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A rapid diffusion immunoassay in a T-sensor

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We have developed a rapid diffusion immunoassay that allows measurement of small molecules down to sub-nanomolar concentrations in <1 min. This competitive assay is based on measuring the distribution of a labeled probe molecule after it diffuses for a short time from one region into another region containing antigen-specific antibodies. The assay was demonstrated in the T-sensor, a simple microfluidic device that places two fluid streams in contact and allows interdiffusion of their components. The model analyte was phenytoin, a typical small drug molecule. Clinically relevant levels were measured in blood diluted from 10- to 400-fold in buffer containing the labeled antigen. Removal of cells from blood samples was not necessary. This assay compared favorably with fluorescence polarization immunoassay (FPIA) measurements. Numerical simulations agree well with experimental results and provide insight for predicting assay performance and limitations. The assay is homogeneous, requires <1 μl of reagents and sample, and is applicable to a wide range of analytes.

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A microfluidic system for manipulating and analyzing biological samples offers many potential advantages over conventional instrumentation, including small volumes, continuous monitoring, and low-cost mass production of devices[1,2]. Although several macroscale techniques have been miniaturized to the microscale (including PCR[3], DNA analysis[4], and cell sorting[5]), most have not. Quantitative immunoassays are primarily conducted in large instruments in centralized laboratories; they are used for therapeutic drug monitoring[6–8], screening for disease or infection with molecular markers[6,7], screening for toxic substances and illicit drugs[7,9], and monitoring of environmental contaminants[7,9]. Immunoassays have been available for years in inexpensive "test strip" format for use in point-of-care settings, but these assays are only qualitative. A quantitative point-of-care immunoassay could reduce the cost of medical immunodiagnostics and improve health care[10,11]. Capillary electrophoresis-based immunoassays are a promising new technology for point-of-care analysis[11,12] but have not been subjected to the rigorous standards set for clinical instrumentation, and their potential as a broadly useful alternative has yet to be demonstrated[12].

This report describes an assay based on features of low Reynolds number flow in a simple microfluidic structure, the T-sensor. The T-sensor (Fig. 1A) allows measurement of analyte concentrations during the early stages of diffusive mixing of two or more laminar flow streams. This has been demonstrated by measuring the concentrations of analytes including pH, calcium, and albumin[13]. A numerical model of these processes has been shown to allow extraction of diffusion coefficients, concentrations, and reaction kinetics from experimental results[14,15]. Previous T-sensor measurements were based on detecting signal intensity changes of indicator molecules (such as a shift in the quantum efficiency of a fluorophore) upon binding of a specific analyte. Here, it is shown that changes in the diffusive transport of molecules upon binding allow measurement of the concentrations of those molecules. This approach is not limited by the functional requirements of an indicator molecule and allows the study of a broad range of binding interactions.

Small ligand molecules diffusing through a microchannel are slowed as they migrate into a region containing larger molecules to which they bind. This results in the transient accumulation of ligand in the region of binding. Depending on the assay format, the extent of accumulation can be used to measure either the concentration of analyte or the affinity of molecules involved in the binding reaction. Unlabeled analyte can be characterized by measuring the diffusive transport of labeled probe molecules that compete for the same binding sites.

This analysis format was used to develop a competitive diffusion immunoassay (DIA) to measure the concentration of phenytoin, an anti-epileptic drug. It is necessary to monitor individual responses to phenytoin treatment in a narrow therapeutic concentration range, typically between 40 and 80 μM in plasma[16]. Many testing formats, both homogeneous and heterogeneous, have been developed for therapeutic monitoring of phenytoin, including the FPIA (refs 17,18), spin immunoassay[19], radioimmunoassay[16], and enzyme immunoassay[20].

Very little time is necessary to conduct a DIA because it is based on changes in diffusive transport during the early stages of binding. Assay conditions will be described that require only 18.5 s of diffusive mixing in the T-sensor for determination of phenytoin concentration in blood samples. It was not necessary to separate blood cells before measurement, as is generally required for FPIA and other methods of immunoanalysis. Preliminary experimental and simulated DIA data demonstrate the usefulness of this technique for clinical applications. This method was compared to FPIA, and consideration of limiting parameters of the assay and its application as a general analytic tool will also be discussed.

