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Investigation of reagent delivery formats in a multivalent malaria sandwich immunoassay and implications for assay performance

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Abstract

Conventional lateral flow tests (LFTs), the current standard bioassay format used in low-resource point-of-care (POC) settings, have limitations that have held back their application in the testing of low concentration analytes requiring high sensitivity and low limits of detection. LFTs use a premix format for a rapid one-step delivery of premixed sample and labeled antibody to the detection region. We have compared the signal characteristics of two types of reagent delivery formats in a model system of a sandwich immunoassay for malarial protein detection. The premix format produced a uniform binding profile within the detection region. In contrast, decoupling the delivery of sample and labeled antibody to the detection region in a sequential format produced a non-uniform binding profile in which the majority of the signal was localized to the upstream edge of the detection region. The assay response was characterized in both the sequential and premix formats. The sequential format had a four- to ten-fold lower limit of detection than the premix format, depending on assay conjugate concentration. A mathematical model of the assay quantitatively reproduced the experimental binding profiles for a set of rate constants that were consistent with surface plasmon resonance measurements and absorbance measurements of the experimental multivalent malaria system.

Graphical abstract

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Supporting Information. This document contains a description of the surface plasmon resonance experiments, the search algorithm, UV-VIS absorbance experiments, the simulation to independently assess the significance of the difference in LOD estimates, and additional comparison of experimental and model binding profiles.

Introduction

Heterogeneous microfluidic immunoassays have been developed for a number of analytes in a variety of implementations that range from laboratory-based immunoassay systems¹ to portable microfluidic systems². Sandwich immunoassays, in particular, are useful for the detection of targets that can support multiple antibody binding sites, and have been used extensively with a variety of detection methods³. The sandwich immunoassay lateral flow test (LFT) has many characteristics that make it appropriate for use in low-resource point-of-care (POC) settings, including a low cost, a rapid time to result, and being equipment-free^{4,5}. However, inadequate test sensitivity and limits of detection have prevented the wider application of LFTs in these settings^{4,6}. There has been much interest in improving the sensitivity and limit of detection of these tests. Efforts have included the use of higher-sensitivity labels such as fluorescent and chemiluminescent species³, the use of alternative materials to nitrocellulose^{7,8}, the use of alternative affinity binders to antibodies^{8,9}, varying the dimensions of the nitrocellulose segment¹⁰, and the incorporation of signal amplification^{11,12}. Efforts in the last category rely on changing the physical layout of the conventional LFT such that multiple reagents can be automatically delivered sequentially to perform multistep sample processing^{5,12,13}. This capability allows for the implementation of alternative reagent delivery protocols for LFTs that is the topic of the current study.

Computational modeling is being used to better understand microfluidics-based binding assays. Previous studies have focused on the direct binding of an in-solution analyte to its surface-immobilized binding partner to investigate the effects of surface feature size, flow rate, and binding constant¹⁴ and real-world volume and time constraints¹⁵ on the amount of captured analyte in pressure-driven flow systems. Other work has focused on a comparison of analyte binding in electrokinetic vs. pressure-driven flow systems¹⁶ and porous bead sensors¹⁷. Most relevant to the current study is the modeling work focused on lateral flow competitive¹⁸ and sandwich¹⁹ immunoassays. In the latter study, Qian et al. investigated the binding signal in a conventional LFT format of premixed sample plus detection antibody conjugated to label (commonly referred to as 'conjugate') for different analyte and conjugate concentrations for a single set of representative rate constants.

In this study, we decoupled the introduction of the sample and conjugate in a sandwich immunoassay for the detection of the malaria analyte *PFHRP2* and investigated the effects of

