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Citation

Published Version
doi:10.1021/ja211788e

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Accessibility
Measuring Binding of Protein to Gel-Bound Ligands with Magnetic Levitation

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Supporting Information Placeholder

ABSTRACT: This paper describes the use of magnetic levitation (MagLev) to measure the association of proteins and ligands. The method starts with diamagnetic gel beads that are functionalized with covalently bound small molecules (putative ligands). Binding of protein to the ligands within the bead causes a change in the density of the bead. When these beads are suspended in a paramagnetic aqueous buffer and placed between the poles of two NbFeB magnets with like poles facing, the changes in the density of the bead result in predictable and measurable changes in the levitation height of the bead. This paper uses a reaction-diffusion model to examine the physical principles that determine the values of rate and equilibrium constants measured by this system, using the well-defined model system of carbonic anhydrase and aryl sulfonamides. By tuning the experimental protocol, the method is capable of quantifying either the concentration of protein in a solution, or the binding affinities of a protein to several resin-bound small molecules simultaneously. Since this method requires no electricity and only a single piece of inexpensive equipment, it may find use in situations where portability and low cost are important, such as in bioanalysis in resource-limited settings, point-of-care diagnosis, veterinary medicine, and plant pathology.

INTRODUCTION

Bioassays that involve the binding of proteins to resin-bound small molecules are often used to screen for inhibitors of proteins, and to identify cellular targets of bioactive small molecules.¹ On-bead binding assays are also used to determine the concentrations of specific proteins in solutions.² Current methods for on-bead binding assays often use fluorescent or radioactive labels to quantify the binding of a receptor to its ligand.³ The installation and measurement of these labels often requires access to relatively expensive equipment and materials. While these methods are very broadly useful, we believe that a low-cost, label-free alternative would be valuable for many applications (for example, in point-of-care diagnosis, especially in resource-limited environments).⁴

This article describes a method for measuring the association of proteins and ligands that uses magnetic levitation (MagLev).⁵ The method employs porous diamagnetic beads that are functionalized with covalently bound small molecules (putative ligands). We suspend these diamagnetic beads in a paramagnetic aqueous solution, which is placed in a cuvette between two permanent NdFeB magnets oriented with like poles facing each other (Figure 1). The balance of gravitational and magnetic forces acting on the diamagnetic bead causes beads within a range of densities to levitate; the density of the bead determines the equilibrium position of the bead in the magnetic field.⁶ With an appropriate choice of bead, binding of protein to ligands within the bead causes a change in the overall density of the bead, and results in a change in the position at which it levitates.⁶a

Figure 1. Schematic representation of the device and method based on MagLev for detecting proteins binding to small molecules immobilized a diamagnetic bead. The device consists of two 0.4 T NdFeB magnets oriented with like poles facing each other. A diamagnetic bead suspended in a paramagnetic solution levitates in the presence of the magnetic field when the gravitational force (Fg) acting on the bead is balanced by the magnetic force (Fm). Binding of protein to the bead alters its vertical position within the device.

This analytical method has the potential to be very simple operationally. The underlying physical chemistry is, however, more complicated: it requires partitioning of protein from solution into the bead, diffusion of protein within the bead, and binding of the protein to the immobilized ligands.⁷ To examine
the fundamental principles that determine rates and equilibrium constants as measured by this system, we have developed a model that enables us to explore all of these characteristics. This model generates data in the form of density of the bead as a function of time. We use this model to define the range of physical parameters (e.g., concentration of protein, dissociation constant of the protein-ligand complex) over which we expect the assay to work. Detailed experimental work using a model system supports the validity of this analysis and leads to predictions about how the system may be improved.

The objective of this paper is to summarize a physical-organic study of a MagLev-based analytical tool for detecting protein and analyzing protein-ligand interactions, rather than to prove the generality and limits of this method in practical analysis. This study is thus aimed at defining the characteristics of the system, and building an analytical model for it, with the goal of constructing the physical-chemical foundation for future use in bioanalysis.

Our model system consists of poly[acryloylated O,O’-bis(2-aminopropyl) polyethylene glycol] (PEGA) beads that are covalently labeled with known inhibitors of bovine carbonic anhydrase (BCA; E.C. 4.2.1.1). We levitate these beads in solutions of BCA and monitor the change in levitation height — and hence the change in density of the beads — over time, as protein binds to the beads. We explain the observed kinetics using a reaction-diffusion model, and validate this model by using it to explain the effects of varying several experimental parameters (the concentration of protein, the concentration of immobilized ligand, the protein-ligand binding constant, and the size of the beads) on the rate of protein binding to the beads and the total amount of protein that binds to the beads.

This Maglev-based technique has six attributes that make it an attractive approach for detecting protein-ligand binding events: (i) It is inexpensive (requiring only a capillary tube or a cuvette filled with a paramagnetic solution, and two NdFeB magnets that cost $5–20 each). (ii) It is easy to use: the results can be visualized with the unaided eye. (iii) It is sensitive: association or dissociation of picomoles of protein to or from an appropriate bead results in an easily measurable change in its levitation height. (iv) It is quantitative: with the correct orientation and separation of magnets, the amount of protein bound per bead correlates linearly with the levitation height of the bead. (v) It can be multiplexed, and offers a method for comparing binding constants of different ligands. (vi) It enables monitoring of association and dissociation of proteins to and from beads in real time.

