NEW APPLICATIONS FOR THE KINETIC EXCLUSION ASSAY (KINEXA)

by

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DEDICATION

I dedicate the study and labor that this work represents to my loved ones suffering with type 1 diabetes: My brother-in-law Arlan Potts, my nephew Jared Potts, and both of my children, Amy Smith Colgan and Matthew Smith. I undertook these graduate studies to better understand the science behind your affliction. I wish I could do more.

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ABSTRACT

This dissertation examines the fundamental principles and applicability of the kinetic exclusion assay (KinExA), developed and marketed by Sapidyme Instruments of Boise, Idaho, since 1995. Chapter One reviews and consolidates the manufacturer's guidance and many early papers that delineate the practical and theoretical aspects of the technology. In brief, KinExA is a two stage analytical system. In stage one, a number of solutions are prepared, whereby one of the partners is kept constant (the constant binding partner, or CBP), while the other (the titrant) is varied, usually in serial dilution. As the titrant is increased, the free CBP decreases, and is analyzed by a sophisticated and precise microfluidic fluorometric device (stage two). The resulting signal can be related mathematically to the affinity (K_D) of the two molecules for each other, and to the kinetic parameters of binding (k_{on}) and dissociation (k_{off}). A comparison of KinExA with other current technology available for quantification of interactions is provided.

In Chapter Two, I investigate the use of KinExA technology with DNA aptamers. DNA aptamers are short nucleotide oligomers selected to bind a target ligand with affinity and specificity rivaling that of antibodies. These remarkable features make them promising alternatives for analytical and therapeutic applications that traditionally use antibodies as biorecognition elements. Numerous traditional and emerging analytical techniques have been proposed and successfully implemented to utilize aptamers for sensing purposes. In this work, I exploited the analytical capabilities offered by the KinExA technology to measure the affinity of fluorescent aptamers for their target

molecule thrombin, and quantify the concentration of analyte in solution. Standard binding curves constructed by using equilibrated mixtures of aptamers titrated with thrombin were fitted with a 1:1 binding model and provided an effective K_D of the binding in the sub-nanomolar range. However, the experimental results suggest that this simple model does not satisfactorily describe the binding process; therefore, the possibility that the aptamer is composed of a mixture of two or more distinct K_D populations is discussed. The same standard curves, together with a four-parameter logistic equation, were used to determine “unknown” concentrations of thrombin in mock samples. The ability to identify and characterize complex binding stoichiometry, together with the determination of target analyte concentrations in the pM–nM range, supports the adoption of this technology for kinetics, equilibrium, and analytical purposes by employing aptamers as biorecognition elements.

In Chapter Three, I explore complex capture agents in the KinExA system. Liposomes made from purified reagents, or from natural cellular membranes, are attached to the beads used in the KinExA process to capture the analyte. Model molecules representing lipophilic dyes, antibodies, and bacterial toxins were successfully captured by the beads for measurement. Residual free ligand captured after pre-equilibration with membrane components, presented as either liposomes or whole cells, could be quantified, and kinetic parameters determined. By this process the “bi-molecular” interaction of the B subunit of cholera toxin for the ganglioside GM1 incorporated into artificial membranes could be quantified, and shown to be dependent upon the presence of the ganglioside in the membrane. The diffusion into artificial membranes of the lipophilic dye DID could be quantified and shown to be dependent upon the amount of lipid

available in the equilibration step. In addition, the bulk affinity of a commercial polyclonal antibody for the surface antigens of their target red blood cells could be evaluated. This membrane immobilization process appears to be generally applicable to any membrane system. Thus, it promises to be valuable for the study of signaling molecules for which purified soluble target cellular components may result in misleading information, or for which soluble fragments are unavailable. Likewise, this process should aid in the search for drugs which mimic or antagonize these signaling ligands.

TABLE OF CONTENTS

DEDICATION.....	iv
ACKNOWLEDGMENTS.....	v
ABSTRACT	vii
LIST OF FIGURES	xiii
LIST OF ABBREVIATIONS.....	xvi
CHAPTER ONE: THE KINETIC EXCLUSION ASSAY	1
The Importance of Affinity	4
Principles of KinExA Operation	5
The KinExA Sensogram	11
Theoretical Foundation	14
The Theory Curve.....	16
N-Curve Analysis	19
KinExA Mode	20
Cooperativity.....	22
Whole Cell Analysis	25
Concentration Measurements.....	26
Kinetics Measurements.....	28
Comparison to Other Methods.....	29
Surface Plasmon Resonance	29

Isothermal Calorimetry	32
Enzyme-Linked Immunosorbent Assay	34
References	37
CHAPTER TWO: KINETIC EXCLUSION ASSAY OF BIOMOLECULES BY APTAMER CAPTURE	44
Abstract	44
1. Introduction.....	45
2. Materials and Methods	47
2.1. Solid Phase and Sample Preparation, and Data Collection with the KinExA 3200 Instrument.....	48
2.2. Data Analysis	50
3. Results and Discussion.....	50
4. Conclusions.....	59
Appendix A	60
Kinetics Exclusion Assay Principles	60
Signal Equations and Modeling	62
References	64
CHAPTER THREE: AFFINITY MEASUREMENTS OF LIGANDS TO IMMOBILIZED NATURAL AND ARTIFICIAL MEMBRANES BY KINETIC EXCLUSION ASSAY.....	74
Introduction	74
Materials and Methods:.....	78
Materials:	78
Preparation of Liposomes from Lipids	80
Preparation of Liposomes from Red Blood Cells	81

Bead Preparation.....	83
Results and Discussions.....	84
Measuring the Interactions Between Gangliosides and CTB-AF647.....	84
Interactions Between Antibodies and Membranes	89
Determining the Affinity of Lipophilic Dyes for Lipid Membranes	93
Conclusions.....	98
References.....	100
CHAPTER FOUR: CONCLUSIONS AND PERSPECTIVES	107
Aptamer Studies	107
Membrane Studies	109
References.....	112

LIST OF FIGURES

Figure 1-1	Examples of reversible interactions important to life.....	2
Figure 1-2.	A. KinExA 3200 with autosampler. B. KinExA 4000. C. Flow cell camera	6
Figure 1-3.	Diagram of bead preparation and labeling for signal generation.....	8
Figure 1-4.	Schematic principles of equilibration step.....	9
Figure 1-5.	Conceptual flow diagram of the KinExA biodetector.....	10
Figure 1-6.	The fluorescent signal.....	12
Figure 1-7.	Sensogram with secondary label.....	13
Figure 1-8.	Notional time sequence of the KinExA sensogram.....	14
Figure 1-9.	Determination of K_D from signal data.....	17
Figure 1-10.	Error Curves.....	18
Figure 1-11.	Simulation of theory curve in relation to the ratio of K_D to CBP.	19
Figure 1-12.	N-Curve analysis.....	20
Figure 1-13.	The KinExA mode.....	21
Figure 1-14.	Effect of flow rate on a weak binding bimolecular pair.	22
Figure 1-15.	Cooperativity.....	23
Figure 1-16.	Suspicious activity calculations.	24
Figure 1-17.	N-curve analysis for a system exhibiting positive cooperativity.	25
Figure 1-18.	K_D measurements of anti-human IL13 (hIL13) mAb to native hIL13.	28
Figure 1-19.	Operating principle of surface plasmon resonance.	30

Figure 1-20.	Surface effects on Biacore analyses.....	32
Figure 1-21.	Basic schematic illustration of the isothermal titration calorimeter instrument.	33
Figure 1-22.	The operating principles of enzyme-linked immunoassay (ELISA).	35
Figure 1-23.	Comparison between SPR, ELISA and KinExA ²⁶	36
Figure 2-1.	Bead preparation for equilibrium and concentration measurements with the kinetics exclusion assay technology.....	49
Figure 2-2.	Preliminary testing of the fluorescent aptamer's suitability for equilibrium and concentration measurements by kinetic exclusion assay.....	52
Figure 2-3.	The evolution of the raw fluorescence signal recorded from capturing the free FA left in solutions equilibrated with various thrombin concentrations.	54
Figure 2-4.	Analysis of FA binding data.....	55
Figure 2-5.	Equilibrium measurements of thrombin concentration by kinetic exclusion assay.	58
Figure A1.	Using aptamers as biorecognition elements for kinetic exclusion assay measurements.	62
Figure 3-1.	Lipids used in this study.....	78
Figure 3-2.	Chol-PEG-Btn. Cholesterol (left side) is linked to biotin (right side) through a polyethyleneglycol (PEG2000) linker (middle).....	79
Figure 3-3.	The lipophilic dye, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (DiD)	80
Figure 3-4.	Dynamic light scattering characterization of artificial liposomes.	81
Figure 3-5.	Dynamic light scattering characterization of liposomes made from RBC.	82
Figure 3.6.	Schematic representation of liposome attachment to PMMA beads.	84
Figure 3-7.	KinExA sensogram of CTB captured by GM1 liposomes.....	86
Figure 3-8.	Theory curve for CTB binding to GM1 liposomes.....	87

Figure 3-9.	Cholera toxin is not captured by non-GM1 liposomes.....	88
Figure 3-10.	Logistic fit of data for CTB captured by GM1 liposomes.....	89
Figure 3-11.	KinExA sensogram for anti-RBC antibodies equilibrated to RBCs.	91
Figure 3-12.	Theory curve for anti-RBC Ab and RBC liposomes.....	93
Figure 3-13.	KinExA sensogram of DiD equilibrated to liposomes.	96
Figure 3-14.	Theory curve of dye accumulation by liposomes.....	97

LIST OF ABBREVIATIONS

Ab	Antibody
AF647	Alexa Fluor 647
Aso	Asolectin
BSA	Bovine serum albumin
BSA-B-A	Bovine serum albumin-biotin-avidin
BSA-B-A-cDNA	Bovine serum albumin-biotin-avidin-complementary DNA
btn-BSA	Biotinylated bovine serum albumin
C	Antigen level/cell, with respect to whole cell analysis
CBP	Constant binding partner. Reagent whose concentration is kept constant throughout a KinExA experiment
cDNA	Complementary DNA
Chol	Cholesterol
Chol-PEG-Btn	A cholesterol molecule attached to a biotin molecule through a polyethyleneglycol linker
CM	Carboxymethylcellulose
CTB	Cholera toxin subunit B
DI	Deionized (water)
DiD	Tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt
DLS	Dynamic Light Scattering
DNA	Deoxyribonucleic acid

EL	Expression level of surface ligand, with respect to whole cell analysis (molecules per cell)
ELISA	Enzyme-linked immunosorbent assay, a method to determine equilibrium constants
FA	Fluorescent aptamer
Fc	Fragment crystallizable region in the constant region of an antibody. Species unique and therefore useful for secondary staining of primary antibodies
FITC	Fluorescein isothiocyanate
GM1	Monosialotetrahexosylganglioside; a prototype ganglioside
ITC	Isothermal calorimeter(try), a method to determine equilibrium constants and thermodynamic properties of reactions
K_D	Equilibrium constant equal to $\frac{k_{off}}{k_{on}}$
K_{DEFF}	Effective equilibrium constant for a system displaying cooperativity
KinExA	Commercial line of instruments designed to measure rate and equilibrium constants by the <u>kinetic exclusion assay</u>
k_{off}	Dissociation rate constant
k_{on}	Association rate constant
L	Ligand
mAb	Monoclonal antibody
N_A	Avogadro's number ($6.02 \times 10^{23}/\text{mol}$)
PBS	Phosphate buffered saline

PBS	Phosphate buffered saline
PDI	Polydispersity index
PEG	Polyethylene glycol
PMMA	Polymethylmethacrylate
R	Receptor
RBC	Red blood cells
RCF	Relative centrifugal force
SELEX	Systematic Evolution of Ligands by Exponential Enrichment. A method of creating aptamers with selective affinity
SPR	Surface plasmon resonance, a method to determine rate and equilibrium constants
ssDNA	Single-stranded DNA
T	Titer of cell concentration, with respect to whole cell analysis (cells/mL)
T _A	Antigen activity level, with respect to whole cell analysis

CHAPTER ONE: THE KINETIC EXCLUSION ASSAY

Since the discovery of the first enzyme, diastase, in a cell-free solution of malted barley in 1833¹, there has been a steady accumulation of detail in the fascinating functions of individual molecules in biology. The discovery of allosteric control of enzymatic activity by, among others, Jacques Monod, and further elucidation of the workings of the *E.coli* lac operon (again spearheaded by Monod, for which he and François Jacob were awarded the 1965 Nobel Prize in physiology) highlight the fact that reversible, non-covalent binding is paramount to the activity and regulation of the molecular functions that make life possible.

Countless examples of reversible binding abound in biology. These include (but are not limited to) gene expression, metabolic regulation, signaling across membranes, self/non-self recognition, intercellular communication, interaction of organisms with their environment, and antigen-antibody interaction. In each of these examples, reversible binding provides a means for intricate control of the biological process (Figure 1-1).

Figure 1-1 Examples of reversible interactions important to life.

A. Competitive binding in enzyme catalysis. Reversible binding of an inhibiting molecule to the active site of an enzyme precludes binding of the intended substrate and prevents progress of the reaction. The reversible nature of the binding allows for the reaction to proceed as the concentration of the inhibitor decreases.

B. Control of the *lac* operon. A repressor molecule prevents the binding of RNA polymerase to the *lac* gene and forestalls the synthesis of mRNA. Reversible binding of lactose alters the affinity of the repressor for the operator sequence, which reverses the blockage and allows lactase synthesis to proceed.

C. Notch-mediated juxtacrine signal between adjacent cells. Reversible binding of the cell surface protein Notch to a Notch receptor (Delta) on an adjacent cell initiates a cascade of metabolic consequences. The precise binding ensures that only the two cells respond to each other's proximity in a very specific manner.

D. Control of glucose transport by insulin. The activity of glucose transporter-4 is carefully controlled by the activity of the insulin receptor, which is in turn finely tuned by the reversible binding of insulin.

In each case, a strong permanent binding would result in a binary, on/off control switch. The elegance of reversible binding is that intermediate control can be attained, depending on the strength of the interaction and the concentration of the reactants².

Consider the reversible binding of a ligand (L) to its receptor (R). The bimolecular reaction may be represented as

Another representation is

At equilibrium, the rate of association and dissociation of the complex are equal, proportional to the concentration of reactants and the rate constant in each direction:

$$k_{\text{on}}[L][R] = k_{\text{off}}[LR] \quad (\text{Equation 1})$$

The concentrations of the reactants are, therefore, in a dynamic steady state.