this decoupling compared to the conventional premix format, using both experiments and modeling. We observed a marked spatial inhomogeneity of the assay signal in the sequential delivery format, while the premixed format produced a spatially uniform signal. We hypothesized that the high concentration of signal at the upstream edge of the detection region for the sequential format case would lead to a lower limit of detection compared to the uniform, lower-level binding for the premix format case. We experimentally characterized the sandwich immunoassay with signal amplification for each of the reagent delivery protocols and we report a four- to ten-fold difference in the limit of detection depending on conjugate concentration. In order to better understand the observed binding profiles in terms of the effective rate constants in the assay system, we implemented a coarse-grained mathematical model of our experimental assay that quantitatively reproduced the experimental binding profiles for the two reagent delivery formats. The model binding profiles were consistent with the experimental binding profiles when the rate constants input into the model satisfied two conditions: (1) the rate constant for analyte association with conjugate in solution was greater than the rate constant for analyte association with the immobilized capture species, and (2) the rate constant for binding of the analyte-conjugate complex to the immobilized capture species was smaller than the rate constant for analyte binding to the immobilized capture species. The model's rate constants were consistent with the microscopic rate constants measured using surface plasmon resonance detection.

Materials & Methods

Card fabrication, image acquisition, and analysis

Devices were designed using AutoCAD (Autodesk, San Rafael, CA). Porous materials including nitrocellulose (Millipore, Billerica, MA), glass fiber (Ahlstrom, Helsinki, Finland), and cellulose (Millipore, Billerica, MA), as well as Mylar plus adhesive composite (Fralock, Valencia, CA) were cut to appropriate dimensions using a CO₂ laser cutting system (Universal Laser Systems, Scottsdale, AZ)²⁰. Glass fiber source pads were elevated with various thicknesses of Mylar plus adhesive composite to ensure adequate contact with the membrane after folding. Card designs were tested for uniform fluid delivery using colored fluids and modifications were made based on the results. Image data (1200 dpi) was acquired using a high-resolution flatbed scanner (Epson, Nagano, Japan). Analysis was performed using either custom MATLAB (Natick, MA) programs or ImageJ (NIH Research Services Branch, Bethesda, MD).

Sandwich immunoassay with signal amplification

A murine antibody (IgM) to *Plasmodium falciparum* histidine rich protein 2 (P_fHRP2) (Immunology Consultants Laboratory, Portland, OR) was patterned at 1 mg/ml with a piezoelectric inkjet printer (Sciencion, Berlin, Germany) to create a test region 3 mm wide by 1.25 or 2.5 mm in length on a nitrocellulose strip 3 mm wide by 31.5 mm in length. An anti-mouse antibody (IgG) was printed at 0.5 mg/ml for a control line 3 mm wide by 0.25 mm, 8 mm downstream of the test region. Membranes were placed in a 37°C oven for 2 hours and then stored in a desiccator. Printed membranes were blocked the day following printing in 5% sucrose, 2.5% polyvinylpyrrolidone (PVP), 2.5% bovine serum albumin, and 0.1% Tween-20 in phosphate buffered saline blocking solution to minimize nonspecific

absorption. Known concentrations of the recombinant malaria protein, *PfHRP2* (CTK Biotech, San Diego, CA) were spiked into fetal bovine serum (Invitrogen, Carlsbad, CA) to serve as mock sample solutions. Tris-buffered saline with 0.1% Tween 20 (TBST) was used as both the wet-out and rinse buffers. A second murine (IgG) antibody to *PfHRP2* (Immunology Consultants Laboratory, Portland, OR) passively conjugated to 40-nm gold nanoparticles (BBInternational, Cardiff, UK) served as the detection species. A commercially-available gold enhancement reagent (Nanoprobes, Yaphank, NY) was used for signal amplification. For the sequential delivery format, 10 μ l of TBST, 30 μ l of mock sample, 40 μ l of conjugate, 40 μ l of rinse buffer, and 40 μ l of gold enhancement solution were applied to the lateral flow strips. The sequential format assay strips were scanned at ~56 minutes. For the premix format, 10 μ l of TBST, 30 μ l of the premix mock sample (mock sample was combined with conjugate and 1% BSA in TBS in volume ratios of 4:5:1, respectively), 40 μ l of rinse buffer, and 40 μ l of gold enhancement solution were applied to the lateral flow strips. The premix format assays were scanned at ~40 minutes. Final sample concentration, BSA concentration, and optical density (OD) of the conjugate were identical for the two reagent delivery formats.

