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Exploring minimal biotinylation conditions for biosensor analysis using capture chips

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ABSTRACT

Using Biacore's new regenerateable streptavidin capture (CAP) sensor chips, we investigated a number of biotinylation conditions for target ligands. We explored standard amine as well as the less commonly used carboxyl biotinylation methods. We illustrate the time scales required for efficient biotinylation as well as the hazards of overbiotinylation. We evaluated a range of desalting methods, including spin columns, dialysis membranes, and filters. Finally, we tested the effects of common buffer components, such as Tris and glycerol, on the biotinylation process. Together, our results serve as a general guide of the steps to consider when minimally biotinylating a target ligand.

A key requirement for a successful biosensor study is to tether the target ligand in an active conformation to the sensor surface. And for small molecule analysis, an added burden is the need to achieve a high ligand density. The commonly used direct amine coupling method [1] often produces high-density surfaces that are active for analyte binding. We have, however, worked with a number of systems that become inactive on amine coupling. Although many investigators may jump to the conclusion that the ligand is inactivated because a reactive amine in the binding site has been modified, it is also possible that the ligand is not amenable to the low-pH and low-salt conditions required for the preconcentration step (typically, pH < 5.0 in 10 mM sodium acetate). It is also important to note that with this method, the ligand may be coupled via more than one primary amine, resulting in surface heterogeneity. If we find that the ligand loses binding activity with direct amine coupling, we will often try minimal biotinylation as an alternative coupling method.

Although biotinylation has been used for decades as a method of immobilizing ligands for a variety of applications, some investigators need time to get accustomed to the concept of minimal biotinylation. For example, even though biotinylation is often used as part of the detection method in an enzyme-linked immunosorbent assay (ELISA),[1] many groups will try to achieve a biotinylation level of 3–4 biotins per target molecule. Although this ratio enhances the signal produced by the ELISA's streptavidin reagent, this overbiotinylation is in fact exactly the opposite of what we want to achieve for a biosensor study.

In biosensor work, ideally one would want only one biotin attached to each ligand molecule (overbiotinylation reduces the ligand capture level because the ligand cross-links on the sensor surface). Because achieving single biotinylation is impossible with random chemical biotinylation, we prefer to intentionally produce a target preparation that is actually underbiotinylated; we use a molar concentration of biotinylating agent that is below that of the target. This means that with our minimal biotinylation approach, a significant amount of the ligand is not even biotinylated. But this is fine. Nonbiotinylated ligand will just flow past the biosensor surface during the capturing step. Although this may seem like an egregious waste of material, minimal biotinylation often produces surfaces that are more active than those produced by amine coupling. The other advantage of chemical biotinylation is that it is easy to do. It is relatively quick (particularly compared with engineering in a biotin tag), can be carried out at low temperature, and can be performed in a variety of buffers.

For years, we have been interested in using the biosensor to systematically explore the variables in minimal biotinylation, but we were hampered by the extremely high stability of the streptavidin/biotin interaction. After capturing a biotinylated target onto a standard streptavidin (or avidin or NeutrAvidin) surface, it is virtually impossible to then strip the target away. This has significantly limited our ability to test a variety of biotinylation conditions efficiently. Recently, however, GE Healthcare Biosciences released

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[1] *Abbreviations used:* ELISA, enzyme-linked immunosorbent assay; CAP, capture; CAII, carbonic anhydrase isozyme II; ATP, adenosine triphosphate; NHS, *N*-hydroxysuccinimide; LC, liquid chromatography; MWCO, molecular weight cutoff; EDC, 1-ethyl-3-(3-dimethylaminpropyl)-carbodiimide hydrochloride; BSA, bovine serum albumin; bCAII, biotinylated CAII; RU, resonance units.

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a new Biacore Biotin CAPture Kit that allows the regeneration of streptavidin-based surfaces. These chips contain an oligo surface that readily hybridizes with specially prepared oligo-linked streptavidin. With this kit, a biosensor user can repeatedly (i) prepare a hybridized oligo-streptavidin surface, (ii) capture a biotinylated target and test analyte binding, and (iii) strip away the analyte/biotinylated ligand/oligo-streptavidin complex. As a first step toward widespread application of this new capturing system, we wanted to establish the reproducibility and capacity of these capture (CAP) sensor chips.

