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Empirically optimized flow cytometric immunoassay validates ambient analyte theory

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ABSTRACT

Ekins' ambient analyte theory predicts, counterintuitively, that an immunoassay's limit of detection can be improved by reducing the amount of capture antibody. In addition, it also anticipates that results should be insensitive to the volume of sample as well as the amount of capture antibody added. The objective of this study was to empirically validate all of the performance characteristics predicted by Ekins' theory. Flow cytometric analysis was used to detect binding between a fluorescent ligand and capture microparticles because it can directly measure fractional occupancy, the primary response variable in ambient analyte theory. After experimentally determining ambient analyte conditions, comparisons were carried out between ambient and nonambient assays in terms of their signal strengths, limits of detection, and sensitivity to variations in reaction volume and number of particles. The critical number of binding sites required for an assay to be in the ambient analyte region was estimated to be 0.1 VK_d. As predicted, such assays exhibited superior signal/noise levels and limits of detection and were not affected by variations in sample volume and number of binding sites. When the signal detected measures fractional occupancy, ambient analyte theory is an excellent guide to developing assays with superior performance characteristics.

Theoretical guidelines for optimizing immunoassay performance have evolved from an analysis by Ekins more than two decades ago that defined conditions for assaying free thyroxin without perturbing its concentration. Elkins reasoned that the equilibrium between bound and free hormones would be undisturbed if only a trace amount of capture antibody were added [1]. Under these "ambient analyte" conditions, Ekins' analysis also predicted, counterintuitively, that the signal-to-background ratio would be improved. He hypothesized that ambient analyte conditions and maximum occupancy would be attained whenever the amount of antibody was much less than the product of the equilibrium dissociation constant, K_d, and the reaction volume[1] [2]. The insight that large surface areas are not required for high sensitivity[2] not only resulted in accurate tests for free hormones but also led Ekins in 1985 to the concept of high-density multiplexed assays and microarrays [3–9] and a thyroid-stimulating hormone (TSH)[3] assay with a limit of detection of 0.0002 mIU/L or 1.4 aM[4] [10].

Although ambient analyte theory predicts that optimal sensitivity can be attained by maximizing the fractional occupancy of the capture surface, another line of reasoning asserts that it is the total mass of analyte captured that determines the limit of detection. Remarkable sensitivity has been reported with these mass sensing assays where the number of capture sites was far above Ekins' limit [11,12]. For example, Mirkin and coworkers reported attomolar sensitivity with a vast excess of antibody-coated magnetic particles [12].

Sensitivity, however, is determined by a number of factors, including signal intensity, nonspecific binding, and variability in the capture surface. Designers of immunoassay systems assume that the reproducibility of results will be proportional to the volumes of sample and reagents added to the reaction cuvette, and automated analyzers contain sophisticated pipetting systems to ensure that volumes are precisely aliquoted to minimize variability. However, if the detection scheme measures fractional occupancy, as is the case with both planar and suspension arrays [6,13,14], then under ambient analyte conditions Ekins' theory predicts that pipetting precision might not be important.

Ambient analyte theory

For the simplest case where a single species of binding sites captures a homogeneous target analyte, the fraction of binding

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[1] In his analysis, Ekins used the equilibrium association constant, K_a, which is the reciprocal of K_d.

[2] The terms "sensitivity" and "limit of detection" are used interchangeably.

[3] *Abbreviations used:* TSH, thyroid-stimulating hormone; IgG, immunoglobulin G; R-PE, R-phycoerythrin; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FACS, fluorescence activated cell sorter.

[4] 0.0002 mIU/L × 306.7 μg/IU/28,000 g/mol = 1.4 aM.

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sites occupied at equilibrium, f, can be expressed as a function of two dimensionless parameters, a and b:

$$f = \frac{(a+b+1) - \sqrt{(a+b+1)^2 - 4ab}}{2b}, \tag{1a}$$

where

$$f = \frac{\Gamma}{\Gamma_m},\ b = \frac{S\Gamma_m}{VK_d} \quad \text{and} \quad a = \frac{A_o}{K_d}. \tag{1b}$$

For the dimensionless terms defined in Eq. (1b), Γ is the surface concentration of occupied receptors or bound target (in mol/cm^2), Γ_m is the maximum surface concentration of occupied receptors or bound target (in mol/cm^2), f is the fractional occupancy Γ/Γ_m, S is the total capture surface area (in cm^2), V is the reaction volume (in L), K_d is the equilibrium dissociation constant (in mol/L), and A_o is the target analyte concentration (in mol/L).

Ekins showed that Eq. (1) reduces to

$$f = \frac{a}{a+1} \tag{2}$$

whenever the number of binding sites, $S\Gamma_m$, is very much less than VK_d, that is, when $b << 1$. Under these conditions, the assay is said to be in the ambient analyte region. Because all terms containing S and V have been eliminated in Eq. (2), one would predict that the signal representing the fractional occupancy, f, would be independent of the total number of binding sites or target analytes present in the reaction. This would translate into enhanced assay robustness because results would not be affected by errors in sample volume or amount of capture surface added.

