Published in final edited form as:

ACS Appl Mater Interfaces. 2017 September 06; 9(35): 30185–30195. doi:10.1021/acsami.7b11046.

A Microsphere-Supported Lipid Bilayer Platform for DNA Reactions on a Fluid Surface

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Abstract

We report a versatile microsphere-supported lipid bilayer system that can serve as a general-purpose platform for implementing DNA nanotechnologies on a fluid surface. To demonstrate our platform, we implemented both toehold-mediated strand displacement (TMSD) and DNAzyme reactions, which are typically performed in solution and which are the cornerstone of DNA-based molecular logic and dynamic DNA nanotechnology, on the surface. We functionalized microspheres bearing supported lipid bilayers (μ SLBs) with membrane-bound nucleic acid components. Using functionalized μ SLBs, we developed TMSD and DNAzyme reactions by optimizing reaction conditions to reduce non-specific interactions between DNA and phospholipids and to enhance bilayer stability. Additionally, the physical and optical properties of the bilayer were tuned *via* lipid composition and addition of fluorescently tagged lipids to create stable and multiplexable μ SLBs that are easily read out by flow cytometry. Multiplexed TMSD reactions on μ SLBs enabled the successful operation of a Dengue serotyping assay that correctly identified all sixteen patterns of target sequences to demonstrate detection of DNA strands derived from the sequences of all four Dengue serotypes. The limit of detection for this assay was 3 nM.

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ASSOCIATED CONTENT

Supporting Information.

The Supporting Information is available free of charge on the ACS Publications website at DOI:

The Supporting Information document includes all supplementary figures as described in the main text, details on strand design, the non-specific interaction controls, the limit of detection calculations, details on the confocal microscopy experiments, and merging data for multiplex microspheres.

Three movies are included as well.

Author Contributions

All authors contributed to writing the paper. AFW, NAB and MEF conducted experiments. MRL designed sequences. CWB ran initial multiplexing experiments. APS, NFO and GAM assisted with FRAP analysis, membrane characterization, and lipid design. DS, MRL and SWG supervised the project.

SWG, MRL, and DS have financial interest in a company, Helion Scientific, Inc., which is exploring the use of molecular logic technologies for biological assays.

Furthermore, we demonstrated DNAzyme reactions on a fluid lipid surface, which benefit from free diffusion on the surface. This work provides the basis for expansion of both TMSD and DNAzyme based molecular reactions on supported lipid bilayers for use in molecular logic and DNA nanotechnology. As our system is multiplexable and results in fluid surfaces, it may be of use in compartmentalization and improved kinetics of molecular logic reactions, and as a useful building block in a variety of DNA nanotechnology systems.

Table of Contents (TOC) Figure

Keywords

DNA-lipid conjugates; supported lipid bilayers; multiplexing; toehold-mediated strand displacement; DNAzymes; detection platform; molecular computation

INTRODUCTION

The field of DNA nanotechnology uses biomolecules to construct computing components analogous to those in electronic computers,^{1–3} and to build synthetic nanostructures to study molecular interactions.^{4,5} DNA is attractive for these applications as Watson-Crick base pairing enables straightforward programming of molecular interactions.^{1,2} DNA-based biomolecular computation has been used for bioassays targeting pathogen signatures and to integrate detection of multiple pathogen signatures into a single reaction.^{6–13} However, non-specific interactions, arising from background environmental nucleic acids and from molecular crosstalk due to lack of component separation in solution, can severely limit assay performance.⁶

DNA-based molecular computation is typically performed in solution using toehold-mediated strand displacement (TMSD), DNAzymes, or a combination of the two approaches.^{6,10} TMSD molecular computing systems are an enzyme-free mechanism wherein target strands interact with a gate complex composed of a template strand partially hybridized to an incumbent strand.² The gate has a double-stranded DNA (dsDNA) region with a short single-stranded DNA (ssDNA) overhang on the template strand, referred to as a *toehold*, which serves as a nucleation site for target strand binding. The target then displaces the incumbent strand from the template *via* branch migration. Formation of the resulting target/template complex can be programmed to be thermodynamically favored due to a net increase in the number of base pairs. DNAzymes, on the other hand, are single-stranded DNA oligonucleotides that catalyze a variety of chemical reactions in the presence of divalent metal ions.^{14,15} Of particular interest are DNAzymes that cleave partially

complementary chimeric DNA substrate molecules at a single RNA base, such as the 8–17 DNAzyme, chosen for this work due to its small size and high catalytic activity.^{1,6,10,16–18} In DNAzyme-driven molecular computation, input signals activate DNAzymes, each of which can cleave multiple substrate molecules, enabling signal amplification *via* catalysis.

