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Mitigating the hook effect in lateral flow sandwich immunoassays using real-time reaction kinetics

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Abstract

The quantification of analyte concentrations using lateral flow assays is a low-cost and user-friendly alternative to traditional lab-based assays. However, sandwich-type immunoassays are often limited by the high-dose hook effect, which causes falsely low results when analytes are present in very high concentrations. In this paper, we present a reaction kinetics-based technique that solves this problem, significantly increasing the dynamic range of these devices. With the use of a traditional sandwich lateral flow immunoassay, a portable imaging device, and a mobile interface, we demonstrate the technique by quantifying C-reactive protein concentrations in human serum over a large portion of the physiological range. The technique could be applied to any hook effect-limited sandwich lateral flow assay and has a high level of accuracy even in the hook effect range.

TOC figure only

The use of lateral flow assays (LFAs) for point-of-care diagnostics is widespread primarily due to their relative ease of use and low production costs. Qualitative LFAs, which typically

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ASSOCIATED CONTENT

Supporting Information

Supplementary figure and explanation of algorithm (PDF)

Author Contributions

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provide a binary result, have been on the market since the introduction of the first dipstick pregnancy test in 1985¹. The adaptation of LFAs for quantitative detection of analytes has been attempted by many in the last fifteen years^{2–6}. Most of these LFAs provide quantification through the use of the ratio of the intensities of the test and control line after the test has been completely run^{3,7–9}. This method can provide accurate results for many analytes, however the dynamic range is often limited on the high end by the high-dose hook effect^{6,10}.

In traditional sandwich LFAs, the ratio of the test line to control line intensity increases with increasing analyte concentrations. This is because the larger concentration of analytes in solution results in more reporter probes (e.g. gold nanoparticles) becoming bound to the test line, which therefore displays a higher contrast against the test strip background. As analyte concentration increases, the test line signal stops increasing and instead starts to decrease, displaying the hook effect. This is a result of excess unlabeled analyte from the sample binding to the antibodies on the test line, blocking sites which would have otherwise captured the labeled analytes at the test line. The specific concentration at which this occurs can be tuned by adjusting the various on-strip chemical concentrations, but it will always ultimately limit the dynamic range at the upper end and prevent accurate quantification.

To demonstrate our technique, we have chosen to measure C-reactive protein, or CRP. CRP is a part of the acute immune response to infection, inflammation and tissue damage¹¹. Serum levels are elevated in individuals with high cardiac risk¹², infection¹³, and inflammatory diseases such as rheumatoid arthritis. Between 1 and 3 µg/ml, CRP concentrations can indicate risk of cardiac disease¹², while concentrations greater than 10 µg/ml can indicate acute infection¹³. CRP concentrations in serum can be indicative of inflammation in rheumatoid arthritis patients anywhere from 1 µg/ml to >100 µg/ml. In cases of severe infection and sepsis, CRP can reach 250 µg/ml or higher¹⁴.

As a result of this relatively large dynamic range, conventional and point-of-care immunoassay methods of CRP quantification face this problem of the hook effect. With a single test, they can measure only part of the range of relevant CRP concentrations^{4,15–17}. For this reason, CRP diagnostics are sometimes called high-sensitivity CRP (or hsCRP) tests, and typically measure in the 1–10 µg/ml range. The most common method of overcoming this problem is serial sample dilution and subsequent testing of the diluted and undiluted samples^{18,19}. This method, while accurate, increases the time and expense of testing for every sample. Another approach is to increase the number of lines on the LFA, adding a competitive test line in addition to the original sandwich line⁶. Although this technique is effective, it also increases the cost of manufacturing the test strips.

There are also other analytes which have broad physiological ranges and whose assays are impacted by the hook effect. Examples of these analytes include human chorionic gonadotropin (hCG), prolactin, and ferritin¹⁸. Serum and urinary hCG concentrations are indicative of pregnancy status and can be used to determine various conditions related to the pregnancy. Falsely low results could result in failure to diagnose or a slower diagnosis of these conditions²⁰.

