RESEARCH PAPER

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Templating effect in DNA proximity ligation enables use of non-bioorthogonal chemistry in biological fluids

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Abbreviations: StAPL, split aptamer proximity ligation; DCA, deoxycholic acid; PAGE, polyacrylamide gel electrophoresis; PEG, polyethylene glycol; PCR, polymerase chain reaction; TBE, tris-borate EDTA; EDTA, ethylenediaminetetraacetic acid; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; TEAA, triethylammonium acetate; HPLC, high-performance liquid chromatography; TAPS, N-tris(hydroxyl)methyl-3-aminopropanesulfonic acid

Here we describe the first example of selective reductive amination in biological fluids using split aptamer proximity ligation (StAPL). Utilizing the cocaine split aptamer, we demonstrate small-molecule-dependent ligation that is dose-dependent over a wide range of target concentrations in buffer, human blood serum and artificial urine medium. We explore the substrate binding preferences of the split aptamer and find that the cinchona alkaloids quinine and quinidine bind to the aptamer with higher affinity than cocaine. This increased affinity leads to improved detection limits for these small-molecule targets. We also demonstrate that linker length and hydrophobicity impact the efficiency of split aptamer ligation. The ability to carry out selective chemical transformations using non-bioorthogonal chemistry in media where competing reactive groups are present highlights the power of the increased effective molarity provided by DNA assembly. Obviating the need for bioorthogonal chemistry would dramatically expand the repertoire of chemical transformations available for use in templated reactions such as proximity ligation assays, in turn enabling the development of novel methods for biomolecule detection.

Introduction

Nature is often required to carry out specific chemical transformations in the presence of competing reactive groups, given the chemical complexity of biological systems. This challenge can be overcome using enzymes, which hold reactants in close proximity, thus promoting the desired reaction over undesired side-reactions.[1] Chemists have sought to mimic this proximity effect through the use of biological and non-biological templates to direct chemical reactions.[2-6] DNA has proven to be particularly effective in reaction templation, as DNA assembles with high affinity and selectivity, and can be used to bring reactants into close proximity, providing a dramatic increase in effective molarity.[7-10] DNA-templated reactions typically rely on the sequence defined affinity of the nucleic acid strands for one another. In contrast, we have recently reported the first DNA-templated reaction that is promoted by small-molecule binding rather than inherent Watson-Crick affinity between the DNA strands.[11,12] This process, which we call split aptamer proximity ligation (StAPL), utilizes a DNA split aptamer as the recognition element. Split aptamers are comprised of two DNA strands that only assemble in the presence of a specific small-molecule or protein target.[13] In StAPL, the target small-molecule triggers assembly of the split aptamer, dramatically increasing the effective molarity of reactive groups appended to the termini of the split aptamer fragments, and thus promoting a chemical reaction (**Fig. 1**). Here we explore StAPL using reductive amination between amine and aldehyde functional groups, and demonstrate that this reaction is efficient and dose-dependent, even in biological fluids where competing reactive groups are present. This is the first example of selective reductive amination in biological fluids, and highlights the power of the enhanced effective molarity provided by DNA templation.

In our initial investigations of StAPL, we utilized strain-promoted azide-alkyne cycloaddition for the ligation step. However, we observed significant background ligation arising from unwanted assembly of the split aptamer in the absence of the target small molecule. StAPL, like most templated reactions, is a two-step process comprised of a reversible assembly step followed by a non-reversible covalent trapping step. The use of reductive amination for the covalent trapping step in other DNA-templated reactions has been shown to increase fidelity,[14-16] as the amine and aldehyde functional groups undergo reversible

