Chapter 2

Measuring Novel Protein-Protein Binding with Surface Plasmon Resonance in the Physical Chemistry Lab

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In the laboratory for Survey of Physical Chemistry, students proceeded through a five-week project in which they measured protein-protein binding. This project engaged the students in learning physical chemistry and laboratory teachniques as they took ownership of a particular, novel protein-protein interaction. First students purified new proteins by size-exclusion chromatography and learned about separation and diffusion. Then students measured the binding strength of new protein-protein combinations using surface plasmon resonance (SPR) as they learned about SPR physics, experimental design, equilibrium binding, and data fitting using integrated rate laws. The web-based platform GENI provided protocols to the students and collected data, organizing projects spanning multiple classes. In the space of an academic year, students asked a question, then found the answer in the lab. Together, by expressing new proteins and measuring binding thermodynamics and kinetics, we found that the NKG2D immunoreceptor and its MIC ligand proteins show remarkable cross-reactivity among human, rabbit, and gorilla orthologs.

Introduction

For the things we have to learn before we can do them, we learn by doing them.
— Aristotle, *The Nicomachean Ethics*

Teague and Gardner; Engaging Students in Physical Chemistry
ACS Symposium Series; American Chemical Society: Washington, DC, 2018.

Students best learn science through its practice (*1*, *2*). Engaging students in the processes of science promotes applied learning that has value beyond the subset of students destined for work in academic or industry science. The goal of collecting unique and novel data is a powerful source of motivation that invests students in their projects personally. As they complete their projects, students must apply their learning to solve novel problems, think critically, and communicate their findings in professional settings. In doing so, both students and faculty are motivated by the understanding that they are making valued contributions to the scientific community, leading to more significant commitments to learning.

More students can glean these benefits if authentic research projects are implemented in the blocks of time allotted to teaching labs (*3*). Scientists at academic institutions also benefit, because we are expected to both conduct research and teach courses. This approach allows teaching faculty to do both activities at the same time, and in a manner that improves student learning and accomplishes genuine scientific investigation.

Authentic research in the teaching lab, also described as course-based undergraduate research experiences (CUREs) have been adapted to many disciplines at many institutions, including general/inorganic chemistry (*4*), analytical chemistry (*5*), and physical chemistry (*6*, *7*). These publications demonstrate that this approach to teaching is both powerful and practical. The best way to teach students to become scientists is by training them to do the work of scientists. However, it can be difficult to fit an authentic research experience into the constraints of limited classroom resources and time while teaching students new protocols and procedures (*8–10*).

A review of research literature on teaching physical chemistry included a recommendation to introduce students to original research to engage them through "the feeling of ownership and responsibility" that comes from authentic inquiry (*11*). The same review notes that the subfield of thermodynamics is one of the most important areas of physical chemistry for students to learn (*11*). Here is described an authentic research project addressing this particular subfield, which has been carried out in a Survey of Physical Chemistry class for multiple years. This project teaches students thermodynamic and kinetic concepts as they gather data on novel protein-protein interactions using advanced and widely used preparatory and analytical techniques that employ interesting physical chemistry.

The techniques used are protein separation by size-exclusion chromatography and protein-protein interaction analysis by surface plasmon resonance (SPR). Both techniques involve physical chemistry theory that the students can apply while collecting and interpreting their data. Students apply physics-based equations for molecular diffusion (*12*) to their size-exclusion chromatogram to explain why larger molecules elute first. The theory of SPR combines many physical chemistry concepts: the collective behavior of electrons as plasmons, the physics of light and lasers, the conversion of energy (in the form of resonance removing certain wavelengths), the determination of a thermodynamic binding constant (K_D) at equilibrium, and the use of integrated rate laws in determining the best fit for the observed protein-protein binding kinetics. Multiple chapters in the physical chemistry text that we cover can be related to one of these laboratory techniques. A comprehensive review of how SPR theory can be applied to the undergraduate

physical chemistry class and the nature of the available instrumentation is given elsewhere (*13*).

The project students undertook in Survey of Physical Chemistry required five weeks of laboratory time. Students learned through authentic research by purifying proteins by size exclusion in a one-week exercise and then measuring the binding thermodynamics and kinetics of those proteins by SPR in a four-week exercise. The proteins were previously selected and/or designed by students as independent projects, and then were expressed and purified by students in the context of a Biochemistry II course (Figure 1). Some students take Survey of Physical Chemistry after Biochemistry II, and such students can analyze the protein they made in the previous course, giving them additional ownership, responsibility, and engagement.