Equation 1 can be rearranged to establish the ratio of reactants to products at equilibrium,

$$\frac{k_{\text{off}}}{k_{\text{on}}} = \frac{[L][R]}{[LR]} \quad (\text{Equation 2})$$

At equilibrium, the association constant, K_A , is defined as

$$K_A = \frac{[LR]}{[L][R]} = \frac{k_{\text{on}}}{k_{\text{off}}} \quad (\text{Equation 3})$$

while its reciprocal, the dissociation constant, K_D , is

$$K_D(M) = 1/K_A = \frac{k_{off}(s^{-1})}{k_{on}(M^{-1}s^{-1})} = \frac{[L][R]}{[LR]} \quad (Equation\ 4)$$

In common usage, K_D is referred to as the affinity, in units of molarity. The smaller the magnitude of K_D , the greater the affinity, i.e., a molecule with a K_D of 1 nM has 1000 times greater affinity than one of 1 μ M.

The Importance of Affinity

In molecular biology, the affinity of a ligand for its receptor is directly related to its activity. In medicine, small molecule drugs or antibodies often exert their therapeutic action by inhibiting or activating a receptor, and, likewise, the activity and efficacy of the drug is directly related to its affinity for the receptor³. In biological science, a reagent is more potent when it has a higher K_D for its target. As can be seen from Equation 3, $[LR]$ is maximized when the association rate constant, k_{on} , is greater than the dissociation rate constant, k_{off} ⁴.

Zuckier et al.⁵ investigated the visualization of beads (as surrogate for cellular antigens) coated with high or low density of antigen, with radiolabeled antibodies of various affinities. They demonstrated in their mouse tumor model an inverse relationship between the affinity of an antibody and the amount of antibody needed to visualize the beads. Beads coated with the lowest density of antigen could be best observed with antibodies of high affinity.

A number of studies have found a correlation between affinity and effectiveness *in vivo* and *in vitro*. Liddament⁶ studied the efficacy of monoclonal antibodies against interleukin-5a in a human-IL-5-dependent cell proliferation assay. Those monoclonal antibodies (mAb) with greater affinity for IL-5 also exhibited greater cell proliferation activity *in vitro*. Li et al.⁷ investigated the efficacy of antibodies of varying affinity to

protect SCID mice^a infected with the intracellular bacterium *Ehrlichia chaffeensis*. They concluded that, “Although it is not known whether high affinity is essential, it was the most important correlate of efficacy.” Zhu et al.⁸ found correlation between antibody affinity and the inhibition of human leukemia in an animal model, using human antibodies directed against vascular endothelial growth factor receptor 2 (VEGF2). Mice treated with antibody of the highest affinity survived longer than those treated with antibodies of lesser affinity.

There may be limits to the affinity-activity paradigm, as increased affinity may actually impede the penetration of a therapeutic agent to its target, such as a tumor mass⁹. Adams et al.¹⁰ found that affinities greater than one nM offered no further advantage to the penetration of single-chain variable antibody fragments into a mouse tumor model.

Principles of KinExA Operation

A number of methods exist to measure the various parameters of Equation 4, including surface plasmon resonance (SPR) and enzyme-linked immunosorbent assay (ELISA). The KinExA line of biodetectors was introduced by Sapidyne, Inc. (Boise, ID) in 1995 as an alternative to these and other methods. The salient operating principle of KinExA, which also affords its chief advantage, is that the ligand and receptor may be contacted in true solution. This feature avoids any perturbation of the equilibrium due to the proximity of any feature of the immobilizing matrix^{11–22}. It also avoids conflation in the kinetic measurements of intrinsic affinity with limitations due to mobility of the molecules in the solution^{23,24}. The detection method is highly accurate and repeatable with a large dynamic range^{25,26}. It has been shown in multiple comparisons to be superior

^a Severe combined immunodeficient mice lack B and T cells.

to SPR and ELISA in accuracy, sensitivity, versatility, and reliability^{11,15,16,25,27–29}.

Molecular weight of the analyte poses no limitations^{24,28,30}, as it has been employed in studies ranging from ions^{28,30} and small molecules^{18,31,32}, to whole cells^{16,27,31,33}. In addition, no separations are necessary. This can be a great advantage, both for logistical considerations for the experiment, and for the system itself. Amizadeh and Van Regenmortel³⁴ showed that physical separation of a bivalent reactant, like an antibody, also removes the second reactant site, which may be unoccupied, and important information can be lost. In the KinExA system both binding sites of the antibody remain available for capture, but must be accounted for in the analysis³⁵.

Two models of the KinExA apparatus are shown in Figure 1-2. The key component of the KinExA apparatus is a flow cell (Figure 1-2C) approximately 8 μL in volume, packed with beads functionalized to react with one partner of a reversible, bimolecular reaction (capture beads). The total surface area is over 2.5 cm^2 , with a void volume of roughly 4 μL . A sophisticated fluid handling system delivers reagents to the flow cell with precise timing, while a highly sensitive epifluorescent illumination and detection system provides the signal for analysis (Figure 1-5). The capture beads are delivered to the flow cell, and the analytical solution is flowed over the cell. The beads

are functionalized so that only free molecules will be bound to the beads. These molecules may be fluorescent themselves, or are subsequently exposed to fluorescent secondary labels, and the fluorescent signal is a function of concentration (Figure 1-3)³⁶. By knowing initial conditions, the amount of free molecule can be measured, and kinetic parameters calculated, as further detailed below.

The typical KinExA experiment consists of three parts. First is preparation of the functionalized beads which will capture the analyte for measurement (Figure 1-3). Second is preparation of a series of solutions consisting of a constant initial concentration of one component of the binary reaction and serial dilutions of the other reactant (Figure 1-4). The component that is kept constant is the constant binding partner (CBP), and is the one which will be analyzed. Third, each reaction mixture is sampled and the fluorescence of free CBP bound to the capture beads is obtained for subsequent numerical analysis.

Figure 1-3. Diagram of bead preparation and labeling for signal generation
 An equilibrium mixture of a constant binding partner (CBP) and its ligand (L) will exist as free CBP, free L, and some proportion of bound L-CBP (A). When this mixture is flowed over beads derivatized with the ligand, a proportion of the free CBP will bind to the ligand on the beads (B). If necessary (e.g., when CBP is not fluorescent), a fluorescent label can be attached to the bead-bound CBP to generate the eventual analytical signal (C).

Figure 1-4. Schematic principles of equilibration step.

A series of mixtures is prepared with one component at the same concentration in all of the mixtures (constant binding partner, or CBP), while the other reactant is present in serial diluted in concentrations ranging two orders of magnitude or more.

After equilibration, most of the CBP in the low-L mixture is still in its unbound state, while very little free CBP exists in the high-L mixture. The concentration of CBP in the intermediate mixtures provides information between the extremes for numerical analysis.

Figure 1-5. Conceptual flow diagram of the KinExA biodetector. Samples and reagents are delivered to the flow cell detection system through a series of tubing, valves and pumps. Beads are first loaded to the flow cell. Samples are then passed over the beads, where a proportion of unreacted (free) molecules from the sample mixture are captured. If necessary, visualization reagent (fluorescent-functionalized antibody, HIS tag, etc.) is then passed over the beads. Fluorescence of the bound molecules are measured with a photodetector.

A number of detection schemes have been used, most frequently fluorescent labeled secondary antibody. A common use of the KinExA platform is the evaluation of the affinity of mAb^{36,37}. In a setting producing antibodies from a single species, say, mouse, a routine protocol can be established using the same secondary antibody, say, goat anti-mouse IgG, for all assays. In some cases, the analyte may be a protein modified with biotin, which can be detected with fluorescent avidin. Proteins labeled with polyhistidine (His-tag) can be visualized with fluorescent anti-His antibody, but results can vary³⁸.

Burden et al. (in press) measured the K_D of the fluorophore RNA mango as it bound to immobilized biotin on the bead surface. The fluorescence response of this aptamer to photoexcitation is several orders of magnitude greater when bound to biotin than when unbound. We herein³⁹ used a fluorescent anti-thrombin single-stranded DNA aptamer to investigate aptamer-thrombin binding, and to propose an aptamer-based procedure to measure biomolecules (see Chapter 2).

The KinExA Sensogram

The fluorescence detected in a given moment in the flow cell is the sum of the fluorescence present in the fluid being passed over the beads, plus the fluorescence of the analyte captured on the surface of the beads themselves. This can be best illustrated by comparing the sensograms of two different experiments differing in the manner of visualization.

In our thrombin aptamer experiments, detailed later in this dissertation, the analyte was an aptamer labeled with Alexa Fluor 647, so that it constantly generated a fluorescent signal to the photodetector. In these experiments, thrombin and a single-stranded DNA (ssDNA) anti-thrombin aptamer were mixed together and allowed to come to equilibrium. These beads would only capture aptamer which was not complexed with thrombin. The equilibrium mixture was then passed over beads which had been coated with a polynucleotide complementary to the anti-thrombin aptamer. The detector response increased monotonically as the sample passed over the beads and a portion of free aptamer bound to the beads. Once the sample had been delivered and a rinsing solution was introduced, the rinse solution no longer contributed to the fluorescence, and

the fluorescence was due only to bound aptamer (Figure 1-6). Similar traces were observed with labeled primary antibodies to biotin, challenging biotin-coated beads³⁶.

Figure 1-6. The fluorescent signal.

Thrombin and a ssDNA anti-thrombin aptamer were mixed together and allowed to come to equilibrium. Beads coated with a polynucleotide complementary to the anti-thrombin aptamer captured only aptamer which was not complexed with thrombin.

The resulting sensogram suggests that most of the free aptamer was captured, as there is only a slight decrease in fluorescence once the rinse solution is introduced.

In the mixtures, the labeled anti-thrombin aptamer was held constant (the CBP) while the concentration of thrombin was varied as threefold dilutions. At the highest thrombin concentration, the equilibrium solutions contained the least free aptamer, while at very low thrombin concentrations most of the aptamer remained free.

KinExA experiments are more commonly performed by binding unlabeled free CBP to the beads, followed by a visualization step, typically labeling with a specific fluorescent antibody. Under these circumstances, the outward appearance of the sensogram is significantly different, while the eventual information is the same. Figure 1-7 depicts a sensogram under these circumstances. Here, a mouse anti-digoxin antibody

was mixed with serial dilutions of digoxin and brought to equilibrium. These equilibrium solutions were flowed across beads functionalized with digoxin, which captured the free antibody. This step was followed with a fluorescently labeled goat anti-mouse IgG antibody, and a final buffer rinse. The final fluorescence is proportional to the bound antibody, which is inversely proportion to the digoxin present in the equilibrium solutions.

Figure 1-7. Sensogram with secondary label.

In this instance, an anti-digoxin antibody was used to quantify digoxin. Mouse antibody and dilutions of digoxin were mixed and brought to equilibrium. The equilibrium solutions were flowed across beads functionalized with digoxin, which bound the free antibody. This was followed with a labeled goat anti-mouse IgG antibody and a final buffer rinse. The change in the curve at the red arrow indicates the introduction of fluorescently labeled secondary antibody; and the change in the curve at the blue arrow indicates the end of labeled secondary antibody and commencement of a buffer wash. The final fluorescence represents the bound antibody, in inverse proportion to the digoxin challenge.

A notional superposition of these two circumstances is depicted in Figure 1-8.

Here, it can be seen that regardless of the manner of fluorescent visualization, the final

photocell response corresponds to the amount of analyte captured by the beads, in turn proportional to the concentration of free analyte in the equilibrium mixture.

Figure 1-8. Notional time sequence of the KinExA sensogram. Equilibrated solution is injected into the apparatus at time 1, and reaches the flow cell at time 2. The dashed line denotes the analyte being captured on the surface of the beads in the flow cell, and also corresponds to the photocell response for a labeled analyte, such as a primary antibody or fluorescent aptamer. By time 3, the analyte is fully loaded on the beads. The solid line represents the introduction of a visualizing agent at this point, such as a labeled secondary antibody. The fluorescence is due to the presence of fluorophore both in free solution and attached to the bound analyte. At time 4, a rinsing buffer reaches the flow cell, removing the free visualizing agent. The fluorescence eventually stabilizes and its value at time 5 is the data point.

Theoretical Foundation

The fundamental principle of kinetic exclusion is predicated on the premise that, as long as $k_{\text{off}} \ll k_{\text{on}}$, then dissociation of a bimolecular complex into its component entities contributes a negligible amount of unbound molecule during the brief period of contact with the beads in the flow cell. If that is the case, then the signal is proportional only to the free molecule (say, L) in the solution being analyzed.

Equation 4 can be rearranged to give

$$[L] = \frac{K_D [LR]}{[R]} \quad (\text{Equation 5})$$

Invoking the conservation of mass, we find that

$$[L] = [L]_0 - [LR] \quad (\text{Equation 6})$$

and

$$[R] = [R]_0 - [LR] \quad (\text{Equation 7})$$

Combining these three equations and solving the resulting quadratic for [R] gives

$$[R] = \frac{R_0 - K_D - L_0 + \sqrt{[R]_0^2 + 2[R]_0 K_D - 2[R]_0 [L]_0 + K_D^2 + 2[L]_0 K_D + [L]_0^2}}{2} \quad (\text{Equation 8})$$

A step-wise derivation of this equation can be found in the KinExA user manual⁴⁰.

The fluorescence detected by the instrument detector (in volts, V) is linearly proportional to the receptor concentration, [R]³⁶, which allows us to describe the relationship

$$V = \frac{(V_{100\%} - V_{0\%})}{[R]_0} [R] + V_{0\%} \quad (\text{Equation 9})$$

where

V = voltage for [R]

V_{0%} = voltage when there is no R in the solution

V_{100%} = voltage when there is no L in the solution.

Substituting Equation 8 for [R] in Equation 9 gives the equation for the signal

$$\text{Signal} = V_{0\%} + \frac{V_{100\%} - V_{0\%}}{2[R]_0} \left(([R]_0 - [K_D] - [L]_0) + \sqrt{R_0^2 + 2[R]_0 K_D - 2R_0 L_0 + K_D^2 + 2[L]_0 K_D + L_0^2} \right) \quad (\text{Equation 10})$$

$[L]_0$ is known from initial conditions, and $[R]_0$, K_D , $V_{100\%}$ and $V_{0\%}$ are determined with aid of KinExA software that minimizes the squared error between the theoretical data curve and the measured data²⁷.