Here, we have explored the use of supported lipid bilayers on silica microspheres (μ SLBs) as a scaffold for both TMSD networks and DNAzyme reactions. In this system, our nucleic acid components are covalently linked to head groups of the supported lipid bilayer. This approach provides a mechanism to easily segregate or compartmentalize elements of these reactions from each other and from nucleic acids in solution. This offers the potential to reduce nonspecific crosstalk within the system and to improve the scalability of DNA-based reactions by permitting the reuse of nucleotide sequences without causing crosstalk. Alternatively, we sought to improve scalability through the incorporation of fluorescently tagged lipids into the μ SLB system, which provides a straightforward method for multiplexed analysis that can be easily monitored *via* flow cytometry.¹⁹ This approach offers benefits for many practical applications, such as bioassay development. Analogous technology (fluorescently multiplexed polystyrene microspheres) has simplified pathogen assays by enabling the detection of multiple signatures in a single solution *via* flow cytometry²⁰ and provides highly quantitative and rapid analysis.²¹ However, lipid bilayers are not readily formed on polystyrene microspheres, making our μ SLB system an excellent alternative. While others have used lipid bilayer based systems for nucleic acid based reactions, these systems were not easily analyzable or multiplexable.^{22–24} To demonstrate the value of our approach, we developed a bivariate fluorescence tagging approach to increase μ SLB multiplexing levels and we demonstrated multiplexed μ SLBs for a TMSD assay that accurately types DNA sequences derived from the genomes of the four Dengue virus serotypes.

Although the spatial isolation of molecular computation components *via* immobilization to fixed surfaces (e.g., direct attachment to polymer microspheres²⁵ and onto DNA origami scaffolds^{26–28}) has been explored, reactants in these systems are constrained to their anchor points and cannot interact with molecules outside of the reach of their initial location. This limitation is most relevant to DNAzyme systems, which could otherwise provide signal amplification *via* multiple turnover cleavage of substrates. Therefore, the final goal of this work was to overcome this issue by linking DNAzymes and their substrates to lipid head groups incorporated into multiplexable microsphere-supported lipid bilayers (μ SLBs). The intent of this approach is to enable surface diffusion of DNAzymes so that they can interact with substrate molecules independent of their attachment position. Surface diffusion might also improve reaction kinetics, as diffusion on a two-dimensional surface is more constrained than the three dimensions available to solution phase reactions.^{26,29–31}

In summary, we present a μ SLB system that enables the compartmentalization of DNA-based molecular computing components. This system is multiplexable, which allows components to be independently monitored in a single tube reaction. Thus, by restricting DNA-based molecular computing components to a μ SLB, we are able to design a detection platform that targets specific pathogenic signatures and uses flow cytometry for analysis.

This has the potential to greatly reduce non-specific interactions, which would enable the creation of more complex DNA-based molecular reaction networks.

EXPERIMENTAL SECTION

Materials

Lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Oligonucleotides were purchased from Integrated DNA Technologies (IDT, Coralville, IA). Ethylenediaminetetraacetic acid (EDTA), disodium phosphate, sodium chloride, and citric acid were purchased from EMD Millipore (Billerica, MA). Nucleosil (50-10) silica microspheres were purchased from GFS Chemicals (Powell, OH). Illustra NAP-5 Columns Sephadex G-25 DNA Grade were purchased from GE Healthcare Life Sciences (Pittsburgh, PA). All other reagents were purchased from Sigma Aldrich (St. Louis, MO).

Silica Microsphere Preparation

To prepare the surface, silica microspheres (SiMS) were treated in a basic solution containing 4% NH_4OH and 4% H_2O_2 at 80 °C for 10 minutes. The SiMSs were rinsed three times with distilled water and treated with an acidic solution containing 4% HCl and 4% H_2O_2 at 80°C for 10 minutes. Finally, SiMSs were rinsed five times with distilled water. After this final wash the SiMSs were suspended in citric acid buffer (CAB6.5) (20mM citric acid, 35 mM disodium phosphate, 108 mM NaCl , 1 mM EDTA at pH 6.5). In addition, the SiMSs were preloaded with an inert oligo (Blocking strand_TSF) to prevent migration of the DNA-lipid conjugates into the porous SiMS by rotating at room temperature for 12 hours with a strand concentration of 10 μM .

Preparation of μSLBs for Multiplex

Four separate lipid mixtures were dried overnight under vacuum, and then hydrated in citric acid buffer (20mM citric acid, 35 mM disodium phosphate, 108 mM NaCl , 1 mM EDTA at pH 6.5 (CAB6.5)). The lipid solution was then extruded to generate monodispersed unilamellar vesicles (100 nm). These unilamellar vesicles were then used to coat 6.7×10^6 SiMS, by vortexing on high for 10 minutes, followed by low for 30 minutes at 37 °C. The lipid-coated SiMSs were washed 3 times in CAB6.5 to remove the 100-fold excess of vesicles. The first vesicle population (P1) was composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), with 10 mol% 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl) butyramide] (MPB PE) incorporated into the lipid cake. No fluorescently labeled lipids were included in P1. The second population (P2) contained the same lipid mixture as 1, plus 0.5 mol% 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(carboxyfluorescein) (PECF). The third population (P3) was the same as 1, plus 0.5 mol% 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (PELR). The fourth population (P4) contained the same as P1, plus 0.5 mol% PELR and 0.75 mol% PECF.

Preparation of μSLBs for DNAzyme Reactions

The above procedure was used for the DNAzyme experiments, minus the fluorescently tagged lipids. DOPC was used in place of POPC to increase diffusivity of the bilayer, and

the amount of MPB PE was reduced from 10 mol% to 1 mol% (representing 4.5×10^6 DNA-lipid conjugates/ μ SLB). This change was made to reduce the density of conjugated components on the surface: at 1 mol% MPB PE there is approximately 1 DNA-lipid conjugate per 70 nm² of bilayer, as opposed to 1 per 7 nm² if 10 mol% MPB PE is used.

