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The views, opinions and/or findings contained in this report are those of the author(s) and should not contrued as an official Department of the Army position, policy or decision, unless so designated by other documentation.

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RPPR Final Report
as of 08-May-2019

Agency Code:

Proposal Number: 63738LS **Agreement Number: W911NF-15-1-0195**
INVESTIGATOR(S):

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Final Report for Period Beginning 01-Jul-2015 and Ending 31-Oct-2018
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Begin Performance Period: 01-Jul-2015 **End Performance Period:** 31-Oct-2018
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Submitted By: Ph.D. Eric Kool Email: eric.kool@stanford.edu
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STEM Degrees: **STEM Participants:**

Major Goals: Major goals of project:
DNA-based self-assembling nanotechnology is a highly promising approach to making programmable structures and devices on the nanometer to micron scale. Many laboratories have made impressive progress on designing large static structures (e.g. origami, tubes) and structures with moving features (e.g. capsules, robots). However, some limits of DNA place constraints on this technology: namely, low hybridization affinity, and mishybridization due to low sequence complexity. This project in the long term was aimed at addressing these issues by testing xDNA as an alternative “genetic” polymer to replace DNA. xDNA forms hydrogen-bonded base pairs similar to DNA, but the bases are larger, rendering the helix wider by 2.4Å. xDNA hybridizes much more stably than DNA, and it contains 8 nucleotides rather than 4, increasing sequence complexity greatly.

Our initial specific aims for this project included the following
(1) identifying the helical repeat, rigidity, and optimal loop residues as basic structural features of xDNA;
(2) studying chemical and enzymatic ligation-based methods for constructing larger xDNAs, and incorporating them into DNA nanostructures;
(3) testing the assembly and structure of an even larger proposed new genetic form, xxDNA.

As described in our early progress reports, our early work revealed that synthesis of the xDNA nucleoside components was highly rate limiting; the compounds require many steps, and this places a constraint on what sequences could be made, since they would need to be assembled on a DNA synthesizer. To address this issue first, we focused on:
(4) synthesizing monomers on larger scale (esp. dxG, the most difficult monomer);
(5) testing whether DNA polymerases might be used to synthesize xDNA on very small scales, thus conserving monomers.

Accomplishments: File - accomplished_kool.pdf attached under Upload

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Training Opportunities: This work supported the research and training of multiple postdoctoral associates. They received training in organic synthesis and in nucleic acids chemistry. In addition, they have learned about analytical methods associated with DNA, including gel electrophoresis and MALDI-mass spectrometry.

Additional training opportunities have come from presenting their work orally at multiple research group meetings.

The P.I. has worked closely as a mentor to the postdoctoral associates, giving them both research advice as well as career training, including advice on networking and even advice on managing finances over a research career.

The P.I. is working with the associates for career placement.

Results Dissemination: Results dissemination

Publications:

Kent T, Rusanov TD, Hoang TM, Velema WA, Krueger AT, Copeland WC, Kool ET, Pomerantz RT. DNA polymerase ? specializes in incorporating synthetic expanded-size (xDNA) nucleotides. Nucleic Acids Res. 2016, 44, 9381-9392.

Invited research talks:

Invited speaker: Department of Chemistry, Miami University (Oxford, OH), “Designer DNA Bases: Probing Molecules and Mechanisms in Biology”, September 17, 2015.

Invited Speaker: “Small Molecule Strategies for Mapping RNA Structure and Sequence,” 251st American Chemical Society National Meeting, San Diego, March 16, 2016.

Keynote Address: “Fluorescent Sensors Built from DNA”, FB3: Annual International Meeting (Fluorescent Biomolecules and Their Building Blocks), Tianjin, China, July 13-17, 2016.

Invited Speaker: “Designer DNA Bases with Biological Function”, 252nd American Chemical Society National Meeting, Philadelphia, August 21, 2016.

Keynote Address: “Probing DNA Repair Pathways with Designer Nucleotides”, Pharmaceutical Society of Japan Annual Meeting, Sendai, Japan, March 25, 2017.

Department of Medicinal Chemistry, Daiichi Sankyo, Inc. (Tokyo, Japan), “Designer Nucleotides for Probing DNA Repair Mechanisms”, March 27, 2017.

