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affiliée à l'Université de Montréal

**Optimization of Lectin Biotinylation for the Assessment of Therapeutic
Protein Glycosylation**

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Institut de génie biomédical

Mémoire présenté en vue de l'obtention du diplôme de *Maîtrise ès sciences appliquées*

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Protein Glycosylation**

présenté par **Benjamin SERAFIN**

en vue de l'obtention du diplôme de *Maîtrise ès sciences appliquées*

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RÉSUMÉ

La glycosylation est un attribut de qualité critique pour les protéines thérapeutiques, et est également un biomarqueur de plusieurs maladies humaines. Pour évaluer l'état de glycosylation des protéines, les lectines sont souvent utilisées car leurs spécificités pour les sucres sont bien caractérisées. Les lectines ont été proposées pour la détection et la caractérisation de plusieurs produits biothérapeutiques, tels que les vaccins viraux et les anticorps monoclonaux. Dans les tests à base de lectines, il est commun de conjuguer celles-ci avec de la biotine. Les étiquettes à base de biotine permettent aux lectines de recruter des complexes à base de streptavidine capables de générer des signaux, ou de capturer des lectines sur des surfaces de biocapteurs fonctionnalisées avec de la streptavidine. Dans ce travail, nous explorons l'application de lectines pour la détection de produits biothérapeutiques dans les biocapteurs à base de résonance de plasmons de surface et dans des tests à base d'enzymes liés aux lectines. En particulier, on démontre que le ratio de conjugaison biotine-lectine peut avoir un impact important sur la performance de ces deux tests à travers la modulation de l'interaction lectine-streptavidine. Dans les biocapteurs à base de résonance de plasmons de surface, une biotinylation excessive peut réduire la capture à des surfaces de streptavidine et peut modifier les cinétiques d'interactions entre les lectines et des glycoprotéines. Dans le test ELLA (« enzyme-linked lectin assay »), un haut niveau de conjugaison amplifie le signal spécifique et baisse la limite de détection. A la lumière de ces résultats, des recommandations sont formulées quant au niveau optimal de biotinylation des lectines selon l'application envisagée.

ABSTRACT

Protein glycosylation is a crucial attribute for therapeutic proteins and a biomarker for many human diseases. For assessing protein glycosylation, lectins are often used as they have well characterized sugar specificities. Lectins have been proposed for the detection and characterization of many biotherapeutic products, including viral vaccines and monoclonal antibodies. In lectin-based assays, it is common to tag lectins with biotin. Biotin-tags allow lectins to recruit signal generating streptavidin-based compounds or capture lectins to streptavidin-functionalized biosensing surfaces. In this work, we explore the use of lectins for the detection of biotherapeutic products in surface plasmon resonance and the enzyme-linked lectin assay. In particular, we demonstrate that the biotin-lectin conjugation ratio can have a significant impact on the performance of these two assay types, via the modulation of the lectin-streptavidin interaction. In surface plasmon resonance assays, excess biotinylation can reduce capture to streptavidin surfaces and modify lectin-glycoprotein binding kinetics. In the enzyme-linked lectin assay, high biotin conjugation amplifies the specific signal and lowers the limit of detection. Finally, we present a workflow for the assessment of optimal lectin biotin conjugation ratio.

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LISTE OF SYMBOLS AND ABBREVIATIONS

[A]	Analyte concentration
[A] _{bulk}	Analyte concentration in the bulk flow
[A] _{surface}	Analyte concentration in the surface layer
[AL]	Analyte – ligand complex concentration
[L]	Ligand concentration
ADCC	Antibody dependent cellular cytotoxicity
Asn	Asparagine
ATIR	Attenuated total internal reflection
BAP	Biotin acceptor peptide
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CBP	Carbohydrate binding protein
CDC	Complement dependent cytotoxicity
CMD	Carboxymethylated dextran
CQA	Critical quality assurance
CRD	Carbohydrate recognition domain
Da	Dalton
DB	Dilution buffer
DNA	Deoxyribonucleic acid
EDC	1-Ethyl-3-(3- dimethylaminopropyl)carbodiimide
ELISA	Enzyme linked immunosorbent assay
ELLA	Enzyme linked lectin assay
ER	Endoplasmic reticulum
Fc	Crystallizable fragment
FcγR	Crystallizable fragment receptor gamma
HA	Hemagglutinin
HBS	HEPES buffered Saline
HCl	Hydrochloric Acid

HEPES	4- (2-Hydroxyethyl)piperazine-1-ethane-sulfonic acid
HILIC	Hydrophobic interaction liquid chromatography
HRP	Horseradish peroxidase
IAV	Influenza A virus
IIV	Inactivated Influenza Vaccines
IMAC	Immobilized metal affinity chromatography
IOP	Initial oligonucleotide pool
IUPAC	International Union of Pure and Applied Chemistry
K _A	Thermodynamic association constant
K _D	Thermodynamic dissociation constant
K _{D,app}	Apparent thermodynamic dissociation constant
k _m	Mass transfer coefficient
k _{off}	Dissociation rate constant
k _{on}	Association rate constant
LAIV	Live attenuated Influenza vaccines
LCA	Lens culinaris agglutinin
LOD	Limit of detection
M2	Matrix protein 2
mAb	Monoclonal antibody
MAL-II	Maackia Amurensis Lectin II
mRNA	Messenger ribonucleic acid
MTL	Mass transport limitation
NA	Neuraminidase
NaBH ₃ CN	Sodium Cyanoborohydride
NHS	N-Hydroxysuccinimide
NTA	Nitrilotriacetic acid
PAMP	Pathogen associations molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDEA	2-(2-pyridinyldithio)ethaneamine
PEG	Polyethylene glycol

PRR	Pattern recognition receptors
QA	Quality assurance
RB	Running buffer
R _{eq}	Resonance units at equilibrium
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
RTX	Rituximab
RU	Resonance units
SELEX	Systematic evolution of ligands by exponential enrichment
Ser	Serine
SNA	Sambucus Nigra Agglutinin
SPR	Surface plasmon resonance
SRID	Single radial immunodiffusion
ssDNA	Single stranded deoxyribonucleic acid
Thr	Threonine
TMB	3,3',5,5'-Tetramethylbenzidine
VP	Viral polymerase
vRNA	Viral Ribonucleic acid
vRNP	Viral ribonucleoprotein
WHO	World health organization

CHAPTER 1 INTRODUCTION

Surface Plasmon Resonance (SPR) biosensors are highly versatile platforms for the detection and characterization of biological interactions in real-time, without the need for molecular labels. They are highly regarded in the field of quality assurance (QA), as they operate in a largely autonomous manner, and require very little sample pre-treatment prior to analysis. As the field of biological therapeutics grows, the need for robust and high-throughput QA platforms increases.

While the field of proteomics has grown significantly in the last century, the sister field of glycomics has lagged far behind. Initially, protein glycosylation was thought to participate primarily in metabolism and nutritional storage. However, glycosylation has since been identified as a key regulator of intra- and extra-cellular interactions, and in many cases contributes to protein stability and conformation [1]. Aberrant glycosylation is a marker of diseases, associated with chronic inflammation, autoimmune disorders and cancer [2]. Glycosylation is equally a critical quality attribute (CQA) of biotherapeutic medicines. Glycosylation is particularly important for monoclonal antibodies (mAbs), a powerful and versatile class of biotherapeutics. For mAbs, glycosylation modulates the binding strength between the Fc domain (the antibody's signaling domain) and the Fc γ receptor (Fc γ R) [3], and ultimately the effectiveness of the biotherapeutic treatment. Numerous techniques have been developed for the characterization of protein glycosylation, but many of these are technically complex and require significant hands-on time. As such, SPR biosensors, relatively user-friendly and autonomous, are an attractive choice for the evaluation of protein glycosylation. Among the ligand options for the evaluation of protein glycosylation by surface plasmon resonance, lectins are a promising option [4]. Lectins are a class of non-catalytic sugar binding proteins whose sugar binding specificities have been well characterized. Since their discovery in plants over a century ago, lectins have been widely used as analytical tools for the assessment of protein glycosylation, histological and cytological studies, and in the study of host-virus interactions [5].

SPR biosensors gained further attention in the field of biomanufacturing following the most recent Influenza pandemic, the 2009 H1N1 "Swine Flu". Following the pandemic, a conference of representatives from numerous national and international public health authorities identified inadequate vaccine quality control as the primary cause for delayed vaccine availability worldwide

[6]. Specifically, reagents required for the gold standard Single Radial Immunodiffusion (SRID) assay were unavailable for weeks following the initial outbreak, resulting in bottleneck in the vaccine distribution pipeline. In this same conference, SPR biosensors were identified as a potential candidate for a novel assay to replace SRID, provided these SPR assays can be performed without anti-Influenza antibodies on which the SRID assay relies [7]. Among others, lectins have been proposed as potential ligands for Influenza detection and quantification by SPR.

Lectin-based techniques like the enzyme linked lectin assay (ELLA) and certain SPR assay configurations employ lectins conjugated with biotin tags. Biotin molecules are used as affinity tags which display a very strong binding affinity for streptavidin [8]. While present in many assays, the biotin-streptavidin affinity system plays different roles depending on assay configuration, and the optimization of this system is therefore assay specific. Despite it being an important modulator of lectin-assay performance, distributors of biotinylated lectins do not divulge the degree of biotinylation of their lectin products,.

In this work, we explore of lectins as analytical tools in SPR biosensors for the detection of Influenza virions and compare them against other classes of ligands, based on the published literature. Later in the work, we evaluate the impact of the lectin biotinylation ratio on the performance of lectin-based protein glycosylation assays, including the ELLA and SPR assays.

CHAPTER 2 Organization of the Dissertation

As a precursor to the two articles contained in this dissertation, chapter 3 contains a literature review on surface plasmon resonance, including subsections on the underlying physical phenomenon, SPR surface design and introduction to binding and transport kinetics that contribute to signal generation. Chapter 3 will also include a brief review of protein glycosylation, and finally a review of the Influenza virus.

The original objective of the research was the detection and quantification of Influenza virions in crude cell lysates by surface plasmon resonance. Specifically, this project aimed to detect Influenza using lectins, a class of non-catalytic sugar binding proteins. Ultimately, the detection of crude Influenza samples using lectins was not pursued in a research capacity. However, a relevant review article was written on the subject of Influenza detection by SPR. This review article, entitled “Antibody-Independent Surface Plasmon Resonance Assays for Influenza Vaccine Quality Control”, was accepted for publication in the journal *Applied Microbiology and Biotechnology*, following a submission in January 2024. This review article is included, in full, in Chapter 4.

When performing initial screening experiments to determine an appropriate lectin to use for the detection of the influenza strain A/Puerto Rico/8/1934 (H1N1), we noted difficulty in achieving high lectin immobilization levels required to produce mass-transport limited interactions, which are favored for quantification by SPR (see Section 3.1.6). Since we were employing streptavidin capture of biotinylated lectins, we hypothesized that the presence of multiple biotin tags on a single lectin was prematurely saturating streptavidin surfaces due to multi-point binding. As a result, maximal lectin immobilization levels were reduced. Communications with distributors of biotinylated lectins yielded no information on the biotin conjugation levels, as these were not routinely determined by distributors for each batch. We hypothesized that excessive lectin-biotinylation was the source of the low capture levels, and that the different lectin assays which employ the biotin-streptavidin affinity system will benefit from different conjugation levels depending on their specific configurations.

The influence of biotin conjugation levels on the analytical performance of lectin proteins was further examined, and produced a research article, entitled “Impact of Lectin Biotinylation for Surface Plasmon Resonance and Enzyme-Linked Lectin Assays for Protein Glycosylation”, which was submitted for publication in the journal *Analytical Biochemistry* in March 2024. The research

article represents the core of the laboratory work executed during the Master's project, and is included, in full, in Chapter 5.

CHAPTER 3 Literature Review

3.1 Surface Plasmon Resonance

3.1.1 Kretschmann Geometry

Surface Plasmon Resonance (SPR) biosensors were born from work of physicists Erich Kretschmann and Heinz Raether, who published their landmark articles on the topic in 1968 [9]. The Kretschmann and Raether configuration (often referred to as simply “Kretschmann configuration”) is the most popular design for SPR optical biosensors. This configuration is present in the BIAcore line of SPR biosensors, first released in the 1990s by Pharmacia Biosensor. Today, the BIAcore is distributed by Cytiva, formerly GE Healthcare, and is by far the most common SPR biosensor found in the literature.

Kretschmann geometry (Figure 3-1) relies on a phenomenon known as attenuated total internal reflection (ATIR). Herein, a thin film of noble metal, usually silver or gold, rests atop a glass prism. Above the metal film, there is a dielectric medium that provides the optimal environment for bio-interactions, but also bears special optical properties [10]. During detection, a polarized beam of incident light scans through the prism and strikes the metal film. At a certain angle of incident light, called the SPR angle, electrons in the metal film are excited into clouds called plasmons. Due to plasmon generation, the light that is reflected back to a detector will be of lower intensity. The angle of incident light at which this intensity dip occurs, SPR angle, is the basis of the signal generation in the Kretschmann configuration [11, 12]. While some SPR biosensors provide the SPR angle as a raw signal, the BIAcore line of SPR biosensors instead converts this angle into arbitrary resonance units (RU), for ease of interpretation.

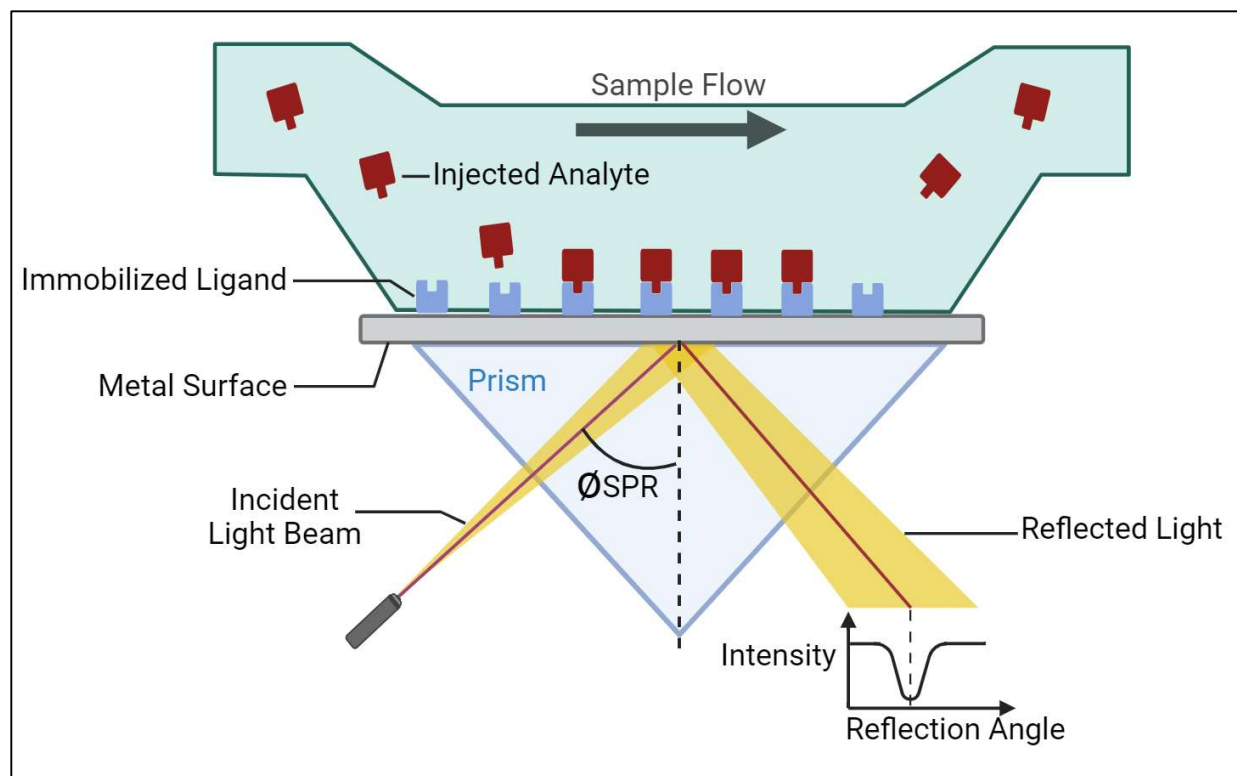


Figure 3-1 Schematic of a SPR biosensor based on Kretschmann geometry.

3.1.2 Producing an SPR Signal

Assuming no changes in the wavelength or intensity of the incident light, the SPR angle is determined by the refractive index of the dielectric medium opposite the glass prism, within a distance of approximately 300 nm from the metal surface. SPR biosensors take special care to control the temperature of the liquid flowing over the surface, as temperature can influence the refractive index of the medium. When no bio-interactions are taking place, the refractive index of the medium (and thus the SPR angle) is determined by the composition of the running buffer (RB). The RB usually contains a pH buffer, such as HEPES or phosphate buffered saline (PBS) and salts, to mimic physiological conditions [13].

The composition of the RB is changed as proteins, peptides or other biomolecules of interest are introduced at the surface. The first of these biomolecules of is called the ligand. Despite the nomenclature, SPR ligands do not require an active binding domain necessary for a bio-interaction. Passive ligands, or “bait” ligands, instead rely on the binding domain of the injected analyte molecule for initiation of the bio-interaction. The ligand is affixed to the sensor surface by covalent

coupling or reversible capture. As the ligand becomes surface bound, entering the detection limit of the biosensor, the refractive index of the medium changes and the SPR angle is shifted. This shift is proportional to the mass accumulated at the surface, wherein 1 RU is roughly equivalent to 1 pg/mm² of protein at the surface [14].

During detection, a second biomolecule known as the analyte is injected into the flow cell. As the analytes accumulate at the surface due to interactions with the ligand, the refractive index of the medium changes and the SPR angle is shifted (Figure 3-1). Tracking the SPR angle in real time enables us to characterize the kinetic and thermodynamic properties of the analyte–ligand interaction.

3.1.3 Ligand Immobilization

The majority of SPR surfaces are decorated with chemically functional matrices, which enable the immobilization of ligands through the formation of covalent bonds. The most common is carboxymethylated dextran (CMD), though some surfaces instead use chemically functional polyethylene glycol (PEG). In addition to chemical availability, these matrices reduce non-specific signal generation due to their low-fouling properties [15]. Some surfaces contain no matrices, and instead rely on passive adsorption or thiol-coupling directly to the gold sensor surface [16].