Conjugation for the Multiplexing

The 5' Thiol Modifier C6 S-S strand was deoxygenated and reduced in CAB (pH 5.0) (CAB5.0) containing 50 mM dithiothreitol (DTT) for 90 minutes at room temperature. The DTT was removed using a NAP-5 column and the sulfhydryl solution was stored at 4 °C in CAB5.0. The various μ SLB populations were then deoxygenated and the reduced thiol oligonucleotide was added into the deoxygenated μ SLB solution at a final concentration of 500 nM, in 500 μ L CAB6.5. This solution was further deoxygenated for 15 minutes and then sealed with parafilm and rotated at 4 °C for 12 hours. The μ SLBs were washed 3 times with 1 $\times$ phosphate buffer saline (PBS). The μ SLBs were coated with bovine serum albumin (BSA) (100 μ g/mL, rotated for 30 minutes). BSA is negatively charged at the physiological pH used in our assays³² and is used to stabilize the membrane.³³ The μ SLB were washed another three times in PBS, resulting in a final concentration of 500 nM thiol strands available for hybridization to the TMSD gates (for 10 mol% MPB PE).

Conjugation for DNAzyme Experiments

The above procedure was modified to include two of three thiol strands, one that hybridizes the DNAzyme (TSD), or one that hybridizes neither the DNAzyme nor the substrate (TSF), and a second strand that hybridizes to the substrate molecule (TSS). In addition, the Na⁺ concentration in all buffers was doubled (from the PBS level of 138 mM) to a final concentration of 276 mM (E276-0). The thiol strands were added at different concentrations to achieve a 4-fold excess of TSS. The final concentration of thiol strands available for hybridization is 50 nM, for 1 mol% MPB PE. These μ SLBs were also blocked with BSA using the above protocol.

Strand Design for TMSD Reactions

The sequences of the viral genomes for the four dengue serotypes were obtained from the National Center for Biotechnology Information (NCBI) website. Target oligomer subsequences of length 18 nucleotides from each of the four serotypes were then selected using a custom algorithm, as follows. A sliding window of size 18 nucleotides was used to compare each candidate target sequence for a given serotype with all 18-mers from the other three serotypes. A candidate target sequence was rejected if it contained a contiguous subsequence that matched any 18-mer sequence from any other serotype longer than a specified threshold length of 8 nucleotides. This test eliminated potential targets with long sequences shared with any other serotype genome. For each candidate sequence that passed this initial test, the Hamming distance (defined as the number of positions at which two sequences of same length differ) was computed between the candidate and each of the 18-mer windows from the other three serotype genomes. For each candidate target, the minimum Hamming distance observed was recorded, and the candidate targets were sorted in descending order of the minimum observed Hamming distance. Thus, the targets with larger minimum observed Hamming distances were those which showed the least similarity

with subsequences from the genomes of the other dengue serotypes. All sequences are listed in Table S1. For each candidate target sequence, the NUPACK secondary structure prediction algorithm³⁴ was used to compute the individual base-pairing probabilities of each base in the target oligomer at equilibrium, along with the minimum free energy structure and the associated free energy. These were recorded and manually inspected to identify target oligomers that displayed the minimal secondary structure, as oligomers without significant secondary structure can more efficiently initiate and execute TMSD reactions. Template strands are fully complementary to each of the selected targets. The incumbent strands match the corresponding target sequences, minus the toehold region at the 5' end of the target sequences, and including 25 bases, which are complementary to the 3' end of the standardized thiol strand. For each TMSD gate, the NUPACK secondary structure prediction algorithm³⁴ was used to compute the individual base-pairing probabilities, to ensure minimal secondary structure.

Strand Design for DNAzyme Reactions

Previously reported DNAzyme and substrate sequences were modified to include a subsequence that hybridizes to the TSD and TSS strands, respectively.^{6,10,17} All sequences are listed in Table S2.

Hybridization of TMSD Gates

For each μ SLB population, the corresponding ATTO647-labeled incumbent strand [1 μ M] was rotated at room temperature for 15 minutes, to allow complete hybridization to the standardized thiol strand. The μ SLBs were washed three times in PBS. Next, the Iowa Black RQ-labeled template strand [1 μ M] was rotated with the μ SLB population at room temperature for 15 minutes, and the μ SLBs were washed a final three times. The μ SLB concentration was then cut by $\frac{1}{4}$ to 1.5×10^6 μ SLBs/mL, before addition to the multiplex.

Hybridization of DNAzyme Components

For the solution phase DNAzyme experiments the Blocking strand_TSF at a final concentration of 50 nM was rotated at room temperature for 15 minutes, to create a dsDNA region on TSF. The μ SLBs were washed three times and the fluorescently labeled substrate strand [50 nM] was then rotated for 15 minutes at room temperature, to allow hybridization to TSS. Finally, the μ SLBs were washed three times in the E276-0 buffer (in the absence of ZnAc_2). For the surface bound DNAzyme experiments, the DNAzyme strand at a final concentration of 50 nM was rotated with the μ SLB population at room temperature for 15 minutes to allow hybridization to TSD, and the μ SLBs were washed three times in E276-0. Next, the fluorescently labeled substrate strand [50 nM] was rotated at room temperature for 15 minutes, to allow hybridization to TSS. Finally, the μ SLBs were washed three times in E276-0.