Results and discussion

Laminar flow conditions and diffusion-dependent mixing achieved with a two-inlet T-sensor were used to test the diffusion immunoassay concept, as illustrated in Figure 1A. Two solutions, one containing antibody (Ab) and the other containing both labeled and sample antigen, were pumped through the two inlets using positive-displacement syringe pumps at equal, constant flow rates. Under low Reynolds number conditions (Re ≈ 0.06 in this case), the flow streams run parallel to each other in the main channel and do not mix except by diffusion. Transport in the diffusion (*d*) dimension is dependent on the average residence time, which is determined in the T-sensor by flow rate and the traversed length (*l*) of the main channel. Concentrations of all components along the *d* dimension, the "diffusion profile", can be held constant at any position *l* by main-

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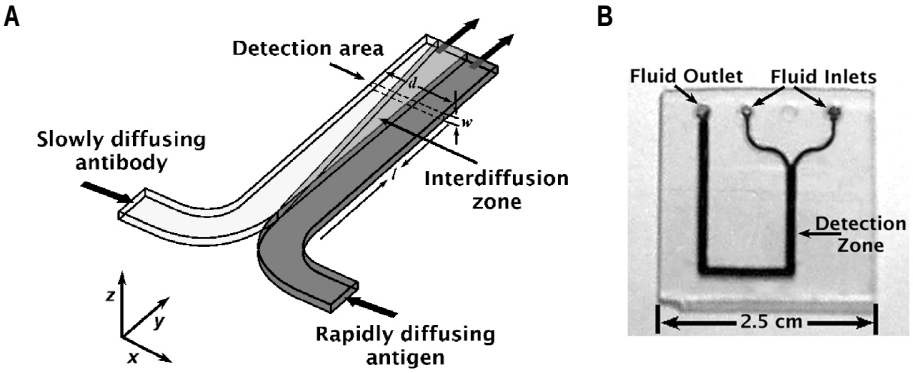

Figure 1. Diffusion immunoassay in a T-sensor. (A) Schematic of a diffusion immunoassay (DIA) implemented in the simplest possible T-sensor. Reagents are pumped separately through device inlets and meet at the junction. At this point, under laminar flow conditions, reagents flow side by side and mix only by diffusion. For a competitive binding assay, sample containing antigen to be measured spiked with a fixed concentration of labeled antigen is pumped through one inlet and a fixed concentration of antibody (Ab) through the other. Assay measurement is made at one location (*l*) downstream where reagents have had sufficient time to interdiffuse and react. Diffusive transport of analyte is measured across the *d* dimension. The diffusive transport of antigen is altered depending on the fraction that binds to Ab and is the basis for determination of sample antigen concentration. (B) Photograph of a microfluidic device used for testing. The flow channel is filled with dye for visualization.

taining the input conditions. This allows continuous monitoring of the diffusion profile using one- or two-dimensional detector arrays such as the charge-coupled device (CCD) camera used here. The T-sensor used for evaluating the DIA is shown in Figure 1B.

Small antigen molecules (relative molecular mass (M_r) <10,000 (<10K)) diffuse ~10-fold faster than Ab molecules (M_r ~150K). Binding of labeled or sample antigen to Ab slows their diffusion rate to approximately that of Ab. If Ab binds antigen molecules as they diffuse along the *d* dimension, the diffusion profile changes in comparison with that expected for freely diffusing antigen (Fig. 2). In the T-sensor, bound antigen accumulates where antigen and antibody interdiffuse.

In a competitive assay for measuring sample antigen concentrations, the diffusion profile of labeled antigen (the observed signal) is affected by the concentration of competing sample antigen. Ab and labeled antigen concentrations are held constant at suitable levels for detecting the expected range of sample antigen. As labeled antigen and sample antigen interdiffuse with Ab, they compete for a limited number of binding sites. To determine the concentration of sample antigen, the diffusion profile of labeled antigen is measured at a position, *l*, sufficiently far downstream that measurable interdiffusion and binding have taken place. For low concentrations of sample antigen, binding sites are readily available and the diffusion of labeled antigen is maximally slowed by binding to Ab (as in Fig. 2D). At higher concentrations of sample antigen, binding sites saturate, so the diffusion profile of labeled antigen is relatively unaffected (as in Fig. 2B).

