L-DNA Oligonucleotide Exchange Enables the Replenishment of Coatings on Blood-Contacting Devices

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Accessibility
L-DNA Oligonucleotide Exchange Enables the Replenishment of Coatings on Blood-Contacting Devices

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A Thesis in the Field of Bioengineering & Nanotechnology
for the Degree of Master of Liberal Arts in Extension Studies

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Abstract

The aim of this project was to determine if L-DNA oligonucleotide toehold exchange would be a viable mechanism for developing refillable anti-thrombogenic coatings for blood-contacting devices. Medical devices that interface with blood, such as catheters and stents, are susceptible to thrombus formation which can limit the lifespan of the device and lead to dangerous patient outcomes. Anti-thrombogenic coatings have been developed to address this problem, but their efficacy is limited by the degradation of these coatings over time; therefore, a system in which degraded coatings could be replenished would increase the longevity of these devices.

One such system using toehold mediated oligonucleotide exchange has been described. In this system, two oligonucleotide strands, one carrying an anti-thrombotic agent as a payload, are immobilized on the surface of the device. When this anti-thrombotic agent degrades, a third strand carrying a fresh payload is introduced displacing the previous strand. This process of strand replacement can be repeated with either strand. While this system is promising, it is potentially limited by degradation of oligonucleotide strands by circulating nucleases.

In this study, DNA and L-DNA, a mirror-image form of DNA with greater stability from enzymatic degradation, were fluorescently labeled in order to study the kinetics of strand replacement reactions. DNA and L-DNA oligonucleotides were also treated with nuclease solutions, whole blood, and serum to investigate their stability in these contexts. Both DNA and L-DNA strands showed similar abilities to perform
multiple strand replacements. Furthermore, these replacements occurred in timescales compatible with their intravenous delivery and predicted plasma half-life times. Additionally, the use of L-DNA oligonucleotides significantly improved stability when exposed to nuclease solutions or serum when compared to DNA.

These findings suggest that L-DNA oligonucleotide replacement represents a promising strategy for refilling anti-thrombogenic coatings of blood-contacting devices.
Acknowledgments

I am grateful to have benefited from an enormously supportive research environment. Specifically, I want to acknowledge Dr. Yevgeny Brudno and Stephanie McNamara for their significant contributions to the formative stages of this project and for Steph’s continued guidance throughout the project. Additionally, I’d like to acknowledge Dr. Simon Matoori and Dr. Junzhe Lou for their frequent help in troubleshooting and Dr. Irene de Lazaro del Rey for contributing the mice needed for whole blood and serum experiments. I especially want to thank Professor David Mooney for his mentorship throughout my entire time at Harvard, for fostering of a generous and collaborative culture within his research group, and, of course, for his support of this thesis project.

Finally, I want to thank my wife, Christine Lindberg, for her unwavering support. Lab work always has the potential for long, strange working hours, but this was doubly true during a global pandemic. Her willingness to readjust our lives to accommodate my ever-changing schedule combined with her constant encouragement made this work possible.
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Chapter I.

Introduction

Medical devices, such as catheters and stents, which interface with human blood are frequently used in patient care, but are associated with risk due to thrombosis formation. The mechanisms of thrombogenesis on device surfaces have been thoroughly characterized and this understanding has led to a number of strategies for the development of anti-thrombotic surface coatings. Because degradation of these coatings has been a limiting factor in their effectiveness, strategies to replenish these coatings have been investigated. One system for coating replenishment is mediated by oligonucleotide strand replacement; however, this system may be limited by nuclease degradation, necessitating the use of modified oligonucleotides to increase stability. This study investigates L-DNA-based toehold mediated oligonucleotide replacement as a system for replenishing surface coatings while resisting degradation by circulating nucleases.

Blood-Contacting Medical Devices: Uses and Risks

Blood-contacting medical devices are ubiquitous in patient care. Their purposes range from maintaining blood vessel patency, as is the case with coronary artery stents, to redirecting blood flow past occluded vessels, as with vascular grafts, to improving cardiac function in severe heart disease, as with left ventricular assist devices, or for long-term venous access for blood sampling and drug delivery, as with central venous
catheters. In each case, use of these devices is associated with risk of complications due to thrombosis resulting in device fouling, infection, or potentially fatal pulmonary embolism (Gorbet & Sefton, 2004, Wall et al., 2015, and Evans & Ratchford, 2018). Strategies to ameliorate the risks associated with long-term use of blood-contacting medical devices have not yet been achieved; however, biomaterials-based solutions engineered to interrupt the process of thrombus formation have been explored with the aim of prolonging the effectiveness and increasing the safety of these devices (Jaffer et al., 2015).

Mechanisms of Thrombus Formation on Blood-Contacting Devices

Thrombi are formed on blood-contacting medical devices through a specific cascade of events which is thoroughly described by Jaffer et al. (2015). The process is initiated by the adsorption of plasma proteins on the blood-contacting surface of the device mediated by the hydrophobicity of the device and electrostatic interactions between the device surface and plasma proteins, eventually leading to the formation of a monolayer around the lumen of the catheter. Of the plasma proteins adsorbed onto the device surface, fibrinogen plays the most important role by promoting platelet adhesion to the protein monolayer surface through integrin \( \alpha_{\text{IIb}}\beta_3 \) binding while simultaneously initiating the contact activation system. This pathway directly promotes thrombin generation and activates the complement system further enhancing thrombin generation. Thrombin both converts adhered fibrinogen to fibrin monomers and recruits more platelets. Polymerized fibrin strands provide structural support for platelet aggregates in a
process which culminates in the formation of a platelet aggregate-fibrin thrombus which can foul the device or worse, break off the device, travel through the pulmonary or systemic circulation, and lodge within a vessel interrupting blood flow causing significant tissue and organ damage (Jaffer et al., 2015).

Anti-Thrombotic Surface Coatings for Blood-Contacting Devices

This understanding of the mechanisms of thrombus formation has been leveraged to develop novel approaches to the prevent catheter fouling. Strategies typically involve either modifying the device such that it is less thrombogenic by preventing protein and cell adsorption onto the surface, or systemically delivering anticoagulant or anti-platelet drugs (Jaffer et al., 2015).

PEO Coated Devices

As previously mentioned, hydrophobicity of the device surface is associated with the initial plasma protein adsorption stage of thrombogenesis; therefore, polyethylene oxide (PEO) coatings, which exhibit strong hydrophilicity and unique, water-like properties, have the potential to inhibit thrombogenesis (Wang & Tsai, 2010). A number of surface modification strategies including bulk incorporation and covalent bonding of PEO to device surfaces have been explored yielding promising results in vitro; however, in vivo success has been largely elusive in part because of rapid oxidation of PEO, eliminating the device coating and, thus, antithrombogenic effect (Wang & Tsai, 2010).
Albumin Coated Devices

Albumin coated devices have been considered as a potential anti-thrombotic biomaterial. This approach is motivated by the finding that albumin does not promote platelet adhesion like fibrinogen does and is therefore less thrombogenic (Jaffer et al., 2015). Strategies have involved both the covalent bonding of albumin to the device surface as well as surface modifications with factors recruiting albumin to the surface, both with success in vitro (Marois et al., 1996). In an in vivo study implanting vascular grafts in dogs, differences were seen between coated and uncoated devices with respect to platelet and fibrin surface adhesion initially; however, this difference was short lived as albumin degraded from the device after four weeks (Marois et al., 1996).