The Theory Curve

As one varies the initial concentration of receptor ($[R]_0$) in a series of experiments, while keeping $[L]_0$ constant, a set of data can be accumulated corresponding to Equation 11.

$$\frac{[R]}{[R]_{0(x)}} = \frac{[R]_0 - [L]_0 - K_D + \sqrt{[L]_0^2 - 2[L]_0[R]_0 + 2[L]_0K_D + [R]_0^2 + 2[L]_0K_D + [R]_0^2 + 2K_D[R]_0 + K_D^2}}{2[R]_0}$$

(Equation 11)

In our discussion, which binding partner is the ligand and which the receptor is ambiguous, as it could go either way. In KinExA parlance, the partner which is kept constant throughout the experiment is called the “constant binding partner” (CBP). The equations above could have been derived for $[L]$ with no material difference. When this data set is plotted in a semi-log fashion, a theory curve can be generated like that shown in Figure 1-9⁴¹. Simultaneous iterative solutions of the data set, minimizing the squared error between the theoretical data curve and measured data, will best fit the curve to the data (example, Figure 1-9).

Figure 1-9. Determination of K_D from signal data.
Data collected as described in the text was fit to a least squares model of Equation 11. The K_D value of 0.14 nM is shown by the vertical dotted line²⁴.

The iterative curve-fitting algorithm integral to the KinExA software is also capable of determining the confidence interval for the calculated K_D , and the amount of CBP that is active in solution (Figure 1-10). This information is valuable not only to establish the precision of the measurements, but to alert the user to possible anomalies in the reaction system (see below).

Figure 1-10. Error Curves.

The KinExA curve-fitting software calculates error curves for both K_D (left) and percent activity of the CBP (right). For each data point represented in Figure 1-9, a fit is performed by fixing the variable (K_D) and by determining the other variables ([CBP], $\text{Signal}_{0\%}$, and $\text{Signal}_{100\%}$) by best fit. The standard deviation calculated by this process is then divided by the value of K_D obtained independently in order to generate the percent variation. The error curve for percent activity is determined in similar fashion, except that [CBP] is fixed. The 95% confidence intervals for K_D and [CBP] are denoted by the gray regions²⁴.

The shape of the curve reflects the concentration of the CBP relative to the K_D of the bimolecular reaction. The slope of the sigmoidal curve increases as the concentration of the CBP increases (Figure 1-11, Sapidyne simulation⁴²). The most accurate estimate of K_D will be obtained when $[\text{CBP}] = K_D$. When $[\text{CBP}] < K_D$, the slope of the curve will decrease, and the curve is said to be a “ K_D controlled curve.” The slope and shape of the curve will not change appreciably below $[\text{CBP}] = 0.20K_D$. At low ratios an accurate K_D measurement may be obtained, but the uncertainty in [CBP] will be high. To the right of K_D in the graph, increasing [CBP] will increase the slope of the curve and also shift it to the right, conditions described as “concentration controlled.” In this region, an accurate measurement of [CBP] can be obtained at the expense of certainty in measured K_D . Exceeding $[\text{CBP}] = 5K_D$ will move the measurement of [CBP] into the shoulder of the curve and reduce reliability.

Figure 1-11. Simulation of theory curve in relation to the ratio of K_D to CBP. In this instance, K_D is 100 pM. For the blue curve, $CBP = K_D$. For the red curve, CBP is 10% of K_D , or 10 pM, while the green curve represents the situation where $CBP = 10 \times K_D$ ⁴².

N-Curve Analysis

In a new experiment, K_D is not known *a priori*. It is therefore recommended that two or more dilution series be prepared at a range of $[CBP]$ ^{12,33,37,43}. KinExA software allows simultaneous analysis of the data with its “n-curve analysis” module⁴².

Two or more data sets for the same bimolecular system are loaded into the module, differing only in the concentration of the CBP. The analytical algorithm will constrain the difference in CBP values for the curves to those that are input, and then apply the data to find the best fit for all the curves loaded (Figure 1-12). The analysis will usually result in narrower confidence intervals, and anomalies can suggest to the operator on off-model behavior, such as cooperativity (see below).

Figure 1-12. N-Curve analysis.

Albumin competes with LT3015 (an anti-lysophosphatidic acid (LPA) monoclonal antibody for binding the various lysophosphatidic acids. Here, the competition of albumin with monounsaturated form is analyzed with KinExA 4-curve analysis.

Antibody was kept constant at 1 nM, and brought to equilibrium with serial dilutions of LPA(18:1) in the presence of varying concentrations of fatty acid-free-

BSA: blue 3 μ M, green 30 μ M, orange 150 μ M, and pink 300 μ M. The n-curve analysis shows that the response curves shift toward higher lipid concentrations in response to the BSA sequestering the LPA(18:1). Fitting any of these curves in the absence of the additional information would result in an incorrect estimate of the LT3015-LPA(18:1) K_D value²¹.

KinExA Mode

As noted above, the fundamental principle which enables KinExA technology is that $k_{\text{off}} \ll k_{\text{on}}$. If such is the case, then the system is said to be operating “in the KinExA mode⁴⁴.” This condition can be verified by running samples at different flow rates. A “tight” system ($k_{\text{off}} \ll k_{\text{on}}$) will exhibit a constant signal at all flow rates, while a “weak”

system will exhibit a decreasing signal as flow rate increases (Figure 1-13)⁴⁵. If a flow rate can be established where a plateau of signal is reached (red curve in Figure 1-13), then that flow rate is appropriate for ensuing experiments.

Figure 1-13. The KinExA mode.
Comparison of KinExA data analysis for a “tight” system ($k_{\text{off}} \ll k_{\text{on}}$) and a “weak” system where such is not the case. The tight system will exhibit a constant signal at all flow rates, while a “weak” system will exhibit a decreasing signal as flow rate increases. The flow rate for the weak system should be set where the response is observed to plateau⁴⁴.

The consequence of an insufficient flow rate for the system will be an erroneously weak K_D (Figure 1-14).

Figure 1-14. Effect of flow rate on a weak binding bimolecular pair. For a weak binding pair, a significant portion of bound partner is washed from the capture element at slower flow rates, leading to a false measurement of CBP in the equilibrated solution. Analyses should be conducted at flow rates where further increases in rate do not change the signal⁴⁴.

Cooperativity

In many biological systems, the binding of a ligand can result in morphological changes that alter the activity of a remote portion of the molecule. This allosteric change can confer greater or lesser activity. For 5-10% of antibodies, even though each arm of the bivalent molecule is identical, the binding of one ligand can increase the affinity of the other arm for that ligand⁴⁶⁻⁴⁸. In such a case, the usual binding model is insufficient, but anomalies can be recognized and dealt with^{49,50}.

Under these circumstances, there are two populations of molecules with different affinities for the ligand: the native antibody, and the antibody with one ligand attached (Figure 1-15).

Figure 1-15. Cooperativity.

The identical arms of a bivalent antibody usually have equal affinity for its ligand ($K_{D1} = K_{D2}$), but a significant fraction of antibodies exhibit cooperativity, where binding of one ligand changes the affinity of the free arm ($K_{D1} \neq K_{D2}$)⁵⁰.

The descriptive equilibrium equations are as follows. The affinity constants for the ligand and the unbound and singly bound antibody are given by equations 12 and 13, respectively, and the mass balances for each reactant in equations 14 and 15.

$$K_{D1} = \frac{[L][R]}{[LR]} \quad (\text{Equation 12})$$

$$K_{D2} = \frac{[LR][R]}{[LRL]} \quad (\text{Equation 13})$$

$$R_T = R + 2LR + LRL \quad (\text{Equation 14})$$

$$L_T = L + 2LR + 2LRL \quad (\text{Equation 15})$$

Potential cooperativity can be suspected by significant deviation of reported K_D and percent activity of a system investigated at two different CBP. The data points can also suggest a different slope than the calculated best fit (Figure 1-16).

Figure 1-16. Suspicious activity calculations.

On the left, the reported percent activity of the CBP is an impossibly high 187%. On the right, an n-curve analysis of relatively high (red) and low (blue) CBP reports a more reasonable percent activity of 75%. In addition, the data points on the blue curve seem to suggest a steeper slope for the theory curve than what is reported⁵⁰.

Invoking a cooperativity model to the data resolves the discrepancy, resulting in a markedly better fit of the data to theory (Figure 1-17). The parameters returned from the model report an effective dissociation constant (K_{DEFF}) along with a value for the Hill coefficient^b, a measure of cooperativity, where

$$K_{D1} = \frac{(K_{DEFF})(Hill)}{(2 - Hill)} \quad (\text{Equation 16})$$

$$K_{D2} = \frac{(K_{DEFF})(2 - Hill)}{(Hill)} \quad (\text{Equation 17})$$

^b The Hill coefficient (n) indicates the degree of cooperativity. A value of 1 denotes no cooperativity. A value $n > 1$ denotes positive cooperativity, while $n < 1$ denotes negative cooperativity. See discussion on concentration measurements, below.

Figure 1-17. N-curve analysis for a system exhibiting positive cooperativity. The theory curve fit to the data is markedly better than seen in Figure 1-16. The parameters returned from the model report an apparent dissociation constant (effective K_D), as well as a Hill coefficient for the system, from which the separate dissociation constants of the native and allosterically modified antibodies can be derived (see text)⁵⁰.

Conformational change resulting in allosteric binding behavior of antibodies may extend into the Fc region of an antibody⁴⁷. Since antibodies directed against the constant region are commonly used to immobilize the antibody being analyzed, (e.g., SPR), unsuspected cooperativity may account for differences in K_D measurements between KinExA and SPR⁵⁰.

Whole Cell Analysis

KinExA is well suited to the analysis of whole cells, both for affinity measurements and to determine receptor density on the surface of cells^{16,27,31,33,51}. CBP and whole cells can be mixed and allowed to come to equilibrium, then separated

gravimetrically. Only unbound CBP will remain in solution and analysis is straightforward.

The expression level of an antigen on the surface of a population of cells may be denoted by

$$EL = \frac{C \times T_A \times N_A}{1000 \times T} \quad (\text{Equation 18})$$

where:

EL: expression level (molecules per cell)

C: antigen level/cell; a reasonable but arbitrary value⁵² is $10^3 - 10^6$

T_A : antigen activity level, determined by KinExA software

N_A : Avogadro's number ($6.02 \times 10^{23}/\text{mol}$)

1000: convert mL to L and align units

T: Total cells/mL

Using this relationship, and n-curve analysis, Xie et al.²⁷ found the abundance of human insulin-like growth factor I receptor to be between 6×10^6 and $5 \times 10^7/\text{cell}$.

Rathanaswami et al.³³ determined the K_D of an antibody and integral surface proteins, by analyzing residual free antibody in solution to KinExA after equilibrium had been established between the antibody and whole cells.

Concentration Measurements

The unknown concentration of a ligand may be determined by creating a standard curve of voltage response to known concentrations and comparing the unknown to that, but the same principles that are used to generate the theory curve can be used to evaluate the unknown. The KinExA software package will generate the binding curve by

averaging and fitting with a four-parameter logistic equation, whereby the signal, as defined in Equation 19, is a function of concentration^{26,36,39,53}:

$$Signal = \frac{B_1 - B_2}{1 + \left(\frac{x}{B_3}\right)^{B_4}} + B_2 \quad (Equation 19)$$

where B_1 is the upper asymptotic plateau (maximum signal), B_2 is the lower asymptotic plateau (NSB), B_3 is the inflection point (the concentration at which the signal is halfway between the extreme values), B_4 is the Hill coefficient (slope)⁵⁴, and x is the titrant's concentration.

The same equation, rearranged to solve for x , allows calculation of the unknown concentrations from the corresponding signals:

$$[x] = \exp \left[\frac{\ln \left[\frac{Signal - B_1}{B_2 - Signal} \right]}{B_4} \right] \cdot B_3 \quad (Equation 20)$$

Rathanaswami et al.⁵⁵ were able to measure serum levels of interleukin 13 (IL13) in serum samples, using a specific anti-IL13 and when no IL13 standards were available, i.e., using only the minute amounts of IL13 available in the serum. By challenging identical concentrations of the anti-IL13 with various dilutions of serum and applying n-curve analysis, both K_D and [IL13] could be determined (Figure 1-18).

Figure 1-18. K_D measurements of anti-human IL13 (hIL13) mAb to native hIL13. Human anti-hIL13 mAb was serially diluted 1:3 from a binding site concentration of 25 nM to 42 fM and incubated with 11x (curve 1), 22x (curve 2), 275x (curve 3), or 550x (curve 4) diluted hIL13 conditioned medium generated from CD8-depleted peripheral blood mononuclear cells. After 72 hours of equilibration, the free hIL13 present in the equilibrium mixtures was measured using mAb-coated PMMA beads and detected with a Cy5-labeled secondary antibody. The percentage free hIL13 was determined with KinExA n-curve analysis software. Optimal values for K_D and the native hIL13 concentration were determined by n-curve analysis⁵⁵.

Blake et al. have employed KinExA to analyze mAbs which were directed toward specific metal chelates^{56–58}. A prototype KinExA protocol was able to detect Pb(II) in a spiked groundwater sample down to 6 nM, a level that was beyond the feasible limit of detection for a corresponding ELISA protocol²⁸.

Kinetics Measurements

The progress of a reaction (kinetics) can be directly observed by choosing “Kinetics Direct” as the experiment type from the KinExA protocol menu. Because of the bead handling and sample loading routines, successive data points will necessarily be five minutes or more apart. For a relatively slow reaction, that may be satisfactory. If equilibrium is substantially achieved within this time frame, early data points must be obtained in separate experiments with manual mixing, and the data combined gathered

together for analysis⁵⁹. Kinetic parameters k_{on} , k_{off} , and K_D (equations 1-4) will all be determined by best fit to the mathematical model.

Comparison to Other Methods

Several other methods are available to measure molecular interactions, notably surface plasmon resonance (SPR, commercially available from Cytiva under the trade name Biacore, or more recently by Carterra as LSA); isothermal titration calorimetry (ITC; commercially available from TA Instruments and Microcal/Malvern Instruments); and enzyme-linked immunosorbent assay (ELISA, widely available).

