonyl groups at 1780 cm⁻¹ and 1807 cm⁻¹ as measured by infrared spectroscopy (Figure 5-2). The number of available NHS groups is determined by allowing the polymer to react with an excess amount of fluorescein 5-thiosemicarbazide. Following purification, optical absorbance is taken, which is used to calculate the number of NHS groups. Approximately 10 NHS units are available for conjugation on each polymer.

The triblock brush is next coupled to DNA-1 by amidation chemistry at 0 °C in a pH=8.3 sodium bicarbonate buffer. This conjugate is characterized and purified by agarose gel electrophoresis. Fluorescein excitation/emission filters are applied when the gel containing the conjugates and free DNA is imaged (Figure 5-3A). The gel image clearly shows that a much higher molecular weight species is present after the reaction. The high MW band correlating to the conjugated product was removed from the gel and the conjugates were recovered using GenElute™ agarose spin column. The number of DNA-1 strands per polymer was determined by fluorescence measurements to be ca. 4.2.
Figure 5-2. Overlaid IR spectra of polymers 1 with monomers (top) and polymer 2 with monomer (bottom).
Figure 5-3. (A) Gel image of the hairpin DNA-polymer conjugate and free DNA-1 (fluorescein filters were applied). (B) DLS data of the conjugate before (top) and after (bottom) annealing. (C) TEM image of the free DNA-polymer conjugate, showing a sphere-like morphology. (D-F) Condensed brushes following annealing. The inset in D shows the worm-like structure as it is being formed; the scale bar is 100 nm. Arrows point to branching (E) and displacement (F) defects.
Figure 5-4. Additional TEM images of assembled DNA-1-polymer conjugate.

To initiate the self-assembly process, we subjected the DNA-polymer conjugate to an elevated temperature (80 °C) in the presence of 0.15 M NaCl, to fully dehybridize the duplexes. The solution was then allowed to cool down to room temperature over a period of 10 hours. This
annealing process should produce thermodynamic DNA duplexes in predominant proportions, which are the self-dimers as opposed to hairpins. As a result, it is expected that the macromolecules will connect in a head-to-tail fashion, to form linear supramolecular chains of polymer brushes. Before the self-assembly, the DNA-polymer conjugates are of a sphere-like morphology as shown by Transmission Electron Microscopy (TEM), and have a dry-state diameter of ca. 15±3 nm (Figure 5-3C). This is expected from the relative length of the PEG side chain (45 repeat units) and the brush backbone (48 repeat units), and is consistent with solution-state observations by Dynamic Light Scattering (DLS), which indicates that the conjugates have a number-average hydrodynamic diameter of 16±5 nm. After the thermal treatment, the hydrodynamic diameter increases to 127±29 nm (Figure 5-3B). TEM shows that the spherical polymer molecules have been assembled into one-dimensional, worm-like nanostructures. The worms have a crosssection diameter of 13±3 nm, which is consistent with the width of the brush polymer. The lengths of the worms vary significantly, ranging from hundreds of nm to several microns, as expected from polycondensation reactions (Figures 5-3D and 5-4). The micron-sized worms have a degree of polymerization of several hundred, suggesting that the DNA-mediated self-assembly process is highly efficient and not prone to errors. Nonetheless, we observed a small amount of defects in the assembled product. For example, branching (Figure 5-3E) and displacement, which creates a point that could lead to branching (Figure 5-3F), have been observed, and there are a small amount of free brush monomers that are not incorporated into the worm (Figure 5-3D, inset). The DNA-mediated assembly process is fully reversible. Upon removal of NaCl and/or increasing the temperature above the duplex T_m followed by rapid cooling, the worms revert to discreet brush polymers (Figure 5-5). In addition, in the presence of an excess of a free, complementary strand
(chain terminator), the poly-condensation process is hindered, and only free “macromonomers” are observed by TEM after annealing (Figure 5-6).

![Figure 5-5](image1.png)

**Figure 5-5.** TEM images of DNA-1-polymer conjugate following disassembly by dialysis.

![Figure 5-6](image2.png)

**Figure 5-6.** When an excess of T-DNA, which is fully complementary to the hairpin DNA-1, is added to the DNA-1-polymer conjugate, the formation of the worm structure is blocked and only spherical particles are observed in TEM.