Although Eq. (2) provides the fractional occupancy for a single binding site, it is common for receptors to display heterogeneous binding characteristics, especially after immobilization onto a solid phase [15]. In this case, Eq. (2) can be modified to accommodate more binding sites. Such an equation for two binding sites would be

$$f = f_1 \frac{a_1}{a_1+1} + (1-f_1)\frac{a_2}{a_2+1}, \tag{3}$$

where $a_1 = A_o/K_{d1}$ and $a_2 = A_o/K_{d2}$ are dimensionless variables derived from the equilibrium dissociation constants of the two sites, K_{d1} and K_{d2}, and f_1 is the fraction of total binding sites that are of the first kind, $f_1 = S_1\ \Gamma_{m1}/(S_1\ \Gamma_{m1} + S_2\ \Gamma_{m2})$. Furthermore, the degree of heterogeneity of binding sites can be assessed using the Sips isotherm:

$$f = \frac{a^h}{1+a^h}, \tag{4}$$

where h is the heterogeneity index [15].

In this article, using a two-component assay system, we demonstrate the ability of ambient analyte conditions to enhance assay performance and robustness. We examine the binding of labeled anti-goat immunoglobulin G (IgG) to immobilized goat IgG in a suspension array format to show that the assay not only exhibits higher sensitivity and signal strength but also is much more resistant to deviations in sample volume and the number of particles when in the ambient analyte regime.

Materials and methods

Materials

Carboxyl polystyrene particles (1.27 μm diameter and 1.05 g/ml density) and Rainbow Calibration particles (3 μm diameter) were generously provided by Spherotech (Libertyville, IL, USA). R-phycoerythrin (R-PE)-conjugated AffiniPure F(ab′)$_2$ fragment donkey anti-goat IgG H + L (product no. 705-116-147) was purchased from Jackson Laboratories (West Grove, PA, USA). Immunopure whole molecule biotin-conjugated goat IgG (product no. 31734) was purchased from Pierce (Rockford, IL, USA). Phosphate-buffered saline (PBS, pH 7.4) was obtained from Sigma (St. Louis, MO, USA) and used as the standard buffer for particle coating and analyte binding assays. IgG-free bovine serum albumin (BSA), also obtained from Sigma, was diluted in PBS and used as a blocker. BD QuantiBrite PE Beads were obtained from BD Biosciences (San Jose, CA, USA). Assays were carried out in 0.5-ml polyethylene microcentrifuge tubes.

Particle coating procedures

Goat IgG was passively adsorbed to the carboxyl-modified surface of the polystyrene particles (1.27 μm diameter). A 50-μl aliquot of stock particles (5%, w/v, 2.22×10^9 particles) was washed three times in PBS and resuspended in 50 μl of the same. The particles were sonicated for 1 min to prevent aggregation, and then 0.15 mg of goat IgG and enough PBS was added to make a final volume of 100 μl. The mixture was immediately vortex-mixed to ensure a homogeneous coating on the particles. The coupling was allowed to occur overnight at room temperature with constant mixing in a rotator. The particles were then washed in PBST (PBS containing 0.05% Tween 20) and stored at 4 °C in PBS (containing 1% IgG-free BSA) at 1% (w/v).

Binding assays

General

Capture particles were sonicated for 1 min and added to aliquots of target analyte (PE-labeled anti-goat IgG) as described below. The assay was incubated overnight (17 h) in a rotator at room temperature. This ensured that equilibrium was achieved for all of the assays. At the end of the incubation period, tubes were centrifuged and unbound PE–anti-goat IgG was separated as supernatant from the particles. The particles were washed three times in PBST and analyzed on a flow cytometer. Although flow cytometry assays do not require any wash steps, it was found that it was necessary because it helped to remove loosely bound analyte molecules (that may have bound nonspecifically).

Particle dilution assay

Particle dilution curves were obtained by incubating a series of particle dilutions ($2.67 \times 10^7 - 1.63 \times 10^3$ particles, 5.07×10^{-8} cm^2/particle, 1.35–8.26×10^{-5} cm^2) overnight with target analyte (final concentration of 2.50×10^{-10} mol/L diluted in PBS containing 1% IgG-free BSA) in a reaction volume of 100 μl.

We compared the effects of changes in volume and the number of binding sites in ambient and nonambient analyte assays. For the change in volume experiment, the analyte concentration and the total number of particles were held constant. This experiment was carried out by incubating 8.88×10^5 particles (surface area 0.045 cm^2, $b = 4.75$) or 8.88×10^3 particles (surface area 0.000445 cm^2, $b = 0.047$) with a fixed target analyte concentration of 2.50×10^{-10} mol/L at a final volume of 100 or 200 μl.[5]

The dependence of the response on the total number of particles was determined from the particle dilution assays described above.