Varying conjugate concentration experiments

A premixed sample solution containing mock sample, conjugate, and 1% BSA in TBS in volume ratios of 4:5:1, respectively, was created immediately prior to assay application. Six different premixed sample solutions were made; the final ODs of the conjugate were 5, 1, 0.5, 0.1, 0.01, and 0. Conjugate solutions were serially diluted in TBS from the starting OD of 10.2. Assays were run simultaneously with OD 5, 1, 0.5, 0.1, 0.01, and 0 premix solutions and a 20 ng/ml final analyte concentration ($N=4$). An OD 5 conjugate solution was made by combining conjugate with TBS and 1% BSA in TBS in volume ratios of 5:4:1, respectively. A 30 μ l volume of premixed sample and conjugate was delivered, followed by a 10 μ l rinse, and a 40 μ l rinse. The assays were scanned and 40 μ l of an OD 5 conjugate solution was subsequently delivered, followed by a 10 μ l rinse, 40 μ l rinse, and final scan. The assays were scanned to acquire image data at 17 minutes and 40 minutes. The images were processed using a custom MATLAB program to create line profiles of the signal intensity across the detection region in the direction of fluid flow as described below.

Sensitivity and limit of detection estimation for signal-amplified sandwich immunoassays

All assays were run on the same day using the same stock reagents and batch of patterned membranes. For the sequential delivery format, four replicates at the non-zero concentrations and 12 replicates at the zero concentration of analyte were run with an OD 5 conjugate solution. For the premix format, four replicates at the non-zero concentrations and eight replicates at the zero concentration of analyte were run at both OD 5 and OD 0.5. However, for the premix OD 5 format, only three replicates for the non-zero concentrations and six replicates for the zero concentration were included in the analysis. The other replicates were rejected due to visible aberrations in capture IgM patterning. For the premix OD 0.5 format, one of the zero concentration replicates was excluded from analysis due to damage to the strip. The scanned 16-bit images were processed using a custom MATLAB analysis program as described previously¹². The region of interest analyzed measured 0.2 mm (in the direction of flow) by 1.7 mm wide. Response curves were created with the signal

intensity defined as the average grayscale intensity in the test region of interest subtracted from the average grayscale intensity in a downstream background region of interest of the same size. The slope of each response curve provided a measure of the sensitivity of the assay under those conditions. The concentration limit of detection (LOD) for each case was then estimated as follows. First, the signal LOD was calculated using the formula *signal LOD* = $\mu_{zc} + 1.645\sigma_{zc} + 1.645\sigma_{lc}$, where μ_{zc} is the mean of the zero concentration sample measurements, σ_{zc} is the standard deviation of the zero concentration sample measurements, and σ_{lc} is the average of the standard deviations from the two lowest non-zero concentration sample measurements²¹. This signal LOD was then converted to a concentration LOD using a linear calibration model for intensity vs. concentration, for a range of concentrations near the LOD. Specifically, a regression of a subset of concentration data near the LOD (4 to 6 points) was used to obtain a linear calibration with associated slope, m , and intercept, b . The concentration LOD was then calculated according to *concentration*

$$LOD = \frac{\text{signal LOD} - b}{m} = \frac{(1.645\sigma_{zc} + 1.645\sigma_{lc}) + (\mu_{zc} - b)}{m}. \text{ Note that when } \sigma_{zc} \cong \sigma_{lc}, \text{ and } \mu_{zc} \cong b,$$

this reduces to the well-known estimate, *concentration LOD* $\cong \frac{3 * \sigma_{zc}}{m}$. The 95% confidence interval of the concentration LOD was estimated using bootstrapping²². At each concentration, replicate signal values were obtained by sampling with replacement from the corresponding experimental data, and the LOD for each sampled data set was calculated. This was repeated for N=1,000, and the 95% confidence interval was obtained directly from the quantiles of the LOD values generated from the resampled data sets. The bootstrap method could be used because the number of distinct resampled data sets that could be generated from the experimental data (greater than 4.6×10^6) was much larger than the number of bootstrap resamplings (N=1,000). Empirical p -values were calculated for the comparison of each of the premix format LODs with the sequential format LOD by tabulating the fraction of cases in which the bootstrapped sequential format LOD was greater than or equal to a bootstrapped premix format LOD and multiplying by 2.