Pyrolytic Carbon and Phosphorylcholine Coated Devices

Pyrolytic carbon and phosphorylcholine coated surfaces have both been explored as well. Initial findings suggested that carbon coatings on coronary artery stents promoted reendothelialization while reducing thrombogenicity; however, further studies showed no significant benefit over bare-metal stents (Sick et al., 2004). The anti-protein and anti-cell adhesion properties phosphatidylcholine exhibits as a cell-membrane lipid inspired biologically similar coatings with a similar pattern of in vitro success and in vivo failure in animal models of angioplasty (Kuiper et al., 1998) as well as in human studies (Galli et al., 2000).
Elastin-Inspired Polymer Coated Devices

A growing source of motivation for blood contacting biomaterials has been the development of elastin-inspired polymers. Blood vessel walls contain elastin which shows little platelet adhesion (Woodhouse et al., 2003). The challenge of isolating elastin from biological tissue sources has led to difficulties in leveraging elastin’s anti-platelet properties to engineer biomaterials; however, this problem has been circumvented by engineering materials with the five amino acid sequence understood to be responsible for its anti-thrombogenicity (Woodhouse et al., 2003). Devices with repeated units of this sequence along the surface have spurred a growing field of elastin-inspired devices which appear to maintain their biological efficacy in vivo as observed in arteriovenous shunt studies in baboons (Jordan et al., 2007). Elastin-inspired devices, therefore, represent the promise of a materials-based solution to device fouling.

Bioinspired Surface Coatings

Just as elastin-inspired devices ultimately derive their inspiration from the blood vessel lining, other approaches have been explored drawing from the anti-coagulative properties of endothelial cells. In some instances, this involves seeding device surfaces with whole endothelial cells (Jaffer et al., 2015). There are many challenges with such an approach. For instance, cellular viability and function can differ depending on environment; therefore, seeded cells may not behave similarly to their native counterparts (Jaffer et al., 2015). To avoid these challenges, devices have been engineered with biologically active surface coatings to mimic their anti-thrombogenicity. Promising
examples include corn trypsin inhibitor, a factor XII inhibitor which prevents contact system activation (Alibeik et al., 2011), heparin, an anticoagulant (Klement et al., 2006), and direct inhibitors of thrombin and platelet aggregation (Lu et al., 2012). Even the most promising approaches show limitations because of their biological degradation over time. Because of the potential need for long-term use of such devices, an adequate solution not only needs to maintain its biological efficacy from in vitro to in vivo, but it also needs this efficacy to persist throughout the lifetime of the device.

Enzyme Mediated Replenishment of Catheter Coatings

To prolong the efficacy of anti-thrombogenic factors immobilized to device surfaces, researchers have begun developing systems where these factors can be replaced after their degradation. One promising technology uses Staphylococcus aureus Sortase A (SrtA) to facilitate multiple replacements of thrombomodulin presented on a device surface (Ham et al., 2015). SrtA recognizes a five amino acid sequence (Leu-Pro-X-Thr-Gly, where X is any amino acid), cleaves the peptide bond between Thr and Gly, then binds a Gly-Gly-Gly motif to ultimately produce a Leu-Pro-X-Thr-Gly-Gly-Gly final product (Ham et al., 2015). Ham et al. immobilized Gly-Gly-Gly groups on the surface of a catheter and systemically co-delivered Leu-Pro-Glu-Thr-Gly peptide bound to thrombomodulin and a mutated version of the SrtA enzyme engineered for greater catalytic activity (2015). The Leu-Pro-Glu-Thr-Gly-thrombomodulin complex homed to the immobilized Gly-Gly-Gly sequence, mediated by mutant SrtA activity, thereby presenting the thrombomodulin payload on the device surface (Ham et al., 2015). After
some time, it is expected that the thrombomodulin would degrade and the device would lose efficacy. In anticipation of this, the system could be stripped by co-delivering Gly-Gly-Gly and SrtA to reverse the previous reaction allowing for an additional cycle of Leu-Pro-Glu-Thr-Gly-thrombomodulin charging (Ham et al., 2015). This process could theoretically be repeated indefinitely allowing the catheter to maintain an anti-thrombogenic state for the duration of its lifetime.

Oligonucleotide Replacement Mediated Replenishment of Catheter Coatings

McNamara et al. proposed another system for multiple replenishments of antithrombogenic coatings which used a system of three oligonucleotide strands (ODNs): one anchor strand and two exchange strands (2020). In their system, the anchor strand had a length of 29 bases while the exchange strands were 22 bases long (2020). Exchange strands 1 and 2 were complementary to the anchor strand; but because they were slightly shorter than the anchor strand, they were complementary to different portions of the anchor strand with exchange strand 1 (XS 1) being complementary to the 3’ most region of the anchor strand and exchange strand 2 (XS 2) being complementary to the 5’ most region of the anchor strand (McNamara et al., 2020). When one of the exchange strands was bound to the anchor strand, their difference in length results in a 7 base long, unbound overhang, referred to as the toehold region. If the other exchange strand was then introduced, it would bind the toehold region, at which point, the invading strand begins replacing the bound strand via branch migration in a process which is reversible with either XS 1 or 2 acting as the invading strand (McNamara et al., 2020). In the
system proposed by McNamara et al., complexes of anchor strand bound to an exchange strand modified to carry an anti-thrombogenic agent can be immobilized onto the surface of a blood contacting medical device (2020). As the anti-thrombogenic agent immobilized to the surface begins to degrade and its efficacy wanes, the opposing exchange strand can then be introduced resulting in the replenishment of the coating, much like in the SrtA mediated replacement system previously described (Figure 1). A noted advantage of this system, however, is that it obviates the necessity of a stripping step before recharging the device as we see in the SrtA system.
Figure 1. Replenishment of Coatings by Toehold Mediated Strand Replacement

A system for replenishing anti-thrombotic coatings on blood contacting devices has been proposed by McNamara et al. (2020). (1) Exchange strand 1 is carrying an anti-thrombotic agent and is bound to the anchor strand. (2) The anti-thrombotic agent is eventually degraded, at which time, exchange strand 2 is introduced, replacing exchange strand 1. (3) Exchange strand 2 is now bound to the anchor strand and presenting its own fresh anti-thrombotic cargo. (4) Once degradation of the agent occurs again, exchange strand 1, with a fresh anti-thrombotic payload, is reintroduced, restarting the cycle.

