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The Revolution of Real-Time, Label-Free Biosensor Applications

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1.1 INTRODUCTION

Initially, we had planned to discuss the revolution of real-time, label-free biosensor applications. This revolution has been monumental. In the early days, biosensors were used as immunosensors to characterize antibody/antigen interactions. It didn't take long for researchers to

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exploit the technology's capabilities to examine other biological systems, including receptors, nucleic acids, and lipids. Once people recognized that low intensity signals were reliable, the biosensor quickly became a tool for characterizing small molecules and even membrane-associated systems.

Upon reflection, we realized a greater development was in users' understanding of how to apply biosensor technology. How we design experiments and analyse data today is different than in years past. Improvements in data processing and global fitting have eliminated much, but not all, of the confusion biosensor users experience when interpreting binding responses. With these advances it is now easier to recognize well performed experiments. So a better title for this discussion may be "Evolution in Our Understanding of Biosensor Analysis".

When we look at how people use biosensors today, we realize that many users still don't know what they are doing with the technology and the problems are not because of the biosensor (it's a poor craftsman that blames his tools). Instead, far too often, users don't employ basics tenets of the scientific method. They don't include controls, test replicates, or even show data when presenting results. As a result, they end up publishing experimental artifacts or misinterpreting the interaction. Unfortunately, poor quality analysis gives all biosensor technology a bad name. In fact, based on the published data, we wonder if a better title for this chapter might be "Why are Biosensor Users Such Poor Scientists?"

Before we examine why most biosensor users aren't good scientists, let's have a short review of where the technology came from. In 1990, a Swedish company called Pharmacia released Biacore, the first commercially viable biosensor. As depicted in Figure 1.1a, the system was operated by a 486 Hz personal computer (PC for short) – boy, does that bring back memories. To put things into perspective, Figures 1.1b–1.1f pictorially depict other significant advances that occurred in 1990. You might not remember it but the World Wide Web (Figure 1.1b) was launched then and changed forever how we gather information and communicate. The Super Nintendo Entertainment System (Figure 1.1c) revolutionized home video gaming, making it possible to play sports without going outside. Researchers who had been using Perrier water as a solvent in their chromatography systems (presumably because of its high level of purity) found some bottles were actually contaminated with benzene (Figure 1.1d). In one of the biggest upsets in boxing history, James Buster Douglas knocked out Mike Tyson (Figure 1.1e). And Pons and Fleischmann discovered cold fusion (Figure 1.1f); thanks to them we now have an

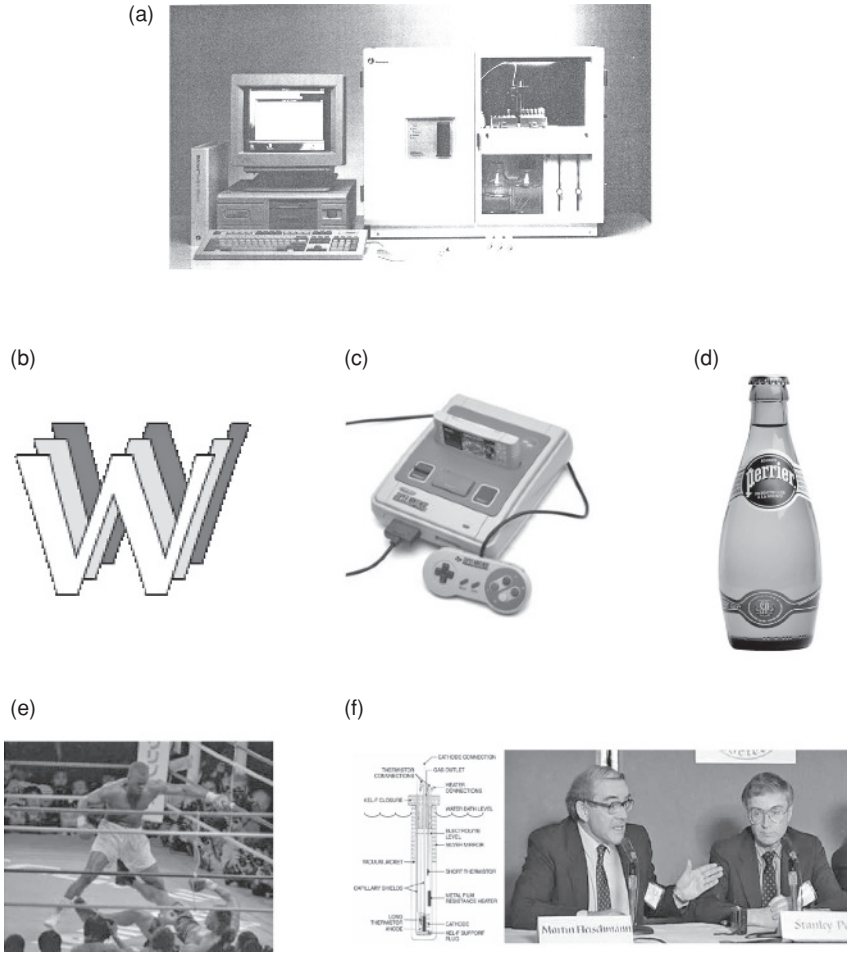

Figure 1.1 Memorable events in 1990. (a) The first commercial optical biosensor, Biacore, was released by Pharmacia. Advent of (b) the World Wide Web and (c) Nintendo's SuperNES gaming console; (d) the Perrier scandal; (e) the Douglas/Tyson boxing match; and (f) Pons and Fleischmann's announcement of cold fusion.

endless supply of cheap, clean energy but of course the cost of Perrier has skyrocketed.

Since the release of the first biosensor, we have seen an explosion in the number and variety of commercial biosensors. Today there are around twenty different instrument manufacturers and about forty different platforms available. These numbers fluctuate as established companies offer new products, old companies falter, and new companies acquire old

companies' products (the circle of biosensors cannot be broken). This diversity in instrumentation is a godsend for bench-top scientists because it means there is a system available to meet each user's sensitivity, throughput, and cost requirements.

While it is true that today's biosensors often employ a variety of detection methods (e.g., surface plasmon resonance, reflectometric interference, evansescent wave, acoustic wave, and dual polarization interferometry to name a few), we think people are too often distracted by a particular platform's detection method. It is not necessary to understand the physics of how a detector works to use it properly. It is far more important to understand how to set up a biosensor experiment and analyse the data properly.

1.2 SPR PESSIMISTS

Unfortunately, there is still significant skepticism in the general scientific community about the validity of biosensor data. Most people can be classified into one of the three categories (Figure 1.2). There are the naysayers who say biosensors don't work (Figure 1.2a), users who think they are experts (Figure 1.2b), and scientists who really love the technology and will do what it takes to get reliable biosensor data (Figure 1.2c).

Let's start with the first group. The naysayers often declare the biosensor has insurmountable problems with instrument drift, nonspecific binding, mass transport, and avidity effects. (Actually, these effects can be minimized and/or accounted for if an experiment is performed properly.) But their fundamental claim is that immobilizing one binding partner on a surface produces artificial binding constants. Sure, taking something

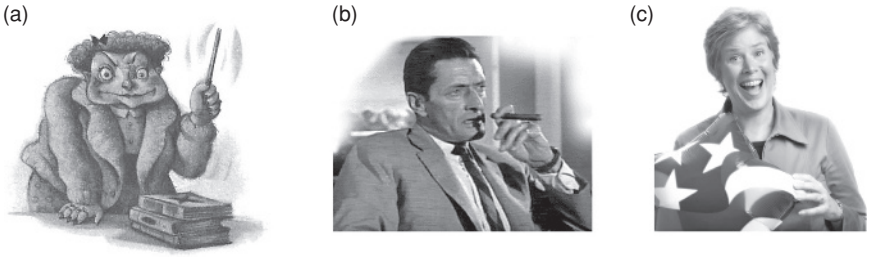

Figure 1.2 Opinions of biosensor technology. (a) "Biosensors don't work." (b) "I'm an expert. I've been using biosensors for years and am not going to change how I do an experiment." (c) "I think biosensors are great and I'm eager to learn about the latest developments."