The technique, however, has at least five characteristics that are either of uncertain value, or are limitations: (i) The detection is carried out in non-physiological medium containing a non-natural ion at moderate ionic strength (e.g., 300 mM chelating buffer using density standard beads (see Supporting Information). We quantified the magnetic susceptibility \( \chi_{\text{Bead}} \approx \chi_m \approx 8.400 \times 10^{-5} \) and density \( \rho_m = 1.099 \, \text{g mL}^{-1} \) of this levitation buffer using density standard beads (see Supporting Information). We used two magnets separated by 45 mm in an anti-Helmholtz configuration. In this configuration the mag-
netic field strength was 0.38 T at the surface of the magnets; this value provided a dynamic range in density of 1.056 – 1.143 g·mL⁻¹.

We used a ruler with a millimeter scale to measure the distance from the bottom magnet to a levitating bead (i.e., the levitation height of the bead). Using a digital camera outfitted with a macro lens, we could measure this distance with an uncertainty of ±0.1 mm; measuring the levitation height with the unaided eye increased this uncertainty to ±0.3 mm. The greatest source of uncertainty in calculating the density of a bead from the levitation height of that bead is the measurement of its levitation height. Using a camera, we could, therefore, measure the change in density of a bead with an uncertainty of ±0.0002 g·cm⁻³; without the camera the uncertainty increased to ±0.0006 g·cm⁻³.

Choice of Model Protein-Ligand System. We defined the basic biophysical chemistry of protein binding using bovine carbonic anhydrase (BCA) as a model system because: (i) BCA is inexpensive and commercially available. (ii) Numerous inhibitors of BCA are known; many are commercially available and have well-characterized binding constants. Many inhibitors of CA contain aryl sulfonamides, which bind to the active site Zn(II) ion as anionic ligands, and several of the reported inhibitors can be covalently attached to the polymer support using standard coupling chemistry. (iii) BCA is a small protein (~ 30 kDa) and will diffuse in and out of the PEGA beads (vide supra) used in this study. (iv) BCA has a very stable tertiary structure, and is not adversely affected by the levitation media. (v) There is extensive background on the use of carbonic anhydrase in physical organic studies of protein binding. In particular values of $k_{on}$, $k_{off}$, and $k_{on/off}$ are known for a number of ligands.

Choice of Solid Support (Resin). We used commercially available poly[acryloylated O$_2$O'-bis(2-aminopropyl) polyethylene glycol] (PEGA) beads for this study (300-500 µm diameter in water). This resin is synthesized from 1900 MW PEG. These beads present amine functionality (0.2 mmol·g⁻¹); this functional group makes chemical modification straightforward. In addition, previous studies have demonstrated that PEGA beads resist non-specific adsorption of proteins.18-21 This combination of properties has resulted in their widespread use for applications including: the identification of target proteins of resin-bound small molecules,18 the screening of libraries of inhibitors,19 the synthesis of peptides20; and the covalent immobilization of proteins.21

The density of PEGA beads ($\rho_{\text{PEGA}} \approx 1.07$ g·cm⁻³) is significantly different from the density of the protein ($\rho_{\text{protein}} \approx 1.3$ – 1.5 g·cm⁻³).22 This difference in density is required if binding of protein to the bead is to cause a usefully quantifiable change in the overall density of the bead.23

The main disadvantage of these commercial PEGA beads is that their small pores (as a cross-linked acrylicamide gel) slow the mass transport of proteins into and through the interior of the bead, and excludes proteins with molecular weight greater than ~ 40-70 kDa.24 We used fluorescein-labeled BCA (FITC-BCA) to estimate both the diffusion coefficient of BCA in PEGA beads ($D_{\text{bead}} \approx 5 \times 10^{-13}$ m²·s⁻¹) and the partition coefficient of BCA between the beads and solution ($K_{\text{bead/sol}} \approx 0.4$, see the supporting information). This value for the diffusion coefficient of BCA within the bead agrees well with data from the literature,25 and is approximately two orders of magnitude slower than the diffusion coefficient in water (~ 9 x 10⁻¹¹ m²·s⁻¹).26

Unmodified PEGA beads are also difficult to visualize during levitation because their refractive index is close to that of the solution. To improve the visibility of these beads, we functionalized a small portion of the amines on them with dyes (e.g., by reaction with the isothiocyanates of rhodamine, malachite green, and 7-dimethylamino-4-methylcoumarin). These modifications make the beads easily visible under ambient or UV light.

Model for Quantifying the Amount of Protein Bound Per Bead with MagLev. Using Eqn. 1, we derived Eqn. 2 to correlate changes in the amount of protein bound to the beads and changes in the levitation height of the bead ($\Delta h$, m).

$$\Delta h = \frac{(\rho_{\text{bead}, p} - \rho_{\text{bead}}) g \mu g d^2}{(\chi_{\text{bead}} - \chi_{\text{n}}) 4 B_0^2}$$

The change in density, $\rho_{\text{bead}, p} - \rho_{\text{bead}}$ is proportional to the difference in the amount of protein present in the bead, after displacing an equivalent volume of the buffer solution out of the bead (Eqn. 3). Here, $[P]$ is the concentration of protein within the bead (M), $MW_{\text{protein}}$ (g·mol⁻¹) and $V_{\text{protein}}$ (m³) are the molecular weight and volume of a protein molecule, $N_A$ (mol⁻¹) is the Avogadro constant, and $\rho_{\text{sol}}$ (kg·m⁻³) is the density of the solution. Using this relationship, $\Delta h$ can be expressed linearly in terms of $[P]$ (Eqn. 4).

