Investigating substrates Amplifu Red and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) in the Colorimetric Detection of DNAzyme Activity Localized to DNA Condensates

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Abstract: Through the NSF Future Manufacturing research program at Pasadena City College (PCC), students engaged in authentic research to explore aspects of DNA nanotechnology and gain experience in the research process. Emphasizing the scientific method and workforce development, students collaborated with our scientific community at UCLA, UCSB and Caltech as they learned how to use the tools of synthetic biology to build nanoscale bioreactors. Toward this goal, students set out to investigate various parameters to couple a DNAzyme-catalyzed redox reaction to DNA condensates with the aim of localizing the reaction. DNAzymes, guanine-rich sequences of DNA that fold into a G4 quadruplex structure, bind hemin, and catalyze a peroxidation reaction, were formed *in vitro* and used to catalyze a colorimetric redox reaction. Substrates ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and Amplifu Red were explored for their ability to 'turn on' or change color when oxidized by hydrogen peroxide in the presence of the peroxidase-like DNAzyme. In efforts to compartmentalize this reaction, the sequence for the G4 quadruplex was extended from one arm of a fluorescent 4-armed DNA nanostar, which contained either 15, 20, or 25 base pairs per arm and palindromic sticky ends. Upon annealing the DNA strands to form 4-armed DNA nanostars, with one of the strands containing the G4 sequence, the folded G4 quadruplex was tested for its ability to catalyze colorimetric peroxidation localized to DNA condensates. Students made important choices regarding the concentration of DNAzyme that would result in observable color change when localized to condensates; they carefully studied buffer compatibility between peroxidation and condensate formation; they tested two fluorogenic substrates in DNAzyme-catalyzed peroxidation, ABTS and Amplifu Red; and they meticulously analyzed the results, using what they learned to inform future decisions. The results of these localization studies will be leveraged in the next steps of this research project aimed at building nanoscale bioreactors from DNA. This high-impact educational experience taught students about the iterative nature of science and the significance of exploring the literature. Through research, they learned the important higher-order skills of experimental design and effective scientific communication, facilitating their development as scientists. This synthetic biology research was translated into lessons and implemented in PCC courses and through outreach, which inspired the students taught in outreach and the PCC researchers who served as learning assistants in this equitable and accessible STEM education.

Keywords: Undergraduate research, DNA nanotechnology, Synthetic Biology, STEM Workforce Development, Biological Redox Chemistry, DNA condensates, experimental design, equity in STEM education, STEM outreach, women in science

Introduction

This article showcases the PCC Future Manufacturing undergraduate research program, which is aimed at training a diverse, resilient, and creative STEM workforce [1]. DNA nanotechnology holds great promise for future manufacturing and is quite versatile due to the programmable nature of nucleic acids [2]. The ability to associate, separate, sort, and spatially organize components, while mastered in industrial biochemical processes, remains a challenge to embed in biochemical reactors at the microscale. Achieving the power to model such components with synthetic materials has the potential to revolutionize biomanufacturing by using microreactors that biochemically organize molecules through membrane permeability and bulk transport. Previous work showed that synthetic polymers, designer proteins, nucleic acids, and mixtures of these biomolecules can create host structures capable of recruiting and localizing molecules and coordinating chemical pathways [3,4]. The programmable nature of nucleic acids allows for the facile design of functional nanostructures in an accessible manner. Synthetic DNA condensates can be designed to compartmentalize biochemical reactions, paving the way toward nanoscale bioreactors.

Through the NSF Future Manufacturing undergraduate research program at Pasadena City College (PCC) in collaboration with UCLA, UCSB, and Caltech, students participated in a novel research project that employs the tools of synthetic biology to localize a biochemical redox reaction to synthetic DNA condensates. Specifically, 4-armed DNA nanostars with either 15, 20, or 25 base pairs per arm, palindromic sticky ends, and a fluorophore attached to arm 1—for visualization via microscopy—were designed to form DNA condensates. By extending arm 4 of a DNA nanostar with the sequence for a G4 quadruplex, a G-rich sequence of DNA capable of folding into a tetraplex structure that can bind hemin to yield a peroxidase-mimicking DNAzyme, we hypothesized that DNAzyme-catalyzed peroxidation could be localized to DNA condensates (Figure 1), which we could confirm through microscopy (Figure 2). Through the research process, PCC 'nanostar' student researchers made important decisions regarding experimental design and data collection (Figure 3). Students acquired essential research skills as they learned how to conduct science in the laboratory, guided by professors, postdocs, and graduate students engaged with similar research in DNA nanotechnology/synthetic biology.

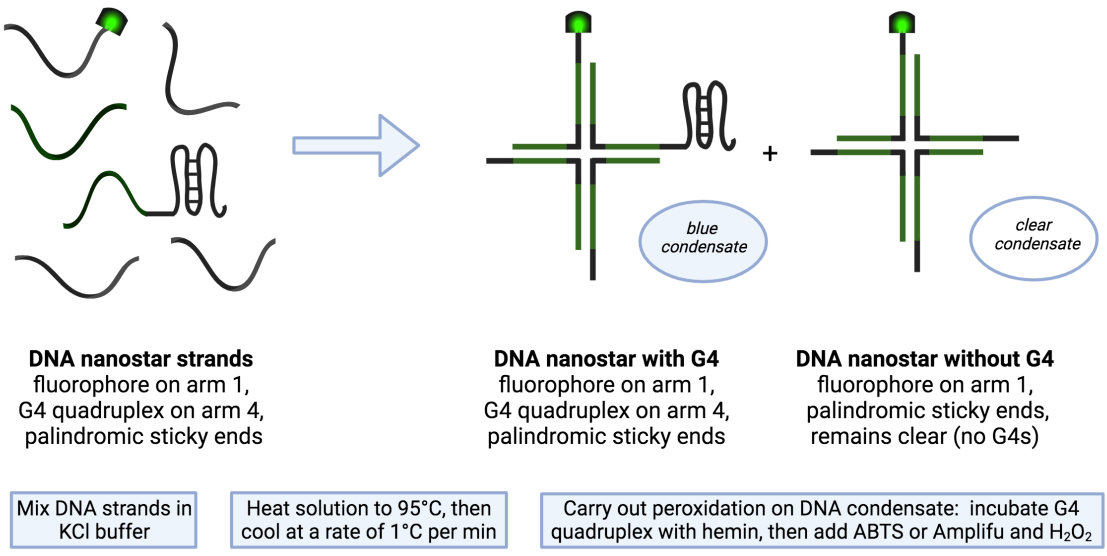


Fig. 1. Overview of strategy to localize DNAzyme-catalyzed peroxidation to fluorescent DNA condensates. DNA strands to form a 4-armed DNA nanostar with either 15, 20, or 25 base pairs per arm, with 5% of DNA nanostars containing a fluorophore for visualization and a portion of nanostars extending the DNA sequence for a G4 quadruplex, are mixed together in buffer containing KCl. This DNA nanostar solution, which includes a DNA strand encoding the G4 quadruplex, is heated and then slowly cooled. Hemin is incubated with DNA condensates hosting the folded G4 quadruplex to form the active DNAzyme catalyst. Adding substrates ABTS or Amplifu Red and H_2O_2 results in a color change upon oxidation [5].