DNA Modifications for Increased Oligonucleotide Stability

Because circulating nucleases in the bloodstream degrade unmodified DNA in the order of minutes, the DNA based replenishment system proposed by McNamara et al. may have limited in vivo viability (Agrawal et al., 1995). While there are a number of potential
DNA modifications that promise protection from degradation, stability must not come at the expense of the kinetics of the strand exchange reaction. Phosphorothioate modification, in which one phosphate oxygen atom in the phosphodiester backbone is replaced with a sulfur atom, allows for protection against nuclease degradation allowing for longer plasma half-lives of 0.5 - 2.0 hours; however, these ODNs are also prone to non-specific protein interactions which may have the effect of altering exchange/anchor strand binding kinetics (Agrawal et al., 1995, Geary et al., 2009, and Bennett et al., 2017). Another possible strategy is to replicate these studies using L-DNA versions of the ODNs previously investigated. L-DNA is the enantiomeric form of DNA; therefore, the two species have identical binding properties, but with L-DNA being less susceptible to nuclease degradation (Hauser et al., 2006 and Kim et al., 2014).

**Research Objective**

The objective of this study was to determine if an L-DNA-based ODN exchange system would retain the ability of the previously described DNA-based system to perform multiple replenishments of surface coatings while resisting degradation in the presence of blood.
Chapter II.

Materials and Methods

The following is a detailed outline of the methods used to modify DNA and L-DNA ODNs with biotin or fluorescent labels, study the kinetics of ODN exchange reactions, and assess the viability of the ODN replenishment system in conditions more similar to the *in vivo* environment.

**DNA & L-DNA Sequences**

DNA and L-DNA ODNs were reconstituted in Buffer EB to create a stock concentration of 500 µM. The anchor strand had a 29-base sequence of GACGTAAGGTTATTGAATGAGTGATGCAG with a thiol modification and an 18-atom hexa-ethyleneglycol spacer at the 5’ terminus (Figure 2). XS 1 had a 5’ thiol modification and a 22-base sequence of CTGCATCCACTCATTCAATACC. XS 2 had a 3’ thiol modification and a 22-base sequence of CACTCATTCAATACCCTACGTC. XS 2 is complementary to the 5’ region of the anchor strand while XS 1 is complementary to the 3’ region of the anchor strand.
Figure 2. ODN Sequences and Modifications

The anchor strand is depicted in purple with a 29-base sequence and a 5’ thiol modification separated from the nucleotide sequence with an 18-atom hexa-ethyleneglycol spacer. This thiol group was used to modify the strand with biotin-maleimide which allowed for immobilization on streptavidin coated surfaces. XS 1 and 2 have 22-base sequences and are depicted in green and red, respectively. XS 1 is complementary to the 3’ end of the anchor strand has a 5’ thiol modification. XS 2 is complementary to the 5’ end of the anchor strand and has a 3’ thiol modification. Exchange strand thiol modifications are used for conjugation with dye-maleimides.

DNA & L-DNA Modification Protocol

Maleimide-Thiol reactions were performed to fluorescently label exchange strands and to biotinylate the anchor strand, allowing for immobilization on streptavidin coated 96-well plates. Exchange strands were labelled with either Sulfo-Cyanine3 (SulfoCy3) or Sulfo-Cyanine5 (SulfoCy5) modified with maleimide groups.

Both DNA and L-DNA ODNs were shipped with thiol modifications protected by disulfide bonds; therefore, a reduction step was required before maleimide-thiol reactions could take place. This was achieved by incubating ODNs with 30 molar equivalents of tris(2-carboxyethyl) phosphine (TCEP) in a 100 mM Tris HCl solution buffered to a pH
of 8.0 at room temperature for 2 hours. To biotinylate the anchor strand, 175 molar equivalents of biotin-maleimide were added to the solution and allowed to incubate at room temperature for 2.5 hours. To fluorescently label exchange strands, 20 molar equivalents of SulfoCy3-maleimide or SulfoCy5-maleimide were added to the solution instead. Solutions were then added to 3 kDa molecular weight cut off centrifuge tubes and centrifuged at 5,000 rcf for 35 minutes. Three wash cycles were then performed in which 400 µL of pH buffered 10 mM Tris HCl was added to the product before centrifugation. The final product was then dissolved in 100 µL of deionized water. DNA concentrations were assessed by absorbance at 260 nm.

Confirmation of Fluorescent Labelling of ODNs

Fluorescence intensities of ODN samples were measured after fluorophore conjugation to determine the percentage of ODNs successfully labelled. A standard curve was first generated by measuring the fluorescence intensity of unbound 0, 0.1, 0.2, 0.5, and 1.0 µM SulfoCy3-maleimide and SulfoCy5-maleimide. ODN samples were then diluted to 1.0 µM and nucleic acid quantification was performed to confirm the DNA concentration of the diluted sample. Fluorescence intensity was then measured, and the molarity of dye in solution was calculated using a linear regression of the generated standard curve. This value was then divided by the measured DNA molarity to determine the fraction of ODN in the sample that was fluorescently labelled.
Kinetics of Exchange Strand Replacement

The kinetics of the strand exchange reaction on immobilized anchor strands were then assessed. Biotinylated anchor strands were diluted to 1 µM in Ca\textsuperscript{2+} and Mg\textsuperscript{2+} free phosphate-buffered saline (PBS) and 30 µL per well was added to the surface of streptavidin coated 96-well plates for 5 minutes at room temperature. Wells were then washed three times with PBS to remove any unbound ODNs. Either fluorescently labeled XS 1 or 2 (30 µL of 1 µM solution) was then added to anchor strand-coated wells for 5 minutes before another PBS wash. Fluorescence was then measured to determine peak fluorescence for both exchange strands. For XS 1 treated wells, XS 2 was then added and vice versa. Fluorescence intensity was measured after 5, 30, 60, 300, 600, 900, and 1,200 seconds of application with PBS washes before each measurement. Fluorescence intensities were normalized to peak fluorescence before analysis. These studies were performed with both XS 1 and 2 as the initial strand and with both DNA and L-DNA ODNs. Additionally, studies were performed with DNA XS 1 bound to SulfoCy3 and XS 2 bound to SulfoCy5 as well as the reverse condition with XS 1 and 2 bound to SulfoCy5 and SulfoCy3, respectively.

For each experiment, non-linear regression analysis was used to determine the replacement limit, \( T_{50\%-\text{limit}} \), and \( T_{90\%-\text{limit}} \). The replacement limit was defined as the coefficient given by the regression curve representing its upper plateau. \( T_{50\%-\text{limit}} \) was defined as the time it takes to reach a value halfway between the lower and upper plateaus of the regression. \( T_{90\%-\text{limit}} \) was defined as the time needed to reach a value that is 90% of the upper plateau of the regression.
Characterization of Multiple Cycles of Exchange Strand Replacement

We then studied exchange reactions through multiple cycles of strand exchange. Anchor strand immobilization was achieved as previously described followed by a 5-minute treatment with either XS 1 or 2 (30 µL of 1 µM solution). After a PBS wash to remove any unbound ODNs, fluorescence intensity was measured to determine peak fluorescence for each strand. The opposite strand was then applied for 5 minutes (30 µL of 1 µM solution) and washed with PBS before another fluorescence intensity measurement. This process was repeated for six total cycles of exchange. Intensity values were normalized to the measured peak intensities before analysis.