The regenerateability of the CAP chips is particularly advantageous for optimizing the parameters for target biotinylation. In this study, we varied the concentration of cross-linking reagent and the time of the cross-linking step, compared coupling via amines versus carboxyls, and evaluated different methods of removing free biotin from the ligand sample. To keep things relatively simple, we chose to work primarily with one buffer system, carried out the biotinylation reactions at 4 °C to improve ligand stability, and held the target concentration constant at 1 mg/ml; altering any of these conditions may impact the biotinylation chemistry further. Our results demonstrate the benefits of the CAP surface, highlight this surface's utility in exploring biotinylation methods, and provide a general guide for efficient minimal biotinylation of target ligands.

Materials and methods

Carbonic anhydrase isozyme II (CAII) from bovine erythrocytes, adenosine triphosphate (ATP), and general laboratory reagents were purchased from Sigma Chemical. Biotinylation reagents EZ-Link sulfo-NHS-LC-LC-biotin (Cat. No. 21338), EZ-Link pentylamine-biotin (Cat. No. 21345), Pierce Slide-A-Lyzer Dialysis Cassettes (molecular weight cutoff [MWCO] = 7 kDa), Pierce Slide-A-Lyzer MINI Dialysis Unit (MWCO = 7 kDa), and Pierce protein desalting spin columns were obtained from Pierce Co. D-Tube Dialyzer Mini (MWCO = 6–8 kDa) units were obtained from Novagen. "V"-series membranes (0.025 μM filter pore size) were purchased from Millipore. Dialysis buttons (100 μl capacity) were purchased from Hampton Research. Spectra/Por 1 dialysis membranes (MWCO = 6–8 kDa) were obtained from Spectrum Laboratories. The Biotin CAPture Kit (containing CAP chips, Conjugate Biotin CAPture [CAP] solution, and regeneration reagents), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), and the Amersham Biosciences Mini Dialysis Kit (MWCO = 6–8 kDa) were obtained from GE Healthcare.

Before use, CAP chips were conditioned and stabilized according to the manufacturer's instructions. Loading of CAPture reagent and regeneration were also performed following the manufacturer's recommendations. Biosensor experiments were performed at room temperature using Biacore 2000 and T100 instruments equilibrated with running buffer (10 mM Hepes, 150 mM NaCl, 0.005% P20, and 0.1 mg/ml bovine serum albumin [BSA], pH 7.4).

EZ-Link sulfo-NHS-LC-LC-biotin was typically used at a 1:1 ratio with CAII except in the biotinylation ratio experiment (for which we used a 3-fold dilution series starting at 3.4 mM). EZ-Link pentylamine-biotin was used at a 100:1 ratio. EDC was used at a 10,000:1 ratio for the time course experiment and in a 3-fold dilution series starting at a 10,000:1 for the biotinylation ratio experiment.

A stock solution of CAII was biotinylated overnight (unless otherwise stated) at 4 °C at a concentration of 1 mg/ml (34 μM) in 10 mM Hepes, 150 mM NaCl, and 0.005% P20. In experiments that tested the effects of various buffer components, the enzyme was also biotinylated in 10, 33, and 100 mM Tris–HCl (with 150 mM NaCl, pH 7.4); in 10, 33, and 100 mM glycine (with 150 mM NaCl, pH 7.4); in 1.7%, 5.0%, and 15% glycerol; and in 0.1, 0.3, and 1.0 mM ATP.

Prior to target capture, free biotin was removed using a Pharmacia fast desalting column except for experiments designed to test different biotin removal methods. In these experiments, 100-μl aliquots of 470 nM biotinylated CAII (bCAII) were spiked with free biotin to a final concentration of 1 μM. Some bCAII aliquots were then dialyzed for 0.5, 1, 2, 4, 8, 16, or 24 h. bCAII aliquots designated for desalting with Pierce spin columns were spun according to the manufacturer's instructions (which required passing one bCAII aliquot through two separate columns). In the spin column experiments, some of the bCAII aliquots contained 0.5 mg/ml BSA and some of the columns were pretreated with 0.5 mg/ml BSA. In the biotin inhibition experiments, aliquots of 470 nM bCAII were spiked with 0.4, 1.2, 3.6, 11, 33, 111, 333, and 1000 nM free biotin.