Given its robust base-pairing forces and customizability, DNA serves as a strong candidate for achieving compartmentalization at the nanoscale to drive biochemical activity. Previous studies that utilize DNA to localize reactions have underscored the relevance of G-quadruplex tertiary structures (G4s), which are higher-order DNA structures formed from guanine-rich sequences [6]. G-quadruplex structures can have a strong affinity to bind hemin, and this resultant G4-hemin complex exhibits peroxidase-like activity [7,8]. These DNAzymes and other enzymatically active interactions can be integrated into aforementioned DNA structures, such as hydrogels [8] or synthetic condensates [9,10], with the aim of localizing biochemical reactions. A key experimental advantage of peroxidase-like DNAzymes is the colorimetric readout of the peroxidation reaction when using substrates ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) or Amplifu Red, which yield reaction products that are blue-green and pink, respectively (Figure 2). This provides a visual readout of the DNAzyme-catalyzed reaction and helps the experimenter correlate a blue/pink DNA condensate to a fluorescent condensate. This makes it possible to determine whether the DNAzyme-catalyzed reaction is localized when analyzing the DNA condensates post-peroxidation using a fluorescence microscope.

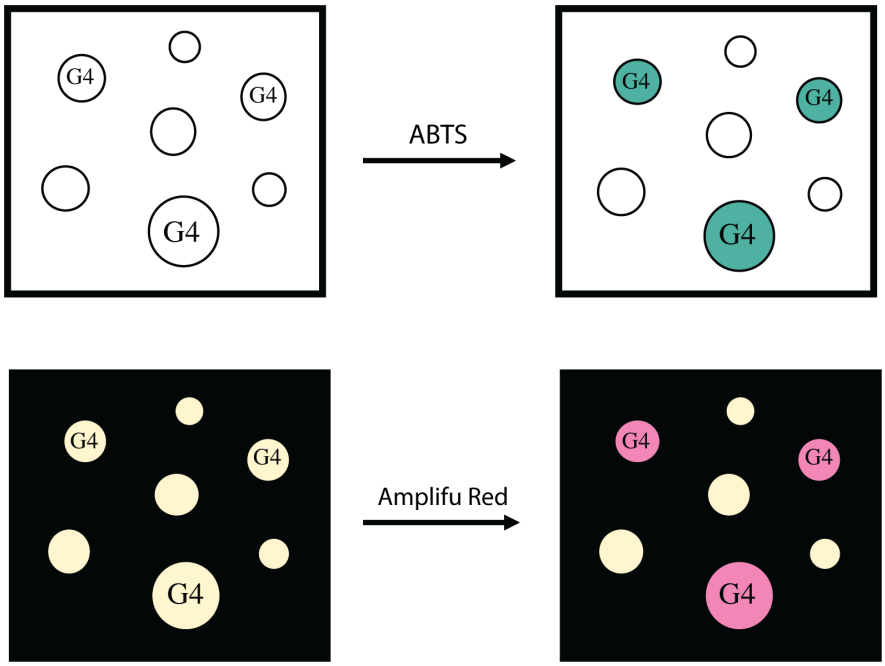


Fig. 2. Expected experimental outcome of DNAzyme-mediated peroxidation localized to microscale DNA condensates with ABTS (top), which results in blue-green condensates, or Amplifu Red (bottom), which yields pink condensates.

In coupling DNA-catalyzed peroxidation to microscale DNA condensates, various experimental parameters and questions were addressed throughout the undergraduate research program (Figure 3). By systematically adressing each point and using the results to inform their next steps, the students moved the research project forward in a very short time period of 6 weeks, working in the laboratory 20 hours per week. Discussions with collaborators and colleagues taught students about the community aspect of science and resourcefulness when confronting challenges. Students learned the importance of sound experimental design, reagent/buffer choice, troubleshooting, and creativity in scientific research. Throughout this paper, figures will be presented that were designed by the research students to display the results of their experiments, which they also presented in poster sessions; this provided students the opportunity to develop important scientific communication skills. Notably, students learned how their thoughtful and informed decisions led to significant progress toward the overall aim of compartmentalizing biochemical reactions using DNA, ultimately toward developing nanoscale bioreactors.

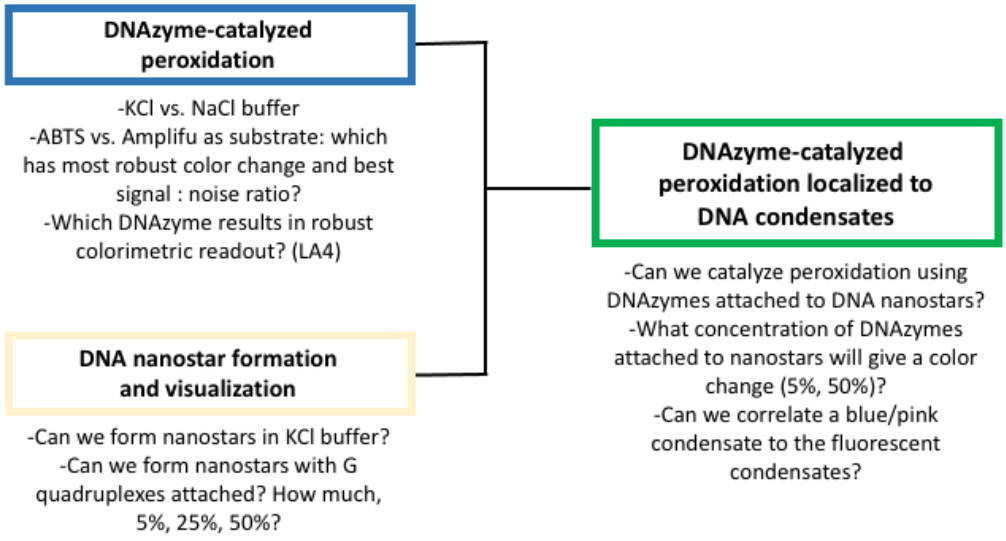


Fig. 3. Overview of major research questions addressed by PCC nanostar researchers in localizing DNAzyme-catalyzed peroxidation to DNA condensates.

Various factors dictate the intensity and duration of the colorimetric change induced during DNAzyme-mediated catalysis, including the identity of the reducing agent and concentrations of other reactants. As a first step toward the use of DNAzymes to catalyze redox reactions at the microscale, students set out to test two different reducing agents and their corresponding colorimetric activity. They achieved this by focusing on two fluorogenic substrates, ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and Amplifu Red, chosen for their stability and high sensitivity [8] when oxidized by hydrogen peroxide in the presence of a peroxidase. Interactions with the peroxidase result in a colorless-to-blue-green change for oxidation of ABTS to $ABTS^{+\cdot}$ [11] and a colorless-to-bright pink change for oxidation of Amplifu Red to Resorufin (Figure 4).

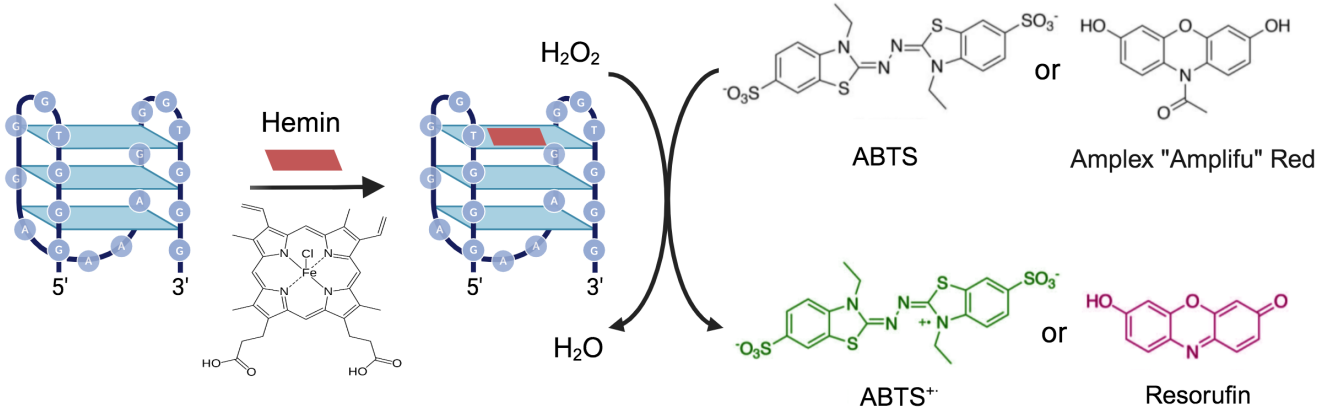


Fig. 4. DNAzyme-catalyzed peroxidation. Upon binding of a hemin cofactor to the folded G4 quadruplex, the resultant DNAzyme is capable of catalyzing a colorimetric peroxidation. Oxidation of ABTS results in the ABTS radical cation, which changes the colorless solution to blue-green, and oxidation of Amplifu Red results in Resorufin, giving a pink colorimetric readout [12].