Department of Chemistry, National Technological University (Singapore), “Designer Nucleotides for Probing DNA Repair Mechanisms”, March 29, 2017.

Invited Speaker: “Measuring and Modulating the Activity of Oxidative DNA Damage Repair Pathways”, Biological Chemistry Division Symposium, 254th American Chemical Society National Meeting, San Francisco, April 2, 2017.

Invited Speaker: “Small Molecule Probes for Study and Control of RNA,” Chemical Society of Canada National Meeting, Edmonton, AB, May 30, 2018.

Invited Speaker: Department of Chemistry, University of Southern California, “Designer Nucleotides for Probing DNA Repair Mechanisms”, February 1, 2018.

Invited Speaker: Department of Chemistry, San Jose State University, “Designer Nucleotides”, April 13, 2018.

Invited Speaker: Arrakis Therapeutics, Waltham, MA, “Small Molecules for Study and Control of RNA”, April 19, 2018.

Invited Speaker: SystemX Alliance Conference, Stanford University, “Molecular Probes of DNA and RNA Biology”, May 2, 2018.

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Honors and Awards: Nothing to Report

Protocol Activity Status:

Technology Transfer: Patent application:

R. T. Pomerantz, E. T. Kool, Compositions and Methods of Treatment Using Expanded-size DNA Base Analogs. U. S. serial # PCT/US17/47508, filed Aug. 19, 2016.

PARTICIPANTS:

Participant Type: Postdoctoral (scholar, fellow or other postdoctoral position)
Participant: Anastasia Kadina
Person Months Worked: 1.00 **Funding Support:**
Project Contribution:
International Collaboration:
International Travel:
National Academy Member: N
Other Collaborators:

Participant Type: Postdoctoral (scholar, fellow or other postdoctoral position)
Participant: Willem Velema
Person Months Worked: 3.00 **Funding Support:**
Project Contribution:
International Collaboration:
International Travel:
National Academy Member: N
Other Collaborators:

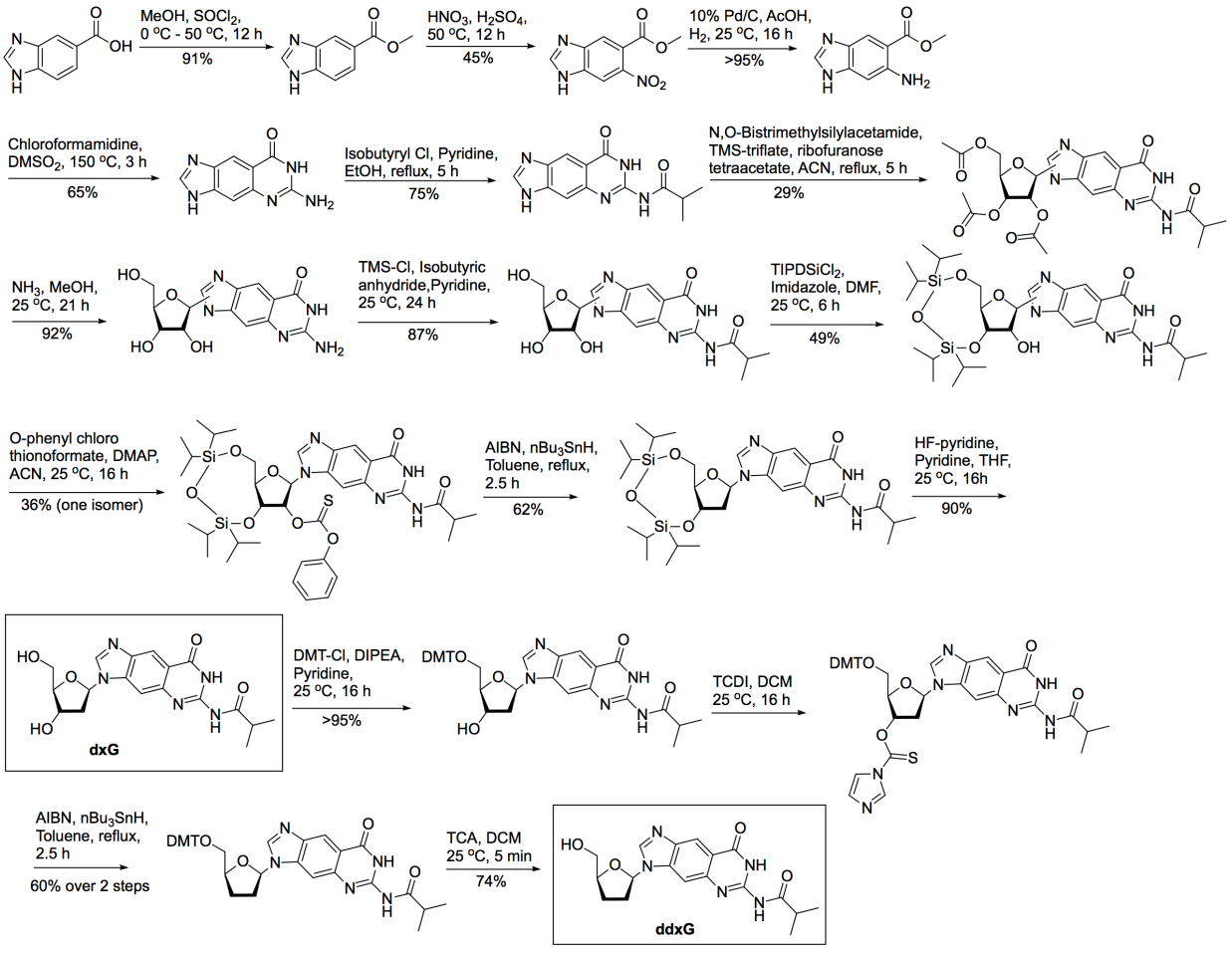
W911NF-1510195 (P.I. Kool)