All data processing was carried out using Scrubber2 (BioLogic Software). For accurate comparison of the responses, all responses or sensorgrams (unless otherwise stated) were normalized for the amount of CAPture reagent loaded and, when necessary, were normalized again to a maximum response within the experiment for ease of interpretation.

Results

To investigate biotinylation conditions, we chose to work with the enzyme CAII from bovine erythrocytes. This protein is well behaved and well characterized; in addition, we have successfully used it in a number of previous biosensor benchmark studies [2–4].

We examined several aspects of biotinylation, including the required molar ratios of biotinylating reagent to protein, the required times for biotinylation, the use of both amine and carboxyl biotinylation chemistries, the extent to which free biotin must be removed after biotinylation, and the effectiveness of various biotin removal methods. The various aspects of this system were tested using the new Biacore Biotin CAPture Kit, which is composed of a CAP chip, the CAPture reagent, and regeneration solutions.

Our first goal was to test the reliability of the new CAP surfaces. To do this, we tested the reproducibility of repeated captures of bCAII. Fig. 1 shows the overlay from several cycles of coating, capture, and regeneration. First, the four flow cell surfaces were simultaneously coated with the oligo-streptavidin CAPture reagent. bCAII was then sequentially captured on the four surfaces, after which the surfaces were fully regenerated. Within a given flow cell, the captured levels varied by approximately 3%.

Although we saw a slight lag in the start times of the CAPture reagent injections and slight variability in the density of CAPture reagent coated across the four surfaces, this is not unexpected given the use of serial injections (flow cell 1 was exposed first and flow cell 4 was exposed last) and a slow flow rate (2 μl/min). On average, the CAP surfaces captured approximately 2000 resonance units (RU) of CAII, with the ligand capture level varying by 13% between the first and last flow cells (this difference in capture levels between flow cells is easily compensated for by normalizing the responses after the capture step, e.g., at $t = 2400$ s in Fig. 1).

Next, we used the CAP surfaces to study how the concentration of the amine-mediated biotinylation reagent EZ-Link sulfo-NHS-LC-LC-biotin affected protein biotinylation efficiency. We tested biotin/protein ratios ranging from 100:1 to less than 0.05:1, and the biotinylation time was held constant at 24 h to ensure that all of the reactions went to completion. Fig. 2 shows that, as expected, capture levels improved as the ratio of biotinylating reagent to protein increased from approximately 0.05:1 (orange sensorgram) to 1:1 (red sensorgram). However, the effect of using ratios above 1:1 is quite striking; capture levels decreased as the biotin/protein ratio increased (black and green sensorgrams correspond to ratios of 10–100:1) because at higher ratios of biotinylating reagent the protein becomes overbiotinylated. Overbiotinylation likely leads to one

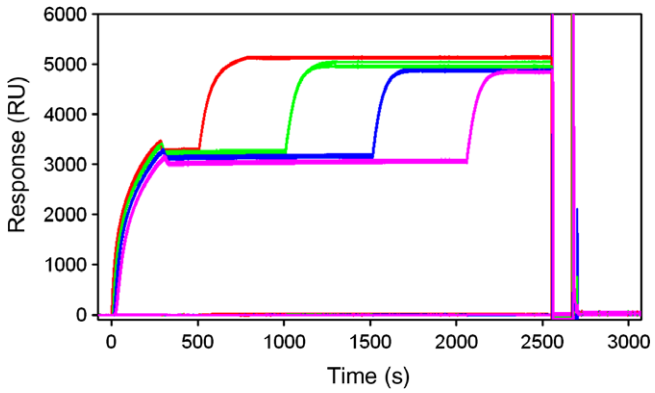

Fig. 1. Reproducibility of biotinylated protein captures using CAP surfaces. Overlaid responses were obtained from repeated cycles of CAPture reagent coating, CAII captures, and CAP surface regeneration. At $t = 0–300$ s, the oligo-streptavidin CAPture reagent was injected simultaneously across the four flow cell surfaces. Starting at $t \sim 500$ s, bCAII (biotinylated using a 1:1 molar ratio of sulfo-NHS-LC-LC-biotin/CAII and desalted using a fast desalting column) was captured sequentially in the four separate flow cells. At the end of each binding cycle ($t \sim 2600$ s), the surfaces were regenerated with 3:1 8-M guanidine HCl/1 M NaOH.