Analyte dilution assay

To obtain analyte dilution curves, 2.66×10^6 particles (surface area 0.135 cm^2, $b = 14.25$) and final analyte concentrations ranging

[5] Values of b were based on parameter estimates, $\Gamma_m = 3.8 \times 10^{-12}$ mol/cm^2 and $K_d = 3.6 \times 10^{-10}$ mol/L, and calculated surface area, $S = 8.88 \times 10^5\ 5.07 \times 10^{-8} = 0.045$ cm^2, using Eq. (1b): $b = S\Gamma_m/VK_d = 0.045\ 3.8 \times 10^{-12}/0.0001\ 0.36 \times 10^{-9} = 4.75$.

between 5.00×10^{-8} mol/L and 3.66×10^{-11} mol/L (diluted in 1% IgG-free BSA) were incubated overnight in a reaction volume of 100 μl. The fractional occupancy was calculated from the ratio of median particle fluorescence intensity, F (in relative fluorescence units), corrected for background fluorescence, F_b, divided by the background-corrected median particle fluorescence obtained with excess target, F_m. Assuming that fluorescence intensity is linearly related to the mass of target bound, $f = \Gamma/\Gamma_m = (F - F_b)/(F_m - F_b)$. The maximum surface concentration, Γ_m, and the equilibrium dissociation constant, K_d, were determined by nonlinear regression (JMP IN release 5.1), which determined values of dimensionless parameters a and b in Eq. (1) that minimized the sum of squared differences between each experimental data point and the model. The physical parameters were then calculated from $K_d = A_o/a$ and $\Gamma_m = b\ VK_d/S$ using the known values of A_o, V, and S.

The value of K_d obtained was verified with an analyte dilution assay carried out using a number of binding sites that was within the ambient analyte region. Approximately 1.78×10^4 coated particles (surface area 9.00×10^{-4} cm^2, $b = 0.095$) were assayed with serial dilutions of analyte (9.60×10^{-8} mol/L to 5.72×10^{-13} mol/L). Estimates of K_d were obtained by fitting Eq. (2) to the observed data.

The value of Γ_m was confirmed by quantifying the signal at full occupancy using the BD QuantiBrite PE Beads. QuantiBrite beads contain four PE intensities at known numbers of PE molecules per bead to provide a simple way of quantifying the PE-conjugated antibodies via flow cytometry.

Flow cytometry

Fluorescence from bound target was measured using a FACS (fluorescence-activated cell sorter) flow cytometer (Becton Dickinson, San Jose, CA, USA). For a standard sample, approximately 10,000 particles were excited at 488 nm and fluorescence emission was collected in the FL2 channel (585/42 nm). However, for more dilute samples, only 2500 particles were scanned. Flow cytometry data were analyzed using WinMDI (version 2.8), and median intensity values were recorded for each sample.

To standardize the measured fluorescence intensities for day-to-day comparison, we read Rainbow Calibration particles on the flow cytometer prior to our samples. Rainbow Calibration particles contain six fluorescence intensities and, thus, exhibit multiple peaks when seen in the flow cytometer. Each median fluorescence intensity value from our sample was divided by the intensity of the third peak of the calibration particles. Fractional occupancy was then calculated by dividing these values by the calibrated intensity corresponding to full occupancy (F_m).

Results

Determination of maximum occupancy

Ambient analyte theory predicts that when the number of binding sites is much less than VK_d, fractional occupancy is maximized. As the capture surface area decreased from approximately 1 to 0.0001 cm^2 in the particle dilution assay for a final analyte concentration of 2.50×10^{-10} mol/L (Fig. 1), the fractional occupancy increased from 0.006 to a plateau value of 0.47. The analyte concentration was selected to give a plateau fractional occupancy less than 0.5, indicating that $A_o < K_d$.[6] Maximum fractional occupancy was obtained with only 2×10^4 particles, which was still a sufficient number to achieve good counting statistics in the flow cytometer.