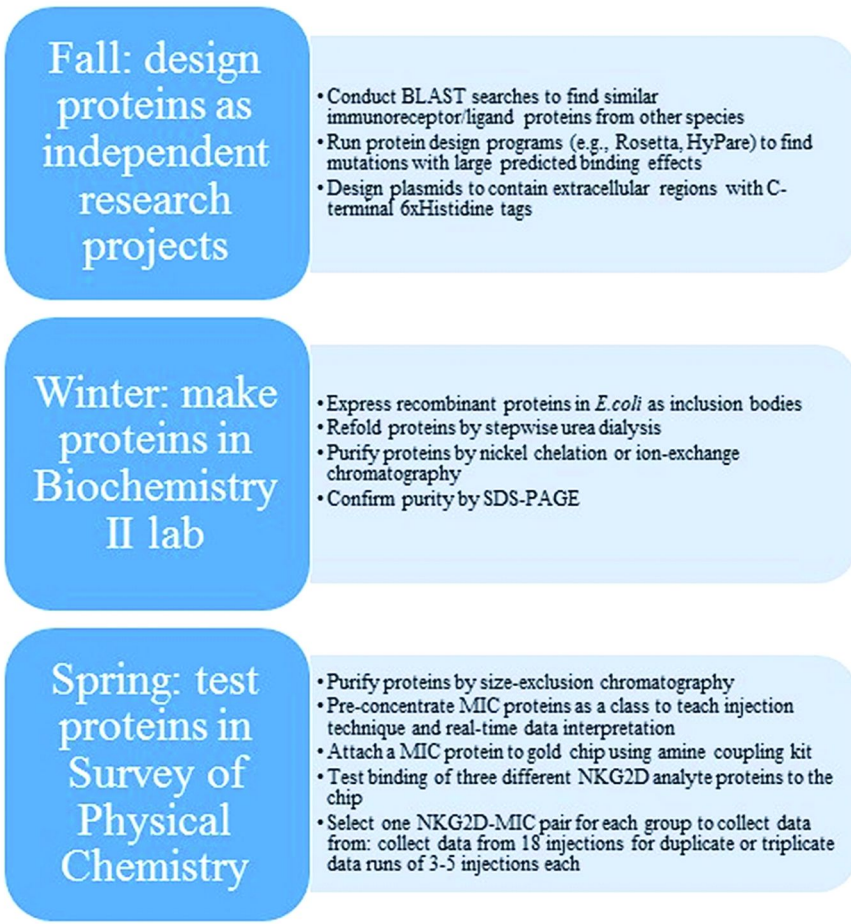

Figure 1. Scaffolding of authentic research projects throughout the three-quarter academic year, in which proteins are made in Biochemistry II and analyzed in Survey of Physical Chemistry. (see color insert)

This specific project used proteins from a previous biochemistry project, but binding projects can also be designed for any macromolecular pair amenable to SPR analysis. Because SPR is a label-free technology, it requires that one binding partner (the analyte) be soluble in buffer and that the other binding partner be tightly bound to the functionalized surface of the chip while maintaining binding activity. Because the SPR response is proportional to the molecular weight of the analyte, experiments are most successful with analytes that are 10-100 kDa in mass.

Within these constraints, this five-week project could be adapted to many different potential binding pairs and does not have to be restricted to protein chemistry. Commerically available antibody-antigen pairs are commonly used for SPR analysis, for example, and "Getting Started" kits for BIAcore instruments available for purchase (GE Life Sciences, Issaquah, WA) include validated antibody-antigen reagents for training scientists, which can be used for teaching students.

Methods

Online Protocol Delivery and Data Collection

This sequence of experiments is organized by the Guiding Education through Novel Investigation (GENI) website at geni-science.org, which was developed by a consortium of biologists and biochemists to facilitate authentic research projects like this in undergraduate teaching laboratories. GENI is a website that distributes protocols to students and collects data from students. I have used GENI for multiple years to organize a bioinformatics project in Biochemistry I, a protein purification project in Biochemistry II, and this protein analysis project in Survey of Physical Chemistry (*14*). GENI is especially useful for collecting data from multiple years of projects carried out in multiple courses, such as the experiments described here, because the results are archived and standardized on the GENI website. Students can access the protocols online in the laboratory using laptops and/or print the protocols depending on their preference and institutional policies on the use of laptops in the lab.