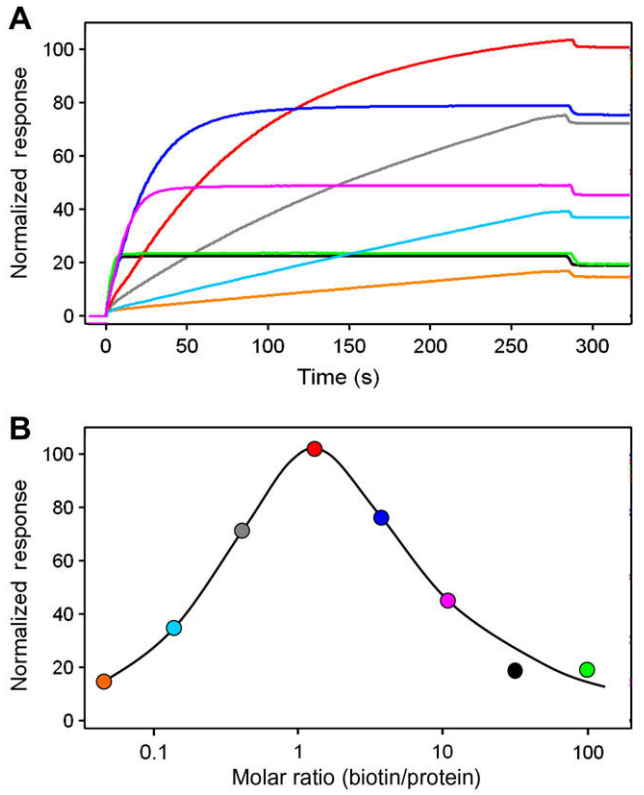

Fig. 2. Optimal amine-biotinylating reagent/protein ratio. (A) Responses for CAII were biotinylated using different concentrations of sulfo-NHS-LC-LC-biotin. Sensorgrams were normalized for CAPture reagent surface density and to the responses observed using 1:1 molar ratios of biotinylating reagent/protein. (B) Responses at $t = 300$ s in (A) plotted against the molar ratio of biotinylating reagent/protein.

ligand molecule occupying multiple streptavidin sites (cross-linking), reducing the capacity of the streptavidin surface.

Also of interest are the differences in the shapes of the responses in Fig. 2A. For example, the capture levels obtained using the lowest (shown in orange) and highest (shown in green) concentrations of biotinylating reagent are comparable, but the plateau in the response for the highest concentration indicates that the surface became saturated (due to the cross-linking). In contrast, the response for the lowest concentration continued to increase throughout the injection. Therefore, protein biotinylated at ratios of less than 1:1 could achieve capture densities similar to the capture density observed for the 1:1 ratio shown in Fig. 2; it would simply require a longer injection time.

We then investigated how time affects the biotinylation reaction itself. Using a 1:1 biotin/protein ratio, we incubated the samples on ice for 3 min to 16 h and then tested the capture of each using an injection time of 280 s. Fig. 3 shows the normalized response levels versus the biotinylation time. Maximum biotinylation was achieved in approximately 3 h. (Of course, biotinylation efficiency for a specific target system may depend on protein structure and concentration, biotin concentration, temperature, and buffer conditions.) It would also be possible to shorten the biotinylation time and then extend the injection time to achieve high-density captures (in cases where the surface was not saturated due to cross-linking). Therefore, the results from Fig. 3 should provide a guide for the amount of time required under our standard conditions. In general practice, with any new ligand, we let the reagents incubate on ice for 2–3 h.