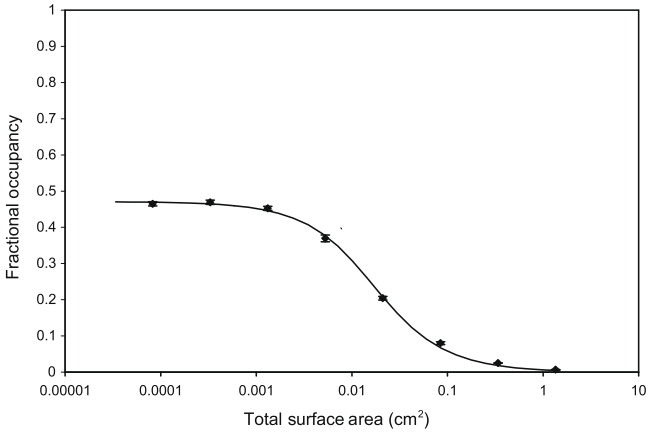

Fig. 1. Particle dilution assay (antibody fractional occupancy vs. surface area) at final target analyte concentration of 2.5×10^{-10} mol/L. The antibody fractional occupancy can be estimated as the measured signal divided by the maximum signal measured at high target analyte concentration. Using nonlinear regression to fit Eq. (1a) to the equilibrium binding data, the best-fit estimates for the dissociation constant (K_d) and capture probe density (Γ_m) were 0.28 ± 0.004 nmol/L and 4.00 ± 0.13 pmol/cm^2, respectively.

Estimation of Γ_m and K_d

To verify that maximum binding is obtained when $b << 1$, it is necessary to determine maximum captured target density, Γ_m, and the equilibrium dissociation constant, K_d, for the binding pair. Both parameters were estimated by fitting Eq. (1) to the analyte dilution data (Fig. 2) that had been normalized to the maximum signal. The best-fit values were 0.27 ± 0.06 nmol/L for K_d and 3.80 ± 0.09 pmol/cm^2 for Γ_m.

We also fit the data obtained from the particle dilution curves to Eq. (1) and again obtained estimates for K_d and Γ_m. The best-fit values and confidence intervals were 0.28 ± 0.0035 nmol/L for K_d and 4.00 ± 0.13 pmol/cm^2 for Γ_m (Fig. 1), which were within the confidence intervals of our initial estimates.[7]

When an assay is in the ambient analyte range, the K_d value can be more easily estimated with a one-parameter model. We fit Eq. (2) to analyte dilution curves obtained using number of particles that were within the ambient analyte range as determined from Fig. 1. The approximated best-fit value for K_d was 0.36 ± 0.03 nmol/L (Fig. 3A). However, a one-site model did not seem to appropriately fit our data. Instead, a two-site model fit (Eq. (3)) was found to be more accurate (Fig. 3B). The best-fit values for K_{d1} and K_{d2} were 0.11 ± 0.02 and 1.57 ± 0.29 nmol/L, respectively. The data can also be fit with the Sips isotherm (Eq. (4)), which is often used to study heterogeneity in solution. The best-fit values using the Sips isotherm were 0.41 ± 0.02 nmol/L for K_d and 0.74 ± 0.02 for the heterogeneity index (Fig. 3C).

The value of the capture probe density estimated from dilution curves was verified by calibrating the intensity signal of the flow cytometer using BD QuantiBrite PE Beads. The fluorescence intensity signal corresponding to full occupancy was quantified as 2.54 pmol/cm^2. The estimates for K_d and Γ_m are compiled in Table 1.

Robustness of ambient analyte assays

Although ambient analyte conditions enhance performance by improving signal levels and minimizing background, they also impact precision by eliminating potential sources of error. In analytical methods that measure fractional occupancy, as is the case with flow cytometric assays, ambient analyte theory predicts that the

[6] In the plateau region where f is independent of b, $a = f_a/1 - f_a$ and $f_a < 0.5$ implies $A_o < K_d$.

[7] f_a, the asymptotic level to which f converges in the plateau region, can also be used to estimate K_d given that $f_a = a/(1 + a)$. This gave a value of 0.29 nmol/L.

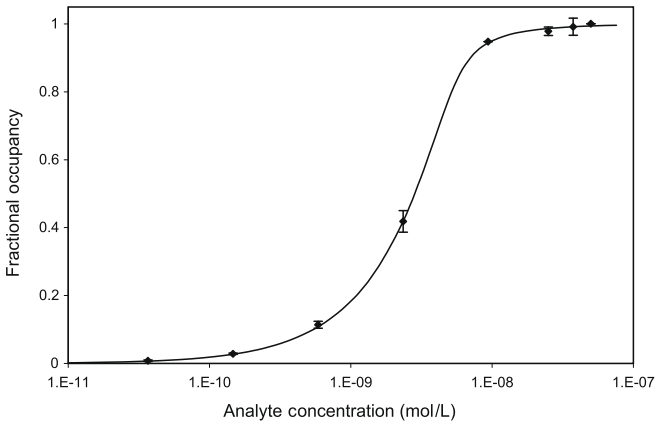

Fig. 2. Analyte dilution assay (antibody fractional occupancy vs. target analyte concentration) at a total particle surface area of 0.135 cm^2 ($b = 14.25$). The best-fit values for K_d and Γ_m were estimated to be 0.27 ± 0.06 nmol/L and 3.80 ± 0.09 pmol/cm^2, respectively (Eq. (1a)).

response will not be dependent on either the reaction volume or the number of particles added.