Protein Design and Production

Multiple student protein design projects have been analyzed by students in Survey of Physical Chemistry. For inter-species binding, the human NKG2D sequence from the Protein Data Bank structure 1HYR was used in an NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) search to find other mammalian sequences, with a six-histidine C-terminal tag added to MIC proteins. MIC8_R64G was designed for high-affinity binding to human NKG2D using the programs RosettaDesign and HyPare as described previously (*15*). The sequence for the MICA-B2 isoform was isolated from alternative splice transcripts and recombinantly produced as described previously (*16*). The protein labeled "single-chain NKG2D" was designed as a student project using linkers from the Linker database (http://www.ibi.vu.nl/programs/linkerdbwww/) in the

1HYR sequence, bridging residue Val216 in one copy of the human NKG2D sequence to Glu93 in a second copy (a length of 13.3Å measured from the 1HYR structure). Several linker sequences were tested computationally and experimentally, and we chose the linker sequence VTNTEKL (from the PDB code 2QIL) because it produced the highest yields of active protein. Finally, the sequence labeled "single-chain mutant NKG2D" here was a design project in which RosettaDesign (*17*) optimized the NKG2D 1HYR homodimer structure at five interior hydrophobic positions in each chain, using Rosetta's APOLAR designation for possible replacement residues. Rosetta optimized the protein sequence 200 times and consistently selected five mutated positions in the single-chain NKG2D construct: I26G, L67Y, L70W, I157F, and L198Y. A sequence combining these five mutations was synthesized into an expression plasmid and prepared in the Biochemistry II course as described previously (*18*).

Size-Exclusion Chromatography

Students separated proteins by size-exclusion chromatography using an AKTAprime Plus chromatography system and a HiLoad 16/60 Superdex 200 prep grade column from GE Healthcare Life Sciences (Figure 2A). The AKTAprime Plus instrument was purchased for $10,000 in 2004. Students injected 4-mL samples of refolded protein using a 5-mL loop onto the column equilibrated in HBS-EA buffer (10 mM HEPES [pH 7.4], 150 mM NaCl, 3 mM EDTA, and 0.02% sodium azide; HBS-EP is the same buffer without azide and with 0.02% P20 detergent), ran the column for 150 minutes at a 1 mL/min flow rate, and collected 5-mL samples of protein using the fraction collector. Protein concentrations were determined by Nanodrop ND-1000 (Thermo Scientific) absorbance at 280nm and by bicinchoninic (BCA) assay (Thermo Scientific).

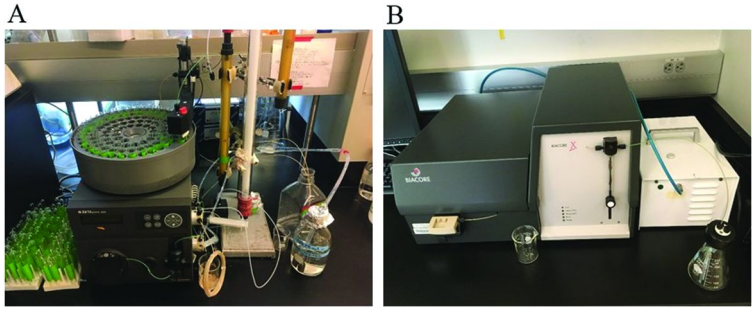

Figure 2. Photos of instrumentation used by students. A) AKTAprime Plus for size-exclusion chromatography; B) BIAcore X for surface plasmon resonance. [Photos taken by the author.] (see color insert)

Surface Plasmon Resonance (SPR)

Students measured protein-protein binding using a BIAcore X instrument (Figure 2B). MIC proteins were coupled to a CM5 chip using procedures and

reagents from an Amine Coupling Kit from GE Healthcare Life Sciences, for 8 minutes at a 10 μL/min flow rate, in a 1:5 or 1:10 ratio with 10mM sodium acetate, pH 5.0 or 5.5, resulting in a permanent 2000-10000 rise in response units. Serial dilutions of NKG2D proteins (the "analyte" protein) were flowed over the chip at nanomolar and micromolar concentrations using flow rates of 40-60 μL/min and with association times of 60-120 s and wash delays of 150-180 s for measuring dissociation kinetics. Data were collected with the BIAcontrol software package and processed with the BIAevaluation software package, using a plot of average response at end of injection vs. analyte concentration (for equilibrium fits), or a 1:1 Langmuir kinetic model (for kinetic fits). For comparison data, a BIAcore T100 at the Fred Hutchinson Cancer Research Center in Seattle, WA, was used to collect data, with CM5 chips, similar coupling chemistry, and similar flow rates and injection times, as described previously (*16*).