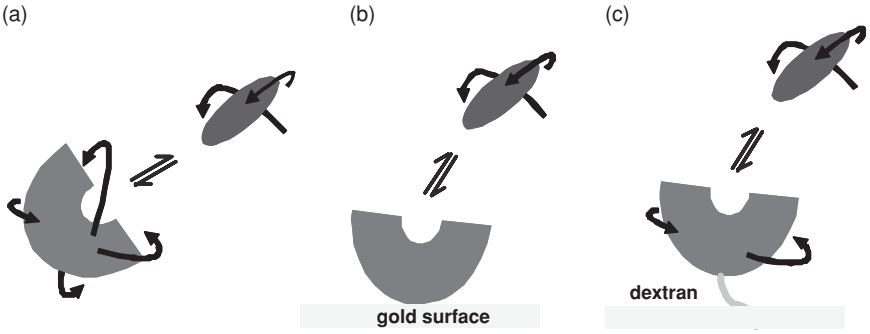

Figure 1.3 Rotational freedom in solution (a) and when the target is immobilized on a flat surface (b) or tethered to a dextran matrix (c).

in solution, as shown in Figure 1.3a, and putting it on a surface could change its entropic properties; perhaps then it cannot freely rotate and would be accessible in only two dimensions (Figure 1.3b) rather than three dimensional space by an approaching binding partner. But, for the vast majority of binding studies the immobilized partner is not actually stuck directly on the flat surface. It is suspended in a dextran layer (Figure 1.3c), which provides a solution-like environment. Maybe the problem with understanding this concept is the word "immobilize". When the ligand is linked to the dextran-coated surface, the binding partner is not immobile. Instead, it is tethered: it is still free to rotate and is accessible in three dimensions for binding.

Relying on its experience using dextran in column chromatography resins, Pharmacia recognized the advantages of using this surface matrix. The dextran layer provides a hydrophilic environment and reduces nonspecific binding. Often the dextran layer is illustrated as a homogeneous forest of seaweed but in reality it is more like cotton candy, whose height depends on buffer conditions, for example, salt concentration. Not only does the dextran layer permit target mobility, but it also introduces a "pre-concentration effect" (1), which allows targets to be readily immobilized, um,... we mean tethered. Coupling a protein on a planar carboxyl surface, for example, requires a higher protein concentration, but with the dextran's capacity to pre-concentrate material through charge effects, a protein could be extracted from a solution of comparably lower concentration and still immobilized at high surface densities. Of course, high densities may not always be optimal (read on).

Coating the sensor surface with dextran was a brilliant decision by Pharmacia when it was developing the biosensor for commercial release. It turned out that the dextran layer is one of the primary reasons its

technology has been so successful. Several manufacturers have produced novel biosensor detection systems but have stumbled in surface chemistry development. Pharmacia's (later Biacore, now GE Healthcare) longevity in the biosensor field is due to its proprietary dextran surfaces. As patents on the use of dextran surfaces begin to expire in 2010, we should see other manufacturers quickly adopt this surface chemistry.

Naysayers often claim that solution- and sensor-determined binding parameters do not match up. To counter this charge, we demonstrated that rate constants and affinities determined using the two approaches do in fact agree when the experiments are done properly. In one study, we determined the kinetics of a small molecule binding to an enzyme using both Biacore technology and a stopped-flow fluorescence instrument (2). The rate constants obtained from the two experiments correlated well. We expanded this investigation to include other biosensor platforms and a panel of compounds that display different affinities for the enzyme and compared results with those obtained from calorimetry measurements (3–8).

A few years ago we began a series of benchmark studies to show that other users can get reliable data from biosensors (2, 3, 7–10). In each study, a panel of participants tested the same interaction. For example, in one study, twenty-two different biosensor users determined the affinities of four compound/target interactions at six temperatures. From these numbers we calculated interaction enthalpies and entropies and compared these values with thermodynamic parameters determined using calorimetry (8). Once again, results from the two approaches matched and the coefficient of variation in the biosensor-determined rate constants was about 10%.

In another benchmark study, we examined a high-affinity antibody/antigen system (9) to demonstrate that even systems with slow off rates could be interpreted reliably. Others have also compared the binding constants for mAb/antigen interactions obtained from Biacore and Kinexa (11), again demonstrating the kinetics and affinity matched between methods.

Recently, we expanded these comparisons to include even more biologically relevant assays. In collaboration with Anthony Giannetti, we compared the biosensor-determined K_Ds of about a hundred kinase inhibitors to the IC_{50}'s measured in biochemical and cellular analyses (Figure 1.4) (12). In both panels the data points lie along a diagonal, which indicates excellent correlation between the biosensor and other methods.

These (and other) comparative studies we have overseen span the range of biosensor variables: testing both small and large analytes

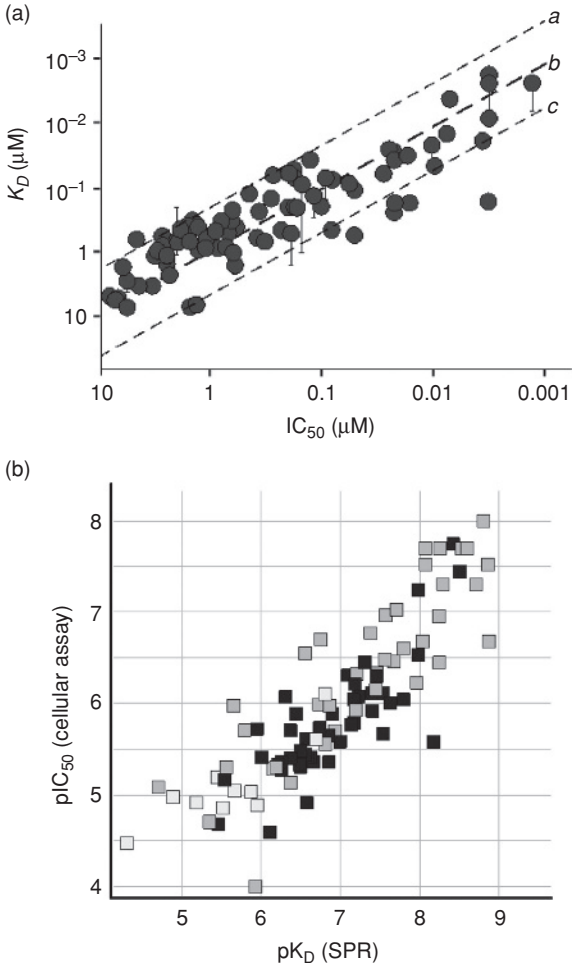

Figure 1.4 Correlation between parameters determined for ~100 kinase inhibitors using biosensor vs. biochemical (top panel) and cellular (bottom panel) assays. In the top panel the heavy dashed line (b) corresponds to $K_D = IC_{50}$. The shorter dashed lines (a and c) correspond to $K_D = 1/5 \times IC_{50}$ and $K_D = 5 \times IC_{50}$, respectively. Error bars not visible are smaller than the symbols used. (Top panel reproduced from (12) with permission from Elsevier © 2008.)

having affinities that differ by more than 100 000-fold, including users (more than 200 to date) of widely different skill levels, and evaluating instruments from the highly automated, high throughput platforms to the manual bench-top models. Across this array of variables, the parameters determined using the biosensor compare well with kinetics, affinities,

thermodynamics, and even activity data measured using solution-based biophysical, biochemical, and cellular assays.