This article follows the research of the PCC nanostar students over a six-week period in the Summer of 2023, where they worked in the lab 20 hours per week, guided by the principal investigator and with the support of professors, postdocs and graduate students at UCLA, UCSB, and Caltech. Following a research techniques bootcamp at UCLA, where students learned invaluable skills in DNA nanotechnology research, such as making and diluting solutions, quantifying nucleic acids, setting up microscale biochemical reactions, and

forming and visualizing DNA nanostars, the students brought their new knowledge and skills back to the PCC research labs and applied it in their own research. They worked together to imagine and design experiments in regard to the overall goal of localizing biochemical reactions to DNA condensates, ultimately toward the development of nanoscale bioreactors. The PCC students systematically addressed various parameters to move the research project forward; and many of the proposed research objectives were met. The students achieved remarkable results in a short research time period, which was facilitated by our scientific community. The positive environment in the laboratory made the research enjoyable and allowed the students to feel safe and supported as they shared their ideas; mentorship also had a significant impact on the PCC research students. Students developed character traits that contribute to success in STEM, began to develop a science identity, and had fun working together in the laboratory doing cutting-edge research in synthetic biology. The PCC students learned to communicate their science through written and oral presentations, and they became mentors to their peers and younger students as they taught what they had learned through research in education and outreach efforts. We conclude by showcasing poster presentations given by the PCC nanostar researchers and highlighting various educational outreach activities designed to teach synthetic biology research in an accessible manner.

Methods

Oligonucleotides, reagents, and buffers

DNA oligonucleotides were purchased from Integrated DNA Technologies (IDT). Following a thorough literature search that resulted in a panel of G quadruplexes which were screened for peroxidase activity (SI), we selected LA4 as the DNAzyme to optimize for localization to DNA condensates and polyT18, composed entirely of thymine bases, as a negative control (Tables S1, S2). DNA strand concentrations were calculated using the Beer-Lambert Law; a NanoDrop 2000 Spectrophotometer was used to measure UV absorbances of DNA strands at 260 nm; these were divided by molar extinction coefficients provided in IDT oligonucleotide specification sheets (Tables S2-S3). Hemin purchased from Frontier Specialty Chemicals was dissolved in DMSO and diluted to 50 μM in reaction buffer. The solution was protected from light and stored at 16°C until use. ABTS tablets were acquired from Sigma Aldrich and dissolved in MilliQ water to 18.3 mM, then diluted to 6 mM in reaction buffer. Similarly, Amplifu Red (Sigma Aldrich) was dissolved in DMSO to 3 mM and later adjusted to 1.5 mM in reaction buffer. Lastly, hydrogen peroxide was diluted in Tris-HCl buffer to 6 mM. Solutions were prepared in 10 mM Tris-HCl (pH 7.0-7.5), with 100 mM KCl, 0.05% (V/V) Triton X-100, and 1% (v/v) DMSO [13]. All buffer reagents besides KCl (ThermoFisher) were purchased from Sigma-Aldrich.

Formation of DNAzymes by the 'snap-cool' method and DNAzyme-catalyzed peroxidation

DNA strands were diluted with deionized MilliQ water to a final concentration of 10 μM in reaction buffer and then folded into G-quadruplexes through a method coined as "snap cooling" (Figures S1, S2). This procedure, as established in the literature [13], involves heating the G4 strands in Table S1 to 95°C for 10 min and then 'snap cooling' in an ice bath for 30 min to yield the G4 quadruplex structure. The folded G4 strands were mixed with hemin and buffered to a final concentration of 5 μM. This mixture was incubated at room temperature for 30 min to form the G4-hemin complex. In determining the ideal concentration of hemin for further studies in localizing DNAzymes to DNA condensates, the concentration of hemin in the assay was modified from 10 μM to 15 μM. The incubated G4-hemin solution was diluted to 0.5 μM in buffer and mixed with the freshly prepared substrate (ABTS or Amplifu Red). As the final step, 6 mM H_2O_2 (final concentration of 600 μM) was added to the solution. The resulting concentration of the substrate was consistently 600 μM for ABTS, and it was adjusted between 150 and 300 μM for Amplifu Red by altering the stock concentration. The colorimetric change was monitored for solutions containing both ABTS and Amplifu Red, with the intensity of color change carefully documented at various time points (2 min, 10 min, 15 min, 20 min, and 64 hr).

Formation of DNA condensates in NaCl or KCl buffer

To form control DNA condensates *in vitro*, the DNA strands were first diluted tenfold from their stock concentrations of approximately 1000 μM (Tables S2, S3). Depending on the DNA nanostars being formed, either with 15, 20, or 25 base pairs per arm, the corresponding DNA strands were used, and their individual concentrations can be referenced in the Supplemental Information (SI). To create the 4-arm DNA nanostar/condensate solution, all four nsDNA strands were mixed in equimolar ratios with either NaCl (5000 μM stock) or KCl (2000 μM stock) and Tris-HCl pH 7.5 (200 μM stock) at final concentrations of 350 μM salt

and 20 μM Tris-HCl. A fluorophore was attached to strand 1 of DNA nanostars for visualization using an Echo Revolve fluorescence microscope. NS15-strand 1, NS20-strand 1, and NS25-strand 1 contained 5% FAM, 5% Texas Red, or 5% Yakima Yellow, respectively. nsDNA strands were pipetted to a final concentration of 20 μM and each molecular species' volume was calculated such that the final volume of the solution would be 50 μL. The DNA nanostar solution was annealed by heating to 95°C for 10 min, then gradually cooled at a rate of 1°C per min, after which it was visualized using fluorescence microscopy.

Formation of DNA condensates +/- G4 sequences extended from arm 4

The DNA nanostar solution that includes the nsDNA strand 4 encoding the G4 quadruplex in KCl buffer was heated to 95º C for 10 min and then snap-cooled by placing it in an ice water bath for 30 min. Hemin was incubated with the DNA condensates/folded G4 quadruplexes for 30 min at room temperature to form the active DNAzyme catalyst attached to a portion of the DNA condensates. Adding substrates ABTS or Amplifu Red and H_2O_2 resulted in a color change upon oxidation, which was carefully documented. The final reaction mixture contained 100 mM KCl, 10 mM Tris-HCl (pH 7.5), 0.05% (v/v) Triton-X 100, 1% DMSO, 0.5 μM G4-hemin catalyst, 600 μM ABTS (or Amplifu Red) and 600 μM H_2O_2, conditions modified from [14].

