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Development of a Linker Mediated Immunoassay Using Chemically Transitioned Nanosensors

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ABSTRACT: Sensitive and specific quantification of protein biomarkers is important in medical diagnostics, academic research, and pharmaceutical development. However, multiple binding steps in conventional sandwich immunoassay protocols result in high assay hands-on-time and delayed results. This is particularly relevant for medical diagnostics, where assay turn-around-time can have an immense impact on patient outcomes. To address this limitation, we report the assembly of nanosensors prepared using DNA-antibody conjugates, which combine capture and detection antibody binding steps by facilitating rapid antigen capture. Following antigen binding, detection antibodies are released using chemically induced complex rearrangement. A panel of 12 chemical additives are characterized to identify melting point depressants capable of rapidly denaturing double stranded DNA (dsDNA) linkers, and 8 compounds are demonstrated to be capable of disrupting dsDNA while maintaining the integrity of protein binding. This technique is then validated for the measurement of the heart attack indicator cardiac troponin I, and is shown to successfully combine antigen binding steps while also increasing detection sensitivity 42×. Linker mediated immunoassays are also demonstrated to provide robust quantification in human serum and are shown to be compatible with each of the most commonly used immunoassay detection modalities.

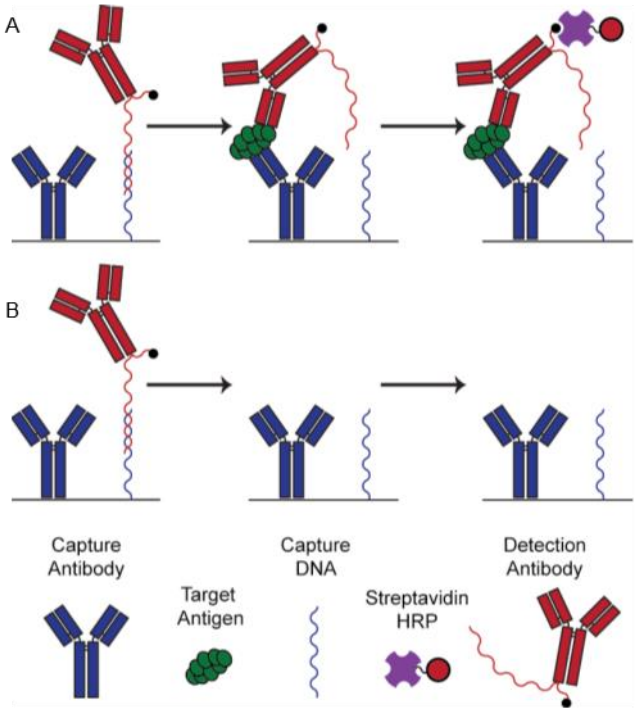
Since its initial use by Engvall and Perlmann in 1971, immunoassays have had an immense impact on academic research, pharmaceutical development, food safety, and medical diagnostics.[1–4] Despite thousands of new publications relating to protein biomarkers each year,[5] immunoassays remain dependent on the inherent weaknesses of highly complex and labor-intensive steps that contribute to high cost and difficult automation. Conventional sandwich immunoassays (SIA) are performed using a process consisting of 2 main incubation steps: antigen capture and detection antibody binding. Each of these steps requires a 30-60 min incubation period as well as 3-6 labor-intensive wash steps. While the effect of washes on user hands on time can be partially addressed through the use of plate washers, squirt bottles, and multichannel pipettes, each additional step contributes to higher error, longer assay turnaround times, additional reagent consumption, and reduced ease of automation.[6] Despite the substantial drawbacks of this protocol, SIAs are still used for the vast majority of protein detection applications as a result of low cost, large linear range, high sensitivity, and broad compatibility with a variety of detection methods.

In recent years, the demand for assays with fewer steps has resulted in a modest transition to label free detection platforms capable of producing signal without detection antibodies, allowing antigens to be detected following a single series of binding and wash steps when functionalized surfaces are pre-prepared[6]. These label free methods include the use of microcantilevers, surface plasmon resonance, voltammetry, potentiometry, and impedimetric detection.[7,8] While these methods address many of the primary drawbacks of conventional immunoassays, limits of detection thousands of times higher,[8,9] drastically increased cross reactivity in multiplexed assays,[10] and larger equipment and disposable costs have prevented any of these systems from gaining substantial commercial traction.

As a result of the limitations of label free immunoassays, there remains an immense need for methods that can reduce the number of steps without dramatically impacting assay cost or compromising test sensitivity.[6] A main factor contributing to this problem is the dependence of label based detection platforms on independent capture antibody and detection antibody binding steps. Here we describe a new technique developed to combine these binding steps to provide a simplified method of performing highly sensitive immunoassays. This is achieved in the linker mediated immunoassay (LMI) through the use of double-stranded DNA (dsDNA) linkers, which temporarily attaches capture and detection antibodies on a functionalized surface to form nanoscale detection complexes. When an antigen is bound by either antibody, the close proximity of the second antibody facilitates rapid binding (Scheme 1). The dsDNA linker is then denatured, which allows for rapid linker dissociation and the removal of unbound detection antibodies. Protein levels within a sample can then be quantified using common detection methods without the need for an independent detection antibody binding step.