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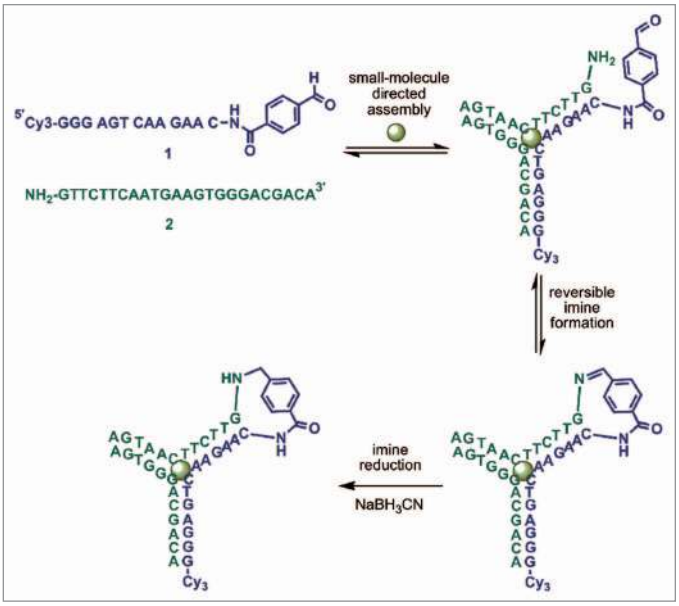

Figure 1. Small-molecule-dependent split aptamer ligation using reductive amination. The small molecule directs assembly of the split aptamer fragments, bringing the aldehyde and amine in close proximity to one another. The aldehyde and amine react reversibly to form an imine intermediate, which is reduced to an amine by sodium cyanoborohydride.

imine formation prior to reduction. Thus, we hypothesized that the background ligation observed in StAPL could be reduced by using reductive amination in place of strain-promoted azide-alkyne cycloaddition.

Results

To test the use of reductive amination in StAPL, we appended a benzaldehyde reactive group to one fragment of the previously reported cocaine split aptamer (aptamer fragment 1),[17,18] and synthesized the second aptamer fragment having a terminal amine reactive group (aptamer fragment 2). The aldehyde-functionalized aptamer fragment was synthesized having a Cy3 fluorophore to enable calculation of ligation yields using denaturing PAGE. An important consideration in DNA-templated reactions is the ability of the reactants to span the ~20 Å diameter of the DNA duplex. Thus, we explored attachment of the amine reactive group using linkers having varying length and hydrophobicity (**Table 1**). Specifically, we used DNA 2a–d in which the amine was attached to the 5' phosphate via a PEG_2, PEG_5, PEG_8, or C_{12} linker. DNA 2b having the PEG_5 linker affords the highest ligation yield (50%) in the presence of 1 mM cocaine, with DNA 2a and 2c having the PEG_2 and PEG_8 linkers providing significantly lower yields of 17% and 6%, respectively (**Fig. 2**). We were surprised to find that in DNA 2d, having the hydrophobic C_{12} linker provided almost no ligated product, as the length of this linker is similar to that of PEG_5. We hypothesize that the C_{12} linker may undergo hydrophobic collapse in aqueous solution, dramatically reducing its effective length and flexibility, and thus preventing the amine from reaching

Table 1. Split aptamer sequences used for ligation reactions

Number	Sequence (5'-3')
1	Cy3-GGG AGT CAA GAA C-C_6-NH-CHO
2a	NH_2-PEG_2-GTT CTT CAA TGA AGT GGG ACG ACA
2b	NH_2-PEG_5-GTT CTT CAA TGA AGT GGG ACG ACA
2c	NH_2-PEG_8-GTT CTT CAA TGA AGT GGG ACG ACA
2d	NH_2-C_{12}-GTT CTT CAA TGA AGT GGG ACG ACA

the benzaldehyde reaction partner.[19,20] Utilizing DNA 2b, we observed dose-dependent split aptamer ligation for cocaine concentrations of 1 μM–1 mM, but with overall slightly lower yield than was achieved in previous studies using strain-promoted azide-alkyne cycloaddition (**Fig. 3**).

Cinchona alkaloids have been reported in the literature to bind to the cocaine aptamer with significantly higher affinity than cocaine,[21-23] with quinine and quinidine having at least an order of magnitude increase in association constant.[21,22] Therefore, we decided to test four alkaloids and an unrelated steroid molecule (DCA) to determine whether the cocaine split aptamer has the same ligand selectivity as the regular aptamer. Aptamer fragments 1 and 2b were incubated with 100 μM of each ligand along with 100 mM $NaBH_3CN$ for 1.5 h, then analyzed by denaturing PAGE (**Fig. 4**). Quinine and quinidine afforded the highest ligation yields of 52% and 53%, respectively, while cinchonine and cinchonidine provided lower ligation yields of 33% and 36%, respectively. In comparison, cocaine and DCA provided ligation yields of 27% and 6%, respectively. This suggests that the cocaine split aptamer primarily binds to the amine-containing bicyclic structure that is common to all of the alkaloids, but that the structural characteristics of the cinchona alkaloids impart higher overall binding affinity relative to cocaine.