Treatment of blood samples. Whole-blood samples are problematic for many methods of detection[17]. Blood proteins, particularly albumin, can interfere with an assay by binding and quenching fluorescently labeled indicator molecules. To prevent quenching of fluorescein–phenytoin conjugate, we adopted a competitive displacement approach reported to enable FPIA measurements[18]. Spiking blood samples with iophenoxate, a molecule known to bind strongly to human serum albumin[21], allowed a functional DIA for phenytoin. Blood cells can also be problematic and are usually separated from the plasma by centrifugation before measurement. Although it is possible to separate and manipulate components of blood without centrifugation using microfluidics[13], it was not necessary to separate the blood cells for measuring phenytoin with the DIA. It was possible to measure the fluorescence intensity profile of labeled antigen even with a final blood concentration of 10% despite the presence of the cells (Fig. 3).

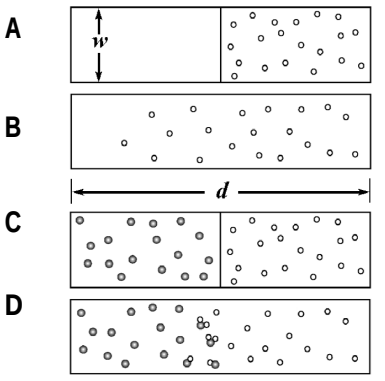

Figure 2. Antibody binding affecting diffusive transport of antigen. (A) Schematic representation of initial conditions in a T-sensor (at the inlet junction, *l* = 0) where the solution on the right contains antigen and the solution on the left contains buffer. The direction of flow is into the page. (B) Schematic representation of the diffusive transport of antigen at a point downstream in the T-sensor from that shown in (A). (C) Initial conditions for a case where Ab is initially on the left and antigen is on the right. (D) The diffusive transport of antigen at a point downstream in the T-sensor from that shown in C. The presence of slowly diffusing Ab limits the diffusive transport of antigen, causing it to accumulate in the center of the channel.

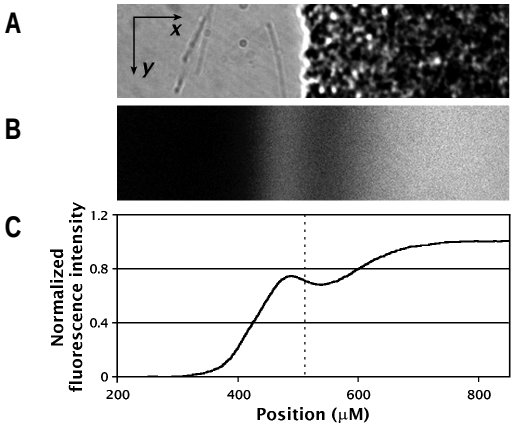

Figure 3. Phenytoin DIA with blood samples. (A) Bright-field image of a DIA with a 10% blood solution spiked with labeled antigen and treated with iophenoxate in the flow stream on the right. The flow stream on the left contains antibody specific for phenytoin. (B) Fluorescence image of the DIA at the same location as in (A). (C) Plot of the fluorescence intensity from image (B) indicating the concentration of labeled antigen across the diffusion dimension of the T-sensor. The dashed line represents the position of slowly diffusing blood cells. Labeled antigen has diffused into the Ab flow stream where binding has caused an accumulation.

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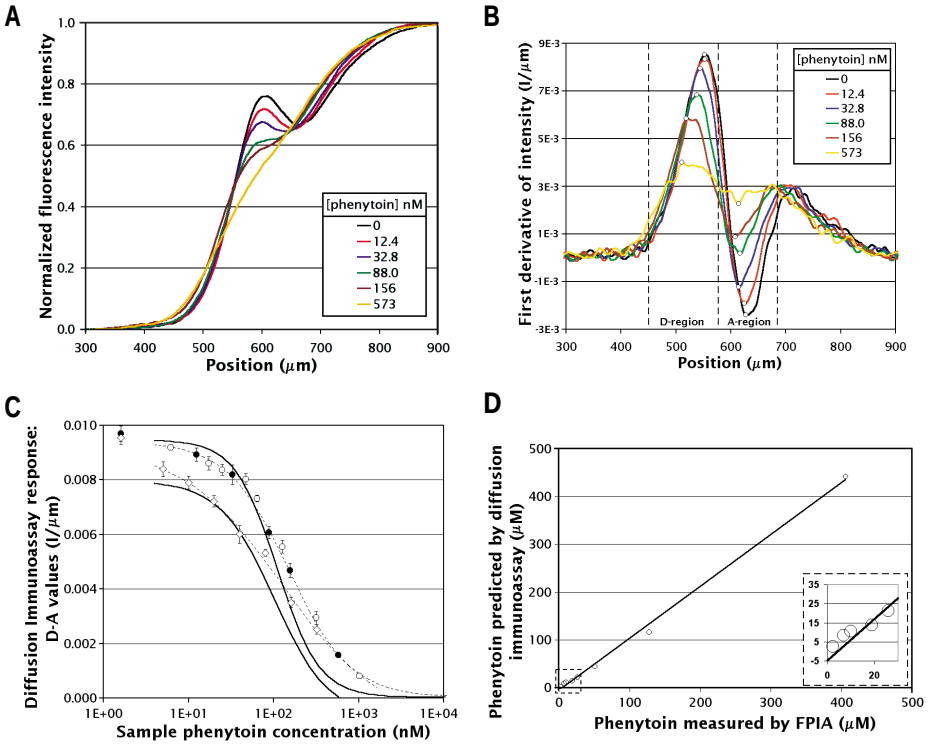