Of course, the key to getting the parameters to agree is to do both of the experiments properly. We find the biggest problem is that most users do not take the time to do the biosensor experiment right. And it's not just new users. We often see in the literature data from more seasoned users who are not setting up the experiment properly. They have the attitude "I've been doing this for years so I know what I'm doing" (Figure1.2b). They have stagnated, not realizing the application of the technology has evolved well past what they consider to be state-of-the-art.

In general we find that many biosensor users have absolutely dreadful technique. We can defend this statement because we read the literature – every single article containing commercial biosensor results that has been published – and for the past decade we have written an annual review of the year's literature (13–22). Most often the problems arise from poor experimental design and execution, as well as inadequate or inappropriate data analysis. It is common to read a paper that suffers from one (or more) of these problems, which renders the authors' conclusions suspect.

1.3 SETTING UP EXPERIMENTS

Bad data often start with bad reagents. The number-one issue to worry about in any biosensor experiment is the quality of the reagents. Unfortunately, the problem is that this is often out of the biosensor user's control. For example, sometimes we are asked to analyse two proteins that were prepared by someone else or simply purchased from a vendor, so we don't have any information about their activities. Remember that what the biosensor is measuring is the activity of the reagents. Unlike mass spectrometry measurements, in which the results are independent of whether the sample is active or inactive, we need two properly folded, conformationally homogeneous, active binding partners for a successful biosensor experiment. There is no way to get meaningful biosensor data from inactive proteins. And the argument that a protein appears as a single band on a SDS-PAGE gel is not good enough. We don't care about purity. We care about ACTIVITY!

But what is the cutoff for being a bad or good reagent? Would we do an experiment if only 50% of each sample was active? Maybe. But if we collect binding data and we see aggregation, complexity, or nonspecific binding in the data set then we really need to consider how the quality of the reagents is affecting the responses. The issue is not that the

technology requires ultra-high-quality reagents. Rather, expectations have to be adjusted based on the quality of reagents. For example, if someone wants to study small molecules binding to an enzyme and they tell us their enzyme preparation can be crystallized, we feel a little more confident that we will be able to get good quality data for that interaction, since the protein has been shown to be well behaved. If, instead, someone brings us protein that has precipitated at the bottom of a centrifuge tube and they cannot see any enzymatic activity but want us to do a binding assay on the sample, we may still do the experiment but we already know to proceed with caution. If we do not see binding or if we see very complex binding we think "Aha! The unusual responses may relate to the quality of the reagents." All too often in the literature we see people over-interpret complex binding responses as something meaningful. The latest fad in interpreting complex binding responses is what we call the "Look everyone, I've got a conformational change" syndrome (more about this later).

Now, assuming your reagents are good, you can move on to starting the biosensor experiment. But stay alert. There are a number of potential pitfalls in each of the steps needed to get good biosensor data. Firstly, consider ligand immobilization. When the biosensor was first released everyone immobilized ligands via amine coupling because it is fast and easy. While this method works great for a number of systems, it does have limitations. For example, the drop in pH and salt concentration required for effective preconcentration may inactivate some ligands. So, a number of alternative chemistries and capturing methods have been developed over the years. Unfortunately, what we most often see users do (and what is presented in the literature) is only one approach: they immobilized the target using amine coupling and got some data, so they stopped optimizing the assay conditions. But if you only do it one way, you are not considering if/how immobilization may affect binding. How do you know you are getting native activity of your ligand without trying other methods?

The opportunity to use several immobilization approaches is one example of how the technology and its applications have evolved over time. With any new system, we recommend trying multiple methods of immobilization. In our laboratory, we set up a preliminary experiment in which we prepare surfaces of the same target immobilized by amine coupling, minimal biotinylation and, if possible, capture via a suitable tag; we then test them side by side for analyte binding.

Another issue with amine coupling is that it is random, which can cause trouble. Amine coupling can produce a heterogeneous ligand

population. If you use random coupling, the ligand on the surface may not all be equally accessible for binding. If, instead, the ligand is captured or otherwise homogenously tethered to the surface, the population should be equally accessible for binding. Remember, we are talking about being chemically, not physically, similarly oriented. A big misconception people have is that oriented immobilization leads to a uniform physical presentation of the molecules on the surface, like all the binding sites are facing up. But, in fact, the dextran layer is flexible and mobile, so an oriented population of immobilized ligand is not all necessarily pointed in the same direction; it is all tethered to the dextran via the same functional group. This beauty of the dextran layer brings to mind an ancient haiku:

Ligands hung in fluid breeze
Some face up, some may face down
Now bind damn it, bind.

The next step is to consider how much target to immobilize. As the technology evolved we showed that for kinetic studies it is important to use lower density surfaces (23,24). In the old days (and you still see this sometimes) people measured very large responses but the binding was mechanistically very complex because they introduced effects like crowding, aggregation, and/or mass transport. We found that as you lowered the surface density the binding responses became simpler and could be described by a single exponential.

We often are asked: "How low in surface density should you go?" The answer is, let the sensor be your guide. Immobilize your ligand at a density that produces low analyte binding signals and run something we call "replicates". Replication is the art of taking the same sample and analyzing it more than one time to determine how reproducible a response is.

It is indeed shocking just how few examples there are in the biosensor literature of doing replicate experiments, even the simple test of injecting the same analyte twice over the same chip. This is even more disappointing given that most commercially available biosensors are fully automated, so you could set up the assay to run ten times while you go to lunch or leave for the day. When you come back to the laboratory, you have an answer about how reproducible your data set is.

Figure 1.5a shows the data obtained from a simple reproducibility test in which analyte was injected across the same surface three times. The triplicate responses overlay, indicating the binding was reproducible