Results

This sequence of protocols can be applied to many different types of proteins, and has been used for many different student-led protein design projects, as described in the Methods section. These data were collected by a single class of 10 students in Spring 2014, and the same techniques of using GENI, size-exclusion, and SPR have been carried out for three years with other projects (*14*). The results shown here represent projects that used the design tools RosettaDesign, HyPare, and the Linker database as described in the Methods section, which were on a scale that students could accomplish in a short time, using freely available online resources. These projects can be adapted to many other proteins and provide a chance for students to imagine and build new structures on the macromolecular level.

One project shown here involved a more biological approach, using evolution as a protein designer. It was overseen by a single student and carried out from research question to data interpretation within a single academic year. This project serves as a model for how a complete research project can be carried out within the time and resource constraints of undergraduate research. The research question was formulated by a student, who started with her interest in both veterinary medicine and biochemistry. Given the cross-reactivity of the NKG2D-MIC immunoreceptor-ligand system as observed previously between human and mouse proteins, The student asked whether other mammalian NKG2D proteins might bind MIC proteins from other mammalian species. She searched genomes using the NCBI BLAST search tool and selected genes from mammalian species with ~90% identity to human MIC-A and ~35-70% identity to human NKG2D (using the human sequences from the 1HYR NKG2D-MIC-A structure). We chose sequences with such high identities to increase the likelihood that they would be expressed well in the series of experiments that had already proven to express the human proteins and designed mutants with high yields (*18*). Rabbit NKG2D had perfect identity with human NKG2D hot-spot residues Tyr152, Lys197, Tyr199, and Glu201 (*19*) as well as high overall identity (66%), so the student chose to order both rabbit NKG2D and MIC-A orthologs, and a more

closely related gorilla MIC-A ortholog. These sequences were expressed and purified by students in Biochemistry II according to the previously described sequence of protocols (*18*) published on the GENI website. These students made eight different proteins for the analysis projects taking place in Survey of Physical Chemistry in the Spring.

In Biochemistry II, these proteins were refolded from inclusion bodies and purified by ion-exchange or affinity chromatography. In Survey of Physical Chemistry, they were polished by size-exclusion chromatography before binding thermodynamics were measured. Separation of proteins through FPLC is a common technique in the biotechnology industry, and the AKTA line of chromatography systems is commonly used by alumni employed in local biotechnology jobs. In this course, students learned to operate the AKTAprime system as they manually injected 4-mL samples onto the preparatory size-exclusion column. They compared their data to a previously run set of size standards with known molecular weights and hydrodynamic radii, and constructed a standard curve from these to calculate the apparent molecular weight of their protein. Students observed a major peak around 90 minutes after injection, corresponding to the expected 30-kDa MIC monomer or the 30-kDa NKG2D homodimer (Figure 3).

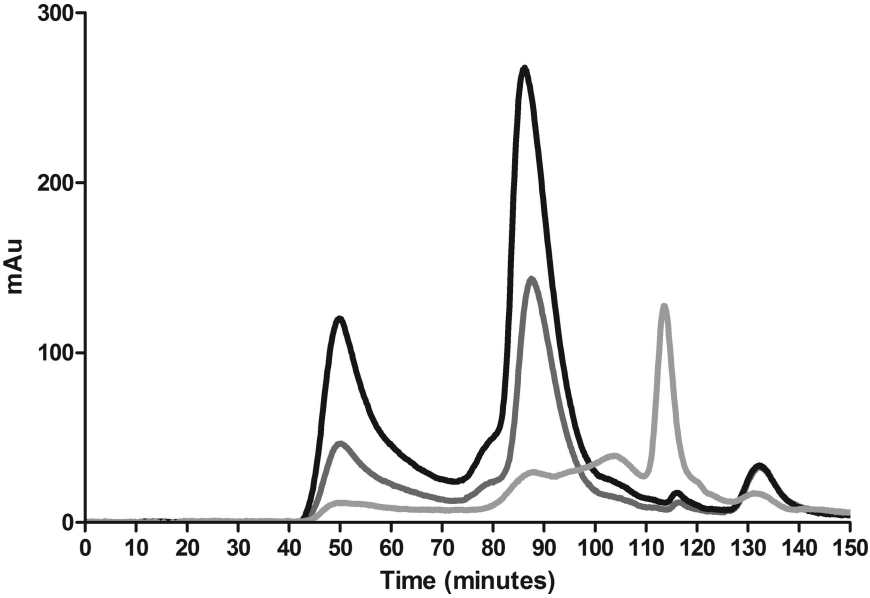

Figure 3. Size-exclusion chromatogram of recombinant mammalian immunoproteins. Black line, absorbance at 280nm for designed human MIC; dark gray line, for rabbit MIC; light gray line, for gorilla MIC.