$$\rho_{\text{bead}, p} - \rho_{\text{bead}} = [P](MW_{\text{protein}} - V_{\text{protein}} N_A \rho_{\text{sol}})$$

$$\Delta h = [P] \frac{(MW_{\text{protein}} - V_{\text{protein}} N_A \rho_{\text{sol}}) g \mu g d^2}{(\chi_{\text{bead}} - \chi_{\text{n}}) 4 B_0^2}$$

BCA has a molecular weight of 29.1 kg·mol⁻¹ and a volume of $3 \times 10^{-26}$ m³ (the protein is assumed to be a sphere with radius ~ 20 Å).27 Eqn. 4, therefore, predicts that, under our standard levitation conditions, a 1-mM increase in the concentration of protein within a bead will result in a 5-mm decrease in levitation height. Experimentally, we obtained a similar value ($\Delta h/[P] = -8.6$ mm/M, see Supporting Information); the difference between these experimental and theoretical values is likely due to inaccuracies in the volume of the protein, $V_{\text{protein}}$, and/or measurements of the volume of the beads.

Model for Kinetic Analysis of Binding. MagLev enables the detection of a change in density that occurs when a soluble protein associates with or dissociates from a ligand that is covalently immobilized on a gel or solid support (provided that there is a difference in density between the protein and the gel/solid support). We model the binding of protein to ligands immobilized in a gel matrix by a three step process (Figure 2A): (i) partitioning of the protein from solution to the gel (thereby displacing an equivalent volume of buffer solution from the bead into the bulk solution), (ii) diffusion of protein within the gel, (iii) binding and unbinding of protein to the ligands immobilized in the gel.

Before each experiment, the gel beads are first equilibrated with a buffered solution of Gd(DTPA). Using a magnetic susceptibility balance, we found that the magnetic susceptibility of these equilibrated beads is similar to the magnetic susceptibility of the buffer; this observation indicates that the concentration of Gd(DTPA) in solution is approximately the same both inside and outside the bead (see Supporting Information). At the beginning of each experiment ($t = 0$), the beads are
transferred to a solution of protein dissolved in the same Gd(DTPA) buffer. The process that follows, involving protein diffusing into the beads and binding to the ligands immobilized there, can be described mathematically as follows.

The partitioning of the protein between the bead and the solution is described by the partition coefficient, $K_{\text{bead/sol}}$ (Eqn. 5), where $[P]_{\text{bead}}$ denotes the concentration of proteins at the external boundary of the bead and $[P]_{\text{sol}}$ denotes the concentration of protein in the bulk solution. Once inside the bead, the protein, $P_{\text{bead}}$, diffuses toward the center of the bead, reacting with the immobilized ligands, $L$, to form protein-ligand complexes, $PL$, according to Eqn. 6 (throughout this text, * is used to indicate a species immobilized on the gel).

$$K_{\text{bead/sol}} = \frac{[P]_{\text{bead}}}{[P]_{\text{sol}}}$$  \hspace{1cm} (5)

$$P_{\text{bead}} + *L \xrightarrow{k_{\text{off}}} *PL$$  \hspace{1cm} (6)

**Figure 2.** Reaction-diffusion model describing the binding of protein from solution to ligands immobilized in a gel bead. (A) The model consists of three distinct steps: (i) partitioning of the protein into the bead, (ii) diffusion within the bead, and (iii) binding of protein to the ligands immobilized on the bead. (B) In a diffusion-limited process (fast/instantaneous reaction), reaction only occurs at the propagating front, $I_F$, when the flux of protein (before the front) encounters the unreacted ligands at the front. This approximation of the diffusion-limited process allows us to derive a simplified characteristic time, $\tau$, for the reaction-diffusion process.

Together, the dynamics of all these three steps can be expressed mathematically in terms of a system of reaction-diffusion equations (Eqns. 7, 8). In these equations, $r$ is the radial coordinate of the bead, $t$ is time, and $D_{\text{bead}}$ is the diffusion coefficient of the protein in the bead.

$$\frac{\partial[P]_{\text{bead}}(r,t)}{\partial t} = D_{\text{bead}} \frac{\partial^2 [P]_{\text{bead}}(r,t)}{\partial r^2}$$  \hspace{1cm} (7)

$$\frac{\partial[L](r,t)}{\partial t} = -k_{\text{on}}[P]_{\text{bead}}(r,t)[*L](r,t) + k_{\text{off}}[*PL](r,t)$$  \hspace{1cm} (8)

The initial parameters (at $t = 0$) are defined as follows: (i) The concentration of ligand within the bead, $[*L]_0$, is defined as $[*L]_c$. (ii) The concentration of protein in solution ($[P]_{\text{sol}}$) is equal to $[P]_{\text{sol}}$. (iii) There is no protein within the bead ($[P]_{\text{bead}} = 0$). (iv) At the external boundary of the bead, the concentration of protein is described by the partition coefficient and the concentration of protein in solution (Eqn. 9).

$$[P]_{\text{bead}} = K_{\text{bead/sol}}[P]_{\text{sol}}$$  \hspace{1cm} (9)

The volume of the bead is assumed to be constant, and therefore that $[*PL] = [*L]_0 - [*L]$, Eqn. 10 describes the process of penetration of protein into the bead at its external boundary. In this equation, $A$ is the surface area of the bead, $r$ is the radial coordinate, $V_{\text{sol}}$ is the volume of solution and $N_B$ is the number of beads used.

