Journal of Immunological Methods 371 (2011) 165–169

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Technical note

A strategic and systematic approach for the determination of biosensor regeneration conditions

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ARTICLE INFO

Article history:
Received 20 April 2011
Received in revised form 1 June 2011
Accepted 6 June 2011
Available online 17 June 2011

Keywords:
Biacore
Biosensor
Regeneration

ABSTRACT

Determining the optimal conditions for surface regeneration is fundamental for performance of efficient and robust protein–protein interaction kinetic studies. We devised a systematic methodology comprised of an automated seven-cycle analyte and buffer injection Biacore scheme and data interpretation algorithm. The efficiency and utility is illustrated using an antigen/monoclonal antibody interaction that required ultimately six pulses of acid for regeneration. This technique has broad applicability to any biosensor assay that requires regeneration of a surface.

1. Introduction

Determining the ideal surface-regeneration conditions for a typical Biacore experiment often can consume an inordinate amount of time and materials for investigators. To address these limitations, we have devised a systematic and strategic methodology to determine a suitable regeneration solution. Regeneration of a Biacore surface between analyte injections is necessary because it is normally impractical to wait for the dissociation phase of a sensorgram to completely decay, especially when studying highly stable complexes. Since collecting sensorgram data from multiple replicates of several different analyte concentrations injected over surface-immobilized ligand greatly improves the statistical power behind estimating values for the association (k_a) and dissociation (k_d) rate constants, proper regeneration of the surface between injections of analyte is essential to ensure the biosensor surface remains reproducible and stable throughout an entire kinetic experiment (Morton and Myszka, 1998; Myszka, 1999). Herein we describe a Biacore schema and data interpretation algorithm for performing short automated experiments to efficiently determine the ideal conditions needed for regeneration. This strategy also has broad utility for other biosensor instruments.

To assess surface regeneration, some investigators attempt to closely match the baseline resonance units (RU) with the post-regeneration baseline RU (Andersson et al., 1999; Murphy et al., 2006; van der Merwe, 2001). This practice, however, is usually difficult because the fluid-like, negatively-charged carboxymethyldextran matrix on Biacore chips is most susceptible to expansion and/or contraction when exposed to the extreme pH conditions commonly associated with regeneration reagents that can result in a baseline RU shift (Löfås and Johnsson, 1990; Karlsson and Fält, 1997). Instead, the search for the correct regeneration conditions when ligand is either covalently coupled to a Biacore chip, or any other similar biosensor surface, should focus on establishing *reproducibility* of analyte binding prior to commencing a full kinetic study. GE Healthcare (2008) does suggest performing experiments to evaluate reproducibility in their "regeneration scouting" approach, but this is only after a user is instructed to plot several baseline and analyte injection report points of RU from manually testing

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doi:10.1016/j.jim.2011.06.003

several different regeneration reagents. Instead, automated test experiments involving only seven cycles of analyte and buffer injections, where each cycle is followed by a proposed regeneration scheme, can be performed in a specific sequence that will provide the investigator a wealth of information regarding the effectiveness of the regeneration conditions. This sequence and an example of how this strategic approach assisted in determining a regeneration scheme for a Biacore experiment studying an antigen–antibody complex are detailed below. It should be noted that these test experiments, as well as a full kinetic Biacore study, should be performed using the most optimal experimental design and data processing (Morton and Myszka, 1998; Myszka, 1999). It also is important to remember that the reproducibility observed with a short regeneration experiment does not necessarily guarantee that the surface ligand will maintain a reproducible activity throughout a complete kinetic experiment involving 30 or more regeneration cycles. However, quite often the optimal regeneration condition found by the methodology described below will be sufficient for most kinetic experiments commonly consisting of 30 to 40 cycles.

2. Materials and methods

2.1. Reagents

Monoclonal antibody was provided in-house. Monomeric antigen was purchased from R&D Systems (Minneapolis, MN). All antigen samples for the Biacore studies were prepared in vacuum-degassed HBS-P buffer (0.01 M Hepes, 0.15 M NaCl, 0.005% surfactant P-20, GE Healthcare, Piscataway, NJ) with 100 μg/ml of bovine serum albumin (Fisher Scientific, Pittsburgh, PA). This buffer was also used as the Biacore instrument running buffer. Biacore amine coupling reagents, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), and ethanolamine were purchased from GE Healthcare. Glycine-HCl buffers at pH 1.5, pH 2.0, and pH 2.5 were purchased from GE Healthcare.