3.1.3.1 Covalent Coupling

The most common approach to ligand immobilization is covalent coupling. This method is the simplest to perform, though has a few notable disadvantages. Firstly, covalent coupling will result in randomly oriented immobilization, wherein the immobilization level (in RU) will not actually reflect the bioactivity of the surface as some of the ligands will be misoriented and unavailable for binding. Secondly, covalent coupling is irreversible. The lifespan of the sensor chip is therefore limited by the bioactivity of the ligand molecule, which can be damaged by harsh regeneration solutions or inadequate storage conditions. Covalent ligand immobilization include amine coupling, thiol coupling or aldehyde coupling (Figure 3-2) [17].

Amine coupling (Figure 3-2a) is the simplest and by far the most common immobilization strategy found in the literature. A disadvantage of amine coupling is the potential for multi-point attachments, wherein the ligand might become attached to the surface via multiple lysine residues, which are relatively plentiful in proteins. Amine coupling is achieved via the formation of a reactive

N-Hydroxysuccinimide (NHS) ester, which reacts with available amine groups on ligands to form amide bonds, directly onto the N-terminal or lysine side chains of the protein or peptide ligand [18].

Thiol coupling (Figure 3-2b) employs a bi-functional linker, PDEA, which contains both an amine group and a disulfide bond. This linker is bound by amine coupling. Once bound, PDEA can form a disulfide bridge with a thiol group on the ligand molecule, commonly via a cysteine amino acid residue [18]. If a cysteine residue is not naturally exposed on the ligand, one can be liberated via reduction of an existing S-S bond, though this will modify the ligand's quaternary protein structure and potentially alter its binding behaviour. Alternatively, cysteine residues can be added to a ligand in a site-specific manner via genetic engineering [19].

Aldehyde coupling (Figure 3-2c) relies on aldehyde chemical groups, which are not naturally found in proteins or peptide. Aldehyde functional groups can be added to a protein via chemical conjugation, prior to the SPR immobilization. While this requires an extra step, it also offers an added layer of customization, which could help prevent random orientation and multi-point attachment. Following creation of a reactive NHS-ester, a hydrazine linker is covalently bound. Subsequently, the aldehyde-conjugated ligand reacts with the hydrazine to form a hydrazone linkage, which is reduced to a secondary amine by NaBH_3CN [18].

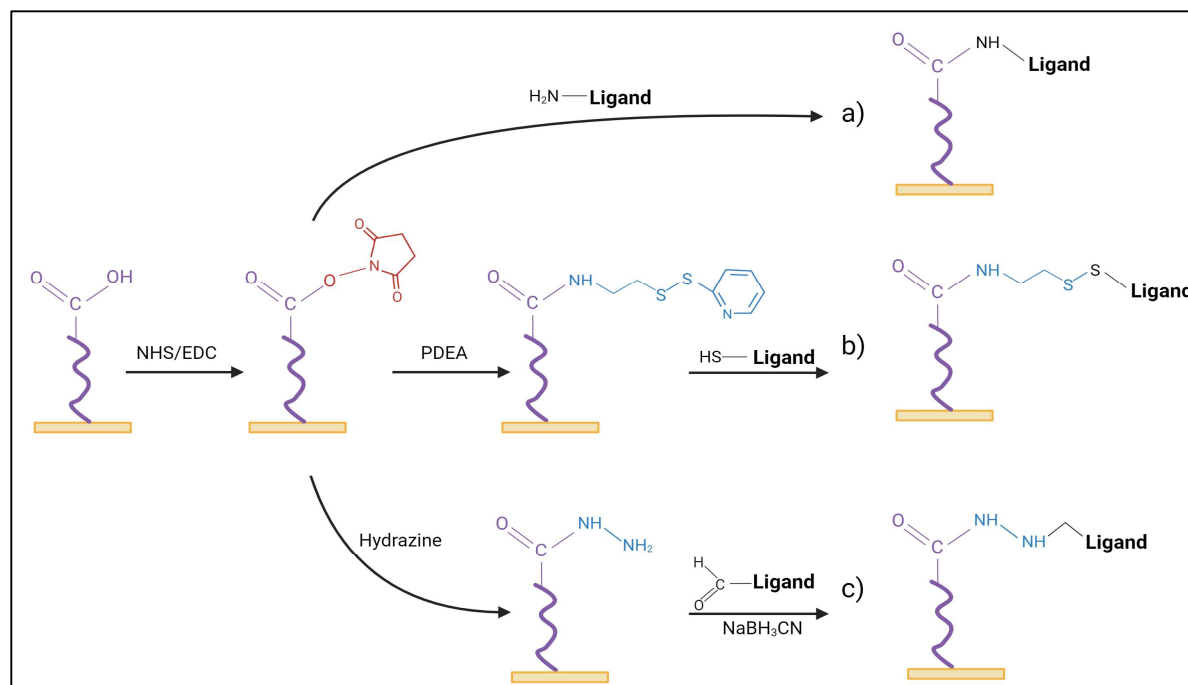


Figure 3-2 Chemical structures depicting the covalent immobilization of ligands to carboxymethylated dextran hydrogel strands. Purple: carboxymethylated dextran. Red: NHS Ester derivative, highly reactive to amine groups and used as the first step in each immobilization strategy. Blue: linker molecule used to present the ligand with the appropriate functional group for immobilization. Black: ligand bearing the required functional group for conjugation. Abbreviations: NHS: N-Hydroxysuccinimide

EDC: 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide

PDEA: 2-(2-pyridinyldithio)ethaneamine NaBH_3CN : Sodium Cyanoborohydride

3.1.3.2 Ligand Capture

Ligand capture is a popular option for ligand immobilization, as prefabricated immobilization kits provide a user-friendly experience. Capture first requires the immobilization of a separate capture ligand. This capture ligand can either bind directly to the SPR ligand (ex. Protein A is a capture ligand for SPR ligands expressing an Fc domain) (Figure 3-3a), or to a molecular tag. One major advantage of capture is that, in some cases, it is reversible. Surfaces can therefore be entirely regenerated via the dissociation of the capture ligand–SPR ligand interaction, providing a platform for the capture of fresh SPR ligands. This can significantly extend the lifespan of an SPR sensor chip, at the cost of greater material consumption.

The streptavidin-biotin interaction is the strongest non-covalent interaction observed in nature, with a thermodynamic dissociation constant (K_D) on the order of 10^{-15} M [8]. Frequently, streptavidin is immobilized to the sensor surface via covalent immobilization, and biotin-tagged ligands are injected over the surface to be captured. Biotinylation of ligands can be achieved via chemical conjugation, though this results in randomly oriented attachments and therefore a randomly oriented capture to the surface. Alternatively, biotinylation can be site-specific via the genetic engineering of a biotin acceptor peptide (BAP) directly to the ligand, for subsequent conjugation using a biotin-ligase enzyme [20]. Capture of biotinylated ligands to covalently coupled streptavidin is functionally irreversible, and therefore presents many of the same disadvantages as covalent coupling (Figure 3-3b).

Single stranded DNA or RNA oligonucleotides are another option for ligand capture, as they display immense specificity for complementary oligonucleotide sequences. Additionally, the affinity of DNA/DNA or RNA/RNA interactions can be finely tuned via the modulation of strand length, or via the inclusion of base mismatches [21]. Complimentary oligonucleotide strands are the favored immobilization method for aptamer ligands [22-24]. This capture ligand can also be used for the capture of proteins, provided they are conjugated with a complimentary oligonucleotide strand [25]. Unlike streptavidin-biotin interactions, oligonucleotide strand-based capture can be regenerated, significantly extending sensor chip lifespan (Figure 3-3c).

Complementary peptides, otherwise known as affinity peptides, are a lesser known but highly versatile option for ligand capture. Complementary peptides will interact with one another much like secondary structures within the tertiary structure of a protein [26]. One example is the E/K coiled-coil peptide system, which consists of two 35 amino acid long peptides, [EVSALEK]₅ and [KVSALKE]₅ named Ecoil and Kcoil, respectively. Alone, the penta-heptads have no organized structure. Together, they dimerize into two coiled alpha-helical secondary structures via a combination of hydrophobic and electrostatic interactions [27]. While one peptide is covalently immobilized to the sensor surface via covalent coupling, the other is genetically engineered to the terminus of the SPR ligand [28] (Figure 3-3d). Like with oligonucleotides, the affinity of coiled-coil interactions can be tuned by changing the length of the peptide or by introducing specific amino acid substitutions [29].

While poly-histidine tags are common tools for the purification of recombinant proteins in immobilized metal affinity chromatography (IMAC), poly-histidine tags have also been employed for the capture of ligands to SPR surfaces. Sensor chips designed for this purpose are first functionalized with nitrilotriacetic acid (NTA). The chips are then primed with a buffer containing Ni^{2+} , which is chelated by NTA and shows an affinity for poly-histidine chains. The relatively low affinity ($K_D = 10^{-6}$ M) can be compensated with a high-density immobilization of the NTA, to encourage rapid rebinding. NTA surfaces have a high capacity for regeneration, which occurs at the level of the NTA- Ni^{2+} interaction [30] (Figure 3-3e).

The techniques described above can be combined so as to exploit the advantages of many systems. The most popular example is the CAP sensor chips, distributed by Cytiva. In CAP chips, streptavidin conjugated to ssDNA is captured to a surface decorated with complementary ssDNA strands. Streptavidin can then capture biotinylated ligands, combining the capacity for regeneration of oligonucleotide capture with the ease of use of the biotin-streptavidin system [31] (Figure 3-3f).

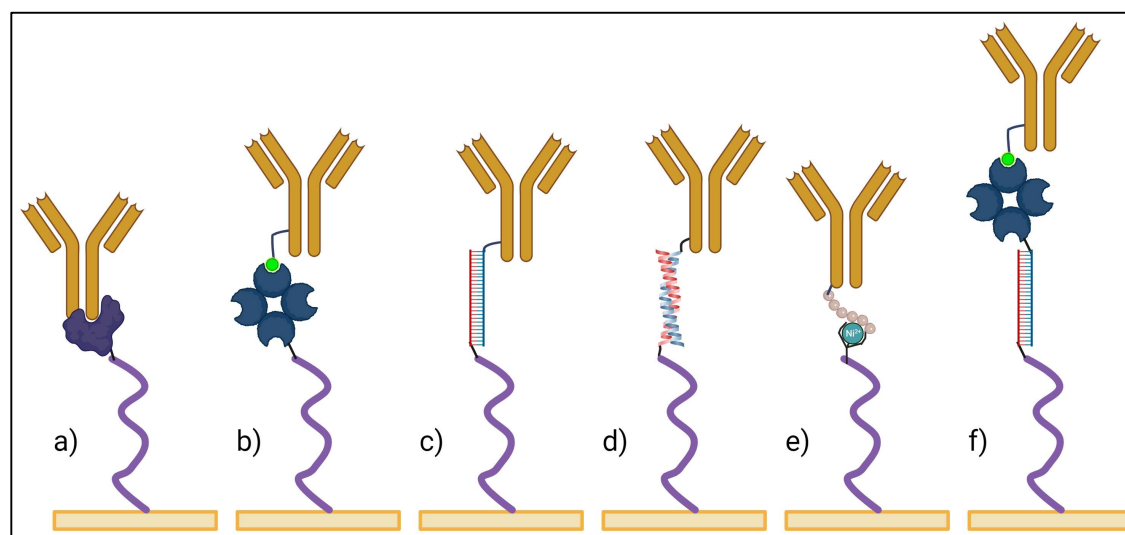


Figure 3-3 Schematic depiction of common SPR surface designs which employ capture ligands for the immobilization of an antibody ligand. a) Direct capture of the antibody's Fc domain with Protein A. b) Capture of a biotin-tagged antibody via a covalently bound streptavidin protein. c) Capture of an oligonucleotide tagged antibody via a covalently bound complementary oligonucleotide strand. d) Capture of an affinity peptide tagged antibody using a complementary affinity peptide. Together these peptides organize into a coiled-coil motif. e) Capture of a poly-histidine tagged antibody using an NTA- Ni^{2+} complex, as seen in IMAC purification platforms. f)

Combinatorial approach found in Cytiva's CAP series of sensor chips. Herein, streptavidin is captured by complementary oligonucleotides, providing a platform for the biotin-streptavidin capture of a biotin-tagged antibody.

3.1.3.3 Hydrophobic Stabilization

Hydrophobic stabilization involves the immobilization of a lipid membrane, which itself contains the ligand of interest. Immobilization of membrane-bound carbohydrate structures [32] or transmembrane receptor proteins [33] is achieved via the immobilization of pre-functionalized lipid vesicles, which already contain the desired structure or protein. For the study of lipid-protein interactions, a nude membrane is immobilized.

Lipid coated surfaces can be constructed via a supported lipid monolayer (HPA chips) or via intact lipid vesicles (L1 chips), both of which are captured to hydrophobic SPR sensor surfaces via hydrophobic interactions. Intact vesicles bearing chemical functional groups or capture tags can also be immobilized to dextran coated sensor chips via the strategies described in the previous sections [34] (Figure 3-2, Figure 3-3). One disadvantage of lipid decorated SPR surfaces are their incompatibility with any RB that contains detergents, as these can destabilize the lipid surface. Without detergents, non-specific adsorption of analyte proteins to the walls of the liquid handling systems would increase, necessitating more frequent maintenance [35].

3.1.4 Double Referencing an SPR Signal

Since the shift in SPR angle is uniquely due to changes in the refractive index of the medium at the surface, proper referencing strategies must be employed to ensure that only the signal coming from the analyte–ligand interaction is being reported. Two referencing strategies are commonly used to isolate the analyte–ligand signal: blank sample and blank slide referencing.

Blank sample referencing is used to eliminate signals arising from the dilution buffer (DB). If the DB (used to prepare analyte samples) differs in composition from the RB, the sudden shift in refractive index will generate a bulk-shift artefact, characterized by a large, nearly instantaneous signal shift that can be positive or negative. To reference bulk shift artifacts, a signal obtained from injecting a blank sample (DB only) over the surface is subtracted from the experimental signal [36].

Blank slide referencing is used to eliminate signals which arise from non-specific interactions with ligands immobilized at the sensor surface. These interactions occur between the

analyte and the non-ligand components of the surface, such as capture molecules or the hydrogel matrix. Blank slide referencing is crucial as the generation of the SPR signal is solely dependent on mass accumulation at the surface: it does not distinguish between specific (analyte–ligand) and non-specific interactions. Blank slide referencing is achieved by injecting the analyte over a reference surface, which is prepared identically to the active surface with the exception of ligand immobilization or capture [36].

3.1.5 SPR Sensorgrams

3.1.5.1 The Langmuir Model of Protein-Protein Interactions

Plotting the signal in resonance units vs. time produces the characteristic graphic of SPR: the sensorgram (Figure 3-4). Sensorgrams can be used to elucidate the kinetic rate constants and thermodynamic equilibrium constants that describe the analyte–ligand interaction.

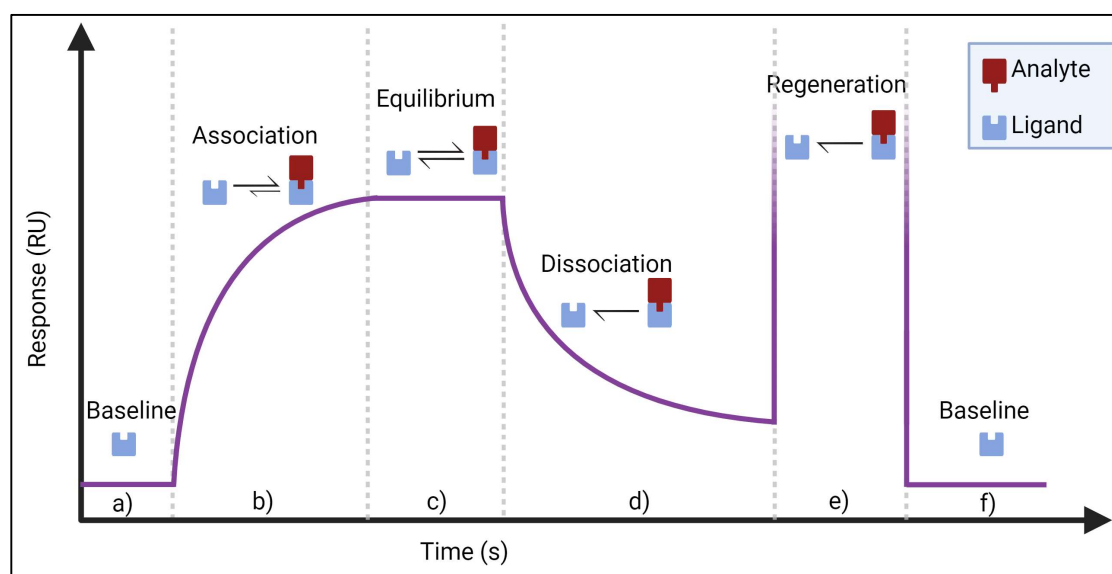


Figure 3-4 A model SPR sensorgram of a single experimental cycle, overlaid with schematics of the interaction dynamics in each phase. a) Baseline. b) Association. c) Equilibrium. d) Dissociation. e) Regeneration. f) Baseline.

The simplest kinetic model to describe ligand–ligand interactions is the Langmuir adsorption interaction model (Equation 3.1). This model describes a 1:1 interaction between an analyte protein (A) and a ligand protein (L) [37]. More complex interaction models, including multivalent ligands, heterogeneous analytes and biphasic interactions require unique characteristic

equations, and cannot be solved using this relatively simple approach [38]. However, the Langmuir adsorption model is the most useful for understanding the sensorgram.