Here, we present a method which utilizes real-time assay kinetics monitored with a low-cost and lightweight device to quantify an analyte over a wide range on an LFA, including the range of the hook effect. We demonstrate that by measuring the speeds at which each of the lines develop, we could distinguish between real and artificially low measurements of the CRP concentration. In this work, we compare results obtained by traditional test to control ratio methods and those obtained through kinetic measurements, demonstrating the utility of our technique in overcoming the hook effect.

Experimental Section

Gold nanoparticle conjugation

We obtained InnovaCoat® GOLD – 40nm gold nanoparticle conjugation kits from Innova Biosciences (Cambridge, UK) and mouse monoclonal anti-human CRP antibodies from Biorbyt LLC (Berkeley, CA, USA). The anti-human CRP antibodies were conjugated to the gold nanoparticles according to the kit instructions, using a 0.1 mg/ml concentration of anti-human CRP antibodies. From an initial optical density of 20, the gold nanoparticle conjugates were diluted to 0.5 OD in conjugate buffer. Conjugate buffer was made up of 2mM borate buffer (Thermo Scientific, Waltham, MA, USA) with 5% (w/v) sucrose (Sigma Aldrich, St. Louis, MO, USA).

Lateral flow strip manufacture and assembly

We purchased nitrocellulose cards with adhesive backing from EMD Millipore (Part no. HF180MC100, Billerica, MA, USA). We obtained glass fiber diagnostic pads for dry storage of gold nanoparticle-antibody conjugates (Part no. GFDX103000) and cellulose fiber sample pad strips for use as absorbent pads from EMD Millipore (Part no. CFSP203000). For sample application, we used sample pad type FR-2 from MDI Membrane Technologies (Part no. FR-2(0.7), Ambala Cantt, India). We acquired goat polyclonal anti-human CRP antibodies for use on the test line from CalBioReagents (San Mateo, CA, USA). We obtained anti-mouse IgG antibodies for use on the control line from Sigma Aldrich.

We dispensed test and control lines at 6.4 μ L/min at a concentration of 1 mg/ml diluted in 1 $\times$ PBS using the Automated Lateral Flow Reagent Dispenser produced by ClaremontBio Solutions (Upland, CA, USA). After we dispensed the lines, the nitrocellulose cards were allowed to dry for at least 2 hours in an incubator at 37°C. We soaked glass fiber conjugate pads in a solution of 0.5 OD gold nanoparticle conjugates for approximately 30 seconds and then dried them in an incubator at 37°C for at least 2 hours.

We then assembled the lateral flow strips as shown in Figure 1(a). Individual test strips were cut to approximately 4mm width using a rotary paper trimmer (Dahle North America, Inc., Peterborough, NH, USA). Individual strips were placed in plastic cassettes (Chongqing Hexijinhong Pharmaceutical Packaging Co., Ltd, Chongqing, China) for use in testing. Strips were stored in a chamber at <10% relative humidity to avoid exposure to varying levels of environmental moisture.

Serum testing on lateral flow assays

Running buffer was made up of 1× tris-buffered saline (Thermo Scientific) with 1% (w/v) bovine serum albumin (Sigma Aldrich), 1.5% (v/v) Tween 20 (Sigma Aldrich), and 0.02% (w/v) sodium azide (Sigma Aldrich). Pre-calibrated serum samples of known concentration were obtained to create calibration curves (Linearity LQ RF/CRP, Audit Microcontrols, Eatonton, GA, USA). Human CRP used to spike buffer for initial testing was obtained from Innovative Research, Inc. (Novi, MI, USA).

Serum samples at known concentrations were diluted 100× in running buffer before testing. 40 µl of diluted serum was pipetted onto the sample pad followed by 60 µl of running buffer. The strip was immediately placed into the portable reader device to begin imaging.

Test strip imaging

Test strips were imaged by a portable imaging device similar to one previously described by Lu et al.²¹. Briefly, the device consists of a 5 megapixel 1080p HD CMOS camera (Raspberry Pi, Cambridge, UK), focusing lens and LEDs (Thorlabs Inc., Newton, NJ, USA), Raspberry Pi computer board, rechargeable lithium ion battery pack (Adafruit Industries, New York, NY, USA), and cassette tray, all surrounded by a 3D-printed light-tight container. The device used in this work is different from the one used by Lu et al.²¹ in that it does not have any filters, as the measurements in this paper are all colorimetric rather than fluorescent.