For condensates with LA4 attached, the only element that was changed from the general condensate formation protocol was the concentration of strand 4 +/- G4 quadruplex added. To achieve condensate solutions that were composed of nanostars with 5% G4, the quantity of control strand 4 was scaled down to 95% concentration (or 19 μM), and the remaining 5% (1 μM) of strand 4 with G4 was added. To form a condensate solution with 50% G4, strand 4 with and without the G4 was added so each would obtain final concentrations of 10 μM (SI).

Imaging DNA condensates using microscopy

DNA nanostars were formed by mixing 10 μM of each component oligomer plus 0.1 μM of one of the DNA strands carrying a 5′ FAM, Yakima or Texas Red fluorophore label in a solution containing 10 mM Tris-HCl (pH 7.0-7.5), with 100 mM KCl or NaCl, 0.05% (v/v) Triton X-100, and 1% (v/v) DMSO in peroxidation reactions. DNA sequences are given in SI *Appendix*, Table S3. This solution was placed in a thermocycler, held at 95°C for 10 min, and then cooled to room temperature at a rate of 1°C/min. 2.5 μL droplet solution was then added to a clean glass coverslip coated with a parafilm chamber and visualized on an Echo Revolve microscope (San Diego, CA) with images captured by a 4× or 10× objective lens. Prior to visualization, glass slides were washed twice, first with distilled water and then with isopropyl alcohol, and then dried under airflow. A square parafilm (Parafilm® M, Fisher Scientific) piece with a punched hole in the middle was adhered to the glass by heating the slides to 50°C for 1 min before imaging. After the coverslips returned to room temperature, 2.5 μL of sample was pipetted into the center of the punched hole. Another smaller coverslip (Fisherbrand™, cat: 12-545-AP) measuring 30 × 22 mm, with a thickness between 0.13 and 0.17 mm, was placed on top of the parafilm to avoid evaporation of the sample solution during the observation period.

Peroxidation on microscale DNA condensates +/- DNAzyme

Once DNA condensate formation (+/- LA4 attached) was confirmed through fluorescence microscopy, peroxidation was carried out on the DNA condensates by adding hemin directly to the DNA condensate solution at a final concentration of 0.5 μM G4-hemin catalyst and incubating at room temperature for 30 min. Then, either 600 μM ABTS or Amplifu Red was added, followed by 600 μM H_2O_2, swirling the tube slowly to mix the reagents, and carefully to not create air bubbles. Images were captured at increasing time intervals using a standard iPhone camera to show the colorimetric change resulting from the peroxidation reaction.

Results and Discussion

Through undergraduate research in DNA nanotechnology/synthetic biology, PCC nanostar student researchers made significant progress toward the overall research objectives in localizing a biochemical reaction to DNA condensates. They learned invaluable research skills, developed character traits such as resilience and leadership, and had fun doing science together in the lab as they made critical decisions to design, implement, and analyze experiments to move the research project forward. The observable colorimetric response upon peroxidation enabled students to evaluate whether biochemical localization had occurred, which was confirmed through microscopy. The design of this research project showcases the accessibility of our science, which is a major aim of the research program and education and workforce development effort.

Overview of major experimental outcomes

Toward the research goal of localizing DNA-mediated reactions to DNA-based 'compartments', DNA strands were designed using well-known kinetic and thermodynamic properties of DNA nanostars and DNAzymes. A thorough literature review resulted in a panel of DNAzyme candidates (Table S1), which were screened for peroxidase activity. LA4 and FC3 emerged as excellent candidates, as their colorimetric response was most robust (Figure S3), and LA4 was ultimately selected for incorporation into condensates due to the consistency of LA4-catalyzed peroxidation. In testing two common reducing reagents, ABTS and Amplifu Red, as colorimetric detectors of DNAzyme peroxidase activity, ABTS was found to be more reliable than Amplifu Red, which sometimes displayed false positive or negative outcomes (Figures 5, 6). As K^+ has been shown to enhance the activity of DNAzymes such as LA4 [16], a significant research outcome was the formation of DNA condensates in KCl buffer (Figure S4), displaying compatibility between the two processes. As a step to localization, peroxidation was carried out on nanostar strand 4s/LA4 to determine whether the extra nsDNA would have an effect on DNAzyme activity, and indeed, the color change was consistently more faint with the strand 4/LA4 samples as compared to the standalone LA4 DNAzyme (Figure 7). To test whether DNA condensates could form with DNAzyme attached and determine the amount of DNAzyme/condensate that results in a visible color change, students formed DNA condensates with increasing amounts of DNAzyme. They determined that 50% was an ideal concentration of DNAzyme (LA4), as condensates with 50% LA4 form *in vitro* (Figure 9) and DNA condensate-localized peroxidation can be observed by eye (Figures 10, 11). Localization was confirmed via microscopy by correlating blue-green condensates in brightfield to fluorescent condensates, which was observed for NS15/50% LA4 and NS20/50% LA4 (Figure 12).

Selection of LA4 for localization studies

Before DNAzymes can be considered for incorporation into complex macroscale nucleic structures, it is important to optimize their utilization at the microscale. A literature search resulted in a panel of DNAzyme candidates (Table S1); and collaborators at UCLA and Caltech carried out the initial screen of DNAzymes for peroxidase activity (Figure S1), testing two different methods of formation, annealing and snap-cooling (Figure S2), adapted from a standard protocol from reference [13] (*see Methods*). The goal was to determine which DNAzymes had the most robust color change and how long it took for the solution to change color.

DNA sequences encoding G4 quadruplexes (Table S1) were ordered from Integrated DNA Technologies, quantified using a NanoDrop spectrophotometer (Table S2), and the procedure from reference [13] was modified according to conditions described in the SI to assess peroxidase activity. Peroxidase-mimicking DNAzyme LA4 was selected for investigations, as it was determined that LA4 has a $V^0 = 164.4$ nM/s [14] and the optimal sequence in studies investigating nucleotide repeats on G4-hemin / DNAzyme activity [15]. As expected, LA4 turned dark blue-green almost immediately, which was followed by FC3 and AS1411; 5xG3T produced a light blue-green color in both G4 formation methods tested (Figure S3). LA4 was selected to incorporate into DNA condensate localization studies, particularly for its robust color change in peroxidation.

Investigating ABTS and Amplifu Red as substrates in colorimetric detection of DNAzyme activity

G4 quadruplexes bind hemin to form DNAzymes that exhibit peroxidase-like activity, catalyzing the oxidation of fluorogenic probes to generate reaction products that result in a color change [7,8]. This colorimetric response, characterized by its distribution, intensity, and duration, provides qualitative insights into DNAzyme activity. The colorimetric profiles of two peroxidation substrates, ABTS and Amplifu Red, were initially assessed. A color change indicated successful G4 formation and hemin binding, as both substrates showed similar color intensity post peroxidation (Figure S5). The DNAzyme-catalyzed reactions exhibited significantly greater color intensity compared to hemin alone, demonstrating the DNAzyme's enhanced catalytic efficiency [16]. However, a faint pink color was consistently observed in the Amplifu Red polyT18 negative control samples, suggesting the presence of unbound hemin catalyzing the reaction. Reasoning that this issue could be resolved by modifying the hemin concentration, we opted to use two hemin concentrations, 10 μM and 15 μM, in peroxidation experiments with ABTS and Amplifu Red. Figures 5 and 6 show LA4-catalyzed peroxidation using 10 μM hemin or 15μM hemin and ABTS or Amplifu Red, respectively. There was a slight observable difference in colorimetric response between the two hemin concentrations using ABTS. In both experiments, the signal-to-noise ratio was very good; the negative polyT18 control remained clear, while the LA4-catalyzed reaction turned blue-green (Figure 5).