Next, we investigated the ability of each alkaloid to promote split aptamer ligation in a dose-dependent manner. The templated ligation in buffer using DNA 1 and 2b is dose-dependent over the concentration range of 100 nM to 1 mM for quinine and quinidine, providing ligation yields of 7–62% and 8–61%, respectively (**Fig. 5**). Dose-dependent ligation was also observed with cinchonine and cinchonidine over the concentration range of 1 μM to 1 mM, providing ligation yields of 12–52% and 11–53%, respectively. Thus, our detection limit is 100 nM for quinine and quinidine and 1 μM for cinchonine and cinchonidine. These detection limits reflect the relative binding affinities of the ligands, suggesting that ligand affinity is the limiting factor in small-molecule detection using StAPL.

A central goal of our research is the use of StAPL to develop assays capable of small-molecule detection in biological fluids. We have demonstrated cocaine dose-dependent ligation in human blood serum using strain-promoted azide-alkyne cycloaddition. This ligation chemistry is highly bioorthogonal, as azides and cyclooctynes do not react with the functional groups found in biological fluids.[24-26] In contrast, reductive amination is not considered to be bioorthogonal, as one reaction partner is an amine, and numerous competing amines are present in the proteins and small-molecules present in biological fluids. Thus,

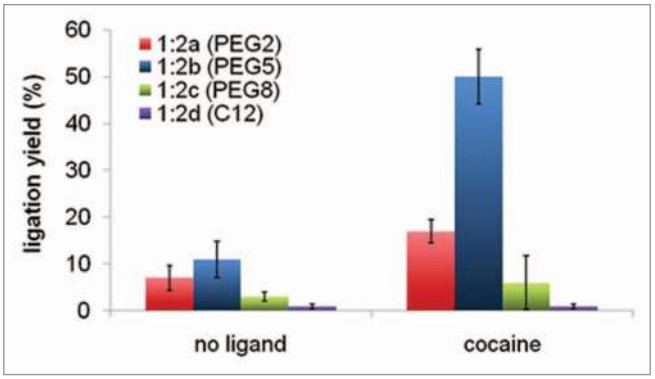

Figure 2. Effect of linker length and hydrophobicity on ligation efficiency. Conditions: 0.5 μM 1, 2.0 μM 2a-d, 30 mM TAPS, pH 8.2, 15 mM NaCl, 100 mM $NaBH_3CN$, 1 mM cocaine, 1.5 h at 22°C.

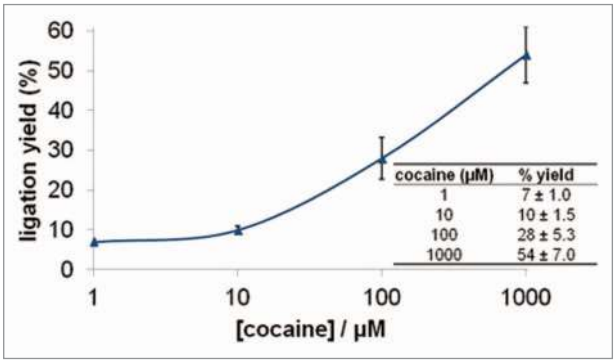

Figure 3. Yield of ligated product as a function of cocaine concentration. Error bars represent standard deviation of three independent trials. Conditions: 0.5 μM 1, 2.0 μM 2b, 30 mM TAPS, pH 8.2, 15 mM NaCl, 100 mM $NaBH_3CN$, 1.5 h at 22°C.

we were unsure whether dose-dependent split aptamer ligation could be achieved using reductive amination in human blood serum. However, we hypothesized that the enhanced effective molarity provided by split aptamer assembly could provide sufficient rate enhancement to enable selective reductive amination between the aptamer fragments despite the presence of competing reactive groups.