After the test strip cassette was placed in the device, we started the imaging. We ran the imaging program via a laptop connected to the imaging device through Wi-Fi. The device then took an image approximately every 10 seconds for a total of 100 images. We processed the images using a Python script which cropped each picture to contain the appropriate portion of the image and determined the test and control line intensities.

Results

Test to control ratio

The final test to control ratio (T/C) is a widely-used metric in the quantification of lateral flow assays^{3,7-9}. Figure 1(b) depicts the final T/C schematically at low, high, and very high concentrations, demonstrating the state of binding at the test and control lines. The resulting hook effect in final T/C is seen in Figure 1(d). Examples of tests with very different final T/Cs can be seen in Figure 2(a) and (c), with the signal intensity graph shown in Figure 2(b) and (d). In Figure 2(e), we see that final T/C provides us with a way to quantify CRP in samples with concentrations less than approximately 50 µg/ml. A logistic curve fit to the initial part of the T/C versus CRP concentration curve up to 120 µg/ml yields an R^2 value of 0.93. After this point, however, we cannot distinguish whether a sample has CRP concentration between 50 and 100 µg/ml or 100 and 250 µg/ml from the final T/C alone. This decrease in signal at very high concentrations is a result of the high-dose hook effect.

Kinetics

The hook effect that is evident in the final T/Cs necessitates an alternate method to determine concentration at high levels of CRP. Our method utilizes the rate of development of the test and control lines, or their reaction kinetics. An example of two tests which have a similar final T/C but very different kinetics can be seen in Figure 3. While the final T/C is close to 1.5 for both tests, the rate of development is completely different. A plot of the T/C ratio over time for all the concentrations spanning the hook effect regime can be seen in Figure 4(a). To monitor the kinetics of the reactions, we first fit bounded exponential functions to the intensities of the test and control lines over time. By doing so, we could eliminate the possibility that single images which were incorrectly processed would have a large impact on the results. (See Supplementary Information for more detail.) We then used the fits to each of these curves to calculate a test to control line ratio (T/C) for each point. From Figure 4(a), we see that at high concentrations of CRP the T/C value starts high and decreases, while at low concentrations of CRP the T/C value starts low and increases. We then analyzed the rate of change of the T/C with time using a geometric derivative of the T/C with respect to time. The geometric derivative of a function is given by Equation 1,

$$\tilde{D}f(a) = \lim_{x \rightarrow a} \left(\frac{f(x)}{f(a)} \right)^{\frac{1}{x-a}} \quad [1]$$

where $f(x)$ and $f(a)$ are the values of the function at two different points²². Taking this derivative for our T/C function at times $x = 120$ and $a = 121$ seconds, we get an approximate geometric derivative. This gives us a measure of the rate of change of the T/C at a time point near the beginning of the curve. We calculated this value for a range of CRP concentrations and found that this rate of change value varies linearly with CRP concentration. This can be seen in Figure 4(b). The R^2 value of the linear fit to this data is 0.85. The figure demonstrates that at a point near 100 $\mu\text{g/ml}$ the value of the geometric derivative crosses over unity. If the value of the geometric derivative is greater than 1, the T/C is increasing over time and the CRP concentration will be low. In this case, the final T/C value could be used to quantify levels of CRP. If the value of the geometric derivative is less than 1, the T/C is decreasing over time and the CRP concentration will be very high. The CRP concentration can then be approximately quantified using the linear fit to the geometric derivative data. In this way, we can quantify low values of CRP with high sensitivity and provide approximate quantification at very high CRP concentrations.

Discussion

The high-dose hook effect is an issue that plagues many sandwich immunoassays measuring analytes at high concentrations^{6,10,18,19}. As mentioned above, typically, methods of overcoming the hook effect require the use of more assay materials through methods such as serial dilution of the sample¹⁸ or the addition of one or more extra test lines to the strip⁶. While these methods will be able to give an accurate result over the desired range, they require more time and expense in making and running the tests. Our method observes the hook effect in the final T/C values, and uses the kinetic data from the same test to determine

concentration over the desired range. The amount of reagents needed to make the test, the amount of sample input, and the amount of work the user must put in remain the same as an ordinary lateral flow assay. With the use of our portable imaging device and imaging software, we can expand the range of measurement without altering the test or procedure.