Response to changes in volume

Under ambient analyte conditions, we would expect that changes in the reaction volume would not affect the response for any given concentration of analyte. We chose two assay conditions; one in the ambient analyte range with $b = 0.047$ (surface area 0.000445 cm^2) and the other at $b = 4.75$ (surface area 0.045 cm^2). At each point, the required number of particles was added to final reaction volumes of 100 and 200 µl. From the data (Fig. 4A), it is apparent that the signal measured for the ambient analyte case is insensitive to changes in the reaction volume. For the nonambient analyte case, however, there is a significant increase in signal when the volume of the reaction is increased from 100 to 200 µl.

We compared the two groups using an unpaired Student's *t* test assuming equal variance. The *P* value when the assay was performed in the ambient range was 0.16, which is not significant at the 0.05 level. Under nonambient conditions, $P < 0.0001$, indicating a significant difference.

We also obtained particle dilution curves for the assays carried out in final volumes of 100 and 200 µl while maintaining a constant analyte concentration (Fig. 4B). The responses from the two curves converge as the assay enters the ambient analyte range (surface area <0.00131 cm^2), showing that the response is robust to changes in analyte volume.

Response to changes in number of particles

When an assay is run under ambient analyte conditions, theory predicts that the fractional occupancy of the capture antibody is not dependent on the total number of binding sites and, hence, a change in the number of particles should not result in a change in signal. The theory is confirmed by the particle dilution response curve (Fig. 1). At particle concentrations corresponding to $b > 0.1$ (total surface area >0.000947 cm^2), the signal is dramatically decreased by increases in the number of particles present in the assay. However, in the plateau region where $b < 0.1$, changes in the number of particles do not affect the signal.

In a particle-based assay, the number of capture sites, $S\Gamma_m$, is reduced by changing the total surface area, *S*, while keeping constant the density of capture sites, Γ_m. The calibration factors, F_b and F_m, used in calculating fractional occupancy, $f = \Gamma/\Gamma_m = (F - F_b)/(F_m - F_b)$, are constant because median background fluorescence per particle, F_b, and maximum median fluorescence