Surface Plasmon Resonance

The operating principle of SPR is shown in Figure 1-20. The change in refractive index imparted by binding of an analyte to a thin film of gold on one surface of a prism is detected by a beam of light directed on that surface. By measuring the spectrum shift of the beam of light, the concentration of analyte can be measured, and the progress of the binding reaction can be followed in real time. The method is sensitive to about 10 pg/mL⁶⁰. The required immobilization of one ligand in the Biacore system could be problematic, especially for small molecules, if the immobilization scheme interferes with the binding site. Since SPR depends on binding directly from the analytical solution, mass transfer limitations, especially for large molecules with high association rate, may obscure the observation of actual K_D . These limitations may be overcome by careful manipulation of the flow conditions or by including a mass transport term in the data fitting step. In terms of cost, the Biacore equipment can cost 3-4 times as much as KinExA, and the cost of Biacore chips is quite high. The Biacore may be configured to

have a higher throughput than KinExA, which may offset the high cost in appropriate circumstances²⁴.

Figure 1-19. Operating principle of surface plasmon resonance.

A thin metal film (typically gold) is evaporated onto the surface of a high-reflective index glass prism. Biorecognition molecules are attached to the gold film, and that surface is brought into contact with the solution to be analyzed. The refractive index of the gold layer is acutely sensitive to the attachment of analyte to the biorecognition elements, which can be detected by a shift in the wavelength of light shone through the prism onto the gold-coated surface^{c,d}. Lower right: Solution containing analyte is flowed over the detector and the change of refractive index can be followed as a function of time to directly measure the progress of the equilibration^e. Subsequently flowing an analyte-free solution reveals the progress of dissociation. The association and dissociation constants can thus be directly measured, and K_D calculated by Equation 3.

Drake et al.¹² performed an extensive study comparing Biacore and KinExA analyses with regards to dextran density on the Biacore chip used in the analysis. Biacore chips can be obtained pre-coated with carboxymethylcellulose (CM). Although the

^c Diagram by Sari Sabban - Sabban, Sari (2011) Development of an in vitro model system for studying the interaction of Equus caballus IgE with its high- affinity FcεRI receptor (PhD thesis), The University of Sheffield, CC BY-SA 3.0, <https://commons.wikimedia.org/w/index.php?curid=18139885>

^d By Original uploader was Tomio- at en.wikipedia - Transferred from en.wikipedia, CC BY-SA 3.0, <https://commons.wikimedia.org/w/index.php?curid=9881864>

^e Figure from Narayane at the English Wikipedia, CC BY-SA 3.0, <https://commons.wikimedia.org/w/index.php?curid=9881873>

absolute value of CM on a chip is proprietary information, they were able to estimate relative values from other available information. They determined that the CM4 chip contains approximately 30% of the CM as the standard CM5 chip, while the C1 chip (while not having any dextran layer) has about 10% of that amount of carboxyl groups. By preparing analytical chips by amine coupling of the Ag to the carboxyl groups on the surface of the chip, and observing the K_D values returned by the SPR protocol, they noted that as the relative carboxyl density decreased, the reported K_D approached that determined by KinExA solution-phase analysis (Figure 1-20). Liddament⁶ measured the affinity of two mAbs using both KinExA and Biacore. While they found the relative affinities of the two antibodies to be the same by both methods, they found KinExA analysis returned absolute K_D values approximately 10-fold lower than Biacore, which they attributed to surface effects for the Biacore method without further investigation.

Figure 1-20. Surface effects on Biacore analyses.
As dextran (carboxyl) density decreased on the Biacore chips used to perform the assay, the reported K_D decreased, approaching the value reported by the purely solution-phase KinExA assay¹².

A recent study by Brown⁶¹ compared the response of newer SPR flat chips to the ones studied by Drake, and found a closer correlation to true solution kinetics. It is instructive to note that the standard of comparison was the KinExA.

Isothermal Calorimetry

ITC is based upon measuring the heat released or absorbed as a reaction proceeds⁶² (Figure 1-21). The apparatus consists of an adiabatic chamber containing a reaction cell and a reference cell. A bolus of analyte is delivered to a solution of its ligand, and the resulting heat released to or absorbed from the chamber is monitored. It is universally applicable to any reaction in solution, without any labeling or other chemical modification to the reactants required in order to facilitate detection. There are no

molecular weight limitations. Instrument analytical software can determine the change in enthalpy (ΔH), binding constant K_D and fractional saturation (n), from which the Gibbs free energy and entropy change can be calculated from the relationships $\Delta G = nRT \ln K_D = \Delta H - T \Delta S$, where R is the universal gas constant, and T is the absolute temperature. Disadvantages of the technique are the relatively large quantities of reactants required, and the fact that the reactants must be quite pure. Reactants must also be stable and sufficiently soluble to provide enough mass to generate a signal. Sensitivity is sufficient to measure K_D s in the millimolar to low nanomolar range, several orders of magnitude less sensitive than KinExA^{24,62,63}. Darling and Brault²⁴ found that, while the ITC apparatus costs just a bit more than KinExA, due to the large quantities of reagents required, the cost of consumables in comparison to KinExA is quite high.

Figure 1-21. Basic schematic illustration of the isothermal titration calorimeter instrument.

A sample and reference cell are contained within an adiabatic chamber. Analyte is injected into the sample cell, which contains the ligand to the analyte, and the heat released or consumed by the reaction is followed by monitoring the temperature and heating requirements necessary to maintain the isothermal conditions, as compared to the reference cell^f. The top right panel shows the sequence of peaks, each of which corresponds to an injection of the solution in the syringe. This example corresponds

^f Left, public domain, <https://en.wikipedia.org/w/index.php?curid=6536395>

to an exothermic reaction. The bottom right panel shows the integrated heat plot. The areas under each peak, calculated and normalized per mole of ligand injected in each injection, are plotted against the molar ratio of reactants. Curve fitting to these data can determine the thermodynamic parameters of K_D , enthalpy, and stoichiometry^g.

Enzyme-Linked Immunosorbent Assay

ELISA is widely available in many variations from multiple manufacturers. In general principle (Figure 1-22), a specific antibody is conjugated to an enzyme, frequently horseradish peroxidase. This conjugated antibody is used to detect its antigen in a manner proportional to its concentration. A plethora of variations abound, differing in the way the analyte is initially captured, the way the enzyme linkage is introduced, and detection method, and other iterations. In the first step of the process, the wells of a multi-well plate are functionalized to capture the analyte. Then, solutions containing the analyte are added to the wells, along with appropriate standards. Next, the bound analyte is marked with the conjugated antibody, and an appropriate substrate is added to react with the enzyme and generate a color for quantification. (Rinse steps are not described in detail in the preceding discussion.) After an appropriate amount of time, the plates are placed in a plate reader for analysis. Information may be obtained commensurate with the other processes presented herein⁶⁴. Darwish et al.⁶⁵, compared KinExA competitive assay to ELISA analysis of four cancer markers in biological specimens. They found KinExA to have superior limits of detection by one to three orders of magnitude for three of the four, while the detection limit for the fourth was roughly the same. They attributed the difference to the inherent sensitivity of the KinExA system as well as to the fact that the

^g Right, by Simon Caulton - Own work, CC BY 4.0,
<https://commons.wikimedia.org/w/index.php?curid=82487783>

antibody-antigen analytical mixture was allowed to come to equilibration for the KinExA system, while the corresponding ELISA contact time was truncated to one hour in accordance with the protocol used.

Figure 1-22. The operating principles of enzyme-linked immunoassay (ELISA). The wells of a multi-well plate are functionalized so as to capture the analyte when exposed. In this case, a virus particle has been captured, proportional to the amount in the sample. This is followed by a virus-specific antibody which has been conjugated to an enzyme, such as horseradish peroxidase (HRP), whose activity will be used to generate the signal. A plethora of variations exist^h. Right: An example of a multi-well plate developed with HRP and the chromophore TMB, showing the color gradient proportional to the analyte concentrationⁱ.

Glass et al. compared the sensitivity of KinExA, ELISA and SPR²⁶. Four antibodies to estradiol were evaluated, and experiments were performed by expert technicians in the companies that manufactured the equipment. Least detectable concentration and dynamic range were determined. Four antibodies specific to estradiol were evaluated in an estradiol assay on each platform. The least detectable concentration (LDC) and dynamic range (DR) of each platform were determined, as well as an estimate of the K_D of the interactions (Figure 1-23). The SPR biosensor was able to measure inhibition curves for all four antibodies, but could measure K_D for only two of the antibodies. The other two antibodies provided insufficient response for K_D estimation.

^h Diagram y Cavitri - Own work, CC BY 3.0, <https://commons.wikimedia.org/w/index.php?curid=9627246>

ⁱ photo of plate By Ajpolino - Own work, CC0, <https://commons.wikimedia.org/w/index.php?curid=88819005>

The KinExA biosensor was able to measure inhibition and K_D for all four antibodies. Inhibition for all four antibodies was detectable by ELISA, but K_D was elusive. In all cases, KinExA provided superior sensitivity and range as compared to the other two platforms.

The kinetic exclusion assay is an accurate, sensitive, versatile, and reliable method to determine kinetic parameters for bimolecular interactions, and to determine concentrations of analytes to minute quantities. In this study, we investigate further its properties and potential for further use.

Figure 1-23. Comparison between SPR, ELISA and KinExA²⁶. Least detectable concentration (LDC), dynamic range (DR) and K_D of SPR, KinExA (KE) and ELISA challenged with four antibodies. Large open circles indicate the measured K_D for the antibody and technique. The vertical lines in panel A indicate the range from lowest to maximum (i.e., usable range) detectable concentration, calculated using three times the standard deviation. The vertical lines in panel B show the LDC (minimum symbol) and DR calculated from noise theory. K_D was not obtainable if the open circle is missing.

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CHAPTER TWO: KINETIC EXCLUSION ASSAY OF BIOMOLECULES BY APTAMER CAPTURE^j

Abstract

DNA aptamers are short nucleotide oligomers selected to bind a target ligand with affinity and specificity rivaling that of antibodies. These remarkable features recommend aptamers as candidates for analytical and therapeutic applications that traditionally use antibodies as biorecognition elements. Numerous traditional and emerging analytical techniques have been proposed and successfully implemented to utilize aptamers for sensing purposes. In this work, we exploited the analytical capabilities offered by the kinetic exclusion assay technology to measure the affinity of fluorescent aptamers for their thrombin target and quantify the concentration of analyte in solution. Standard binding curves constructed by using equilibrated mixtures of aptamers titrated with thrombin were fitted with a 1:1 binding model and provided an effective K_D of the binding in the sub-nanomolar range. However, our experimental results suggest that this simple model does not satisfactorily describe the binding process; therefore, the possibility that the aptamer is composed of a mixture of two or more distinct K_D populations is discussed. The same standard curves, together with a four-parameter logistic equation, were used to determine “unknown” concentrations of thrombin in mock

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samples. The ability to identify and characterize complex binding stoichiometry, together with the determination of target analyte concentrations in the pM–nM range, supports the adoption of this technology for kinetics, equilibrium, and analytical purposes by employing aptamers as biorecognition elements.

1. Introduction

Aptamers are short nucleotide oligomers selected and isolated by the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) technique^{1–4} to present high specificity and affinity for a large variety of targets ranging from ions^{5–8} and small molecules^{9–12} to whole cells^{13–15}. Their ability to selectively recognize and bind targets recommends them as promising alternatives to antibodies for scientific and medical purposes^{9,13,16–25}. Aptamers have been investigated as potential analytical and diagnostic tools with approaches and technologies that traditionally employ antibodies as recognition elements. Compared to antibodies, DNA aptamers are cost effective, present similar specificity and affinity for targets, have greater stability either lyophilized or in solution at room temperature, and are readily amenable to chemical modifications^{17,25–28}.

Numerous traditional and emerging analytical techniques have been proposed for aptamer-based qualitative and quantitative assessments of molecular and cellular interactions^{23,25,28–32}. Aptamer-thrombin systems that use the 15-mer³³ and/or 29-mer³⁴ aptamer as biorecognition elements are among the most used in scientific investigations focused on affinity and concentration determination^{29,35–41}, which is justified by the essential physiological role of thrombin⁴². Nonetheless, both aptamers are far from fully characterized in terms of affinity and binding models³⁵. For example, the reported affinity of the 29-mer aptamer varies by more than four orders of magnitude, and is also strongly

dependent on the instrumental method and adopted binding model^{29,34–37,39–41}. Only a few studies reported affinities in the nM range^{40,41}, close to the ~0.5 nM first reported by Tasset³⁴, while many others indicated affinities up to a few hundred times larger^{29,35–37,39}. It is not clear if such discrepancies originate in the instrumentation and mathematical models employed, or whether particular experimental conditions and methodologies also influence the measurements³⁵. To better understand the aptamer-thrombin interactions and pave the way towards analytical applications, we used the kinetics exclusion assay (KinExA) technology, developed by Sapidyn, Inc. (Boise, ID, USA) to determine the 29-mer aptamer affinity for thrombin, and thrombin concentration in solutions.

This technology provides highly accurate analyses of molecular interactions in true solution phase systems^{43–54}. The principle of operation relies on specifically assessing only the unbound partner in a mixture of bound and unbound molecules^{55–58}. The KinExA instrument is an automatic solution-handling system equipped with a sensitive and versatile fluorescence detection system^{55,59–61}. To perform measurements, a small volume of a bi-molecular reaction mixture is flowed into a low-volume flow cell over a solid phase consisting of small beads functionalized to specifically bind one of the partners^{52,56,62}. The contact time between the beads and solution is brief enough that any dissociation of the bi-molecular complex is insignificant. The beads specifically capture a small fraction of the free partner, proportional to total free molecule in solution, which is quantified by either intrinsic fluorescence of the captured molecules or by using fluorescent secondary probes (i.e., specific labels, anti-tag, or anti-species secondary antibody) that do not participate in the primary interaction in the solution phase^{63,64}. Depending on the experiment goals, the resulting fluorescence signal measured directly

on the beads is analyzed using various binding models and protocols to determine kinetics, affinity, or concentration^{48,50,55,58,65–69}.