Binding profiles from the detection region of the sandwich immunoassays

Binding profiles were taken from scanned images, along the direction of flow and over an extended distance that was greater than the extent of the capture region. First, the average grayscale intensity was averaged across the lateral dimension perpendicular to flow, approximately 2.1 mm (using custom MATLAB code). Then, the pixel location of the maximum of each experimental binding profile was used as a reference point to align the profile to the other binding profiles. For the data from the varying conjugate concentration experiments, the binding profiles resulting from the premixed solution with conjugate at OD 5 did not have distinct maxima and were shifted by the average shift of the binding profiles from the OD 0.5 case. For the signal-amplified assay data (at 5 ng/mL analyte and OD 5 conjugate) that was used for direct comparison with the model data, one premix binding profile did not have a distinct maximum, and was shifted by the average shift value of the other signal-amplified binding profiles. Shifts were within 0.3 mm of one another. Next, each binding profile was background subtracted using a value obtained by averaging along a 0.2 mm length of the extended profile, downstream of the capture region. Finally, the

aligned, background-subtracted experimental binding profiles were averaged across replicates for a given condition. The error bars in the plots represent the standard deviation.

Calibration between gold nanoparticle concentration and intensity

Pieces of nitrocellulose membrane, each 12 mm × 12 mm in size and of capacity 17 µl, were blocked in commercially available Invitrogen blocking solution (Life Technologies, Grand Island, NY) to minimize non-specific adsorption. Conjugate solutions at seven different concentrations (OD 0, 0.3125, 0.625, 1.25, 2.5, 5 and 10) were added to the nitrocellulose (N=4) and the nitrocellulose was scanned after 15 minutes. A custom MATLAB program was used to extract the average grayscale intensity in a 5 mm × 5 mm region of interest for each piece of nitrocellulose in the image data. OD values were converted to concentrations of gold nanoparticles using a calibration provided by the vendor, BBIInternational. Data of gold nanoparticle concentration (C) versus intensity (I) was fit to a power law of the form $I = AC^B$ ($R^2 \sim 0.93$) where $A = 2.0 \times 10^7$ and $B = 5.6 \times 10^{-1}$. This function was then used to convert the model data from units of moles/m³ into intensity units for direct comparison to the experimental data.

Mathematical model of the assay system

A model of the assay was created in COMOSL Multiphysics (Burlington, MA). The model assumed Darcy flow within the channel (1.4×10^{-2} m region) and a uniform distribution of immobilized capture sites within a downstream detection region (1.25×10^{-3} m detection region). In the model, the value of the bulk porosity was 0.8, consistent with the porosity of the nitrocellulose membrane of the experimental system (specification from Millipore). In the model, the effect of pore diffusion was not modeled explicitly, but was instead indirectly taken into account through effective rate constants. Association and dissociation reactions were modeled using mass action kinetics. Values for the model parameters were estimated from experimental data as described in the following. The initial concentrations of analyte and detection species in the model were set based on the known concentrations of analyte and conjugate in the experimental system. The timing of the reagent pulses and the assay end point (2,260 s and 1,440 s for the sequential and premix formats, respectively) in the model, were based on the reagent volumes used in the experimental system and an average flow rate of 1.5×10^{-4} m/s. For simplicity, analyte was assumed to be capable of binding up to one capture species and up to one detection species at a time, for a total of six possible species with four pairs of rate constants. Each of the six species in the coarse-grained model represented a subpopulation of related species in the multivalent experimental system. As an example, the complete complex of surface-bound capture-to-analyte-to-detection species in the model represented the collection of surface-bound complexes containing one or more detection species (and possibly multiple molecules of analyte) in the experimental system. Effective association and dissociation rate constants were input into the model based on surface plasmon resonance (SPR) measurements of the microscopic rate constants for three of the interactions (Table S1 in Supporting Information Section I). Specifically, SPR measurements of the rate constants for analyte interaction with immobilized capture IgM antibody, and for detection IgG antibody interaction with analyte bound to immobilized capture IgM antibody, were used directly as model inputs. The SPR-measured dissociation rate constant for the interaction between analyte and immobilized detection IgG antibody