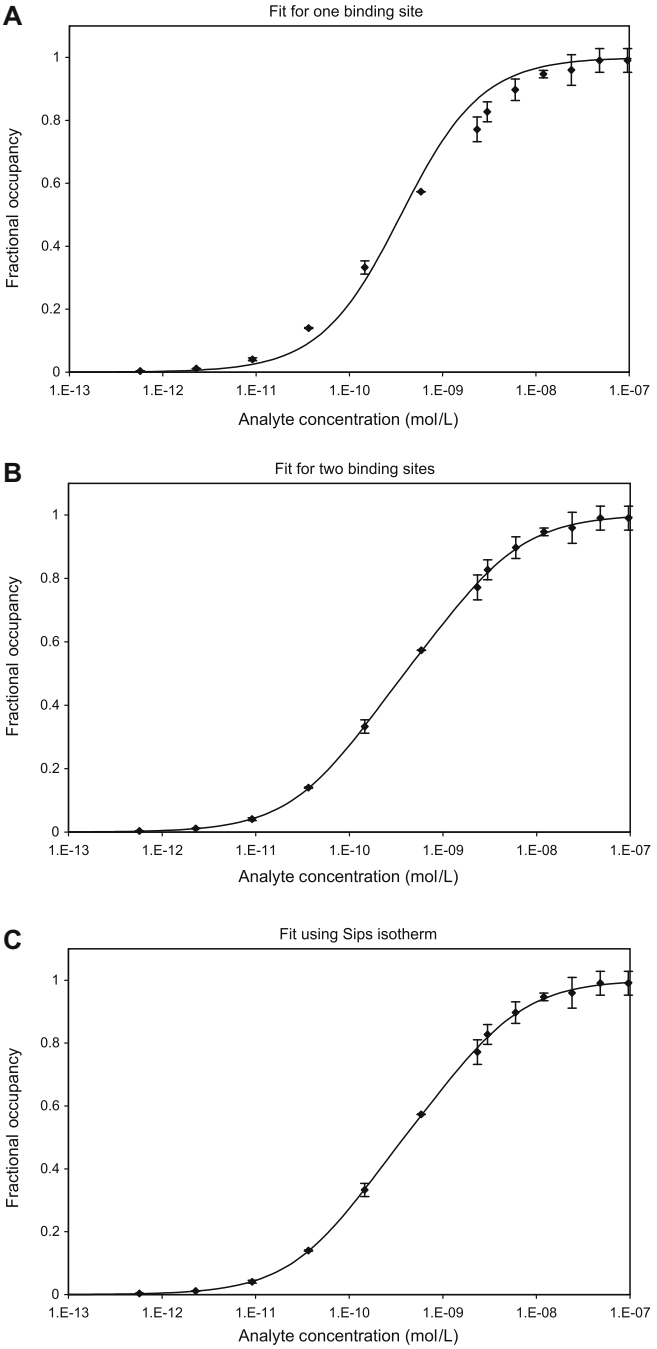

Fig. 3. Analyte depletion curve carried out under ambient analyte conditions (surface area 0.0009 cm^2, $b = 0.095$). (A) A one-site model (Eq. (2)) is used to fit the data. The best-fit value for K_d is 0.36 ± 0.03 nmol/L. The one-site model does not fit the data very well. (B) A two-site model (Eq. (3)) is a better fit for the data. The best-fit values for the two classes of binding sites are 0.11 ± 0.02 and 1.57 ± 0.29 nmol/L for K_{d1} and K_{d2} and 0.53 ± 0.06 for f_1. (C) The Sips isotherm (Eq. (4)) is used to fit the data. The best-fit values are 0.41 ± 0.02 nmol/L and 0.74 ± 0.02 for K_d and heterogeneity index, respectively. An *F* ratio of 89.6 confirms that the two-site fit is much better than the one-site fit.

per particle, F_m, do not vary with the total number of particles. This behavior might not be seen in other assay formats where the number of capture sites is modulated by varying antibody density.

Discussion

When an assay is performed under ambient analyte conditions, not only is fractional occupancy maximized but also the response

Table 1
Parameter estimation through curve fitting.

Curve	Parameters	
	Γ_m (pmol/cm^2)	K_d (nmol/L)
Particle dilution	4 ± 0.13	0.28 ± 0.004
Analyte dilution (one-site model in the ambient analyte region)		0.36 ± 0.03
Analyte dilution (two-site model in the ambient analyte region)		K_{d1} = 0.11 ± 0.02 K_{d2} = 1.57 ± 0.29
Analyte dilution (nonambient analyte region)	3.8 ± 0.09	0.27 ± 0.06
BD QuantiBrite PE beads	2.54	

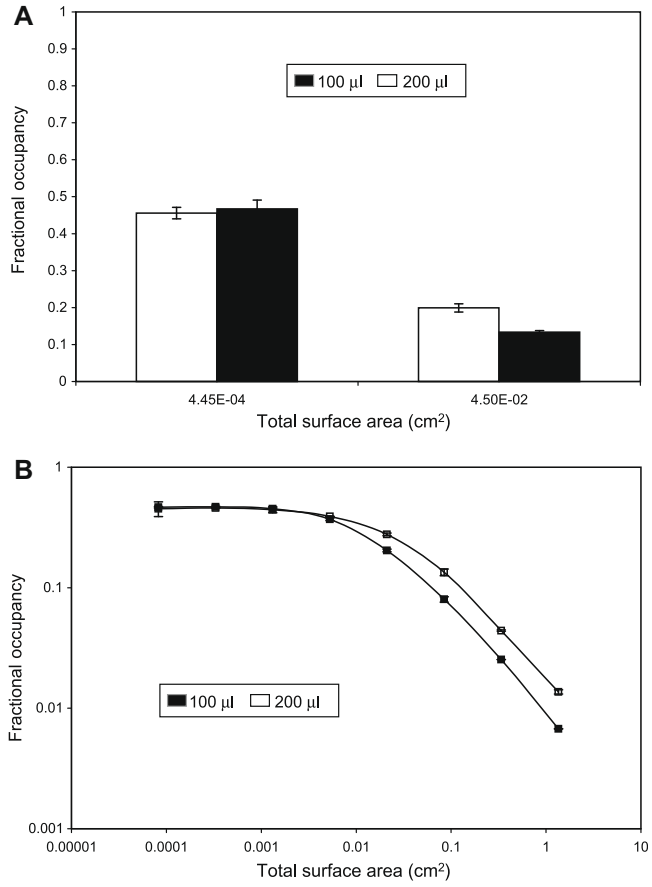

Fig. 4. (A) Robustness to changes in total volume (■, 100 µl; □, 200 µl) between an assay performed in the ambient analyte region (surface area 0.000445 cm^2, b = 4.75) and one performed outside the region (surface area 0.045 cm^2, b = 0.047). At the higher particle concentration, fractional occupancy increases significantly (P value <0.0001) as a result of doubling the total reaction volume, whereas at low particle concentration, there was no significant difference in fractional occupancy (P value =0.16). Data shown represent the averages of four replicates ± 2 standard deviations. (B) Response to changes in volume (■, 100 µl; □, 200 µl) for assays with surface areas ranging from 1.35 to 8.24 × 10^{-5} cm^2 at a final analyte concentration of 2.5 × 10^{-10} mol/L. The response at higher surface areas (nonambient analyte conditions) is dependent on volume, whereas at low surface areas (<0.00131 cm^2), the two responses converge, showing that the response is solely a function of the analyte concentration to which the antibody is exposed.

is unaffected by either the volume of sample or the mass of antibody added. These advantages of operating in the ambient analyte regime have been experimentally verified with a two-component fluorescent assay read on a flow cytometer that directly measures binding site occupancy.