Several other methods have been previously developed to detect proteins using DNA sensors. In one design, this was achieved by conjugating antigens directly to DNA and detecting antibodies in solution by measuring binding-mediated conformational changes to molecular beacon fluorescence.[11,12] Other platforms

have demonstrated that binding of DNA-antibody conjugates can induce detectable changes in electrophoresis gel mobility.[13,14] Still other strategies use DNA aptamers to directly bind and detect proteins.[15] While these methods provide excellent demonstrations of how conformational changes in DNA sensors can be used to detect binding events, each of these strategies necessitate the use of detection modalities with inherent limitations including low detection sensitivity, as is the case with molecular beacon based approaches, or the need for lengthy protocols such as gel separation, staining, and band analysis. LMI technology aims to leverage similar principles to improve upon existing immunoassay methods using the convenient and highly optimized detection modalities already in use.



Scheme 1. Linker mediated immunoassay. Assembled molecular complexes are initially displayed on functionalized surfaces. Complexes are then shown following incubation in the presence (A) or absence (B) of a target antigen and complex washing with linker dissociation buffer. When the antigen is present, cooperative linker mediated binding to capture and detection antibodies occurs (A). In the absence of antigen binding, detection antibodies are removed (B). As a final step, streptavidin conjugated detection agents are bound to biotin-conjugated detection antibodies.

A main challenge in successfully developing multi-state nanosensors is the need to identify methods for regulating the controlled transition of dynamic complexes without compromising the overall complex stability. While existing actuation methods including photoactuation,[16–18] DNA binding,[19,20] and electroactivation[21] exist, these strategies necessitate added equipment and lengthy incubation times.[22,23] Alternatively, the use of high temperatures can rapidly melt dsDNA, but has the potential to interfere with protein binding.[24,25] To address these limitations, the impact of 12 known melting point depressants on dsDNA melting temperature was characterized, and the most promising candidates were evaluated based on their impact on protein binding.

A new protein-friendly protocol for initiating rapid chemical transition of DNA nanosensors is then demonstrated in conjunction with the LMI. The common myocardial infarction biomarker,[26] Cardiac troponin I (CTNI), was selected as an initial target analyte to emphasize the benefits of simplified methods for assays where turnaround time has an immense impact on patient management. Using nanoscale complexes and the optimized strategy for chemical state transition, LMIs are shown to outperform conventional immunoassays using each of the most common detection methods as well as when measuring proteins in buffer and in complex media.

Experimental Section

DNA Melting Point Evaluation

Melting point determination experiments were prepared using Unmodified Detection DNA and its compliment Unmodified Capture DNA (Table S1) mixed to a final concentration of 5 µM along with a 1:5,000 dilution of SYBR Green I (Thermo Fisher, Carlsbad, CA), and an indicated concentration of chemical additives in low salt phosphate buffer (10 mM NaH_2PO_4, 1.8 mM KH_2PO_4, 1 mM ethylenediaminetetraacetic acid (EDTA)). Mixed DNA was then heated to 80 °C for 10 min and was allowed to incubate in white 96-well thin walled PCR plates (Sigma-Aldrich, St. Louis, MO) at room temperature for 1 h to allow DNA to hybridize. Melting points were then determined using an Eppendorf Realplex 4S Real-time PCR system with a ramp rate of 0.5 °C /min and a 520 nm emission filter. Data was analyzed using Eppendorf Realplex software version 2.2 with a threshold value of 5%.

Microplate DNA Surface Functionalization

DNA coated 96-well plates were prepared using N-oxysuccinimide ester functionalized DNA-BIND plates (Sigma-Aldrich, St. Louis, MO). Primary amine modified capture DNA was covalently attached to the plate surface through incubation at a concentration of 120 nM for 1 h in plate linker binding buffer (500 mM NaH_2PO_4, 1 mM EDTA, pH 8.5). Unbound DNA was then removed, and unreacted N-oxysuccinimide ester groups were quenched by washing wells 4 times with 200 µL tris-buffered saline (50 mM Tris(hydroxymethyl)aminomethane, 150 mM NaCl, pH 7.4). Modified plates were then allowed to dry and were stored at 4 °C until use.

Linker Mediated Streptavidin Detection Experiments

Streptavidin detection complexes were assembled using DNA functionalized plates prepared as described above with Biotin Capture DNA 1,2,3,4, or 5, which were designed with 13, 12, 11, 10, and 9 bp of complementarity with Biotin TYE563 Detection DNA, respectively (Table S1). Biotin Capture DNA 4 was used for surface functionalization in all streptavidin detection experiments unless otherwise indicated (Table S1). Following DNA attachment, plates were incubated with blocking buffer (2% bovine serum albumin, 150 mM Na_2CO_3, 350 mM $NaHCO_3$, pH 9.6). Biotin TYE563 Detection DNA was then added to each plate at a 60 nM concentration for 30 min at room temperature in hybridization buffer (900 mM NaCl, 20 mM

Tris(hydroxymethyl)aminomethane, 1 mM EDTA, 0.1% tween 20, pH 7.4)(Table S1). Plates were then washed 6 times with 200 µL hybridization buffer and stored at 4℃ until use. Streptavidin was then added in 100 µL phosphate buffered saline (PBS)(137 mM NaCl, 10 mM NaH_2PO_4, 1.8 mM KH_2PO_4, 1 mM EDTA, pH 7.4) for 30 min at room temperature. Following streptavidin binding, samples were washed 6 times with indicated buffers to facilitate linker dissociation. The fluorescence signal of Biotin TYE563 Detection DNA was then quantified using a Synergy-HT spectrophotometer (BioTek Instruments, Winooski, VT) with excitation at 530±25 nm and emission at 590±35 nm.