Multiplex Formation

To generate the multiplex 50 μ L of each μ SLBs population, at 1.5×10^6 μ SLBs/mL, was added into 300 μ L PBS, for a total volume of 500 μ L, bringing the total μ SLB concentration to 6×10^5 μ SLBs/mL. If target was added, only 250 μ L of PBS was added, and 50 μ L of

target at a final concentration of 50 nM was added to bring the final volume to 500 μ L. The multiplexed μ SLBs were rotated at room temperature for 1 hour and then tested on a Becton Dickinson (Washington, D.C.) LSR Fortessa flow cytometer. All μ SLB populations were initially plotted on a forward scatter (FSC) versus side scatter (SSC) plot (see Supporting Information Figure S1a), the μ SLBs within this gate were then plotted on a green PE CF (488/515-545 nm) versus yellow PELR (561/575-590 nm) plot (Supporting Information Figure S1b).

TMSD Reactions

Initial strand tests were run on individual TMSD gates, hybridized to the corresponding μ SLB population, using the same parameters as those used for the multiplex experiments. Three separate samples were prepared for each TMSD set. A positive control was established by hybridizing the fluorescently labeled incumbent strand, Supporting Information Figure S2, in the absence of template strand. Next the incumbent and template strand, which comprised the complete TMSD gate, were hybridized to the corresponding μ SLB, the second bar (black) of Supporting Information Figure S2. Finally the corresponding target sequences were added to the μ SLB population with the complete TMSD gate hybridized, shown in red with black stripes of Supporting Information Figure S2.

DNAzyme Reactions

For the solution phase DNAzyme and surface-bound substrate reactions, the substrate is added in excess to the μ SLB solution. After 15 minutes of rotation at room temperature we wash away the excess substrate and add solution phase DNAzyme at a given concentration. Our surface-bound DNAzyme and substrate setup begins by adding DNAzyme in excess to the μ SLBs with TSD covalently linked to the MPB PE, which creates a surface that has DNAzyme bound to all available TSD strands. These μ SLBs are washed and resuspended to remove free DNAzyme in solution. The substrate molecule is added in excess, and washed. This process creates a surface that has all TSS sites taken up by substrate and the DNAzyme molecules either bound to the surface-bound substrate or an excess substrate. After hybridization of the substrate and DNAzyme, the μ SLBs were rotated at room temperature in 25 μ M Zn^{2+} (E276-25) buffer.

Confocal Microscopy

To verify that DNA-lipid conjugates were freely diffusing on the surface of μ SLBs, a multi-photon laser scanning confocal microscope (Olympus FV1000, Tokyo, Japan) with a fluorescence lifetime imaging attachment (Becker & Hickl, Berlin, Germany) was used for fluorescence recovery after photo bleaching (FRAP) experiments. The DNA tag (Cy5, 647/670 nm) laser was run at 15% transmissivity. Run mode was 40 μ s/pixel, and stimulus mode was one pre-activation frame, and 5 activation frames. The FRAP interval was 3.264 seconds/frame (see Movie S1). For the FRAP experiments the Cy5-tagged oligo was hybridized to a thiol strand, which was conjugated to MPB PE (incorporated at 0.5 mol%). The FRAP experiments were run with one batch of MPB PE lipid, the cost of which prohibited replicates across batches; a second data set, from this batch, is included (Supporting Information Figure S3).

RESULTS AND DISCUSSION

Preparation of μ SLBs

The μ SLBs consist of a 10 μ m mesoporous silica microsphere bearing a supported POPC or DOPC phospholipid bilayer that contains up to two fluorescently tagged phospholipids for multiplexing and maleimide phospholipids for covalent coupling of DNA molecules that serve as linker strands (Figure 1A). Above the transition temperature of the base lipid (-2°C for POPC, and $<-2^{\circ}\text{C}$ for DOPC), bilayers formed on mesoporous silica microspheres maintain the ability to diffuse laterally.^{35,36} Multiplexed μ SLB populations are created by incorporation of lipids with fluorescently-labeled headgroups (one with a yellow rhodamine dye (PELR) and one with a green fluorescein dye (PECF)) at varying percentages (Figure 1B). μ SLB populations were analyzed *via* multi-parameter flow cytometry and demonstrated a well resolved 4-plex (Figure 1C). We have created up to 9-plex μ SLB populations (Supporting Information, Figure S1b). The approach here represents an advance over our recently reported single fluorophore indexing approach,¹⁹ as it allows facile creation of bivariate populations and higher multiplexing levels. Standard flow cytometry gating (Figure 1C) let us use a single reporter fluorophore to monitor reactions taking place on individual microsphere populations, thereby enabling multiplexed reporting with a limited number of fluorophores. Within the time frame of our experiments the microsphere populations remain discretely separated (Supporting Information, Figure S4).

