Protein–Ligand Interactions

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# Assessment of Molecular Interactions through Magnetic Relaxation**

*Oscar J. Santiesteban, Charalambos Kaittanis, and J. Manuel Perez**

Molecular interactions, particularly those involving a ligand and a protein, play a vital role in multiple biological processess.[1–5] The study of these interactions, notably those associated with diseases, is important for the development of novel therapeutics and sensing technologies. These interactions are typically evaluated by a dissociation constant ($K_D$), an equilibrium constant that describes how strongly a ligand binds to a particular target protein, which is determined by measuring the propensity of the ligand to dissociate from the protein. The smaller the $K_D$ value, the higher the affinity between the ligand and protein. Techniques such as surface plasmon resonance (SPR),[1] titration calorimetry,[6,7] and radioligand binding assays[8] among others have been developed to assess these molecular interactions and measure $K_D$ values. However, these techniques have some drawbacks. For example, SPR requires the binding of either the ligand or the protein to a solid support, which affects the binding kinetics and it is not representative of the binding affinity in solution. Titration calorimetry and radioligand assays, even though they are performed in solution, use either expensive instrumentation, radioactive materials, or solubilized receptors instead of living cells. Furthermore, as most reported $K_D$ values have been determined using different methods, comparative studies are difficult as variations in $K_D$ values can be seen using different techniques.[9]

The conjugation of targeting ligands to iron oxide nanoparticles has been extensively utilized to fabricate nanosensors and targeting imaging agents for the detection of various molecular targets.[10–13] In particular, we recently reported that the binding of a protein to a ligand attached to magnetic iron oxide nanoparticles resulted in an increase in the spin–spin relaxation times ($T_2$) of the water protons in solution.[14] This observation facilitated the development of magnetic relaxation nanosensors that can quantitatively sense the presence of a target by measuring the increase in the water $T_2$ upon target binding. To differentiate our nanosensors from previously described magnetic nanosensors that cluster upon target addition resulting in a decrease (not an increase) in $T_2$, we herein denote our nanosensors as binding magnetic relaxation (bMR) nanosensors and explore their utility as sensors to interrogate molecular interactions. Specifically, we hypothesized that a targeting bMR nanosensor can be used in a competition assay format to determine the $K_D$ of a particular molecular interaction as it occurs on the surface of a nanoparticle.

In our magnetic-relaxation-based competition assay, a constant amount of bMR nanosensors in a series of aqueous solutions containing increasing amounts of free competing ligand is mixed with the targeting protein. In the absence of free ligand, the target protein binds to ligands on the bMR nanosensors, causing an increase in the $T_2$ of the solution. This change in $T_2$ represents the initial state of our assay and herein we denote it as $\Delta T_{2(\text{initial})}$. The addition of increasing amounts of the free ligand (competitor) in the sample lowers the observed change in $T_2$, as the added free ligands in solution compete with the bMR nanosensors for binding to the target protein. We denote this change in $T_2$ in the presence of a competitor as $\Delta T_{2(\text{competitor})}$. As the amount of free ligand increases, the change in the magnetic relaxation signal ($\Delta MR$ signal) defined by the equation shown in Scheme 1, increases, reaching a plateau when the $\Delta MR$ signal approaches a value of one (Scheme 1). The initial $\Delta MR$ signal in the absence of a competitor will be zero, since there is no competing ligand present in the sample to compete for binding to the target protein. Thus, low competitor concentrations will yield low $\Delta MR$ signals while high competitor concentrations will cause a disruption in the interaction between the target protein and the bMR nanosensors causing larger $\Delta MR$ signal values.

Therefore, as the concentration of the competitor increases in the system, the values of the $\Delta MR$ signal are expected to increase, reflecting the competition that the free ligand is effecting on the system. The concentration of free ligand at which a 50% change in the MR value is observed is then defined as the dissociation constant. Specifically at this concentration, an equilibrium is achieved, where 50% of the target protein interacts with a free ligand and the remaining portion associates with the nanosensors, inducing quantifiable MR changes.