The proteins were analyzed for binding by surface plasmon resonance. An SPR instrument was brought into the undergraduate laboratory on a limited budget by purchasing an older, pre-owned BIAcore X (Figure 2B). This early model is

less than a tenth of the price of the current BIAcore instruments, because it lacks automatic injection capabilities or multiple flow cells (in 2009 the price for a refurbished model was $25,000). The instrument is therefore low-throughput, but its requirement for direct user injection and attention during data collection benefits student engagement and education. The instrument required a separate compressor and voltage transformer for operation, and the software required an older operating system. Its internal optics are similar to the more advanced instruments, and they produced data with similar responses and baselines containing more noise and drift. Within these parameters, using the BIAcore X, we have been able to measure binding for several different protein-protein interactions in the micromolar to nanomolar scale of affinities.

The experimental determination of protein binding was divided into four parts on the GENI website:

a.) Amine-coupling the MIC protein to the SPR chip;
b.) Testing multiple NKG2D analyte proteins against that surface;
c.) Measuring binding characteristics of multiple concentrations of one of the NKG2D analytes; and
d.) Data processing both for binding at equilibrium and for kinetic fits to the binding sensorgram curves.

These four steps provide a general structure that can be adapted to many other analytical instruments or techniques for proteins with unknown interaction characteristics. We did not know how cross-reactive the NKG2D would be, and this experimental structure allowed us to adapt to unexpected results in the classroom. In particular, we could choose the best protein-protein interaction from multiple candidates and/or to reassign high-affinity protein-protein interactions among student groups if one of the groups was assigned a poor-binding MIC protein. In this manner, 11 physical chemistry students divided into five groups were able to analyze five different protein-protein interactions within the space of a month in Spring 2014.

The division of protocols into four steps on GENI was fit into the constraints of the physical chemistry lab schedule of three-hour weekly labs by dividing the tasks into group tasks and individual tasks. Before a protein can be amine-coupled to an SPR chip, it is placed into solutions with different pH values and tested for the ability to approach the negatively-charged functionalized dextran surface of the chip. This process is called “pH scouting” or “pre-concentration,” and it has several benefits as an introductory exercise: it is quick, taking only a few minutes per injection, and it is reversible, meaning that student mistakes do not have expensive consequences. Therefore, it was scheduled for the first three-hour lab period, and all students learned how to inject samples into the BIAcore X and interpret data in real time together.

After this, students scheduled time to use the instrument in independent research groups over the following two weeks. Some performed the amine-coupling chemistry by following the kit instructions, and sometimes these steps have been carried out by the instructor. Students then tested 2-3 different types of NKG2D against their MIC-coupled surface and noted which gave high

responses, indicating specific binding. One of these was chosen for triplicate investigation, and each group collected a few dozen data points of the response of the surface to this protein, consisting of three sets of six serial dilutions each, in addition to 2-3 blank injections. Each injection required 5-10 minutes to complete, so data collection required about six hours of laboratory time per group. This time could be scheduled flexibly in 1-2 hour time periods depending on the students' schedules. Because BIAcore involves injecting microliters of protein solutions into non-hazardous buffers, it is a safe procedure for independent work and does not consume much protein.

Another aspect of the experimental organization that helped the experiment fit into the Survey of Physical Chemistry schedule was the separation of data collection from data analysis. In most physical chemistry survey textbooks, thermodynamics and equilibrium binding is discussed in the middle of the course, and kinetics is discussed at the end, because kinetics builds on thermodynamic concepts. The micromolar-strength NKG2D-MIC interaction can be analyzed for binding both at equilibrium and by kinetic fits. Students analyzed data using the simpler equilibrium binding graphs immediately. At the end of the course, they re-analyzed their data using more complex kinetic fits, applying the integrated rate laws that they learned in the penultimate part of the course.

By following these protocols, students collected results on the BIAcore X that compared well to results collected for the same protein-protein interaction on a BIAcore T100 at the Fred Hutchinson Cancer Research Center (Figure 4). The values given for dissociation constants and kinetic constants also compare well, with more variation between equilibrium and kinetic measurements of binding constants than between the two instruments (Table 1). BIAcore X data is inherently more variable, resulting in additional air bubbles that appear as spikes in the data and more baseline drift, but the students learned to account for these features during data analysis, and these features make up only a small fraction of the data points collected overall.