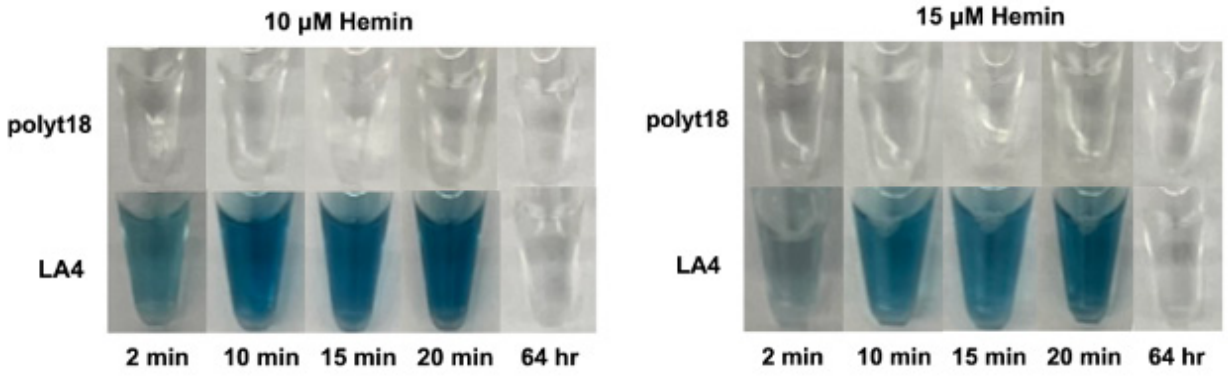


Fig. 5. Colorimetric detection of DNAzyme activity using ABTS and varying concentrations of hemin at increasing time intervals. Either 10 µM or 15 µM hemin was added to the snap-cooled LA4 (or polyT18 negative control) to yield a DNAzyme that was used to catalyze colorimetric peroxidation of ABTS.

150 µM and 300 µM Amplifu Red were tested to determine the optimal concentration for peroxidation (Figure 6). Both concentrations showed comparable color intensity in LA4 samples within each hemin concentration system, attributed to constant DNAzyme concentration leading to similar amounts of Resorufin end product. However, in polyT18 negative controls, 150 µM Amplifu Red exhibited a fainter color change compared to higher substrate concentrations. This suggests that higher substrate levels enhance hemin-mediated redox reactions, resulting in a brighter color. Negative controls with 15 µM hemin were significantly darker than those with 10 µM, likely due to more hemin molecules acting as catalysts. Initially, samples with 10 µM hemin showed a more intense pink color, but this reversed after 10 minutes as the reaction darkened. This suggests that hemin initially faced steric hindrance but eventually maximized interactions with G4s in solution. Based on these observations, 10 µM hemin with 150 µM Amplifu Red were chosen as reaction conditions, as they produced an immediate, intense color change. The strong pink color persisted for 64 hours, showing a longer colorimetric response duration than ABTS. While Amplifu Red's longer-lasting color change is advantageous for extended monitoring of DNAzyme activity, the observed inconsistencies and pink color in polyT18 negative controls suggest fine-tuning is necessary.

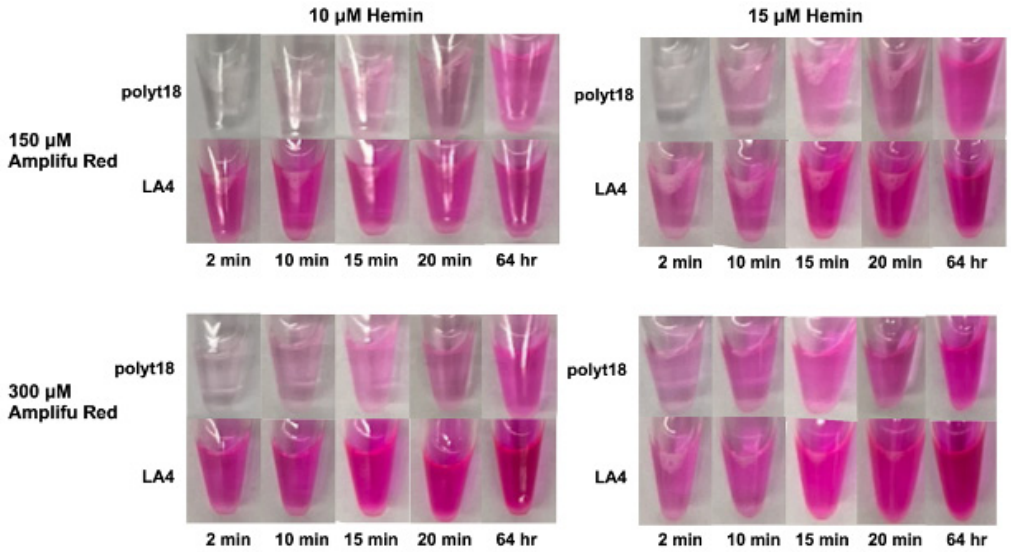


Fig. 6. Colorimetric detection of DNAzyme activity using Amplifu Red and varying concentrations of hemin at increasing time intervals. Either 10 µM or 15 µM hemin was added to the snap-cooled LA4 (or polyT18 negative control) to yield a DNAzyme that was used in colorimetric peroxidation with 150 µM or 300 µM Amplifu Red substrate added to the reaction to generate a colorimetric response.

Buffer compatibility between DNAzyme-catalyzed peroxidation and DNA condensate formation

Designing a buffer system that provided enough stability within the G4 for housing hemin and catalyzing oxidation was a prerequisite to performing substrate comparison experiments. A K^+ containing buffer system proposed by another study was adopted to provide optimal G4 stability [13]; the general protocol for G4 folding and peroxidation outlined in the same work was used. Hemin was another important experimental component to consider due to its central role as a cofactor promoting G4 peroxidase activity and providing stability [15]. As DNAzyme LA4 had not been previously evaluated for its hemin-binding affinity, the goal was to determine a hemin concentration that would result in a robust and consistent color change. Two hemin concentrations were used, 10 μM and 15 μM, suggested by preliminary efforts. In buffer compatibility studies, LA4 and FC3 quickly turned a darker color in KCl than NaCl buffer in peroxidation (Figure S4), which was expected [16]. However, the question remained as to whether DNA condensates could form in KCl buffer, which was required for DNAzyme-mediated peroxidation. Importantly, it was determined that DNA condensates could form in KCl buffer, a key step toward localizing DNAzyme-mediated peroxidation to condensates (Figure S6).

DNAzyme-catalyzed peroxidation using NS15, NS20, and NS25 strand 4 / LA4

As a next step in achieving localization of a colorimetric redox reaction to DNA condensates, peroxidation was carried out on DNA nanostar strand 4s with LA4 extended to observe the effect of extra DNA on the G4 quadruplex's ability to catalyze peroxidation. Both ABTS and Amplifu Red were used in peroxidation reactions with LA4 extended from DNA nanostar NS15, NS20, and NS25 strand 4s. These reactions were set up in comparison to standalone DNAzyme LA4, a positive control, whereby the solutions were heated to 95°C for 10 min, then snap-cooled by placing them in an ice-water bath for 30 min; hemin was incubated with the solutions for 30 min and then peroxidation was carried out with either ABTS (Figure 7) or Amplifu Red (Figure 8). Interestingly, when using ABTS as a peroxidation substrate, the strand 4/LA4 reactions were much more faint blue-green as compared to the dark blue-green hue of the LA4 positive control; whereas, Amplifu Red turned all reactions the same intense pink color. The negative polyT18 control turned a slight pink when using Amplifu Red, whereas the negative control in ABTS experiments remained clear.