As with exchange rate experiments, these studies were performed with both XS 1 and 2 as the initial strand, with both DNA and L-DNA ODNs, and with DNA XS 1 and 2 bound to SulfoCy3 and SulfoCy5, respectively, as well as with XS 1 and 2 bound to SulfoCy5 and SulfoCy3, respectively. Exchange efficiencies were calculated for each exchange cycle to compare conditions. Exchange efficiency was defined as the difference between fluorescence intensity after treatment and the residual fluorescence intensity from the previous cycle.

Degradation of ODNs by DNase I

Degradation of ODNs by increasing concentrations of DNase I was studied by comparing fluorescence intensity before and after treatment. As previously described, anchor strands were immobilized onto the surface of streptavidin coated plates, followed by an application of SulfoCy3-labelled DNA or L-DNA. Additionally, a Biotin-PEG-Cy3
marker was immobilized onto the surface of the plate. A PBS wash was then performed to remove all unbound materials before measuring fluorescence to determine baseline values.

Solutions of 0, 0.008, 0.016, 0.032, 0.080, and 400 U/mL DNase I were prepared in a DNase buffer consisting of 2.5 mM MgCl$_2$ and 0.5 mM CaCl$_2$ dissolved in a pH buffered solution of 10 mM Tris HCl. ODNs were then treated with 100 µL of prepared DNase I for 20 minutes. A PBS wash was then performed, and fluorescence intensity was measured and normalized to baseline values. Within each DNase I concentration, statistical analysis comparing DNA ODNs, L-DNA ODNs, and Biotin-PEG-Cy3 was performed using one-way ANOVA with Tukey’s multiple comparisons post-test.

Degradation of ODNs by Whole Blood and Serum

Degradation of ODNs by mouse blood and serum over time was studied by comparing fluorescence intensities. First, blood was collected from euthanized mice in accordance with active animal protocols approved by Harvard University’s Institutional Animal Care and Use Committee. Blood was collected in ethylenediaminetetraacetic acid (EDTA) coated tubes to prevent coagulation for whole blood experiments. For serum experiments, blood was collected in uncoated tubes and allowed to coagulate before centrifugation at 2,000 rcf for 10 minutes at 5º C. The separated liquid serum was then transferred to a separate tube for immediate use.

As previously described, DNA or L-DNA anchor strands were immobilized on the surface of streptavidin coated plates followed by application of SulfoCy3-labelled DNA or L-DNA XS 1. Following a PBS wash to remove any unbound ODNs,
fluorescence intensity was measured to determine baseline values. ODNs were then treated with 100 µL of PBS, whole blood, or serum and incubated at 37º C. Wells were washed and fluorescence was measured after treatment times of 1, 6, 12, and 24 hours. Fluorescence values were normalized to the previously measured baseline value. PBS, whole blood, and serum treated wells for DNA and L-DNA were then compared using one-way ANOVA with Tukey’s multiple comparisons post-test.
Chapter III.

Results

The following experiments characterize the kinetics of DNA and L-DNA ODN exchange reactions. Additionally, ODN stability was investigated after treatment with nuclease, whole blood, and serum.

Fluorescent Labelling of DNA and L-DNA ODNs

Sulfonated cyanine dye-maleimides were conjugated to thiolated ends of DNA and L-DNA ODNs to monitor strand exchange and degradation behavior. Specifically, SulfoCy3-maleimide was bound to the 5’ end of exchange strand 1 (XS 1) and SulfoCy5-maleimide was bound to the 3’ end of exchange strand 2 (XS 2).

To determine the fraction of ODN bound to dye-maleimides, the molarity of dye in solution was divided by the molarity of DNA in solution (Table 1). For 3’ modification of DNA XS 2 with SulfoCy5, this value was $1.1 \pm 0.1 \mu M_{\text{dye}}/\mu M_{\text{DNA}}$ while 5’ modification of XS 1 with SulfoCy3 gave a value of $2.8 \pm 0.9 \mu M_{\text{dye}}/\mu M_{\text{DNA}}^{-1}$. For L-DNA, these values were $1.2 \pm 0.02 \mu M_{\text{dye}}/\mu M_{\text{DNA}}^{-1}$ and $2.3 \pm 0.8 \mu M_{\text{dye}}/\mu M_{\text{DNA}}^{-1}$ for XS 2 and 1, respectively. Therefore, in both DNA and L-DNA ODNs, the ratio of dye concentration to DNA concentration was roughly 1:1 for XS 2-SulfoCy5 conjugates, but more than double in XS 1-SulfoCy3 conjugates.
<table>
<thead>
<tr>
<th></th>
<th>Dye Concentration (µM) / DNA Concentration (µM)</th>
</tr>
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<tbody>
<tr>
<td>DNA XS 1-SulfoCy3:</td>
<td>2.8 ± 0.9</td>
</tr>
<tr>
<td>DNA XS 2-SulfoCy5:</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>L-DNA XS 1-SulfoCy3:</td>
<td>2.3 ± 0.8</td>
</tr>
<tr>
<td>L-DNA XS 2-SulfoCy5:</td>
<td>1.2 ± 0.02</td>
</tr>
<tr>
<td>DNA XS 1-SulfoCy5:</td>
<td>1.1 ± 0.03</td>
</tr>
<tr>
<td>DNA XS 2-SulfoCy3:</td>
<td>2.7 ± 0.05</td>
</tr>
</tbody>
</table>

Note: Multiple combinations of dye-DNA conjugation were tested. For comparison between conditions, dye concentration was divided by DNA concentration in the same sample. Values represent mean and standard deviation (N = 3).

To further investigate this difference in the ratio of molar concentration of dye to molar concentration of DNA, dye-maleimides were conjugated to the opposite strands as before. When SulfoCy3 was bound to DNA XS 2, this value was 2.7 ± 0.05 µM<sub>dye</sub> µM<sub>DNA</sub>⁻¹ while SulfoCy5 conjugation to DNA XS 1 gave a value of 1.1 ± 0.03 µM<sub>dye</sub> µM<sub>DNA</sub>⁻¹. These data show that regardless of which exchange strand was being bound, SulfoCy3 use was associated with a nearly 3-fold higher dye:DNA ratio than with SulfoCy5.

**Kinetics of Exchange Strand Replacement**

The rate at which each exchange strand replaced the other was tested with anchor strands immobilized on well plates. These tests were performed with exchange strand 1 (XS 1) replacing initially bound exchange strand 2 (XS 2) and vice versa with both DNA and L-DNA ODNs. Initially, experiments were done with DNA and L-DNA XS 1 bound
to SulfoCy3 and XS 2 bound to SulfoCy5. Additional experiments were performed with DNA XS 1 bound to SulfoCy5 and XS 2 bound to SulfoCy3 to uncouple effects related to the strand from effects related to the dye.