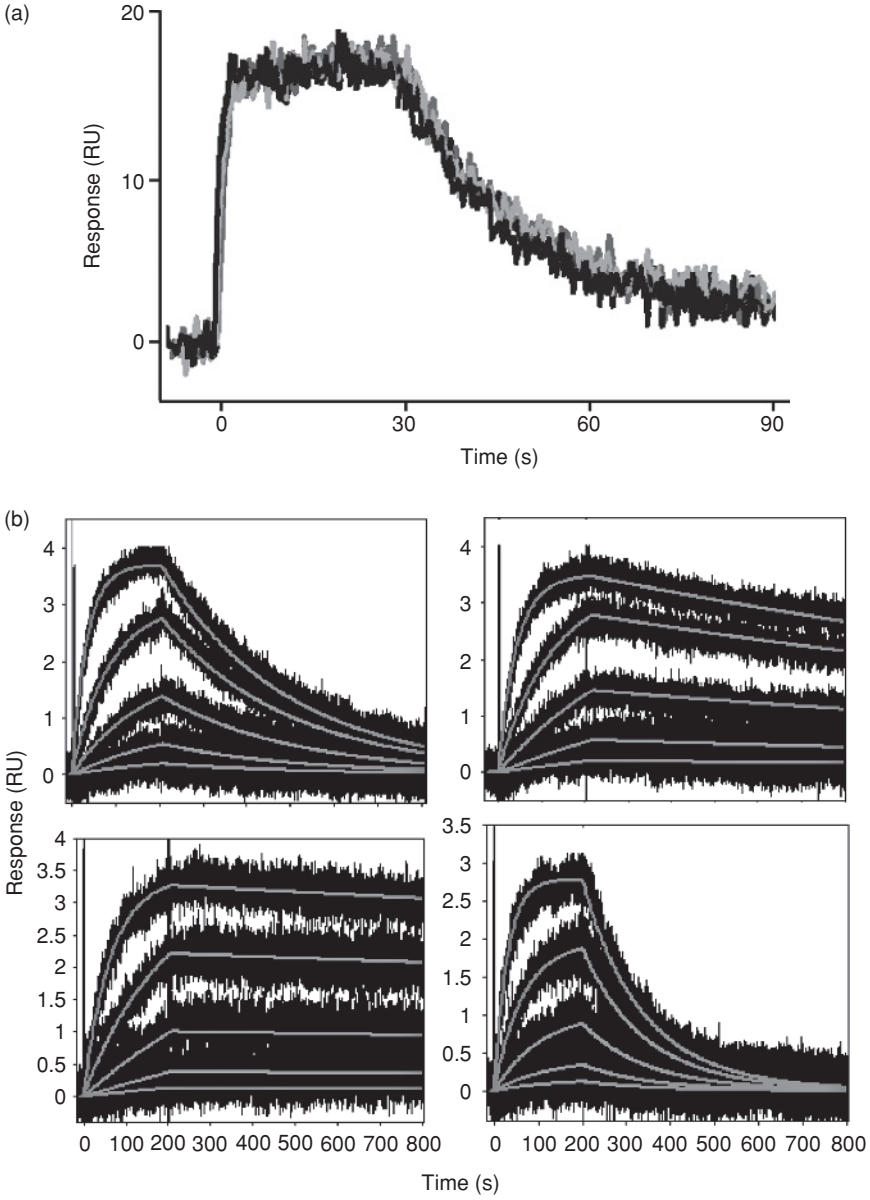

Figure 1.5 Reproducible responses obtained from low density surfaces. (a) Overlay of the responses obtained for an analyte tested three times. (b) Light gray lines depict the fit of a 1:1 interaction model; black lines are the responses (triplicates overlaid) from each analyte concentration.

and therefore reliable, even at this low response level. Figure 1.5b shows the full analyses of antigen binding to four low density antibody surfaces. In each panel, every antigen concentration was tested three times. The responses are so reproducible you cannot see that there are three individual curves overlaid. While the responses in Figure 1.5 are only a few RU (resonance units) in intensity, they are easily discernable above background and the overlay of the replicates, as well as their fit to a 1:1 interaction model, demonstrate these data are reliable.

Keep in mind that there is a lot of information in replicate data sets, even if the responses are not exactly reproducible. First of all, we do not believe any response until we see it at least twice. Then, if the responses overlay, we know the binding partners are stable and the regeneration condition is working. If the replicate responses decrease over time, one (or both) of the partners may be losing activity during the experiment. Working with unstable reagents is still possible in some experiments, but again we would need to adjust our expectations of the quality of data we could obtain. Knowing the reproducibility of a binding event is a critical first step to evaluating an interaction.

How many replicates do you really need? The data set in Figure 1.6 consists of 80 different analyte concentrations measured four times each. Admittedly, this is an extreme example – you don't need to do this many replicates of such a narrow dilution series to prove a mechanism or define

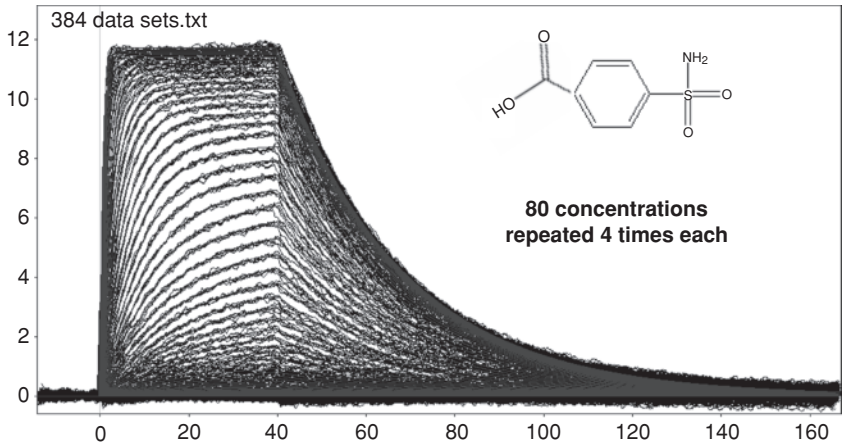

Figure 1.6 Overlaid responses obtained for eighty concentrations of a small molecule (shown in the inset) binding to an immobilized target. Each analyte concentration was tested four times. The light gray lines depict the fit of the responses (black lines) to a 1:1 interaction model.

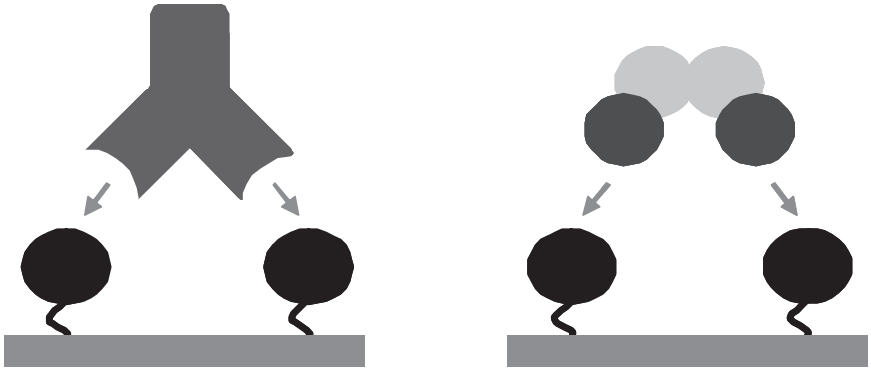

Figure 1.7 Poor experimental design: testing bivalent analytes (left: antibody; right, GST-tagged protein) in solution binding to immobilized binding partners introduces avidity.

the binding constants. We ran this experiment years ago because at the time naysayers were claiming that nothing measured using the biosensor ever fits a simple interaction model. But Figure 1.6 contains over 600 000 data points, spanning more than a 1000-fold concentration range, all fit simultaneously to a 1:1 interaction model, which proves that some data sets can all be fit by a simple interaction model if you know what you are doing.

The key to getting data to fit a simple model is to get good quality data. The problem is that too many experiments are poorly designed. One classic problem is testing a bivalent system in solution (for example, antibodies or GST-tagged proteins) against a monomeric partner immobilized on the surface (Figure 1.7). With this set-up you will get avidity effects. People who design an experiment this way either don't understand avidity or choose to ignore it, but as a consequence they end up reporting an artificially tight affinity for the interaction. Unfortunately, this is just one example of scientific carelessness. There are a number of other experimental factors to consider and we have tackled them in a number of publications (25–27). The bottom line is: if you set up the experiment incorrectly in the beginning you cannot expect to get good data out.

1.4 DATA PROCESSING AND ANALYSIS

The next big issue is proper data processing, which can account for systematic noise, instrument drift, and even nonspecific binding. Over the years there have been a number of advances in data-processing tools. In

2001, we launched Scrubber, which significantly automated data processing. Briefly, Scrubber allows one to zero data before injections, crop data, perform x-alignment, subtract reference data, and perform double referencing. Double referencing, which we introduced in 1999, is the process in which buffer is injected over the surfaces to determine the systematic differences between reference and reaction surfaces (27). By subtracting out this difference data quality can be significantly improved, particular when responses are very low. Artifacts in data that are poorly processed are misinterpreted too often as interesting binding events.