$$-D_{\text{bead}} \frac{\partial[P]_{\text{bead}}(r,t)}{\partial r}|_{r=R} = \frac{\partial[P]_{\text{sol}}}{\partial t} \frac{V_{\text{sol}}}{N_B}$$  \hspace{1cm} (10)

In analogy to our experiments, we used the following parameters for our calculations: (i) The radius of each bead, $R$, is 170 \mu m. (ii) There are 15 beads in each experiment ($N_B$). (iii) The concentration of ligand within the bead, $[*L]_c$, is 3.2 mM. (iv) The initial concentration of ligand in the solution, $[P]_{\text{sol}}$, is 0.6 mM. (v) The diffusion coefficient, $D_{\text{bead}}$, is $\sim 10^{-12}$ m$^2$s$^{-1}$, as observed in a previous study and confirmed experimentally by us (see Supporting Information). Solving this system of equations using finite-difference method in spherical coordinates (implemented in Matlab), gives the concentration profiles of protein, ligand and bound protein-ligand complex with respect to both space and time. Integrating the concentration profile of bound protein-ligand throughout the bead gives the total amount of protein-ligand complex in the bead, $n_{PL}$ (Eqn. 11).

$$n_{PL} = 4\pi R^2 \int_0^R [*PL] r^2 dr$$  \hspace{1cm} (11)

Experimentally, we have determined that the change in levitation height, $\Delta h$ (mm), is proportional to $n_{PL}$ (mmol) and inversely proportional to the volume of the bead, $V_{\text{bead}}$ (L) according to the relationship $\Delta h = 8.6 n_{PL}/V_{\text{bead}}$ (see Supporting Information; Eqn. 4 provides a theoretical approximation of this relationship).

**The Characteristic Time, $\tau$, of the Reaction-Diffusion Process.** We describe here our derivation of a characteristic time parameter, $\tau$, which describes the time required for the reaction-diffusion process to reach equilibrium; we begin by proving that this process is diffusion-limited. Eqn. 12 describes the characteristic time for the binding of protein to ligands within a bead, $t_{\text{res}}$. In this expression, $k_{\text{off}}$ (M$^{-1}$s$^{-1}$) is the reaction rate constant and $[P]_{\text{bead}}$ is a characteristic concentra-
tion of protein in the bead; this concentration can be represented by the product of the partition coefficient, $K_{\text{bead/sol}}$, and the initial concentration of protein in the solution, $[P]_{0\text{sol}}$. Based on the known values of $k_{\text{on}} \approx 10^7 \text{M}^{-1}\text{s}^{-1}$, $K_{\text{bead/sol}} = 0.4$, and $[P]_{0\text{sol}} \approx 100 \mu\text{M}$, the characteristic time ($\tau_{\text{on}}$) for the forward reaction is, therefore, $\approx 0.25 \text{s}$. Eqn. 13 describes the characteristic time for diffusion of the protein within the bead ($\tau_{\text{diff}}$).

$$\tau_{\text{on}} \approx \frac{1}{k_{\text{on}}[P]_{\text{bead}}} \approx \frac{1}{k_{\text{on}}K_{\text{bead/sol}}[P]_{0\text{sol}}}$$

(12)

$$\tau_{\text{diff}} \approx R^2 / D_{\text{bead}}$$

(13)

Given the diffusion coefficient of the protein in the bead, $D_{\text{bead}} \approx 10^{-12} \text{m}^2\text{s}^{-1}$, and a bead of radius $R \approx 170 \mu\text{m}$, we estimate $\tau_{\text{diff}}$ to be on the order of 8 hours. Since $\tau_{\text{diff}} > \tau_{\text{on}}$ (8 hrs $\gg 0.25$ s), the reaction-diffusion process is diffusion-limited. We, therefore, make the following two assumptions in order to derive a simple, analytical expression for the characteristic time of the reaction-diffusion process: (i) the reaction to form the protein-ligand complex is instantaneous in comparison to the rate of diffusion, and (ii) once formed, the protein-ligand complex, *PL, does not dissociate (the rate of dissociation is typically much slower than the rate of association). Given these assumptions, there exists a propagation front of the protein (Figure 2B), that is a boundary where the concentration of diffusive protein penetrating into the bead is zero. At the propagation front, the incoming flux of protein is immediately consumed to form the protein-ligand complex (Eqn. 6) according to Eqn. 14.

$$D_{\text{bead}} A_F \left( \frac{\partial [P]_{\text{bead}}}{\partial r} \right)_{l_F} \text{d}t = [L]_0 A_F \text{d}l_F$$

(14a)

$$\frac{\text{d}l_F}{\text{d}t} = \frac{D_{\text{bead}} \left( \frac{\partial [P]_{\text{bead}}}{\partial r} \right)_{l_F}}{[L]_0}$$

(14b)

In this equation, $r$ is the radial coordinate, $A_F$ is the cross-sectional area of the front, $l_F$ is the radial position of the front, and $\text{d}l_F/\text{d}t$ is the velocity of the propagating front. We derive an estimate of the term $\left( \frac{\partial [P]_{\text{bead}}}{\partial r} \right)_{l_F}$ by considering the region before the propagating front, where the incoming protein is subject only to diffusion. In this region, we can apply a pseudo-steady approximation to the concentration profile of diffusing protein (Eqn. 15).

$$\frac{1}{r^2} \frac{\partial}{\partial r} \left( r^2 \frac{\partial [P]_{\text{bead}}}{\partial r} \right) = 0$$

(15)