Ambient analyte conditions exist whenever the number of binding sites, $S\ \Gamma_m$, is much less than the product of the reaction

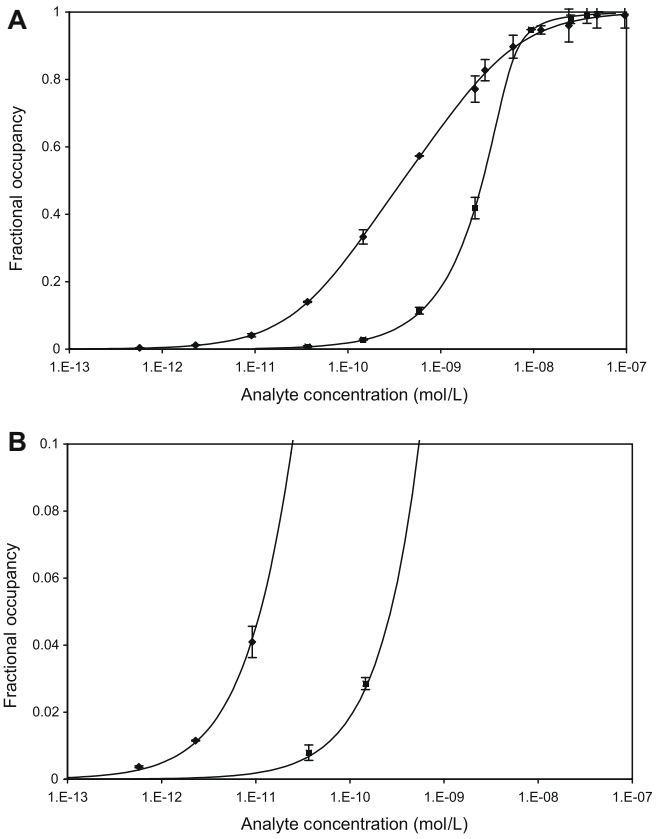

Fig. 5. Comparison of the analyte dilution curves at ambient (♦) and nonambient analyte (■) conditions. (A) The assay carried out in ambient conditions (area 0.0009 cm^2, b = 0.095) showed a much larger dynamic range compared with nonambient analyte conditions (area 0.135 cm^2, $S\ \Gamma_m/V\ K_d$ = 14.25). (B) Expanded view of the bottom of the analyte dilution curves. The detection limit for ambient analyte conditions was 0.11 pmol/L, whereas that for nonambient analyte conditions was 2.23 pmol/L.

volume and the equilibrium dissociation constant, $V\ K_d$.[8] In our two-component assay, it was shown that the fractional occupancy increases to a plateau as the number of binding sites is reduced. After estimating K_d and Γ_m, it was apparent that fractional occupancy was maximized when b was less than 0.1 (1.87 × 10^4 particles, total surface area 0.00095 cm^2). Although this was larger than the value that Ekins predicted [2], smaller differences from the maximum could not be detected because of experimental error.

It should be noted that for assay developers desiring to formulate their assays in ambient analyte conditions, the determinations of Γ_m and K_d are not required. These determinations were performed only to validate Ekins' predictions of the region. In practice, a particle dilution curve (Fig. 1) is adequate to provide an estimation of the number of particles required to maximize fractional occupancy.

Our experiments also showed the effects of changes in assay volume and number of particles on fractional occupancy under ambient and nonambient conditions. We found, as predicted (Eq. (2)), that assays in the ambient analyte region were insensitive to changes in volume and the number of particles. This could be particularly important for point-of-care assays where precise volumes may be difficult to obtain due to lack of operator expertise and equipment.

[8] More generally, ambient analyte conditions exist whenever the total number of binding sites is much less than the greater of VK_d or VA_o. Because A_o is generally less than K_d for high-sensitivity assays, the VK_d limit applies in most cases.

In suspension arrays, sensitivity is a function of the number of fluorophores bound on a particle and the particle background. For our system, it was estimated to be 0.11 pmol/L in the ambient analyte range (Fig. 5A and B).[9] As the number of particles increases beyond the ambient analyte region, fractional occupancy decreases, thereby decreasing sensitivity. Assay sensitivity can be further improved by using higher affinity antibody pairs or smaller particles.

Although our assays were at equilibrium when measurements were acquired (data not shown), theoretically, ambient analyte conditions should also enhance nonequilibrium end points and will be the subject of a future study. The kinetics of assays carried out on microparticles and planar arrays has been reviewed elsewhere [16–20].

The ambient analyte principle can be applied to all forms of immunoassays that measure a response proportional to the fraction of binding sites occupied. Saviranta and coworkers [8] and Dandy and coworkers [9] demonstrated that planar microarrays operate in the ambient analyte regime. In planar arrays, the ambient analyte region can be reached by reducing the capture spot size. We demonstrated that suspension arrays can also be formulated in the ambient analyte region by reducing the number of particles used in the assay.