Two Step Streptavidin Detection

DNA surfaces were initially functionalized using biotin conjugated Non-Complimentary Capture DNA covalently attached to microplate surfaces (Table S1). Streptavidin was then added for 30 min at room temperature, washed with hybridization buffer 4 times, incubated for 30 min at room temperature with Biotin TYE563 Detection DNA, and washed 4 times with the low salt phosphate buffer at 37 ℃. TYE563 fluorescence was then quantified using a Synergy-HT spectrophotometer (BioTek Instruments, Winooski, VT) with excitation at 530±25 nm and emission at 590±35 nm.

Antibody-DNA Conjugation

Antibody conjugations were conducted using Thunder-Link® conjugation kits (Innova Biosciences, Cambridge, UK) using the supplied protocol.[13] In short, Biotin Detection Ab DNA was diluted to 100 µM in Thunder-Link wash buffer and was added to DNA activation vials (Table S1). Ab115704 (Abcam, Cambridge, UK) or ab19615 (Abcam, Cambridge, UK) was then diluted to 1 mg/mL and added to antibody activation vials. Desalting columns were equilibrated by allowing storage buffer to flow through and then washing each column 5 times with 3 mL of Thunder-Link wash buffer. After a 1h incubation at room temperature, activated antibodies and DNA were added to the top of independent columns. Wash buffer was then added to push activated material to the base of each column. 200 µL of wash buffer was then added to the top of each column and eluate was collected. Activated antibodies were then added to activated DNA and allowed to incubate overnight at room temperature. Conjugations were performed at a 1:16 antibody to DNA molar ratio unless otherwise indicated. Reactions were then transferred to 4 ℃ and Thunder-Link clean up reagent was added. After a 10 min incubation, tubes were centrifuged at 15,000g for 5 min, supernatant was removed, and antibody conjugates were resuspended in Thunder-Link antibody suspension buffer. Antibody conjugates were then aliquoted and stored at -20 ℃ until use.

Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 10% TGX precast polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA). All samples were prepared in reducing conditions using 2X Laemmli Sample Buffer (Bio-Rad Laboratories, Hercules, CA) containing 5% 2-mercaptoethanol. Samples were heated for 5 min at 95℃ and were then run at 100V for 1.5 h in a Mini-PROTEAN® Tetra Vertical Electrophoresis Cell containing Tris-Glycine SDS running buffer (25 mM Tris(hydroxymethyl)aminomethane , 192 mM glycine, 0.1% SDS, pH 8.3). Gels were then removed and incubated in Tris-Glycine SDS running buffer containing a 1:10,000 dilution of SYBR Gold with shaking for 1 h at room temperature. Following DNA visualization, gels were evaluated using a Pierce Silver Stain Kit (Thermo Scientific, Carlsbad, CA) using the suggested protocol. Gels were imaged using a Bio-Rad Gel Doc XR+ System.

Linker Mediated Immunoassay Surface Preparation

White corning 96-well DNABind plates were covalently conjugated as described above using 20 bp Capture DNA, 40 bp Capture DNA, 80 bp Capture DNA, or 80 bp Nuclease Resistant Capture DNA (Table S1). Following capture DNA binding, plates were washed 4 times with 200 µL PBS to remove all unbound DNA. Capture antibodies were then added at a 1 µg/mL concentration in capture antibody binding buffer (150 mM Na_2CO_3, 350 mM $NaHCO_3$, pH 9.6) and were allowed to incubate for 1 h at room temperature. Plates were then washed 4 times with 200 µL PBS and were allowed to incubate overnight at 4℃. Linker conjugated detection antibodies were then bound to the plate surface for 1 h at room temperature at a concentration of 1 ug/mL in hybridization buffer. Plates were then washed 6 times with 200 µL hybridization buffer and were stored at 4℃ until use. Unless otherwise indicated, 80 bp Nuclease Resistant Capture DNA was used as capture DNA, ab19615 (Abcam, Cambridge, UK) was used as a capture antibody, and ab115704 (Abcam, Cambridge, UK) conjugated to Biotin Detection Ab DNA was used as a detection antibody.