The next level of challenge in using biosensors is data analysis. The two common types of analysis are equilibrium analysis, to extract affinities, and kinetic analysis to extract reaction rates. Firstly, equilibrium analysis. The key word in equilibrium analysis is "equilibrium". When an interaction is at equilibrium, which happens when the same number of complexes are forming as are breaking down, the binding response is flat. Figure 1.8a is an excellent example of an equilibrium analysis. The responses (left panel) for every concentration reach a plateau before the end of the injection, so each response reached equilibrium and can be fit to a binding isotherm (right panel) to determine K_D. The biggest problem we see in the literature regarding equilibrium analysis is that users do not allow the interaction to come to equilibrium before taking a measurement; a few examples are shown in Figure 1.8b. An equilibrium analysis cannot be done unless the responses are at equilibrium. We call this problem "end-of-injection analysis" and we see it in the literature all the time.

Kinetic analysis is more involved than equilibrium analysis but it starts by visualizing the data. As shown in Figure 1.9, even child psychologists begin with images to understand, in this case, family dynamics. Interpretation of these data is a bit subjective. For example, when analyzing this drawing of a family at play, one viewer may focus on the mother and daughter playing catch while another sees the son appearing to throw knives at his father. But unlike kinetic family drawings, biosensor responses are not open to interpretation. If two people analyse the same data set, they should get the same results. But this was not always the case because older methods of data analysis were very subjective.

In the early days of biosensor data analysis people used what is referred to as linear analysis. Firstly, the binding response, which is normally a curve (Figure 1.10a, left panel), would be transformed to create a plot to which a line was then drawn (Figure 1.10a, right panel) and then these slopes were plotted in a third plot to which another line was drawn. From this third plot, the slope gave the on rate and the intercept gave

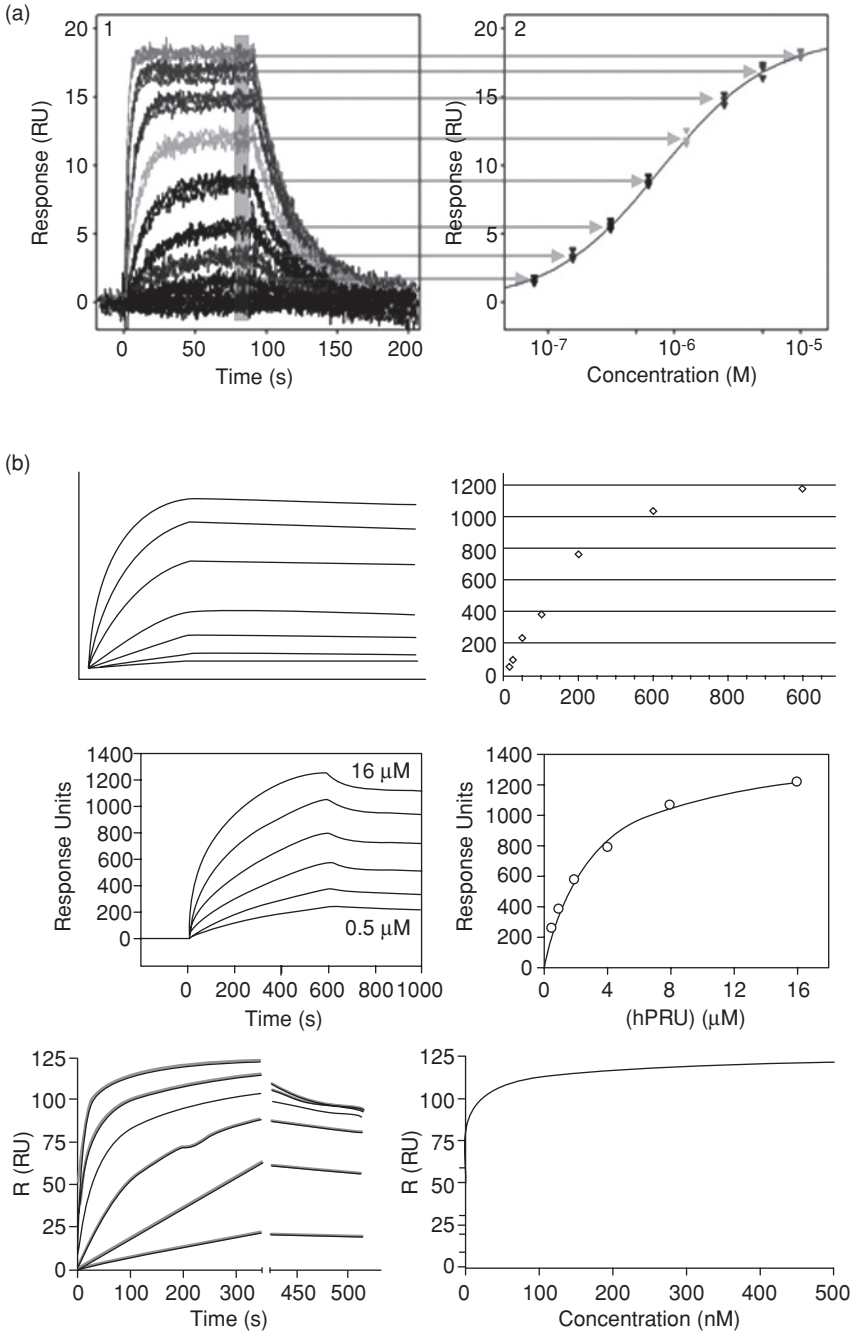

Figure 1.8 Examples of (a) good and (b) bad equilibrium analyses. (Panels in (b) reproduced from (28–30) with permission from Elsevier © 2009.)

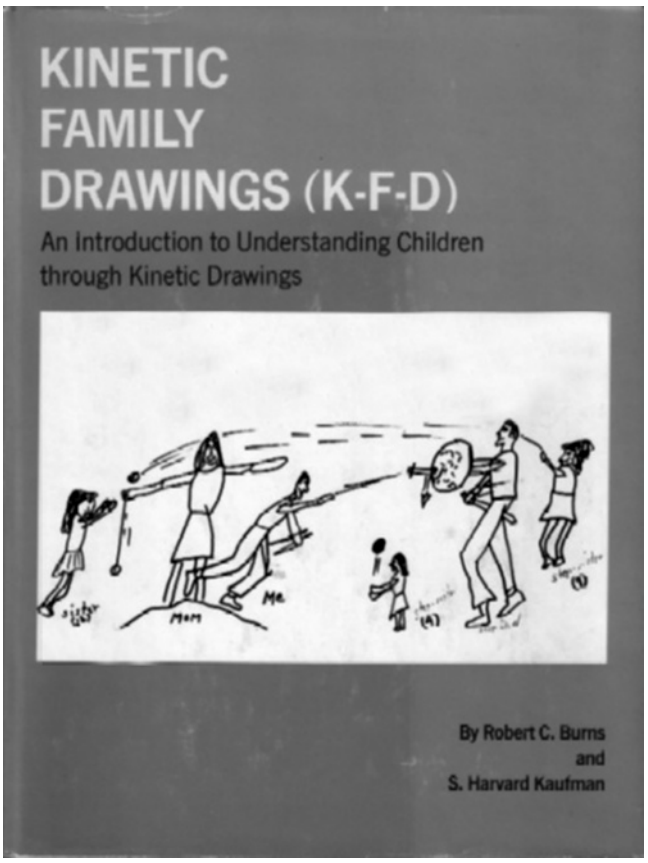

Figure 1.9 Book cover that demonstrates the variable interpretations of a kinetic drawing.

the off rate (Figure 1.10a, inset in right panel). Confusing and slow, we know.