was also used directly as a model input. The SPR-measured association rate constant for this interaction provided a lower limit for the relevant in-solution interaction of analyte and detection species in the model, due to the constraints on the orientation and the organization of the surface-bound partner in the binding pair compared to the in-solution case²³. Thus, the model association rate constant for the in-solution interaction was assumed to be greater (22-fold) than the SPR-measured value for the surface-binding case. The model dissociation rate constant for the interaction between analyte-detection species complexes in solution and the immobilized capture species (for which there was no direct SPR data), was set to $7.1 \times 10^{-4} \text{ s}^{-1}$, consistent with the reasonable lower bound provided by the SPR measurement for the dissociation rate constant for analyte only from the immobilized capture IgM antibody. A search algorithm (see Supporting Information Section II) was then used to identify the values for (i) the effective association rate constant for that last interaction and (ii) the initial concentration of capture species, that produced the best-observed agreement of model and experimental binding profiles. The effective association rate constant for interaction of analyte-detection species and immobilized capture species was allowed to vary between $1 \times 10^3 \text{ (M}\cdot\text{s)}^{-1}$ and $1 \times 10^5 \text{ (M}\cdot\text{s)}^{-1}$ and the initial concentration of capture species was allowed to vary between $1.7 \times 10^{-2} \text{ moles/m}^3$ (the number of binding sites deposited within the test region volume was $\sim 1.7 \times 10^{-2} \text{ moles/m}^3$) and $1.7 \times 10^{-4} \text{ moles/m}^3$.

Results and Discussion

The striking differences in the binding profiles for the two reagent delivery formats are shown in Figure 1. In the case of sequential delivery of (1) analyte in the sample and then (2) detection antibody conjugated to label (i.e., conjugate) to the detection region, the binding profile has a sharp maximum near the upstream edge (with respect to fluid flow) of the detection region and drops off rapidly to a low level of binding downstream. In contrast, the delivery of premixed sample and conjugate to the detection region results in a more uniform binding profile. Note that a critical difference between the two reagent delivery formats is the population of species available for binding within the detection region as a function of time. In the case of the sequential delivery format, the introduction of analyte and conjugate are separated in time, and there is no appreciable complex formation, while in the case of the premix format, the population of species that arrives at the upstream edge of the detection region is composed of free analyte, free conjugate, and complexes of analyte bound to conjugate. These two reagent delivery formats are shown schematically in Figure 2 (as well as the subsequent signal amplification). Thus, differences in the binding kinetics of the various species could lead to differences in the final binding profiles from the two reagent delivery formats. For the malarial antigen system used in this study, the analyte, *PHRP2*, contains 18 epitopes recognized by the detection antibody²⁴, and each conjugate species contained approximately 12 IgG for a maximum of 24 binding sites (communication from BBIInternational). This multivalency could lead to the formation of higher-order solution complexes of analyte and conjugate in the premix format case with significantly smaller effective association rate constants for interaction with immobilized capture species compared to the effective association rate constant for interaction between analyte only (or analyte bound to one conjugate molecule) and the immobilized capture species.

To investigate the effect of the premix solution species composition on the binding profile, different concentrations of conjugate (7.5×10^{-7} , 1.5×10^{-7} , 7.5×10^{-8} , 1.5×10^{-8} , 1.5×10^{-9} , and 0 moles/m³ corresponded to OD 5, 1, 0.5, 0.1, 0.01, and 0, respectively) were premixed with analyte to a final analyte concentration of 20 ng/mL, input into the assay, and followed by a rinse step. The binding at this first stage depicted the pattern of analyte-conjugate complex binding to the capture species in the detection region (see images of Figure 3A). To visualize surface-bound antigen, this was followed by the application of excess conjugate at OD 5 to all cases (see images of Figure 3B). Binding profiles were extracted from a subset of the image data at each of the stages to allow for clear comparison (Figures 3C). The binding pattern from the premix solution with conjugate at OD 5 indicated uniform binding throughout the detection region, as observed previously. As the concentration of conjugate in the premix solution was decreased, OD 1, OD 0.5, and OD 0.1, a decreasing gradient of analyte-conjugate complex binding along the direction of flow was observed, with decreasing intensity for decreasing conjugate concentration. Excess conjugate at OD 5 was then applied, in order to visualize analyte that had been captured within the detection region, but had not already been labeled by conjugate. The no initial conjugate case, OD 0, showed high intensity binding at the upstream edge of the detection region, consistent with the sequential delivery binding profiles observed previously. The cases of premixed solution with conjugate at OD 0.01, 0.1, and 0.5, also showed high intensity binding at the upstream edge of the detection region, which is consistent with there having been binding of free analyte to the capture species. The premix cases with highest concentration (OD 1 and OD 5) did not change appreciably in binding profile, indicating that most of the analyte had been bound to conjugate in complexes (rather than in free form).