To test this hypothesis, we reacted DNA 1 and 2b with 100 mM $NaBH_3CN$ and varying concentrations of quinine in 30% human blood serum. After a reaction time of 1.5 h, we observed dose-dependent ligation with yields of 24–62% for quinine concentrations of 100 nM to 1 mM (**Fig. 6A**, green line). Human blood serum contains sodium ion concentrations of 137–147 mM,[27] and we have found that this increased ionic strength can promote split aptamer assembly, leading to increased background ligation in the absence of ligand. We have previously circumvented this problem via mutation of the split aptamer sequence.[11,12] However, in this case, we found that simply reducing the reaction time from 1.5 to 1 h reduced the background ligation from 21% to 8%, but still enabled dose-dependent ligation for quinine concentrations of 100 nM to 1

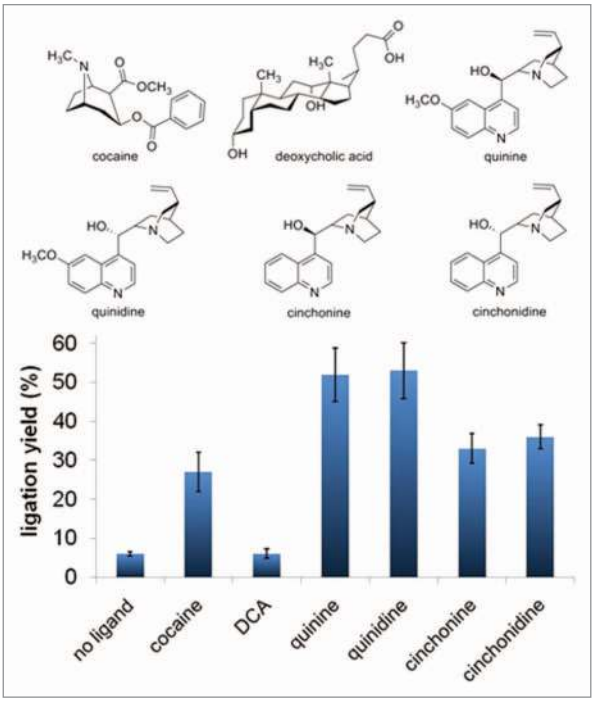

Figure 4. Substrate selectivity of split aptamer ligation. Conditions: 0.5 μM 1, 2.0 μM 2b, 30 mM TAPS, pH 8.2, 15 mM NaCl, 100 mM $NaBH_3CN$, 100 μM ligand, 1.5 h at 22°C.

mM with yields of 10–41% (**Fig. 6B**, green line). These results demonstrate that despite the presence of numerous competing reactive groups, we can achieve selective reductive amination between split aptamer fragments in human blood serum. Under reaction conditions that minimize background ligation, the maximum ligation yield observed with 1 mM quinine is moderate at 41%. However, the 62% yield achieved using a 1.5 h reaction time is identical to that observed in buffer, suggesting that the reduced yields in the case of the 1 h reaction time are not a result of side-reactions between the aldehyde-functionalized aptamer fragment and competing functional groups in serum.

We were also curious to explore StAPL using reductive amination in a solution containing urine media. We had not previously explored StAPL in urine media, but given the frequent use of urine drug testing to detect illicit drug use,[28,29] adapting split aptamer ligation for use in urine would be extremely valuable in future assay development. Thus, we reacted DNA 1 and 2b with 100 mM $NaBH_3CN$ and varying concentrations of quinine in 30% artificial urine media.[30] Urine media contains high concentrations of both urea and inorganic salts. Inorganic salts promote DNA assembly, whereas urea promotes DNA denaturation. Thus, we were intrigued to observe how these components would impact the efficiency of split aptamer ligation. Using a reaction time of 1.5 h, we observed dose-dependent ligation with yields of 34–66% for 100 nM to 1 mM quinine (**Fig. 6A**, red line). We hypothesized that despite the presence of urea, the high salt concentration in the urine media was promoting aptamer assembly leading to increased background ligation. However, this problem was again easily circumvented by reducing the reaction