Equation 3.1 describes an equilibrium between association (the forward reaction) and dissociation (the reverse reaction). Considering this equilibrium reaction, we can express Equation 3.2.

$$\frac{d[AL]}{dt} = k_{\text{on}}[A][L] - k_{\text{off}}[AL] \quad (3.2)$$

In Equation 3.2, we assign $[A]$ as the concentration of free analyte in solution, and $[L]$ as the concentration of free ligand at the surface. As the ligands and analytes interact, they form a complex whose concentration is described by the factor $[AL]$. The association and dissociation kinetic rate constants are respectively denoted as k_{on} and k_{off} . The signals generated from association is therefore equal to $k_{\text{on}}[A][L]$, whereas the signal generated by dissociation is equal to $k_{\text{off}}[AL]$. The rate of complex formation $d[AL]/dt$ is the slope of the SPR sensorgram. Apart from the regeneration phase, the slope of each phase can be described using Equation 3.2.

3.1.5.2 Baseline

In the first baseline phase (Figure 3-4a), only the ligand is present at the surface. The absolute value of the signal is still a function of the refractive index, though without analyte molecules in solution this simply reflects the composition of the experimental running buffer and the ligand immobilized at the surface. In practice, the baseline signal is normalized to zero. During baseline, both the analyte concentration ($[A]$) and analyte–ligand concentration ($[AL]$) are null, and therefore the slope remains flat (Equation 3.2a) [39].

$$\frac{d[AL]}{dt} = k_{\text{on}}[A][L] - k_{\text{off}}[AL] = 0 \quad (3.2)$$

$$[A] = 0, [AL] = 0$$

$$\frac{d[AL]}{dt} = 0 \quad (3.2a)$$

3.1.5.3 Association

In the association phase (Figure 3-4b), the analyte is injected over the surface and begins to associate with the immobilized ligand. During this phase, both association and dissociation occur, though association is greater since the concentration of the analyte–ligand complex ([AL]) is initially small. The slope is positive (Equation 3.2b) and will trend towards a plateau as the system approaches equilibrium [39].

$$\frac{d[AL]}{dt} = k_{on}[A][L] - k_{off}[AL] \quad (3.2)$$

$$k_{on}[A][L] > k_{off}[AL]$$

$$\frac{d[AL]}{dt} > 0 \quad (3.2b)$$

3.1.5.4 Equilibrium

If equilibrium is reached, the slope is null (Equation 3.2c) as the rates of association and dissociation are equal and opposite. In the graphical sensorgram, this appears as a plateau in the association phase (Figure 3-4c). This phase is critical for the determination of thermodynamic association constants [39]. The plateau represents an equilibrium, not a saturation of all the available ligand binding sites.

$$\frac{d[AL]}{dt} = k_{on}[A][L] - k_{off}[AL] \quad (3.2)$$

$$k_{on}[A][L] = k_{off}[AL]$$

$$\frac{d[AL]}{dt} = 0 \quad (3.2c)$$

3.1.5.5 Dissociation

During dissociation, RB is injected into the flow cell. With this disruption in the equilibrium ([A] drops to 0), analyte-ligand complexes dissociate and the newly liberated analyte are removed. The analyte concentration ([A]) is zero, resulting in a negative slope in the sensorgram (Equation 3.2d). The dissociation phase (Figure 3-4d) is used to determine the kinetic dissociation constant, k_{off} [40].

$$\frac{d[AL]}{dt} = k_{on}[0][L] - k_{off}[AL] \quad (3.2)$$

$$[A] = 0$$

$$\frac{d[AL]}{dt} = -k_{off}[AL] \quad (3.2)$$

$$\frac{d[AL]}{dt} < 0 \quad (3.2d)$$

3.1.5.6 Regeneration

The regeneration phase is the only portion of the SPR sensorgram that cannot be adequately described by the characteristic Equation 3.2. In the regeneration phase (Figure 3-4e), a solution designed to disrupt the analyte–ligand interaction is injected to ensure complete dissociation, returning the surface to baseline (Figure 3-4f) for the following cycle. Since regeneration solution usually has an ionic concentration (and therefore refractive index) that is significantly different from that of the experimental running buffer, a bulk shift artifact is produced. This signal artifact is completely independent of the analyte-ligand interaction [41]. While regeneration is useful for restoring the baseline, these harsh solutions can be damaging to the ligands at the surface and will diminish the bioactivity of the surface over time.

3.1.6 Mass Transport Limitation (MTL)

The flow cell is a microfluidic chamber that houses the SPR surface, and through which the running buffer and analyte solutions are injected. Taking a cross section of the flow cell, we can distinguish two separate layers: the surface and the bulk flow. The surface is the section of the flow cell within the ~300 nm detection limit, while the bulk flow is the remainder of the flow cell above this threshold. Only the refractive index of the surface layer contributes to signal generation [42].

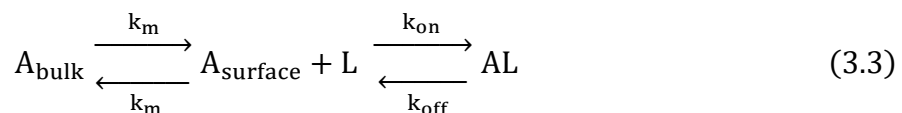
As analytes diffuse from the bulk flow to the surface and bind to the immobilized ligands during the association phase, their concentration in solution at the surface is depleted. In kinetically limited experiments, analytes from the bulk flow quickly diffuse to the surface to equilibrate the surface depletion. However, there are cases when the diffusion of analytes from bulk flow to surface is too slow to compensate and the concentration of free analytes at the surface becomes depleted. When this occurs, the diffusion of analytes from the bulk flow to the surface becomes the

rate limiting step in analyte–ligand complex formation, and the system suffers from a mass transport limitation (MTL) [42].

For kinetically limited interactions, we neglect the influence of mass transport and simply describe the interaction using Equation 3.1.



If we consider the diffusion of analytes from the bulk flow (A_{bulk}) to the surface (A_{surface}), we can modify our equilibrium accordingly (Equation 3.3).



When diffusion to the surface (left) is slower than the formation of the analyte ligand complex (right) the overall reaction is said to be mass transport limited. The rate of analyte diffusion from the bulk flow to the surface depends on the mass transfer coefficient (k_m) and the concentration (Equation 3.4) [43].

$$\frac{d[A_{\text{surface}}]}{dt} = k_m [A_{\text{bulk}}] \quad (3.4)$$

In MTL conditions, the association portion of the SPR sensorgram is linear (Figure 3-5a), with the slope being proportional to the analyte concentration in the bulk flow (Equation 3.4). Using a standard curve constructed using samples of known concentration (Figure 3-5b), one can accurately quantify samples of unknown concentration using the slope of their linear association rate, assuming their mass transport coefficient is the same as that of the standards [44].

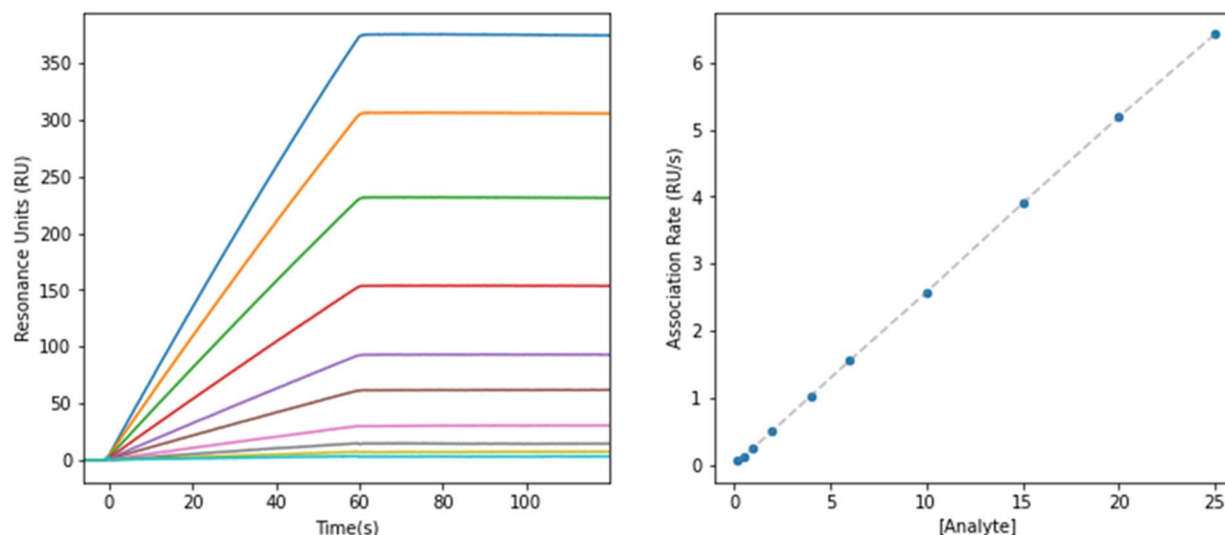


Figure 3-5 a) Mass-transport limited sensorgram used to construct a b) standard curve of association rate vs analyte concentration, useful for quantification using SPR.

3.1.7 Challenges in Surface Plasmon Resonance

3.1.7.1 Non-Specific Binding

One of the primary qualities of SPR biosensors is their capacity to detect bio-interactions without the need for molecular labelling. Rather than through labelling, signals are produced as mass accumulates in proximity to the sensor surface [13]. Due to signal generation being label free, the signal produced from analyte-ligand interactions cannot be distinguished from the non-specific signals.

When detecting analytes in crude samples, such as cell culture lysates or biological fluids, the complex mediums tend to display more non-specific binding to the sensor surface. When non-specific binding occurs on the reference surface, this signal is subtracted from the specific signal, producing a biased sensorgram which is of lower amplitude than one produced by a “clean” sample with the same analyte concentration [45]. The complexity of these mediums also presents sample-sample variability, which introduces more uncertainty with respect to the bias produced from non-specific binding.

Two approaches are available for the reduction of non-specific binding. Firstly, increased purification can be performed upstream of SPR detection to reduce the complexity of the medium. Secondly, low-fouling matrices such as polyethylene glycol self-assembled monolayers,

zwitterionic surface layers and biomimetic surface layers can be used to reduce protein absorption for detection of analytes in complex mediums [46]. From a bioprocess monitoring standpoint, low-fouling surfaces are an attractive option as these would enable detection in complex mediums without additional upstream sample preparation.

3.1.7.2 Ligand Bioactivity Loss

Between SPR detection cycles, it is often necessary to regenerate the cycle to ensure that all of the analyte molecules have been cleared from the surface before the next analyte injection. For ligands which are covalently coupled to the surface, regeneration occurs at the level of the ligand-analyte interaction. For captured ligands, regeneration instead removes the ligand, which must be recaptured before the next detection cycle [47].

With certain exceptions, regeneration solutions typically have high / low pH or extreme ionic concentrations, depending on the particular interaction to be regenerated. Harsh regeneration solutions can sometimes permanently annihilate the binding activity of ligands or capture ligands immobilized at the surface. Conversely, weak regeneration solutions will produce insufficient dissociation, resulting in carry-over from cycle to cycle [48]. In either case, poor regeneration conditions will result in inconsistent sensing surfaces and poor repeatability between replicates. Optimization of regeneration is a critical and often challenging step in the development of reliable assays. Due to the potential of ligand bioactivity loss, errors during optimization could also prove very costly.

Some SPR experiment configurations completely negate the use of regeneration solutions, therefore avoiding the associated risk of lost ligand bioactivity. Kinetic titration involves the sequential injection of higher and higher analyte concentrations, without regeneration steps between injections. Kinetic titration, sometimes called “single cycle kinetic”, configurations should be favored for ligands which are particularly difficult to regenerate or sensitive to bioactivity loss [49].

3.1.7.3 High Cost

Due to advanced liquid handling systems and high sensitivity, SPR biosensors require very small sample consumption for adequate detection. With respect to consuming chemical reagents and biological samples, SPR biosensors are highly cost-effective. However, the cost of SPR

biosensors themselves is high, as is the cost of the gold-coated biosensing chips. While good practices can extend the operational life of certain materials, the high cost of SPR biosensors, from a labware standpoint, is largely beyond the operator's control.

3.2 Glycobiology

3.2.1 Glycosylation

Glycosylation is a post-translational modification which vastly complexifies the human proteome. At the most fundamental level, glycosylation involves the attachment of oligosaccharide chains to proteins and lipids, transforming them into glycoproteins and glycolipids, respectively. Glycosylation is the result of the combined actions of glycosidase, glycotransferase and other enzymes which operate in the endoplasmic reticulum (ER) and Golgi. This cellular glycosylation machinery executes glycosylation in response to intracellular or extracellular signals [50].

Glycosylation plays many roles, which can be generalized to a few categories: structural and modulatory roles, intraspecies communication, and interspecies communication [1]. Structural and modulatory roles include any functions which contribute to the organization, stabilization or biophysical properties of protein structures. Glycan-dependent conformational modifications can modulate protein-protein interactions, though the glycan is not directly used in signaling [51]. Intraspecies communication involves glycan participating in signaling pathways on the protein or cellular scale [1]. Herein, the glycan chains are directly used in the signaling through interactions with carbohydrate binding proteins (CBP) [50]. Interspecies communication regards the recognition of pathogens, such as bacteria or viruses, through the specific recognition of pathogenic glycans considered to be pathogen associations molecular patterns (PAMP) and subsequent activation of the innate immune system [52]. Glycosylation-mediated interspecies communication can also be exploited by pathogens: Influenza viruses express their own carbohydrate binding proteins, which they use to bind to human glycoproteins to initiate host cell infection [53].

The nearly ubiquitous presence of glycosylation in human physiology has made it a valuable biomarker for disease. Aberrant glycosylation has been associated with chronic inflammatory disease and immune disorders, as glycans play a role in the regulation of immune cell differentiation [54]. Similarly, aberrant glycosylation has been associated to numerous cancers [55-57] including ovarian [58, 59], gastric [60], pancreatic [61], breast [62] and prostate [63].

As the role of glycosylation in disease has been expanded, many novel treatments based on the modulation of glycosylation pathways have been developed. Glycosylation engineering, via the modulation of cellular glycosylation pathways, is a promising avenue for antiviral treatments and has been proposed as a preventative measure against viral infections [64]. Modulation of glycosylation pathways is also being used for the glycan engineering of therapeutic proteins, most prominently monoclonal antibodies (mAb) [65].

3.2.1.1 N-Linked Glycosylation

N-linked glycosylation is so named because the oligosaccharide chain is linked to an Asparagine (Asn) amino acid residue via an N-glycosidic bond. More specifically, N-glycosylation can occur at sequences defined by Asn-X-Ser/Thr, where X is any amino acid except for proline. The presence of an amino acid other than Serine (Ser) or Threonine (Thr) at the third position is possible, but uncommon. All N-linked glycans share a common core sequence consisting of two linear N-Acetylglucosamine, followed by three mannose residues which form a fork. The remainder of the N-glycan structure depends heavily on the cell type and the role of the glycan [66].

3.2.1.2 O-Linked Glycosylation

O-linked glycosylation is so named as it is initiated by the formation of an O-glycosidic bond to the -OH groups present in the amino acid side chains of serine or threonine. O-linked glycans are less complex in structure than N-linked glycans, and all share a common core sugar: N-acetyl galactosamine [67]. O-glycosylation was previously described as mucin-type O-glycosylation. Mucin proteins are heavily O-glycosylated, and are key components of mucosal membranes, which play a role in the regulation of gas exchange, lubrication, adsorption, chemical sensing and resistance to pathogens at the luminal membranes. The O-glycosylation of mucins is crucial to regulating the biophysical and biochemical properties of mucosal membranes [68].

3.2.2 Lectins

Lectins are a broad class of sugar binding proteins that are specific to mono- or oligosaccharide structures found on glycoproteins and glycolipids. By binding to cell surface or circulating glycostructures via their carbohydrate recognition domains (CRD), lectins behave as the effectors of intra- and interspecies signaling. The unifying trait of lectins is their binding

specificity towards sugar structures; they are an otherwise highly diverse class of proteins. While animal and plant lectins differ widely in primary amino acid structure, they share similar tertiary protein structures, particularly in the CRD [69].

Lectins, by definition, do not have any catalytic or enzymatic properties, but instead behave only as signaling proteins through the specific recognition of mono- or oligosaccharides. The specific role of lectins depends largely on the organism, tissue or cell-type in which they are expressed. In bacteria, lectins act as mediators of cellular adhesion, allowing bacteria to aggregate onto host cells for infection, or to other bacteria for colonization [70]. In plants, lectin-signaling is active in macromolecule trafficking and the regulation of growth. Plant lectins also recognize exogenous glycans, important for immune defense as well as the regulation of plant-microorganism symbiosis [71].

In animals, lectins play a critical role in the innate immune system, acting as pattern recognition receptors (PRR). Immune lectins initiate responses when they recognize PAMPs, or if they encounter endogenous glycosylation signals associated with cellular distress. Essentially, any glycosylation patterns which differ from healthy or self-associated glycosylation will result in lectin-mediated immune reactions. Immune system lectins are expressed both on the cell-surface and in the bloodstream, playing a similar role to circulating antibodies [50]. In addition to pathogen recognition, lectins recognize aberrant glycosylation on-host cells, and participate in the initiation of apoptosis and the regulation of the cellular life cycle [69].

Lectins are widely used in bioresearch pertaining to protein glycosylation. Lectin-based methods are favored for glycoprotein characterization as they exhibit well-characterized specificity for mono- or oligo-saccharide structures, and do not usually require specialized equipment. Lectin-based glycosylation studies can usually be performed on unmodified glyco-analytes, whereas some other glyco-profiling techniques require chemical or enzymatic modifications for glycan characterization [72]. Lectins have also been widely used in affinity chromatography, to specifically purify proteins based on their glycosylation profile [73].

The affinity between a lectin CRD and a single monosaccharide is rather weak, with a thermodynamic dissociation constant (K_D) in the 10^{-3} M range. To overcome the low affinity, lectins often present as di-, tri- or tetrameric proteins with multiple CRDs. Alternatively, lectins are expressed at a high density on the membrane such that multivalency arises from interactions

with multiple monomeric lectins. Similarly, many glycan structures often present branched conformations, or are expressed at a high density on a given cell or tissue to increase the avidity of lectin-sugar interactions via multivalent binding [50]. This so-called “cluster effect” increases the apparent affinity of lectin-sugar interactions, bringing the apparent affinity constant ($K_{D,app}$) to the $10^{-7} - 10^{-8}$ M range [74] in some lectins. In many cases, lectins rely on multivalency to achieve biologically significant interaction strength, adding an additional degree of specificity, at the tissue level, to otherwise broadly specific proteins.