The worm-like morphology is targeted in our study because it has been recognized to exert a profound impact on the behavior of nanomaterials in biological systems.\textsuperscript{35-37} For example,
filomicelles of several microns in length showed prolonged blood circulation times up to several days. Worm-like polymer micelles that were modified with folate could enter KB cells with nearly five-fold higher selectivity compared with their spherical counterparts modified with equal amounts of folate. This method of worm-formation, to the best of our knowledge, is a new strategy yet unreported, which can potentially lead to programmable assemblies of macromolecules.


In addition to using temperature to initiate the self-assembly process, we were also interested in assembling the brushes with an added DNA sequence as a linker strand. This strategy requires two different DNA strands to be connected to the two ends of polymer brush in a regioselective fashion. In order to achieve such a structure, we synthesized a thiol-reactive monomer (norbornenyl maleimide, N-MI) and incorporated it into the triblock brush as the third
block (p(N-NHS)$_{10}$-$b$-p(N-PEG)$_{29}$-$b$-p(N-MI)$_{10}$, **Scheme 5-2**). This “heterotelechilic” polymer features NHS ester units which can couple with amine-modified DNA strands on the first block, and maleimide groups for reaction with thiol-modified DNA strands on the third block. The successful synthesis of the polymer is verified by MALDI-ToF MS ($M_n$=67.5 kDa, PDI=1.20, Figure 5-1, for GPC see Figure 5-1B). In addition, the incorporation of the maleimide units was confirmed by $^1$H NMR (Figure 5-7), which shows the resonance of the maleimide double bond protons at 6.63 ppm.

![Molecular structure and NMR spectra](image)

**Figure 5-7.** Top: $^1$H NMR spectrum of polymer 1. Bottom: overlaid spectra of polymer 2 (blue) and norbornenyl maleimide (black).
Figure 5-8. (A) Multiplex gel image of the “heterotelechelic” DNA-polymer conjugate and free DNA-2 and DNA-3 strands (fluorescein and Cy3 channels are overlaid) (B) DLS measurements before (top) and after (bottom) assembly. (C) TEM images of free conjugates and (D) self-assembled structure following addition of the linker strand.

The two DNA strands to be conjugated to the polymer are designed to be free of self-dimers or hairpins, but can both hybridize with a common linker strand (DNA-2: 5’-Cy3-GAG GGT AAG GAG TTT-SH-3’, DNA-3: 5’-NH2-TTT GGA AAG GTT AGT-F-3’, and linker DNA: 5’-CTC CTT ACC CTC ACT AAC CTT TCC-3’). Again, fluorescent dyes (Cy3 and fluorescein) are incorporated to allow convenient tracking, quantification, and multiplex imaging of the DNA. The regioselective conjugation of the two different DNA strands to the polymer brush proceeds via a
two-step process. We first incubated DNA-2 with the polymer at 4 °C and pH 7.0 for 1 h. This consumes the maleimide groups selectively (the reaction rate of thiols to maleimide is three orders of magnitude higher than that of amines to maleimides at pH 7.0). The short reaction time and lowered temperature are to preserve the NHS esters from hydrolytic degradation. Thereafter, amine modified DNA-3 was added, and the pH of the solution was increased to 8.3. This step leads to the conjugation of the second DNA strand. The conjugate is again purified by agarose gel electrophoresis. The gel image shows a high MW band corresponding to the conjugate, which emits both fluorescein and Cy3 fluorescence, indicating that both DNA strands are successfully conjugated (Figure 5-8A). Quantification by fluorescence shows that ca. 3 DNA-2 strands and ca. 6 DNA-3 strands are conjugated to each brush.
**Figure 5-9.** Additional TEM images of assembled “heterotelechilic” DNA-polymer 2 conjugate. Arrows in A and B point to defects in assembly.
**Figure 5-10.** When T-DNA, which is non-complementary to either DNA-2 or DNA-3, is added to the DNA-2, 3-polymer conjugate, the formation of the networks is not triggered and only spherical particles are observed in TEM.