The really big problem with linear analysis is that when binding responses are complex due to mass transport, heterogeneity or drift (Figure 1.10b, left panel), deciding which region is linear becomes subjective: one person might pick a different region to fit than someone else (the authors' selection of which region to fit is shown in Figure 1.10b, right panel). Figure 1.10c illustrates that, in many cases, linear transformation of the dissociation phase could also produce a curve instead of a straight line. Here the authors reported two dissociation rates for this complex but it could easily be argued that there was a third rate which was completely ignored. So, when users found their data could not conform to

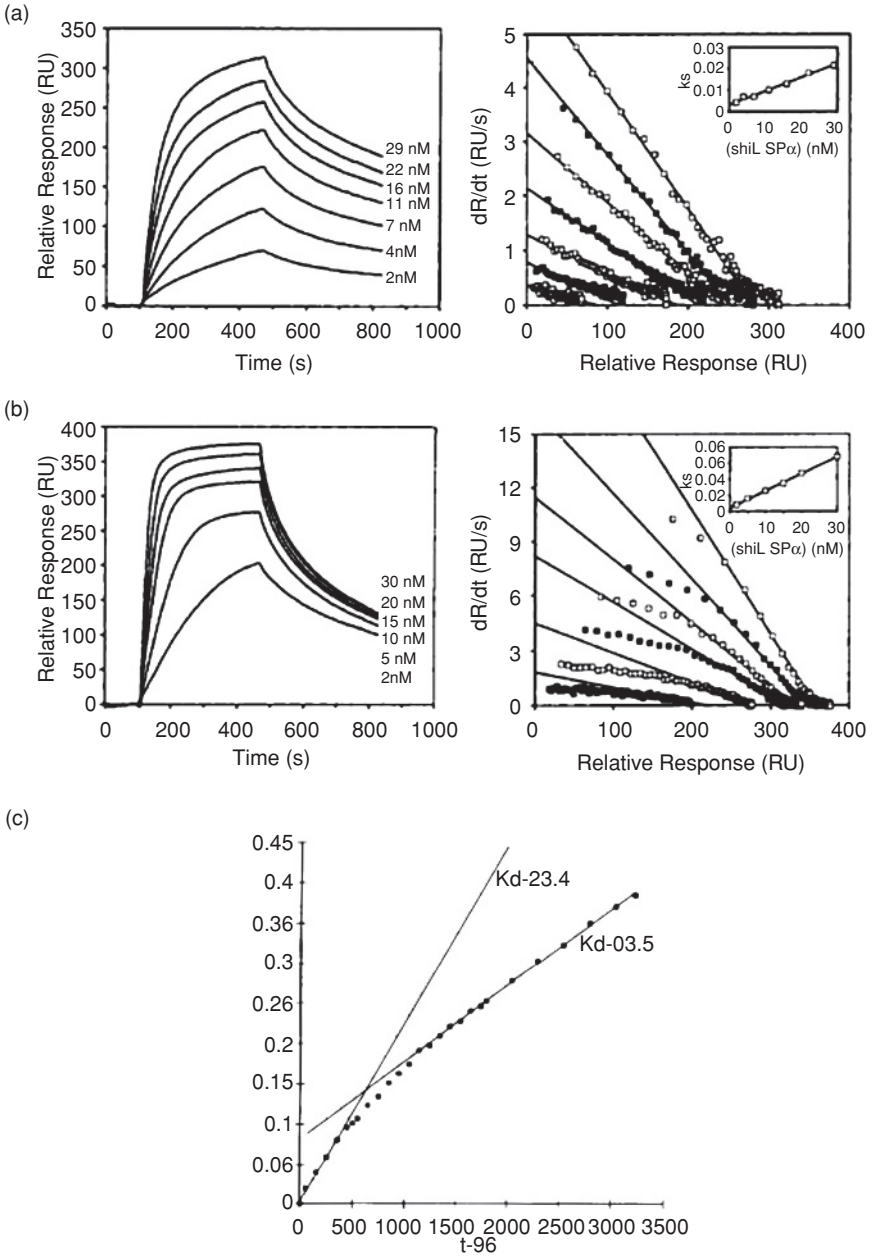

Figure 1.10 Linear analyses of biosensor data from (a) simple and (b and c) complex interactions. In (a) and (b), responses in the left panels were transformed to the linear plots in the right panels, with slope/intercept plots to determine rate constants shown in the insets. In (c), the nonlinear plot was fit to two rate constants. [(a) and (b) reprinted with permission from [31] Copyright 1994 John Wiley and Sons, Ltd and (c) reprinted from [32] with permission from Elsevier]

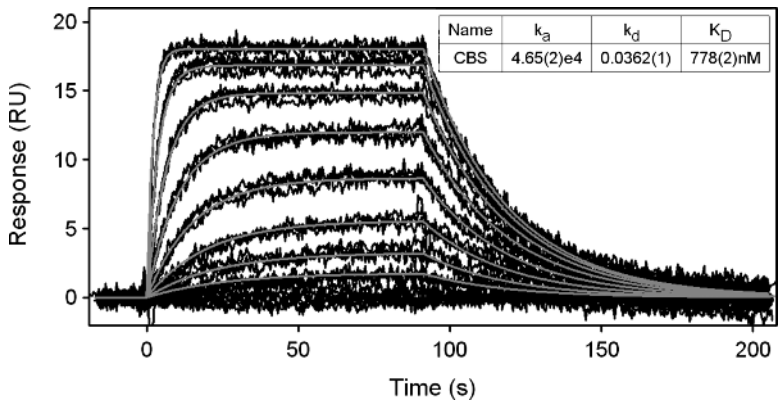

Figure 1.11 Global analysis of an interaction. The light gray lines depict the fit of the responses (black lines) to a 1:1 interaction model. Binding parameters determined from the fit are shown in the inset.

a straight line, everyone got wrapped up in developing binding scenarios that involved multiple rate constants. Unfortunately, the complexity was not due to interesting biology. Instead the experimental design was sub-optimal. That brings us back to setting up the experiment carefully.

We were always bothered by the fact that scientists were choosing which portions of their data to analyze. Some would argue to take the early phase of binding, and some the late. So in the mid 1990s we introduced the concept of globally fitting all of the data at the same time with a software program called CLAMP (33). With this approach, all the data (at each concentration and during both association and dissociation) are fit simultaneously to a single reaction model. Figure 1.11 depicts a global analysis of a small molecule binding to an immobilized target. The fit of a 1:1 interaction model (shown as the light gray lines) is overlaid on the binding responses (measured in triplicate; black lines). The overlay of the data and fit indicate this interaction is well described by the parameters listed in the inset.