3.3 Influenza

Influenza viruses belong to the *Orthomyxoviridae* family. While there exist 4 types of influenza viruses (A, B, C and D), only Influenza A and B are a threat to humans. The infectious properties of Influenza A viruses (IAV) are governed by the activity of two major surface proteins, hemagglutinin (HA) and neuraminidase (NA), two proteins which both display a specificity for sialic acid terminated glycostructures expressed on the luminal surface of animal epithelial cells [75, 76]. It is the identity of the HA and NA proteins that give rise to Influenza A subtypes. For example, H1N1 is decorated with the subtypes H1 and N1.

In addition to many minor epidemics, Influenza has caused 3 major global pandemics since the 1918 “Spanish Flu”, an H1N1 outbreak. These occurred in 1957 (H2N2), 1968 (H3N2) and most recently in 2009 (H1N1) [77, 78].

3.3.1 Influenza Replication

As influenza enters an animal body, the viral lectin HA binds to sialic acid terminated glycans expressed on the luminal surface of respiratory or digestive tissues. The affinity of an HA monomer and a single sialic acid monosaccharide is weak, having a K_D in the mM- μ M range. Through trimerization in the quaternary structure, as well as a high density of HA trimers on the viral membrane, multivalent binding can bring the avidity of HA-sialic acid interactions into the low nM range in certain tissues [79]. The dependence on multivalency for physiologically relevant interaction strength gives Influenza virions specificity for the respiratory lumen in mammals or the enteric lumen in avian species [75].

HA binding to the surface of epithelial cells results in the virus being internalized into an endosome. Due to the low pH of the endosome, a conformational change occurs in the viral HA,

resulting in fusion of the viral membrane to the endosome membrane, and the release of viral contents into the cytoplasm. Genetic information, stored within viral ribonucleoprotein complexes consisting of viral RNA (vRNA) wrapped around multiple viral ribonucleoprotein (vRNP) units, is imported into the host-cell nucleus. Once in the nucleus, viral replication is achieved via the hijacking of host-cell transcription and translation machinery, initiated by viral polymerase (VP). Replicated viral proteins gather at the host cell membrane, where they bud off into newly replicated virions. To facilitate viral release from the infected host cell, NA cleaves sialic acid residues from the cell surface to prevent viral HA from rebinding [53, 80]. Since newly formed virions bud from the host cell membrane, the replicated viruses also contain several proteins originating from the host, though these are of relatively low abundance compared to viral proteins [81].

3.3.2 Antigenic Shift vs. Antigenic Drift

Antigenic shift arises from random mutations during replication, causes in a sudden change in the antigenicity of a circulating seasonal influenza strain. When this occurs, animal immune systems lack the adaptive immune preparations to respond to infection [53]. In some cases, changes in the structure of HA can cause avian Influenza strains to become infectious to mammalian cells, or vice-versa, via modifications in the specificity of HA's carbohydrate recognition domain. Usually, HA expressed in avian influenza strains are specific to sialic acids linked in an α 2-3 configuration, whereas human Influenza strains recognize α 2-6 linked sialic acids. These specificity shifts produce dangerous new Influenza strains with pandemic potential [75].

Influenza viruses also exhibit antigenic drift, small mutations that result from immune pressure. While slower and less drastic, antigenic drift results in novel strains that overcome the defenses of the adaptive immune system. Antigenic drift necessitates seasonal vaccinations, even if the dominant circulating strains do not necessarily change from year to year [53].

CHAPTER 4 ARTICLE 1: ANTIBODY-INDEPENDENT SURFACE PLASMON RESONANCE ASSAYS FOR INFLUENZA VACCINE QUALITY CONTROL

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4.1 Author Contributions

BS: Primary research and writing of the manuscript. AK: Conception of the review topic and critical reviewing of scientific content. GDC: Research supervision and critical reviewing of scientific content. OH: Research supervision, funding acquisition and critical reviewing of scientific content.

4.2 Keywords

Surface Plasmon Resonance, Influenza, Sialic acid, Hemagglutinin

4.3 Key Points

- Antigenic drift of Influenza's hemagglutinin protein necessitates the seasonal production of antibodies to be used in the single radial immunodiffusion (SRID) quantification assay.
- The delay in Influenza immunoassay availability is a major bottleneck in the Influenza vaccine pipeline.
- Surface plasmon resonance is a valuable alternative for the bioprocess monitoring of Influenza vaccine production.

4.4 Abstract

Surface plasmon resonance (SPR) based biosensors have emerged as a powerful platform for bioprocess monitoring due to their ability to detect bio-interactions in real-time, without the need for labelling. Paramount for the development of a robust detection platform is the immobilization of a ligand with high specificity and affinity for the in-solution species of interest. Following the 2009 H1N1 pandemic, much effort has been made towards the development of quality control platforms for Influenza A vaccine productions, many of which have employed SPR for detection. Due to the rapid antigenic drift of Influenza's principal surface protein, hemagglutinin, antibodies used for immunoassays need to be produced seasonally. The production of these antibodies represents a 6–8-week delay in immunoassay, and thus vaccine, availability. This review focuses on SPR based assays that do not rely on anti-HA antibodies for the detection, characterization and quantification of Influenza A in bio-productions and biological samples.

4.5 Introduction

Since the 1918 Influenza pandemic (H1N1 “Spanish Flu”), the Influenza virus has been responsible for 3 major pandemics, i.e, those of 1957 (H2N2 “Asian flu”), 1968 (H3N2 “Hong Kong flu”) and 2009 (H1N1 “Swine flu”) [77, 78]. Following the most recent Influenza pandemic in 2009, the World Health Organization (WHO) made a call for the improvement of vaccine production workflows. Despite major improvements on the production end, robust and rapid quality control methods are still lacking [82, 83]. Since 1979, the single radial immunodiffusion (SRID) assay has been the gold standard for the quantification of Influenza particles, due in part to its simplicity, reproducibility and high degree of specificity [84]. The SRID assay relies on antibodies to detect the primary antigenic protein, hemagglutinin (HA). However, due to the rapid antigenic drift of hemagglutinin's head domain, novel anti-HA antibodies need to be produced each season once the dominant strains are selected in anticipation of the next Influenza season. While vaccine production is unaffected, delays in SRID assay availability arising from lengthy antibody manufacturing process, represent a major bottleneck for Influenza vaccine distribution [85]. Following the 2009 H1N1 pandemic, the WHO and other regulatory health agencies identified the unavailability of SRID reagents as the primary cause of delayed vaccine distribution worldwide. In the same workshop, these agencies recommended improvements of the SRID assay, as well as the development of alternative methods for Influenza vaccine quality control [6].

Influenza refers to a group of viruses which all belong to the Orthomyxoviridae family. There exist 4 types of Influenza viruses, termed Influenza A, B, C and D, though only Influenza A and B pose a pathogenic threat to humans [80]. The infectious properties of Influenza viruses are governed by the activity of two major surface proteins, hemagglutinin (HA) and neuraminidase (NA), which both recognize sialic acid terminated glycostructures, expressed on the luminal surface of host epithelial tissue. Human Influenza strains preferentially bind to sialic acids linked in an α 2-6 “bent” conformation, whereas avian strains tend to bind to sialic acids linked in an α 2-3 “straight” conformation [75, 76]. Following viral entry and vRNA replication, the neuraminidase protein cleaves sialic acid residues to release newly replicated Influenza virions from the infected host cell [75, 80].

Influenza A viruses (IAV) are further subclassified into strains, which are named based on the identity of the HA and NA proteins, which in turn reflect genetic and antigenic properties. For example, the H1N1 IAV is decorated with H1 and N1 surface proteins. Other surface proteins include the matrix protein 2 (M2) [80], as well as proteins originating from the membrane of the host cell in which the Influenza virion replicated [81].

Since HA is the most abundant viral membrane bound protein, and the dominant antigen, the quantity of HA is the critical quality attribute of Influenza vaccine doses, and is standardized around 15 μ g/dose [85]. The amount of NA present for the standard 15 μ g/dose of hemagglutinin can vary widely, as different Influenza strains vary in their relative expression of HA and NA [86]. While Influenza vaccination does produce an increase in circulating anti-NA, which reduces disease severity, anti-NA do not inhibit viral infection as effectively as anti-HA antibodies [87]. In a randomly controlled trial, various commercially available inactivated Influenza vaccines were found to produce significant differences in circulating anti-NA for vaccinated subjects [88]. Due to its greater impact on viral immunogenicity, HA is favored as a target for the detection of Influenza by regulatory health agencies.

The strategies addressed in this review will be focused on the detection of fully expressed proteins, as these are the primary immunogenic structures used in inactivated Influenza vaccines (IIV), live attenuated Influenza vaccines (LAIV) and recombinant Influenza vaccines [89]. While effective mRNA based Influenza vaccines have been demonstrated in animal models [90], these will not be addressed in this review.

4.6 SPR Biosensors

SPR is a powerful tool for the detection of bio-interactions in real-time, without the need for a molecular label. Most popular SPR biosensor designs rely on Kretschmann geometry, developed in the late 1960s [91]. Since its commercialization in the 1980s, SPR has been widely used for the quantification of proteins and the kinetic evaluation of protein-protein interactions.

In the Kretschmann configuration, a sensor chip, consisting of a thin film of gold or silver, sits atop a prism. On the opposite side, the sensor surface, a dielectric medium provides an adequate milieu for bio-interactions, and also contributes to detection due to its specific optical properties [10]. During detection, an incident beam of light scans across the metal film from beneath the sensor surface and excites electrons from the metal into oscillation clouds known as plasmons. Due to plasmon generation, the light reflected off the sensor chip is of lower intensity. The angle of the reflected light beam at which the peak intensity dip occurs, is known as the SPR angle. The SPR angle is a function of the refractive index of the dielectric medium above the surface, and therefore of the concentration of matter in proximity (300nm) of the surface (Figure 4-1, top).

Onto the surface of an SPR sensor chip, a biomolecule referred to as the ligand is covalently immobilized or reversibly captured. During the association phase, a second biomolecule known as the analyte is injected into the flow cell. As analytes interact with ligands and mass accumulates at the surface, the refractive index of the dielectric medium changes, resulting in a change in the SPR angle, expressed in arbitrary resonance units (RU). Plotting RU versus time provides an insight on kinetics of biomolecular interactions between the surface-bound ligand and the injected analyte [11, 13, 92, 93] (Figure 4-1, bottom). Alternative signal generation methods and sensor chip designs, intended to increase sensitivity, are beyond the scope of this review [94].

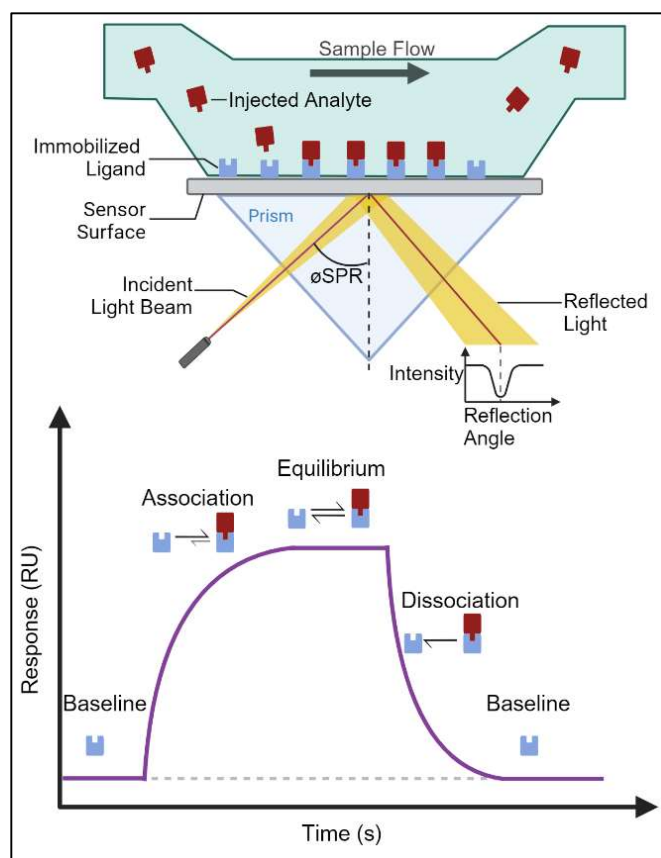


Figure 4-1 : Top: schematic representation of the flow cell, prism and light emission and detection system in the Kretschmann configuration. Bottom: phases of a SPR sensorgram, overlaid with the ligand-analyte interactions taking place.

Beyond these basic principles, each SPR experiment is designed to satisfy the experimenter's objectives. For any given ligand-analyte pair, the assay configuration, ligand immobilization method, ligand density, analyte flow rate, analysis temperature and reference surface design can be modified to accomplish the desired objectives. This review will focus specifically on the selection of SPR ligands for the detection of Influenza. The ligands presented can be separated into two broad classes. "Hook" ligands bind to Influenza via the ligand's binding domain (Figure 4-2, top), while "bait" ligands capture influenza by presenting a target for the viral surface protein hemagglutinin, mimicking the infection mechanism (Figure 4-2, bottom).

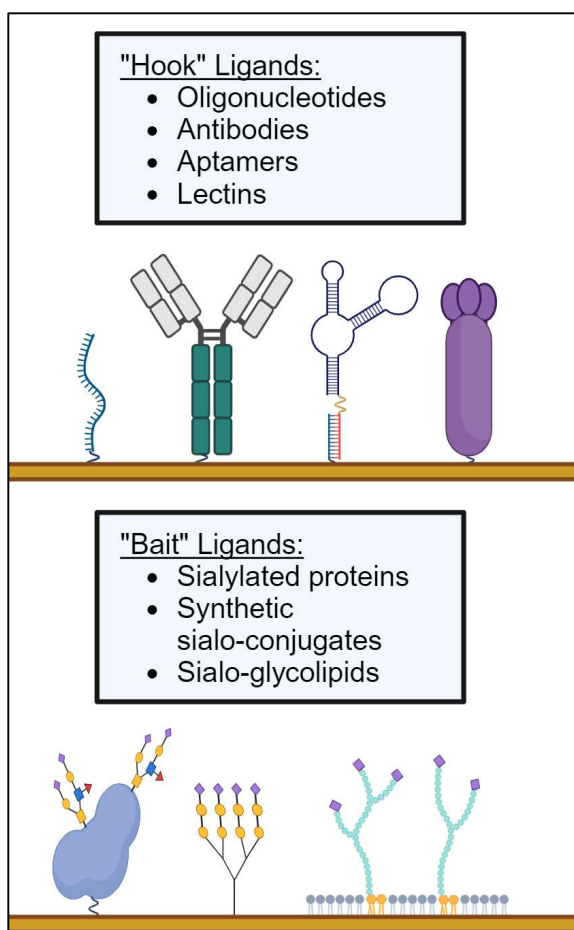


Figure 4-2 : Summary of "Hook" and "Bait" ligands suitable for the detection of Influenza using SPR biosensors.

4.7 Oligonucleotides

Oligonucleotides have been demonstrated to be an effective tool for the detection of Influenza in complex biological samples. The direct detection of viral RNA (vRNA) would solve the problem of ligand availability, as the isolation and amplification of vRNA could be achieved via a few rounds of reverse-transcription polymerase chain reaction (RT-PCR), whereas animal immunization and subsequent antibody proliferation is a lengthy process. In the vRNA approach, viruses are first lysed, and the viral RNA contained within are amplified by RT-PCR. The amplified vRNA serves as the analyte, whereas a complimentary RNA strands serves as the immobilized ligand. One intrinsic disadvantage of detecting oligonucleotides is their relatively low molecular weight. The SPR signal is proportional to the accumulation of mass at the sensor surface [13], and

thus interactions with low molecular weight analytes result in rather weak signal generation. To overcome this limitation, one approach is the inclusion of a tag, like biotin, to the terminus of the amplified vRNA product. Following the initial vRNA-RNA interaction, a molecule with specificity towards the tag, streptavidin in this example, can be injected to amplify the specific signal [95] (Figure 4-3).

Another disadvantage of vRNA based detection is that only live attenuated influenza vaccines (LAIV) containing complete vRNA sequences can be analyzed. Inactivated influenza vaccines (IIV) contain damaged vRNA, whose molecular weight as well as specificity for the immobilized oligonucleotide would be highly inconsistent, depending on the inactivation strategy employed. Recombinant vaccines are devoid of complete vRNA strands [96], and thus are unsuitable for this oligonucleotide-based approach.

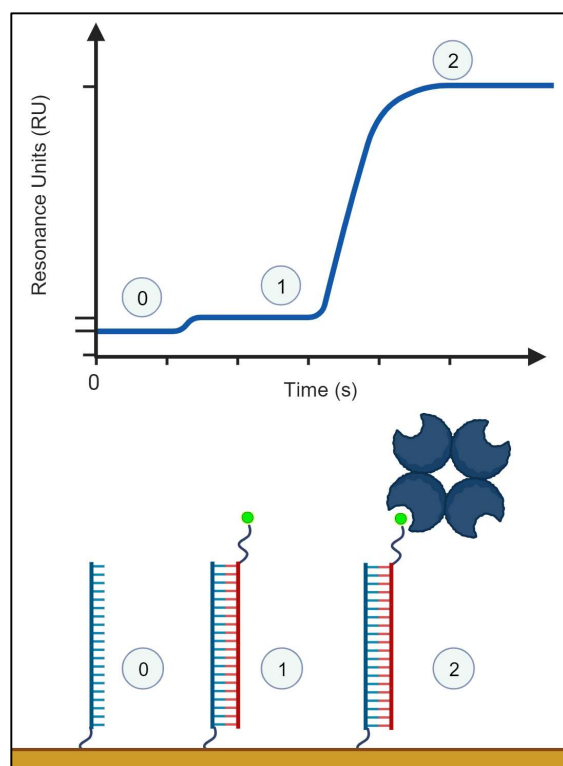


Figure 4-3 Schematic representation of signal amplification using a biotin tag to recruit a high molecular weight protein (e.g., streptavidin). (0): The baseline, where only the immobilized ligand is present at the surface. (1): Binding of the low molecular weight biotin-tagged analyte. Since the SPR signal is proportional to mass accumulated at the surface, the resulting signal is weak. (2):

Binding of a high molecular weight protein (streptavidin) to the biotin tag, resulting in an analyte-specific signal amplification.