We next investigated the assembly of the DNA-polymer conjugate in the presence of the linker sequence. Before the self-assembly, the conjugates are discreet, spherical particles similar to the hairpin DNA-polymer conjugates (Figure 5-8C), with a mean hydrodynamic diameter of 17±6 nm as determined by DLS (Figure 5-8B). The addition of the linker DNA (3.0 equiv. to polymer) increases the size to 308±71 nm. TEM reveals that the brushes have formed cross-linked networks (Figures 5-8D and 5-9). In contrast, a non-complementary dummy linker has no effect on the assembly state of the brushes (Figure 5-10). On closer examination of the networks, it is observed that the degree of branching is much more significant when compared with the self-dimer constructs, with branching every one to two repeat units (Figures 5-9A and B). This is likely due to the added length of the linker strand, which creates room for more than two brushes to connect at each junction. Such a scenario may be further favored by the rigidity of the duplexes, which creates kinks in the assembly, facilitating branching. A third possibility would be the longer length (10 repeating units) of the oligomeric blocks used for DNA conjugation. These factors restrict the polymer brushes from forming very long linear structures, and instead guided them to yield cross-
linked networks. Of note, it is important to have a strict stoichiometry of the linker strand. With a large stoichiometric imbalance (i.e. less than 0.5 equiv. or more than 10 equiv. of the linker DNA), assembly is greatly hindered. This is expected as each junction requires at least one linker strand, but excessive amounts would quickly populate all of the brush chain ends, which disfavors further assembly of the brushes.

5.3 Conclusion

This study opens up new promising possibilities to create tailored polymer assemblies. We expect that with the synthetic availability of various types of polymer architectures, the diversity of the assembled polymer structures should increase significantly. The hybridization-controlled self-assembly also has the potential to be mediated by innate mRNA in an \textit{in vivo} setting, and therefore has important implications in nanomedicine.
5.4 Materials and Experimental procedures

Table 5-1. Oligonucleotide sequences used in this study.

<table>
<thead>
<tr>
<th>Name of Strand</th>
<th>Application</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA-1 (hairpin)</td>
<td>Couples with p(N-NHS)$<em>8$-b-p(N-PEG)$</em>{35}$-b-p(N-NHS)$_5$</td>
<td>5’-NH$_2$-TTT TTA ATC CGT AGC GCT AGC CAT T-fluorescien-3’</td>
</tr>
<tr>
<td>DNA-2</td>
<td>Couples with maleimide groups of p(N-NHS)$<em>{10}$-b-p(N-PEG)$</em>{29}$-b-p(N-MI)$_{10}$</td>
<td>5’-Cy3-GAG GGT AAG GAG TTT-SH-3’</td>
</tr>
<tr>
<td>DNA-3</td>
<td>Couples with NHS ester groups of p(N-NHS)$<em>{10}$-b-p(NB-PEG)$</em>{29}$-b-p(N-MI)$_{10}$</td>
<td>5’-NH$_2$-TTT GGA AAG GTT AGT-fluorescein-3’</td>
</tr>
<tr>
<td>DNA-4 (linker)</td>
<td>Serves as a linker for DNA-2 and DNA-3.</td>
<td>5’-CTC CTT ACC CTC ACT AAC CTT TCC-3’</td>
</tr>
<tr>
<td>T-DNA</td>
<td>Serves as the terminating strand and the dummy strand (controls in Figure S6 and S9)</td>
<td>5’-AAT GGC TAG CGC TAC GGA TTA AAA A-3’</td>
</tr>
</tbody>
</table>

Syntheses of norbornenyl N-hydroxysuccinimidyl ester 2 and norbornenyl maleimide 5

The synthesis is based on published procedures. Briefly, 2 was synthesized by esterification reaction between norbornenyl carboxylic acid 1 and N-hydroxysuccinimide (NHS) with a coupling reagent ethyl(dimethylaminopropyl) carbodiimide (EDC). This monomer was
used as the starting material to synthesize the other two monomers. Norbornenyl NHS ester 2 reacted with ammonia for 24 h to give norbornenyl amide 3, which was further reduced by lithium aluminum hydride (LiAlH₄) to form norbornenyl amine 4. The monomer norbornenyl maleimide 5 was successfully synthesized by reaction between 4 and maleic anhydride.