Although this technology has been chiefly used with antibodies as biorecognition elements^{46,54–56,70}, the only restriction on recognition molecules is that at least one of them needs to be fluorescently detectable, either directly or indirectly⁵⁵. We used this technology to interrogate an aptamer-thrombin binding system, and our protocols make full use of the advantages offered by the KinExA technology in terms of automation, accuracy, reliability, and adaptability. In addition, the use of aptamers enables using a complementary DNA on a solid phase^{71–74} to specifically capture the free aptamer in solution (unbound to the target thrombin). By introducing a fluorescently-labeled aptamer as a specific biorecognition element for thrombin, the detection of the unbound aptamer can be achieved without using a secondary label. Our results demonstrate that the KinExA platform is directly applicable to aptamers for determining their affinity for target, whether simple or complex binding stoichiometries are considered, and for measuring concentration of target molecules.

2. Materials and Methods

The 29 nucleotide thrombin-specific aptamer (sequence 5'-AGTCCGTGGTAGGGCAGGTTGGGGTGACT-3'), identified by Tasset et al.³⁴ was obtained by custom order from Integrated DNA Technologies, Inc. (IDT, Coralville, IA, USA). At our request, the supplier provided the aptamer modified at the 5' end by the addition of the fluorophore Alexa Fluor 647 to enable direct fluorescent detection. For the capture DNA (cDNA) strand, we selected a 16-nucleotide sequence complementary to bases 11–26, with two additional thymine residues and biotin at the 3' end (5'-

CACCCCAACCTGCCCTTT-biotin-3'). This material was also obtained by custom order from IDT. The DNA strands were reconstituted with nuclease-free water to a stock concentration of 100 μ M. Sample solutions consisting of fluorescent aptamer (FA) with various concentrations of thrombin (MilliporeSigma, St. Louis, MO, USA) being prepared in sample buffer (SB), consisting of phosphate buffered saline with 0.02% sodium azide (PBS, Sapidyne Instruments) supplemented with 1 mg/mL bovine serum albumin (BSA, Sapidyne Instruments). The running buffer consisted of PBS alone.

2.1. Solid Phase and Sample Preparation, and Data Collection with the KinExA 3200 Instrument

The multi-step preparation of functionalized beads as solid phase for capturing the free FA in solution is shown schematically in Figure 2-1. Following the manufacturer's guidance, 1 mL of 20 μ g/mL BSA-biotin (MilliporeSigma) in PBS was mixed with 200 mg of polymethylmethacrylate (PMMA) beads of 98 μ m average diameter (Sapidyne Instruments). The beads were rotated for two hours at room temperature, after which we performed five steps of gravity pelleting/PBS washing. Next, the beads were exposed to 100 μ g/mL egg white avidin (MilliporeSigma) in PBS containing 10 mg/mL BSA and rotated for two hours to functionalize them for anchoring the biotinylated cDNA molecule through the strong biotin-avidin bond. After five steps of gravity pelleting/PBS washing, one vial of beads functionalized with BSA-biotin and avidin (BSA-B-A) was reserved for preliminary control experiments, while another vial underwent a last step of functionalization by immobilizing the biotinylated cDNA on the beads, following similar equilibration and washing procedures in which we used as bathing solution 1 mL of 2 μ M

biotinylated cDNA in PBS. The cDNA functionalized beads (BSA-B-A-cDNA) were further used for control, equilibrium, and concentration measurement experiments.

Figure 2-1. Bead preparation for equilibrium and concentration measurements with the kinetics exclusion assay technology.

Polymethylmethacrylate (PMMA) beads functionalized with bovine serum albumin (BSA)-biotin and avidin serve as anchoring elements for the DNA strand with sequence complementary to a portion of the fluorescent aptamer. The free aptamer is able to bind the complementary strand anchored to the bead, while binding of the aptamer-thrombin complex is prevented.

For the control experiments, we utilized the BSA-B-A and BSA-B-A-cDNA functionalized beads, which were automatically injected into the flow cell of the KinExA 3200 instrument with autosampler (Sapidyne Instruments). The controls included samples consisting of PBS alone (for baseline) or 50 pM FA prepared in SB. The equilibrium measurements (see Figure A1 and the explanations provided in Appendix A) employed a constant concentration of FA (i.e., 10 pM, 50 pM, and 10 nM) in SB, mixed with thrombin at final concentrations up to 1 μ M, and equilibrated for at least two hours at room temperature. To demonstrate the potential of the technology for concentration and

diagnostic testing using aptamers, we prepared mixtures of FA (50 pM) and thrombin (5 nM, 2.5 nM, and 0.5 nM). These samples were equilibrated at room temperature for two hours.

Aliquots of samples were passed over the functionalized beads in the flow cell of the instrument. A fresh aliquot of functionalized beads was used for each sample and replaced automatically using the default protocol supplied with the instrument. Sample measurement steps consisted of initiation with a flow of 100 μ L of PBS, injection of 1 mL of the analytical sample (or control), and injection of 1 mL of PBS, all at a flow rate of 0.5 mL/min. This was followed by a final rinse with 1.5 mL of PBS at a rate of 1.5 mL/min. The fluorescence data from the detector (expressed in volts) was recorded at a rate of one sample/second during the above steps and plotted for data interpretation (see Figure A1 in Appendix A for reference) and further analysis. The same measurement steps were followed throughout for concentration measurements.

2.2. Data Analysis

Details of the signal equation used for fitting the 1:1 binding model^{55,59,75,76}, n-curve analysis module^{54,67,70,76}, mixed model^{75,77}, and logistic equation for concentration determination^{61,78} are provided in Appendix A. The data were analyzed and plotted with KinExA Pro software (version 4.4.36, Savidyne Instruments), and Origin 8.5.1 software (Origin Labs, Northampton, MA, USA).

3. Results and Discussion

In this work, we investigated the suitability of the kinetic exclusion assay technology for measuring an aptamer's binding affinity for its designated target, and for using the aptamer as a recognition element in a bioassay for thrombin concentration

measurements. Our proposed investigative platform employs BSA-B-A-cDNA functionalized beads to capture a fraction of the free FA remaining in equilibrated mixtures of analyte (thrombin) and FA. A potential impediment to this approach would be an unexpected strong, non-specific binding of the aptamer molecules to functionalized beads in the absence of cDNA immobilized on their surface. To address this issue, we performed preliminary investigations of specific and non-specific binding by employing functionalized beads without and with cDNA immobilized on their surface (control and capture beads, respectively) and running the experiments with 1 mL of either FA-free SB or SB containing 50 pM FA.

The analysis of the evolution of the raw fluorescence signal (Figure 2-2) shows steady, almost identical baselines (~ 1 V absolute signal value) recorded for both types of beads in the absence of FA in the solutions. When the FA sample reached the flow cell containing control beads, the fluorescence started to slowly increase and reached a maximum value of ~ 1.1 V (~ 0.1 V above baseline). Such a small change of the signal was expected since the FA concentration in the flow solution was very low (50 pM). During the rinsing step, the free FA was washed away with the buffer, as inferred from the gradual decrease of the fluorescence signal. The small difference from the baseline recorded at the end of the trace versus baseline indicated a weak, non-specific binding (NSB).

Figure 2-2. Preliminary testing of the fluorescent aptamer's suitability for equilibrium and concentration measurements by kinetic exclusion assay. The tests investigated specific and non-specific binding of 50 pM fluorescent aptamer (FA) to control and capture beads. All the data in the graphs represent experimental values, with the symbols added for identification. Each curve shows the average values of experimental data ($n = 3$, $\pm$ SD).

When the FA-containing sample was flowed over capture beads (BSA-B-A-cDNA), the fluorescence signal increased significantly (Figure 2-2) and reached an absolute value of ~ 2.9 V (~ 1.9 V above baseline). This much larger signal may be explained by a continuous accumulation of the FA in the flow cell as it was captured by the cDNA immobilized on the capture beads. The signal decreased by only a small fraction during the rinsing process, suggesting that the FA bound very strongly to the capturing beads. This was anticipated since the resulting duplex DNA, although relatively

short, has a predicted melting temperature of 58.4° C and the estimated free energy for hybridization is large ($\Delta G = -39$ kcal/mole; supplier data).

The specific binding of free FA resulted in a strong increase in the fluorescence signal (~ 1.9 V above the baseline), while the non-specific binding led to a change in the signal of less than 0.1 V. Consequently, we concluded that the experimental system was adequate for initiating further investigation of affinity and concentration measurements.

Having established the existence of specific FA binding to our functionalized solid phase, we turned our attention to determining the affinity of the aptamer for thrombin from kinetic exclusion assay experiments. We assumed that the FA-thrombin complex will not be able to form a duplex with the cDNA; therefore, specific capture of the complex will be prevented. In accordance with standard practice for n-curve analysis, we set up three experimental curves, for which we adjusted the concentration of the FA in SB (i.e., 10 pM, 50 pM, and 1 nM, respectively), used as a constant binding partner (CBP) for each curve. The thrombin amount in the sample tubes was adjusted (titrated) for final concentrations ranging from 5 pM to 1 μ M, and we used up to 18 thrombin concentrations (including no thrombin samples) for the experiments. The 1 nM and 50 pM FA samples were incubated for two hours, and the 10 pM FA samples were incubated for 6.5 h before measurements with the KinExA 3200 instrument, and each sample set was run at least in duplicate. A typical run for the sample set that utilized 50 pM FA as CBP is shown in Figure 2-3. The plot clearly indicates that the fluorescence signal, indicative of free FA in the equilibrated solutions decreased significantly as the titrant (thrombin) concentration increased. The slope of the traces increased with the amount of free FA in the equilibrated samples (therefore, it decreased as more thrombin was added

to the reaction tubes), and confirmed the signal rate's dependency on the concentration of binding partner⁷⁴.

Figure 2-3. The evolution of the raw fluorescence signal recorded from capturing the free FA left in solutions equilibrated with various thrombin concentrations. The free FA availability decreased as the titrant concentration in the samples increased. For this experiment we utilized 50 pM FA as constant binding partner (CBP).

The curves corresponding to the three different FA concentrations were simultaneously analyzed with the n-curve analysis module^{54,67,70,76} included with the KinExA Pro software package to determine the equilibrium dissociation constant K_D . The calculated percent of free FA vs. thrombin concentration and the theoretical curves for the three FA concentrations for a 1:1 binding model (Equation (A1)) are shown in Figure 2-4a.

Figure 2-4. Analysis of FA binding data.

(a) The n-curve analysis provided the calculated percent free FA (symbols) and theoretical simulations (dashed lines) for 10 pM, 50 pM, and 1 nM FA in the thrombin-titrated solutions for a 1:1 binding model. **(b)** The 10 pM and 1 nM FA signal data (symbols) were fitted with a binding model (dashed lines) that comprised a hypothetical mixture of two competing aptamers, characterized by different K_D values. The experimental data represents average values $\pm$ SD ($n = 3$ for 50 pM FA, and $n = 2$ for 10 pM and 1 nM FA).

The n-curve analysis provided a K_D of 298 pM (+111/−81 pM), which is in good agreement with previous estimates of the same aptamer-thrombin system of ~ 0.5 nM³⁴, or a few nM^{40,41}. We also noted that the 10 pM and 50 pM FA curves were nearly identical (Figure 2-4a), which can only occur when the thrombin concentration corresponding to the 50% inhibition point (approximately 300 pM) of both curves is equal to the K_D ⁵⁹. In addition to data overlapping, the 10 pM and 50 pM FA concentrations exhibited a better fit than the 10 nM FA samples. Even so, one may observe a distinct deviation of the data from the 1:1 binding model used for analysis (i.e., Equation (A1)) for the higher thrombin concentrations. All the additional curves we obtained and analyzed showed the same systematic error in the same range. Aware that mixtures of antibodies can generate standard binding curves of similar appearance^{75,77}, we wondered if the fit discrepancies in Figure 2-4a could be the result of competition between two or more populations of FA,

each with its own affinity for thrombin. To examine this hypothesis, we followed the example provided by Glass et al.⁷⁷ and set up a model that accounted for the competition of two FA of differing K_D for thrombin^{59,75}. With this competitive binding model, we obtained a qualitatively better fit to the measured data (Figure 2-4b), which corresponded to a hypothetical two component FA mixture, in which 84% has a calculated K_D for thrombin of 298 pM, and the remaining 16% has a K_D of 500 nM.

A better fit is not sufficient to prove that the dual binding model is the correct one, and we have no reason to believe that the mixture, if it exists, would have only two components. In this regard, we note that the fitted FA activity in Figure 2-4 was only ~47% for all FA concentrations, suggesting that half of the FA was actually inactive with regard to binding thrombin. Previous investigations on the same aptamer considered either a 1:1 binding model or models that imply multiple binding sites, including cooperativity and induced fit mechanisms^{29,35-41}. Nonetheless, almost all of those approaches report significantly lower affinities, and the possibility of a mixed population was not addressed. The fluorescent label that we added to the aptamer may alter the binding and equilibrium, but most of the studies cited herein as comparison have also used labels or spacers as modifiers. We do not believe that this particular label addition led to such a significant increase in affinity, although this potential effect is worthy of further investigations. The complex binding observed in our case may be inherent to this aptamer, or may originate in a potential duplex formation between a shorter segment of the cDNA and a complementary sequence of the FA outside the thrombin binding site. Either way, it is a strength of the KinExA technology that deviation from 1:1 binding can

be discerned in the collected data, which we anticipate will prompt further inquiry into mechanisms that may explain the observed binding curves for this and other aptamers.

Our next experiments aimed at evaluating the FA as a bioassay recognition element using kinetics exclusion assay technology. This task does not require a well-defined K_D , only that we have a reproducible standard curve. To measure thrombin concentration by employing kinetic exclusion assay using aptamers as ligands, we included measurements of mock unknowns (i.e., 5.0 nM, 2.0 nM, and 0.25 nM thrombin concentration) in the 50 pM FA assay. Similar to the experimental results plotted in Figure 2-3, the resulting raw signal curves (Figure 2-5a) recorded with the equilibrated mock samples show that the end signal decreased substantially when the thrombin concentration increased, indicating reduced availability of FA in the equilibrated mixtures.

Figure 2-5. Equilibrium measurements of thrombin concentration by kinetic exclusion assay.

(a) The raw fluorescence signal recorded for thrombin concentrations of 0.25 nM, 2 nM, and 5 nM ($n = 3$, $\pm$ SD) in equilibrated mixtures at a constant FA concentration of 50 pM. The symbols were added to facilitate curve discrimination and identification. (b) The end signal values measured against the baseline for standard and mock samples (symbols) were plotted and fitted (dashed line) with the four-parameter logistic equation (Equation (A2)) to determine the unknowns. The range of concentrations represented by the x error bars on the “measured” data points represent concentrations corresponding to the average measured signal ($n = 3$) $\pm$ SD of the measured signals.