Accomplished under goals

Synthesis of xDNA nucleosides and nucleotides

The preparation of xDNA nucleotides – the first step in making xDNAs - is considerably more complex and low-yielding than is the case for natural nucleotides. We spent much of our efforts in this project synthesizing these xDNA monomers. Our goals were two-fold: synthesis of monomer nucleoside triphosphates, and synthesis of dideoxynucleoside triphosphates. The former were needed for polymerase synthesis of xDNA, and the latter are needed to sequence xDNA and confirm polymerase activity.

Scheme 1 shows the route we employed to make the most difficult nucleotide, dxG, and the dideoxy variant, ddxG, which were the greatest focus of the project since they were the biggest rate-limiting step. We ultimately succeeded in making both dxG nucleoside and ddxG nucleoside triphosphates, and these were tested for their use in making xDNA (see below).



Scheme 1. Synthetic methods used to prepare dxG nucleoside (the precursor of triphosphate dxGTP) and the ddxG nucleoside.

Overall, we found multiple chemical challenges in this synthesis: first, it requires a relatively long synthetic route that takes much time and lowers yields by attrition. Second is some regio- and stereochemistry challenges that lower yields and make purification difficult. The biggest issue on this topic is glycosylation of the heterocycle (see the 6th step in this scheme), which gives a difficult-to-separate mixture of isomers. The third difficulty is that the ribose sugar works most efficiently for glycosylation than deoxyribose; this fact necessitates the late removal of the 2' and 3' oxygens, which also adds time and lowers yields. We believe that future studies of xDNA will benefit from alternative synthetic strategies for making the components – either altered synthetic chemistry approaches, or biochemical (enzymatic) methods.

Discovery of a polymerase that can accept xDNA nucleotides

Polymerase synthesis of xDNA base pairs is challenging because most polymerases have evolved a tight active site, fitting closely around DNA, which is considerably smaller than xDNA. However, some DNA repair polymerases are known to be more sterically flexible. Previous studies have shown, for example, that the bacterial repair DNA polymerase Dpo4 could perform relatively efficient nucleotide incorporation opposite template xDNA bases in vitro, in comparison to A family Pol I (Klenow fragment) which exhibits higher fidelity DNA synthesis. Before this project, however, the incorporation of dxNMPs into a primer strand by a template-dependent DNA polymerase had not been tested.