2.2. Biacore protocol

All Biacore experiments were performed using a Biacore 2000 instrument (GE Healthcare). The monoclonal antibody was immobilized to one flow cell of a CM4 biosensor chip (GE Healthcare) via conventional EDC/NHS coupling. Excess activated carboxyl groups on the biosensor surface were blocked with ethanolamine. A reference flow cell on each CM4 chip was activated and blocked with no mAb immobilization to serve as a reference surface. The reproducibility of analyte binding to the surface was tested by injecting four replicates of a single concentration of antigen and three buffer replicates in the sequence described below. Each injection was followed by a regeneration step described below. For the full kinetic experiment, six concentrations of antigen were randomly injected in triplicate and several cycles of buffer injections were interspersed for double-referencing the sensorgram data. All replicates of antigen and buffer were injected for two minutes followed by ten minutes of dissociation at a flow rate of 100 μl/min. The surface was regenerated with six 15-second pulses of 10 mM glycine-HCl, pH 2.5. Sensorgrams for all experiments were double-referenced using Scrubber 2.0 software (BioLogic Software, Australia).

3. Results and discussion

Once ligand has been covalently coupled to a Biacore chip, four identical concentrations of analyte and three buffer injections should be performed in the seven-step sequence shown in Fig. 1 to assess the regeneration scheme. Each injection of analyte (steps 1, 4, 5, and 7) or buffer (steps 2, 3, and 6) in this sequence must be followed with the identical regeneration scheme in question. The analyte concentration used should be the highest concentration that will be utilized in the proposed full kinetic experiment because regeneration of the surface is most difficult at the highest concentration of analyte. Biacore control software allows the user to program this sequence of cycles into an automated method. The flow chart in Fig. 1 explains inferences from all possible relative binding responses between injections of analyte. Furthermore, the deductive scheme encompassed by the flow chart allows for experimental evaluation of the effectiveness of each regeneration protocol and then leverages existing experimental outcomes to assist in designing subsequent regeneration conditions for testing. In short, the strength of this regeneration strategy lies in the fact that a single automated experiment assesses the reproducibility of a Biacore surface when the ligand is exposed to one, two, and three regeneration cycles between injections of analyte, thus eliminating the need to perform these scenarios individually.

Notably, the first injection of analyte (step 1) actually serves two purposes, depending on whether the regeneration experiment is the first to be performed on a prepared surface or if it follows the first regeneration experiment on the same surface. In the former case, the first analyte injection serves to ensure that the analyte actually shows binding to the ligand and consequently establishes the R_{max} of the surface as expected when the analyte concentration is sufficiently (~10-fold or greater) above the expected K_D. Therefore, it is important that no regeneration reagent be injected over the newly immobilized ligand before the initial test experiment. If the ligand is first exposed to a regeneration reagent and no binding is observed with the first analyte injection, then it would be unknown if the regeneration reagent deactivated the ligand or if there were truly no binding specificity between the analyte–ligand pair. The ligand's first exposure to the regeneration reagent should occur after this first analyte injection. In addition, even if the first regeneration experiment shows reproducible sensorgrams with the three subsequent analyte injections (indicating a successful regeneration protocol), the sensorgram from the first analyte injection will often show an R_{max} that is slightly greater than those of the other three reproducible sensorgrams. This is because the immobilization procedure often leaves some nonspecifically bound ligand on the surface that is normally removed once the surface is treated with a regeneration cycle after the first injection of analyte.

The second purpose of the first analyte injection is to act as an indicator of the condition of the surface if subsequent seven-step regeneration experiments are necessary on the same surface. For example, a prior seven-step regeneration