Table 1. Comparison of thermodynamic and kinetic data collected on BIAcore T100 and BIAcore X.[a]

	Equilibrium K_D *(/10^{-6} M)*	*Kinetics* K_D *(/10^{-6} M)*
BIAcore X	10.4 ±2.8	4.6 ±0.2
BIAcore T100	14.7 ±2.7	4.8 ±0.1

[a] Values are averages of duplicate runs of 3-5 injections each of serial dilutions in the nanomolar to micromolar range of analyte concentrations.

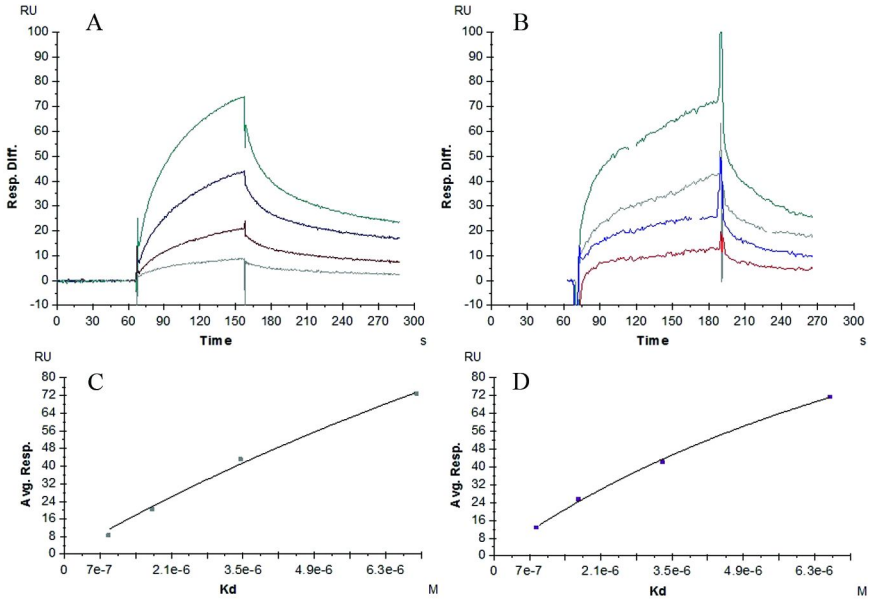

Figure 4. Comparison of data collected on BIAcore T100 and BIAcore X. Sensorgrams collected for similar concentrations of serial dilutions of analyte protein A) on the T100 instrument and B) on the X instrument. Concentrations of NKG2D injected across the MICA-B2-coupled surface were as listed on the y-axes of the equilibrium fits in BIAevaluation software using data C) from the BIAcore T100 and D) from the BIAcore X. These fits are hyperbolic, with the upper asymptote located at the Rmax value. (see color insert)

Most of the protein-protein pairs tested by the students gave measurable SPR responses (Table 2), meaning that the data showed extensive cross-reactivity for NKG2D proteins from different mammalian species binding different types of MIC proteins. The best binding was observed for the two cognate pairs tested: rabbit NKG2D binding rabbit MIC (which presumably have evolved to bind well), and single-chain mutant NKG2D binding MIC8_R64G (which were designed to be more stable molecules that bind well). Single-phase kinetic fits to a 1:1 Langmuir binding model approximated the experimental data (Figure 5).

Table 2. Comparison of free energies of binding for recombinant mammalian immunoproteins[a]