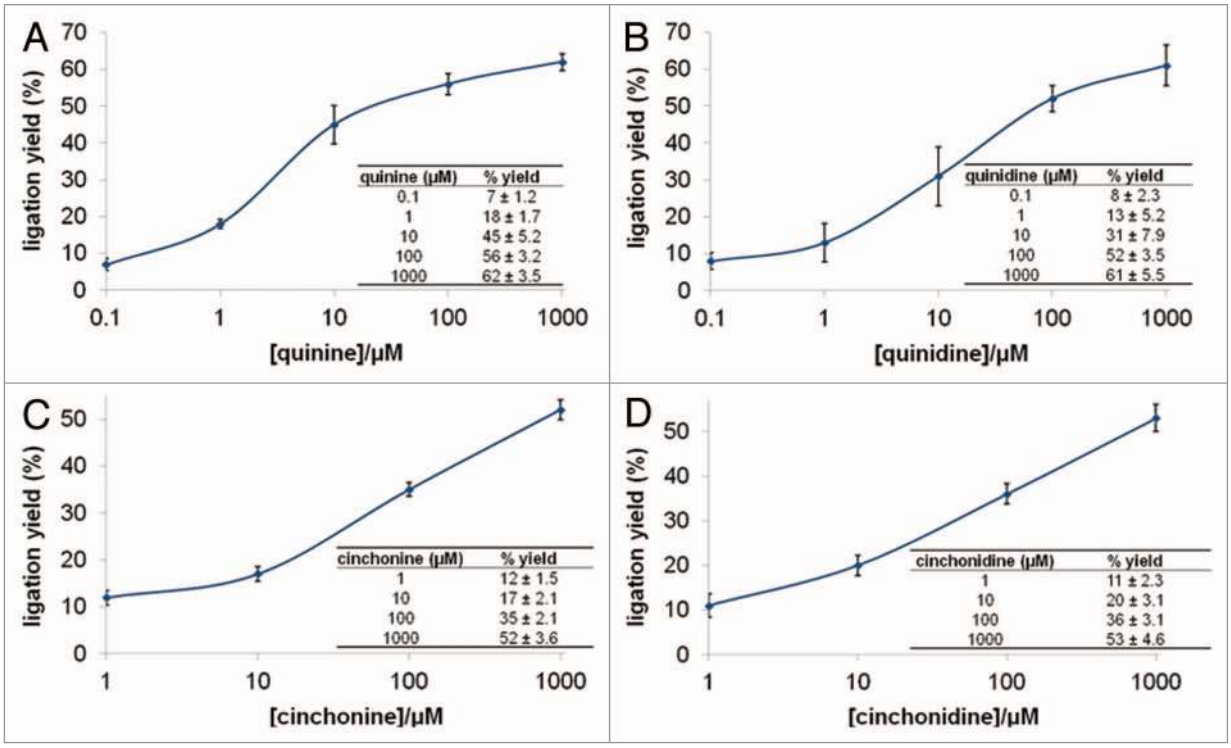

Figure 5. Yield of ligated product as a function of ligand concentration for (**A**) quinine, (**B**) quinidine, (**C**) chinchonine and (**D**) cinchonidine. Error bars represent standard deviation of three independent trials. Conditions: 0.5 μM 1, 2.0 μM 2b, 30 mM TAPS, pH 8.2, 15 mM NaCl, 100 mM $NaBH_3CN$, 1.5 h at 22°C.

time from 1.5 to 1 h. After a 1 h reaction time, we observed dose-dependent ligation with yields of 12–35% for 100 nM to 1 mM quinine (**Fig. 6B**, red line). Again, these yields are lower than those observed in buffer, but the ability to achieve a 66% yield with extended reaction time suggests that the reduction in yield does not result from side reactions between the aptamer fragments and amine-containing molecules present in the urine media.

As reflected in the data above, as well as our previous reports, the target-dependent ligation yields in StAPL can be increased by increasing either reaction time or the inherent affinity of the split aptamer fragments for one another.[11,12] However, these changes also result in an increase in background ligation of the split aptamer fragments in the absence of the target small molecule. Thus, the reaction conditions and split aptamer sequences used in the current study were optimized to provide the greatest relative difference in ligation yield between the background and the highest target concentration (1 mM). This resulted in reaction times of 1.5 h for reactions in buffer and 1 h for reactions in serum or urine. Maximizing this relative increase in ligation yield provides the optimum signal over background ratio for small-molecule detection.