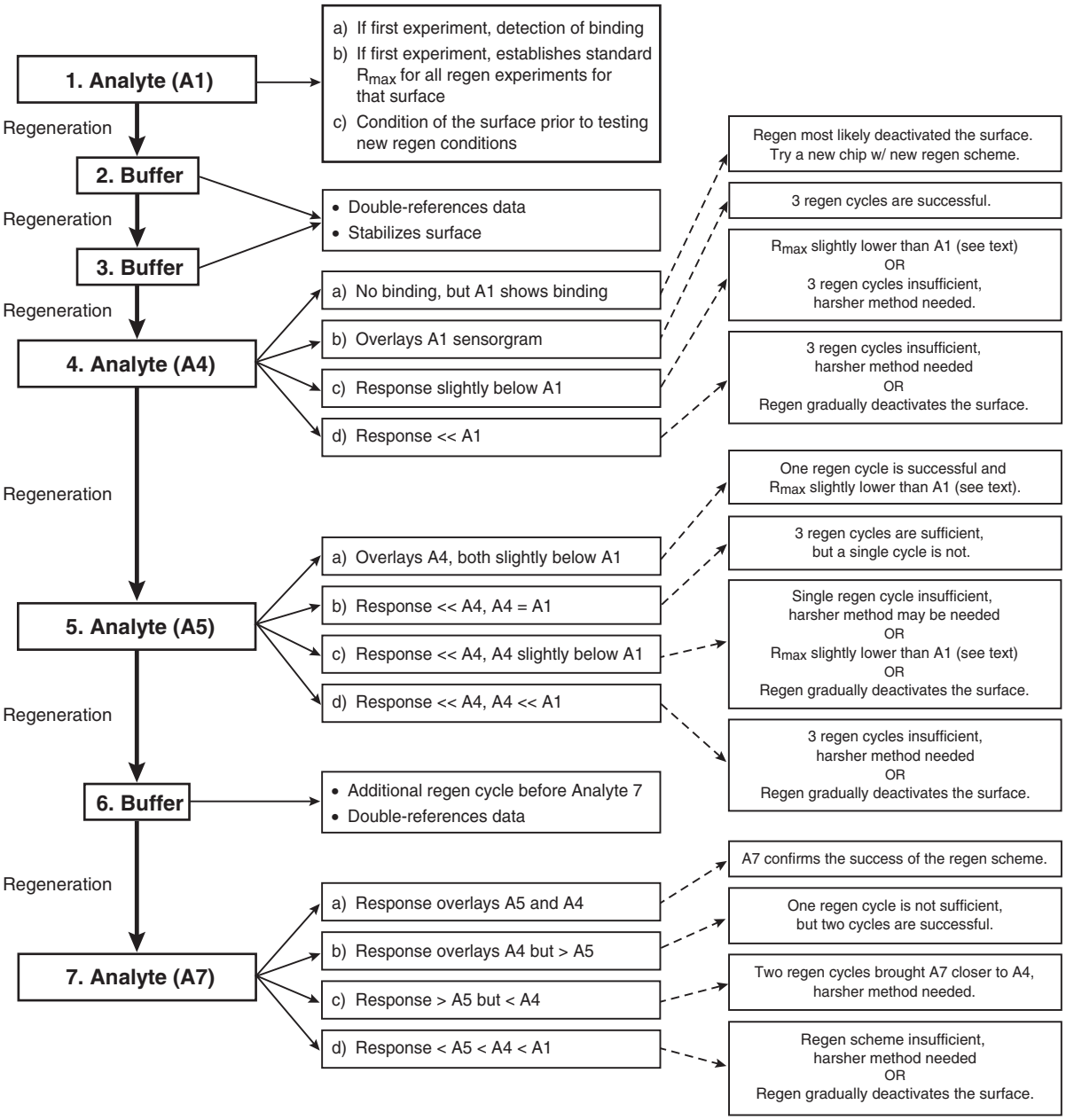

Fig. 1. The Drake–Klakamp methodology, presented as a sequential flow chart of a seven-cycle Biacore experiment designed to assess how well a regeneration scheme provides reproducible binding responses. All possible relative binding responses between four analyte injections can be followed left-to-right with the solid arrows from each step. The dotted arrows signify the possible conclusions based on the relative binding responses and how these conclusions can be used to assess regeneration conditions. Regeneration is abbreviated as regen.

experiment may not have been able to remove all analyte from the surface. In that case, the binding response for the first analyte injection in an experiment testing a new regeneration scheme may be lower than the second injection of analyte if the updated regeneration conditions are more successful at removing bound analyte.

The sequence of buffer injections within the seven-step regeneration schema is strategic. The two buffer injections (steps 2 and 3) following the first analyte injection expose the ligand to two more cycles of regeneration after the first injection of analyte. These additional cycles allow for an assessment of three regeneration cycles between the first and second analyte injections (steps 1 and 4, respectively). The buffer injection in step 6 exposes the ligand to one additional regeneration cycle between the third and fourth injections of analyte (steps 5 and 7, respectively) so that two regeneration cycles can be assessed between analyte injections. Importantly, the sensorgram data from all three injections of buffer provide a means to double-reference all analyte sensorgrams (Morton and Myszka, 1998; Myszka, 1999).