In order to test our assay, we determined the $K_D$ values of a broad range of protein–ligand interactions with different degrees of affinities by measuring the magnetic relaxation upon binding. As a model system, we first examined the avidin–biotin interaction, a well-studied strong interaction, with reported $K_D$ values in the femtomolar range.[15] For these studies, we designed a bMR nanosensor consisting of avidin-conjugated iron oxide nanoparticles (76 nm and an $r_2$ relaxivity of 116 $mM^{-1}s^{-1}$ at 0.47 T). Samples containing the bMR nanosensor (0.015 mg Fe $mL^{-1}$) in a solution containing different concentrations (10 fM–1 pM) of free avidin (competitor) were prepared, followed by the addition of biotin (200 nM). Within 15 min, $T_2$ measurements and calculation of the $\Delta MR$ signal values at various concentrations of inhibitor

[*] O. J. Santiesteban,[+] Dr. C. Kaittanis,[+] Prof. J. M. Perez
Nanoscience Technology Center, Department of Chemistry
University of Central Florida
12424 Research Parkway, Suite 400, Orlando, FL 32826 (USA)
E-mail: jmperez@ucf.edu
Homepage: htpp://www.nanoscience.ucf.edu/faculty/perez.php
[+] These authors contributed equally to this work.

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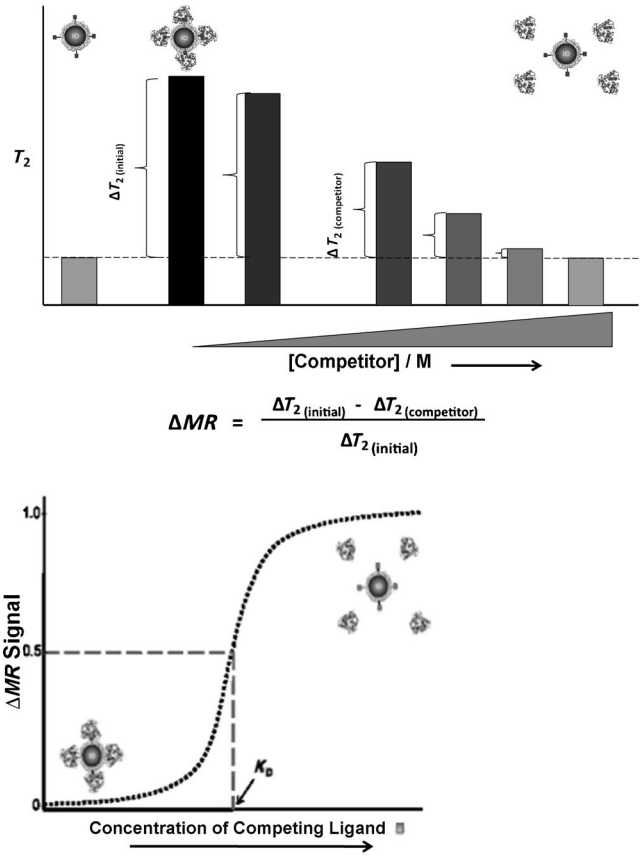


***Scheme 1.*** Approach for the determination of the dissociation constant ($K_D$) through changes in magnetic relaxation. In the absence of a competing ligand, the target protein interacts with ligands on the bMR nanosensors, resulting in a low $\Delta MR$ signal. As the concentration of the competing ligand increases, the interaction between the protein and the nanosensor is disrupted, resulting in an increase in the $\Delta MR$ signal.

revealed a concentration-dependent trend (Figure 1 A). A 50% change in the $\Delta MR$ signal values was observed at an avidin concentration of 3 fM, indicating that the $K_D$ value avidin–biotin binding was equal to this value. This $K_D$ value is in close agreement with a reported $K_D$ value of 1 fM determined by titration calorimetry studies.[15] In control studies with bovine serum albumin (BSA), the bMR nanosensors yielded nominal changes, suggesting that the observed changes in magnetic relaxation were target-specific.

Next, we investigated the affinity between Protein G and IgG (Figure 1 B), as well as between concanavalin A and dextran (Figure 1 C), as model interactions for protein–protein and protein–carbohydrate interactions, respectively. For the Protein G–IgG interaction, a bMR nanosensor composed of Protein G carrying iron oxide nanoparticles was incubated with various amounts of free Protein G (0.43 fM–0.43 pM), before addition of mouse IgG (50 nM) as target protein. Our magnetic relaxation results indicated that the $K_D$ of this interaction is in the picomolar range (0.4 pM). The reported $K_D$ value for a similar interaction, using a rabbit IgG in a fluorescence binding assay, is also in the picomolar range (50 pM), although slightly higher.[16] This is expected as the interaction with Protein G and IgG has been reported to be different depending on the source of IgG used (rabbit versus mouse).[17] Meanwhile, for the interaction between dextran and the carbohydrate-binding protein concanavalin A (Figure 1 C), dextran-coated iron oxide nanoparticles were used as bMR sensors and introduced to a solution containing various amounts of free dextran (1.25 nM–62.5 nM), before addition of concanavalin A (50 nM). Our measurements indicated that a $K_D$ value for this interaction is in the nanomolar range (18.8 nM), within the same range as the literature value (90 nM).[18] The use of different sources and batches of concanavalin A (a lectin) and dextran (a complex carbohydrate) might affect the interaction between