These results indicated that there was a significant difference in the effective binding kinetics for the different species in the premix solution created with varying concentrations of conjugate. For premixed solutions with low concentrations of conjugate (low OD), the fraction of free analyte in the solution was relatively high and resulted in high intensity binding at the upstream edge of the detection region, characteristic of sequential delivery in the malaria system. As the concentration of conjugate in the premix solution was increased, the formerly free analyte was instead found within analyte-conjugate complexes that bound in a decreasing concentration in the detection region along the direction of flow.

An interesting result in this system was the change across the binding profiles for the premix solutions that were created with the highest concentrations of conjugate, OD 0.5, 1, and 5. In the absence of any multivalency, it would have been expected that additional conjugate in the premixed solution would lead to a uniform higher intensity binding profile as observed for the cases of premix OD 0.5 and premix OD 1. However, a comparison of the binding profiles for the cases of premix OD 1 and premix OD 5 showed uniformly lower intensity binding for premix OD 5 than for premix OD 1 (or premix OD 0.5). These binding profiles suggested a difference in the type of species present in the premix OD 5 solution. Given the multivalency of the system, the results were consistent with the presence of higher-order analyte-conjugate complexes, i.e., complexes containing multiple gold nanoparticles (which appear to have even lower effective association rates for the capture species in the detection region than the single gold analyte-conjugate complex) in the premix OD 5 solution that were not present in the premix OD 1 solution. This scenario is supported by absorbance

spectra of conjugate-only compared to the premix solution of conjugate plus analyte for conjugate concentrations corresponding to OD 1 and OD 5 (Figure S1 in Supporting Information Section III). The absorbance spectrum of the premix solution at OD 5 was shifted to higher values from the baseline spectrum of the conjugate-only solution at OD 5 indicating that the addition of analyte produced a change in the species population, such as gold nanoparticle aggregates that were not present in the conjugate-only solution. In contrast, the absorbance spectra from the conjugate-only solution at OD 1 and the premix solution with conjugate at OD 1 (i.e., conjugate solution at OD 1 plus the addition of analyte) showed no difference due to the formation of complexes between analyte and conjugate.

We hypothesized that the higher final concentration of detection label at the upstream edge of the detection region in the case of sequential delivery would lead to a higher sensitivity and a lower limit of detection for the sequential format case than for the premix format case. Three signal-amplified assays were run; sequential at OD 5, premix with conjugate at OD 5, and premix with conjugate at OD 0.5. In all cases, signal amplification was performed using a gold enhancement solution that produced a dark purple color in the label-containing areas of the detection region. Representative images of a concentration series for each of the three cases are shown in Figure 4. The results from the sequential format with conjugate at OD 5 (Figure 4A), the premix format with conjugate at OD 5 (Figure 4B), and the premix format with conjugate at OD 0.5 (Figure 4C), were consistent with the data of Figure 2. Assay response curves, using the average grayscale intensity in a narrow region of interest, similar to that in conventional LFTs, as the signal are shown for the three cases (Figure 4D). The response curve for the sequential format at OD 5 had a significantly higher slope than for the premix format at OD 5, i.e., a higher sensitivity. The assay response curve for the premix format at OD 0.5 also had a significantly higher slope than for the premix format at OD 5 (Figure 4E), and was comparable to the sequential case. In addition, the standard deviations of the zero and low-concentration sample measurements were lower for the sequential format than for the premix formats. The limits of detection for sequential format at OD 5, premix format at OD 5, and premix format at OD 0.5, were estimated to be 0.6, 8.1, and 2.4 ng/mL, respectively, as shown in Figure 4F. Empirical p -values were calculated for the comparison of each of the premix format LODs with the sequential format LOD and indicated that the null hypothesis, sequential format LOD = premix format LOD, could be rejected at $p < 0.01$. To independently assess the statistical significance of the observed difference between the two closest LOD estimates (OD 0.5 premix and OD 5 sequential), a simulation was carried out in which signal data were generated for the two assays based on the empirically estimated signal response relationships and based on empirically determined signal sample standard deviations, adjusted to ensure that the two models have *equal* underlying LODs (see Supporting Information Section IV). Across 300,000 simulations of the assays under the “equal LODs” model, less than 2% of simulation realizations resulted in LOD estimates for the two assays that differed by an amount greater than or equal to the observed difference in LOD estimates based on the real signal data. The results of the simulation were consistent with the bootstrap analysis in indicating that the observed difference in LODs between the assays is unlikely to occur by chance under an “equal