		Equilibrium			*Kinetics*				
MIC	*NKG2D*	*Rmax*	K_D *(/10^{-6} M)*	$\Delta G°$ *(/kcal mol^{-1})[b]*	*Rmax*	k_a *(/M^{-1} s^{-1})*	k_d *(/s^{-1})*	K_D *(/10^{-6} M)*	$\Delta G°$ *(/kcal mol^{-1})[b]*
MICA-B2	single-chain	176 ±25	8.5 ±1.9	-6.9 ±0.2	100 ±2	1710 ±43	0.0081 ±0.0002	4.7 ±0.1	-7.2 ±0.1
MICA-B2	single-chain	202 ±27	12.3 ±2.3	-6.7 ±0.2	83 ±2	1990 ±52	0.0087 ±0.0002	4.4 ±0.1	-7.3 ±0.1
	AVERAGE	189 ±29	10.4 ±2.8	-6.8 ±0.2	91 ±9	1850 ±148	0.0084 ±0.0003	4.6 ±0.2	-7.3 ±0.1
MIC8_R64G	sing.chn.mut.	106 ±38	6.1 ±2.8	-7.1 ±0.5	59 ±1	2740 ±81	0.0032 ±0.0001	1.2 ±0.1	-8.1 ±0.1
MIC8_R64G	sing.chn.mut.	74 ±17	2.8 ±1.0	-7.5 ±0.4	51 ±1	4300 ±131	0.0042 ±0.0001	1.0 ±0.1	-8.2 ±0.1
	AVERAGE	90 ±33	4.5 ±2.7	-7.3 ±0.5	55 ±4	3520 ±788	0.0037 ±0.0004	1.1 ±0.1	-8.1 ±0.1
MIC8_R64G	rabbit	161 ±23	14.2 ±2.9	-6.6 ±0.2	71 ±1	2040 ±48	0.0118 ±0.0001	5.8 ±0.1	-7.1 ±0.1
MIC8_R64G	rabbit	150 ±15	12.4 ±1.9	-6.7 ±0.2	78 ±2	2040 ±65	0.0129 ±0.0002	6.3 ±0.1	-7.1 ±0.1
MIC8_R64G	rabbit	143 ±19	11.8 ±2.3	-6.7 ±0.2	73 ±1	2320 ±63	0.0130 ±0.0002	5.6 ±0.1	-7.2 ±0.1
	AVERAGE	151 ±19	12.8 ±2.4	-6.7 ±0.2	74 ±3	2133 ±111	0.0126 ±0.0006	5.9 ±0.2	-7.1 ±0.1
rabbit	rabbit	129 ±52	5.5 ±2.8	-7.2 ±0.5	67 ±1	3750 ±72	0.0074 ±0.0001	2.0 ±0.1	-7.8 ±0.1
rabbit	rabbit	97 ±47	4.7 ±3.0	-7.3 ±0.6	60 ±2	4240 ±214	0.0102 ±0.0002	2.4 ±0.1	-7.6 ±0.1
rabbit	rabbit	113 ±50	5.3 ±3.0	-7.2 ±0.6	55 ±2	4370 ±178	0.0080 ±0.0001	1.8 ±0.1	-7.8 ±0.1

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Table 2. (Continued). Comparison of free energies of binding for recombinant mammalian immunoproteins[a]

		Equilibrium			*Kinetics*				
MIC	*NKG2D*	*Rmax*	K_D *(/10-6 M)*	*ΔG° (/kcal mol-1)[b]*	*Rmax*	k_a *(/M-1 s-1)*	k_d *(/s-1)*	K_D *(/10-6 M)*	*ΔG° (/kcal mol-1)[b]*
	AVERAGE	113 ±50	5.1 ±3.0	-7.2 ±0.6	61 ±5	4120 ±208	0.0085 ±0.0012	2.1 ±0.1	-7.7 ±0.1
gorilla	rabbit	87 ±10	14.9 ±2.5	-6.6 ±0.2	*inconc.*	*inconc.*	*inconc.*	*inconc.*	*inconc.*
gorilla	rabbit	27 ±14	5.5 ±4.6	-7.1 ±0.8	*inconc.*	*inconc.*	*inconc.*	*inconc.*	*inconc.*
	AVERAGE	57 ±32	10.2 ±6.0	-6.9 ±0.7					

[a] Values and errors are the result of data fits using BIAevaulation 3.0 to data from 3-5 injections of serial dilutions in the nanomolar to micromolar range of analyte, with each student contributing all runs for an interaction pair. Average errors were calculated according to the method of Tatebe (*20*). "inconc." = data fit inconclusive. [b] $\Delta G^o = -RT\ln K_D$, T = 298K.

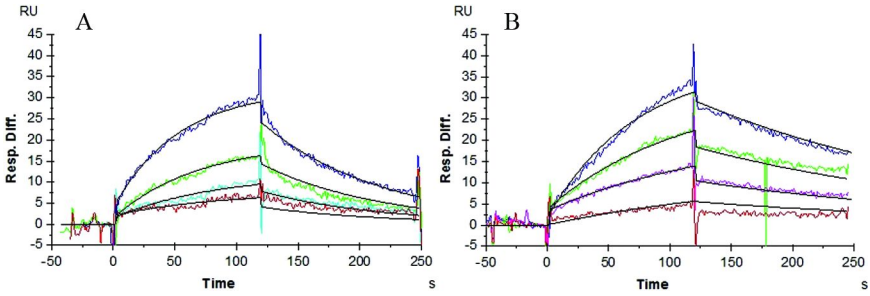

Figure 5. Kinetic fits for cognate pairs of proteins. Sensorgrams and 1:1 kinetic fits for serial dilutions of A) 2.0-μM rabbit NKG2D injected over a surface of rabbit MIC; and B) 2.3 μM-single-chain mutant NKG2D injected over a surface of designed MIC8_R64G. (see color insert)