Demonstration of Lipid Bilayer Fluidity *via* Fluorescence Recovery After Photobleaching (FRAP)

Figure 2 shows DNA-lipid conjugate diffusion on a μ SLB with 0.5 mol% MPB PE. While this concentration is predicted to provide $\sim 10^6$ conjugation sites per μ SLB, typical conjugation efficiencies to the surface are expected to result in a small fraction of available sites to be covalently linked to the available thiolated lipids within the μ SLB. Less than optimal conjugation efficiencies, along with potentially uneven partitioning of the MPB PE between the two leaflets of the bilayer³³, make it difficult to provide a precise calculation of the number of Cy5 molecules on the surface. Regardless, Figure 2 shows photobleaching (panel A) and then recovery (panel B), respectively, on the surface of a μ SLB. For the FRAP experiments the Cy5 bearing DNA oligo was used because ATTO647 does not readily photobleach. The fluorescence data were plotted as a function of time (Figure 2C), and using the ImageJ plug-in simFRAP³⁷, the diffusion coefficient was calculated as $0.25\ \mu\text{m}^2/\text{second}$, which falls just below the expected range of $0.5 - 5\ \mu\text{m}^2/\text{second}$ for fluid phospholipid bilayers at room temperature.^{33,38} While multiple factors, such as variance between lipid preparations or differing conjugation efficiencies, might result in variations in this calculated value, the value is comparable to diffusion coefficients reported for DNA oligos tethered to vesicles.³⁹ While further studies are underway to confirm the effect of DNA conjugation on lipid diffusion, it is evident from this data that DNA-lipid conjugates do diffuse and will allow DNA molecules to move from their original attachment point.

Multiplexed TMSD Reactions

To demonstrate the utility of multiplexed μ SLBs, we constructed a Dengue serotype specific TMSD gate on each of four microsphere populations. The four gates were designed to target

one of four single-stranded DNA (ssDNA) target sequences chosen from the genomes of the four dengue virus serotypes (Dengue 1 – 4, from GenBank sequence accession numbers NC_001477.1, NC_001474.2, NC_001475.2, and NC_002640.1 respectively). Figure 3 presents the mechanism of a basic TMSD reaction in the context of the μ SLB system. For the TMSD reactions, the linker strand sequence is complementary to a binding region on the TMSD gate structures. For each microsphere population, the corresponding TMSD gate was annealed and hybridized to the linker strand and washed (see Supporting Information, Figure S5 for depiction of individual TMSD gates, and Figure S2 for functionality of individual TMSD gates). The four μ SLB populations were combined in a single tube and various combinations of target strands were added at 50 nM, and the solution was rotated at room temperature for 1 hour. In the presence of the correct target sequence, the corresponding template strands, bearing quenchers, were displaced and fluorescence of the ATTO647 tagged incumbent strand increased due to dequenching. To demonstrate specificity, we used the four multiplexed receptor populations to detect all 16 possible combinations of the four target strands in 16 single tube assays with high signal-to-background ratios (Figure 4). Using established methods,⁴⁰ we calculated the limit of detection as 3 nM (see Supporting Information page 3). We include this value, not in direct competition with current DNA or RNA techniques that use target amplification (e.g., PCR),^{41,42} but to establish the limits of the system, which compare favorably with the nanomolar dissociation constants typically encountered in many bioassays. This system demonstrates the feasibility of using multiplexed μ SLBs to monitor specific reactions in a multi-reaction system individually.

Optimization of Conditions for DNAzyme Reactions

To demonstrate the modularity of the μ SLB platform, we also used it to perform DNAzyme reactions. To optimize experimental conditions for DNAzyme reactions, we began by studying the interactions of a fluorescent substrate analog with the μ SLB surface in conditions typical of solution phase DNAzyme reactions (1 M NaCl, 1 mM ZnAc₂, and 50 mM HEPES).^{6,10} The inclusion of ZnAc₂ in this buffer was crucial because the 8–17 DNAzyme requires Zn²⁺ ions as cofactors for its catalytic activity.¹⁶ We show schematic scenarios in Figure 5 and report results in Figure 6. The blank μ SLB (Figure 5A) had a non-complementary strand (TSF) conjugated to the bilayer, but was not exposed to the fluorescent substrate analog. The negative control μ SLB (Figure 5B) contained the fluorescent substrate analog in solution with TSF. The experimental μ SLB (Figure 5C) contained the fluorescent substrate analog in solution with the correct linker strand (TSS) conjugated to the bilayer. Thus, nonspecific binding is likely occurring in both systems, and the equivalent binding levels suggest that at higher Zn²⁺ concentrations nonspecific binding is dominant. The purpose of including TSF in the negative control was to ensure that the total amount of DNA conjugated to the μ SLB surface (and thus the electrostatic environment) was constant. Figures 5D and 5E depict the system in the absence of Zn²⁺ and Figure 5F depicts the system with Zn²⁺ at 0.1 mM. With 1 mM Zn²⁺, we saw the same amount of DNA binding to the negative control μ SLB as the experimental μ SLB (Figure 6A). The lack of specific binding is consistent with the literature, which shows that divalent cations, such as Zn²⁺, form “salt bridges” between DNA and zwitterionic lipid headgroups (here, DOPC), leading to non-specific binding of DNA to the bilayer.^{43,44} Our results show that as Zn²⁺ is increased from 0 to 100 μ M nonspecific binding increases. In the absence of

Zn^{2+} and at Zn^{2+} concentrations of 100 mM and below we see that non-specific binding is greatly reduced (Figure 6B), presumably due to reduced salt bridge formation. This data suggested that 25 μM was the optimal Zn^{2+} concentration for specific TSS hybridization.