4.8 Anti-HA2 Antibodies

While the globular HA1 “head” domain exhibits rapid antigenic drift, the HA2 “stalk” domain, which anchors HA to the viral envelope, is more conserved over time and across Influenza A subtypes (Figure 4-4). Immunization against the stalk domain would produce broad protection against numerous Influenza strains, and these antibodies last longer than a single Influenza season [53].

Anti-stalk domain antibodies could similarly be a valuable tool for detection of Influenza in biosensor assays. In one work, bioinformatics was used to identify and synthesize a peptide sequence in the HA2 stalk domain that is highly conserved across Influenza subtypes. Mice immunized against the peptide immunogen produced anti-HA2 antibodies that were specific for 39 of the 40 Influenza subtypes tested, which dated back to 1933 [97]. While a significant improvement over anti-HA1 domains, large mutations of the HA2 domains would introduce the same pipeline bottleneck that occurs seasonally with anti-HA1.

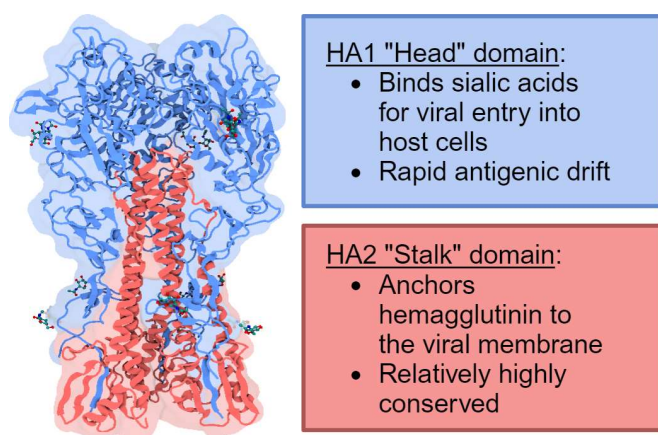


Figure 4-4 3D conformation of the homo-trimeric hemagglutinin protein from Influenza A H1N1. In blue, the HA1 “head” domain, susceptible to rapid antigenic drift and the target for immunodetection in the traditional SRID assay. In red, the HA2 “stalk” domain, whose structure is less susceptible to antigenic drift. Protein Data Base ID: 3HTO [98]

4.9 Aptamers

Aptamers are short, single stranded oligonucleotide sequences that adopt 3D conformations and can interact with biomolecules. Their specificity and affinity for their biological partners are on par with antibodies, with thermodynamic dissociation constants (K_D) on the nM-pM range, though they are far simpler and faster to produce. The conventional process of aptamer design is known as systematic evolution of ligands by exponential enrichment (SELEX). Herein, an initial oligonucleotide pool (IOP) consisting of nearly every nucleotide sequence of a given length (30-80bp), is incubated with a solid-phase target analyte. Due to competition for the analyte protein, the aptamers with the greatest affinity for the analyte will remain bound, while those with weaker or null interactions will be washed away. Following an elution step, the aptamers with high affinity will be amplified by PCR. This sequence of competitive binding followed by amplification continues until only a few aptamers remain, and their affinities for the analyte have reached the nM-pM range [99]. Using high affinity ligands is beneficial as it lowers the limit of detection for SPR assays [42]. SPR biosensors can even be used in the SELEX workflow thanks to their bio-functional sensor surfaces and precise liquid handling systems. In this setup, the SELEX target plays the role of the immobilized ligand in the SPR biosensor, whereas the aptamers are injected as analyte and compete for binding [100].

Due to the presence of amine functional groups in guanine, adenine and cytosine, traditional amine coupling via carbodiimide chemistry is unsuitable for aptamer immobilization to SPR surfaces. Rather, oriented immobilization strategies relying on specific interactions are favored. Two main strategies include the capture of biotinylated aptamers to streptavidin decorated surfaces [45, 101, 102], or capture of aptamers bearing a poly-adenine tail to a surface decorated with poly-thymine strands [22-24, 100].

SELEX derived aptamers have been employed in numerous SPR assays for the detection of influenza A [22, 24, 45, 100-102] and B [23]. HA-specific aptamers have displayed superior specificity to antibodies when it comes to differentiating between IAV subtypes [102], and have been used for the detection of Influenza virions in complex biological samples [45]. Their binding affinity for HA is on par with antibodies and can be further increased through chemical modifications. In one work, aptamers constructed using 2'-fluoropyrimidine in place of pyrimidine

base pairs displayed a thermodynamic dissociation constant (K_D) on the femtomolar scale ($K_D \sim 10^{-14}$ M) [22].

One of the most interesting approaches to aptamer selection is negative SELEX, or counter SELEX. In this approach, the aptamers are first incubated with the non-target contaminants that may be present in the final sample (ex. host-cell proteins). The aptamers which display no specificity for these non-targets are then collected and then used for a conventional SELEX-based aptamer selection. The result is a highly selective aptamer that can distinguish between different IAV subtypes [101], or even within the same subtype from different seasons [22, 24].

4.10 Lectins

Lectins are sugar-binding proteins that are biological antagonists to glycans, primarily playing a role in cellular signaling and adhesion via interactions between sugar structures and the lectin's carbohydrate recognition domain (CRD) [50]. Protein glycosylation is achieved via the combined actions of host-cell glycotransferases and glycosidases [2], and thus the glycosylation patterns will depend largely on the cell lines and culture conditions in which the viruses replicate [103]. In turn, identical Influenza strains will bind differently to identical lectin surfaces depending on the production platform [104]. Further, due to inter-lot variability of HA glycosylation, as well the presence of multiple glycans within the same glycostructure, multiple lectin candidates can viably act as ligands for the same Influenza batch [105].

The main advantage of lectins is their indifference towards the antigenic drift of the HA head domain, though the variability in viral glycosylation is not insignificant. For reliable detection using lectins, strict control of culture conditions and medium components is required. Host-cell gene modification or addition of glycosylation inhibitors could be used to further control glycosylation and improve consistency from batch to batch [106]. Control of glycosylation in vaccine production could be a valuable research avenue, as HA glycosylation has been demonstrated to play an important role in protein folding, stability, pathogenicity and affinity for sialic acid decorated glycostructures. Certain HA glycosylation patterns are also associated with increased innate immune response from an infected host [107]. However, the broad specificity of lectins towards any correctly glycosylated macromolecule makes them a poor choice for detection in complex media, such as cell culture supernatants or biological samples.

4.11 Sialic Acid Decorated Surfaces

The most common ligand choice for the detection of Influenza is a sialic acid decorated surface. In this approach, sialic acid residues serve as “bait” for viral HA proteins and present a surface that mimics the natural infection mechanism.

The affinity between HA and a single sialic acid residue is in the mM- μ M range. Due to the trivalency of HA, as well as high density of HA on the viral membrane [108], Influenza virions display multivalent binding, increasing the affinity to the low nM range [79]. Additionally, IAVs display a preference for tissues which express a high density of sialic acid terminated glycostructures such as the respiratory lumen in humans and swine, or the enteric lumen in avian species [75]. This “cluster effect”, wherein multivalency increases apparent affinity of lectin-sugar interactions, is a feature of most animal, plant and viral lectins [50, 109]. To exploit this cluster effect, sialic acid tends to be immobilized at a high density on SPR surfaces. One major disadvantage is that sialic acid decorated surfaces are susceptible to degradation by neuraminidase, which cleaves sialic acids from host surfaces during influenza replication.

Occasionally, avian Influenza strains acquire specificity for α 2-6 linked sialic acid residues. The sudden shift introduces highly pathogenic Influenza strains to the human immune system [77, 110]. Sialic acid terminated ligands are effective ligands for the detection of avian Influenza strains that have acquired interspecies infectivity, as was the case for the 2009 H1N1 “Swine Flu” pandemic [111].

4.11.1 Sialylated Glycoproteins

Sialic acid terminated glycans fall under the category of “complex type” glycans, which tend to be branched and are expressed only in higher order organisms, such as vertebrates [112]. This branching, in combination with the potential for multiple glycosylation sites on a single protein, contributes towards the cluster effect. For example, the sialylated human serum glycoprotein protein α 1-AGP, bearing 5 potential N-glycosylation sites, was successfully used to detect HA from a human Influenza strain [105].

In another work, bovine serum fetuin, a glycoprotein bearing both α 2-3 and α 2-6 linked sialic acid residues, was used to quantify the HA present in a vaccine lot. More impressively, this same surface design was selective enough to detect H1N1 in a clarified cell supernatant. In the

same work, the authors reported no binding between immobilized fetuin and monomeric recombinant HA, likely due to the absence of avidity binding. An adequate reference surface was prepared with asialofetuin, to subtract the signals arising from non-specific interactions [113].

4.11.2 Synthetic Glyco-Conjugates

Synthetic sialylated ligands are highly versatile tools, as they can be precisely designed to accommodate experimental requirements. High avidity interactions can be ensured via branching, such that a single synthetic ligand contains multiple terminal sialic acids [111, 114]. These synthetic ligands can also be chemically conjugated to capture tags, like biotin, for immobilization to an SPR surface.

Another approach for exploiting avidity effects is chemical conjugation of synthetic glycans to a carrier protein. The conjugation ratio of glycan to carrier protein can be used to fine-tune the apparent affinity of the Influenza-sialoglycan interaction. Low to moderate affinity ligands are an attractive choice for in-line applications, as they would dissociate quickly and would not require surface regeneration, in turn increasing the lifespan of the SPR surface [105].

The synthetic approach provides the ability to control the attachment orientation of the terminal sialic acid, for the detection of avian or human Influenza strains. One such strategy involved selective use of either α 2-3-sialyltransferase or α 2-6-sialyltransferase to control the linkage of sialic acid to the subterminal galactose residue [115]. Alternatively, linkages for which no Influenza strains display specificity, like α 2-8 attachment, can be produced and used as reference ligands to construct “blank slide” control surfaces [116].

4.11.3 Sialoglycolipids

To even further emulate physiological conditions, sialoglycan decorated lipid monolayers can be immobilized to hydrophobic sensor chips. In one work, naturally sialylated bovine brain lipid neomembranes were immobilized to a SPR sensor chip. While the surface successfully interacted with injected IAV, the sialoglycolipids were highly susceptible to degradation neuraminidase, giving rise to baseline drifts over the course of repeated injection cycles [32].

4.12 Perspectives / Conclusions

The development of novel, antibody independent Influenza quantification assays is critical for the improvement of the vaccine distribution pipeline, and will reduce the time to vaccine availability by weeks in a pandemic scenario. Among the alternative strategies proposed by regulatory health agencies following the most recent Influenza pandemic, SPR biosensors have already been demonstrated to be powerful tools for in-line and off-line monitoring of biomanufacturing. To adequately replace SRID, SPR assays for influenza detection will require highly selective, high affinity ligands immobilized to the sensor surface. Crucially, these ligands must either be rapidly produced once a dominant Influenza strain has been identified, or must target structures other than the HA1 “head” domain and therefore be independent of Influenza’s rapid antigenic drift. While not discussed in this review, functional SPR surfaces for Influenza quantification should also be resistant to regeneration solutions. For in-line applications, surface designs should also consider reduction of non-specific binding from contaminants present in complex solutions such as cell culture medium. Additionally, SPR quality control assays should be aimed at multiple stages of the vaccine production pipeline, from crude in-line samples to final vaccine formulations in the presence of adjuvants.

4.13 Statements and Declarations

This work was supported by the Fonds de Recherche Nature et Technologies (FRQNT), grant number PR-300890, the Natural Sciences and Engineering Research Council of Canada (stipends allocated to Benjamin Serafin via the NSERC-CREATE PrEEmiuM program) (NanoBio Technology Platform) and its main funding partner, the Canada First Research Excellence Fund. The authors declare no competing interests that are relevant to the content of this article.

4.14 Ethical Statement

This article does not contain any experiments with animals or human participants performed by any of the authors.

CHAPTER 5 ARTICLE 2: Impact of Lectin Biotinylation for Surface Plasmon Resonance and Enzyme-Linked Lectin Assays for Protein Glycosylation

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5.1 Author Contributions

Benjamin Serafin: conceptualization, methodology, investigation, formal analysis, writing, reviewing, and editing original draft. **Amine Kamen:** reviewing and editing the original draft. **Olivier Henry:** funding acquisition, supervision, reviewing and editing the original draft. **Gregory De Crescenzo:** funding acquisition, conceptualization, project administration, supervision, reviewing and editing the original draft.

5.2 Declaration of Interests

None

5.3 Statements

This work was supported by the Fonds de Recherche Nature et Technologies (FRQNT), grant number PR-300890, the Natural Sciences and Engineering Research Council of Canada (stipends allocated to Benjamin Serafin via the NSERC-CREATE PrEEmiuM program) (NanoBio Technology Platform) and its main funding partner, the Canada First Research Excellence Fund.

5.4 Subject Category

Enzymatic assays and analysis

5.5 Highlights

- High lectin biotinylation reduces capture to streptavidin SPR surfaces
- High lectin biotinylation affects glycoprotein-lectin binding affinity
- High lectin biotinylation effectively amplifies ELLA signals

5.6 Keywords

Lectin, Glycosylation, Biotin, Streptavidin, Surface Plasmon Resonance, ELLA

5.7 Graphical Abstract

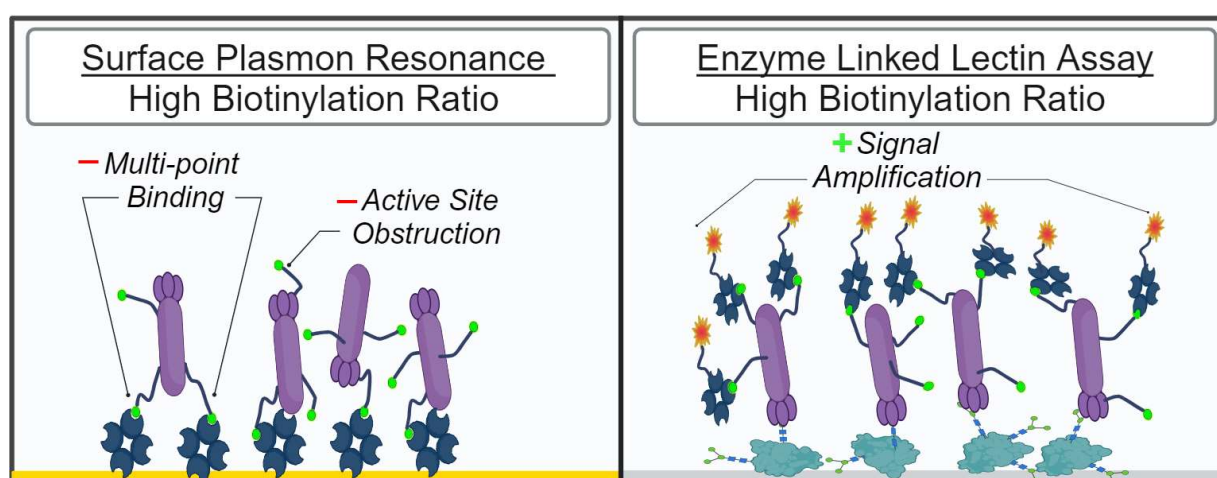


Figure 5-1 Graphical abstract depicting the impact of a high biotinylation ratio on the performance of lectins in protein glycosylation assay.

5.8 Abstract

Protein glycosylation is an abundant post-translational modification that plays a critical role in the immune system and is a biomarker for disease. Lectins are widely employed for the assessment of protein glycosylation as their carbohydrate binding specificities have been well characterized. It is common in glycosylation assays to covalently conjugate lectins with biotin tags, which serve to capture lectins to streptavidin decorated biosensing surfaces or recruit streptavidin-based signal generating molecules, depending on the assay configuration. The degree of biotin conjugation to lectins is rarely explored as an avenue for glycosylation assay improvement. We here demonstrate that biotin conjugation levels modulate the capture of lectins to streptavidin surfaces while also influencing the affinity of lectin for glycoproteins. The combination of these

effects of biotinylation in turn results in different biotinylation ratios being optimal for biosensor and enzyme linked-lectin assay. Finally, we present an experimental workflow for the determination of optimal biotinylation conditions for lectins, depending on the protein glycosylation assays they are used for.

5.9 Introduction

Glycosylation is a form of post-translational modification that occurs in all eukaryotic cells, and involves the attachment of polysaccharide chains, via the sequential addition of monosaccharides, to a protein. Glycosylation is not encoded by a protein's primary structure, but is instead the product of cellular glycosylation enzymes, which function in the endoplasmic reticulum (ER) and the Golgi apparatus [2]. Glycans play many biological roles, participating in glycoprotein folding, water solubility, protease resistance, in addition to cell-cell signaling, adhesion, trafficking, immune system activation, nutritional storage and more [1].

The monitoring of glycosylation is essential in the quality control of therapeutic proteins, especially in the case of monoclonal antibodies (mAbs). The identity of the glycans added to the mAbs impacts the overall conformation of the Fc domain, the affinity of their interactions with Fc receptors [51, 65] and therefore the effectiveness of antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) pathways [117]. Antibody glycosylation also affects their circulating half-life [3], and their potential to elicit adverse immune responses [3, 106].

Due to their well-characterized sugar specificities, lectins are often used for the evaluation of protein glycosylation. Lectins are a class of non-catalytic sugar-binding proteins which participate in cell-cell signaling, adhesion and protein trafficking. In the immune system, lectins participate in pathogen recognition, regulation of the immune response and activation of the apoptotic pathway [50]. Lectins recognize glycans via their carbohydrate recognition domains (CRD). The selectivity of CRDs varies widely from lectin to lectin, with some showing rather broad range (specific to monosaccharides) and others being more narrow (specific to oligosaccharide chains with a defined sequence and specific linkages).

Lectins are employed in the enzyme linked-lectin assay (ELLA), a protein glycosylation assay derived from the enzyme linked-immunosorbent assay (ELISA). The ELLA does not require chemical or enzymatic modifications to the glycoproteins prior to analysis, and does not require

any specialized equipment [118]. In ELLA assays, where the lectin replaces the primary antibody, it is common to conjugate the lectins with a universal label such as biotin, rather than use a specifically designed secondary antibody for each lectin in the assay [118, 119].