Certainly, the most commonly used biotinylation method is via primary amines. However, we have found that a number of systems are not amenable to amine biotinylation (it is likely that reactive amines may be present in the binding site or binding interface) yet yield highly active surfaces using the less common carboxyl biotinylation method. Biotinylating through carboxyls first involves activation of the ligand's carboxyl groups with EDC and then coupling with an amino-biotin reagent (e.g., EZ-Link pentylamine-biotin). To avoid ligand oligomerization, we used a 100-fold molar excess of the pentylamine-biotin reagent. To avoid overbiotinylation, the biotinylation level was controlled by the amount of EDC added to the sample; we tested EDC/protein molar ratios from 0.1:1 to 10,000:1. As shown in Fig. 4A, relatively high EDC concentrations are required for effective biotinylation. And even with a 10,000-fold excess of EDC and a 100-fold excess of pentylamine-biotin, no overbiotinylation was observed. Most important, the capture densities of carboxyl-biotinylated CAII are comparable to those obtained using traditional amine biotinylation. When incubated over the streptavidin surfaces for a longer period of time, we were able to achieve a capturing level of 2000 RU for carboxyl-biotinylated CAII.

We also investigated the time dependence of carboxyl biotinylation. In these experiments, we used a 100-fold excess of pentylamine-biotin and a 10,000-fold excess of EDC and incubated the samples for 3 min to 16 h. As shown in Fig. 4B, maximum biotinyl-

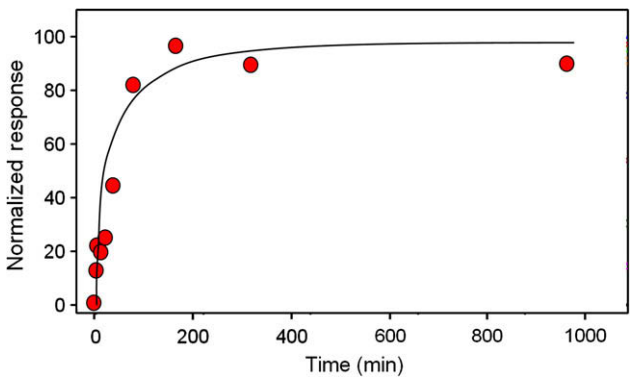

Fig. 3. Time required for optimal biotinylation. Shown are capture levels (normalized for the amount of CAPture reagent loaded and to capture levels observed at maximum biotinylation) of CAII biotinylated for various times using sulfo-NHS-LC-LC-biotin. Biotinylation reactions were quenched with glycine (pH 8.5) added to a final concentration of 100 mM, and free biotin was removed using a fast desalting column.

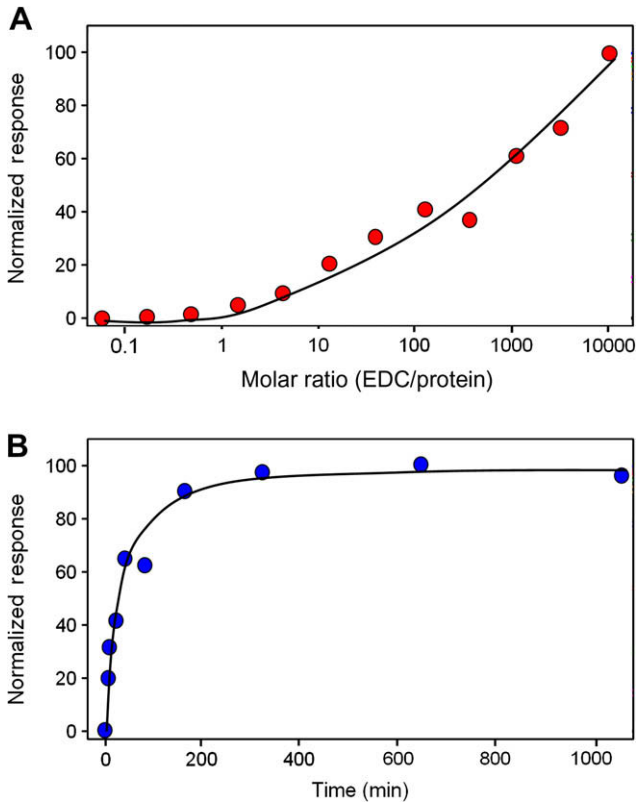

Fig. 4. Biotinylation using carboxyl coupling reagents. (A) Capture levels of CAII biotinylated using a 100-fold excess of pentylamine-biotin and varying concentrations of EDC. (B) Capture levels of CAII biotinylated for various times using pentylamine-biotin. Responses were normalized for the amount of CAPture reagent loaded and to capture levels observed at maximum biotinylation.