Since the 1:1 binding model (Equation (A1)) proved unsatisfactory to best describe the experimental data, we used the four-parameter logistic (Hill slope) equation (Equation (A2))^{61,78} to fit the signal data for the standard curve (Figure 2-5b). The best fit parameter values calculated with this model are: upper asymptotic value $B_1 = 2.0$ V, lower asymptotic plateau (NSB) $B_2 = 0.38$ V, inflection point $B_3 = 0.32$ nM, and a Hill slope $B_4 = 0.87$. These parameters were also used to compute the “unknown” concentrations from the average values of the respective signals using Equation (A3). Our calculations provided average concentration values equal to 5.4 nM for the 5 nM sample, 2.2 nM for the 2.0 nM sample, and 0.32 nM for the 0.25 nM sample (Figure 2-

5b). It is interesting that although only one of the samples (i.e., 0.25 nM thrombin) had a concentration near the middle of the curve, while the more concentrated samples were intentionally outside this region, the determined average concentration values were satisfactory. As expected, the uncertainty in the determined concentration is larger and asymmetrical for the higher concentrations because of the more gradual slope and curvature of the standard curve at these concentrations. From the signal curve (Figure 2-5b) one may easily observe that for this FA concentration, visible changes in the binding signal occurs for thrombin concentrations in the pM range; further optimizations of the FA concentration in relation to affinity may provide improved sensitivity, even in the sub-pM range⁵⁹.

4. Conclusions

This work strongly supports the hypothesis that the kinetic exclusion assay technology is suitable for kinetics and equilibrium measurements employing aptamers as specific biorecognition elements. Our experimental results demonstrate that both aptamer characterization and analyte concentration measurements are achievable through this methodology. All the advantages presented by this technology are anticipated to be maintained when aptamers are used as ligands for analytes. The KinExA technology does not suffer from surface matrix affinity effects^{45,79}, mass transport limitations, or mobility effects^{55,80}, does not require radioactive labels, avoids any surface-immobilization procedure that may alter binding constants compared to those measured in true solution phase, is highly accurate for repeated readings⁸¹, and has no molecular weight limitations^{55,82,83}. Multiple studies have revealed its superiority to SPR or ELISA in terms of accuracy, sensitivity, versatility, and reliability^{45,48,49,76,81,82,84}. The instrument may assess,

in various formats, the binding of partners ranging from ions^{82,83} and small molecules^{50,85,86} to large structures (e.g., whole cells, including fixed cells)^{49,76,85,87}, which makes aptamers an excellent choice for numerous kinetics, equilibrium, and concentration measurements by using the KinExA technology. The ability to identify complex binding stoichiometries may provide valuable insights into fundamental analyses of aptamer–analyte interactions, while its high sensitivity and accuracy recommend this technique for numerous bioanalytical applications.

Appendix A

Kinetics Exclusion Assay Principles

The principles of measurement and the anticipated evolution of the fluorescence signals during the various steps of the adapted protocol are briefly presented in Figure A1. The instrument automatically handles the packing of beads and the injection of all the required solutions into the microfluidic system, while monitoring the fluorescence signal of the label captured by the beads. In our case, the beads (Figure A1, panel A) are functionalized for capturing the free FA by following the procedures presented in Materials and Methods and Figure 2-1, and introduced into the flow cell. The reactants (FA and thrombin) are equilibrated in reaction tubes in which we kept constant the concentration of FA and varied the amount of thrombin (Figure A1, panel B). The concentration of free FA in pre-equilibrated solutions is in inverse proportion to the amount of thrombin added to the reaction tube. A fraction of the free FA is captured by the functionalized beads upon injection into the flow cell (Figure A1, panel C). For otherwise identical settings, the amount of captured FA is proportional to the amount of free FA; more thrombin in the equilibrated sample leads to less FA available for capture.

FA injection after bead handling leads to an immediate increase in fluorescence signal owing to specific capture on the beads (Figure A1, panel D). The subsequent rinsing step washes away any non-captured FA, with the end signal representing only captured FA. This end signal is proportional to the amount of free FA that was injected in the previous step. It is assumed that the FA-thrombin complex (bound FA) will not bind to the cDNA, and the rate of dissociation of the pre-equilibrated complex is slow enough to be negligible during the brief residence time in the flow cell. Consequently, the end signal represents only binding of a fraction of FA free in equilibrated FA-thrombin solutions. An affinity value may be derived from further analysis of the relative changes in the end signal versus baseline (Figure A1, panel D) of a series of equilibrated solutions^{55,59}.

Figure A1. Using aptamers as biorecognition elements for kinetic exclusion assay measurements.

(A) A pack of beads functionalized with cDNA for FA capture is introduced in the flow cell. (B) Samples consisting of constant FA concentrations and variable thrombin amounts are equilibrated in test tubes. (C) Aliquots of equilibrated samples are flowed over the beads in the flow cell and a fraction of free FA is captured. This fraction is inversely proportional to the amount of thrombin added during the equilibration step. (D) The expected evolution of the fluorescence signal in response to different concentrations of free FA in solution. FA injection produces an increasing fluorescence signal owing to its capture and accumulation onto beads.

The end signal recorded after the unbound FA is rinsed from the flow cell is proportional to the amount of free FA present in the equilibrated sample (not complexed with thrombin). For identical label (FA) concentrations in equilibrated solutions, the end signal decreases as the concentration of the target molecule (thrombin) increases, indicative of a reduced availability of the free, unbound label.

Signal Equations and Modeling

The instrument end signal as a function of concentration for a 1:1 binding model is described by Equation (A1)^{55,59,75,76}:

$$Signal = \frac{Sig_{100\%} - NSB}{2[FA]_0} \left(([FA]_0 - K_d - [T]_0) + \sqrt{[FA]_0^2 + 2[FA]_0 K_d + K_d^2 + 2[T]_0 K_d + [A]_0^2} \right) + NSB \quad (A1)$$

where $Sig_{100\%}$ represents the end signal recorded in the absence of thrombin in the mixture, NSB is the end signal corresponding to non-specific binding (or the signal when all of the aptamer is complexed with thrombin), and $[FA]_0$ and $[T]_0$ are the initial concentrations of FA and thrombin in the mixtures. This equation is fitted by the method of least squares to the experimental data obtained by using FA as CPB and thrombin as titrant. The n-curve analysis simultaneously fits Equation (A1) to multiple signal curves generated by using different CBP concentrations^{54,67,70,76}.

For the two-population aptamer mixture, the total concentration of FA , $[FA]_0$, is assumed to be composed of two fractions $[FA]_1 = f \times [FA]_0$, and $[FA]_2 = (1 - f) \times [FA]_0$, where f is the fraction of the tightest binder in the population. We assumed that an FA molecule will provide the same signal irrespective of the population to which it belongs. For this fit, we used the 298 pM reported in the KinExA n-curve analysis as the tighter K_D , and varied both the fraction f of the tightest binder and the K_D of the second fraction. The competitive equilibrium concentrations of FA_1 and FA_2 were calculated using a model described elsewhere^{75,77}. We scaled the resulting combined free fraction of FA using a maximum binding signal ($Sig_{100\%}$) and offset it using a non-specific binding variable (NSB), as has been reported previously^{59,75}.

For the mock sample concentration measurement, the standard binding curve was obtained by averaging and fitting with a four-parameter logistic equation^{61,78}:

$$Signal = \frac{B_1 - B_2}{1 + \left(\frac{x}{B_3}\right)^{B_4}} + B_2 \quad (A2)$$

where B_1 is the upper asymptotic plateau (maximum signal), B_2 is the lower asymptotic plateau (NSB), B_3 is the inflection point (the concentration at which the signal is halfway between the extreme values), B_4 is the Hill coefficient (slope), and x is the titrant's concentration.

The same equation, rearranged to solve for x , allows calculation of the unknown concentrations from the corresponding signals:

$$[x] = \exp \left[\frac{\ln \left[\frac{-(-Signal + B_1)}{(-Signal + B_2)} \right]}{B_4} \right] \cdot B_3 \quad (A3)$$

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CHAPTER THREE: AFFINITY MEASUREMENTS OF LIGANDS TO IMMOBILIZED NATURAL AND ARTIFICIAL MEMBRANES BY KINETIC EXCLUSION ASSAY

Introduction

The cellular membrane, with its myriad inclusions, provides a communication channel as well as a boundary and barrier between the cell and its surroundings. Both the barrier function and communication are essential to the health or disease for all of life ¹. The two major membrane components, i.e. lipids and proteins, create and maintain electrochemical gradients; transport nutrients and wastes; and monitor the extracellular environment and facilitate the cell's response to nutrients, toxins, signaling molecules, adjacent cells and surfaces, and other exogenous stimuli¹⁻⁴. These numerous and complex functionalities rely on primary, specific interactions between ligands of various types and components of cell membranes. The characterization of these interactions is of utmost importance for a large variety of scientific, biotechnological, and biomedical applications.

Membrane components constitute preferred targets for more than 60% of drugs currently on the market⁵⁻⁸. Therapeutic antibodies target membrane proteins to treat cancer, inflammatory and autoimmune diseases⁹⁻¹³. Viruses must breach the cell membrane barrier to enter the cell, and many bacterial toxins manifest their mischief by disrupting the integrity of the cell membrane¹⁴⁻²¹. The development of any chemotherapeutic strategy aimed at hindering or potentiating these processes at the

membrane level must incorporate a quantitative measurement of those interactions. Specific components of cell membranes are also of great scientific interest for applications such as imaging, manipulation of cellular functions, or to better understand the complex membrane environment^{22–29}. Therefore, understanding vital cellular functions and screening for new drug targets and more potent therapeutics requires affinity measurements to these membrane components.

Quantification of membrane-ligand interactions poses particular challenges to both experimental design and theoretical modeling^{22,23,25,30–33}. Cell membranes are unique mixtures of lipids and proteins, self-assembled into a dynamic matrix described as a fluid mosaic¹. The affinity of membrane components with their ligands can be influenced by the particular chemical composition of the membrane, mutual lipid-protein interactions, physical state (i.e., particular thermodynamic phase), internal organization into domains, or membrane curvature^{16,18,20,23,34–40}. Isolated from this intricate structure, lipids and membrane proteins may interact very differently or not at all with their ligands. These considerations argue for measuring affinities of target molecules presented in a functional membrane.

A number of analytical techniques, including radioimmunoassay (RIA)⁴¹, enzyme-linked immunosorbent assay (ELISA)^{42,43}, surface plasmon resonance (SPR)^{20,31,37}, electron spin resonance (ESR)^{31,44}, optical spectroscopy^{2,17,20,28,31,33}, and imaging assays^{28,29,31,33,45}, have been applied to describe interactions with biological membranes both qualitatively and quantitatively. Current methodologies generally measure the interaction between one soluble reactant and the other immobilized on a solid surface⁴⁶. The immobilization of one component may introduce surface matrix

artifacts to the measurement^{47,48}, or may present an unsatisfactory orientation of the molecule^{49,50}. Dedicated techniques have been recently developed to quantify binding at membrane interfaces but their utility was demonstrated only for particular experimental systems^{15,51}. In addition, these experimental explorations are accompanied by a large variety of theoretical and computational approaches unique to the experimental conditions^{5,23,25,30,32}. Among these techniques, the kinetic exclusion assay (KinExA) presents an appealing choice for assessing membrane-ligand interactions in various experimental formats^{9–12,52}.

The KinExA technology measures the free amount of a binding partner left in an equilibrated solution that contains a specified amount of one partner (constant binding partner, CBP) and a variable amount of the other (titrant, T). The first step involves equilibration of the binding partners in solution, therefore closely resembling physiological conditions, and does not require any labeling or immobilization that may obscure the true binding constants. As the initial concentration of titrant increases, the equilibrium concentration of free CPB will decrease. The equilibrated solution is then flowed over a bead pack in a small flow cell, functionalized to specifically bind the CBP molecules. A portion of the free CBP is captured on the beads, and the captured fraction is quantified by fluorescence provided either from the captured molecules themselves or by secondary labels. The fluorescence signal is analyzed by fitting the experimental data to a binding model that provides exact analytical solutions to accurately determine affinity. This technology has been employed to accurately measure affinities of antibodies for cell surface receptors by using live cells as binding partners^{9,11}. The use of live cells may introduce uncontrolled irregularities such as batch variability, artifacts

presented by persistent growth of cells during equilibration and measurements, or secretion of compounds or even a physiological response that may interfere with the interactions under scrutiny. To alleviate some of these problems, a “lifeless” membrane system made from ruptured membranes was recently used for binding affinity determination of therapeutic antibodies to membrane targets¹². However, this methodology required multiple isolation and purification steps to eliminate potential artifacts arising from a low purity of the analyzed membranes.

In this work we use artificial and natural membranes as capture elements to extend KinExA technology into this realm. KinExA-based measurements generally use immobilized binding partners or antibodies as capture elements^{9,10,12}, which are not always available for the intended measurements. A common approach to preparing these capture elements is to first coat polymethylmethacrylate (PMMA) beads (among other materials) with biotinylated bovine serum albumin (btb-BSA), followed by attachment of a biotinylated molecule through an avidin sandwich process. Previous reports have documented the use of cholesterol-based anchor lipids to modify the surface properties of cells and lipids⁵³ and to anchor cells to solid surfaces⁵⁴. With this knowledge, it was reasonable to suppose that this linker could be used to anchor liposomes, made from both natural and artificial membranes, to the beads used in the KinExA process.

Using the improved approach described in this work, the natural receptor of the ligand we are measuring is also the capture element. We tested this experimental system to quantify the interactions between lipophilic dyes and artificial liposomes, cholera toxin subunit B (CTB) and artificial liposomes containing gangliosides, and sheep red blood cells and their antibodies. Our results demonstrate that this technology may be used to

precisely determine affinities for biological membranes of large and small molecules while eliminating many of the shortcomings plaguing alternative approaches.

Materials and Methods:

Materials:

The lipids asolectin (Aso) and cholesterol (Chol) were purchased from Sigma-Aldrich, and ovine brain ganglioside (GM1) from Avanti Polar Lipids as powders (Figure 3-1). Stock solutions from the powders were prepared by dissolving the lipids in chloroform at concentrations up to 100 mg/mL.

Figure 3-1. Lipids used in this study.