Most of the student-collected data gave K_D values in the low-micromolar range of previously observed NKG2D-MIC affinities (*15*, *16*, *19*), with overall error in the free energy of binding (*20*) in the range of 0.2-0.7 kcal mol^{-1} for equilibrium measurements and around 0.1 kcal mol^{-1} for kinetic measurements (Table 2). These errors are typical but constrain interpretation of the data. For example, the errors range from 0.05 to 0.6 kcal mol^{-1} for equilibrium measurements collected from the BIAcore 3000 instrument using similar proteins (*21*). Factors that may increase error for these experiments include the lesser optics of the BIAcore X instrument, student error in injection or sample preparation, or inherent error in weak binding given the concentrations of analyte used (in this case, mid-micromolar binding constants involve closer spacing at equilibrium, and therefore result in higher errors for the data fits). The data for rabbit NKG2D binding gorilla MIC could not be fit to a simple kinetic model, which may indicate multiphase kinetics and/or weak binding. With these limitations in mind, data with error values on the order of half of a kcal mol^{-1} should be interpreted conservatively and qualitatively, with the errors serving as an indication of the limits of data comparison.

Discussion

The project described here began with a question asked by a student in the Fall quarter of the academic year: would an NKG2D immunoreceptor bind a MIC ligand from another species? Three specific pieces of technology or instrumentation (GENI, FPLC, and SPR) helped to answer this question in the context of a physical chemistry course. It was answered in the affirmative: the rabbit immunoreceptor and the gorilla ligand as well as a redesigned human ligand bound with low-micromolar affinities (Table 2). Non-cognate pairs bound with affinities around 10 μM, while cognate pairs bound with affinities around 2-4

times more tight. Overall, off-rates varied by a factor more than on-rates, indicting that specific interactions may affect the former more than the latter, which matches a theory that on-rates are driven by nonspecific electrostatic interactions (*22*). The slowest off-rate was found with the cognate human designed protein pair, while the fastest on-rate was found with the cognate rabbit protein pair.

The fact that rabbit NKG2D binds with measurable micromolar affinity to even gorilla and redesigned human MIC proteins confirms the remarkable cross-reactivity of this immunoreceptor-ligand system, already observed in other instances for multiple NKG2D ligands within a species (*23*) but here confirmed even between different species. This project also confirmed that the protein production techniques that worked well for human and mouse versions of these proteins can be extended to gorilla and rabbit proteins, meaning that this platform may be an effective way to explore the role of cross-reactivity and the limits of interfacial variation in the immune system.

The placement of protocols on the GENI website allowed for integration of student results with standard assessment surveys, which have been added to a pool of data currently being collected from hundreds of students at more than a half-dozen institutions using GENI for various research projects. On the scale of this particular physical chemistry course, the course sizes were so small that standard assessments could be carried out while guaranteeing student privacy. However, individual anecdotal narratives have confirmed that students are engaged by this project. One has enrolled in an M.D./Ph.D. program; another has credited this course with inspiring her to apply to graduate study in chemistry. The student who developed this project was admitted to a top veterinary program.

This project occupies about half a quarter or a third of a semester, but it can be expanded in several directions if a single project carried out through the entire academic term is desired. The BIAcore X and other SPR instruments can change the temperature of the flow cells, allowing van't Hoff plots of binding vs. temperature to be constructed (*21*). The phenomenon of surface plasmon resonance can be investigated with color-changing gold nanoparticles (*24*) or thin gold films (*25*), both of which have been demonstrated as suitable for undergraduate research. Undergraduate students can even construct a "homemade" SPR instrument for protein binding measurement (*26*), which would illustrate SPR concepts at a deep level.

Overall, the benefits to this project are a level of student engagement that is more than the sum of its parts. When research is accomplished in the teaching lab, the beneficial synergy from this collaborative and integrative approach is considerable. Students actively participate in the processes of science, become more fully engaged in the learning process, and generate novel results as part of the science curriculum. Instructors advance research projects and answer novel questions in their field. The scientific community benefits, both from the results of these investigations and by gaining well-prepared graduates ready to apply scientific knowledge and skills. In the teaching lab, students engage physical chemistry topics as scientific collaborators and learn by doing as they produce data that constitutes new scientific knowledge.

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