Another common approach for characterizing protein glycosylation employs surface plasmon resonance (SPR) biosensors. SPR biosensors are a powerful tool for the study of biomolecular interactions, as they provide information on the binding between two biomolecules in real-time, without the need for any label. Briefly, a biomolecule termed the ligand is covalently immobilized or reversibly captured to the sensor surface. A second biomolecule termed the analyte is then injected onto the surface and the binding of the analyte to the ligand is recorded in real-time to produce a sensorgram. SPR sensorgrams can be used to evaluate the affinity and kinetic constants of the ligand-analyte interaction, or for the determination of analyte concentration, depending on the conditions of the assay [13]. SPR has been widely employed to assess glycosylation, with lectins acting either as the immobilized ligand [105, 120-124] or the injected analyte [125-128]. A common approach to the immobilization of lectins is the capture of biotinylated lectins to streptavidin-decorated surfaces.

For use in ELLA and many SPR assays, lectins are conjugated with biotin via covalent coupling. Covalent coupling of biotins to lectins is achieved via several chemical reactions, which can target amine, carboxyl or thiol functional groups present in amino acid side chains. Beyond selecting the target functional group, the chemical conjugation of biotin to proteins is random. Considerations should be taken to avoid coupling a biotin tag proximal to a protein's binding domain, as this could sterically hinder interactions with a second protein. The position of the biotin tag can be tightly controlled via the addition of an engineered biotin acceptor peptide (BAP) within the protein for BirA-mediated enzymatic conjugation [20]. Alternatively, cysteine residues [19] or aldehyde bearing amino acids [129] can be genetically engineered at specific locations in a protein to act as chemical handles for site-specific conjugation. While viable, these options are considerably more expensive, difficult and time consuming than chemical conjugation to naturally present side chains.

The biotin conjugation ratio plays an important role in the ELLA and SPR glycosylation assays. The ELLA uses streptavidin-HRP to produce a signal, and thus a high biotin to lectin ratio may amplify the signal strength for a given glycoprotein:lectin interaction (Figure 5-2). In SPR,

ligands with high biotinylation ratios achieve lower overall immobilization levels on streptavidin surfaces due to multipoint binding [130]. Additionally, a high biotin ratio at incubation is more likely to tag the lectins near their carbohydrate recognition domain, which can sterically hinder interactions or modify binding kinetics (Figure 5-2). The biotin conjugation ratio of commercially available lectins is not provided by the manufacturers; it is thus impossible to estimate its impact upon assay performance. Conjugating lectins in-house would enable optimization of an otherwise uncontrolled variable.

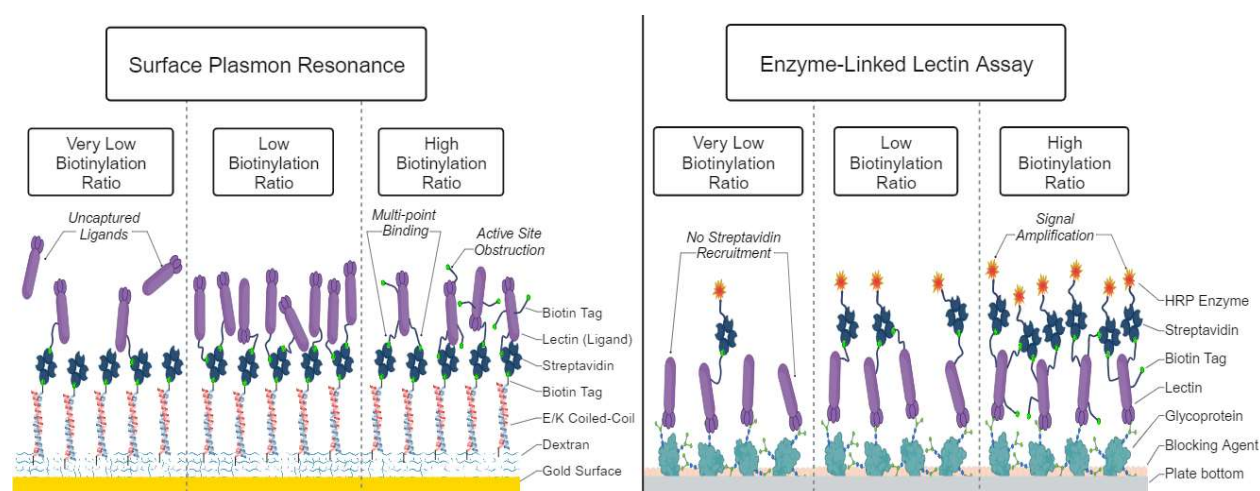


Figure 5-2 Schematic illustration of the impact of the lectin biotinylation ratio on the performance of protein glycosylation assays using a surface plasmon resonance biosensor (left) or the enzyme linked lectin assay (right).

5.10 Materials

4mL-capacity Amicon centrifugal filter units (molecular weight cut-off = 30 kDa) Im(#UFC8030), bicinchoninic acid (BCA) assay kit (#71285-3), phosphate buffered saline packs (#P5368), $MgCl_2$ (#M8266), $Mn(II)Cl_2$ (#244589), Tween 20 (#P7949), streptavidin (#189730), sulfuric acid (#258105) and TMB substrate (#T0440) were all purchased from Millipore Sigma. HEPES (#329850500) and $CaCl_2$ (#033296-A3) were purchased from Thermofisher. Streptavidin-HRP (#DY998) was purchased from R&D Systems. All lectins used in this study (LCA: #21510006, MAL-2: #21511098, SNA: #21510104) were purchased unconjugated from GlycoMatrix. The biotinylation reagent NHS-dPEG4-biotin was purchased from Quanta Biodesign Ltd. (#10200). Cysteine-tagged Kcoil and biotin-tagged Ecoil peptides (peptide sequences being

CGG-[KVSALKE]₅ and CGG-[EVSALEK]₅, respectively) were chemically synthesized at the University of Colorado's peptide facility [131]. Ecoils were biotinylated at their terminal cysteine residue using biotin-maleimide, as described previously [132]. The Biacore T200 SPR biosensor, thiol coupling kit (#BR100557), HBS-EP buffer solution (#BR100669) and CM5 Series S sensor chips (#29149603) were purchased from Cytiva.

5.11 Methods

5.11.1 Lectin Conjugation

Lectins were chemically conjugated using NHS-PEG4-biotin at a range of molar ratios: 0, 0.3, 1, 3, 10 and 30 mol of NHS-dPEG4-biotin per mol of lectin, diluted in 10 mM PBS (amine-free buffer solution). Lectins were incubated with the reagent for 30 min at room temperature (RT). Excess unreacted NHS-dPEG4-biotin (589 Da) was removed via filter centrifugation (cut off = 30 kDa) for 5 cycles of 15 min at $3500 \times g$. The designation of the biotinylated lectins used throughout the study (R0, R0.3, R1, R3, R10 and R30) are derived from these incubation ratios.

Biotinylated lectins were quantified via the bicinchoninic acid (BCA) assay, using bovine serum albumin (BSA) and unconjugated lectin standards to construct a standard curve. The concentrations of all biotinylated lectins were thus adjusted for the remainder of the work, such that the only variable at play is the relative degree of biotin conjugation.

5.11.2 Relative Biotinylation Assay

The relative biotinylation assay was used to demonstrate the degree of lectin biotinylation resulting from each molar incubation ratio. Biotinylated lectins in PBS were directly incubated at 500 pM in a 96-well, flat-bottomed microplate. Traditional BSA blocking solutions could not be employed, due to the potential presence of contaminant glycoproteins within the BSA solution [118]. Instead, a protein-free blocking solution of 10 mM PBS containing 0.5% v/v Tween (PBS-T) was used for all microplate assays [133]. Following the blocking step, the biotinylated lectins were detected using streptavidin-HRP and a TMB substrate solution, according to the manufacturer's instructions, and absorbance was measured at 450 nm. Absorbance levels were compared using a 1-tailed Student's t-test ($\alpha=0.05$). Since the lectins were all incubated at the same concentration, the relative signal strengths are determined only by the biotin conjugation ratio.

5.11.3 ELLA Assay

ELLA assays adopted the “direct” configuration, as reported in [118]. Model glycoproteins (rituximab for LCA, fetuin for MAL-II and SNA), for which the lectins have a well-documented specificity, were incubated overnight in the plate wells, which were then blocked with PBS-T. Biotinylated lectins diluted in a cation-containing buffer solution (10 mM HEPES buffer, pH 7.4, containing 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM MnCl₂ and 0.05% Tween20) (HBS-I) were then added. Divalent cations such as Ca²⁺, Mg²⁺, Mn²⁺ and Zn²⁺ play an important role in lectin glycan binding through stabilization of the lectin CRD [4], and were therefore included in the buffer. To avoid signal saturation from an excessive number of biotins in the upper ranges of the assay, the concentration of Streptavidin-HRP was increased 2-fold from the manufacturer’s instructions. Absorbance was again measured at 450 nm. Blank signals were obtained from wells containing no glycoprotein, which were otherwise treated the same. Each standard curve contained a blank signal produced with the appropriate lectin, which was subtracted from the other points in the curve.

The reported Limit of Detection (LOD) for each biotinylation ratio was calculated according to standard IUPAC guidelines (Equation 6.1). The slope and blank were unique to each conjugation ratio. LOD were compared using a 1-tailed Student’s t-test ($\alpha=0.05$).

$$\text{LOD}_{\text{Lectin}} = \frac{\text{Blank} + (3.3 \times \text{SD}_{\text{Blank}})}{\text{Slope}} \quad (6.1)$$

5.11.4 Contrsuction of Multi-Capture SPR Surface

Regeneratable Kcoil decorated SPR sensor chips were constructed via thiol coupling, as previously reported [28]. Approximately 1500 RU of cysteine-bearing Kcoil were covalently immobilized to both the active and reference flow cells.

Regeneratable streptavidin surfaces were produced anew at the start of each cycle using a multi-capture approach, ultimately anchored to the surface via the thiol coupled Kcoil [132]. First, 30 nM Ecoil-biotin was injected for 180 s and captured (~130 RU) by surface-bound Kcoil. Second, 250 nM streptavidin was injected for 30 s and captured (~500 RU) via its interaction with the biotin of the previously captured Ecoil. Third, biotinylated lectins were captured by the previously captured streptavidin (Figure 5-3, left panel). At the end of the detection cycle, regeneration was

achieved via five repeated pulses of 6 M guanidium-HCl, each lasting 30 s, to dissociate the stable and high-affinity Ecoil-Kcoil complex [27]. Following regeneration, only the Kcoil remained bound to the surface, and was available to capture biotinylated Ecoil for the next cycle. All injections were performed at 10 $\mu\text{L}/\text{min}$, aside from guanidium-HCl which was injected at 100 $\mu\text{L}/\text{min}$. Via targeted regeneration at the site of the E/K coiled-coil interaction, we constructed over 300 such streptavidin surfaces to evaluate the capture of biotinylated lectin ligands.

Streptavidin and lectins were diluted in HBS-I, which served as the running buffer for all SPR experiments. To avoid aggregation via the formation of chelating ion bonds between negatively charged amino acid residues, the Ecoil-biotin was diluted in a buffer devoid of the divalent cation salts (i.e. HBS-EP).

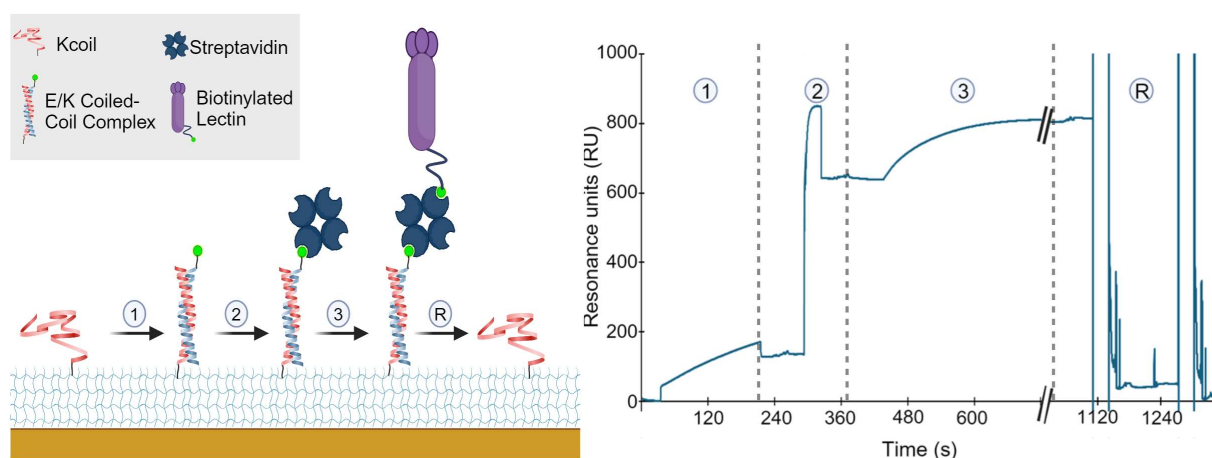


Figure 5-3 : Left: Schematic representation of the multi-capture streptavidin surface. Right: The associated sensorgram. At the beginning of the cycle, only the thiol coupled Kcoil is present at the surface. (1) Biotinylated Ecoil capture via coiled-coil interactions. (2) Streptavidin capture by the exposed biotin. (3) Capture of a biotinylated lectin by streptavidin, which acts as a linker for biotinylated biomolecules due to its homo-tetrameric quaternary structure. (R) Regeneration at the level of the coiled-coil interaction, leaving behind the thiol coupled Kcoil for the next capture cycle.

5.11.5 Influence of Biotinylation on Capture Kinetics

To evaluate how the biotin:lectin ratio affects the biotinylated lectin capture to a streptavidin surface, 100 nM of biotinylated lectins were injected onto a streptavidin surface for 8 min at 10 $\mu\text{L}/\text{min}$. Due to the very low dissociation rate of the streptavidin:biotin complex, the dissociation of the captured lectin is virtually null, especially for high conjugation ratios where

rebinding is more likely to occur. All SPR sensorgrams were double referenced [13]: blank samples contained no lectins, whereas blank surfaces corresponded to surfaces where streptavidin was absent.

The lectin capture rate was derived from the quasi-linear initial portion of the association phase. Its slope, expressed in RU s^{-1} , was then divided by the concentration of the biotinylated lectin, leading to an initial capture rate expressed in $\text{RU s}^{-1} \text{M}^{-1}$.

5.11.6 Influence of Biotinylation on Binding Kinetics

Kinetic titration experiments were performed to determine the impact of biotinylation on lectin-glycoprotein interactions. Herein, biotinylated lectins were immobilized to the biosensor surface via the multi-capture surface described previously. The model glycoproteins were injected at $10 \mu\text{L/min}$ for 300 s at four increasing concentrations (long enough to reach equilibrium for each injection). Regeneration then followed. Again, all SPR signals were double referenced, though in this case blank samples contained no glycoproteins, and blank surfaces had no lectin captured.

Kinetic titration sensorgrams were analysed by Scatchard analysis to estimate the thermodynamic association constant [74]. To reduce bias induced by experimental noise ($0.5\text{-}1.5 \text{ RU}$), the steady state value (R_{eq}) was calculated by taking an average of the signal at equilibrium (Figure 5-4).

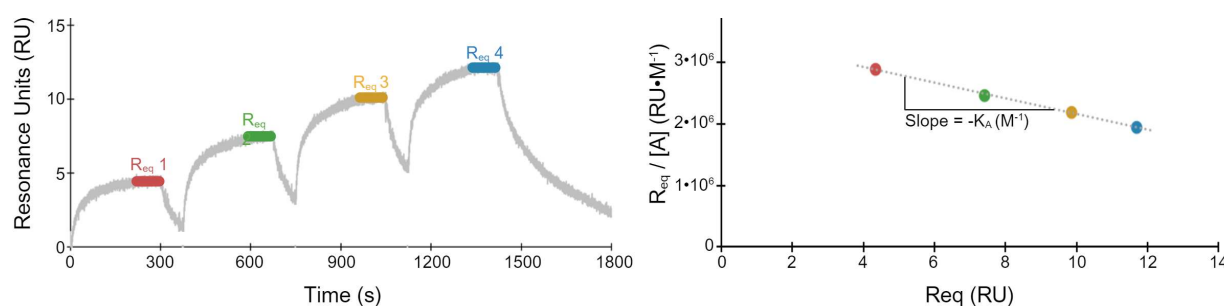


Figure 5-4 Titration protocol for the interaction between immobilized MAL-II (R1) and injected fetuin. Titration sensorgram (Left panel) corresponds to fetuin injections at $1.5 \mu\text{M}$, $3 \mu\text{M}$, $4.5 \mu\text{M}$ and $6 \mu\text{M}$. The signal at equilibrium (R_{eq}) is used to construct the Scatchard plot (Right panel).

5.12 Results and Discussion

5.12.1 Biotin Conjugation Levels produced by different Incubation Conditions

Lectins were incubated at a range of molar ratios with biotin-PEG4-NHS, including 0, 0.3, 1, 3, 10 and 30 moles of biotin reagent per mole lectin. To evaluate the degree of biotin conjugation at the different molar incubation ratios, the lectins were adsorbed in multi well plates and their biotin tags were detected using streptavidin-HRP. In Figure 5-5, we report the relative biotinylation levels (in arbitrary absorbance units) for the three lectins (LCA, MAL-II and SNA), at each biotin reagent-to-lectin molar ratio.

For all three lectins, increasing the incubation ratio produced a higher final biotin conjugation level, as evidenced by a greater absorbance signal. Each threefold increase in conjugation resulted in a statistically significant increase in absorbance, as measured using streptavidin-HRP. The only exception was between R0.3 and R1 for the SNA lectin. Since all lectins were incubated at the same concentration, the only variable which contributed to the signal amplification was the number of biotin tags conjugated to the lectins.

While not the subject of the study, we also observed that the absolute signal produced by the different lectins varied. At the highest incubation ratio R30, the signal from SNA is 3.8-fold greater than that from LCA and 1.7-fold greater than that from MAL-II for the same incubation conditions. According to available primary sequence data, the three lectins contain a similar number of lysine residues (potential conjugation sites) per unit (LCA: 26 [134], MAL-II: 28 [135] and SNA: 26 [136]). The observed differences in signal amplitude more likely results from differences in lysine side chain availability within each lectin quaternary structure.