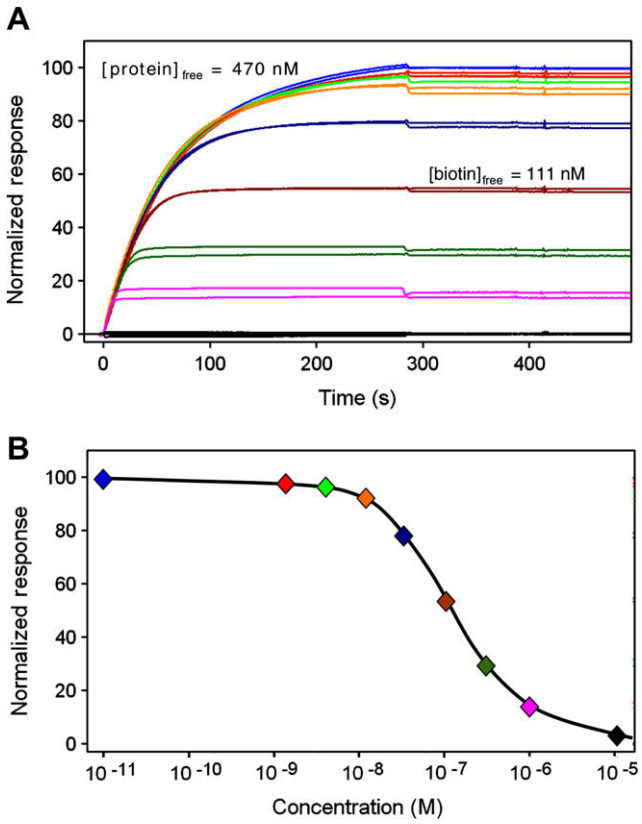

Fig. 5. Inhibition of target capture by free biotin. (A) Responses for capture of 470 nM amine-biotinylated CAII incubated with 0.4–1000 nM free biotin, with duplicate analyses of each sample shown in the figure. Sensorgrams have been normalized for the amount of CAPture reagent loaded and to responses observed at the lowest level (0.4 nM) of free biotin used. (B) Responses from (A) at the end of the association phase plotted as a function of free biotin concentration.

ation was achieved in approximately 3 h (similar to what we observed for amine biotinylation). Together, the results shown in Fig. 4 suggest that carboxyl biotinylation is a viable alternative strategy to biotinylation via amines.

A significant factor in the success of capturing biotinylated target is the ability to separate the target from free biotin after the biotinylation reaction. To demonstrate how unremoved biotinylating agent affects target capture levels, we spiked biotinylated target samples with free biotin (0.4 nM to 1 μM) and tested each sample for capture. As shown in Fig. 5, increasing concentrations of free biotin reduced the target capture levels because of competition for the available streptavidin sites on the surface. Interestingly, the shapes of the responses also change with increasing free biotin, suggesting that low binding responses that rapidly reach saturation (e.g., pink sensorgrams in Fig. 5A) are a diagnostic for insufficient biotin removal.

These results also show that it is possible to capture biotinylated ligand at moderate densities without completely removing the free biotin; at a free biotin/target ratio of approximately 0.25:1, capture was approximately 50% inhibited (brown sensorgrams in Fig. 5A). For some protein/protein interactions, these lower capture densities may be acceptable and so exhaustive biotin removal might not be necessary.

Given the important role that the absence (or presence) of free biotin plays in ligand capture, we explored a number of commonly used methods for removing biotin from protein samples. Using bCAII samples spiked with free biotin, we compared seven biotin removal methods; one involved standard disposable desalting columns, another involved the use of V-series Millipore membranes, and the remaining five represented a variety of standard dialysis methods. Fig. 6 shows an overlay of the results for each biotin removal method. Not only does this figure illustrate the utility of each method, but it also shows the length of time that each method must be employed to be effective.

If we go from the worst method to the best method, we need to start with the V-series membranes (brown curve in Fig. 6). The V-series membranes are disks onto which a droplet of the protein/biotin mixture was pipetted, and then the membranes were set afloat on the dialysis buffer surface. Presumably, the smaller biotin molecule would diffuse more rapidly into the buffer reservoir. We found that evaporation and spreading of the droplet on these membranes made this a difficult method with which to work. In the end, we were not able to recover any biotinylated material using this method.