LODs” assumption for the assays. Thus, the data and analysis indicates that the sequential format results in a lower limit of detection than the premix format in this assay.

Experimental binding profiles were extracted from the sandwich immunoassay data for an analyte concentration of 5 ng/mL in the sequential and premix formats (at OD 5 conjugate) and compared to model binding profiles (model parameter values were based on parameter estimates in the experimental system, as described in Materials & Methods). Comparison of the experimental and model binding profiles showed good agreement, as depicted in Figure 5A. Experimental and model binding profiles for lower analyte concentrations in the sequential format were also consistent (Figure S2 in Supporting Information). The direct comparison of experimental and model data was enabled by an experimentally derived calibration curve relating the concentration of gold nanoparticle labels to intensity units, shown in Figure 5B. The effective association and dissociation rate constants that produced model results in quantitative agreement with the experimental results are shown schematically in Figure 5C. Experimentally measured rate constants (Table S1 in Supporting Information Section I) provided a basis for the assignment of seven (out of eight) of the model rate constants. A search algorithm was used to identify values for the remaining association rate constant and the initial concentration of capture species that produced excellent agreement between the model and experimental binding profiles. The value for the initial concentration of capture species returned by the search algorithm was 1.3×10^{-3} moles/m³, about ten-fold smaller than the estimated concentration of capture species deposited into the substrate. Given that not all of the capture species will be oriented with their binding sites accessible, and accounting for some loss of deposited mass during the initial wet out rinse of the assay, this value is reasonable. The value for the association rate constant returned by the search algorithm was approximately 5×10^3 (M·s)⁻¹. This value is consistent with the scenario of larger analyte-conjugate complexes in solution having a much smaller association rate constant than for the other interactions in the assay system, and would explain the generally lower-level binding observed experimentally for the premix format case compared to the sequential format case. Note that since the lower bound for $k_{\text{off}}^{\text{d}}$ was assumed in the model results of Figure 5A, similar agreement between the model and the experimental data is expected for a family of larger k_{on}^{d} and $k_{\text{off}}^{\text{d}}$. The four model association rate constants satisfied two conditions. First, the model association rate constant for the solution interaction of analyte to the conjugate complex was greater than the model association rate constant for the analyte to the immobilized capture species. In addition, the model effective association rate constant for the conjugate and analyte complex to bind to immobilized capture species was smaller than the model effective association rate constant for the analyte only to bind to immobilized capture species.

Conclusions

We investigated the effects of varying the reagent delivery format on the assay signal in a model system of a sandwich immunoassay for a malaria analyte. In this multivalent system, the sequential and premix reagent delivery formats produced very different spatial binding profiles that resulted in a four- to ten-fold improvement in the limit of detection in the sequential delivery case over the premix case, depending on the conjugate concentration. A simple coarse-grained model of the assay system quantitatively reproduced the experimental

binding profiles with effective binding constants consistent with SPR measurements. The malaria model system demonstrates the significant impact that reagent delivery format can have on assay sensitivity and limit of detection. However, a disadvantage of the sequential format is that decoupling of sample and conjugate delivery increases the number of steps in the assay and, thus, the time to result. The increase in the time to result may be acceptable in cases where the improvement in sensitivity and limit of detection would be needed in order to fulfill the performance requirements of the application.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Varying the reagent delivery format can have a dramatic effect on the signal in a sandwich format immunoassay. (Left) Diagram of the detection region of the immunoassay, consisting of a control region and an extended test region. (Middle) Sequential delivery of reagents resulted in a spatially inhomogeneous signal. (Right) In contrast, delivery of a premixed solution of reagents resulted in a uniform signal in the test region. The concentration of analyte, *PHRP2*, in both cases was 20 ng/mL.