The relative biotinylation assay presented here is thus a measure of functional biotinylation. It represents how biotin conjugation would influence streptavidin interactions in our analytical assays, SPR and ELLA.

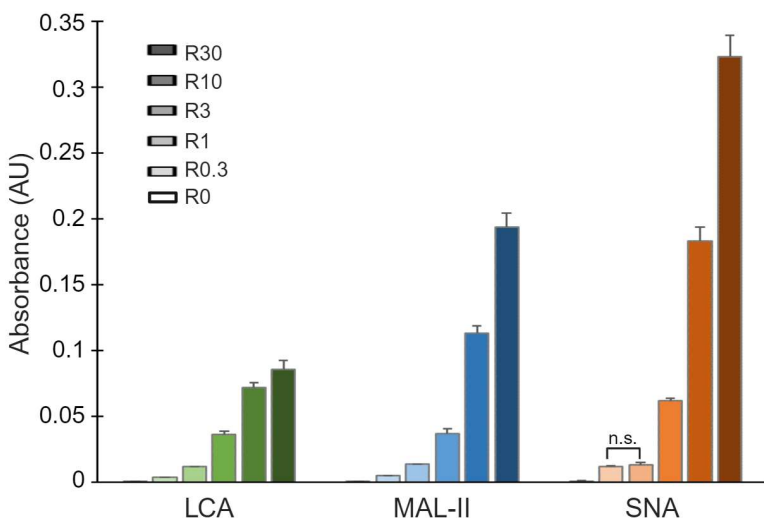


Figure 5-5 Qualitative display of the relative biotin conjugation level produced by the various incubation conditions. For all three lectins, increasing the biotin-to-lectin molar ratio upon incubation produced significantly greater absorbance values, with the exception of R0.3 and R1 for SNA ($\alpha=0.05$).

5.12.2 Biotin Conjugation Ratio impacts the Kinetics of Capture to Streptavidin Surfaces

To evaluate the influence of the conjugation ratio on the capture kinetics, we injected lectins biotinylated at multiple incubation ratios onto a streptavidin surface and recorded the capture over time. These ratios were 0.3, 1, 3, 10 and 30 moles of biotin-PEG4-NHS per mole lectin, denoted as R0.3, R1, R3, R10 and R30 respectively. Figure 5-6a presents the initial capture rate over the first 10 seconds of association (in $\text{RUs}^{-1}\text{M}^{-1}$). For the 3 lectins (LCA; green, MAL-II; blue, SNA; orange), we observe that the capture rate is faster for the higher conjugation ratios, indicating that the degree of biotinylation impacts the kinetics of the biotinylated lectin – streptavidin interaction. The amount by which the capture rate increases is more significant at lower ratios (i.e., between R0.3 and R1) than between higher ratios (i.e., between R10 and R30).

Figure 5-6b, c and d depict the whole capture sensorgrams of the biotinylated LCA, MAL-II and SNA, in RU, over the entire 8 minute injection period. For all 3 lectins, the higher ratios R10 and R30 achieve relatively low capture amounts compared to the lower ratios. The lower ratios achieve greater capture, despite having slower associations, as depicted in Figure 5-6a. The

biotinylated lectin capture to streptavidin was functionally irreversible (Figure 5-9). The lack of a dissociation curve is due to the remarkably strong interaction affinity of biotin and streptavidin [8], and due to a high density of streptavidin on the surface, which promotes rebinding [137].

The diminished capture levels observed at higher conjugation ratios are a demonstration of multipoint binding: a single lectin bearing multiple biotin tags binds to multiple streptavidin sites, and thus prevents the further capture of biotinylated lectins (as schematized in the High biotinylation ratio panel, Figure 5-2). As a result, the total capture achieved on the surface is limited. While the lower conjugation ratios exhibit slower association (Figure 5-6a), the streptavidin surface has a greater capacity over time, and the lower ratios display a higher capture over long injection periods. This is demonstrated by the capture sensorgrams of the 3 lectins (Figure 5-6b, c and d) which show that the lower ratios R0.3 and R1) had not yet reached equilibrium after an 8-minute injection.

The behaviours of the biotinylated lectins at the various biotinylation ratios present an interesting choice when preparing a biotin-streptavidin SPR surface. If low to moderate immobilization levels are desired (eg, lectin screening experiments), it would be preferable to use high conjugation ratios, which would reduce assay time and lower sample consumption. On the other hand, low conjugation ratios should be employed if high immobilization levels are desired (eg, for glycoprotein quantification studies) [13].

5.12.3 Excess Biotinylation Interferes with Lectin-Glycoprotein Binding

The influence of excessive biotin conjugation on the affinity of the lectin-glycoprotein interactions was then evaluated. We injected the glycoprotein Fetuin, which bears glycans terminated by both α 2-3 and α 2-6 linked sialic acids, onto surfaces of MAL-II and SNA (α 2-3 and α 2-6 linked sialic acid specific, respectively) biotinylated at multiple conjugation ratios. The resulting lectin-glycoprotein interactions were recorded over time and the resulting titration sensorgrams were used to determine the thermodynamic affinity constants (K_A). The K_A for the lectins at each conjugation ratio are presented in Figure 5-6e and f.

For MAL-II, we observe that R30 exhibits a significantly lower affinity for the glycoprotein analyte compared to the lower conjugation ratios (Figure 5-6e, darkest bar). Going from R10 to R30 produces a sudden 54% reduction in the calculated K_A , from $1.25 \times 10^5 \text{ M}^{-1}$ to $5.78 \times 10^4 \text{ M}^{-1}$.

¹ , respectively. For MAL-II the lower conjugation ratios (R1, R3 and R10) exhibit very similar glycoprotein binding affinity (Figure 5-6e).

For SNA, we instead report a more progressive reduction in binding affinity as the biotinylation ratio increases (Figure 5-6f). Over the same interval of R10 to R30, a 22% reduction in affinity is noted, from $9.55 \times 10^5 \text{ M}^{-1}$ to $7.45 \times 10^5 \text{ M}^{-1}$. For both lectins, the greatest loss in affinity is observed between R10 and R30. The greater affinity of SNA for fetuin, compared to MAL-II, is consistent with previous findings [138, 139].

Results from lectin-glycoprotein binding experiments indicate that low biotinylation ratios are preferable for thermodynamic binding analysis, more likely as a result of biotin tags near the lectin CRD hindering glycoprotein binding (Figure 5-2, see High biotinylation ratio panel for SPR).

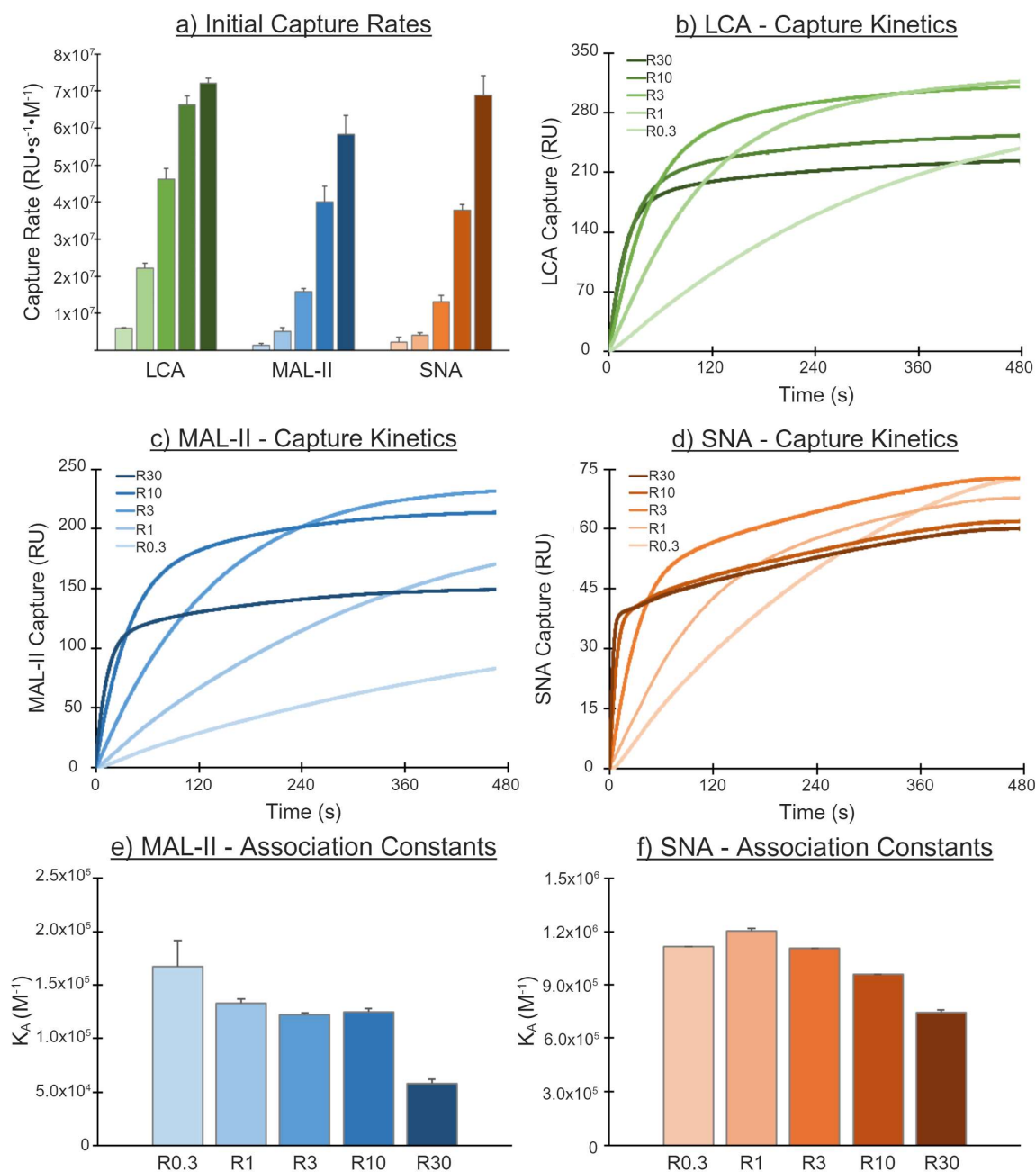


Figure 5-6 a) Capture rates for biotinylated lectins on a streptavidin decorated SPR surface during the first seconds of the biotinylated lectin injection. b, c, and d) SPR sensorgrams displaying LCA, MAL-II and SNA biotinylated to varying degrees being captured on a streptavidin decorated SPR surface. e, f) Thermodynamic association constants of MAL-II and SNA, biotinylated to varying degrees, binding to injected Fetuin.

5.12.4 High Biotinylation Ratio improves the performance of the ELLA

To evaluate the impact of the biotin conjugation ratio on lectins in the ELLA, we compared standard curves of glycoprotein concentration constructed using each lectin at different biotinylation ratio. The signal for each glycoprotein concentration was measured in absorbance units, which is function of the streptavidin-HRP recruited by the lectins (Figure 5-7a, c, and e).

The trends are similar for all three lectins in the study. At lower conjugation ratios (R0.3-R3), we observe a signal amplification as we increase the biotin conjugation, exemplified by the steeper standard curves. This was also observed for the higher ratios (R10-R30), though the amplification was attenuated, as evidenced by the smaller differences between the standard curves, despite evidence of significant differences in biotin conjugation (Figure 5-5). Reduced signal amplification at higher ratios is likely due to greater blank signals for these higher ratios, which was subtracted from the raw absorbances to correct for non-specific adsorption.

An important exception to this trend are the curves of R10 and R30 for MAL-II (Figure 5-7c), for which no significant amplification of the signal was observed, despite a higher degree of biotin conjugation between these lectin preparations (Figure 5-5, last two blue columns). Since the ELLA relies on lectin-glycoprotein binding (Figure 5-2, see ELLA), the amplification effects due to high biotin conjugation (Figure 5-5) is balanced by the reduction in binding affinity at these higher conjugation ratios (Figure 5-6e and f). There is therefore a limit of effectiveness with respect to highly-biotinylating lectins for ELLA.

The limits of detection (i.e., the lowest glycoprotein concentration that can be reliably detected) were calculated from these standard curves (Figure 5-7b, d, and f). The limit of detection was significantly higher, in all cases, for R0.3. The LOD decreased as the conjugation ratio increased, due to an increase in the slope of the standard curve (Equation 6.1). At higher conjugation ratios (R3, R10 and R30), the LOD was not always significantly smaller for higher conjugation ratios (R3 vs. R10 for LCA, R3 vs. R10 vs. R30 for MAL-II, R10 vs. R30 for SNA). Considering Equation 6.1, this is due to the fact that (i) blank sample noise is greater for high conjugation ratios, increasing the LOD, and (ii) a reduced lectin-glycoprotein binding affinity (Figure 5-6e and f) results in less lectin attachment. Our study thus indicates that over-biotinylation would raise the limit of detection and impair the performance of the ELLA.

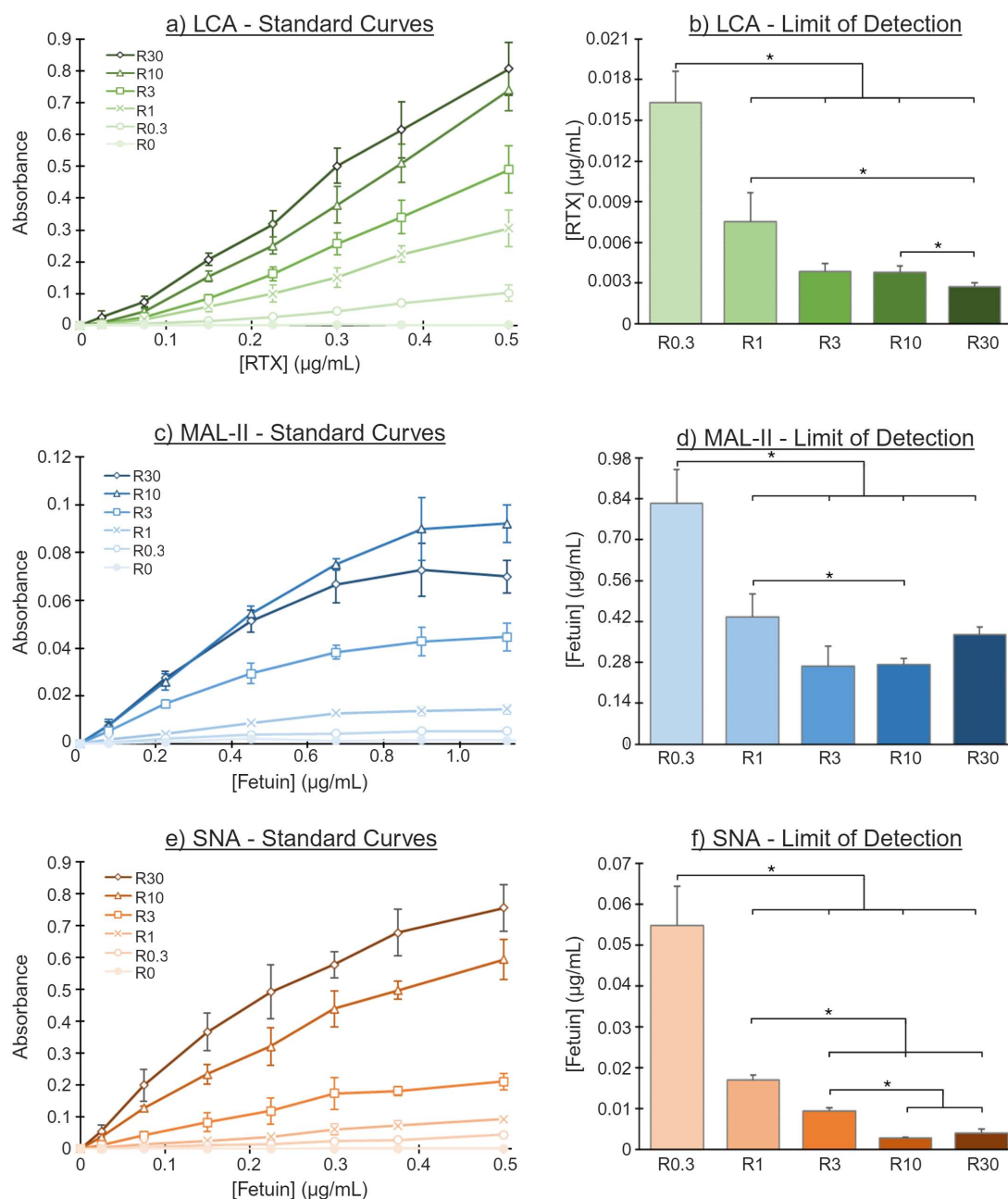


Figure 5-7 Left: Standard curves of incubated glycoproteins constructed using a) LCA, c) MAL-II and e) SNA lectins at multiple biotin conjugation levels. Note that the connecting line between observation points serves as a visual aid and is not intended as a representative model. Right: Limits of detection of the ELLA assay for b) LCA, d) MAL-II and f) SNA at the various biotin conjugation levels. Conditions were compared using a 1-tailed Student's T-test ($\alpha=0.05$). Error bars represent standard deviations between replicates.

5.13 Conclusions

Results from SPR and ELLA assays demonstrate that the appropriate biotinylation ratio can significantly impact the performance of glycoanalytical assays (Figure 5-8). Comparison of capture kinetics at varying biotinylation ratios, measured by SPR, indicates that lower ratios are capable of higher capture levels, as the inhibitory effects of multipoint binding are reduced. This would enable greater lectin immobilization, which would be advantageous for quantification assays. On the other hand, high conjugation ratios demonstrate a much faster association to a streptavidin surface, which would be advantageous for low to moderate capture levels, as it would reduce sample consumption and assay time.

ELLA assays have demonstrated an overall superiority of high biotinylation ratios, which produce significant signal amplifications and lower the limit of detection. This would be especially useful in the detection of very low glycoproteins concentrations, as in diagnostic settings. The limits of quantification would also be effectively reduced by increasing conjugation ratios.