Top: Asolectin is a mixture of phosphatidylcholine (shown), cephalin, phosphatidyl-inositol and a few other minor components, extracted from soy beans; phosphatidyl-choleoline is the major component. **Center:** Cholesterol. **Bottom:** Ganglioside GM1.

The stock solutions were kept frozen until use, and any further dilution was made with chloroform. Whole sheep blood in Alsevers solution (1:1) was purchased from Colorado Serum Company (Denver, CO, USA) and kept refrigerated until used to prepare liposomes from red blood cells (RBCs). A 3 mg/mL stock solution of anti-sheep RBC antibodies labeled with fluorescein isothiocyanate (FITC) was purchased from LifeSpan Biosciences. CTB labeled with Alexa Fluor 647 (CTB-AF647) from LifeTechnologies was used for determining the interactions with liposomes. The cholesterol-polyethylene glycol 2000-biotin linker (Chol-PEG-Btn, Figure 3-2) was purchased from NanoSoft Polymers, and a stock solution of 1 mg/mL in phosphate buffered saline (PBS) was prepared and kept frozen until further use.

Figure 3-2. Chol-PEG-Btn. Cholesterol (left side) is linked to biotin (right side) through a polyethyleneglycol (PEG2000) linker (middle).

The far red-fluorescing lipophilic carbocyanine dye DiD (1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (Figure 3-3), was purchased from ThermoFisher Scientific as a crystalline salt and was dissolved in pure ethanol; any further dilution of the stock solution for kinetic exclusion assay measurements was performed in PBS.

Figure 3-3. The lipophilic dye, 1,1'-dioctadecyl-3,3',3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (DiD)

Dry polymethylmethacrylate (PMMA) beads of 75 μm average diameter (Sapidyne Instruments), functionalized as described below, were used as the solid phase bead pack for measurements with the KinExA 3200 instrument with autosampler (Sapidyne Instruments).

Preparation of Liposomes from Lipids

Liposomes, an excellent biomimetic system for characterization of ligand-membrane interactions^{16,28,31} were prepared from lipids by extrusion²⁸ through filters mounted in an Avanti lipids extruder. Powdered lipids (Aso, Chol, and GM1) were dissolved in chloroform, mixed and dried overnight under vacuum in 20 mL glass vials. The dry weight ratio of the lipids was Aso:Chol:GM1=10:4:0.6. A control preparation contained the same proportion of Aso to Chol, but with no GM1. The total amount of Aso added to each vial was 10 mg. After chloroform evaporation, the dried lipid cake was hydrated for one hour at 55° C in 1.1 mL of a 135 mM KCl solution buffered at neutral pH with 20 mM HEPES. The hydrated lipid cake was subjected to five cycles of freezing-thawing, followed by 30 seconds of sonication in a sonicator bath (Fisher

Scientific) to achieve complete dispersion of the lipid aggregates. The hydrated mixture was introduced into the extruder equipped with 200 nm polycarbonate filters, and extruded at low speed for a total of 40 cycles. After extrusion, the liposomes were assessed by dynamic light scattering (DLS) with a Malvern Zeta-Sizer and found to have an average diameter of 146.3 nM with a polydispersity index (PDI) value ranging from 0.172 to 0.227 (Figure 3-4); the liposomes were kept refrigerated until further use.

Figure 3-4. Dynamic light scattering characterization of artificial liposomes. Representative analysis by Malvern Zeta-sizer. The liposomes were prepared by extrusion.

Preparation of Liposomes from Red Blood Cells

RBCs were separated by centrifugation of 5 mL whole sheep blood in heparinized tubes at 1500 RCF. The RBC pellet was washed with 5 mL ice-cold PBS. After two more

cycles of centrifugation-PBS rinsing, the pellet was resuspended in 5 ml of DI water and refrigerated at 4° C for 1 hour to lyse the cells. After addition of 5 mL PBS, the RBC cellular membranes (ghosts) were separated in five consecutive cycles of centrifugation at 18,000 RCF for 5 minutes at 4° C and rinsed with 5 mL PBS. A final volume of 2 mL RBCs in a glass vial was sonicated with a Misonix S-4000 tip sonicator. The glass vial was placed on ice, and the sonication was performed for 15 minutes at 25% amplitude setting, a power transfer of 6-7 W, and a total energy transfer of 5.4 kJ. The liposomes were sized by DLS in triplicate, which indicated a mean diameter of 144.7 nm and a PDI ranging from 0.161 to 0.182 (Figure 3-5).

Figure 3-5. Dynamic light scattering characterization of liposomes made from RBC.

The liposomes were prepared by extrusion of purified RBC ghosts as described in the text.

Bead Preparation

The procedure for attaching liposomes to beads for kinetic exclusion assay measurements is depicted in Figure 3-6. Two hundred mg of PMMA beads were mixed in a vial with 1 mL PBS containing 30 μ g biotin-BSA (SigmaAldrich) for adsorption coating. After rotating the vial for two hours, the beads were washed five times with 1 mL PBS after gravity pelleting. Next, a coating of streptavidin was achieved by mixing the bead pack with 1 mL PBS containing 100 μ g SA and 10 mg BSA, and rotating the vial for two hours at room temperature⁵⁵. The free proteins were removed with five cycles of pelleting/PBS washing. The next step of functionalization consisted of coupling the Chol-PEG-Btn linker to the surface of the beads via the strong biotin-streptavidin bonds; this was achieved by mixing the beads with 10 μ M Chol-PEG-Btn linker in 1 mL PBS, and rotating the vial for three hours at room temperature. After five cycles of pelleting/PBS washing, the final step of bead preparation was liposome attachment. To anchor the liposomes to the beads, the bead pack was mixed with either 400 μ L of extruded liposomes or 800 μ L of RBC liposomes and rotated overnight in a refrigerator. After removal of free liposomes by pelleting/PBS washing, the beads were placed in a bead vial for the KinExA 3200 instrument, and PBS added for a total volume of 30 mL.

Figure 3.6. Schematic representation of liposome attachment to PMMA beads. PMMA beads were coated with biotin-conjugated BSA by adsorption. Streptavidin was added to activate the surface, and the surface was further functionalized by the addition of Chol-PEG-Btn. The cholesterol moiety self-inserts into membranes to capture the liposomes.

The functionalized beads were used as the ligand-capturing solid state phase for kinetics exclusion assays of the affinity of toxins. All of the ligands used were fluorescent, either intrinsically or by labeling. For this investigation, we employed CTB-AF647, fluorescein-labeled anti-sheep RBC antibodies, and the lipophilic dye DiD, prepared as described in the materials section.

Results and Discussions

Measuring the Interactions Between Gangliosides and CTB-AF647.

To determine the affinity of CTB for GM1, we used beads functionalized with GM1 liposomes to capture the free CTB-AF647 from solutions pre-equilibrated with liposomes. Two mL aliquots of a 10 pM CTB-AF647 solution as the constant binding partner were mixed with varying amounts of liposomes (thus different concentrations of GM1) as titrant. The GM1 concentration in the sample vials varied from 0.1 nM to 200 nM. The solutions were equilibrated for 6 hours at 25° C and introduced for automatic measurements in the autosampler of the KinExA 3200 instrument. It is notable that we

did not perform any additional separation of the free CTB-AF from solutions; we assumed that the CTB-AF647 bound to liposomes would not interact with the liposomes anchored to the PMMA beads, and only the free toxin would bind to the liposomes on the beads. Also, due to the small size of the liposomes (~ 200 nm), we assumed that all the liposomes free in solution would pass through the flow cell during the process. This is a very reasonable assumption since the mesh size of the filter that keeps the beads in the flow cell is ~ 20 μm , which is ~ 100 times larger than the average size of the liposomes.

After equilibration, the evolution of the fluorescence signal for each sample was measured with the KinExA instrument. For GM1 liposomes, the fluorescence of CTB-AF647 started to increase monotonically immediately when the bolus reached the flow cell (Figure 3-7). The increasing signal represents the fluorescence recorded from both free and bound CTB-AF647 (either to liposomes in the equilibrated solutions, or liposomes anchored to the beads). The buffer exchange removes the free ligand and the ligand bound to liposomes in solution, which is seen as a decrease of the fluorescence signal. The end signal is given by the fraction of CTB-AF647 attached to the beads, and is proportional to the amount of free ligand in solution. One may easily observe that increased amounts of GM1 liposomes in the equilibrated solutions resulted in a decreased end signal, indicating a smaller amount of free CTB-AF647.

Figure 3-7. KinExA sensogram of CTB captured by GM1 liposomes. Ten pM CTB was mixed with varying amounts of liposomes (thus of GM1) and allowed to equilibrate. The equilibrated solution was analyzed by KinExA with liposomes immobilized on PMMA beads as the capture element.

The end signal was plotted as percent maximum versus GM1 concentration and the KinExAPro software provided a K_D value of $5.98 \text{ nM} \pm 0.78\%$ for the GM1-CTB interaction (Figure 3-8). This value is well within the range reported for such interactions by employing alternative techniques^{16,20,37,38}. However, this value must be carefully interpreted for its meaning with respect to molecular interactions. The interaction between the CTB ligand and GM1 is not at all a traditional bimolecular interaction since GM1 is actually included in a membrane. It is not known how the physical and chemical properties of the membrane (i.e., composition, curvature, lipid segregation) affect the interaction. The interactions between membrane and ligands might be better described as

a Langmuir absorption process, and the affinity estimated as either a Langmuir constant or partition coefficient.

Figure 3-8. Theory curve for CTB binding to GM1 liposomes. Data shown in Figure 3-7 was analyzed by KinExAPro software to generate a K_D estimate of $5.98 \text{ nM} \pm 0.78\%$.

Next, we asked whether or not GM1 is an essential receptor for CTB interactions with membranes^{16,20,37,38}. To answer this question, we prepared the beads by following the same protocol previously described but used liposomes containing no GM1 lipids. The testing was initiated by flowing only CTB-AF647 over the liposome-functionalized beads. To get an idea of the magnitude of the interactions, we increased the CTB-AF647 100 fold (i.e., 1.0 nM). At this very high ligand concentration, the fluorescence signal increased monotonically during injection and reached a very large value (10 V, Figure 3-

9). Nonetheless, despite this large fluorescence value, the end signal recorded after injecting ligand-free buffer in the flow cell decreased to a negligible value. This very small fluorescence signal, together with the large ligand concentration used in this experiment, indicates that GM1 is an essential component for interactions between CTB and membrane, and for the complete cholera toxin to manifest its effects^{16,20,37,38}.

Figure 3-9. Cholera toxin is not captured by non-GM1 liposomes. A bed of beads coated with liposomes devoid of GM1 was challenged with a 1.0 nM solution of CTB-AF647. The immediate decrease in fluorescent signal to a negligible value upon initiation of the rinse phase shows that very little CTB-AF647 is bound to the liposome capture element.

The fit of experimental data provided a satisfactory value for affinity, but this was calculated by employing a single-step, one-to-one binding model. Although this model is presented in multiple reports, investigators also considered that CTB binding is a multi-

step, cooperative process^{35,36,56}, with a Hill coefficient less than 2. Therefore, we employed the logistic equation to calculate the affinity and cooperativity coefficient (Figure 3-10), which provided a better fit of experimental data (Figure 3-10); the affinity value ($K_D = 11.36 \pm 0.6$ nM) and Hill coefficient ($n = 1.36 \pm 0.15$) are in reasonable agreement with values reported using other techniques^{35,38}.

Figure 3-10. Logistic fit of data for CTB captured by GM1 liposomes. Data shown in Figure 3-7 was analyzed with Origin 8.5 software using a logistical model to generate a K_D estimate of 11.36 ± 0.6 nM, with a Hill coefficient of 1.36 ± 0.15 .

Interactions Between Antibodies and Membranes

To investigate the interactions between antibodies and membranes, we assessed the binding of anti-sheep RBC antibodies to RBCs by employing KinExA. To achieve

this goal, we used PMMA beads functionalized with RBC liposomes as described in Methods. However, the specific target of the antibodies on the membrane surface is unknown, and no quantitative indication of affinity or titer was provided by the supplier. To characterize the antibodies, we attempted fluorescence microscopy imaging; while this approach allowed us to verify specific staining, sustained photobleaching even during short exposure precluded any quantitative analysis. In addition, we were able to surmise that the antibodies do not possess a great affinity for the target since the fluorescent images faded significantly after washing, or by antibody dilution with buffered isotonic solutions added to the bulk.

The great advantage of the KinExA technology is that it requires knowing the concentration of only one partner participating at the binding reaction in the equilibrated samples, and this is essential for assessing membrane-ligand interactions in which only one partner (usually the ligand) has a known concentration. In our analysis, we used the RBC liposome beads to capture the free Ab from solution, while the equilibrated samples comprised a fixed concentration of Ab (100 nM) and a variable number of RBCs. Although the RBC liposomes may be also used as titrant, a more thorough analysis requires knowing the number of liposomes/sample. Owing to their small size, they cannot be easily quantified. A rough estimate of their concentration may be obtained from lipid concentration and average size; nonetheless, one may encounter major deviations between theoretical estimates and experimental values owing to loss of lipids during preparation procedures, polydispersity, addition of small molecules (i.e., cholesterol), and many other factors. In contrast, cellular densities may be accurately determined by

cytometry. Therefore, we used RBCs as titrant to generate the Ab-RBC equilibrated samples for KinExA measurements after 3 hours of equilibration at 25° C.

The equilibrated samples were introduced into the fluidic system of the KinExA 3200 instrument equipped with a set of fluorescein filters. The end signal for each run increased as the concentration of the titrant (RBC) decreased, resulting in more free Ab available to interact with the RBC liposomes anchored to the beads (Figure 3-11).

Figure 3-11. KinExA sensogram for anti-RBC antibodies equilibrated to RBCs. Constant concentrations of Ab were equilibrated against varying amounts of RBCs. The equilibrated solutions were analyzed by KinExA with PMMA beads functionalized with liposomes made from RBCs as capture element.

The binding signal expressed in percent maximum was fitted with the binding equation, accounting for the number of cells present in each sample (Figure 3-12). Data

analysis with KinExAPro software provided an affinity value $K_D = 38.6 \text{ nM} \pm 0.2\%$. The antibodies we used are polyclonal, and thus represent multiple molecular targets on the surface of the RBCs. As a result, the number of binding sites/cell reflects the cumulative number of targets present on cell membranes, irrespective of their chemical identity. Thus, this K_D value certainly needs to be interpreted as Ab affinity for the entire RBC membrane instead of a specific receptor. Despite this apparent shortcoming, there is no doubt that the affinity value is not high, which we anticipated from microscopy imaging experiments. However, since we were able to provide the number of cells as an input parameter, the software can estimate the “expression level” (site density) to be 4.7×10^6 binding sites/cell. Consequently, this technique can also be very useful in determining the expression level for known targets on the surface of cells with monoclonal antibodies.