Linker Mediated Immunoassays

LMI complexes were assembled as described above. CTNI samples were prepared using PBS or pooled human plasma (Innovative Research, Novi, MI) containing 1 mM EDTA. Samples were added to LMI plates and were allowed to incubate for 1 h at room temperature. Unbound detection antibodies were then removed by washing plates 6 times using 1,5 pentanediol wash buffer (2.4 M 1,5 Pentanediol, 10 mM NaH_2PO_4, 1.8 mM KH_2PO_4, 1mM EDTA, 0.1% tween 20, pH 7.4) unless otherwise indicated. Streptavidin conjugated detection agents including Ultra Streptavidin-HRP (Fisher Scientific, Waltham, MA), Quantum dot (Qdot®) 605 Streptavidin Conjugate (Thermo Scientific, Carlsbad, CA), or Phycoerythrin (PE) Streptavidin (BioLegend, San Diego, CA) were then added and incubated for 1 h at room temperature. Plates were then washed 6 times with PBS containing 0.1% tween 20 to remove unbound streptavidin conjugates.

Sandwich Immunoassays

SIA surfaces were functionalized by adding capture antibody ab19615 (Abcam, Cambridge, UK) or ab115704 (Abcam, Cambridge, UK) at 1 µg/mL in capture antibody binding buffer for 1 h at room temperature. Plates were then washed 4 times with 200 µL PBS and were blocked at 4℃ overnight with blocking buffer. CTNI samples prepared using PBS or pooled human plasma (Innovative Research, Novi, MI) containing 1 mM

EDTA were then added and allowed to incubate for 1 h at room temperature. Plates were then washed 6 times to remove unbound detection antibodies using PBS. Biotin conjugated ab115704 (Abcam, Cambridge, UK) or ab19615 (Abcam, Cambridge, UK) detection antibodies were then added for 1 h at room temperature at a concentration of 1 ug/mL in PBS containing 2% bovine serum albumin. Plates were then washed 6 times with 200 µL PBS. Streptavidin conjugated detection agents were then added and incubated for 1 h at room temperature. Plates were then washed 6 times with PBS containing 0.1% tween 20 to remove unbound streptavidin conjugates. Unless otherwise indicated, ab19615 (Abcam, Cambridge, UK) was used as a capture antibody and ab115704 (Abcam, Cambridge, UK) conjugated to Biotin Detection Ab DNA was used as a detection antibody.

Chemiluminescent Detection

ELISABright (Advansta, Menlo Park, CA) peroxide and luminol solutions were mixed in a 1:1 ratio. Plates were then aspirated, and 100 µL of the substrate mixture was added to each well. Luminescence was then measured at 250-850 nm on a SpectraMax M2 Multi-detection reader.

Colorimetric Detection

Ultra 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (Life Technologies, Carlsbad, CA) was allowed to warm to room temperature prior to the addition of 100 µL TMB to each well. The substrate was then allowed to react for 5-15 min until sufficient color developed, at which point reactions were halted through the addition of 100 µL of 160 mM sulfuric acid. Solution absorbance was then measured at 450 nm on a SpectraMax M2 Multi-detection reader.

Fluorescent Detection

Fluorescence was measured in 100 µL PBS directly following streptavidin conjugate binding and subsequent washes. Streptavidin Phycoerythrin was quantified using a 490 nm excitation filter and a 573 nm emission filter, and streptavidin quantum dots were quantified using a 410 nm excitation filter and a 605 nm emission filter using a SpectraMax M2 Multi-detection reader.

Data analysis and Figure Rendering

The limit of detection was determined by calculating the concentration corresponding to the background signal plus 3 times the background standard deviation. For streptavidin detection, all trend lines were created using GraphPad Prism analysis software based on linear fitting with $R^2 \geq 0.99$. For SIA and LMI data, all trend lines were created using nonlinear one site total saturation binding curve fitting using GraphPad Prism software with $R^2 \geq 0.988$. EC_{50} values reported were based on GraphPad Prism EC_{50} analysis software.

Results and Discussion

LMI technology utilizes a number of well-established techniques in the design of a unique immune complex, including the use of DNA as a cleavable linker.[16–18,22,23] In order to completely remove all unbound detection antibodies, DNA linkers must be rapidly denatured and unbound detection antibodies need to be completely removed without disturbing antigen binding interactions. While covalent cleavage methods have been demonstrated to be effective for cleaving DNA,[16–18,22,23,27,28] a main drawback of these strategies is the need for additional equipment and long incubation periods. To address these limitations, we developed a chemical dissociation method to enable extensive linker dissociation in a single series of washes of typical duration.

As an initial means of demonstrating that simultaneous binding of capture and detection agents could be achieved in tandem and to optimize a chemical linker dissociation method, a model system was developed using biotin-conjugated capture and detection DNA used to detect the biotin binding protein, streptavidin.[29] In this model system, two biotin conjugated DNAs were assembled into a detection complex with one capture strand covalently attached to a surface and also bound to a fluorescent TYE563 conjugated detection strand (Scheme S1). Low salt phosphate buffer was then heated to 15 ℃ above ambient room temperature to simulate the predicted impact of melting point depressants.[30,31,32,33,34] To evaluate the optimal length of dsDNA linkers, streptavidin detection complexes were prepared using 5 capture DNA variants with dsDNA lengths from 9-14 bp and predicted melting temperatures ranging from 21.4 ℃ to 62.3 ℃ (Table S1). Following streptavidin binding, complexes were washed with a low salt buffer heated to 37 ℃ to denature dsDNA linkers, and TYE563 fluorescence was quantified. Results of these experiments indicated that capture DNA with 9 bp and 10 bp of complementarity and predicted melting points of 27.9 ℃ and 21.8 ℃ each enabled rapid and thorough linker dissociation even after a single wash step of normal duration (Figure S1A). This same phenomenon was not observed for capture DNA with larger regions of complementarity, which were not completely removed even after 4 washes (Figure S1B).