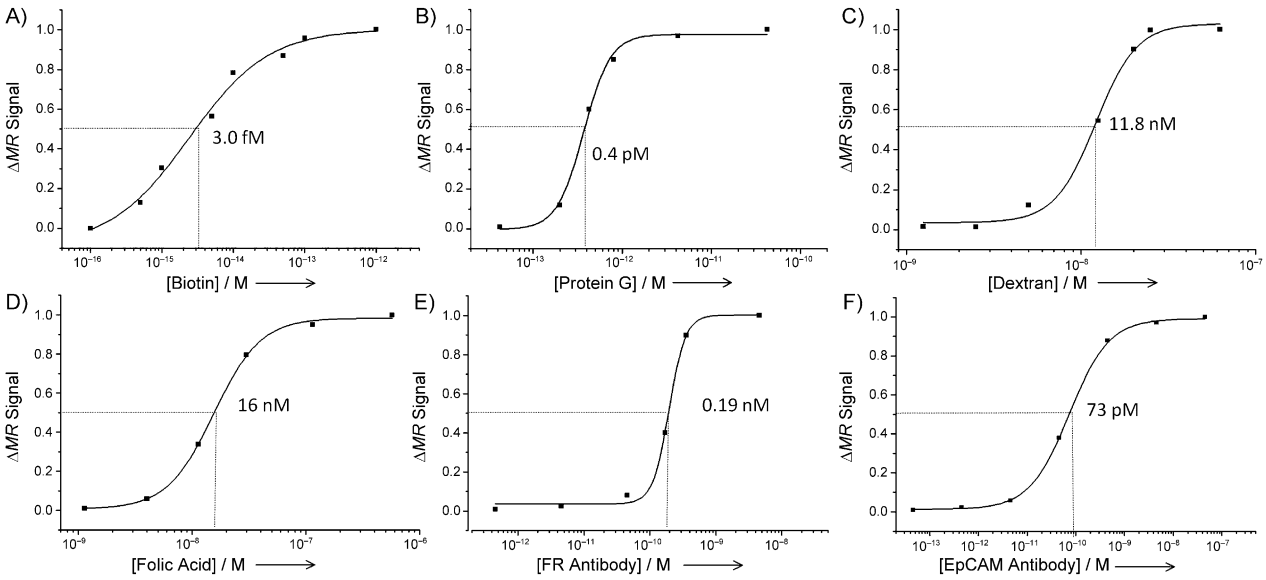


***Figure 1.*** Determination of the dissociation constant for various molecular interactions using magnetic relaxation and bMR nanosensors. A) Avidin–biotin, B) Protein G–IgG, C) dextran–concanavalin A, D) folic acid–folate receptor (FR) expressed on HeLa cells, E) anti-folate receptor antibody–folate receptor on HeLa cells), F) anti-EpCAM antibody–EpCAM Receptor (MCF-7 Cells). (Errors were within 1–2%, which are too small to represent.)

  

these two macromolecules, resulting in different $K_D$ values. Furthermore, control experiments using BSA do not reveal a concentration-dependent signal change confirming the specificity of our assays (see Figure S1 A–D in the Supporting Information). Taken together, these results indicate that our bMR-based method can rapidly measure the dissociation constant of different protein–ligand interactions within a wide range of affinities.