Discussion

Using the cocaine spit aptamer, we have demonstrated small-molecule-dependent split aptamer ligation via reductive amination between amine and aldehyde reactive groups. A survey of various linkers for attaching the amine to the aptamer revealed that both linker length and hydrophobicity impact the efficiency of split aptamer ligation, as the reactive groups must be capable of spanning the diameter of the DNA duplex. Additionally, we have shown that the substrate binding preferences of the cocaine split aptamer mirror those of the regular cocaine aptamer, with cinchona alkaloids such as quinine and quinidine demonstrating enhanced binding affinity relative to cocaine. This enhanced binding affinity in turn provides improved detection limits for split aptamer ligation, as a detection limit of 100 nM was achieved with quinine and quinidine, compared with 1 μM for cocaine.

Most surprisingly, we have found that split aptamer ligation using reductive amination is dose-dependent in solutions containing human blood serum and artificial urine media, despite the presence of competing reactive groups in these solutions. We do observe a narrower range of ligation yields in these biological fluids. However, we do not observe side reactions between the aptamer fragments and other molecules present in the solution, and we maintain a detection limit of 100 nM for quinine in both serum and urine. Thus, the effective molarity provided by split aptamer assembly is sufficient to enable selective reductive amination between the aptamer fragments despite the presence of competing reactive groups.

To our knowledge, this is the first example of selective reductive amination in biological fluids, and demonstrates the power of DNA templation to direct specific chemical transformations in chemically diverse environments. While the current study focuses on small-molecule-dependent ligation of split aptamer

fragments, we hypothesize that the ability of increased effective molarity to overcome the requirement for bioorthogonal chemistry may be highly general. Obviating the need for bioorthogonal chemistry would dramatically expand the repertoire of chemical transformations available for use in templated reactions such as proximity ligation assays. This increased chemical flexibility would in turn be anticipated to enable the development of novel methods for biomolecule detection.

Materials and Methods

General techniques. Unless otherwise noted, all starting materials were obtained from commercial suppliers and were used without further purification. Cocaine was purchased from Sigma-Aldrich as a 1 mg/mL solution in acetonitrile. This solution was diluted with water, lyophilized and redissolved in water at a concentration of 3.3 mM. Cocaine samples were separated into microcentrifuge tubes, lyophilized, and stored at -80°C in 0.1 mg aliquots until needed. Cocaine solutions for ligation experiments were freshly prepared before use. All other cinchona alkaloids were purchased from Sigma-Aldrich as solids. DNA was purchased from the University of Utah DNA/Peptide synthesis Core Facility. Human blood serum was purchased from Sigma Aldrich. Mass spectra were collected using the Mass Spectrometry Core Facility, University of Utah. PAGE gels were analyzed for Cy3 fluorescence using a Typhoon 9400 scanner (Amersham Biosciences) with a 532 nm excitation laser and 580 BP 30 emission filter. Fluorescence volume was analyzed using ImageQuant 5.2 software. Fluorescence volumes were corrected for background by subtracting the fluorescence volume of an identically sized area of the gel in which no bands were present.

Modifiers used for DNA synthesis. All modified phosphoramidites and CPG cartridges were purchased from Glen Research. Amine functional groups for DNA 2a–c were installed using 5'-amino-modifier 5 phosphoramidite (10-1905) with no spacer, spacer phosphoramidite 9 (10-1909), or spacer phosphoramidite 18 (10-1918), respectively. The amine functional group for DNA 2d was installed using 5'-amino-modifier C12 phosphoramidite (10-1912). The amine functional group for DNA 1 was installed using a C6-amino CPG cartridge (20-2956). The Cy3 fluorophore for DNA 1 was installed using Cy3 phosphoramidite (10-5913).