Although we used a direct assay format in this study, our results would be applicable to sandwich immunoassays as well because the limiting step in these formats is the binding of analyte to the capture probe. The detector-labeled antibody, or secondary antibody, is typically in excess concentration.

By reducing the number of binding sites, the total number of target analyte molecules bound decreases, but the signal per unit area increases due to the reduction in total area. Therefore, assays formulated to operate in the ambient analyte regime are able to detect lower concentrations of target molecules with higher precision. The insensitivity of ambient analyte assays to sample volume and mass of binding sites is critical to the performance characteristics of microparticle-based assays and suspension arrays and possibly also of DNA and protein microarrays.

Acknowledgments

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References

[1] R.P. Ekins, Towards immunoassays of greater sensitivity, specificity, and speed: an overview, in: A. Albertini, R. Ekins (Eds.), Monoclonal Antibodies and Developments in Immunoassay, Elsevier/North-Holland, Biomedical, Amsterdam, 1981, pp. 3–21.
[2] R.P. Ekins, Ambient analyte assay, in: D. Wild (Ed.), The Immunoassay Handbook, third ed., Elsevier, Stockton, New York, 2005, pp. 48–62.
[3] R.P. Ekins, Current concepts and future developments, in: W.P. Collins (Ed.), Alternative Immunoassays, John Wiley, New York, 1985, pp. 219–237.
[4] R.P. Ekins, F.W. Chu, E. Biggart, Development of microspot multianalyte ratiometric immunoassay using dual fluorescent-labelled antibodies, Anal. Chim. Acta 227 (1989) 73–96.
[5] R.P. Ekins, F.W. Chu, Multianalyte microspot immunoassay: microanalytical "compact disk" of the future, Clin. Chem. 37 (1991) 1955–1967.
[6] R. Ekins, F. Chu, E. Biggart, Multispot, multianalyte immunoassay, Ann. Biol. Clin. (Paris) 48 (1990) 655–666.
[7] R.P. Ekins, Ligand assays: from electrophoresis to miniaturized microarrays, Clin. Chem. 44 (1998) 2015–2030.
[8] P. Saviranta, R. Okon, A. Brinker, M. Warashina, J. Eppinger, B.H. Geierstanger, Evaluating sandwich immunoassays in microarray format in terms of the ambient analyte regime, Clin. Chem. 50 (2004) 1907–1920.
[9] D.S. Dandy, P. Wu, D.W. Grainger, Array feature size influences nucleic acid surface capture in DNA microarrays, Proc. Natl. Acad. Sci. USA 104 (2007) 8223–8228.
[10] R.P. Ekins, F.W. Chu, Binding assay employing labeled reagent, U.S. Patent 5,516,635, 1996.
[11] J.W. Silzel, B. Cercek, C. Dodson, T. Tsay, R.J. Obremski, Mass-sensing, multianalyte microarray immunoassay with imaging detection, Clin. Chem. 44 (1998) 2036–2043.
[12] J.M. Nam, C.S. Thaxton, C.A. Mirkin, Nanoparticle-based bio-bar codes for the ultrasensitive detection of proteins, Science 301 (2003) 1884–1886.
[13] L.A. Sklar, D.A. Finney, Analysis of ligand–receptor interactions with the fluorescence activated cell sorter, Cytometry 3 (1982) 161–165.
[14] J.P. Nolan, L.A. Sklar, Suspension array technology: evolution of the flat array paradigm, Trends Biotechnol. 20 (2002) 9–12.
[15] R.A. Vijayendran, D.E. Leckband, A quantitative assessment of heterogeneity for surface-immobilized proteins, Langmuir 15 (1999) 6829–6836.
[16] M. Stenberg, L. Stiblert, H. Nygren, External diffusion in solid-phase immunoassays, J. Theor. Biol. 120 (1986) 129–140.
[17] M. Stenberg, H. Nygren, Kinetics of antigen–antibody reactions at solid–liquid interfaces, J. Immunol. Methods 113 (1988) 3–15.
[18] O.G. Berg, P.H. von Hippel, Diffusion-controlled macromolecular interactions, Annu. Rev. Biophys. Biophys. Chem. 14 (1985) 131–160.
[19] M.R. Henry, P.W. Stevens, J. Sun, D.M. Kelso, Real-time measurements of DNA hybridization on microparticles with fluorescence resonance energy transfer, Anal. Biochem. 276 (1999) 204–214.
[20] J. Crank, The Mathematics of Diffusion, Clarendon, Oxford, UK, 1979. p. 102.

[9] Sensitivity is defined as the analyte concentration corresponding to a signal 2 standard deviations above the mean background signal.