As solution phase experiments have routinely used 1 mM Zn^{2+} for optimal DNAzyme activity,^{6,10} we compared the activity of the DNAzyme at 25 μM and 1 mM Zn^{2+} using a FRET substrate in solution. While the DNAzyme had reduced activity at 25 μM Zn^{2+} vs. 1 mM Zn^{2+} , the reaction ran to completion in ~2 hours (Figure 6C). Finally, as lipids aggregate during extrusion in the presence of 1 M NaCl, which complicates the process of vesicle formation by extrusion, we tested the effects of reduced sodium chloride concentration on DNAzyme reactions run at 25 μM Zn^{2+} (Figure 6D; for the kinetic traces see Figure S6). The experiment indicated that 276 mM NaCl performed similarly to the typical solution phase concentration of 1 M NaCl. Therefore, 276 mM was used in our DNAzyme experiments on μSLBs , as this concentration did not cause lipid aggregation.

DNAzyme Reactions

We configured μSLB -based DNAzyme reactions in one of two ways: in the first there is a solution phase DNAzyme and the substrate molecule is surface-bound *via* hybridization to a partially complementary bilayer-conjugated oligomer (TSS), as shown in Figure 7A, and in the second both the DNAzyme and the substrate are surface-bound *via* conjugated linker strands, the substrate to TSS as before and the DNAzyme to an orthogonal linker strand (TSD), as shown in Figure 7B. Both of these approaches are easily monitored through loss-of-fluorescence assays with flow cytometry,^{45,46} which provides excellent discrimination between free vs. bound signals at the surface of microspheres.⁴⁶

Using optimized assay conditions, we performed reactions where solution phase DNAzyme cleaved surface-bound substrates (Figure 7A) at varying concentrations of Zn^{2+} . While we observed signal (i.e., loss of fluorescence) at concentrations greater than 10 μM Zn^{2+} , the best signal-to-noise ratio was observed at 25 μM Zn^{2+} (Figure 8A). The signal becomes unobservable at higher Zn^{2+} as a result of non-specific binding, which overwhelms any loss of fluorescence due to DNAzyme activity. The data presented in Figure 8A, along with the nonspecific interaction experiments (Figure 6B), indicate that the optimal Zn^{2+} concentration is 25 μM . Having optimized the buffer conditions for the μSLBs system, we tested the effect of having higher concentrations of solution phase DNAzyme cleaving surface-bound substrate. Using the same setup as above, we added DNAzyme to the solution at final concentrations of 50 nM, 150 nM, 500 nM, and 1.5 μM . The microspheres were then incubated for 1 hour in the absence of Zn^{2+} prior to initiating the reactions with the addition of 25 μM Zn^{2+} . The results (Figure 8B) show an increased loss of fluorescence at higher concentrations. The rapid initial loss of fluorescence at higher concentrations is likely due to an increased on-rate for the solution phase DNAzyme to the surface-bound substrate. It is also notable that even at high concentrations of DNAzyme the cleavage does not remove all fluorescence from the μSLBs . This is most likely due to either non-specific interaction between the fluorescent substrate and the μSLB or some level of substrate inaccessibility during the cleavage reaction.

Finally, we aimed to demonstrate that surface-bound DNAzymes could cleave surface-bound substrates, as shown schematically in Figure 7B. Here, the ratio of substrate to DNAzyme on the surface is set by the initial conjugation reactions, so we added a 4-fold higher concentration of TSS (which hybridizes to the substrate) than TSD (which hybridizes to the DNAzyme). This estimate of a 4:1 ratio of substrate to DNAzyme concentration on the surface assumes that there is no bias between the efficiency of conjugation for TSS and TSD. We believe that this assumption is valid because the only difference between the TSD and the TSS strands is in nucleotide sequence. However, the potential partitioning of the maleimide lipids^{33,38} and inability to guarantee highly efficient conjugation efficiency, due to low percentages of maleimide on the μ SLB surface and/or charge effects of the phospholipid headgroups, precludes the precise calculation of surface-attached molecules. Regardless of the exact concentration of surface molecules, addition of Zn^{2+} at 25 μM results in rapid loss of fluorescence from μ SLBs compared with the negative control, a μ SLB with TSF conjugated in place of TSD and no DNAzyme present, indicating successful cleavage of surface-bound substrate by surface-bound DNAzyme (Figure 8C). However, the loss of fluorescence is not complete, which is consistent with observations for solution phase DNAzyme cleaving substrate from μ SLB surfaces. Again, this lack of completion may be due to non-specific binding of cleaved substrate to the μ SLB surface or substrate inaccessibility, but it also might be due to more complex effects such as reduced dissociation of DNAzyme from cleaved substrates that are bound to a surface. Our graphical representations of the model show cleavage of the substrate molecule, but they do not show disassociation of the DNAzyme and lower portion of the substrate because we do not have evidence that disassociation is occurring. While it remains possible that reduced or limited diffusion of DNAzyme and substrates on the surface could also result in the observed incomplete cleavage, we do not believe such effects occur since a diffusion coefficient of 0.25 $\mu\text{m}^2/\text{sec}$ is measured for the conjugated DNA molecules on μ SLB surfaces (Figure 3 and Movie S1).