Benzaldehyde modification of DNA 1. Five milligrams (20 μmol) succinimidyl-*p*-formyl-benzoate in 50 μL DMF was added to 58 μL H_2O, 30 μL of 1 M sodium phosphate (pH 8.2), and 75 μL of 300 μM Cy3-DNA-NH_2 (20 nmol) in a microcentrifuge tube. Final concentrations were 100 μM Cy3-DNA-NH_2, 150 mM sodium phosphate, 100 mM aldehyde NHS ester, and 40 vol% DMF. The microcentrifuge tube was wrapped in aluminum foil to protect the Cy3 fluorophore, and agitated on a shaker for 2 h at 22°C. After 2 h, the solution was desalted using a Nap 5 column (GE Healthcare) and purified using reverse phase HPLC (Agilent ZORBAX Eclipse XDB-C18, 5 μM particle size, 4.6 × 150 mM) with a binary mixture of 0.1 M TEAA:acetonitrile. HPLC was set at a 2 mL/min flow rate starting with 95:5 TEAA:acetonitrile and changing to 70:30 TEAA:acetonitrile over a period of 16 min. DNA 1 was collected at a retention time of 12.694 min, and was lyophilized to afford 4–6 nmol of a pink solid (20–32%). The purified DNA was analyzed by MALDI-TOF in linear positive mode cald[M+H]$^+$4859, found 4862 (**Fig. S1**).

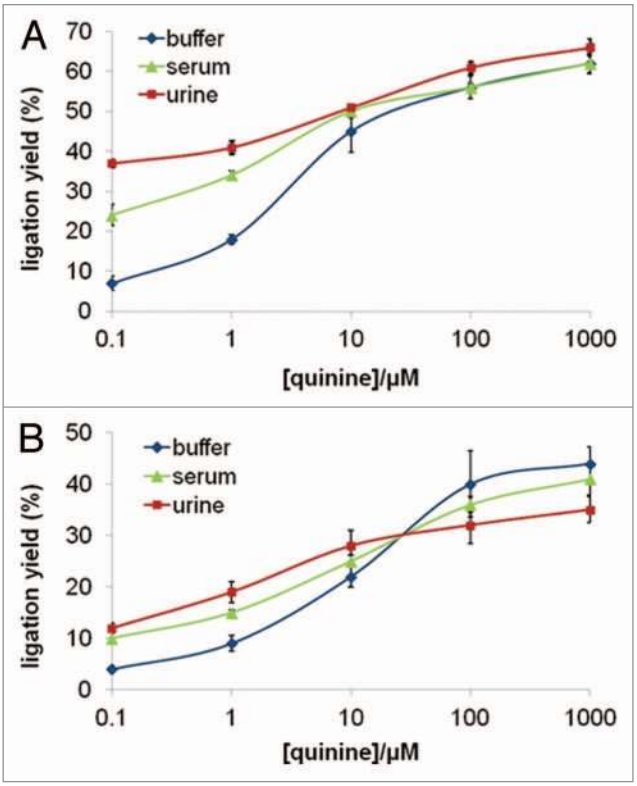

Figure 6. Yield of ligated product as a function of quinine concentration after reaction time of (**A**) 1.5 h or (**B**) 1 h. Error bars represent standard deviation of three independent trials. Conditions: 30% serum or 30% artificial urine medium with 0.5 μM 1, 2.0 μM 2b, 30 mM TAPS, pH 8.2, 15 mM NaCl, 100 mM $NaBH_3CN$, 22°C.

General split aptamer ligation protocol. Ligation of DNA 1, DNA 2 and metabolite was performed in 0.2 mL PCR tubes. The alkaloid solution was added to the PCR tube first, followed by 3.0 μL of 200 mM TAPS, pH 8.2, 1.0 μL of 10 μM DNA 1, and 1.33 μL of 30 μM DNA 2. The total volume in each PCR tube was brought to 19 μL with water, followed by subsequent addition of 1.0 μL of freshly prepared 2 M sodium cyanoborohydride. The PCR tubes were capped and incubated at 22°C in a PCR thermocycler for the specified reaction time, then quenched using 2× PAGE loading buffer containing 7 M urea. The reaction mixtures were analyzed by denaturing PAGE on a 12% TBE/urea polyacrylamide gel. Denaturing polyacrylamide gels were imaged as described above (**Fig. S2**). The yield for ligation reactions was calculated according to **Eq 1:** % yield = 100 × [V_p/(V_p + V_r)] in which V_r is the fluorescence volume of the band for reactant 1 and V_p is the fluorescence volume of the band for 1 + 2.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journal/artificialdna/article/23842

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