While it was confirmed that each strand is able to replace the other, the rate and extent of replacement differed between the two strands. Non-linear regression analysis was used to determine the replacement limit, $T_{50\%-\text{limit}}$, and $T_{90\%-\text{limit}}$ for exchange reactions (Table 2 and Figure 3). This analysis showed that the $T_{50\%-\text{limit}}$ of XS 1 was 1.89 seconds while was $T_{90\%-\text{limit}}$ was in 8.51 seconds. Conversely, $T_{50\%-\text{limit}}$ of XS 2 was 17.4 seconds while $T_{90\%-\text{limit}}$ was 218 seconds.

Table 2. Kinetics of Exchange Strand Replacement

<table>
<thead>
<tr>
<th>Invading Strand:</th>
<th>$T_{50%-\text{limit}}$</th>
<th>$T_{90%-\text{limit}}$</th>
<th>Replacement Limit</th>
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</tbody>
</table>

Note: Non-linear regression analysis was used to compare the kinetics of exchange reactions. These analyses provided a value the Replacement Limit. $T_{50\%-\text{limit}}$ and $T_{90\%-\text{limit}}$ were then calculated to compare rates of reaction. Values represent mean and standard deviation ($N = 3$).
Figure 3. Kinetics of Exchange Strand Replacement

(A) Rates of exchange in DNA ODNs with either XS 2-SulfoCy5 (left) or XS 1-SulfoCy3 (right) as the initial strand. (B) Rates of exchange in L-DNA ODNs with either XS 2-SulfoCy5 (left) or XS 1-SulfoCy3 (right) as the initial strand. (C) Rates of exchange in DNA ODNs with either XS 2-SulfoCy3 (left) or XS 1-SulfoCy5 (right) as the initial strand. Values represent mean and standard deviation (N = 3).
Not only did exchange times vary between the two exchange strands, but the replacement limit achieved by both strands also differed. This value was 88.9% for XS 1 replacing XS 2, but only 59.6% for the opposite condition.

Exchange strand ODNs engineered from the enantiomeric L-DNA form behaved similarly to DNA ODNs. T\textsubscript{50\%-limit} and T\textsubscript{90\%-limit} for replacement of L-DNA XS 2 by L-DNA XS 1 were achieved in 2.31 and 16.6 seconds, respectively, while the T\textsubscript{50\%-limit} and T\textsubscript{90\%-limit} for replacement of L-DNA XS 1 by L-DNA XS 2 were 39.7 and 279 seconds, respectively. The trend of markedly faster exchange by XS 1 compared to XS 2 is maintained in L-DNA studies; however, the kinetics appear to be slightly slower in each condition in L-DNA compared to DNA ODNs. Replacement Limit for L-DNA XS 1 was 99.4% compared to 65.0% for L-DNA Strand 2. Again, the trend of more complete exchange by XS 1 compared to XS 2 observed in DNA ODNs is maintained in L-DNA; however, both L-DNA exchange strands achieved a higher fraction of exchange compared to the corresponding values for DNA exchange strands.

These studies were repeated with DNA exchange strands conjugated to the opposite dye-maleimides (XS 1-SulfoCy5 and XS 2-SulfoCy3). T\textsubscript{50\%-limit} for XS 1-SulfoCy5 replacing XS 2-SulfoCy3 was 0.35 seconds and T\textsubscript{90\%-limit} occurred in 14.4 seconds. In the opposite reaction, T\textsubscript{50\%-limit} occurred in 36.8 seconds with T\textsubscript{90\%-limit} occurring in 764 seconds. Finally, the replacement Limit was 100% with XS 1 as the replacing strand and 80.7% with XS 2 as the replacing strand. These data show that regardless of which fluorophore is conjugated to which strand, the trend of XS 1 exchanging faster and more completely than XS 2 is maintained.
Characterization of Multiple Cycles of Exchange Strand Replacement

The ability for DNA and L-DNA ODNs to replace one another after multiple cycles of 5-minute treatments with alternating strands was assessed. As in the previous experiment, anchor strands were immobilized to the surface of well plates and normalized fluorescence intensities were analyzed to characterize ODN behavior. Initially, experiments were done with DNA and L-DNA exchange strand 1 (XS 1) bound to SulfoCy3 and exchange strand 2 (XS 2) bound to SulfoCy5. Additional experiments were performed with DNA XS 1 bound to SulfoCy5 and XS 2 bound to SulfoCy3 to uncouple effects related to the strand from effects related to the dye.

As observed in the previous exchange rate experiments, XS 1 replaces XS 2 more efficiently than XS 2 replaces XS 1 (Table 3 and Figure 4). After a 5-minute treatment with XS 1, its fluorescent signal reached 82% ± 4.5% on average while XS 2’s signal decreased to 6.6% ± 2.7%. Treatment with XS 2 increases its fluorescence intensity to an average of 51% ± 7.1%; however, residual XS 1 remains with a level of 51% ± 4.2% of peak fluorescence.
Table 3. Characterization of Multiple Cycles of Exchange Strand Replacement

<table>
<thead>
<tr>
<th></th>
<th>Signal when replacing (%)</th>
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<th>Exchange Efficiency (%)</th>
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<td>DNA XS 1-SulfoCy5:</td>
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<td>39 ± 13</td>
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<td>DNA XS 2-SulfoCy3:</td>
<td>53 ± 6.8</td>
<td>17 ± 5.6</td>
<td>39 ± 8</td>
</tr>
</tbody>
</table>

Note: Exchange strand replacement dynamics were described by with three parameters: fluorescence intensity when introduced as the replacing strand, residual fluorescence after being replaced by the opposing strand, the difference between these values, referred to here as Exchange Efficiency. Values represent mean and standard deviation (N = 3).
Figure 4. Characterization of Multiple Cycles of Exchange Strand Replacement

Illumination of the change in fluorescence intensities after multiple exchanges. (A) DNA ODNs with either XS 2-SulfoCy5 (left) or XS 1-SulfoCy3 (right) as the initial strand. (B) L-DNA ODNs with either XS 2-SulfoCy5 (left) or XS 1-SulfoCy3 (right) as the initial strand. (C) DNA ODNs with either XS 2-SulfoCy3 (left) or XS 1-SulfoCy5 (right) as the initial strand. Values represent mean and standard deviation (N = 3).
This trend of near-complete replacement by XS 1 and incomplete replacement by XS 2 is also observed in L-DNA ODNs. After XS 1 treatment, its signal increases to 91% ± 3.0% while XS 2’s signal falls to 11% ± 3%. Upon treatment with XS 2, its fluorescence intensity reaches 67% ± 9.7% while 53% ± 3.9% of XS 1 remains.