Figure 4. Diffusion immunoassay analysis. (A) Plots of fluorescence intensity profiles measured in the *d* dimension of the T-sensor for 0.25% blood calibrator samples. The values of sample antigen were determined by FPIA. (B) Plots of the first derivative of the intensity profiles (from A) with respect to distance in the *d* dimension. Values used for a calibration curve (D – A values) are the maximum values in the depletion region (D-region) minus the minimum values in the accumulation region (A-region) marked with an open circle. (C) DIA dose response curves. Filled circles indicate D – A values of 0.25% blood calibrator samples. Error bars represent 1 s.d. based on repeated measurements (n = 8 except for blank samples where n = 50). The limit of detection (LOD) for this set of conditions is 8.1 nM based on the level of error for blank measurements. Open circles indicate D – A values of 0.25% blood "unknown" samples (plotted here with phenytoin levels determined by FPIA). Open diamonds indicate D – A values for diluted serum calibration standards using lower Ab concentration (7.5% of stock). For these conditions, LOD = 0.43 nM. Broken lines are standard curves generated by four-parameter logit fits to each set of conditions by the method of nonlinear least squares. The equation for the line is as follows: $y = (y_0 - y) / (1 + (x/c)^b) + y_\infty$. Solid lines are fits to each set of conditions as predicted by the numerical model. Curves are based on logit fits to representative D – A values generated by the model. (D) Linear regression analysis of the method comparison data. The equation of the fitted line is as follows: $y = 1.055x - 4.802$, $r^2 = 0.998$.

Phenytoin diffusion immunoassay. As a first step in developing a DIA for phenytoin, the ability of Ab binding to alter the diffusion profile of labeled phenytoin (in the absence of sample phenytoin) was tested. Stock solutions of Ab (polyclonal) and labeled phenytoin (fluorescein–phenytoin conjugate) were from a standard FPIA kit. The average interdiffusion time and the concentrations of Ab and labeled antigen were critical factors in generating a measurable change in labeled antigen diffusion profiles. It is reasonable to limit concentrations of Ab and labeled antigen in order to reduce the limit of detection (LOD) and conserve costly reagents. This may require dilution of clinical samples for sample antigen concentration to fall within the range of detection, as is the case for the phenytoin assay. On the basis of preliminary results (data not shown), we used 10% Ab stock and a 7.5% final dilution of labeled phenytoin stock (~19 nM based on fluorescence intensity measurements) in assays to monitor clinically relevant phenytoin concentrations in blood samples. These choices have not been fully optimized for the phenytoin assay, and a routine procedure for choosing concentrations of Ab and labeled antigen has not yet been developed. Calibration measurements such as these are useful for setting the conditions of an assay or determining binding kinetics for a given antibody–antigen pair, and once established would not need to be repeated.

To compare the DIA with an established FPIA assay for phenytoin, a set of 12 mock samples was prepared by spiking whole blood with a range of phenytoin concentrations that can be measured clinically by FPIA. A small fraction of each sample was diluted to 0.25% (with final concentrations of 7.5% labeled antigen stock and 1 mM iophenoxate) for DIA analysis. The remainder of each sample was centrifuged to remove the blood cells, and the plasma was submitted to the University of Washington Academic Medical Center, Department of Laboratory Medicine, for a routine total phenytoin assay (Abbott AxSym FPIA).