As a result of these findings, Biotin Capture DNA 4 with 10 bp of complementarity was selected as the highest melting point candidate capable of maintaining stable complex at room temperature while also rapidly dissociating in the presence of buffer heated to 15 ℃ above room temperature. The effect of sequential washes was then analyzed, and it was determined that 97% of cleavable detection DNA was removed in the first two washes, with >99% removed after 4 washes (Figure S1C). As a final evaluation of the streptavidin model system, linker mediated detection was directly compared to streptavidin detection with independent capture and detection agent binding steps. Results of this analysis indicated that, in addition to simplifying the assay protocol, linker mediated streptavidin detection resulted in a 3.71 fold increase in fluorescence signal relative to sequential streptavidin detection (Figure S1D). These results provided the first demonstration that dsDNA denaturation can be leveraged to provide rapid and thorough linker dissociation without the need for harsh conditions known to denature proteins and disrupt antibody antigen interactions. While this data demonstrated thorough linker dissociation in a single series of washes using heated buffers, a more consistent and convenient means of linker dissociation would utilize wash buffers containing compounds which facilitate DNA melting at room temperature through chemical disruption of DNA binding.

The effects of chemical additives on dsDNA melting

One of the most simple chemical mechanisms for reducing DNA melting temperature is to reduce the salt concentration, a process which decreases dsDNA stability by increasing charge repulsion between dsDNA strands[35,36,37]. The presence of salt and buffers within a solution are important for maintaining solution pH and protein integrity, however, and elimination of buffer salt was not found to be sufficient to facilitate effective melting of stable dsDNA linkers at room temperature. There are, however, a significant number of chemical additives that have been shown to disrupt dsDNA binding.[30,31,32,33,34] To determine if sufficient melting point depression could be achieved, 12 chemical agents were selected based on known melting point depression as well as previous reports of the effects of these compounds on protein-protein interactions. These reagents included: L-proline,[38] betaine,[39] sarcosine,[39,40] d-sorbitol,[30] trehalose,[40] DMSO,[41,42] methanol,[43] 1-propanol, glycerol,[44,45] ethylene glycol,[37,46] propylene glycol,[47] and 1,5 pentanediol.[48]

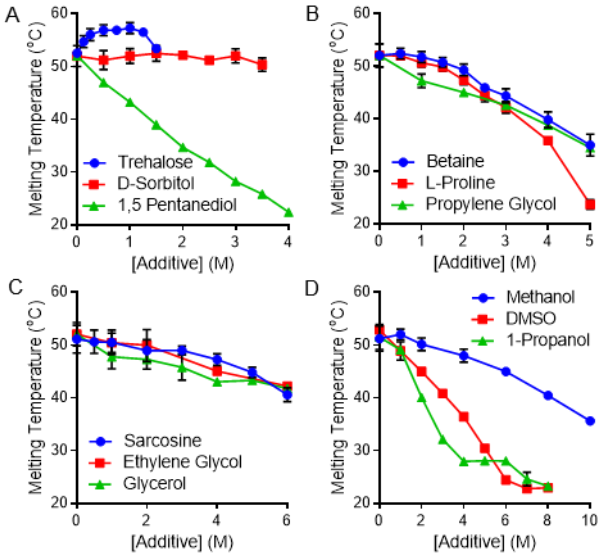


Figure 1. The effect of chemical additive concentration on dsDNA melting temperature. Melting points for indicated chemical additives are shown. All data points and error bars represent the average and standard deviation of 3 technical replicates.

In order to evaluate the effect of each agent on dsDNA, the melting temperature of double stranded DNA was evaluated with different concentrations of chemical additives.[49,50,51] Surprisingly, the results of this analysis indicated that 9 out of the 12 reagents screened were capable of achieving the 15 °C of melting point depression required to facilitate linker denaturation (Figure 1, Table S2). Notably, 1,5 pentanediol was shown to perform extremely effectively, with >30 °C of melting point depression achieved at a concentration of 4M. Alternatively, Sarcosine, d-sorbitol, and trehalose were found to exhibit insufficient melting point depression at concentrations soluble at room temperature to reach a concentration capable of achieving the desired melting point depression.

Characterizing the effectiveness of chemical additives on streptavidin binding

Once chemical additive concentrations necessary to facilitate 15 °C of melting point depression were identified, each buffer was evaluated for use as a linker dissociation buffer for streptavidin quantification. Fluorescently labeled streptavidin detection complexes were prepared, incubated with streptavidin, washed with chemical dissociation buffers, and surface fluorescence was quantified. Buffers were then evaluated based on the capacity to facilitate DNA melting at room temperature without disrupting biotin-streptavidin interactions (Figure 2).