After validating the ability of our magnetic relaxation method to measure $K_D$ values of molecular interactions in solution, we investigated whether our method can be applied to study interactions with transmembrane proteins in intact cells. Membrane proteins and cellular receptors control key biological processes within the cell and are the target for a wide variety of therapeutics.[19–21] Most of the current methods to determine $K_D$ values use purified membrane receptors in solution or attached to a flat surface.[9,22–24] Therefore, we reasoned that one could use the bMR assay to determine the $K_D$ between a ligand and a cell receptor using cells in suspension. To validate our hypothesis, we used the folate receptor (FR) as a model system and investigated the affinity of this receptor to its natural targeting ligand, folic acid, using HeLa cells as the source of FR.[25] For these studies, a folic acid conjugated iron oxide nanoparticle was used as the bMR nanosensor. Following a 30 minute incubation of the HeLa cells with the bMR nanosensors and increasing amounts of free folic acid (1.1 nM–0.56 μM), behavior similar to that observed with the soluble protein targets was observed (Figure 1 D). At a low amount of competitor, a low $\Delta MR$ signal was observed which increased at higher concentrations and eventually reached a plateau, allowing us to calculate the $K_D$ as 16 nM for this particular interaction. This value is within the range of the reported values of 0.1 nM[26] and 30 nM[25] for folate receptor/folic acid interactions. However, these values were obtained using the solubilized receptor in a radioligand-binding assay, instead of using HeLa cells in suspension. It is worth noticing the different $K_D$ values reported in the literature for the same molecular interaction, which suggests that these values depend on the nature of the assay and the experimental conditions.[9]

The interaction between the folate receptor and an anti folate receptor antibody (Santa Cruz Biotechnology, sc-28997) was also studied (Figure 1 E). For this study, anti-folate antibody conjugated nanoparticles were used as bMR sensors. Results revealed a $K_D$ value in the low nanomolar range (0.19 nM), while the reported value in the literature for a system using a recombinant solubilized receptor and a totally different antibody was 2.23 nM.[22] In control studies, substituting the FR antibody with the EpCAM antibody (Santa Cruz Biotechnology, sc-73491) resulted in no significant increase in the $\Delta MR$ signal value with increasing concentration of EpCAM antibody. Similarly, no response was observed when MCF-7 cells were used, as this cell line does not express the folate receptor assays (Figure S1C, D in the Supporting Information). These results indicate that the observed changes in the $\Delta MR$ signal are specific to a folate receptor/anti-folate antibody interaction and not to a non-specific interaction between the designed bMR nanosensors and the HeLa cells. In additional experiments, we used anti-EpCAM antibody conjugated iron oxide nanoparticles as bMR nanosensors to measure the $K_D$ between the EpCAM antibody and EpCAM receptors in MCF-7 cells (Figure 1 F). Our results indicated a $K_D$ of 73 pM while the literature value is 550 pM.[23] Again, these previous studies were performed using the extracelluar domain of EpCAM (expressed and purified from yeast) which was attached to a solid support for SPR studies. Taken together, these results indicate that our magnetic relaxation method can be used to study the interaction of small-molecule ligands and proteins with cell-surface receptors using intact cells with an accuracy comparable to that of current methods, yet at a higher speed and potentially lower cost. As these experiments are performed within 30 min with cells in suspension, any endocytosis of the nanoparticles is minimized.

Next, we utilized the bMR-nanosensor-based competition assay to study the interaction between toxins and small molecules. Recent reports describe the interaction of doxorubicin with the tetanus toxin C fragment (TTC)[27] and galactose or dextran with cholera toxin B subunit (CTB).[28] The study of these interactions is important for the development of small-molecule-based therapeutics. For the TTC–doxorubicin interaction, a doxorubicin-carrying iron oxide nanoparticle was designed. Within 15 min, we were able to observe a sigmoidal response with an increasing $\Delta MR$ signal value upon incubation of the bMR nanosensors with TTC (4 nM) in the presence of increasing amounts (0.9 μM–12 μM) of doxorubicin (Figure S2 A in the Supporting Information). Using this data a $K_D$ of 4.1 μM was calculated, which is close to the reported value of 9.4 μM determined using a competition assay similar to ours with a fluorescence readout instead of magnetic relaxation.[29] Next, we tested if rhein, an anthracycline antibiotic structurally similar to doxorubicin, binds to TTC. Results show that indeed rhein interacts with TTC with a $K_D$ value of 33.6 μM, which is slightly higher (weaker affinity) to the interaction with doxorubicin (Figure S2 B in the Supporting Information). To our knowledge this is the first time that rhein has been reported to bind TTC. This is not surprising since both rhein and doxorubicin possess an anthraquinone group, which in the case of doxorubicin has been reported to play a key role in TTC binding.[25] This observation points toward the use of our bMR-nanosensor-based competition assay in structure–activity relationship (SAR) studies, where the competition of structurally similar compounds in binding to a particular protein or cellular receptor is studied by magnetic relaxation.