The efficiency and utility of this regeneration procedure is best illustrated with an example of how the method aided the determination of a regeneration condition for an antigen binding to an antibody covalently-coupled to a Biacore surface. Fig. 2a shows the sensorgrams for assessing the binding reproducibility of the mAb surface using one 15-second injection of 10 mM glycine-HCl pH 2.0 for regeneration. The numbers indicate the injection of antigen according to the seven-step sequence described in Fig. 1. As shown in sensorgram 1, antigen injection over the Biacore surface prior to surface exposure to glycine-HCl pH 2.0 resulted in a larger binding response than all subsequent injections of antigen. This observation was not unusual because some non-specifically bound ligand likely remained on the surface following the chemical coupling. Subsequent sensorgrams indicate the binding responses steadily decreased for antigen injections 4, 5, and 7. This result indicates that either the glycine-HCl pH 2.0 was slowly deactivating the surface or perhaps it was removing some of the antigen from the surface, but not enough to achieve reproducibility. If the latter were true, one might expect the response level of sensorgram 7 to be above sensorgram 5 due to the extra regeneration cycle from the buffer injection in step 6. As this was not the case, the results suggest that glycine-HCl pH 2.0 may have been degrading the surface. Regardless of the cause for the decreasing binding, it was clear that glycine-HCl pH 2.0 was not reproducibly regenerating the surface and further tests with other regeneration reagents were necessary.

In the next experiment a lower pH glycine buffer was used to determine more definitively if the results seen in Fig. 2a were due to the inability of glycine-HCl pH 2.0 to remove bound antigen. Fig. 2b shows the sensorgrams from the next reproducibility test on the same mAb surface using one 15-second injection of 10 mM glycine-HCl pH 1.5 for regeneration.