Compound 2:

\[
\text{Norbornenyl Maleimide (5)}
\]

\[\begin{align*}
\text{H-NMR (400 MHz, CDCl₃): } & \delta 6.20-6.13 (m, 2H), 3.27 (s, 1H), 3.00 (s, 1H), 2.83 (s, 4H), 2.50 (m, 1H), 2.06 (m, 1H), 1.55-1.50 (m, 1H), 1.46-1.40 (m, 2H); \\
\text{C-NMR (400 MHz, CDCl₃): } & \delta 171.9, 169.5, 138.8, 135.5, 47.4, 46.6, 42.0, 40.5, 31.3, 25.8.
\end{align*}\]

Compound 5:

\[
\text{Norbornenyl Maleimide (5)}
\]

\[\begin{align*}
\text{H NMR (400 MHz, CDCl₃): } & \delta 6.70 (s, 2H), 6.04 (s, 2H), 3.61-3.47 (m, 2H), 2.84 (s, 1H), 2.53 (s, 1H), 1.78 (m, 1H), 1.46-1.37 (m, 2H), 1.24-1.22 (m, 2H); \\
\text{C NMR (400 MHz, CDCl₃): } & \delta 137.2, 136.3, 134.2, 45.2, 44.5, 43.2, 42.0, 38.4, 30.8.
\end{align*}\]
Synthesis of norbornenyl poly(ethylene glycol) 2 kDa 6

\[
\begin{align*}
\text{Norbornenyl NHS ester} & \quad \text{DIPEA} \\
& \quad \text{DCM} \\
\text{Poly(ethylene glycol) methyl ether (mPEG-NH₂)} & \quad \text{N, N-diisopropyl ethyl amine (DIPEA)} \\
\end{align*}
\]

In a 25 mL round bottom flask, a stir bar was placed. Then, \(\omega\)-amine terminated poly(ethylene glycol) methyl ether (mPEG-NH₂, 200 mg, 0.095 mmol) and norbornenyl NHS ester (29 mg, 0.1235 mmol) were dissolved in 5.0 mL dichloromethane (DCM). N, N-diisopropyl ethyl amine (DIPEA, 16 mg, 0.1235 mmol) was added into the reaction mixture. The reaction mixture was allowed to stir for 4 h at room temperature. This reaction was monitored by MALDI-ToF MS, and all mPEG-NH₂ had been consumed. The reaction mixture was concentrated and precipitated into 15 mL diethyl ether for 3 times. The supernatant was removed, and the remaining white solid was dried in vacuo (190 mg obtained, yield=90\%).

\(^1\)H NMR (400 MHz, CDCl₃): \(\delta\) 6.12-6.07 (m, 2H), 3.80-3.45 (m, 185H), 3.36 (s, 3H), 2.91-2.89 (d, 2H), 2.02(m, 1H), 1.91-1.88 (m, 1H).

**General method for synthesizing tri-block copolymers**

The modified Grubbs’ catalyst (0.02 M) was dissolved in DCM in the Schlenk flask. All the monomers (0.04 M) were dissolved in DCM in three different Schlenk flasks. All the reagents were degassed by 3x freeze-pump-thaw cycles. The solution containing modified Grubbs’ catalyst was added into N-NHS ester solution via a microsyringe. The reaction mixture was stirred for 5 min, before the second monomer, norbornenyl-PEG was added. After 7 min of rapid stirring, the third monomer (N-NHS or N-MI) was added and allowed to react for 5 min. Finally, several drops
of ethyl vinyl ether (EVE) were added into the mixture and the reaction was stirred overnight. The reaction mixture was concentrated and precipitated into ethyl ether 3x and the precipitant was dried in vacuo.

**Quantitation of N-NHS groups for p(N-NHS)$_5$-b-p(N-PEG)$_{35}$-b-p(N-NHS)$_8$**

In a round bottom flask, a stir bar was placed. p(N-NHS)$_5$-b-p(N-PEG)$_{35}$-b-p(N-NHS)$_8$ (7 mg, 67 nmol) was dissolved in 2.0 mL DMF, to which fluorescein 5-thiosemicarbazide (1.43 mg, 3.4 µmol) and DIPEA (0.44 mg, 3.4 µmol) were added. The reaction mixture was allowed to stir at room temperature overnight. Then, the reaction mixture was dialyzed against NaCl solution (0.15 M) using dialysis tubing (MWCO 6-8 kDa) for 48 h. Finally, a polymer solution (2.4 mg/mL) was obtained. A UV-Vis absorption standard curve for fluorescein 5-thiosemicarbazide (491 nm) was obtained via serial dilution. The concentration of fluorescein in the polymer solution was determined by UV-Vis spectroscopy, and compared with the standard curve. Approximately 10 fluorescein tags were attached to each polymer molecule (Figure 5-11).