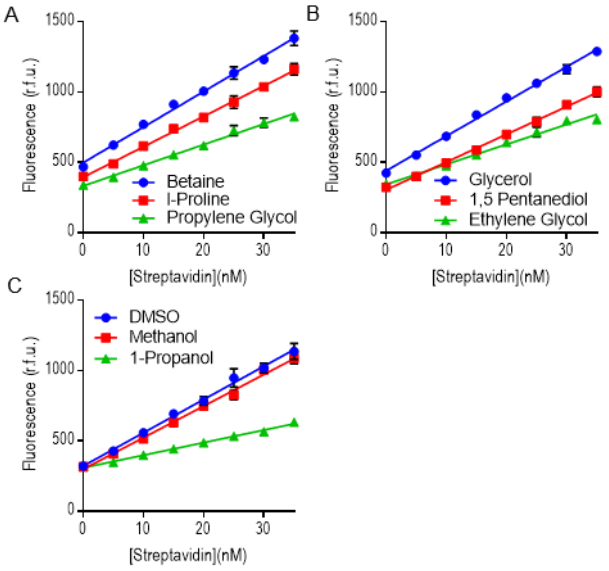


Figure 2. The effect of chemical dissociation buffers on linker mediated streptavidin detection. Assays were performed as described in materials and methods with 4 washes with buffers containing indicated additives at concentrations listed in Table S2. All data points and error bars represent the average and standard deviation of 3 technical replicates. Trend lines were created using GraphPad Prism analysis software to perform linear regression analysis with $R^2 \geq 0.99$ in all instances.

Results of these experiments indicated that each of the 9 selected buffers successfully facilitated linker dissociation and allowed for streptavidin detection with a linear trend (R2 >0.99) between concentrations of 0 and 35 nM. Use of these chemical additives were also found to impact the slope of the streptavidin detection linear fit with the greatest signal being maintained in the following order: betaine>L-proline>DMSO>methanol> glycerol>1,5 pentanediol>propylene glycol> ethylene glycol> 1-propanol. Results also indicated that 1,5 pentanediol, DMSO, and methanol had the highest signal-to-background ratio, indicating that streptavidin binding had remained high while also facilitating a higher rate of linker dissociation and detection DNA removal in the absence of streptavidin.

Of the compounds screened, it was found that 1-propanol had a particularly detrimental effect on the fluorescence signal (Figure 2). This finding suggested that 1-propanol had a substantially more detrimental impact on streptavidin binding than the other additives, and therefore would not likely make an optimal candidate for protein detection. Together, these results demonstrated that 8 of the 12 initially screened compounds effectively

denatured linkers without substantially interfering with biotin-streptavidin binding interactions. While other methods have been demonstrated to successfully denature or cleave stable DNA complexes at room temperature, no other method to our knowledge achieves this goal without requiring an incubation step.[16–18,22,23,27] As the field of DNA nanomachines continues to progress, we anticipate that this rapid method for chemically transitioning DNA nanostructure state will become an increasingly attractive strategy in a variety of emerging platforms.

Characterizing the effectiveness of chemical additives on antibody binding

While streptavidin detection provided a terrific model system, a more applicable use of linker mediated binding is in antibody mediated antigen detection. To this end, antibodies were conjugated to DNA at a variety of DNA-to-antibody molar ratios, and conjugates were visualized using SDS-PAGE in reducing conditions.[52] Silver staining of proteins bands showed the presence of 50 kD antibody heavy chains and 25 kD antibody light chains in all samples containing antibodies (Figure S2A). Consistent with successful conjugations, reactions performed in the presence of DNA showed protein bands with larger molecular weights, corresponding to each chain conjugated to 8.95 kD oligonucleotide linkers. Conjugation products were further characterized using SYBR Gold, a DNA specific dye known to exhibit fluorescence enhancement upon binding to ssDNA.(Figure S2B).[53,54] As expected, SYBR Gold DNA visualization showed strong fluorescence intensity in larger molecular weight bands corresponding to DNA antibody conjugates and minimal signal in smaller bands corresponding to unmodified heavy and light chains.

Each of the chemical additives were then evaluated based on performance in the detection of CTNI using the LMI illustrated in scheme 1. LMI complexes were assembled, incubated with CTNI, and linkers were dissociated using each of the 8 buffers that had performed well in prior experiments (Table S2). Detection antibodies were then labeled using streptavidin conjugated horseradish peroxidase (Streptavidin-HRP), and assay signal was measured using TMB colorimetric substrate. Results of these experiments indicated that each all 8 compounds screened were capable of successfully dissociating linkers and quantifying CTNI (Figure 3).