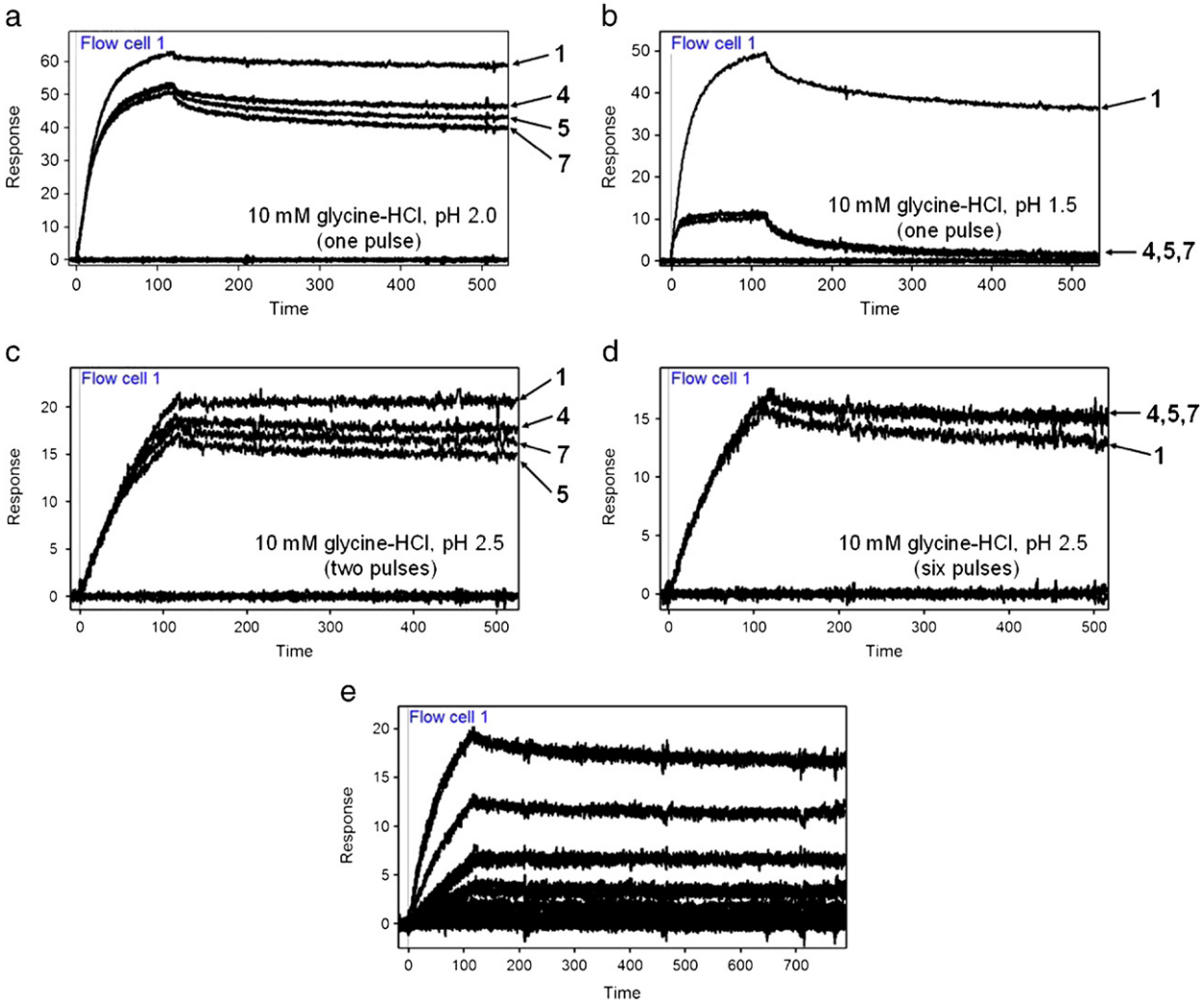

Fig. 2. Biacore sensorgram data from several experiments designed to determine an optimal regeneration condition for antigen (Ag) binding to covalently immobilized mAb. The numbers on the right side of the sensorgrams denote the sequence of antigen injections corresponding with the seven-step test experiment in Fig. 1. The Ag concentrations and regeneration conditions are as follows: a. 49 nM Ag, one 15-second pulse of 10 mM glycine-HCl, pH 2.0, b. 49 nM Ag, one 15-second pulse of 10 mM glycine-HCl, pH 1.5, c. 20 nM Ag, two 15-second pulses of 10 mM glycine-HCl, pH 2.5, and d. 14 nM Ag, six 15-second pulses of 10 mM glycine-HCl, pH 2.5. e. sensorgram data from a full kinetic experiment with Ag injected in triplicate ranging from 470 pM–15 nM demonstrated binding reproducibility after the regeneration scheme from panel d proved to be successful.

The binding responses of sensorgrams 4, 5, and 7 in Fig. 2b all dropped significantly below sensorgram 1, which had been acquired before the surface had been exposed to the lower pH glycine-HCl. These findings were consistent with glycine-HCl pH 1.5 deactivating the surface. Hence, more reproducibility tests were needed using a newly prepared Biacore chip.

For the next experiments, mAb was immobilized on a new CM4 chip at a lower surface capacity than the previous chip because this mAb/antigen interaction appeared difficult to regenerate, and in general lower capacity surfaces are easier to regenerate. Because it appeared glycine-HCl pH 2.0 was gradually degrading the surface and glycine-HCl pH 1.5 had significantly deactivated the immobilized mAb, 10 mM glycine-HCl pH 2.5 was instead tested using the seven-step regeneration schema. It also was surmised that two 15-second pulses of the 10 mM glycine-HCl pH 2.5 might reveal more information about regenerating the surface than a single pulse. Fig. 2c shows the resulting sensorgrams of this regeneration test on the new CM4 chip. Once again, sensorgram 1 established the binding response from a freshly prepared surface prior to regeneration. As described previously, analyte injections 4, 5, and 7 are the most important for determining binding reproducibility because the surface already has been exposed to several regeneration cycles by the time these injections occur, which would have effectively dissociated any nonspecifically bound ligand from the surface. Recall that there were three regeneration cycles between sensorgram 1 and sensorgram 4, so the surface had been exposed to six pulses of glycine-HCl pH 2.5 before the injection of antigen in sensorgram 4. The binding response of sensorgram 5 was significantly below sensorgram 4, indicating the two pulses of glycine-HCl pH 2.5 after the injection of antigen in sensorgram 4 did not reproducibly regenerate the surface. The surface was exposed to four pulses of glycine-HCl pH 2.5 after the injection of antigen in sensorgram 5 and before sensorgram 7. The binding response of sensorgram 7 in Fig. 2c is above that of sensorgram 5, and only slightly below sensorgram 4. This experiment demonstrated that four pulses of glycine-HCl pH 2.5 brought sensorgram 7 closer to reproducing the binding response of sensorgram 4 than two pulses did before sensorgram 5. In addition, because the binding response of sensorgram 7 was above the response in sensorgram 5, multiple pulses of glycine-HCl pH 2.5 did not appear to be deactivating the surface. It was therefore deduced that more pulses of glycine-HCl pH 2.5 after each injection of antigen or buffer should be tested.