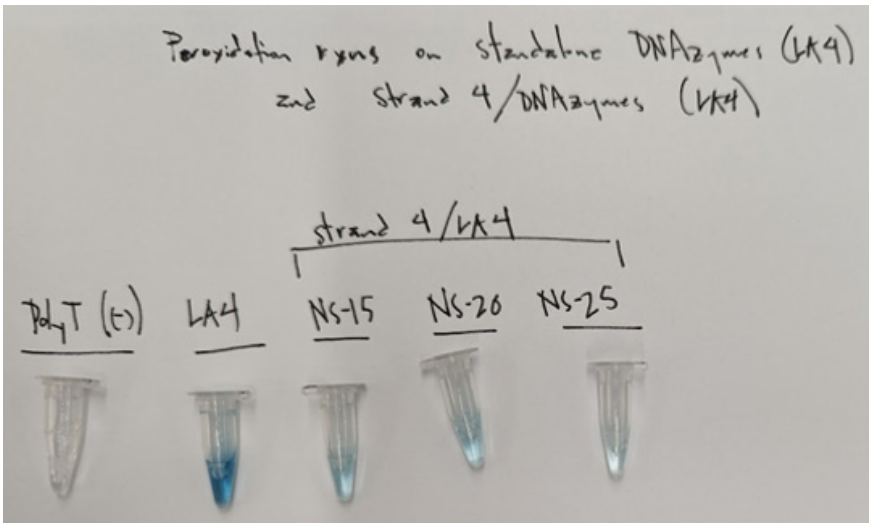


Fig. 7. Peroxidation catalyzed by LA4 and NS15, NS20, NS25 DNA nanostar strand 4s with LA4 extended using ABTS as a substrate. DNA strands for polyT18 (negative control), LA4, NS15 strand 4/LA4, NS20 strand 4/LA4, and NS25 strand 4/LA4 were mixed with KCl buffer, heated to 95 °C, then snap cooled to form folded G4 quadruplexes, which were bound with hemin to form active DNAzymes, to which substrates ABTS and H_2O_2 were added. Image was taken on an iPhone camera ten minutes post peroxidation.

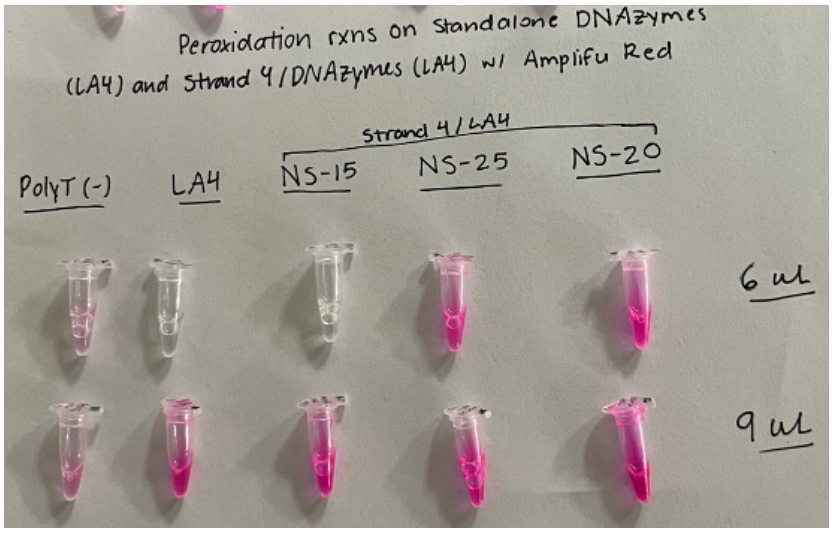
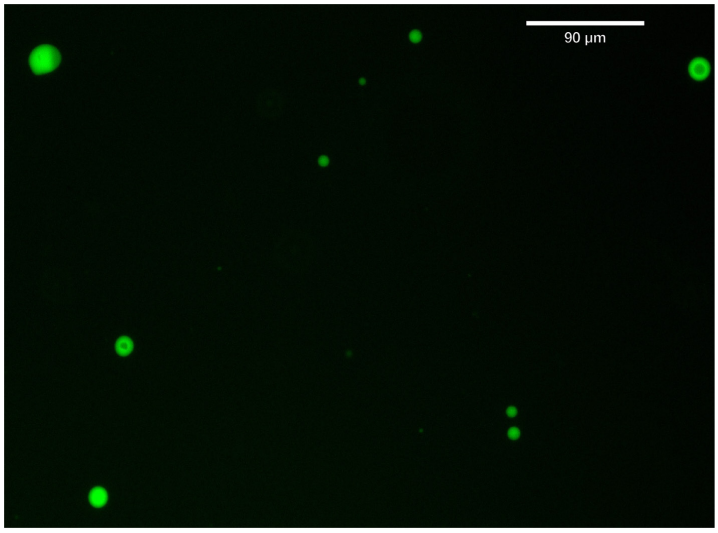


Fig. 8. Peroxidation catalyzed by LA4 and NS15, NS20, NS25 DNA nanostar strand 4s with LA4 extended using Amplifu Red as a substrate. DNA strands for polyT18 (negative control), LA4, NS15 strand 4/LA4, NS20 strand 4/LA4, and NS25 strand 4/LA4 were mixed with KCl buffer, heated to 95°C, then snap cooled to form folded G4 quadruplexes, which were bound with hemin to form active DNAzymes, to which Amplifu Red and H_2O_2 were added. Image was taken on an iPhone camera ten minutes post peroxidation.

Formation of DNA condensates with DNAzyme LA4 attached

The next question addressed by student researchers was whether DNA condensates could form with folded G4 quadruplex/DNAzyme structures attached, and whether there was a limit to the concentration of DNAzyme that could be incorporated without disrupting or preventing condensate formation. It was experimentally determined that DNA condensates formed with up to 50% DNAzyme LA4 in solution, which was confirmed by microscopy (Figures 9, S7-S8).



DNA condensates formed from NS-15 (5% FAM) + 50% LA4 DNAzyme

Fig. 9. Formation of fluorescent DNA condensates from NS15, a 4-armed DNA nanostar with 15 base pairs per arm, with 50% LA4 extended from arm 4 and FAM on the 5' of arm 1, which were used to catalyze peroxidation. Fluorescence micrograph viewing NS15 FAM + 50% LA4, 10x magnification.

Coupling DNAzyme-mediated peroxidation to DNA condensates

After confirming that DNA condensates could form with folded DNAzyme structures attached with up to 50% DNAzyme LA4 (Figures 9, S7-S8), the student researchers wanted to test whether DNA condensates with folded G4 quadruplexes could bind hemin and catalyze peroxidation. NS15 with 0, 5, and 50% LA4 were formed according to procedures outlined in the *Methods* and confirmed through microscopy. Peroxidation was then carried out using concentrations shown in Tables S10-16. A colorimetric response was observed when peroxidation was carried out on NS15/50% LA4 (Figure 10C), whereas NS15 and NS15/5% LA4 remained clear (Figure 10A,B). This result was reproduced with Amplifu Red as compared to ABTS, and notably, in NS15/50% LA4 and NS20/50% LA4, a blue (ABTS) or pink (Amplifu Red) pellet was observed (Figure 11).