All four dialysis methods shown as the red, orange, green, and black data in Fig. 6 yielded comparable results. In these cases, a maximum capture level required approximately 16 h of dialysis. All four of these methods use a single dialysis membrane that is incorporated into the side or bottom of a container. If we compare these results with those obtained using the Pierce cassette (pink data) system, we see that the Pierce cassettes are significantly more efficient in terms of reducing dialysis time. This is consistent with this two-membrane apparatus having more than twice the surface area of the other dialysis devices.

The most efficient biotin removal method involved two passes through a spin column. However, significant protein recovery was achieved only by pretreating the spin columns with BSA (light blue data in Fig. 6) as well as adding BSA (a protein carrier) directly to the ligand sample (blue data) just prior to desalting. Interest-

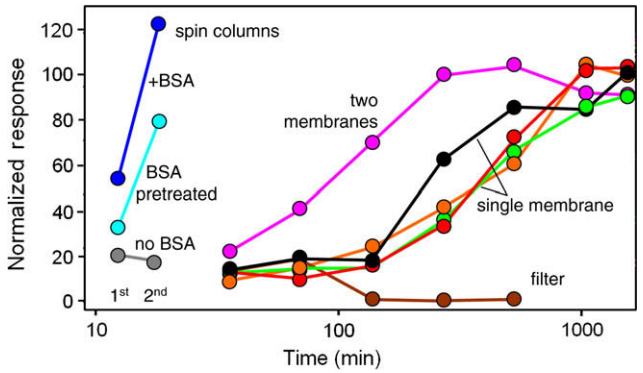

Fig. 6. Comparison of biotin removal methods. Normalized capture levels for 470 nM CAII spiked with 1 μM free biotin were spun or dialyzed for varying times using a range of commercially available products. The dialysis products include the Pierce Slide-A-Lyzer Dialysis Cassette (pink curve), Hampton Research dialysis buttons with Spectrum dialysis membranes (black), the Pierce Slide-A-Lyzer MINI Dialysis Unit (red), the D-Tube Dialyzer Mini (orange curve), the Amersham Biosciences Mini Dialysis Kit (green), and Millipore "V"-series membranes (brown). Using Pierce protein desalting spin columns, CAII was spun once or twice (with two separate columns) with BSA added to the protein solution (blue line), BSA added to the column before sample addition (light blue), or no BSA added (gray). Responses were normalized for the amount of CAPture reagent loaded and to maximal binding observed after using the D-Tube Dialyzer Mini for 16 h. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

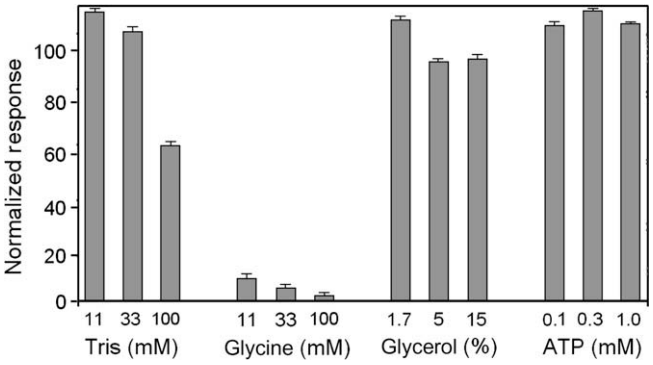

Fig. 7. Effects of common buffer components on protein biotinylation. Capture levels of CAII amine biotinylated in the presence of various concentrations of Tris, glycine, glycerol, and ATP. Responses have been normalized for the amount of CAPture reagent loaded and to maximum capture levels observed of control samples of bCAII.

ingly, we found that the spin columns without BSA pretreatment resulted in very little recovery of CAII (gray curve). Of course, sample loss in these spin columns will likely be dependent on the nature of the protein. But it is important to note that pretreating the spin columns with BSA or adding BSA directly to the ligand solution after a 3-h biotinylation step did not affect ligand capture (unbiotinylated BSA will simply pass over the biosensor surface). Given these results, in general practice, we add 0.1 mg/ml BSA to our biotinylated ligand sample just prior to passing the ligand sample through two spin columns.