This approximation assumes that the concentration profile of diffusing protein is established much more rapidly than the propagating front moves (this approximation is reasonable when $[P]_{0\text{sol}}K_{\text{bead/sol}}[*L]_0 \ll 1$, as is the case in our system). We derive the average value $\langle 1/r^2 \partial [P]_{\text{bead}}/\partial r \rangle_{l_F}$, across the bead by solving Eqn. 15 using the following boundary conditions: at $r = R$, $[P]_{\text{bead}} = [P]_{0\text{sol}}K_{\text{bead/sol}}$, and at $r = l_F$, $[P]_{\text{bead}} = 0$ (Eqn. 16).

$$\frac{\langle 1/r^2 \partial [P]_{\text{bead}}/\partial r \rangle_{l_F}}{[L]_0} = \frac{R}{6D_{\text{bead}}[P]_{0\text{sol}}K_{\text{bead/sol}}}$$

(16)

For our system, the characteristic time $\tau$ of the reaction-diffusion process is proportional to the radius of the bead $R$ over the average velocity of the propagating front, $\text{d}l_F/\text{d}t$ (Eqn. 17). Finally, combining Eqns. 14, 16, and 17 yields an expression that describes the characteristic time $\tau$ as a function of a number of experimental variables (Eqn. 18).

$$\tau \sim \frac{R}{\langle 1/r^2 \partial [P]_{\text{bead}}/\partial r \rangle_{l_F}} \frac{\text{d}l_F/\text{d}t}{l_F}$$

(17)

$$\tau = \frac{R^2[L]_0}{6D_{\text{bead}}[P]_{0\text{sol}}K_{\text{bead/sol}}}$$

(18)

This expression indicates that the time required for the system to reach steady-state is proportional to the square of the radius of the beads, $R^2$, the initial concentration of the ligands in the bead, $[L]_0$, and is inversely proportional to the diffusion coefficient, $D_{\text{bead}}$, the initial concentration of protein in solution, $P_{0\text{sol}}$, and the partition coefficient, $K_{\text{bead/sol}}$.

**RESULTS AND DISCUSSION**

This section outlines a detailed physical-organic study of a MagLev-based analytical tool that can be used for (i) quantifying the amount of protein bound to beads, and (ii) monitoring the kinetics and thermodynamics of association of proteins with resin-bound small molecules. We use this physical-organic study to identify the parameters that ultimately influence the function and practicality of this tool.

Unless specified otherwise, all MagLev measurements were performed in a solution of 300 mM Gd(DTPA), and 0.05% polysorbate 20, dissolved in 6-mM phosphate buffer and adjusted to pH 7.4 using sodium hydroxide (referred to throughout this text as the ‘standard levitation buffer’). This composition provided a buffer with an approximate match in density to that of the PEGA beads. The chosen concentration of gadolinium(III) provided a useful compromise between sensitivity and dynamic range of detection by MagLev. Addition of the non-denaturing surfactant, polysorbate 20, reduced non-specific binding of proteins to the PEGA beads, and prevented the beads from adhering to the cuvette.

MagLev provides a spectroscopy-free method of measuring the binding of protein to ligand-functionalized PEGA beads. Initially, we verified that protein would adsorb to PEGA beads only when these beads were functionalized with a small-molecule ligand that bound specifically to that protein. We reasoned that the binding of most proteins (average density $\approx 1.3–1.5 \text{ g} \cdot \text{cm}^{-3}$) to PEGA beads (density $\approx 1.07 \text{ g} \cdot \text{cm}^{-3}$) should increase the density of the beads, and result in quantifiable changes in levitation height between bound and unbound states.

Throughout these studies, we used a well-characterized system of proteins and ligands: bovine carbonic anhydrase (BCA) and derivatives of benzenesulfonamide. We immobilized 4-carboxybenzene sulfonamides 1 and 2 on Rhodamine-dyed PEGA beads using standard coupling chemistry (Eqn. 20, EDC = N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide, NHS = N-hydroxysuccinimide; see Supporting Information for more information).
Sulfonamide 1, when bound to resin, is expected to bind tightly to BCA (the $K_d$ of an analogous ligand in solution is 0.7 µM, see Supporting Information). Sulfonamide 2 should not associate measurably with BCA,16 this functionalized bead serves as a control. The PEGA beads we used have a density of amine groups of 0.2 mmol·g$^{-1}$ (when dry); this value gives a final concentration of ligand within the bead of 1 - 10 mM (when swollen in water).

We observed only a very small decrease (< 0.1 mm) in the levitation height of beads functionalized with 4-dimethylsulfamoylbenzoic acid 2 after incubation with 0.33 mM BCA for 7 days. In contrast, we observed a time- and concentration-dependent change in the levitation height of beads functionalized with sulfonamide 1.

At least one hour before each experiment, the PEGA beads were placed in a solution of the standard levitation buffer. We then transferred seven to ten beads functionalized with either sulfonamide 1 or 2 into a microcuvette containing 0.6 mL of a solution of BCA in the standard levitation buffer. We observed only a very small decrease (< 0.1 mm) in the levitation height of beads functionalized with 4-dimethylsulfamoylbenzoic acid 2 after incubation with 0.33 mM BCA for 7 days. In contrast, we observed a time- and concentration-dependent change in the levitation height of beads functionalized with sulfonamide 1.