Five of these samples (and one blank sample) were used as standards to establish a calibration curve. The remaining seven samples were treated as unknowns for method correlation to FPIA. The fluorescence intensity profiles measured during the DIA for the calibrator samples are plotted in Figure 4A. Several methods of quantifying these changes in the diffusion profile were compared. The best method for determining phenytoin concentration over the dynamic range of the assay was based on the first derivative of the normalized fluorescence intensity with respect to location in the *d* dimension (Fig. 4B).

Two points on the first derivative curves were used for determining phenytoin concentration. In the "accumulation region" (A-region), accumulation of labeled antigen flattens the slope of the diffusion profile. In the "depletion region" (D-region), the slower diffusion of Ab-bound labeled antigen results in a steeper diffusion profile. A plot of the difference between the first derivative maximum in the D-region and the minimum in the A-region, the D minus A (D–A) value, provides a reproducible high signal-to-noise ratio calibration curve for determining phenytoin concentration (Fig. 4C). The shape of the calibration curve plotted with phenytoin concentration on a log scale is sigmoidal in shape as expected for a competitive immunoassay.

To generate a standard curve, we used a four-parameter logit model frequently used to fit immunoassay calibration curves[22] (Fig. 4C). Concentrations of the unknown samples were determined on the basis of the standard curve, and these values were compared to values measured by FPIA. A linear regression was performed on the resulting data set to determine the correlation between methods (Fig. 4D). These results show that the DIA can be used to quantify unknowns as well as an established method.

An analytical model for experimental measurement and design. To determine the detection range for any DIA and to assist in designing an appropriate device for an assay, we developed a numerical model of the assay based on the previous work of Kamholz *et al*[14]. The input parameters include diffusion coefficients and concentrations of Ab, sample antigen, and labeled antigen, the diffusion dimension

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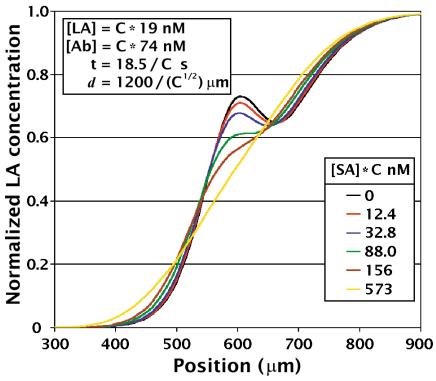

Figure 5. A numerical model for DIA development. (A) Diffusion profiles generated by the model for one set of assay parameters. LA and SA represent labeled and sample antigen, respectively. The variable *C* is a nondimensionalized parameter that can be used to set values for five related parameters to generate the set of diffusion profiles plotted. The five parameters are time, [sample antigen], [labeled antigen], [Ab], and *d*. The model results (with C = 1) are very similar to experimental results (Fig. 4A).

length, channel length, input flow velocity, and both forward and reverse reaction rate constants between the relevant species. The normalized concentration profiles generated by the model (Fig. 5) were similar to the intensity profiles measured experimentally (Fig. 4A).

Also indicated in Figure 5 is the dependence on five related parameters: measurement time (t); initial labeled antigen concentration, [LA]; initial Ab concentration, [Ab]; range of sample antigen concentration, [SA], and size of the channel in the *d* dimension. The value chosen for *C*, a nondimensional constant, indicates the dependent parameter values that would generate the set of curves shown. Values of dependent parameters can be determined by fitting experimental data to the analytical model. D – A values generated by the model are compared to experimental results in Figure 4C. The model results agree quite well with experiments, even though they do not account for the parabolic flow velocity in the *w* dimension, the range of binding kinetics present when using polyclonal Ab, or differences in the affinity of Ab for sample antigen versus labeled antigen. A recent study has shown that a similar model can accurately predict solute diffusion[23].

Limitations. A good indication of the LOD is the error associated with repeated measurements of blank samples[6]. Measurements of 0.25% blood samples (n = 50) indicates an LOD of 8.1 nM phenytoin for the conditions used. This limit was determined by taking the D – A value of the blank sample, subtracting twice the standard deviation, and extrapolating the phenytoin concentration at which this value would fit on the calibration curve. It is possible to improve the LOD by adjusting reagent concentrations, length of the *d* dimension, and time allowed. Reducing the concentration of Ab from 10% to 7.5% of the stock solution reduced the LOD from 8.1 to 0.43 nM phenytoin (Fig. 4C). This lower Ab concentration also produced a wider dynamic range that is probably due to a more optimal balance between the concentrations of Ab and labeled antigen. An even lower LOD may be achieved in the same time frame by further reducing Ab and labeled antigen concentrations. Reasonable accumulation of fluorescein–phenytoin was still observed when Ab was further reduced to 2% of the original stock and labeled antigen to 0.1% of stock (D – A value of 0.0068 for a blank sample). These conditions approached the limitations of the detector to measure labeled antigen and of Ab to bind sufficient antigen. Though not necessary for any routine drug monitoring, it is expected that the LOD would be even lower than 0.43 nM for these conditions, but absolute limitations have not yet been established.