One of the most convenient aspects of our minimal biotinylation approach is that it can often be done directly in the buffer in which the protein is provided. Although we have seen the warning signs, such as one should not use Tris as a buffer for biotinylation because it can quench the biotinylation reaction, we wanted to test how much an effect it would have. We also wanted to test how substrates (e.g., ATP, which we like to include to stabilize some enzymes and to protect their binding sites) might affect the biotinylation chemistry. Therefore, we determined the effects of four common buffer additives—Tris, glycine, glycerol, and ATP—each at a range of concentrations.

As shown in Fig. 7, including a buffer additive that contains a primary amine (e.g., glycine) significantly reduces the efficiency of biotinylation. Tris did reduce biotinylation levels, but even Tris concentrations as high as 100 mM inhibited the reaction by only approximately 40%. In this buffer system, more efficient biotinylation may simply require higher molar ratios of biotin to target protein. And as one would expect, high concentrations of glycerol and ATP had very little effect on the biotinylation levels.

Discussion

Choosing whether or not to prepare biotinylated proteins and use streptavidin surfaces has often proved to be a dilemma for biosensor users. Although biotinylated targets are readily and stably captured by streptavidin, the steps involved in protein biotinylation and the lack of reliable regeneration conditions often outweigh the advantages of this capturing system. To address these concerns, here we have outlined a straightforward biotinylation method, which we termed minimal biotinylation, and evaluated this method using Biacore's new Biotin CAPture Kit.

The kit provides a major leap forward in biosensor protocols for systems involving biotinylated reagents. The CAPture reagent, designed to capture biotinylated ligand, can be reproducibly loaded and regenerated, an essential feature when developing experiments that require numerous loading cycles. The ability to regenerate streptavidin surfaces will be a particularly welcome development to investigators who study high-affinity interactions between analytes and biotinylated ligands.

We used this kit to evaluate several fundamental parameters of minimal biotinylation, including the required molar ratios of biotinylating reagent and protein, the times required for biotinylation, the use of both amine and carboxyl coupling chemistries, the extent to which biotin must be removed, and the effectiveness of various biotin removal methods. It must be stressed that the goal of these methods is not to capture a population of molecules that have been quantitatively biotinylated; instead, it is to create a high-density surface of optimal activity. Any residual sites on the streptavidin surface can be blocked with an injection of soluble D-biotin.

Several interesting results were obtained in this study. For instance, the study highlights the consequences of overbiotinylating. In addition to reducing ligand activity by modifying active site residues, overbiotinylation can have deleterious effects on surface capacity. We also illustrated the method of carboxyl-mediated biotinylation. Although we have been hard-pressed to find an example of carboxyl biotinylation reported in the biosensor literature, we found this method to be simple to apply and to yield highly active surfaces (most notably, for several systems in which amine biotinylation was unsuccessful). So, we encourage others to consider this method in the future. In fact, with any new project, we often will run both biotinylation methods side by side and choose the one that works best for further analysis. As we mentioned in the introductory paragraphs, we decided to biotinylate our target protein on ice. With targets that are stable at higher temperature, this might not be necessary, but when working with any new system, we prefer to be cautious and keep the target at low temperature during the biotinylation and desalting steps.

Overall, minimal biotinylation is simple and lends itself easily to a 1-day investigation regarding its suitability. Our studies show that only a 2- to 3-h period of biotinylation is necessary, and biotinylation can be performed in buffers that include Tris, glycerol, and ATP (the idea here is to run the biotinylation in a buffer that maintains ligand activity and to minimize excessive handling of the protein). Our biotin removal experiments suggest that a dialysis period of as little as 4 h is necessary if dialysis cassettes are used

and that an even shorter time period is necessary if desalting spin columns are used. We caution, however, that one may want to consider prewashing the spin columns with BSA or adding BSA directly to the biotinylated ligand just prior to desalting so as to prevent loss of proteins to the spin column resin.

Certainly, each ligand system may require optimizing biotinylation methods to achieve the most efficient level of capture and the highest activity surfaces. The methods we illustrated here should be viewed as a guide to what conditions to explore and how to take advantage of the ability to regenerate streptavidin surfaces using the CAP chips.

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