We plotted the change in levitation height of beads functionalized with sulfonamide 1 ($\Delta h$, in millimeters) versus time ($t$, in hours) for each concentration of BCA and fit these data to our model of the reaction-diffusion process (Eqns. 7, 8). The comparison demonstrates good agreement (Figure 3A). We estimate the dissociation constant from the model to be $K_d \approx 1.5$ µM. We note that since the process of protein binding to ligands within the beads is diffusion-limited (see the Section on characteristic time for further discussion), we can only determine $K_d$, and not the on and off rates ($k_{on}$ and $k_{off}$).

Eqn. 21 presents an alternative method of calculating the dissociation constant using the equilibrium conditions of the reaction summarized in Eqn. 6, and assuming that protein is in excess.

$$K_d = K_{bead/sol} [P]_{0,sol} \frac{[PL]_{eq} - [PL]_{eq}}{[PL]_{eq}}$$

Experimentally we found that with $[P]_{0,sol} = 100$ µM, $[PL]_{eq} = 3.2$ mM and $K_{bead/sol} = 0.4$, the final change in height of the beads is 26.5 mm; this value indicates that the $[PL]_{eq}$ is 3.08 mM (Eqn. 4). Substituting these values into Eqn. 21 gives a dissociation constant, $K_d$, of 1.5 µM.

These results demonstrate that this MagLev-based method provides reasonable estimates of binding constants for BCA with benzenesulfonamides, without the need for spectroscopy. The small difference between the measurements using Mag-Lev and more conventional spectroscopic means may be due to an increase in the protein-ligand dissociation constant as a result of immobilization of the ligand. We previously found that the observed dissociation constants of BCA with ligands in solution are ~ 10 times lower than the dissociation constants of the corresponding ligands presented on the surface of a monolayer.

The system reaches a state of equilibrium more rapidly when higher concentrations of protein in solution and/or lower concentrations of ligand within the beads are used. Based on our derivation of the characteristic time, $\tau$, we hypothesized that both increasing the concentration of protein in solution, and decreasing the concentration of ligand within the bead, should increase the rate at which the bead would reach an equilibrium concentration of protein and protein-ligand complex ($\tau \sim [L]_0/[P]_{0,sol}$, Eqn. 18). In addition to decreasing the assay time, experimenting with these parameters would also allow us to test the validity of our model. To vary the concentration of ligand within the beads, we combined benzene sulfonamides 1 and 2 in molar ratios of 1:0, 1:2, 1:8, 1:26, and 1:80. We used these mixtures to synthesize batches of PEGA beads containing different concentrations (3.2 - 0.040 mM) of benzene sulfonamide (Eqn. 22).
We placed 7 – 10 of each batch of beads in a microcuvette containing a solution of 100 μM bovine carbonic anhydrase (BCA) dissolved in the standard levitation buffer and plotted the change in levitation height (Δh, in millimeters) versus time (t, in hours) for each concentration of ligand (Figure 3B). Fitting these data to our reaction-diffusion numerical integration model (Eqs. 3, 10) revealed the expected correlations: lower concentrations of ligand within the bead resulted in smaller changes in levitation height (Δh = h₀ - h₁); the equilibration time was, however, inversely proportional to the concentration of ligand in the bead.

In order to verify these results further, we repeated the same experiment with several different concentrations of BCA. Each plot was fit to a first-order exponential curve (Eqn. 19) from which we derived the calculated characteristic times, τ, by fitting a plot of levitation height versus time to Eqn. 18. The combined data substantiate our derivation of the characteristic time (Eqn. 17) by illustrating the predicted linear relationships between (A) the characteristic time τ and the concentration of ligand within the bead (expressed as the percentage of the bead functionalized with sulfonamide 1), and (B) the characteristic time τ and one over the concentration of protein in solution (1/[BCA]₀,sol).

Figure 4. Effect of the concentration of protein in solution, and the ligand concentration within the beads, on rates of equilibration. We calculated each characteristic time, τ, by fitting a plot of levitation height versus time to Eqn. 18. The combined data substantiate our derivation of the characteristic time (Eqn. 17) by illustrating the predicted linear relationships between (A) the characteristic time τ and the concentration of ligand within the bead (expressed as the percentage of the bead functionalized with sulfonamide 1), and (B) the characteristic time τ and one over the concentration of protein in solution (1/[BCA]₀,sol).

Figure 5. Effect of the radius of the bead on the rate of binding of BCA to ligand-functionalized beads. We levitated functionalized PEGA beads in cuvettes in the presence of 130 μM BCA dissolved in the standard levitation buffer. We plot the average levitation height for eight large beads (<R> = 166 ± 5 μm), and eight small beads (<R> = 136 ± 7 μm) versus time, and show the predictions of the reaction-diffusion model (the dotted and dashed lines).

The rate of equilibration is faster for smaller beads. PEGA beads, as supplied commercially, span a range of sizes. For our studies, beads purchased from Polymer Laboratories were reported to range in diameter from 300 – 500 microns (when swollen in water). To quantify the effect of bead size on the rate of binding of protein to ligands within the bead, we isolated the largest and smallest beads by filtering through several sizes of mesh (see Supporting Information).

We levitated eight large beads (average radius of 166 ± 5 microns) and eight small beads (average radius of 136 ± 7 microns) in a solution of 130-μM BCA dissolved in the standard levitation buffer. Figure 5 shows the comparison of the change in levitation height (Δh, in millimeters) versus time (t, in hours) for each batch of beads. When these data were fit to first-order exponential curves (Eqn. 19), the calculated characteristic times, τ, were 14.6 ± 2.7 hours for the small beads, and 26.9 ± 2.1 hours for the large beads. This ratio is in accord with our prediction of the relationship between the radius of the bead (R) and t (τ ~ R⁻²; Eqn. 18).