Another surface reproducibility experiment was performed on the same surface that was used for Fig. 2c wherein the regeneration test had six pulses of glycine-HCl pH 2.5 after each injection of antigen and buffer. The resulting sensorgrams are shown in Fig. 2d. Note how sensorgrams 4, 5, and 7 completely overlay, indicating that six pulses of the glycine-HCl pH 2.5 successfully regenerated the surface. The response in sensorgram 1 was lower than the other sensorgrams, most likely because antigen remained on the surface from the previous regeneration experiment (Fig. 2c) which had tested two pulses of glycine-HCl after each injection.

With a regeneration protocol successfully deduced from several seven-step reproducibility experiments, a full Biacore kinetic study of the antigen-mAb complex was performed using the same chip used in Fig. 2c and d. Fig. 2e shows processed sensorgrams of six different antigen concentrations injected in triplicate. Regeneration after each injection of antigen or buffer involved six pulses of 10 mM glycine-HCl at pH 2.5. Fig. 2e shows the sensorgrams from each of the six antigen concentrations. Note that what appears to be a single sensorgram for each antigen concentration is actually three overlaid sensorgrams, indicating a highly reproducible surface. In fact the determined regeneration conditions were so robust that reproducible sensorgrams were observed on the same surface even after ~220 pulses of glycine-HCl pH 2.5, which had resulted from the two regeneration experiments shown in panels c and d and the full kinetic experiment in panel e. Requiring six pulses of glycine-HCl pH 2.5 is a fairly unconventional regeneration scheme and, of course, may not have been the only means to regenerate the biosensor surface. Other investigators may have preferred to find a reagent, or a "cocktail" of reagents, that could regenerate with fewer pulses. If so, this seven-step regeneration approach would still be the best method to assess further attempts at regeneration.

A reproducible surface is essential for all rigorous biosensor kinetic measurements. Determining an optimal surface regeneration protocol where ligand is covalently immobilized to the biosensor surface typically involves a significant amount of time, reagents and guesswork. In contrast, the systematic regeneration methodology proposed here using seven strategically placed injections of analyte and buffer provides both a means to assess surface reproducibility and renders valuable information on the effect of a proposed regeneration protocol even if that protocol was unsuccessful. More importantly, utilization of this strategic and systematic approach can direct the researcher to a more rational regeneration test procedure in subsequent experiments thereby greatly reducing the time, reagents and guesswork involved in the search for a reproducible regeneration protocol.

Acknowledgments

The authors would like to thank Drs. Greg Landes, Mary Haak-Frendscho, and Giuseppe A. Papalia for critical reading of the manuscript and for supporting this research.

References

Andersson, K., Hamalainen, M., Malmqvist, M., 1999. Identification and optimization of regeneration conditions for affinity-based biosensor assays. A multivariate cocktail approach. Anal. Chem. 71, 2475–2481.

GE Healthcare, 2008. Biacore Sensor Surface Handbook, Edition AB. GE Healthcare Bio-Sciences, Piscataway, NJ. reprinted.

Karlsson, R., Fält, A., 1997. Experimental design for kinetic analysis of protein–protein interactions with surface Plasmon resonance biosensors. J. Immunol. Methods 200, 121–133.

L fás, S., Johnsson, B., 1990. A novel hydrogel matrix on gold surfaces in surface plasmon resonance sensors for fast and efficient covalent immobilization of ligands. J. Chem. Soc. Chem. Commun. 21, 1526–1528.

Morton, T.A., Myszka, D.G., 1998. Kinetic analysis of macromolecular interactions using surface plasmon resonance biosensors. In: Abelson, J.N., Simon, M.I., et al. (Eds.), Energetics of Biological Macromolecules. Methods Enzymol., part B, vol 295. Academic Press, New York, pp. 268–294.

Murphy, M., Jason-Moller, L., Bruno, J., 2006. Using Biacore to measure the binding kinetics of an antibody–antigen interaction. Curr. Protoc. Protein Sci. 45, 19.14.1–19.14.17.

Myszka, D.G., 1999. Improving biosensor analysis. J. Mol. Recognit. 12, 279–284.

van der Merwe, P.A., 2001. Surface plasmon resonance. In: Harding, S.E., Chowdhry, B.Z. (Eds.), Protein–Ligand Interactions: Hydrodynamics and Calorimetry. Oxford University Press, pp. 137–170.