One of the beauties and challenges with global analysis is that it requires very high quality data. Artifacts due to poor experimental design and processing become very apparent when you try to fit the data globally. Maybe this is why so many people do not publish their biosensor data but instead just report the rate constants they determine. Pictures really are better than words and certainly more revealing than a table of numbers. Ideally, every article describing a biosensor kinetic analysis would include at least one figure of replicate responses that span a

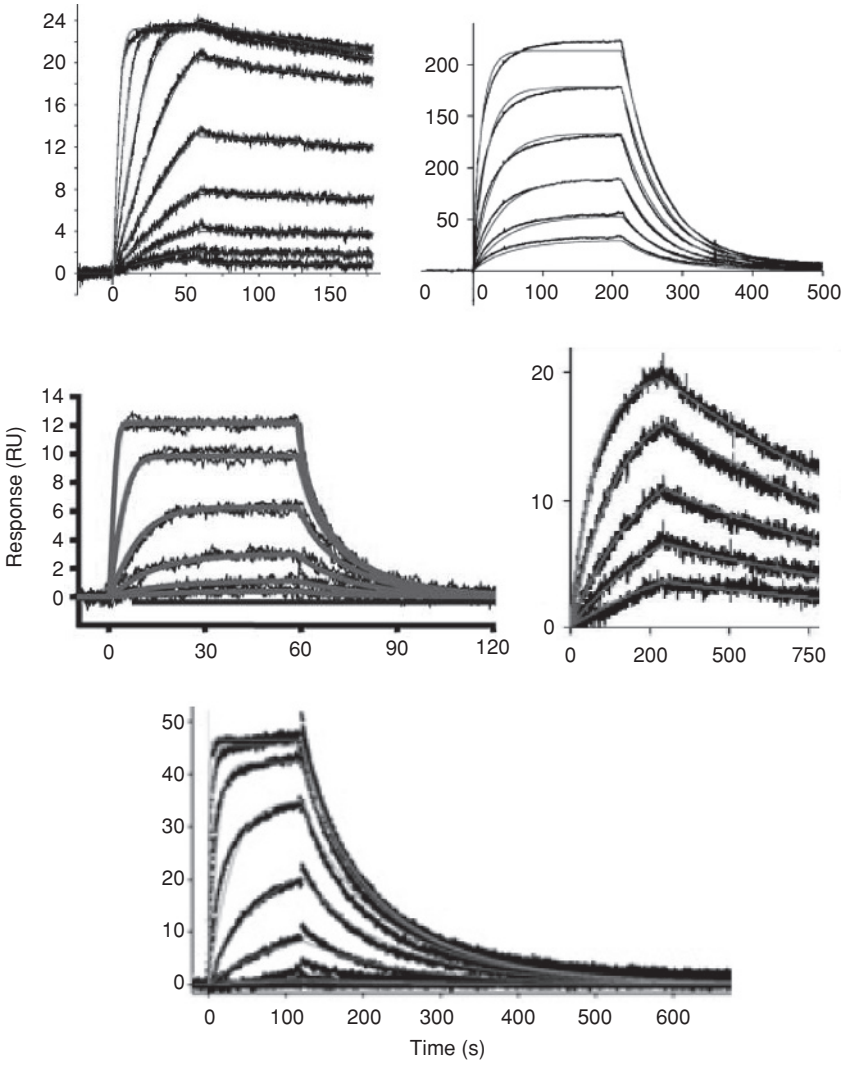

Figure 1.12 Published examples of high quality kinetic analyses. Reprinted from [Refs: 33, 34, 36 and 37] with permission from Elsevier, European Molecular Biology Organization, and National Academy of Sciences.

wide concentration range overlaid with the model fit (like those shown in Figure 1.12). But too often in the literature we see no figures of data – so we cannot trust the reported constants – or we see data that is not interpreted correctly. Figure 1.13 shows two examples from articles that included a figure of responses and reported rate constants but did not show the model fit. Since we doubted the reported rate constants really

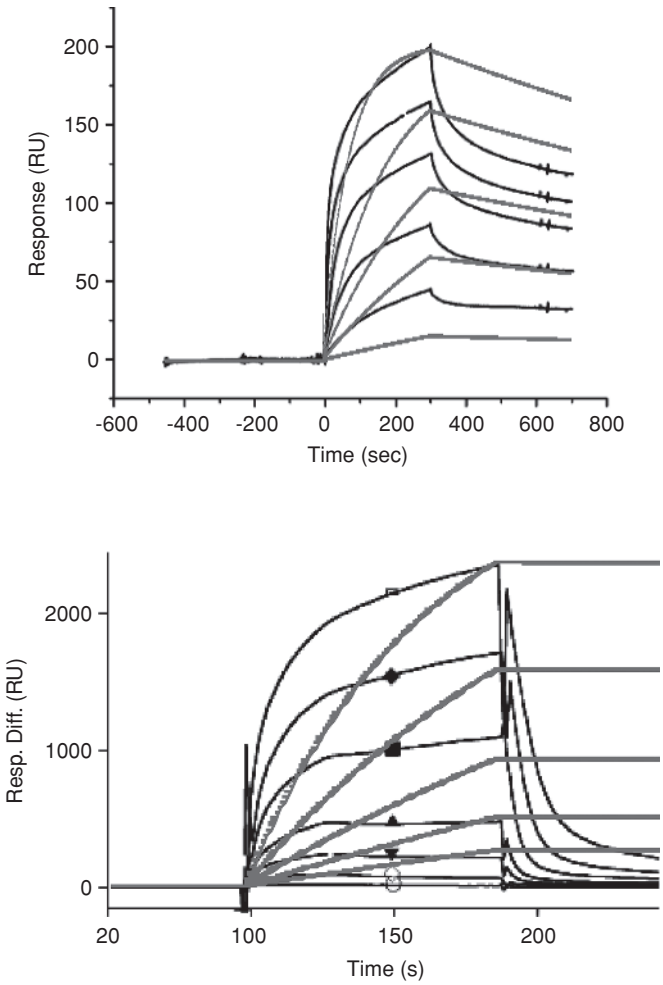

Figure 1.13 Published data sets (black lines) that do not match the accompanying reported rate constants. Fits simulated from the rate constants are shown in light gray overlaid with the responses. Reprinted with permission from [39] Copyright 2007 American Chemical Society., and Reprinted from [40] with kind permission from Springer Science + Business Media.

described these responses, we simulated data derived by the rate constants and overlaid it atop the responses. Our simulated fits (overlaid smooth lines in Figure 1.13) do not even come close to mimicking the responses, so we wonder how these data were actually analysed.

For several years now, we have been asking anyone who reports rate constants to show a data set overlaid with the model they used to fit it.

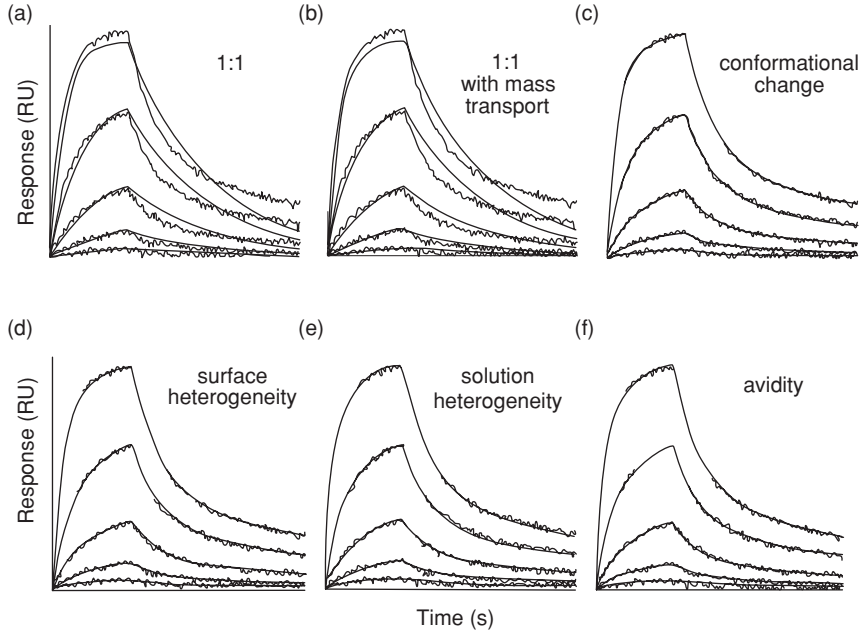

Figure 1.14 A complex data set fit to (a) simple 1:1 interaction, (b) 1:1 interaction that includes a mass transport parameter, (c) conformational change, (d) surface heterogeneity, (e) solution heterogeneity, and (f) avidity models.