**Figure 5-11.** UV-Vis absorbance standard curve for free fluorescein.
**Coupling of DNA-1 to p(N-NHS)$_{5}$-b-p(N-PEG)$_{35}$-b-p(NHS)$_{8}$**

The polymer p(N-NHS)$_{5}$-b-p(N-PEG)$_{35}$-b-p(NHS)$_{8}$ was dissolved in DMSO to obtain a stock solution of 1 mM. DNA-1 (14.7 nmol) was dissolved in 10 μL aqueous solution of NaHCO$_3$ (50 mM) and NaCl (1 M). The polymer solution (1.5 μL) was added to the DNA solution, and the mixture was shaken gently for 4 h at 0 ºC on an Eppendorf Thermomixer. Thereafter, the reaction mixture was loaded into a 1 % agarose gel. The gel was electrophoresed in 0.5x TBE buffer under 100 V for 30 min. The desired conjugate band was cut and recovered from the gel by using a GenElute™ spin column. The final solution was lyophilized to get dim green powder.

**Quantitation of DNA-1 on the DNA-1-polymer conjugate**

A fluorescence standard curve for free DNA-1 in Nanopure™ water was obtained by serial dilution, following excitation at 494 nm and emission at 517 nm. Conjugate solutions of known concentrations were measured for fluorescence at the same excitation/emission settings, and the results were compared with the standard curve. Approximately 4.2 DNA-1 strands were coupled to each polymer molecule (Figure 5-12).

**Figure 5-12.** Fluorescence standard curve for free DNA-1.
Assembly and disassembly of DNA-1-polymer conjugate

The DNA-1-polymer conjugate (0.1 nmol) was dissolved in 0.5 mL 0.15 M NaCl solution in a centrifuge tube (for control, 2.1 nmol T-DNA is added). This tube was incubated in hot water bath and allowed to cool down in a thermally insulated container from 80 ºC to 25 ºC in ca. 10 h. After the annealing process, the conjugate solution was directly used for TEM and DLS analysis. To reverse the assembly process, the annealed DNA-1-polymer conjugate was dialyzed against Nanopure™ water at 70 ºC overnight. The resulting solution was directly used for analysis.

Coupling of DNA-2 and DNA-3 to p(N-NHS)\textsubscript{10}-b-p(N-PEG)\textsubscript{29}-b-p(N-MI)\textsubscript{10}

In a typical synthesis, 50 nmol DNA-2 strands was treated with 100 µL of 100 mM dithiothreitol (DTT) in 50 mM pH 8.0 phosphate buffer for 1 h. Thereafter, the DNA was desalted using a G25 illustra NAP-10 column (GE Healthcare). Polymer solution in DMSO (2 µL 1.25 mM) was mixed with thiol-modified DNA-2 in 2 M NaCl solution (20 µL 1.25 mM) at 4 ºC. After 1 h, an aliquot of the sample was analyzed by gel electrophoresis, which showed successful conjugation of DNA-2. The remainder of the reaction mixture was added to DNA-3 dissolved in 1 M NaCl 50 mM NaHCO\textsubscript{3} solution. The reaction mixture was incubated at room temperature overnight. Thereafter, the final product was purified and lyophilized (vide supra), which exhibited a pale red color.

Quantitation of DNA-2 and DNA-3 on the DNA-p(N-NHS)\textsubscript{10}-b-p(N-PEG)\textsubscript{29}-b-(N-MI)\textsubscript{10} conjugate

Fluorescence standard curves for DNA-2 and DNA-3 in Nanopure™ water were established, using excitation 494 nm/emission 520 nm for DNA-3, and excitation 546 nm/emission 570 nm for DNA-2. Conjugate solutions of known concentrations were measured for fluorescence
at the same excitation/emission settings, and the results were compared with the standard curves. Approximately 5.6 DNA-3 and 2.8 DNA-2 strands were coupled on each polymer molecule (Figure 5-13).