MagLev enables multiple binding interactions to be monitored simultaneously. MagLev provides the basis for an on-bead assay that can measure the affinity of multiple different resin-bound small molecules for the same protein. To make it possible to differentiate resin-bound inhibitors visually, we functionalized a small portion of the available amines on PEGA beads with one of five different dyes (isothiocyanates of rhodamine, fluorescein, malachite green, eosin and 7-dimethylamino-4-methylcoumarin). Using standard coupling chemistry, we attached five different ligands (4-
carboxybenzene sulfonamide, 4-(isothiocyanatomethyl) benzzenesulfonamide 3-carboxybenzene sulfonamide, 3-carboxy-4-methoxybenzene sulfonamide, and 4-dimethylsulfamoylbenzoic acid) of carbonic anhydrase to the differently colored beads.

We then suspended these five color-coded beads, each functionalized with a different inhibitor of BCA, (~15 beads per inhibitor) in a microcuvette containing a solution of 100-µM BCA dissolved in the standard levitation buffer. Initially, the beads all levitate at nearly the same height (Figure 6). In the presence of BCA, the beads begin to sink, and begin to segment based on binding affinities. The relative levitation height of the beads represents the relative differences in binding affinities of the ligands attached to the beads; the bead containing the ligand with the lowest dissociation constant for BCA levitates at the lowest value of h because it has more protein bound to it than the other two beads. Using Eqn. 21, we calculated the dissociation constants of the immobilized ligands from BCA. We also measured the dissociation constants of analogous ligands in solution using fluorescence (see Supporting Information). A comparison of the values measured using both methods demonstrates good agreement (Figure 6).

At the beginning of the experiment, the beads form relatively tight clusters because they have equal densities. As the beads approach 50% equilibration, however, they become increasingly disperse. We hypothesize that this dispersion results from differences in the percentage of ligands that are bound to protein as a result of differences in the size of the beads. As the beads approach their final levitation height, their dispersion in levitation heights decreases because each bead contains the same concentration of ligand, and the final equilibrium levitation height ultimately depends only on the density of the bead, not on its radius.

![Figure 6](image)

**Figure 6.** Using MagLev to measure the binding of BCA to several immobilized ligands. This figure presents a series of photographs showing changes in levitation height over time that result from incubating PEGA beads functionalized with five different inhibitors of BCA in the presence of 100 µM BCA dissolved in the standard levitation buffer. We used Eqn. 21 to calculate the dissociation constants of the immobilized ligands from BCA; also shown, in parentheses, are the dissociation constants of analogous ligands in solution from BCA (see Supporting Information).

MagLev is a useful tool for quantifying the concentration of a specific protein. Methods for the detection of small amounts (nmol – fmol) of protein have revolutionized biotechnology, and comprise some of the most heavily used methods for the diagnosis of disease. In order to adapt the MagLev-based methods described in this paper to the detection of small amounts of protein, we utilize a semi-kinetic approach. We begin by incubating a single bead in a solution of a target protein. To provide optimal detection of small amounts of protein, we fully functionalize this bead with a ligand that binds tightly to the target protein. Specifically, we use PEGA beads functionalized with sulfonamide 1 to detect BCA.

With the goal of detecting low concentrations of protein, it is beneficial to maximize the sensitivity of the system to changes in the density of the bead by performing measurements of levitation height using the lowest practical concentration of paramagnetic salt. For these measurements, we, therefore, utilize a lower concentration of Gd(DTPA) than what is present in the standard levitation buffer: 150 mM Gd(DTPA) provides a practical compromise between sensitivity and equilibration time. With this concentration of Gd(DTPA), the beads reach their equilibrium levitation height in ~30 minutes and we are able to differentiate between differences in density of ± 0.0004 g·cm⁻³. With lower concentrations of Gd(DTPA) sensitivity is increased, but the beads take significantly longer to reach their equilibrium levitation height. Future studies will focus on increasing the rate of adsorption of protein by using smaller beads, lower concentrations of ligand within the beads, or beads with larger pore volumes.

Reaching an equilibrium state at low concentrations of protein requires an impractically long time (for example, a bead functionalized with sulfonamide 1 requires more than a thousand hours to equilibrate fully with a 10-µM solution of BCA). A calibration curve, however, can easily be generated to relate the change in levitation height after a given period of time to the concentration of protein in the sample. Figure 7 demonstrates this relationship.

To generate the data in Figure 7A, we incubated individual beads, functionalized with sulfonamide 1, in solutions (20 µL) of BCA (40 nM - 40 µM) dissolved in 10 mM PBS at room temperature (20 °C), for either 3 or 24 hours. We then transferred these beads into a microcuvette containing a solution of 150 mM Gd(DTPA) and 200 mM sucrose dissolved in 6 mM phosphate buffer and adjusted to pH 7.4 with NaOH (referred to hereafter as the ‘high sensitivity levitation buffer’), and measured their levitation height (in comparison to the levitation height of a bead that had been incubated in a solution free of BCA). After three hours, beads that have been incubated in a 600 nM solution of BCA can be distinguished from beads that have been incubated in a solution free of BCA; after 24 hours the detection limit is lowered to 300 nM. Notably, this method requires only 20 µL of sample (the volume of blood in a typical finger prick).