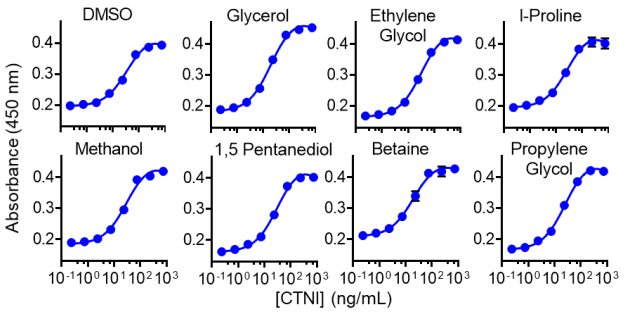


Figure 3. The effect of chemical wash buffer additives on linker mediated immunoassays. Linker mediated immunoassays were performed using the standard protocol and 4 washes conducted with buffers containing the indicated additives at concentrations reported in Table S2. All data points and error bars indicate the average and standard deviation of 3 technical replicates.

Surprisingly, each of the compounds used in wash buffers demonstrated robust binding curves with minimal variation in assay signal for any of the chemicals evaluated. Results of this analysis indicated that Ethylene Glycol, Propylene Glycol, and 1,5 Pentanediol demonstrating the lowest background values and the highest signal-to-background ratios, suggesting that these compounds achieved the most extensive removal of unbound detection antibodies while also maintaining the highest levels of antigen binding. Further, it was found that intra-assay coefficients of variation values ranged from 1.01-4.11%, with 1,5 pentanediol, Ethylene Glycol, and Propylene Glycol demonstrating the lowest coefficients of variation of 1.01%, 2.08%, and 2.33%, respectively (Figure 3). Of the compounds screened, 1,5 pentanediol was identified as the most effective of the linker dissociation agents with the lowest background signal, a minimal assay variation, as well as the lowest limit of detection.

Successful demonstration of LMI represented the first known demonstration that detection and capture antibody binding events can be combined into a single close proximity interaction that can be detected following rapid chemical denaturation of dsDNA linkers. While this finding provides encouraging results, quantification of CTNI alone is not sufficient to determine if the assay illustrated in Scheme 1 is performing as expected. To further validate this process, the effect of eliminating each of the main assay components was evaluated. Unsurprisingly, results indicated that extremely low signal was observed in the absence of capture antibodies, Capture DNA, Biotin Detection Ab DNA, detection antibodies, or streptavidin-HRP (Figure S3A). These results also indicated that the use of a blocking buffer resulted in an increased signal as well as a reduction in background in the absence of CTNI. Further, evaluation of the effect of wash buffers indicated that chemical additives were necessary to successfully remove unbound detection antibodies and to accurately detect antigen signal (Figure S3B).

While many emerging immunoassay platforms require new equipment, the LMI system has the potential for broad applicability across detection platforms presently in use.[2] To demonstrate detection platform compatibility, streptavidin conjugated detection agents for chemiluminescent, colorimetric, and 2 fluorescent based detection platforms were each evaluated. For fluorescence based detection, Phycoerythrin, a bright fluorescent protein, and quantum dots, bright and stable semiconducting nanocrystals, were selected.[55,56] For each of the enzymatic methods, Streptavidin-HRP was used along with the common strate containing peroxide and luminol.[57] For each of these detection methods, LMI assays were directly compared to SIAs performed using identical reagents.

As previously reported, each of the fluorescent detection agents achieved substantially lower sensitivity relative to enzymatic methods. Of the fluorescence methods, Phycoerythrin was found to be most effective, with a limit of detection (LOD) of 4.46 ng/mL in the LMI system and 39.8 ng/mL in the two step conventional SIA (Figure S4A). Likewise, the more stable but less sensitive quantum dots allowed for a LOD of 16.9 ng/mL and 65.3 ng/mL for LMI and SIA, respectively (Figure S4B). As with fluorescent detection, both enzymatic detection platforms demonstrated improved LODs in LMIs when compared to the conventional SIA protocol, with a LOD of 2.69 ng/mL and 1.77 ng/mL for colorimetric and luminescence platforms

(Figure S4 C,D). This data provided important validation of the broad compatibility of the LMI system with each of the most common immunoassay detection methods,[6] with chemiluminescence being identified as the most sensitive.

These experiments represented the first direct comparison of LMIs with conventional immunoassays and provided compelling evidence that capture and detection antibody binding steps can be effectively combined to simplify the assay protocol. Notably, while LMIs do reduce the number of steps necessary to detect target antigens, more extensive plate preparation protocols are required. As a result, this method would provide the greatest value when automated and high throughput machinery can be used to prepare many assays for storage until the time of use.

One surprising outcome from initial LMI experimentation was the observation that in addition to increased assay sensitivity, LMIs exhibited dramatic differences in effective concentration (EC50) for each of the detection methods measured, values which are known to be indicative of the strength of binding interactions.[58–62] When comparing LMIs with conventional SIAs, EC50 values were observed to be 30-233 times lower for LMIs. While further investigation would be required to conclusively demonstrate the cause of shifted EC50 curves, one explanation of these findings is that multivalent binding present in LMIs results in enhanced avidity, a well-established phenomenon resulting from cooperative antigen binding.[63–66] An alternative explanation of these findings is that the improved sensitivity observed could be attributed to differences in specific capture or detection antibody affinity. To explore this possibility, the same experiments were performed with each antibody used for alternative assay functions, with ab19615 and ab115704 utilized as either capture or detection antibody. Results of this analysis indicated that a robust benefit to sensitivity was observed in LMIs independent of which specific antibody was used for capture or detection, a finding which provided additional support for the conclusion that LMI complexes convey a fundamental benefit to antibody binding (Figure S5).