Sensitivity can also be improved by increasing the interdiffusion time and *d* dimension as indicated by the relationship of parameters in Figure 5. For example, on the basis of these criteria, an established LOD of 0.43 nM phenytoin with t = 18.5 s and *d* = 500 µm could be reduced to LOD = 0.43 pM (C = 0.001) by increasing the interdiffusion time to t = 5.14 h and the diffusion distance to 1.58 cm and decreasing concentrations of labeled phenytoin and Ab by a factor of 1,000. Note that the optical detection system used in the work described here would not be sufficiently sensitive for measuring such low concentrations of fluorescein–phenytoin.

The relevant sample concentrations for most therapeutic drugs, drugs of abuse, and pesticides are well within the sensitivity range of the phenytoin DIA. Because these molecules are also similarly small and rapidly diffusing, most should be measurable under the same constraints as the phenytoin assay. However, more time would be necessary to achieve similar sensitivity for larger molecules such as proteins, which diffuse much more slowly. Additionally, the diffusion coefficient of Ab may need to be reduced by conjugation to larger molecules or beads in order to preserve the difference in diffusion coefficient between bound and unbound ligand. For binding interactions in which the rate of association is much lower, such as a protein–substrate interaction that has much lower binding kinetics than can be achieved with an antibody system, more time and/or higher concentrations of protein and substrate would be necessary for binding events to alter the diffusive flux of substrate.

Conclusions. Our homogeneous microfluidic immunoassay format offers some clear advantages over conventional immunoassays: direct analysis of blood samples, potential for portability, the small sample and reagent volumes necessary (< 1 µl), and analysis times of <1 min. Experiments reported here demonstrate that the DIA allows measurement of concentrations of a small molecule as low as 0.43 nM. The dynamic range for one set of assay conditions was as high as three orders of magnitude. The analysis format might also be useful for studying molecular interactions beyond immunochemistry. For example, a similar diffusion-based analysis scheme may allow measurement of the affinity of protein variants for a particular substrate. Another possibility is screening of substances for their potential use as drugs by measuring their affinity for protein binding sites. Microfluidic diffusion-based studies in such areas may be especially desirable for high-throughput screening and conservation of valuable samples. Multiple tests could be performed by sequentially pumping different analytes through a single channel, or multiple T-sensor channels could be fabricated in a miniature device allowing higher throughput. Microfluidics is rapidly becoming a cornerstone technology in chemical diagnostics; the DIA is a tool that could be used in many such applications.

Experimental protocol

Reagents. Fluorescein-labeled phenytoin and specific Ab stock solutions were from a standard FPIA kit (Sigma, St. Louis, MO). All reagents were buffered with 50 mM sodium phosphate/150 mM NaCl, pH 8.0. Sample antigen spiking solutions were prepared by serially diluting sodium phenytoin injectable solution (50 mg/ml phenytoin/40% propylene glycol/10% ethanol, pH 11.0; Elkins-Sinn, Cherry Hill, NJ) with 40% ethylene glycol/10% ethanol, pH 11.0. Each blood sample (1.4 ml) was spiked with 10 µl of the appropriate spiking solution to generate samples with a range of phenytoin conventionally monitored.

Flow cell. Outer layers were two glass coverslips (Fig. 1B). In one, three holes were drilled for fluidic access. Between the coverslips was 50 µm thick Mylar coated on both sides with 25 µm adhesive (Fraylock, Inc., San Carlos, CA), through which channels were cut using a CO_2 laser (Universal Laser Systems, Inc., Scottsdale, AZ). In the main fluid channel, *d* = 1,200 µm and *w* = 100 µm.