Future work will investigate the detailed mechanisms of DNAzyme reactions on μ SLB surfaces, which are expected to be affected by surface transport principles that have been explored in detail for receptor protein interactions on surfaces⁴⁷. Such work will benefit from more robust methods of DNA integration into the bilayer, which include oligomer modifications such as cholesterol tags, or copper(1)-catalyzed conjugation of an azide and an alkyne.⁴⁸ Improving DNA integration into the bilayer will enable precise control over membrane bound DNA concentrations, which is critical to detailed mechanistic studies of such systems. Another fruitful direction may be to investigate programmable control of the diffusion rates of DNA components integrated with the bilayer, which might be achieved by modulating the physical properties of the bilayer or of the components themselves (e.g., to incorporate a second bilayer linker to provide additional drag on the diffusion process). Such a system could then potentially be used to implement two-dimensional spatial patterning on the microparticle surface, such as that achieved *via* reaction-diffusion systems in protein systems on membranes⁴⁹.

CONCLUSIONS

In summary, we implemented DNA-based molecular computing systems, using fluid, microsphere-supported lipid bilayers. These systems provide a multiplexable platform for bioassay development, and as a proof of concept here we designed DNA strand displacement gates for the detection of the four Dengue serotypes and demonstrated that every combination of the four targets was correctly identified. We then optimized the system to allow DNzyme reactions to be carried out on a zwitterionic lipid bilayer, in the presence of divalent cations. The use of a fluid bilayer as the supporting surface allows multi-component reactions to be engineered, which may eventually be used for signal amplification if multiple-turnover reactions can be demonstrated. Thus, this work offers a route toward the implementation of sophisticated, multi-reaction cascades on single particle surfaces and/or compartmentalization of particular reactions on individual particles. Such DNA-lipid conjugate systems, with stable and tunable bilayers that are easily monitored using flow cytometry, could lead to new smart materials with potential applications in targeted drug delivery, advanced molecular computation, and self-assembling nanomaterials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Loreen Stromberg, for training and assistance with the confocal imaging and FRAP experiments at the Center for Integrated Nanotechnologies (CINT), and Milan Stojanovic, for suggestions regarding the diffusion experiments.

Funding Sources

This material is based upon work supported by the National Science Foundation under grants 1525553, 1518861, and 1318833. Assay development for Dengue was in part supported by NIH NIAID 5 R21 AI115105-02. The LRS Fortessa data were collected at the UNM Shared Flow Cytometry and High Throughput Screening Resource Center supported by the University of New Mexico Health Sciences Center and the University of New Mexico Cancer Center with current funding from NCI 2P30 CA118100-11 "UNM Cancer Center Support Grant". This work was performed, in part, at the Center for Integrated Nanotechnologies, an Office of Science User Facility operated for the U.S. Department of Energy (DOE) Office of Science. Los Alamos National Laboratory, an affirmative action equal opportunity employer, is operated by Los Alamos National Security, LLC, for the National Nuclear Security Administration of the U.S. Department of Energy under contract DE-AC52-06NA25396.

ABBREVIATIONS

μSLB(s)	silica microsphere supported lipid bilayer(s)
TMSD	toehold-mediated strand displacement
POPC	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphocholin
DOPC	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphocholine
MPB PE	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl) butyramide]
PECF	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphoethanolamine-N-(carboxyfluorescein)

PELR	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)
BSA	bovine serum albumin
PBS	phosphate buffered saline

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Figure 1. Silica Microsphere Supported Lipid Bilayers (μ SLB)

A) The supported lipid bilayer (SLB), with a POPC (black circles) base coating a 10 μ m SiMS. To generate distinct spectral addresses 0.5 mol% of a lipid tagged with a green fluorophore (PECF) (green circles) and 0.5 mol% of a lipid tagged with a yellow fluorophore (PELR) (blue circles) are added. The maleimide lipid (MPB PE) (magenta circles) is incorporated to enable conjugation of thiolated oligonucleotides. There is a ~2 nm aqueous layer between the microsphere and the bilayer (~5 nm), shown in cyan. The DNA at the surface of the SiMP is shown in blue, and its tag in red. **B)** Four microparticle populations are then combined in a single tube. P1 – 90% POPC, 10% MPB PE. P2 – 89.5% POPC, 10% MPB PE, 0.5% PECF. P3 – 89.5% POPC, 10% MPB PE, 0.5% PELR. P4 – 88.75% POPC, 10% MPB PE, 0.5% PELR, 0.75% PECF. **C)** Bivariate flow cytometry data, showing the 4 microsphere populations in a single tube. The green PECF channel (488/515-545 nm) is plotted on the x-axis, against the yellow PELR channel on the y-axis.

Figure 2. Fluorescence Recovery After Photobleaching (FRAP)

A) Frame directly after photobleaching. The blue circle shows the ROI that was bleached, and the white shows the reference. This image was processed in ImageJ (NIH) and a screenshot was taken to show the ROIs. Brightness and contrast were adjusted to overcome the dimness resulting from low concentrations. **B)** Six frames after photobleaching showing full recovery. The entire video is available for viewing (Movie S1). **C)** With 0.5 mol% MPB PE, which represents $\sim 10^6$ DNA-lipid conjugates/ μSLB . Using simFRAP the diffusion coefficient was calculated to be $0.25 \mu\text{m}^2/\text{sec}$. The decrease in overall fluorescence, seen in the Reference line as well, is due to photobleaching caused by scanning laser confocal microscopy. The error bars represent standard error of mean for 5 replicates.