While LMIs were demonstrated to provide increased sensitivity, early experimentation indicated that the initial protocol resulted in higher background signal than conventional sandwich immunoassays. Despite EC50 values up to 233-fold lower and similar coefficients of variation, background binding resulted in less dramatic changes to overall assay sensitivity. To address the issue, a variety of strategies were evaluated for reducing background binding including the optimization of oligonucleotide-antibody conjugation ratio (Figure S6A) and DNA linker length (Figure S6B), factors which together resulted in a 3.23 fold reduction in background as well as a 4.21 fold improvement in maximum assay signal (Figure S6).

While initial experiments demonstrated the benefits of the LMI platform when compared to conventional sandwich immunoassays performed in buffer, the vast majority of immunoassays are performed in complex matrices which can contribute to assay interference in realistic applications. To evaluate whether LMIs maintained viability in complex matrices, CTNI quantification was compared to conventional SIAs performed in PBS and pooled healthy human plasma. Results of these experiments indicated that a LODs of 0.708 ng/mL and 0.510 ng/mL were achieved using LMI in PBS and plasma as compared with LODs of 30.0 ng/mL and 12.4 ng/mL for conventional immunoassays (Figure 4). As with other sandwich immunoassays, a hook effect was observed at high concentrations as a result of independent antigens saturating both capture and detection antibody binding sites. While this effect can reduce the upper limit of detection, prior studies have shown that the impact can be effectively mitigated through reagent optimization, reduced incubation times, and sample dilution.[67–69]

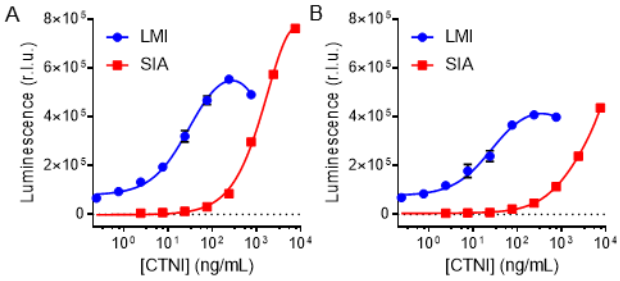


Figure 4. Comparison of LMI with conventional SIA. Experiments were performed using CTNI spiked into either PBS (A) or pooled human plasma (B). All data points and error bars indicate the average and standard deviation of 3 technical replicates.

Direct comparison of LMIs with SIAs indicated that LMIs achieved a 42.4 fold improvement in LOD in PBS and a 24.3 fold benefit in plasma. Much more dramatic differences in EC50 curves were observed, however, with EC50 values 76.3 and 223 fold lower for LMIs in PBS and plasma, respectively (Figure 4). This finding suggests that LMIs may be capable of contributing to even greater improvements in sensitivity if further reductions in background binding could be achieved. Consistent with the hypothesis that cooperative antibody binding occurs in LMIs, these results also indicated that matrix effects had a smaller impact on signal strength in LMIs than in conventional immunoassays. Together these findings provide compelling evidence that LMIs can effectively combine capture and detection antibody binding steps to provide a simplified antigen detection with higher detection sensitivity, especially when measuring biomarkers in complex mediums.

Conclusions

The present study demonstrates a new immunoassay method which combines capture and detection antibody binding steps into a single cooperative binding event. This is achieved using nanoscale complexes assembled on microplate surfaces, which capture antigens using close proximity binding. Following antigen capture, molecular complex structure can be rapidly transitioned using melting point depressants to release and remove unbound detection antibodies while maintaining the integrity of antigen-bound complexes. This technique represents a novel method for chemically transitioning complex state by denaturing dsDNA, a strategy which provides an extremely useful tool in the emerging field of DNA nanomachines. LMIs are then shown to outperform conventional SIAs using each of the most common immunoassay detection modalities and to successfully quantify CTNI with a sensitivity 42X higher than corresponding SIAs as well as improved performance in complex media. Considering the successful elimination of a fundamental binding step, the LMI provides a simplified and user-friendly alternative to conventional immunoassays, especially when high through-

put processes can be employed to facilitate surface functionalization. Together with improved sensitivity and broad compatibility with existing detection platforms, the LMI system represents a platform technology which we believe will provide an important advancement in the field of protein detection.

Supporting Information

Associated Content: tables of DNA sequences used, linker mediated streptavidin detection schematic, capture DNA evaluation experiments, wash buffer additive concentrations, polyacrylamide gel electrophoresis images of DNA-antibody conjugates, an evaluation of the effects of chemical additives on LMIs, effects of different antibodies on assay performance, LMI and SIA comparisons using alternative detection agents, complex assembly and assay reaction controls, LMI performance with alternative DNA:AB conjugations, and LMIs with alternative capture DNA. This material is available free of charge via the Internet at http://pubs.acs.org.

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