These values show the fraction of each exchange strand bound to immobilized anchor strands after a given exchange cycle; however, the fluorophores conjugated to each exchange strand represent a hypothetical anti-thrombotic agent to be presented at the device surface. Because it is expected that the efficacy of such a biological agent will wane necessitating replacement, exchange efficiency (defined as the difference between normalized fluorescence intensity after treatment and the residual fluorescent signal from the preceding exchange cycle) was also analyzed. In DNA ODNs, exchange efficiency of XS 1 was 32% ± 3.2% and 41% ± 4.3% for XS 2. These values were 39% ± 3.2 % and 54% ± 9.7% for L-DNA XS 1 and 2, respectively. Interestingly, L-DNA Strands 1 and 2 have 23% and 31% higher exchange efficiencies than their respective DNA ODN counterparts. This difference is statistically significant.

These studies were repeated with DNA exchange strands conjugated to the opposite dye-maleimides (XS 1-SulfoCy5 and XS 2-SulfoCy3). Treatment with XS 1-SulfoCy5 resulted in an average signal of 79% ± 16%. Replacement with XS 2-SulfoCy3 reduced XS 1-SulfoCy5 signal to 39% ±13% while increasing XS 2-SulfoCy3 fluorescence intensity to 53% ± 6.8%. Subsequent replacement with XS 1-SulfoCy5 decreases XS 2-SulfoCy3 signal to 17% ± 5.6%. Exchange efficiency was calculated to be 35% ± 10% for XS 1-SulfoCy5 and 39% ± 8.0% for XS 2-SulfoCy3. These data suggest that regardless of which fluorophore is bound to which exchange strand, the
trends observed in the preceding experiments are mediated by exchange strand behavior and are not greatly affected by differences in dye-maleimides use.

Degradation of ODNs by DNase I

The effect of DNase I on DNA and L-DNA ODNs was studied to better understand the toehold exchange system’s susceptibility to degradation in environments more closely resembling in vivo conditions with respect to nuclease activity. In this study, SulfoCy3 labeled DNA and L-DNA ODNs were bound to anchor strands immobilized on the surface of streptavidin coated plates before treatment with increasing concentrations of DNase I for 20 minutes. A Biotin-PEG-Cy3 was included in each condition as a negative control. Fluorescence intensities were normalized to pretreatment values and compared (Figure 5).
Figure 5. Degradation of ODNs by DNase I

DNA ODNs, L-DNA ODNs, and a Biotin-PEG-Cy3 control were treated with increasing concentrations of DNase I for 20 minutes. Fluorescence intensities of the three groups were compared by one-way ANOVA with Tukey’s multiple comparisons post-test. Values represent mean and standard deviation (N = 3).

At a concentration of 0.008 U/mL, DNase I reduced normalized fluorescence intensity of DNA ODNs to $52\% \pm 3.3\%$ while intensities of L-DNA ODNs and Biotin-PEG-Cy3 were $97\% \pm 3.3\%$ and $86\% \pm 22\%$, respectively. This difference between DNA ODNs and L-DNA ODNs and Biotin-PEG-Cy3 was statistically significant ($P < 0.05$) while there was no significant difference between L-DNA ODNs and Biotin-PEG-Cy3 when compared to each other or to the untreated condition.
The magnitude of reduction of DNA ODN fluorescence correlated with increased DNase I concentrations. Intensity decreased to 17% ± 3.5% at 0.016 U/mL, 4.0% ± 1.6% at 0.032 U/mL, and 1.0% ± 1.3% at 0.080 U/mL. This reduction was not observed in L-DNA ODNs or Biotin-PEG-Cy3. A final 400 U/mL DNase I treatment was performed to confirm L-DNA ODN integrity at high DNase concentrations. In this condition, DNA signal was reduced to 0.5% ± 0.2% while there was no significant difference between L-DNA and Biotin-PEG-Cy3 with fluorescence intensities of 90% ± 1.8% and 92% ± 1.2%, respectively. These results confirm that bound DNA ODNs are susceptible to DNase I degradation while bound L-DNA ODNs remain intact, even at high concentrations.

Degradation of ODNs by Whole Blood and Serum

The extent to which DNA and L-DNA ODNs are degraded by treatment with whole blood and serum was then studied. In these studies, DNA and L-DNA ODNs were bound to anchor strands immobilized on the surface of streptavidin coated plates before treatment with PBS, whole blood, or serum. Fluorescence intensity was measured 0, 1, 6, 12, and 24 hours post-treatment and values were normalized to 0-hour fluorescence intensities (Figure 6).
Figure 6. Degradation of ODNs by Whole Blood and Serum

DNA and L-DNA ODNs were treated with whole blood, serum, or PBS. (A) Changes in fluorescence intensity were measured at 1, 6, 12, and 24 hours after treatment. (B) The effects of whole blood, serum, and PBS on DNA and L-DNA ODNs were compared at the 12-hour timepoint. Comparisons were made using one-way Anova with Tukey's multiple comparisons post-test. Values represent mean and standard deviation (N = 3).
Over time, fluorescence intensity decreased in all treatment groups; however, beginning at 12 hours, a statistically significant reduction was observed in serum treated DNA ODNs that was absent in all other groups. Serum treated DNA ODNs experienced a reduction in fluorescence to 7.0% ± 0.0% compared to 65% ± 8.8% and 61% ± 7.5% for PBS and whole blood treated DNA ODNs, respectively. Comparatively, fluorescence intensities were 60% ± 1.5%, 62% ± 8.9%, and 72% + 2.9% for serum, whole blood, and PBS treated L-DNA ODNs, respectively; these differences among L-DNA groups were not statistically significant. These studies demonstrate that serum is capable of degrading bound DNA, but not L-DNA, ODNs within 12 hours of exposure. Whole blood treatment did not result in a statistically significant reduction in fluorescence intensity in any timepoints studied.
Chapter IV.
Discussion

Blood contacting medical devices, whether in the form of central venous catheters, coronary artery stents, or vascular grafts, are frequently used in patient care settings. The use of these devices, however, is associated with complications due to thrombus formation in the catheter lumen (Wall et al., 2015). This has led to substantial research into potential antithrombogenic coatings for such devices. While promising technologies have been proposed, the duration of coating effectiveness remains a limiting factor (Jaffer et al., 2015). One potential solution allows for the replenishment of antithrombogenic coatings via toehold mediated oligonucleotide strand exchange (McNamara et al., 2020). In this system, an oligonucleotide is immobilized on the surface of a device. This anchor strand is then available for binding by one of two exchange strands which may carry an antithrombogenic agent. The exchange strands are so named because when one is bound to the anchor strand, introduction of the other results in the replacement of the old strand with the new strand. This system allows for multiple replenishments of a device coating, potentially prolonging the effectiveness of the device. Because this system is based on ssDNA oligonucleotides, it may be susceptible to degradation by circulating nucleases in in vivo conditions. L-DNA, the enantiomeric form of DNA, could provide protection from degradation, increasing the robustness of the system (Kim et al., 2014). Therefore, the aim of this study was to determine if a similar
L-DNA based system would maintain the exchange kinetics of the previous DNA based system while providing protection from nuclease degradation.