Figure 3-12. Theory curve for anti-RBC Ab and RBC liposomes.
 The sensogram data shown in Figure 3-11 were analyzed by KinExAPro software to return a binding K_D of $38.6 \text{ nM} \pm 0.2\%$, and an antigen expression level of 4.7×10^6 binding sites per cell.

Determining the Affinity of Lipophilic Dyes for Lipid Membranes

Lipophilic dyes are molecular tools largely used for membrane imaging and tracing in live cells and artificial membrane systems^{26–29,57,58}. They are well characterized for their optical properties (i.e., fluorescence characteristics), and also their mechanisms of interactions with target membranes and how such interactions change their fluorescence^{27–29}, but their use is not standardized, nor is it clear which one should be used for a particular experiment. All lipophilic dyes, as their name indicates, present a preferential interaction with the lipid portion of cell membranes. As a result of such interactions, many of them undergo a massive hyperchromic effect (increase of

fluorescence), or a spectral shift, in a lipophilic environment, therefore facilitating detection with spectroscopy or microscopy^{27,28}. However, it is not clear how the affinity changes when the target membrane has a different chemical composition from the test membrane. Consequently, a rapid method for measuring the affinity of lipophilic dyes for target membranes may provide a useful means of determining the applicability of such dyes for particular applications. To explore this possibility, we employed KinExA to determine the affinity of the lipophilic dye DiD for artificial membrane systems.

The PMMA beads were functionalized with liposomes composed of Aso and Chol and prepared by extrusion as described in the Methods section. The liposomes were also used as titrant to prepare equilibrated solutions that contained variable amounts of liposomes and a constant concentration of lipophilic dye (10 pM). After two hours of equilibration at room temperature, the samples were centrifuged at 56,000 RCF and the supernatant assessed for the free dye content by using KinExA and the liposome-functionalized beads as the capturing solid phase. The evolution of the fluorescence signal upon sample introduction in the flow cell and subsequent rinse with dye-free buffer solutions is shown in Figure 3-13. The fluorescence signal starts increasing immediately as the sample bolus reaches the flow cell, similar to the previous experiments. This signal is generated by free lipophilic dye molecules interacting with the membranes of the liposomes attached to the beads. Unlike previous experiments, rinsing of the bead pack with dye-free buffer does not elicit a visible decrease of the fluorescence signal. This particular shape is explained by the intrinsic properties of the label. In a hydrophilic environment, this lipophilic dye presents a weak fluorescence. However, when inserted into the hydrophobic core of the lipid membranes the intrinsic

fluorescence increases substantially, hence facilitating its detection. Since the free dye has a weak fluorescence, its contribution to the total signal is negligible, thus its removal from the flow cell does not affect the fluorescence signal. Dye molecules bound to liposomes in the equilibrated samples were removed by centrifugation and, therefore, did not contribute to the fluorescence signal during analysis. The detected signal is chiefly generated by the free dye binding to the liposomes attached to the beads. The flat signal plot recorded during rinsing indicates that the lipophilic dye molecules bind tightly to the target membranes and do not leave them easily. Similar signal shapes are common when the fluorescence of the captured molecule far exceeds the fluorescence of the sample itself⁵⁹.

Figure 3-13. KinExA sensogram of DiD equilibrated to liposomes. Varying amounts of liposomes, reported as lipid concentration, were brought to equilibrium with a constant concentration of DiD. The liposomes and solution were separated by centrifugation, and the residual dye analyzed by KinExA.

The binding curve representing the percent signal as function of label concentration is depicted in Figure 3-14. The best fit performed with the KinExAPro software provided a K_D value of $215 \text{ pM} \pm 0.59\%$, confirming our prediction with regards to a high affinity of this particular dye for lipid membranes. To calculate this K_D value, we used the total lipid concentration employed to produce the liposomes. However, as previously discussed, this analysis is descriptive of molecular absorption at a surface, but this value may better be interpreted as a partition coefficient. However, unlike a classical Langmuir isothermal absorption process, although the ligands may interact at first with only one side of the membrane, they are expected to diffuse into the lipid core of the

membrane, therefore introducing computational complications when utilizing a traditional binding model.

Figure 3-14. Theory curve of dye accumulation by liposomes.
Data shown in Figure 3-13 were analyzed by KinExAPro software, which returned an apparent K_D of 215 pM $\pm$ 0.59%.

The KinExA technology, when used with liposome capture elements, provides an ideal platform for characterizing membrane-ligand interactions with membrane-penetrating toxins or lipophilic dyes, as well as with surface-anchoring molecules. Although the common feature of this and other experimental platforms is the use of membrane systems anchored to the solid phase to capture the free ligand, the KinExA approach is unique in pre-equilibrating the receptor-ligand pair in solution with either cells or biomimetic systems. Nonetheless, one must ponder the interpretation of the K_D

value with respect to molecular interactions, in spite of its extensive use for quantification of membrane-ligand interactions. Interactions with intact membranes have resisted measurement by traditional methods. As we previously stated, the ligands may bind completely differently to the individual target molecules (i.e., lipids, or proteins) when presented as part of an intact membrane than when presented as a free molecule. Having one member of the reaction pair anchored into a relatively large and immobile membrane changes the dimensionality of the system from a tri-dimension to a two-dimension interaction space^{4,36,51}. This may have consequences on the accuracy of the interaction model⁴, which might be better described as a Langmuir absorption process^{20,38}. The influence of membrane composition, curvature, clustering, cooperative binding, and target multivalency^{35–38,40,51,56,60} suggests that avidity or partitioning coefficients may be more appropriate parameters to characterize ligand-membrane interactions.

Conclusions

KinExA is a powerful platform used to characterize intermolecular binding, limited only by the ability to capture ligands in the analytical chamber for analysis. Heretofore, this limitation has been most critical in capturing ligands to transmembrane proteins or for surface proteins for which a soluble fragment is unavailable. In this work, we show that:

- 1) Liposomes produced from natural or artificial membranes can be easily immobilized in a straightforward manner to the beads used in the KinExA flow cell for use as capture elements.

2) Liposomes so immobilized will successfully capture ligands of interest, whether they be target-specific molecules (e.g., antibodies or toxins) or generalized molecules (e.g., dyes).

3) Kinetic parameters of academic and commercial interest can be determined using the liposome immobilization process coupled with KinExA analysis.

Further investigation as to the nature of surface antigens so presented is warranted. Affinity of surface antigens and their ligands is often measured using soluble fragments of the former as surrogates, but these surrogates may misrepresent the native conformation and the resulting measurements may be misleading. Factors such as oligomerization⁶¹, stability⁶², and function⁶³ may be essential to the interaction and may be absent from purified fragments. Some efforts to overcome these obstacles have included detergent micelle preparations⁶³⁻⁶⁵ to mimic the trans-membrane milieu, and formaldehyde fixation¹⁰ to preserve native configurations over time. Binding studies of mAbs to formaldehyde-preserved surface antigens retained full activity for some antigens, complete inactivation of others, and some in between¹⁰. Thus, it can be said that results were mixed. Micellar purification is wrought with numerous confounding parameters, often requiring tedious trial-and-error approaches before finding a satisfactory solution⁶⁴. Such purified components, nonetheless, may still not mimic native configurations as other membrane components, lost during purification, may have significant influence on structure and function.

Our studies presented here promise to extend the use of KinExA analyses to unpurified and even unknown cell surface reactants, together with determining the density of the binding sites present on particular membrane systems.

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CHAPTER FOUR: CONCLUSIONS AND PERSPECTIVES

The KinExA concept, and the instrument designed to exploit it, is a sensitive and versatile method to measure affinity and kinetic data for bimolecular interactions. In the work represented in this dissertation, I have extended the use of KinExA to encompass aptamers as both recognition and capture elements, and also described a means of potentially measuring complex membrane components in a natural setting.

Aptamer Studies

Our experimental results with aptamers demonstrate the utility of using aptamers as capture elements for analytes of interest, and that KinExA can also be used to characterize the aptamers themselves. Certain assumptions were made, and realized, in the design of the experiments presented in Chapter Two and published in the journal *Sensors* in 2020¹. Follow-on work should acknowledge these features, build upon them, and perhaps circumvent any limitations they might impose.

The first, and most important, assumption was that the thrombin-specific aptamer would not bind to its complimentary sequence, the capture aptamer, when bound to the target molecule thrombin. The second assumption was that the free (and only the free) aptamer would bind to the capture aptamer with sufficient affinity to serve as a fluorescent beacon. These two assumptions were by no means assured when we began the study.

In this context, it is worth mentioning that the first thrombin aptamer I investigated was one identified by Bock et al. in 1992². They identified a 15 nucleotide

consensus sequence of several aptamers they isolated as GGTGTTGGTGGTGGTGG.

However, we tested a biotinylated sequence complementary only to a seven nucleotide sequence on the fluorescent aptamer. Although binding competition was observed, a relatively fast washing away during the rinse phase was also observed, most probably due to a fast k_{off} . These results clearly exhibit the “KinExA mode” limitation of the technology (see Chapter One).

This earlier experience, as well as the published results, suggest a number of studies to further investigate the system.

1. The Bock and Tasset thrombin aptamers both include a consensus sequence first identified by Bock. The capture aptamer that we used for the Tasset aptamer has a complementarity of nine nucleotides, although discontinuous, as opposed to the seven contiguous nucleotides for the errant capture aptamer. It would be interesting to see if the Bock aptamer is bound to this capture aptamer, and if so, to compare the affinity.

2. Similarly, what is the minimum complementarity required to efficiently capture the labeled aptamer?

3. The decreasing signal of captured free aptamer from solution in equilibrium with its target represents a competitive binding situation. Can this phenomenon be exploited to identify the key nucleotides of the aptamer binding site? If so, then targeted chemical modification of those nucleotides could result in even greater affinity and/or specificity.

4. Can this process be used to identify drugs that will bind to specific sequences of DNA or RNA and block transcription and translation? One way to achieve the production of specific gene products (rather than suppressing translation overall) is by

introducing small interfering RNA (siRNA) into the cell³. The ability to deliver RNA to a cell is by now well established, witnessed by the mRNA vaccines which, at this writing, are being administered *en masse* for the first time to combat the COVID-19 pandemic^{4,5}. The conceptual methods demonstrated here may be used to quickly assess the affinity of siRNA constructs, and, perhaps more importantly, semi-synthetic modifications which may prove to be more effective.

Membrane Studies

Conceivably, any ligand that can be captured in the analytical chamber for analysis can be measured. A major limitation for this and other systems (e.g., SPR or ELISA) has been capturing ligands to transmembrane or membrane-bound proteins for which a soluble fragment is unavailable or even unknown. In this work, we show that:

1) Liposomes produced from natural or artificial membranes can potentially be easily immobilized in a straightforward manner to the beads used in the KinExA flow cell for use as capture elements.

2) Liposomes so immobilized will successfully capture ligands of interest, whether they be target-specific molecules (e.g., antibodies or toxins) or generalized molecules (e.g., dyes).

3) Kinetic parameters of academic and commercial interest can be determined using the liposome immobilization process coupled with KinExA analysis.

Note that the first assertion states that natural membranes can “potentially” be immobilized. Erythrocytes are significantly less complex than other cells, in that they are essentially devoid of organelles. Purified membranes, or “ghosts,” are entirely composed

of cytoplasmic membrane, and readily form liposomes under the conditions described in Chapter Three.

Numerous preliminary experiments were not successful in attaching whole cells to beads, but led to the concept of forming the membranes into tiny liposomes and attaching the liposomes to the beads through a cholesterol linker. The much smaller liposomes would be expected to have more structural integrity than the larger whole cells, and to be less apt to come off or break up under shear.

Studies presented here promise to extend the use of KinExA analyses to unpurified and even unknown cell surface reactants, together with determining the density of the binding sites present on particular membrane systems. The successful coating of PMMA beads with RBC-derived natural liposomes, and the recovery of anti-RBC antibodies by the same in the KinExA procedure, suggest a number of further studies.

As mentioned above, RBCs are relatively simple cells, devoid of organelles. Membranes recovered from lysed RBCs are, therefore, entirely and uniformly cell membranes. Other cell types will pose additional challenges. Numerous protocols exist for the isolation and purification of cellular and organelle membranes⁶, and preparation of liposomes from membranes so isolated should be thoroughly investigated.

Rathanaswami et al.⁷, used KinExA to conduct affinity measurements of antibodies to unpurified antigen, by equilibrating the antibodies with whole cells, capturing the residual free antibody on beads coated with a heterospecific anti-immunoglobulin. Using the same general scheme, residual ligand could be captured by beads coated with natural liposomes. Would it even be necessary for the resulting

liposomes to be a homogeneous presentation of cellular membrane? Would it matter if nuclear, endoplasmic reticulum or mitochondrial membrane were present? The important parameter is that free ligand from the equilibrium phase of the experiment be captured and measured.

Isolation and purification of membrane proteins frequently involves solubilization of the membrane with detergents, assuming that the membrane anchored or transmembrane protein maintains its native properties when sequestered within the resulting detergent micelle. This may not be the case. Membrane fractions from lysed cells may be isolated by ultracentrifugation on a discontinuous sucrose density gradient⁸. Efforts to form and characterize liposomes from these fractions may prove fruitful.

All the advantages exhibited by KinExA are anticipated to be maintained with novel capture elements. KinExA is highly accurate and reproducible⁹. It avoids matrix interference in analysis, both at the surface of the capture element^{10,11} and in the analytical milieu¹². Mobility effects due to mass transport or molecular weight pose no limitations^{13,14}. As it measures only free ligand in solution, it avoids surface-immobilization procedures that may alter binding constants compared to those measured in true solution phase. The instrument has been used to examine binding partner interactions from chelated ions^{15,16} and small molecules^{17,18} to whole cells^{7,19–21}. The ability to reveal complex binding stoichiometries, demonstrated in Chapter Two and elsewhere^{17,22}, may provide valuable insights into fundamental analyses of aptamer–analyte and surface protein interactions, while its high sensitivity and accuracy suggest that this technique will be useful for even more bioanalytical applications.

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