In particular, the polymerase domain encoded by the human *POLQ* gene - referred to herein as DNA polymerase θ —has been characterized as a highly promiscuous enzyme that exhibits translesion synthesis activity and the unique ability to synthesize DNA across a double strand break. Overall, Pol θ appears to be the most versatile and promiscuous of the error-prone translesion polymerases and therefore might conceivably incorporate size-expanded nucleotides into a polymer.

Here, we tested the ability of this enzyme to incorporate dxNMPs into sequence-encoded polymers.[1] To test this possibility, we first examined the ability of the purified polymerase domain to perform primer extension in the presence of a single complementary dxNTP in vitro. Pol θ was incubated with the indicated radio-labeled primer-templates which respectively encode for one of the four different bases immediately downstream from the 3' primer terminus. Primer-template extension was then initiated by the addition of either the complementary canonical dNTP or the respective large-sized dxNTP at equimolar concentration.

Surprisingly, Pol θ demonstrated efficient use of all four dxNTPs as substrates for primer-template extension. For example, Pol θ exhibited a similar efficiency of primer-template extension in the presence of the complementary dNTP or dxNTP for each template. Interestingly, in the case of dxGTP, Pol θ incorporated two consecutive dxGMPs, but only incorporated a single dGMP on the same template under identical conditions. This suggests that the presence of the incorporated large-sized nucleoside (dxGMP) in the enzyme's active site

facilitates the subsequent misincorporation event, possibly due to increased stacking interactions between dxNMPs. In the case of dxATP, two consecutive incorporation events are also observed. Interestingly, in the presence of dxTTP and dTTP, a different pattern emerged. Here, the canonical nucleotide (dTMP) was incorporated twice, whereas the large-sized nucleotide (dxTMP) was incorporated only once. This suggests that Pol θ may exhibit a reduced efficiency of dxTMP incorporation compared to other dxNMPs, which could reflect the somewhat weaker stacking ability of the xT base compared with the other expanded bases Overall, the results demonstrate that Pol θ effectively incorporates all four size-expanded dxNMPs into DNA, which has not been described before.

To explore this further, we carried out quantitative studies of the enzyme with these unusual substrates, by measuring the relative velocities of dxNMP incorporation by Pol θ under steady-state conditions.[1] The results show that Pol θ exhibits a substantially higher rate of incorporating size-expanded nucleotides derived from purine bases (dxGMP, dxAMP). We speculate that increased base stacking interactions by dxGMP and dxAMP contribute to their higher rate of incorporation. We next compared relative velocities of incorporation of large-sized and canonical purine and pyrimidine nucleotides, again under steady-state conditions. We found that Pol θ exhibits similar steady-state kinetics for dxGMP (V_{max}/K_m = 2.8 x 10^{-2} min^{-1} uM^{-1}) and dGMP (V_{max}/K_m = 5.0 x 10^{-2} min^{-1} uM^{-1}). Thus, these results demonstrate that Pol θ incorporates dxGMP with remarkable efficiency despite its large size.

Overall, the results show a robust ability of Pol θ to make xDNA base pairs from xDNA nucleotides. This is the first report of such an activity. Given that the goals of our ARO project were to prepare and measure the properties of short and long segments of xDNA, our new results suggest the possibility that we could use an enzyme to make duplexes of xDNA rather than exclusively using a DNA synthesizer. The advantage of using a polymerase enzyme is that a polymerase can synthesize DNA with much smaller quantities of nucleotide monomers as compared with DNA synthesizers.

[1]Kent T, Rusanov TD, Hoang TM, Velema WA, Krueger AT, Copeland WC, Kool ET, Pomerantz RT. DNA polymerase θ specializes in incorporating synthetic expanded-size (xDNA) nucleotides. Nucleic Acids Res. 2016, 44, 9381-9392.

Project final outcomes and conclusions.

• We reported the novel discovery that xDNA is a surprisingly good substrate of a newly discovered human DNA polymerase, Pol θ. This was described in a published paper and in a patent application. Enzymatic synthesis of this novel form of DNA is a particularly promising way to make and study xDNA in the future.

• Our studies determined that chemical synthesis of xDNA nucleotides is a limiting factor in assembling them into xDNA. We conclude that future work should focus on novel approaches to making these nucleotides, including potential chemo/enzymatic routes.