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Optimizing Ligand Biotinylation: Conditions for Molecular Interactions Analysis

Technical Note

Scope
This Technical note provides Octet users with guidance on the generation of optimally biotinylated ligands for use with streptavidin biosensors for kinetics and customized quantitation assays.

Introduction

During biologics drug development, the detection and quantification of the expressed biologic and its interaction with its intended target are critical processes that enable the selection and subsequent optimization of lead candidates. Octet® BLI systems utilize a label-free analytical approach, to provide a fast and straightforward means to accurately quantify these molecules and determine binding specificity and affinity to a target. To enable these applications, streptavidin has been deployed as a key biosensor surface that can be used for both kinetics and quantification assays. Streptavidin-based biosensor assays require the biotinylation of the ligand to be used as the capture molecule. After biotinylation, any unreacted biotin molecules must be removed from the biotinylated ligand. The presence of unreacted biotin during the ligand loading step may interfere with optimal capture of the biotinylated ligand. In this technical note, we illustrate ligand binding response features that are exhibited by optimal biotinylation processes that in turn enable successful kinetics or custom quantification assays on Octet® BLI systems. In addition, we recommend the use of Sartorius Vivaspin® desalting columns for the removal of free and unreacted biotins.

Streptavidin Biosensors for Kinetic Analysis

A variety of streptavidin biosensors have been developed at Sartorius for the capture of biotinylated ligands. While these biosensors (Octet® SA, SSA, SAX and SAX2) are all coated with streptavidin molecules and intended for use with biotinylated ligands, they differ in the intended applications (refer to biosensor selection guide[1] for details).

Ligand Biotinylation

Proteins can be biotinylated in-vitro using biotin labelling reagents, which can be targeted against a variety of functional groups such as primary amines and sulfhydryls. The interaction between streptavidin and biotin is widely used as a system for the rapid, stable, and irreversible non-covalent binding of biological molecules. The biotin-streptavidin coupling method creates a stable bond to the surface on the biosensor similar to covalent coupling yet requires minimal optimization.

To capture a ligand onto a streptavidin biosensor, it must first be biotinylated. Ideally one would want only one biotin attached to each ligand molecule as over biotinylation can lead to a reduction in the ligand capture level due to potential cross-linking on the sensor surface[2].

Minimal biotinylation is therefore recommended, despite the risk that it may be expensive due to an increased wastage potential from unbiotinylated ligands. A reduction in the ligand capture levels while not necessarily harmful to kinetics assays may lead to a less-than-optimal biosensor surface for custom quantitation assays where a high capture level often translates into a wider dynamic range for analyte quantitation.

Biotinylation is simple to perform, gentle on proteins and is performed at neutral pH. The reaction is controlled by regulating the number of biotin molecules added to the reaction per target protein. Long chain linkers (reactive biotins with different lengths of PEG molecules are often used) should be incorporated into a biotin tag to minimize steric effects, especially when immobilizing smaller molecules such as peptides. A biotinylated ligand can be prepared in batches and stored for use in multiple experiments.

The protocol below describes the biotinylation process[3] using the EZ Link NHS-PEG4 Biotin kit (Thermo Scientific):

1. Prepare 100 µL of the ligand to be biotinylated at a concentration of 0.5-1 mg/mL. The ligand should be prepared in buffer free of primary amines. Presence of free primary amines such as Tris would compete with the protein for the EZ link NHS Biotin reagent.
2. Calculate the volume of a 1 mM biotin solution needed for a molar coupling ration (MCR) of 1 using the equation given in Formula 1. If the volume is less than 2 µL, recalculate the volume for a 0.2 mM biotin solution to minimize the errors associated with pipetting small reagent volume.
3. Immediately before use, add 170 µL of ultrapure water to one vial of 2 mg of NHS-PEG4-Biotin to create 20 mM stock. Dilute to 1 mM or 0.2 mM stock with ultrapure water.
4. Add the calculated volume of biotin reagent to the ligand solution and incubate on ice for two hours or at room temperature for 30 minutes.
5. Remove non-reacted NHS-PEG4-Biotin using a Vivaspin® 500 Desalting Column using the instructions below[4].

Formula 1: *Calculation volume biotin reagent*

$$\text{Vol 1 mM biotin reagent (µL)} = \frac{\text{Protein Conc (mg/mL)}}{\text{MW Protein (kDa)}} \times \text{MCR} \times \text{volume Protein (µL)}$$

Example 1:
1 mL solution of IgG (150 kDa) at 1 mg/mL with an MCR of 1: µL 1 mM biotin reagent
= (1/150) × 1 × 1,000 = 6.67 µL

Example 2:
0.5 mL solution of IgG (150 kDa) at 0.5 mg/mL with an MCR of 1: µL 1 mM biotin reagent
= (0.5/150) × 1 × 500 = 1.67 µL

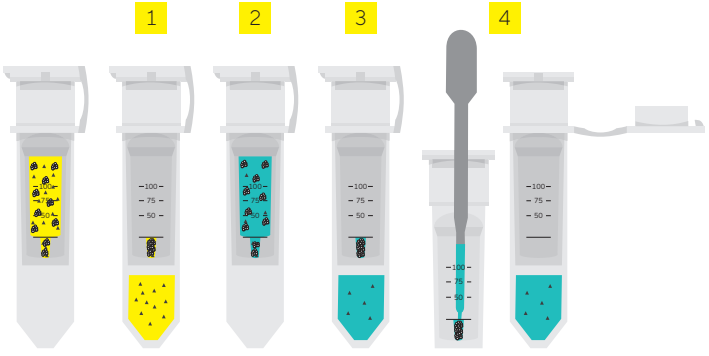
Removal of Excess Biotin

To remove un-reacted biotin after biotinylation, Sartorius Vivaspin® 500 is recommended. The Vivaspin® product family includes a comprehensive range that can be used for a variety of applications including diafiltration, desalting and buffer exchange of amongst other things, antibodies, proteins and viral particles. In addition, the incorporation of a low MW cutoff membrane allows for the removal of any particulates in the sample with molecular weight below the cut-off while the protein is retained. It is designed to maximize protein recovery of up to 98- 99% of the protein.