Fluorescent markers were used to track the behavior of oligonucleotides throughout this study. Oligonucleotides were modified with thiol groups to allow for fluorescent labeling via maleimide-thiol chemistry. This reaction was optimized with the aim of achieving near 100% labeling of ODNs. This was assessed by dividing the measured concentration of dye after labeling by the concentration of DNA. Values close to 1.0 suggest complete labelling of our ODN sample while values less than 1.0 suggest incomplete labelling. This value was 1.1 ± 0.1 \( \mu \text{M}_{\text{dye}} \mu \text{M}_{\text{DNA}}^{-1} \) when the 3’ end of XS 2 was labelled with SulfoCy5 suggesting complete labelling. However, the same value was 2.8 ± 0.9 \( \mu \text{M}_{\text{dye}} \mu \text{M}_{\text{DNA}}^{-1} \) when the 5’ end of XS 1 was labelled with SulfoCy3. Values greater than 1.0 suggest that more than one dye-maleimide molecule is binding a single ODN. This is unlikely, however, as there is only one thiol group available per strand. Another interpretation is that binding to ODNs fundamentally changes the fluorescence properties of SulfoCy3. An in-depth characterization of the properties of a similar Sulfoindocyanine Cy3 dye showed a 2.4-fold increase in fluorescent signal when bound to ssDNA compared to free dye (Sanborn et al., 2007). These data are approximately consistent with our value of 2.8 ± 0.9 \( \mu \text{M}_{\text{dye}} \mu \text{M}_{\text{DNA}}^{-1} \) for SulfoCy3 labelling of XS 1. To confirm that neither differences in ODN sequence nor differences in 5’ versus 3’ modification play a role, the same dyes were used to label opposite strands. In this case, 5’ labelling of XS 1 with SulfoCy5 resulted in a value of 1.1 ± 0.03 \( \mu \text{M}_{\text{dye}} \mu \text{M}_{\text{DNA}}^{-1} \) while 3’ labelling of XS 2 with SCy3 increased this value to 2.7 ± 0.05 \( \mu \text{M}_{\text{dye}} \mu \text{M}_{\text{DNA}}^{-1} \). These data suggest that differences in dye concentration / DNA concentration are related to the
properties of the dye rather than differences in strand sequence or 5’ versus 3’ labelling. These trends were also observed in labelled L-DNA ODNs. Therefore, the following experiments assume near 100% fluorescence labelling of DNA and L-DNA exchange strands; however, further liquid chromatography-mass spectroscopy studies will be performed to test this assumption.

The first step in determining potential in vivo feasibility of this system was to characterize the replacement kinetics of the two exchange strands. In both DNA and L-DNA conditions, XS 2 replaced XS 1 substantially faster than in the opposite case. For both DNA and L-DNA ODNs, XS 1 nearly fully replaced XS 2 in under 20 seconds while the reverse reaction occurred in slightly less than 5 minutes. These findings differ from previous studies using the same ODN sequences which showed more similar exchange rates between the two strands (McNamara et al., 2020). One key difference is that in prior experiments, 5’ FAM and 3’ HEX fluorophores were incorporated into the ODN structure rather than performing modifications via maleimide-thiol chemistry. This conjugation process may be responsible for altering exchange strand behavior. These experiments were also repeated with fluorescent dyes labeling the opposite strands to confirm that behavior observed was related to the strands themselves rather than properties of the dyes used. Although the rates of exchange were slightly slower for both strands in this condition, the same trend of faster exchange by XS 1 compared to XS 2 was observed, confirming that the kinetics of replacement observed are indeed mediated by the strands and not the dyes. With respect to in vivo application, previous studies show a terminal plasma half-life of intravenously delivered ODNs on the order of minutes, while phosphorothioate modified ODNs have plasma half-lives of 30 minutes to 2
hours (Agrawal et al., 1995 and Geary, 2009). These findings suggest that DNA ODNs may be cleared too quickly for sufficient replacement to occur. However, protective modifications to ODNs increase ODN half-life to a timescale compatible with strand replacement in vivo. Therefore, an L-DNA based system may be required for successful coating replenishment in vivo.

Another interesting finding from the replacement kinetics studies was that not only was XS 1 faster at replacing XS 2 than XS 2 was at replacing it, but XS 1 replaced a larger proportion of XS 2 than vice versa. With both DNA and L-DNA ODNs, XS 2 achieved about 65% of the strand replacement achieved by XS 1. This becomes relevant in analyzing exchange efficiency data in experiments performing multiple exchanges. Because XS 2 incompletely replaces XS 1, residual XS 1 remains during the next exchange cycle. In contrast, XS 1 more completely replaces XS 2 leading to lower amounts of residual XS 2 for the next replacement cycle. This allows XS 2 to have a larger exchange efficiency than XS 1, because even though XS 1 is associated with faster and more complete replacement, its exchange efficiency is limited by the incomplete replacement of XS 2 and the residual XS 1 this leaves behind. This point is exemplified by the differences in exchange efficiency after the first cycle of XS 1 delivery when XS 2 is the initial strand compared to exchange efficiency in each subsequent cycle of XS 1 application. Ultimately, exchange efficiencies for XS 1 were 32% ± 3.2% and 39% ± 3.2% for DNA and L-DNA, respectively, and 41% ± 4.3% and 54% ± 9.7% for DNA and L-DNA XS 2, respectively. These values represent the amount of fresh antithrombotic agent available for presentation at the device site after each cycle of exchange. Previous studies coating polyurethane catheters with anchor strands followed by treatment with
exchange strand resulted in a total concentration of $14 \pm 8$ pmol cm$^{-2}$ (McNamara, S. L., et al., 2020). The known concentration of thrombomodulin lining the endothelial cells of blood vessels is $107$ fmol cm$^{-2}$ (Qu, Z. et al., 2013). Comparatively, a 32% exchange efficiency, the lowest observed in any condition, would result in a concentration of approximately $4.5$ pmol cm$^{-2}$ for immobilized ODN and its payload. This concentration is 40-fold greater than the concentration of thrombomodulin lining blood vessels. Therefore, these data suggest that this system is capable of performing multiple replenishments of antithrombotic agents in concentrations sufficient to maintain an antithrombotic state on the device surface.