Our use of the geometric derivative as a measure of the rate of change of the T/C yields a linear decrease in geometric derivative as CRP concentration increases. The geometric derivative gives us a way to observe the relative change in T/C for each concentration. It separates concentrations whose T/Cs are increasing from those which are decreasing at $\tilde{D}f=1$, which coincides with the peak of the final T/C ratio curve at a concentration of approximately 100 $\mu\text{g/ml}$. The geometric derivative creates a way to observe the change in T/C kinetics so that low and high concentrations are distinguishable and concentration is linearly dependent on the geometric derivative. We tested the robustness of the geometric derivative to changes in assay parameters by changing the control line antibody concentration and the optical density of the gold nanoparticles soaked into the conjugate pad. While the sensitivity of the test changed, the geometric derivative of the T/C curve with respect to time still had a fairly linear relationship with the serum CRP concentration (See Supplementary Material). This showed that the trend in geometric derivative remains, even with different assay parameters.

The choice of 120 seconds as the location of the fit is based on the developing signal strength and the differences between geometric derivatives of different concentrations over time. As time tends towards infinity, the geometric derivatives of the different concentrations converge to very similar values, as can be seen in Figure 5. At very early times, the signal is the weakest and therefore the signal to background ratio is least and we see the highest effect of noise. To ensure that the signal was strong enough that any background effects could not significantly affect the measurement, we chose a time when nearly all the tests had intensities of both lines greater than 10. At 2 minutes, over 95% of the tests have intensities greater than 10. While this exact time would not necessarily be the same for tests of different analytes, the signal strength of those tests could be measured and a similar cutoff at an intensity of 10 could be made. In this way, our technique could be applied to many different analytes.

Conclusions

We have created a system which monitors the kinetics of the antibody-antigen reactions in an LFA. Our technique minimizes user input and increases the dynamic range of detectable concentrations of analyte. The hook effect in sandwich immunoassays affects many analytes with wide ranges of relevant concentrations and so our kinetic monitoring technique may be able to increase the range and decrease the cost of measuring numerous analytes. Our algorithm could be extended to other analytes of interest measured on LFA. By simply monitoring the test strip as the test progresses, we can extract more information about the concentration of the analyte in the sample and yield a more accurate result.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Lateral flow assay with hook effect schematic

(a) Lateral flow strip schematic showing (from left to right) sample application pad, conjugate pad containing gold nanoparticles conjugated to mouse anti-CRP IgG, nitrocellulose membrane with test and control lines, and absorbent pad. (b) Schematic depiction of signal development at low, high, and very high CRP concentrations. (c) Schematic depiction of kinetic development of test to control ratio for low, high and very high CRP concentrations. (d) Schematic depiction of final test to control ratio for low, high, and very high CRP concentrations.

Figure 2. Final (t=1000s) test to control line ratios at various concentrations of CRP

(a) Image of test strip run with sample at 1 µg/ml CRP (control line is on top, test line below). (b) Plot of intensity of color across the image in (a). (c) Image of test strip run with sample at 73 µg/ml CRP. (d) Plot of intensity of color across the image in (c). (e) Plot of T/C versus CRP serum concentration demonstrating the high-dose hook effect. Values and error bars shown are mean and standard deviation (n=3).

Figure 3. Examples of variation over time of test and control line intensities for two concentrations

(a) Images of one test with sample concentration 50 µg/ml at four time points (control line is on top, test line below). (b) Test to control line intensity ratio every 10 seconds from 50 to 1000 seconds for the test in part (a). (c) Images of one test with sample concentration 183 µg/ml at four time points. (d) Test to control line intensity ratio every 10 seconds from 50 to 1000 seconds for the test in part (c).

Figure 4. Interpretation of kinetics data

(a) Plot of T/C values for various concentrations of serum CRP over time. (b) Plot of geometric derivative of T/C with respect to serum CRP concentration with line of best fit. Values and error bars shown are mean and standard deviation ($n=3$).

Figure 5. The geometric derivative of T/C over time

Geometric derivative of the test to control ratio with respect to time for all concentrations.