Step by Step Method for excess Biotin removal
Select an appropriate MWCO for your sample. For maximum recovery, select a MWCO of 1/2 the molecular weight of the molecule of interest.

1. Wash column with PBS, centrifuge at 12,000 x *g* for 2 min
2. Load biotinylated ligand onto the column, centrifuge at 12,000 x *g* for 1 min.
3. Perform buffer exchange 3x by adding 100 µL of PBS and centrifuging at 12,000 x *g* for 1 min each time.
4. Collect the sample in the retentate.

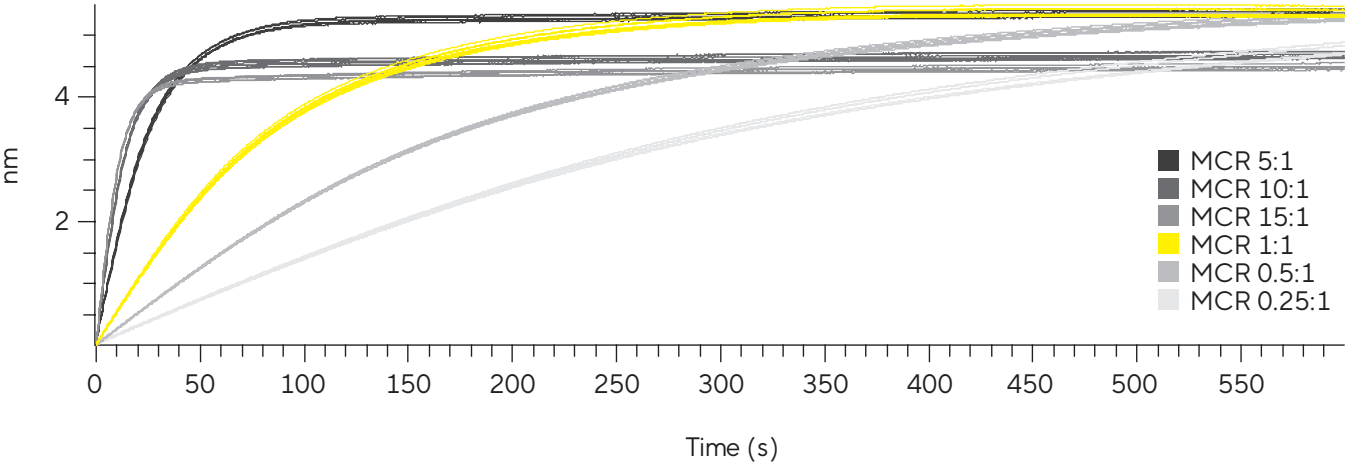
Figure 1: *Biotin removal and sample concentration workflow. Molecules with molecular weight below the membrane cut-off are removed into the filtrate at the bottom of the tube while the protein is retained.*



Conclusion

An evaluation of different levels of incorporation of biotin to ligand ranging from molar coupling ratios of 0.2:1 – 15:1 (biotin:ligand) reveal that minimal biotinylation of approximately 0.5-1 biotin per ligand yields ligand capture response levels that are equivalent over time. These levels are also equivalent to the levels achieved at a 5:1 ratio but significantly higher than incorporation at a 10:1 or 15:1 ratio. In addition, the slopes of the binding response curves are lower and more gradual with lower incorporation ratios. These results suggest that incorporation ratios higher than 5:1 may lead to over biotinylation that in turn may lead to steric non-optimal capture of the ligand that may not be optimal for target binding. In addition, for custom quantitation assays and small molecules characterization assays where high ligand response levels are needed to amplify analyte binding response, minimal biotinylation; ideally from 1:1 – 5:1 is recommended. The Vivaspin® 500 desalting columns are demonstrated as an optimal and appropriate column that leads to efficient removal of excess biotins and yields a high recovery of the retentate (biotinylated ligand). As a Sartorius product, the Vivaspin® 500 columns. In addition to streptavidin-based biosensors, offer Octet® BLI system users a one-stop shop for acquisition of consumables needed to seamlessly perform streptavidin-based kinetics or quantitation assays.

Figure 2: *A comparison of biotinylated ligand binding response levels after removal of excess biotin. Samples reacted at different biotin incorporation levels are compared side by side at normalized ligand concentration.*



References

1. Octet Biosensor selection guide
2. Giuseppe Papalia, David Myszka: Analytical Biochemistry 403 (2010) 30–35
3. Sartorius Technical Note: Biotinylation of Proteins for the Immobilization onto Streptavidin Biosensors
4. Sartorius Application Note: Concentration of Low Molecular Weight Peptides with Vivaspin® 500 Ultrafiltration Devices

Germany
Sartorius Lab Instruments GmbH & Co. KG
Otto-Brenner-Straße 20
37079 Göttingen
Phone +49 551 308 0

USA
Sartorius Corporation
3874 Research Park Drive
Ann Arbor, MI 48108
Phone +1 734 769 1600

For further information, visit
sartorius.com

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