But as people have begun to publish their data with model fits, we have discovered the next level of trouble: using complex reaction models. Too often users fit their data with a complex model simply because it fits the data better than a simple model does. Sometimes authors will even state this in the paper "We used this conformational change model because it fit the data." The funny thing is that you cannot prove a model is correct based on how well it fits the data. We know this sounds counterintuitive, so the data in Figure 1.14 help to illustrate the point.

We can all agree that a simple 1:1 interaction model (Figure 1.14a) and a 1:1 model that includes mass transport (Figure 1.14b) do not describe the data. Therefore, we can unequivocally state that these two models are wrong. What you often see people doing next is to apply a more complex model like a two-step conformational change model (Figure 1.14c), which appears to fit the data well. Then they run off and publish that their interaction undergoes a conformational change. A more prudent scientist would try other models as well. For example, it turns out this data set is fit equally well by a surface- and a

solution-heterogeneity model (Figures 1.14d and 1.14e, respectively), as well as an avidity model (Figure 1.14f). So which model is correct? You actually cannot tell using modeling. The only way to prove a mechanism is through experimentation. And actually deconvoluting through experimentation is not easy given the fact that most users cannot set up a single sensor experiment properly. And now you are expecting them to set up multiple experiments under different conditions to help define the correct mechanism.

Even worse, the conformational change model seems to be inattentive users' favorite choice when fitting complex data. "Hey look at me, I got a conformational change." Keep in mind the biosensor measures events on the time scale of minutes. So if you apply this conformation change model and take the time to really think about the rate constants that come out of the analysis, you will find that they correspond to half-lives of minutes – sometimes up to forty minutes. It is unlikely that events on this time scale are biologically relevant. The proponents of using the conformational change model have never explained to us why an entire protein can refold on a millisecond time scale and yet their binding undergoes a conformational change with a half-life of forty minutes – seems a bit odd. Unless, of course, you consider that the complexity they see in the biosensor data is due to aggregation or nonspecific binding, which could be described by much slower events.

Finally, do not be fooled by people who use a crystal structure showing conformational change as evidence to justify fitting complex sensorgrams to a conformational change model. The crystal structure and biosensor data are completely unrelated pieces of information. Until someone shows us time resolved structural data with a conformational change that matches the slow conformational change supposedly observed in their biosensor data, we will stick to improving the quality of the biosensor experiment and remove the artifact.

1.5 THE GOOD NEWS

Now we are left to discuss the third, and our most favorite, group of scientists (Figure 1.2c), users who believe in the technology and want to use it properly to get high quality data. While their numbers may be small, fortunately this is the most rapidly growing group. The first thing we teach them at our workshops is the shape of an exponential binding response (Figure 1.15a). This "shark fin" profile is not some random shape. True binding data should conform to an exponential in

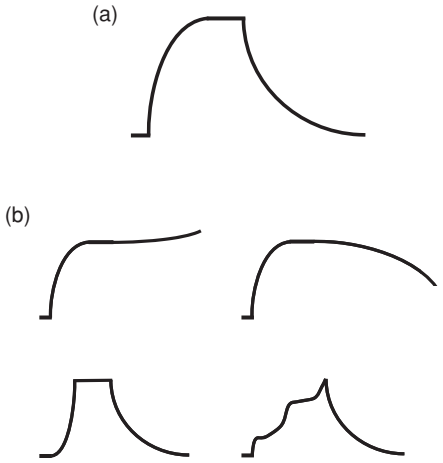

Figure 1.15 Example shapes of published responses. The shape in (A) depicts a plausible binding response, while those in (B) are due to experimental artifacts.

the association and dissociation phase. The association phase is second order, meaning it is concentration dependent. The higher the concentration of analyte, the faster the apparent binding rate. The dissociation phase is independent of concentration. No matter where you start with the response, the rate of decay should be the same. It is like radioactive decay. One ton and one gram of plutonium-238 decay at the same rate. In either case, in 88 years you still have half of a mess to clean up.

With a little practice, everyone can learn to recognize plausible binding signals (Figure 1.15a) and those responses that don't make much sense (Figure 1.15b). Unfortunately, we can find examples of all of the shapes depicted in Figure 1.15b published in the literature. Figure 1.16 shows data sets that display the signature shark fin shape (other examples are shown in Figures 1.5, 1.6, 1.11, and 1.12). Although the shark fin shape varies based on the kinetics of the interaction, by now you should be getting an idea of what a reasonable binding profile looks like. Because these are all simple exponential curves, they are well described by a 1:1 model (light gray lines in each data set).

We don't always get super data the first time we study some interaction and all users need to realize that getting good quality data is often an iterative process. Our initial studies are focused on firstly just seeing some binding between two partners. Once we can confirm that the molecules interact, then we optimize the immobilization chemistry, surface density,

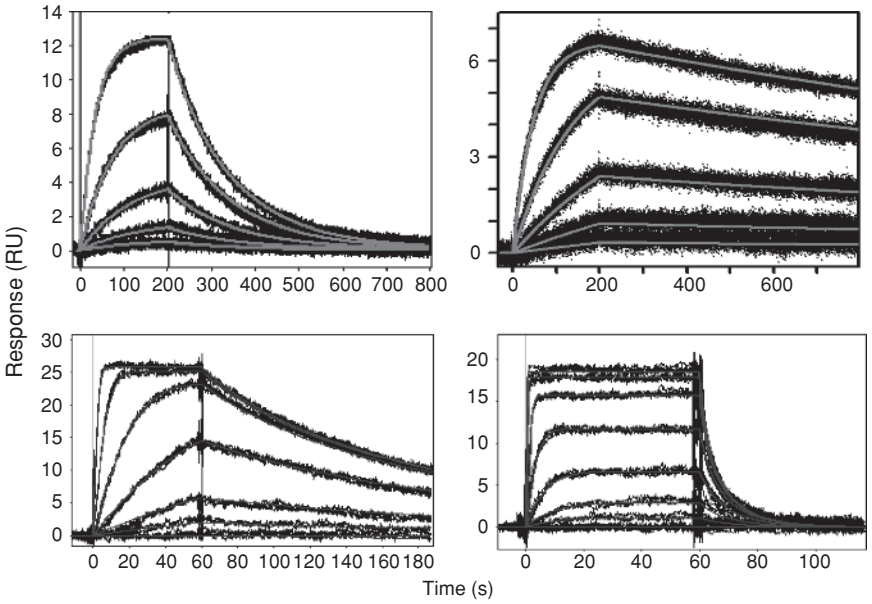

Figure 1.16 Responses of different sharkfin shapes all fit to a 1:1 interaction model. Reproduced from references 41 and 42 with permission from Elsevier © 2004 and 2006.

flow rates, regeneration conditions, and running buffer to produce better data. It is important to realize that the process of generating good data does take time and effort. In fact, a trait that defines the best biosensor users is that they recognize when data are not simple exponentials and they work hard to improve it.

The fact that biosensors are now readily available and easier to use has been one of the biggest problems with the technology as well. Users need to take a step back and really think about what they are doing. They need to be skeptical about their own data and keep an open mind about what the biosensor may be telling them. We have a saying "the biosensor doesn't lie". If you have poor quality reagents, the biosensor will show you that. If you design the experiment incorrectly, it will show you that too. The reason we need to get on top of this problem now is that the number of instruments, as well as the throughput of the technology, keeps increasing, which makes it easier to generate a lot of really poor quality data, and no one wants to see that. By alerting all users now about this problem, we are confident the technology has an exceptionally bright future.

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