The effect of DNase I on DNA and L-DNA based ODN systems was then studied. Since DNase I is the principal nuclease active in blood, resistance to its degradation activity would suggest increased circulation time (Barra, G. B., et al. 2015). After 20 minutes of treatment with increasing concentrations of DNase I from 0.008 U/mL to 400 U/mL, there was no statistically significant difference in any condition between L-DNA ODNs and a Biotin-PEG-Cy3 control in which no effect from DNase I would be predicted; however, there was a statistically significant reduction in DNA ODN signal in every treatment group tested. These results confirm that L-DNA ODNs are protected from DNase degradation. Previous publications report that DNase I activity in human blood plasma ranged from 0.004 to 6 U/mL (Tamkovich et al., 2006). These findings, in combination with this ODN degradation data, suggest that a DNA based system would not be viable in constant contact with circulating blood. In contrast, there was no change in L-DNA signal, even with treatment of highest concentration of 400 U/mL, nearly 100 times greater than the upper range of DNase I measured in human blood plasma.
Finally, DNA and L-DNA ODNs were exposed to whole blood or serum for 24 hours at 37º C. Additionally, both ODNs were treated with PBS as a control. Signal decreased in all groups, including the PBS control, over 24 hours; however, beginning at 12 hours, there was a statistically significant reduction in signal from DNA ODNs treated with serum. This continued into 24 hours of treatment, while there was no significant difference between any of the L-DNA groups, DNA + PBS, and DNA + whole blood groups. The uniform reduction of signal in all treatment groups, besides DNA + Serum, suggests that enzymatic activity was not the only driver of signal reduction over time. It is possible that well plates lose their streptavidin coating over time at increased temperatures. However, it is clear that at the 12-hour timepoint, the DNA + serum group experiences a dramatic loss in signal that is distinct from the uniform, gradual loss of signal in the other treatment groups. Surprisingly, there is no significant loss of fluorescence intensity in the DNA + whole blood group when compared to the PBS treated control. The difference between whole blood and serum data could be explained by increased protein concentration in serum compared to whole blood; however, the short plasma half-lives of ODNs reported in the literature would predict degradation in whole blood treated groups as well. It is likely that the protein dynamics leading to ODN degradation are altered in in vitro conditions without constant circulation; therefore, these data suggest that in vivo studies are required. Nevertheless, these experiments suggest that a DNA based system for device coatings would likely not have long-term in vivo efficacy while an L-DNA based system is protected from degradation by circulating enzymes in the blood.
These experiments show that the proposed L-DNA based system is able to repeatedly replenish device coatings with sufficient concentrations of anti-thrombotic agents. Furthermore, the use of L-DNA provides for protection against degradation by circulating proteins, allowing for more replenishment cycles, and ultimately increasing the lifespan of the device. This work is limited by the use of \textit{in vitro} assays which have limited fidelity to the \textit{in vivo} environment. Future studies will characterize the L-DNA based system when immobilized on the surface of polyurethane catheters. Additionally, \textit{in vivo} experiments are needed to study the viability of the system over longer timescales. One application of interest is venous catheterization where replenishment is achieved by simply flushing the catheter with a solution of L-DNA exchange strand, mimicking a central venous catheter-like application. Venous catheterization without external access to the catheter could also be explored. In this case, the ability for systemically delivered L-DNA ODNs to replenish the device coating would be assessed. This model would more closely resemble a coronary artery stent or vascular graft application. Finally, potential antithrombogenic agents that do not dramatically alter the exchange behavior of the ODN system must be identified and tested.

Ultimately, the findings reported here support the hypothesis that L-DNA oligonucleotide strand replacement is a promising mechanism for multiple replenishments of antithrombotic coatings on blood contacting device surfaces warranting future studies.
A system for replenishing anti-thrombotic coatings on blood contacting devices has been proposed by McNamara et al. (2020). (1) Exchange strand 1 is carrying an anti-thrombotic agent and is bound to the anchor strand. (2) The anti-thrombotic agent is eventually degraded, at which time, exchange strand 2 is introduced, replacing exchange strand 1. (3) Exchange strand 2 is now bound to the anchor strand and presenting its own fresh anti-thrombotic cargo. (4) Once degradation of the agent occurs again, exchange strand 1, with a fresh anti-thrombotic payload, is reintroduced, restarting the cycle.
Figure 2. ODN Sequences and Modifications

The anchor strand is depicted in purple with a 29-base sequence and a 5’ thiol modification separated from the nucleotide sequence with an 18-atom hexa-ethyleneglycol spacer. This thiol group was used to modify the strand with biotin-maleimide which allowed for immobilization on streptavidin coated surfaces. XS 1 and 2 have 22-base sequences and are depicted in green and red, respectively. XS 1 is complementary to the 3’ end of the anchor strand has a 5’ thiol modification. XS 2 is complementary to the 5’ end of the anchor strand and has a 3’ thiol modification. Exchange strand thiol modifications are used for conjugation with dye-maleimides.
Figure 3. Kinetics of Exchange Strand Replacement

(A) Rates of exchange in DNA ODNs with either XS 2-SulfoCy5 (left) or XS 1-SulfoCy3 (right) as the initial strand. (B) Rates of exchange in L-DNA ODNs with either XS 2-SulfoCy5 (left) or XS 1-SulfoCy3 (right) as the initial strand. (C) Rates of exchange in DNA ODNs with either XS 2-SulfoCy3 (left) or XS 1-SulfoCy5 (right) as the initial strand. Values represent mean and standard deviation (N = 3).
Figure 4. Characterization of Multiple Cycles of Exchange Strand Replacement

Illustration of the change in fluorescence intensities after multiple exchanges. (A) DNA ODNs with either XS 2-SulfoCy5 (left) or XS 1-SulfoCy3 (right) as the initial strand. (B) L-DNA ODNs with either XS 2-SulfoCy5 (left) or XS 1-SulfoCy3 (right) as the initial strand. (C) DNA ODNs with either XS 2-SulfoCy3 (left) or XS 1-SulfoCy5 (right) as the initial strand. Values represent mean and standard deviation (N = 3).
Figure 5. Degradation of ODNs by DNase I

DNA ODNs, L-DNA ODNs, and a Biotin-PEG-Cy3 control were treated with increasing concentrations of DNase I for 20 minutes. Fluorescence intensities of the three groups were compared by one-way Anova with Tukey’s multiple comparisons post-test. Values represent mean and standard deviation (N = 3).
Figure 6. Degradation of ODNs by Whole Blood and Serum

DNA and L-DNA ODNs were treated with whole blood, serum, or PBS. (A) Changes in fluorescence intensity were measured at 1, 6, 12, and 24 hours after treatment. (B) The effects of whole blood, serum, and PBS on DNA and L-DNA ODNs were compared at the 12-hour timepoint. Comparisons were made using one-way Anova with Tukey’s multiple comparisons post-test. Values represent mean and standard deviation (N = 3).
Appendix 2. Tables

Table 1. Fluorescent Labelling of DNA and L-DNA ODNs

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<td>DNA XS 1-SulfoCy3:</td>
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<td>DNA XS 2-SulfoCy5:</td>
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<tr>
<td>L-DNA XS 1-SulfoCy3:</td>
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<td>L-DNA XS 2-SulfoCy5:</td>
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<td>DNA XS 2-SulfoCy3:</td>
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*Note: Multiple combinations of dye-DNA conjugation were tested. For comparison between conditions, dye concentration was divided by DNA concentration in the same sample. Values represent mean and standard deviation (N = 3).*
Table 2. Kinetics of Exchange Strand Replacement

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<td>80.7%</td>
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<td>53 ± 6.8</td>
<td>17 ± 5.6</td>
<td>39 ± 8</td>
</tr>
</tbody>
</table>

*Note: Exchange strand replacement dynamics were described by three parameters: fluorescence intensity when introduced as the replacing strand, residual fluorescence after being replaced by the opposing strand, the difference between these values, referred to here as Exchange Efficiency. Values represent mean and standard deviation (N = 3).*
References