Figure 3. TMSD Reaction on μ SLB Surface

The gate comprises an ATTO647-labeled incumbent strand hybridized to the thiol linker that attaches it to the μ SLB. The μ SLB is composed of POPC (black circles), a lipid with a headgroup labeled with a green fluorophore (green circles), and for the TMSD reactions MPB PE (magenta circles) is incorporated at 10 mol%. The part of the incumbent that is not hybridized to the linker is fully complementary to a longer Iowa Black RQ-labeled template strand. Thus the gate complex has a double-stranded DNA (dsDNA) region with a short ssDNA overhang on the template strand. In the template/incumbent duplex fluorescence is blocked *via* fluorescence resonance energy transfer (FRET). Upon binding of the target strand branch migration begins. Eventually the template strand is displaced and fluorescence increases for the microparticle population.

Figure 4. Dengue Serotyping Assay

Response for all multiplexed microsphere populations, in the presence of 16 possible combinations of four ssDNA targets relevant to Dengue serotyping. For each chart the addition of target is indicated by +, lack of target is indicated by -. The lower right (- - -) was used as a baseline, and was subtracted from the remaining 15 charts. This chart is not normalized, but plotted as median fluorescence. The other 15 charts were normalized to an arbitrary number ($1 = 3.6 \times 10^4$ fluorescent units on the ATTO647 channel (640/663-677 nm)), which ensures that all values are plotted on the same scale. The limit of detection is 3 nM. Error bars represent standard error of mean for 3 replicates.

Figure 5. Non-Specific Interactions

A) Blank control, which has not been exposed to the fluorescently labeled (Cy5) strand. **B)** Bead population with non-complementary TSF conjugated to the bilayer in the presence of 1 mM Zn^{2+} . **C)** Bead population with TSS conjugated to the bilayer in the presence of 1 mM Zn^{2+} . TSS is complementary and hybridizes the Cy5 labeled strand. **D)** Bead population with TSS conjugated to the bilayer in the absence of Zn^{2+} . **E)** Bead population with TSF conjugated to the bilayer in the absence of Zn^{2+} . **F)** Bead population with TSF conjugated to the bilayer in the presence of 0.1 mM Zn^{2+} .

Figure 6. Optimization of Buffer Conditions

A) Three bead populations in the presence of 1 mM ZnAc₂. The blank bead population has TSF conjugated to the bilayer and has not been exposed to the fluorescently labeled (Cy5) strand, the second has TSF conjugated to the bilayer, and the third has TSS conjugated to the bilayer. Error bars represent standard error of the mean for 3 replicates. **B)** TSF and TSS bead populations in the presence of increasing ZnAc₂ concentrations. Again TSF will not hybridize the Cy5-labeled strand and so any fluorescence is a result of nonspecific binding to the μ SLB, and TSS does hybridize the Cy5-labeled strand and fluorescence above the TSF level is specific and due to hybridization to TSS. **C)** Solution phase DNAzyme and solution phase substrate reaction with 1 mM ZnAc₂, which is the standard concentration for DNAzyme reactions, and 25 μ M ZnAc₂. The 25 μ M reaction is slower compared to the standard, but is completed at 2 hours. **D)** Relative rates of solution phase DNAzyme and solution phase substrate reactions in the presence of increasing Na⁺ concentrations. The standard DNAzyme concentration is 1 M; however, optimal rates were achieved at 276 mM Na⁺.

Figure 7. DNAzyme Reaction Schemes

A) Solution Phase DNAzyme - For all DNAzyme experiments, the lipid bilayer is composed of DOPC (black dots), which increases the diffusivity of the bilayer as compared to POPC, and MPB PE is incorporated at 1 mol%. For this first set of DNAzyme reactions there are 2 thiol strands conjugated: the thiol strand that hybridizes the substrate (TSS) and a space filler thiol strand (TSF). The DNAzyme hybridizes the surface-bound substrate, and cleavage occurs in the presence of metal ion cofactors, $25 \mu\text{M Zn}^{2+}$. After cleavage of the substrate the DNAzyme dissociates from the substrate-binding arms and returns into solution along with the fluorescently labeled upper portion of the substrate, and fluorescence decreases for the microsphere population. **B) Surface-Bound DNAzyme** - TSS and a thiol strand that hybridized the DNAzyme (TSD) present on the bilayer. The surface-bound DNAzyme and substrate diffuse and hybridize and cleavage occurs in the presence of metal ion cofactors, $25 \mu\text{M Zn}^{2+}$. After cleavage of the substrate the fluorescently labeled upper portion of the substrate moves into solution, and fluorescence decreases for the microsphere.

Figure 8. μ SLB-Based DNAzyme Reactions

A) Solution Phase DNAzyme – DNAzyme, in solution at a final concentration of 50 nM, and surface-bound substrate reactions in the presence of increasing Zn^{2+} concentration. Optimal cleavage was seen at 25 μM Zn^{2+} . **B) Solution Phase DNAzyme** – Surface-bound substrate, with increasing solution phase DNAzyme concentrations. No DNAzyme was added for the black trace; this is our negative control. The blue trace is 50 nM DNAzyme, the green 150 nM, the orange 500 nM, and the red 1.5 μM . Error bars are standard error of the mean for 3 replicates. **C) Surface-Bound DNAzyme** – Reaction with both DNAzyme and substrate surface-bound, showing ~30% decrease in fluorescence. For this reaction there was a fourfold excess of TSS, compared with TSD. The reaction was run with 276 mM Na^+ and 25 μM Zn^{2+} . Error bars are standard error of the mean for 3 replicates.