While not explored in this work, the impact of biotinylation on streptavidin capture or recruitment would also depend upon the length and flexibility of the linker used between the lectin and the biotin. Longer, more flexible linkers would likely spread further away from the lectin, increasing the obstructive effects of multipoint binding (in SPR assays) as well as signal amplification (in ELLA). Crucially, this work has demonstrated that there is no “one size fits all” solution for the preparation of biotinylated lectins, and presents an experimental workflow for the optimization of lectin-based protein glycosylation assays via the modulation of the biotin conjugation ratio.

	Low Biotinylation Ratio	High Biotinylation Ratio
Surface Plasmon Resonance (SPR)	<i>Prevents multi-point binding</i> <i>Prevents bias from sterically hindered binding domain</i>	<i>Faster capture rate to streptavidin surfaces</i>
Enzyme Linked Lectin Assay (ELLA)	—	<i>Decreases limit of detection (LOD)</i> <i>Decreases limit of quantification (LOQ)</i>

Figure 5-8 Summary of the effects of the lectin biotinylation ratio for SPR and ELLA glycosylation assays.

5.14 Acknowledgements

We wish to thank Jimmy Gaudreault, Ilona Métayer and Sofiane Khan for producing rituximab used in this work, as well as Michel Gilbert for characterizing rituximab glycosylation by hydrophilic interaction chromatography (HILIC). The authors would also like to thank Benoit Liberelle and Jimmy Gaudreault for critical proofreading of the manuscript. All figures were created with BioRender.com.

5.15 Supplementary Figures

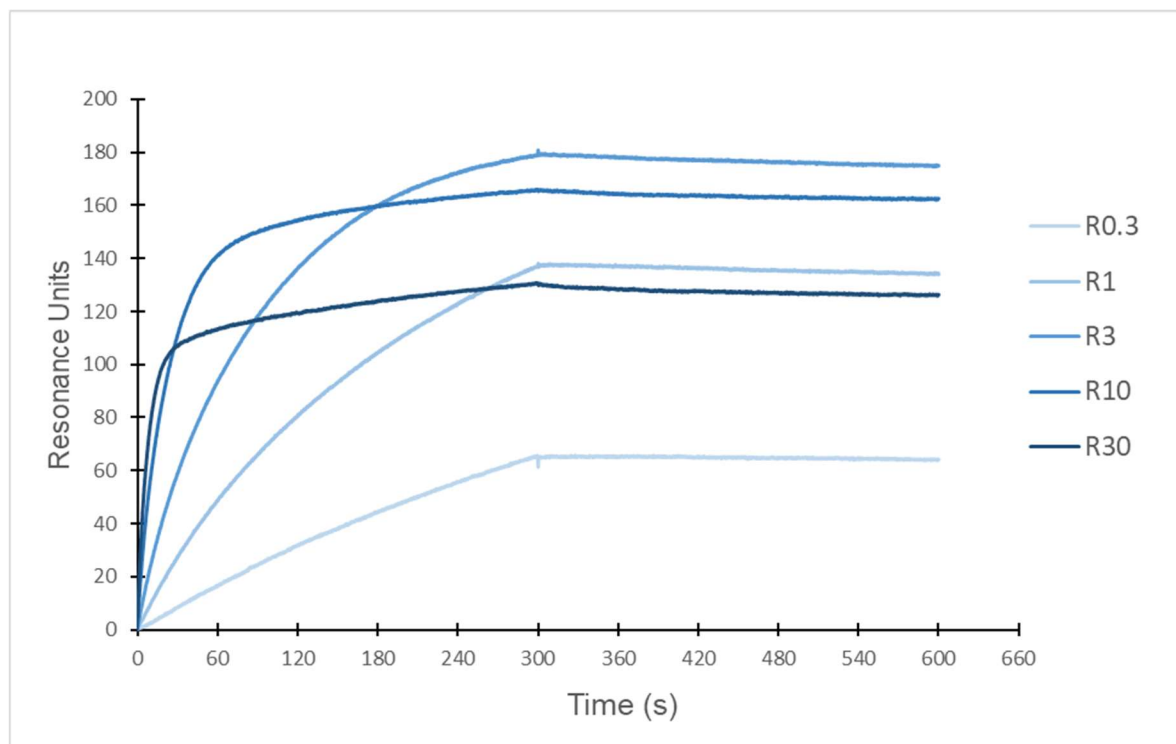


Figure 5-9 Sensorgram of biotinylated MAL-II capture to a 540 RU streptavidin decorated surface, demonstrating no observable dissociation. The association phase ends at 300 seconds, as evidenced by a small injection artifact present in some of the sensorgrams.

CHAPTER 6 General Discussion

6.1 Overview of the Research Activities of the Master's Project

The experimental work presented in this thesis is limited to the avenues of research which proved successful. In reality, many avenues of research were explored and abandoned due to experimental difficulties. The following subsections provide a description of each avenue of research that was explored, the general approach, the outcomes and recommendations for future research in these areas. Conclusions specific to the experimental work performed on the impact of lectin biotinylation are found in chapter 8 of this document.

6.2 Surface Plasmon Resonance for Detecting Influenza Virions in Crude Samples

Originally, the goal of the project was to develop an antibody independent SPR assay for the quantification of Influenza in crude cell lysates. This assay would then be employed by a collaborating research group lead by Professor Amine Kamen of McGill University.

As described in the review article entitled “Antibody-Independent Surface Plasmon Resonance Assays for Influenza Vaccine Quality Control”, SPR based assays have the potential to replace SRID as a gold-standard quality control assay for the quantification of Influenza vaccine preparations. For this purpose, many ligand options are promising, though each have a particular advantage. For detection in crude mediums, aptamers show immense promise as they can be designed, through SELEX, to have high affinity and high specificity for HA. Like anti-HA antibodies, aptamers must be produced on a yearly basis to compensate for antigenic drift and shift, though the chemical synthesis of aptamers occurs on a much shorter timescale and would thus not incur such a substantial bottleneck in vaccine availability.

In cases of time-pressure, such as following the emergence of an Influenza pandemic, sialic acid terminated glyco-structures are the best candidates for Influenza detection, as they require no seasonal preparations. These “bait” ligands have been demonstrated to be effective for HA quantification in both crude and pure samples, as well as final vaccine formulations. The primary disadvantage of sialic acid decorated surfaces is their instability, as these “bait” ligands are susceptible to degradation by Influenza’s NA protein. Implementing reversible ligand capture

would compensate for the bioactivity loss produced by NA degradation, at the cost of a higher sialo-ligand consumption.

Ironically, the ligand option which shows the least promise in the quantification of Influenza vaccine productions is the ligand we spent most time working with: lectins. Lectins are powerful tools for the assessment of protein glycosylation, undoubtedly an important quality control attribute of biotherapeutic proteins. However, lectins display specificity only towards the sugar structures on glycoproteins, not to the protein structure itself. Therefore, any glycoprotein in the crude sample which bears this sugar can bind to a lectin decorated SPR surface and contribute to a “false positive” signal. This is especially problematic as glycosylation is dependent on the choice of host-cell and culture conditions. It is not consistent from batch to batch or even at different times within the same batch. The SPR response of crude Influenza sample binding to lectins is potentially a poor indicator of the quantity of virions in the sample: glycosylated non-target proteins produce false positives, and non-glycosylated target proteins do not contribute to the signal. Furthermore, single lectin detection is unsuitable for quantification, as glycosylation is likely to be heterogeneous within a single batch.

Ultimately, the Influenza project was abandoned, as no Influenza specific signals could ever be identified using lectin-functionalized surfaces. In part, this failure was due to uncertainty of the sample composition. Influenza viruses are produced by infecting host cells (HEK-293 in this case), which provide the necessary machinery for replication. When the viruses accumulate in the host cell, it is eventually lysed and ejects all cellular contents into the growth medium. The sample we were using had only been filtered (0.22 μm) and thus contained an abundance of non-HA proteins that could interfere with the assay in unexpected ways. If this project were to be started anew using lectins, we recommend using highly purified Influenza samples for the initial ligand selection phase. In this same vein, we do not recommend lectins as a biosensing ligand for crude samples, as their specificity towards any appropriately glycosylated proteins makes them susceptible to false positive generation. The performance of lectin-based detection can also be improved via genetic or chemoenzymatic manipulation of host-cell glycosylation pathways to increase glycosylation homogeneity.

6.3 Surface Plasmon Resonance for Assessing Antibody Glycosylation

The next avenue we embarked on was the assessment of mAb glycosylation using immobilized lectins in an SPR biosensor. As mentioned in the text, glycosylation is a critical quality attribute of therapeutic antibodies. Using the lectin *Sambucus Nigra* Agglutinin (SNA), specific to α 2-6 linked sialic acids, we attempted to detect the IgG Trastuzumab via binding to its glycan chain. This particular antibody was produced in an attempt to influence the glycosylation of antibodies via genetic and enzymatic modulation of the cellular glycosylation machinery. During this portion of the work, we assumed this lot of antibodies was heavily α 2-6 sialylated.

Unable to achieve binding with unmodified antibodies, we hypothesized that glycans were obstructed and hidden within the opposing Fc domains of the antibodies, as previously reported in the literature. Indeed, this glycosylation site is highly conserved across antibodies and plays an important role in modulating interactions with Fc receptor proteins. To expose the glycan, we reduced the thiol bonds that held together the opposing Fc domains. Despite reduction, we were unable to bind to the glycan chain using SNA. This line of research was eventually discontinued. Much later, we discovered that the sample of Trastuzumab we were using contained less than 2% sialylated antibodies, and thus appreciative binding signals were unlikely, regardless of the antibody preparation.

Of course, lectin binding to antibodies in SPR has been demonstrated by other researchers. However, using lectins as a method for glycan characterization in SPR platforms still poses significant challenges. Firstly, lectins provide only the identity of the terminal mono or disaccharide in the chain. Elucidation of the whole glycan chain using lectins requires progressive glycan digestion using glycosidase enzymes, which adds significant cost and time to the experiment. Furthermore, many lectins require divalent cations in the running buffer to achieve binding. These divalent cations can facilitate interactions between two negatively charged particles and produce non-specific signals. In fact, many researchers include the chelator EDTA in the SPR running buffer to specifically prevent the formation of such ionic bridges by divalent cations. While lectins are valuable for glycan detection, their particularities make them unattractive for use in SPR biosensors, specifically.

A more interesting avenue for glycan characterization using SPR is via the evaluation of the thermodynamic properties of antibody-receptor interactions. The influence of antibody

glycosylation on receptor binding kinetics is well documented and has been already explored by other members of our research group. This approach is especially promising for the monitoring of productions in-line. In one possible configuration, antibodies could first be captured to the SPR surface by Protein A in mass transport limited conditions, and subsequently bind to injected Fc-receptors in kinetically limited conditions. The two binding events would provide information on the concentration and glycosylation state of the antibodies in solution. With an appropriate sterile sampling setup, this could be performed autonomously, in a few minutes, thanks to the advanced liquid handling systems included with most commercial SPR biosensors.

CHAPTER 7 Conclusions and Recommendations

7.1 Conclusions

In the research article entitled “Impact of Lectin Biotinylation for Surface Plasmon Resonance and Enzyme-Linked Lectin Assays for Protein Glycosylation”, we conclude that the control of lectin biotinylation can significantly improve the performance of glycosylation assays using these proteins. Notably, the optimal degree of lectin biotinylation differs depending on the role of the biotin-streptavidin interaction in each assay.

In SPR, streptavidin was used to capture biotinylated lectins to the surface (Figure 5-2, left). In that case, having a single biotinylated lectin bind to many streptavidin binding sites is undesirable, as this prevents further lectin capture due to surface saturation. To increase the total capture of biotinylated lectins, we desire a lower conjugation level. Conversely, moderate binding levels, suitable for screening (on-off) studies, would benefit from high immobilization levels, as these demonstrate a faster ligand capture (Figure 5-6a). Additionally, we demonstrated that the affinity of lectin-glycoprotein interactions is reduced in cases of high biotin conjugation (Figure 5-6e and f). For studies which are purely focused on evaluating the thermodynamic properties of lectin-glycoprotein interactions, low biotinylation conditions should be favored. To completely avoid this potential bias, kinetic evaluations of lectin-glycoprotein binding should be performed using unconjugated lectins, immobilized by covalent coupling.

The ELLA is essentially the reverse of the SPR assay with respect to the biotin-streptavidin interaction. In this configuration, signal generating streptavidin-HRP is captured to a biotin-presenting surface (Figure 5-2, right). We concluded that increased biotin conjugation produced an amplified signal for the same incubation conditions (i.e., the same concentration of glycoprotein and lectin). As a result, the limit of detection and limit of quantification for these assays was lowered. Modulating the degree of lectin biotinylation is rarely employed as a method of assay optimization, despite it being relatively simple and cheap to perform. High conjugation ratios should be favored for the detection of very diluted samples.

Ultimately there is no “one size fits all” solution for lectin biotinylation. While we presented general trends, the optimal degree of biotinylation will depend on the specific assay being performed. This is especially true for the SPR, where many assay configurations are possible. In the work “Impact of Lectin Biotinylation for Surface Plasmon Resonance and Enzyme-Linked

Lectin Assays for Protein Glycosylation”, we demonstrated the influence of biotinylation for a lectin-immobilized assay, where biotin is used as a capture tag. In the reverse configuration (i.e., lectins are injected as analytes, glycoproteins are immobilized), a high biotinylation ratio might be favorable as it would enable amplification via a secondary streptavidin injection (Figure 4-3), similar to the ELLA. This approach to amplification via secondary binding would be especially useful for low molecular weight analytes.

Finally, this work provides a framework for the evaluation of the optimal biotinylation ratio for any assay type which employs the biotin-streptavidin interaction and is not limited to the two assay types we demonstrate here. While commercially biotinylated lectins remain viable options, in-house evaluation of the optimal biotinylation ratio would significantly improve the performance of the eventual glycosylation assay. From a quality control perspective, in-house preparation of these lectins would enable a greater batch-batch consistency, as the qualities of biotinylated lectins are not provided by lectin distributors. This workflow should also be considered for the preparation of other biotinylated analytical ligands, such as antibodies or aptamers, which are used in assays similar to the ELLA and SPR assays presented in this work.

7.2 Recommendations

7.2.1 Modulating Linker Length

For a continuation of this work, the workflow presented in this research article can be employed to test additional factors surrounding biotinylation in lectin-based assays. Perhaps the most interesting new condition would be the variation of the linker between the biotin tag and the body of the protein. In our work, the linker was kept constant at a length of 4 PEG subunits. Extending the linker to 12 or 24 PEG subunits might significantly increase the amplifying effects of high biotinylation ratios in ELLA assays, as these tags would reach further away from the protein and be less inhibited by steric obstructions. Due to their reach, longer linkers would likely increase the effects of multipoint binding with regards to capture to a streptavidin surface. A study evaluating the combination of biotin conjugation ratio and linker length, using our proposed workflow, would provide a better understanding for optimizing biotinylation of lectins for these assays.

7.2.2 Evaluating Lectins with Various Quaternary Structures

Another potential avenue for the continuation of this research question would be the application of the workflow to other SPR ligands. In particular, we could evaluate the impact of lectin biotinylation on binding kinetics for ligands with a variable number of binding domains. Lectins are usually multimeric, expressing 2-4 identical CRD which serve to increase the apparent affinity of the interaction with a glycoprotein through multivalent interactions. Thanks to multiple binding domains, lectins might be able to “compensate” for obstructive biotinylation via the availability of the other, non-obstructed binding sites. Following this theory, the obstruction/inhibition of ligand analyte binding might be more severe for ligands which bear only one active domain. This continued work would involve the evaluation of impaired analyte-ligand binding, due to biotin conjugation, as a function of the ligand’s quaternary structure. Due to the large variety of the lectin protein class, lectins would be ideal candidates for this research avenue.

7.2.3 Optimizing other assays which use the Streptavidin-Biotin Affinity System

A simpler avenue would involve the extension of the presented workflow to other lectin-based techniques which employ the streptavidin-biotin affinity system. The lectin-blot, a derivative of the Western-blot, would be a good first step. Other biosensors based on optical and electrochemical signal generation would also be valuable targets. While conceptually simple to execute, this work would require access and expertise in each of the different assays to be evaluated. For the evaluation and comparison of multiple biosensors, this would become increasingly expensive.

7.2.4 Site-Specific Tag-Based Signal Amplification

Efforts could be dedicated towards combining tag-based amplification with site-specific labelling. Site-specific labelling, through the introduction of specific conjugation targets via genetic engineering, is valuable for preventing active site obstruction (see Section 6.8). One could introduce many of these conjugation targets to enable both site-specific tagging and tag-based amplification. Since this research avenue is relatively complex and requires genetic engineering, it should be favored for ligand classes with highly conserved domains, like the Fc domain of antibodies, rather than for lectins.

7.2.5 Amplification using Biotin Scaffolds

Rather than modulating the location or number of chemical conjugation sites, amplification via increased biotin conjugation could also be approached through the construction of biotin-scaffolds. These scaffolds would be more complex than the one-dimensional PEG chains used in this work, ranging from flexible branched PEG structures to rigid three-dimensional ssDNA nanostructures, and would each carry multiple biotin tags. By optimizing the geometry of a linker, we could achieve greater biotin-ligand conjugation ratios while avoiding problems associated with over-conjugation (i.e., inhibited binding kinetics). In tandem with site-specific conjugations, these biotin-scaffolds could eventually ensure tag-based amplification and oriented immobilization, particularly for ELLA.

7.2.6 Applying the Workflow to other Molecular Tags

Finally, this workflow could also be extended to biomolecular tags other than biotin, specifically dyes or fluorescent tracers like Cy5, Cy3, Fluorescein, Rhodamine, and other patented dyes. Like biotin, these tags are sold pre-conjugated to lectins for the purpose of histological and cytological studies of tissue- and cell-surface glycosylation. These other tags have variable sizes, which could produce different results based on the location conjugation site relative to the binding domain. Additionally, the mode of signal generation is optical. Rather than multi-point binding with streptavidin, increasing the conjugation of these dyes could increase the signal proportional to the background signals. On the other side of the spectrum, excess tags might result in over-labelling or cell structures, loss of spatial resolution, or obstruction of non-target structures.

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