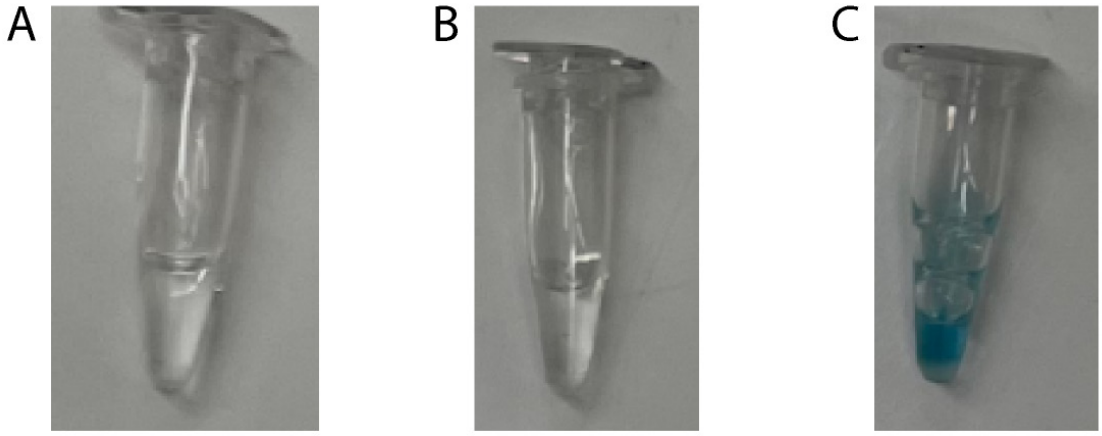


Figure 10. Peroxidation on microscale DNA condensates composed of NS15 with 0, 5, and 50% DNAzyme LA4. (A) NS15, (B) NS15/5% LA4, and (C) NS15/50% LA4 DNA condensates were formed by annealing (see Methods) and to the resultant condensates, hemin was added and incubated for 30 min at room temperature. ABTS and H_2O_2 were added and colorimetric peroxidation was observed. This image was taken with an iPhone camera in lab lighting ten min after the substrates were added to NS15/50% LA4.

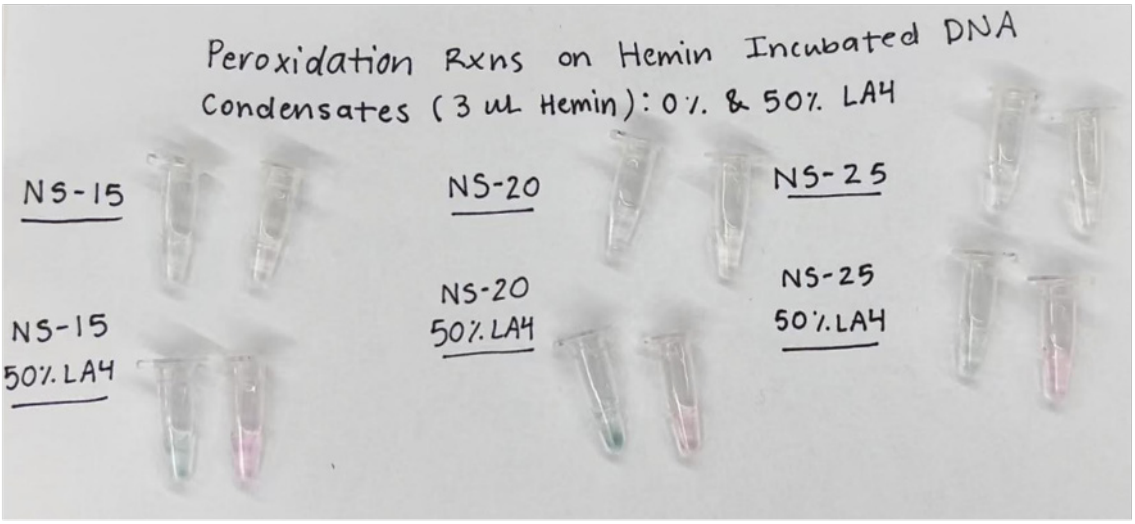


Figure 11. Peroxidation on microscale DNA condensates with LA4. NS15 +/- 50% LA4 (left), NS20 +/- 50% LA4 (middle), NS25 +/- 50% LA4 (right), DNA condensates were formed by annealing and to the resultant condensates, hemin was added and incubated for 30 min at room temperature. Either ABTS or Amplifu Red and H_2O_2 substrates were added, and colorimetric peroxidation was observed. This image was taken with an iPhone camera in lab lighting ten min after the substrates were added to NS25/50% LA4.

Microscopic analysis to correlate DNAzyme-catalyzed peroxidation to DNA condensates

To determine whether DNAzyme-catalyzed peroxidation was localized to DNA condensates, brightfield and fluorescence microscopy were used. Colored pellets observed in solutions containing NS15/50% LA4 and NS20/50% LA4 (Figure 11) were carefully pipetted onto a clean microscope slide and observed using an Echo Revolve microscope. A dark monochrome spot observed under brightfield was correlated to a fluorescent spot under fluorescence for the pellet sequestered from the NS15/50% LA4 ABTS solution, which was confirmed by overlaying the micrographs. The blue pellet within the NS20/50% LA4 ABTS sample was identified under brightfield and clearly correlated to a red fluorescent 'blob' under Texas Red fluorescence, providing strong evidence that the DNAzyme-catalyzed peroxidation was confined to the DNA condensates (Figure 12).

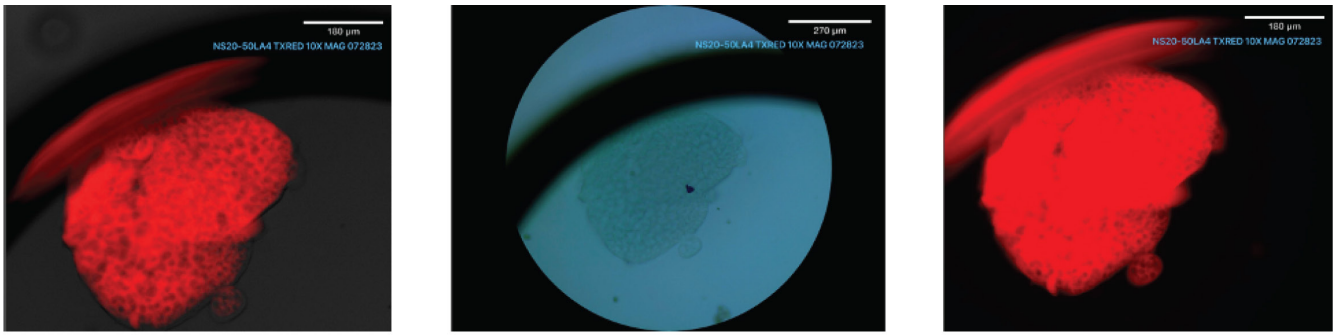


Figure 12. Micrographs post-peroxidation on microscale DNA condensates with 50% DNAzyme (LA4) from Fig. 11. NS20/50% LA4 condensates imaged on an Echo Revolve fluorescence microscope. Overlay of brightfield and fluorescence shows correlation between blue 'blobs' and fluorescent 'blobs' (DNA condensates are referred to here as 'blobs').

Conclusions

The tools of DNA nanotechnology can be used to imagine and build materials of the future, which includes sustainable nanoscale bioreactors. This provides an ideal platform for undergraduate research and accessible STEM education and outreach. This research was conducted at PCC during the Summer of 2023 (6 weeks, 20 hours per week) as part of the NSF Future Manufacturing program, which began with a research techniques training bootcamp at UCLA. Students were guided by graduate students, postdocs, and professors as they engaged with the scientific process, collaborating to make important decisions regarding experimental design and analysis; and they made remarkable research progress in a short time period.