Meanwhile, the interaction of the cholera toxin B subunit (CTB) with dextran was studied using dextran-coated iron oxide nanoparticles as bMR nanosensors (Figure S3 A in the Supporting Information). Using our competition method, we found that the $K_D$ of the dextran–CTB interaction is 4.9 μM. This value is substantially lower than the value we previously determined using SPR (14 mM).[28] In that study, CTB was immobolized to the SPR gold plate using a ganglioside as a linker. Likely, this approach may block sites at which dextran binds, therefore affecting the interaction between CTB and dextran. Hence as the spatial orientation of these entities is constrained by the adhering mechanism, this may

  

affect the $K_D$ values. Our magnetic relaxation method using bMR sensors in solution is a homogeneous assay and therefore more sensitive than those in which the target protein is attached to a solid support.[30,31]

Finally, we investigated whether the dextran-coated iron oxide nanoparticles can be used as bMR nanosensors to study the interaction of CTB with other carbohydates. We reasoned that this bMR nanosensor may be used to determine the $K_D$ values of similar molecules, because of the similarity in structure and chemical composition (i.e. functional groups) between the nanoparticles' coating and the screened molecules. These studies revealed that increasing concentrations of glucose, galactose, lactose, and β-cyclodextrin disrupted the association between dextran-coated iron oxide nanoparticles and CTB (Figure S3 B–E in the Supporting Information). Hence, we determined that glucose had a $K_D$ of 36 μM, whereas the $K_D$ of galactose, lactose, and β-cyclodextrin were 3.5 μM, 88 μM, and 5.6 μM, respectively. Summarizing, we observed that dextran has a lower affinity towards CTB than galactose and β-cyclodextrin, while glucose and lactose have the least affinity in that order ($K_{DGal} < K_{DCyclo} < K_{DDex} < K_{DGlu} < K_{DLac}$). To our knowledge, the interaction of these carbohydates with CTB has not been previously reported. Our study's findings are summarized in Table 1.

In summary, we have developed a novel homogeneous assay to measure the $K_D$ values of molecular interactions on a nanoparticle surface, over a wide range of affinities using a simple NMR tabletop relaxometer. Our method is fast, uses small amounts of targets, and is performed using a magnetic nanoparticle to label the ligand. This is a unique method as it measures the interaction between ligands on the surface of a nanoparticle with either a solubilized proteins or a cell-surface receptor on intact cells. When molecular interactions are studied using ligand-conjugated nanoparticles and intact cells, avidity plays a key role as multiple receptors can interact with multiple ligands on the nanoparticle, strengthening the interaction and yielding a lower $K_D$. As avidity plays a critical role in molecular recognition in nature, and multivalent nanoparticle are being used increasingly in sensing and therapeutic applications, a method like ours that can evaluate the contribution of these parameters in terms of $K_D$ values would provide insights in the molecular dynamics under these conditions. The contribution of avidity and nanoparticle multivalency, as well as differences in proteins used (rabbit versus goat IgG) contribute to the differences between $K_D$ values reported in the literature and those obtained by our nanoparticle-based method. Furthermore, as our assay is performed using low concentrations of the bMR nanosensors with subfetomolar amounts of ligands, it could be utilized in competitive binding assays for quantification of $IC_{50}$ as under these conditions Kd values are similar to $IC_{50}$ values ($K_d = IC_{50}$, where [labeled ligand] $\ll Kd$).

***Table 1:*** Comparison of $K_D$ values determined in this study with those reported in the literature.

| Interaction | $K_D$[a] | $K_D$[b] reported |
|---|---|---|
| avidin–biotin | 3 fM | 1 fM[15] |
| Protein G–IgG | 0.4 pM | 50 pM[16] |
| concanavalin A–dextran | 11.8 nM | 90 nM[18] |
| folate receptor–folic acid | 16 nM | 0.1 nM[26] |
| folate receptor–anti-FR Ab | 0.19 nm | 2.2 nM[22] |
| EpCAM receptor–anti-EpCAM Ab | 73 pM | 550 pM[23] |
| TTC–doxorubicin | 4.1 μM | 9.4 μM[29] |
| TTC–rhein | 33.6 μM | N/A |
| CTB–dextran | 4.9 μM | 14 mM[28] |
| CTB–glucose | 36 μM | N/A |
| CTB–galactose | 3.5 μM | N/A |
| CTB–lactose | 88 μM | N/A |
| CTB–β-cyclodextrin | 3.6 μM | N/A |

[a] Values determined using bMR nanosensor competition assay.
[b] Values reported in the literature, corresponding reference in brackets. N/A = not available.

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