The initial rate of protein absorption correlates linearly with the concentration of protein in solution (τ ~ 1/[P]₀, Eqn. 18); as the ligands become saturated with protein, however, the rate of net protein diffusion into the beads decreases. The result is that at short incubation times, there is an approximately linear relationship between the concentration of protein in the incubation solution and the amount of protein adsorbed on the bead, while at longer incubation times this simple correlation no longer holds true (at very long incubation times the system...
approaches equilibrium, where binding is described by Eqn. 21).

**CONCLUSION**
MagLev provides a new way to detect and measure specific protein-ligand interactions. Its simplicity and independence of electricity and infrastructure suggest that, with development, it has the potential to be useful in resource-limited environments. In this method, the binding of protein to ligands immobilized within a gel bead results in a change in the density of the bead. MagLev allows the density of the bead to be monitored in real time with high accuracy, and therefore provides a direct readout of the amount of protein bound to the bead.

We have developed a mathematical model of the reaction-diffusion processes that result in the binding of protein to ligands immobilized within the bead. This model provides good quantitative agreement between theory and experiment. The magnitude of the change in levitation height as a function of time depends on a number of controllable parameters including: (i) the concentration of protein in solution, (ii) the structure, concentration, and binding constant of the ligand immobilized in the bead, and (iii) the size of the bead. The rate of change in levitation height also depends on a number of factors that are more difficult to manipulate, such as: (i) the partition coefficient of the protein between the bead and the bulk solution, and (ii) the diffusion coefficient of protein within the bead. We developed Eqn. 17, which describes the dependence of the characteristic time, $\tau$, for protein binding to ligands immobilized within gel beads, on all of the reaction parameters. This equation, therefore, allows the performance of the system to be predicted, under a variety of conditions.

Overall, the MagLev-based method for detecting protein-ligand binding has a number of significant advantages: (i) This method requires no electricity, and only a single piece of equipment. It may, therefore, find use in situations where portability and low cost are a high priority; for example, in point-of-care diagnosis, especially in resource-limited, military, and point-of-care environments, or in veterinary medicine, plant pathology, and food safety. (ii) It is easy to use; results can be visualized with the naked eye. (iii) It has the capability to monitor binding accurately in real time. This attribute provides a quantitative means of determining the concentration of a protein in complex biological medium such as blood without the need for initial separation or purification steps.
specific protein in solution. (iv) It can qualitatively distinguish the binding affinities of a protein to several resin-bound small molecules simultaneously. In analogy, MagLev should also be capable of simultaneously measuring the binding of multiple proteins to an array of resin-bound small molecules. This property should allow for the ready design of multiplexed binding assays. (v) Protein-ligand binding directly results in a change in levitation height. This method, therefore, does not require intermediate reagents, such as enzyme-linked secondary antibodies.

In its present form, this method also has several disadvantages, but there are clear development paths to reduce or eliminate these issues. These disadvantages include: (i) The kinetics of protein-ligand binding are relatively slow (on the order of several days). This problem may potentially be resolved through the design and synthesis of beads with improved characteristics (e.g., larger pore size). (ii) This method is only applicable to ligands that are amenable to chemical immobilization on a gel support; this limitation, however, is inherent to most detection methods that occur on solid support. (iii) This method is currently not applicable to large proteins, most notably antibodies, due to the restricted pore size of PEGA beads.

ASSOCIATED CONTENT
Supporting Information Available: General methods and additional experimental information. This material is available free of charge via the Internet at http://pubs.acs.org.

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ACKNOWLEDGMENT
This work was partially supported by the Bill & Melinda Gates Foundation (no. S1308), the Wyss Institute of Biologically Inspired Engineering, the U.S. Department of Energy (no. DE-FG02-00ER45852, funding used for the development of the multiplexed assays), and postdoctoral fellowships from the Damon Runyon Cancer Research Foundation (STP) and NIH (STP and NDS).

ABBREVIATIONS
MagLev, Magnetic Levitation; BCA, bovine carbonic anhydrase; EDC, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide; NHS, N-hydroxysuccinimide; PEGA, poly[acryloylated O,O'-bis(2-amino propyl) polyethylene glycol].

REFERENCES


(8) Mammalian cells typically have intracellular ionic strengths on the order of 200-300 mM, and contain ~200 mg/mL protein, see: Storey, K. B. Functional Metabolism: Regulation and Adaptation; John Wiley & Sons: Hoboken, NJ, 2004, p.403.


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(23) It would also be possible to use beads with density greater than the density of protein. This experimental design, however, would require that the levitation media contain a diamagnetic cosolute of high density in order to match the density of the levitation media ($\rho_m$) with the density of the bead.


(29) Previous studies have demonstrated that protein binding to PEGA beads does not significantly change the volume of the beads, see ref 25.


(31) Although both bound and unbound protein increase the density of the bead, the influence of the unbound protein is negligible because its concentration is significantly lower than the concentration of the bound protein.


(34) We monitored the levitation height of beads functionalized with 100% sulfonamide 1 in the presence of 10-μM BCA for more than 500 hours (3 weeks). Even after this extended period of time, protein-ligand binding had not yet reached its equilibrium state. This fact hindered our ability to calculate accurately the characteristic time for this system; as a result, we have excluded this datum point from further analysis.

(35) The small variations in the initial levitation heights of the color-coded beads are likely due to differences in the densities of the covalently immobilized ligands, see ref 6b.


(37) The plot shown in Figure 7A is also presented in the supporting information with a logarithmic concentration axis.
Magnetic levitation enables label-free quantification of protein-ligand binding, visualized with the naked-eye, monitored in real time, and without electricity.