Students applied theory from UCLA bootcamp lectures, the scientific literature, and their STEM courses in authentic research and learned about emerging concepts in molecular biology and DNA nanotechnology, such as DNA nanostars [17], DNA condensates [9], [10], and DNAzymes [7], [13], [16]. Students learned how these ideas relate to their career interests in medicine and technology. For example, G-quadruplexes have come to light as important biological structures with significant medical applications within the field of DNA nanotechnology. The stacking of G-quartets in a helical fashion to form G4s occurs only under specific physiological conditions, hinting at their involvement in in vivo processes as functional elements [18], [19]. The widely acknowledged high-stability of G4 structures is attributed to electrostatic and hydrogen bonding interactions, paired with the presence of alkali metal cations between each quartet [18]. As such, these structures contain high-affinity hemin binding sites and they can mimic peroxidases to catalyze H_2O_2 oxidation reactions [20]. Previous studies have integrated fluorogenic substrates into their experimental design to qualitatively assess G4 (or DNAzyme) activity and enhance functionality [7], [8]. This study contributes to this larger effort by optimizing the efficiency of the most widely used substrates, ABTS and Amplifu (Amplex) Red, in generating a colorimetric response in DNAzyme-catalyzed oxidation by H_2O_2, applying this assay to DNAzymes localized to DNA condensates. These experiments underscore the importance of substrate selection in optimizing DNAzyme-catalyzed reactions at the microscale. Expanding the range of colorimetric detectors of DNAzyme activity, especially for this widely applicable DNAzyme-catalyzed peroxidation reaction [21], can inform micro- and nanoscale manufacturing systems, enhance our understanding of potential reducing reagents, and provide valuable insights in selecting reagents for other nucleic acid-based reactions.

Although some fine-tuning may be necessary when utilizing Amplifu Red as a fluorogenic substrate in DNAzyme-catalyzed peroxidation, it has the potential to monitor reactions through its strong colorimetric response. Its chemical structure can possibly be further investigated to develop methods for controlling or 'turning off' the reaction post-initiation, potentially leading to advanced programmable reaction systems. An important part of the biochemical manufacturing process involves scaling up small preliminary pilot studies to larger macroscale systems. This research project, which uses ABTS and Amplifu Red as reagents in DNAzyme-catalyzed peroxidation localized to DNA condensates, enhances our understanding of the role of reagent selection in compartmentalization. This presents a promising avenue for further exploration in studies that make use of bulk layering of orthogonal condensate systems to localize chemical reactions. These results could inform future studies that exploit DNA as a programmable material by varying hemin, DNAzyme concentration, peroxidation reagents, and buffer conditions. Nucleic acid-catalyzed reactions, such as a ribozyme-mediated Diels-Alder cycloaddition [22], can also be envisioned in this context.

PCC student researchers developed important skills and characteristics through undergraduate research, such as leadership, collaboration, resourcefulness, and communication; and integrating professional skills into undergraduate research facilitates their development as scientists [23]. Students translated their research results into a poster, which they presented to peers and colleagues at PCC and at the National Conference for Undergraduate Research (NCUR) (Figure 13), where one of the nanostars won the NCUR Engineering Division Video Challenge Award for a one-minute research summary video posted on YouTube [24].

Fig. 13. PCC nanostars research team.The PCC nanostars presented the results of their research at the National Conference for Undergraduate Research (NCUR) and at PCC.

Toward the goal of accessible education and workforce development, lessons were developed based on this research to teach students about DNAzyme-catalyzed peroxidation and localization to DNA condensates; these lessons were implemented in courses at PCC and through STEM outreach to teach basic principles of chemistry and biochemistry and engage students with cutting-edge science. Students were captivated by the color-changing reaction catalyzed by DNAzymes, and they were inspired by the PCC researchers who served as learning assistants (LAs) in these laboratory activities. Providing students with the opportunity to translate their research into lessons and teach as LAs improves their scientific communication skills, builds confidence in the lab, and identifies talent for teaching science using effective, equitable teaching methods.

The PCC nanostar researchers were provided opportunities to participate in various outreach and education efforts, such as the Career and Internship Panel at PCC's Pi Day, Women in Science events at PCC, Girls Science Day, where the PCC nanostars taught young girls how to do their research in synthetic biology, and as learning assistants in General, Organic and Biochemistry (Chemistry 2B) at PCC in the Spring 2023 and 2024 semesters (Figure 14).

Fig. 14. PCC research students engaging in STEM outreach and education. The PCC nanostars participated in a Career and Internship panel at Pi Day at PCC in 2024 (A), a Women in Science event at PCC in 2024 (B), Girls Science Day in 2024 (C,D), and served as learning assitants in Chemistry 2B (General, Organic and Biochemistry) at PCC in the Spring 2024 semester (E,F).

Impact of synthetic biology undergraduate research on PCC students

This undergraduate research program was transformative for the PCC student researchers. They learned how to collaborate on a novel research project in synthetic biology, developed important character traits toward success in STEM, such as resilience and leadership, and applied their knowledge from courses throughout a scientific campaign. Collaborative research helped students develop a science identity, while providing a sense of belonging; these important features of undergraduate research have been shown to increase the success of underrepresented students—such as women—in STEM. In fact, the PCC nanostars reported that female mentorship in science has been a revelation to them; they can now see themselves in this field because of the inspiring female mentors who supported them throughout this research program.

Overall, by participating in the NSF Future Manufacturing program, PCC student researchers engaged in authentic research using the tools of synthetic biology. Students learned about the iterative nature of science, the importance of 'failure' in science and learning, the value of collaboration and mentorship, the impact of equitable STEM education and outreach, and they developed key character traits and professional skills that will facilitate their success in the future biomanufacturing workforce.

Quotes from the PCC nanostar student researchers:

This undergraduate research program has been transformative, catalyzing my research ambitions and earning me placements in multiple labs where I've gained both technical skills and the resilience essential for a future in research and discovery. I've had the freedom to explore my inquiries and passions, develop critical thinking, and sharpen my problem-solving abilities. Being a nanostar has meant being immersed in a supportive, equity-driven scientific community, where I've learned the value of uplifting underrepresented groups in STEM. The opportunities that have spawned from this experience, such as teaching as a Learning Assistant and engaging in workshops, have honed my ability to convey complex concepts. Opportunities to present posters—most recently at NCUR 2024—have built my confidence as a science advocate and communicator at conferences. The skills and growth I've experienced here have even led to my nomination for respected scholarships, underscoring the program's impact on my development as a scientist and a leader.

My overall experience as a PCC nanostar was empowering, confidence-boosting, and enlightening. It provided me with a sense of belonging. The professors made the learning environment safe and supportive for me. The space allowed for creativity, my attitude is now very determined. This research experience was so impactful because I am excited for research now. The idea of pursuing a Ph.D. is very appealing now.

When I think back on this summer, one thing that I truly enjoyed was the collaborative environment that formed in our lab. This provided for a constant flow of ideas and encouraged every member of the group to voice their worries or suggestions to the team. This summer program taught me about teamwork, resilience, critical thinking, and hands-on research skills. Because of this tremendous intellectual experience this summer, I am inspired to continue enriching the rest of my educational path with scientific research that I can contribute to.

I was inspired by the female leadership personalities involved in this research endeavor. Having female PIs and collaborators was important to our experiences because we were always encouraged and made to feel confident in airing our opinions, asking questions, and making suggestions. Being part of a program that stresses the importance of diversity and inclusion for women made me realize how many women can benefit from opportunities like these!

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Disclosures. The authors declare no conflicts of interest.

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