**Figure 5-13.** Fluorescence standard curves of DNA-2 (red) and DNA-3 (green).

**Assembly of DNA-p(N-NHS)$_{10}$-b-p(N-PEG)$_{29}$-b-(N-MI)$_{10}$ conjugate**

Linker DNA (DNA-4, 0.3 nmol) was dissolved in a NaCl solution (0.5 mL 0.2 M), which was added to the DNA-polymer conjugate (0.1 nmol) to give a final salt concentration of 0.15 M (for control, a dummy DNA sequence (T-DNA, 0.3 nmol) is added instead of the linker DNA). The mixture was incubated at room temperature overnight. After the incubation, the product was directly used for TEM and DLS analyses.
5.5 References


Chapter 6

Conclusion and Future Work

In this thesis, we have developed functional polyethylene glycol brush polymers that can be integrated into nanostructured materials or bioconjugates. The functional polymer-based bioconjugates are mainly investigated in two distinct areas of studies.

1. Brush polymers for oligonucleotide therapy. Nucleic acids have long been envisioned as biopharmaceutical agents in many forms of therapies. However, difficult biopharmaceutical characteristics of nucleic acids, such as poor enzymatic stability, rapid clearance by reticuloendothelial organs, immunostimulation, and coagulopathies, limit their clinical application. Many of these side effects are initiated via sequence-specific or non-sequence-specific interactions with proteins. We envisage that a strategy capable of inhibiting protein access but retaining nucleic acid hybridization should bypass many if not all of the side effects associated with oligonucleotide therapeutics. With this goal in mind, we developed a novel form of brush polymer-DNA conjugate, which was able to provide the DNA with such steric selectivity (Chapter 2). This is achieved through the intermediate density of the brush side chain environment, which blocks protein access but allow the slenderer DNA chains to access and hybridize. We demonstrate that the brush protects against nuclease degradation, prevents deactivation of blood components such as thrombin, and bypass the immunogenic side effects of certain oligonucleotide motifs (Chapter 4). Interestingly, the DNA within the brush polymer exhibits nearly identical thermodynamic characteristics as free DNA, and is able to serve as an antisense agent to regulate cellular gene expression without a transfection agent (Chapter 3). The PEG brush also improves the in vivo bio-distribution of the embedded DNA. Therefore, we believe the pacDNA can be a...
viable alternative to the polycationic transfection agent, which has been carefully studied, and is a radically new approach to addressing several long-lasting challenges in oligonucleotide therapeutics. Future research will explore (1) the new chemical structure of pacDNA with increased oligonucleotide loading, (2) cell biology of the pacDNA with respect to cell uptake mechanism and intracellular trafficking pathways in different cell lines. (3) the bio-distribution, immune response and gene regulation efficacy in vivo. (4) the delivery of other forms of oligonucleotide or peptide.

2. DNA-mediated materials assembly. DNA has been explored as a structure component of a variety of nanomaterials, owing to its tailorability and programmability. For instance, origami structures of arbitrary shapes consisting of pure DNA have been created using multiple bespoke DNA sequences. DNA has also been used to mediate the assembly of various inorganic nanoparticles to form novel, complex crystals. Although significant progress has been achieved, a strategy to program the ordered assembly of polymer molecules in a sequence-defined fashion is still very much in the embryonic stage. In Chapter 5, we have investigated the use of DNA in the “polycondensation” of brush copolymers into much larger structures (in one or three dimensions), where the DNA serves as the functional group equivalent in step-growth polymerization. We demonstrate that the steric of the brush allows one-dimensional connection of brush polymer building blocks in a head-to-tail fashion, generating long, linear brush “superpolymers”. Achieving one-dimensional assembly is the first step to realize more complex programmable assemblies that involve branching and length control. Thus, our approach for programmable bottom-up assembly is expected to generate exciting new possibilities for a spectrum of multiscale materials with unique electronic, biological, optical, and magnetic properties that can be tuned by their
dimensions and cooperativity. We plan to explore molecules with multiple bonding characteristics, which should lead to new architectures beyond 1D worms (stars, blocks, etc.) in the future study.