Experimental system. Reagents were manually loaded into fluid lines (polyetheretherketone tubing, Upchurch Scientific, Oak Harbor, WA) and then pushed through the device using syringe pumps (Kloehn, Las Vegas, NV). A 100 W halogen lamp of a Zeiss ICM-405 inverted epifluorescence microscope

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(Carl Zeiss Inc., Thornwood, NY) was used as an excitation source. The emission signal was magnified through a 10× objective and captured using an integrating cooled CCD camera (SBIG ST-7I; Santa Barbara, CA). A flow rate of 41.7 nl/s through the main channel was chosen and measurements of the diffusion profile were taken at l = 6.4 mm, which corresponds to an average interdiffusion time of 18.5 s. Although the equipment used to test the concepts of the DIA could not be used for point-of-care diagnostics, it should be possible to miniaturize the system using excitation and detection sources such as laser-induced fluorescence and a CCD chip. In addition, microfluidic platforms have been demonstrated that would allow fluid handling and pumping.

Curve fitting and linear regression. A four-parameter logit model of DIA response versus phenytoin concentration was used to generate a standard curve. The responses for six standards were fitted by a nonlinear least-squares method. Data transformation to enable linear regression was not done to avoid the problems associated with transformation[24]. Microsoft Excel's Solver function was used to determine the standard curve parameters that produced a minimum value in the sum-squared error by a generalized-reduced-gradient iteration method[25]. The r^2 and individual residual values of the four-parameter logit fit to the blood calibration samples were $r^2 = 0.9995$ and IR% < 7.9%.

Analytical model. The analytical model developed as described elsewhere[14] was implemented using a set of five partial differential equations for the DIA.

$$\partial[LA]/\partial t = D_{LA}(\partial^2[LA]/\partial x^2) - k_1([LA][Ab]-[AbLA]/K^1_{eq}) \quad (1)$$

$$\partial[SA]/\partial t = D_{SA}(\partial^2[SA]/\partial x^2) - k_2([SA][Ab]-[AbSA]/K^2_{eq}) \quad (2)$$

$$\partial[Ab]/\partial t = D_{Ab}(\partial^2[Ab]/\partial x^2) - k_1([SA][Ab]-[AbLA]/K^1_{eq}) - k_2([SA][Ab]-[AbSA]/K^2_{eq}) \quad (3)$$

$$\partial[AbLA]/\partial t = D_{AbLA}(\partial^2[AbLA]/\partial x^2) + k_1([LA][Ab]-[AbLA]/K^1_{eq}) \quad (4)$$

$$\partial[AbSA]/\partial t = D_{AbSA}(\partial^2[LA]/\partial x^2) + k_2([LA][Ab]-[AbSA]/K^2_{eq}) \quad (5)$$

where LA = labeled antigen, SA = sample antigen, $AbLA$ = Ab-bound labeled antigen, $AbSA$ = Ab-bound sample antigen, D_N is the diffusion coefficient for species N, k_1 and k_2 are the forward reaction rate constants for the Ab-labeled antigen reaction and the Ab–sample antigen reaction, respectively, and K^1_{eq} and K^2_{eq} are the equilibrium constants for the same two reactions. Coordinate axes are those used in Figure 1A. A number of simplifying assumptions about the device geometry allows treatment of this problem with a model explicit only in the d dimension[14,15]. Here, [Ab] is the concentration of individual Ab binding sites, and is therefore equal to twice the concentration of Ab molecules. Values used for diffusion coefficients were based on molecular weight with $D_{Ab} = 4.30 \times 10^{-7}$ cm^2/s, $D_{SA} = 5.8 \times 10^{-6}$ cm^2/s, $D_{LA} = 3.2 \times 10^{-6}$ cm^2/s, and because both antigens are small compared to Ab, $D_{AbLA} = D_{AbSA} = D_{Ab}$ was used. To qualitatively match the set of diffusion profiles observed experimentally, values for $k_1 = k_2 = 4 \times 10^6$ M^{-1} s^{-1} and [Ab] = 74 nM (for 10% of stock) were used. The value used for association rate is not unreasonable for a high-affinity antibody–antigen pair[26]. It was determined that rates of dissociation typical for antibodies are not likely to be an important factor for binding events studied over such a short time period (<20 s). A value for $K_{eq} = 4 \times 10^{10}$ M^{-1} was used, although model results were not significantly affected unless values for the dissociation rates were $>1 \times 10^{-2}$ s^{-1}, corresponding to $K_{eq} < 4 \times 10^8$ M^{-1}. Upper limits of K_{eq} are 1×10^{12} M^{-1} for immunoassays[6].

Acknowledgments

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