Figure 2. Schematic of binding species in the malaria immunoassay. (A) In the sequential format, *PfHRP2* analyte binds to *PfHRP2* IgM antibody immobilized to the nitrocellulose substrate. This is followed by conjugate (*PfHRP2* IgG antibody conjugated to gold nanoparticle labels) binding to analyte already bound to the immobilized IgM antibody. (B) In the premix format, analyte and conjugate form a complex that binds to immobilized *PfHRP2* IgM antibody. In addition, the reactions of (A) are also possible. For simplicity, higher order complexes are not depicted. (C) Signal amplification is accomplished using gold enhancement, in which gold salt and a reducer in solution, act to increase the size of the original gold nanoparticle labels captured on the nitrocellulose. The result is a significantly darkened purple signal relative to the light pink of the original gold nanoparticle labels.

Figure 3.

Images and binding profiles from the assays with OD 5, 1, 0.5, 0.1, 0.01, and 0 premixed solutions at a *Pf*HRP2 analyte concentration of 20 ng/mL. (A) Representative images after the addition of the premix sample and a rinse step. (B) Representative images after the addition of OD 5 conjugate solutions and a rinse step. (C) Binding profiles from the assays with OD 5, 0.5, 0.1 and 0. For each case, the binding profile after the addition of the sample premixed with the specified OD and a rinse step (dashes) is plotted with the binding profile after the addition of OD 5 conjugate solution and a rinse (hollow diamonds). Each profile is

an average over replicates, two for OD 0 premix, three for OD 5 premix, and four for the others. The error bars represent the standard deviation.

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Figure 4.

Signal-amplified assay results. Image series of the detection region for different concentrations of *P/HRP2* analyte for (A) sequential format with conjugate at OD 5, (B) premix format with conjugate at OD 5, and (C) premix format with conjugate at OD 0.5. At the far left is a schematic of the membrane. Dashed lines represent approximate regions of analysis. In all cases, the signal from the original gold nanoparticle labels was amplified using a gold enhancement solution. (D) Response curves for sequential and premix formats with conjugate at OD 5 and (E) response curves for the premix formats with conjugate at OD 5 and OD 0.5. Sequential data points are an average of four replicates for non-zero concentrations and 12 replicates for the zero concentration. Premix data points are an average of three (OD 5) or four (OD 0.5) replicates for non-zero concentrations and six (OD

5) or seven (OD 0.5) replicates for the zero concentration. The error bars represent the standard error. (F) Estimates of the limit of detection for the three cases based on the experimental data (left). The 95% confidence intervals for the cases were estimated using bootstrapping and are indicated by the error bars. The resampled data (N=1,000 for each) are shown in the histograms (right).

Figure 5.

Comparison of model and experimental results for the different reagent delivery formats. (A) Experimental binding profiles for sequential (solid circles are the average of four replicates) and premix (solid triangles are the average of three replicates) formats at OD 5 for an analyte concentration of 5 ng/mL and the model binding profiles for sequential (hollow circles) and premix (hollow triangles) formats show excellent agreement. The error bars for the experimental binding profiles represent the standard deviation. (B) Direct comparison of experimental and model data was enabled by a calibration curve relating intensity units and concentration of gold nanoparticle labels. The plot displays the measured intensities for

seven concentrations of gold nanoparticles in porous nitrocellulose (hollow diamonds with four replicates at each concentration) and a power law fit to the data (solid line) that was used to convert the model data. (C) Schematic of the rate constants in the model. The association and dissociation rate constants for the interactions k^a and k^b were relevant for the sequential format. Additionally, k^c and k^d were relevant for the premix format. The values used for the model results of (A) are tabulated.