Biotin coverage

ABSTRACT: Biosensors and other biological platform technologies require the functionalization of their surface with receptors to enhance affinity and selectivity. Control over the functionalization density is required to tune the platform’s properties. Streptavidin (SAv) monolayers are widely used to immobilize biotinylated proteins, receptors, and DNA. The SAv density on a surface can be varied easily, but the predictability is dependent on the method by which the SAv is immobilized. In this study we show a method to quantitatively predict the SAv coverage on biotinylated surfaces. The method is validated by measuring the SAv coverage on supported lipid bilayers with a range of biotin contents and two different main phase lipids and by using quartz crystal microbalance and localized surface plasmon resonance. We explore a predictive model of the biotin-dependent SAv coverage without any fit parameters. Model and data allow to predict the SAv coverage based on the biotin coverage, in both the low- and high-density regimes. This is of special importance in applications with multivalent binding where control over surface receptor density is required, but a direct measurement is not possible.

KEYWORDS: localized surface plasmon resonance (LSPR), quartz crystal microbalance (QCM), streptavidin (SAv), biotin, supported lipid bilayer (SLB), self-assembled monolayer (SAM), surface coverage

1. INTRODUCTION

Streptavidin (SAv) monolayers are widely used to immobilize biotinylated proteins, receptors, and DNA.\(^1\)\(^\text{−}\)\(^5\) The immobilized entities can be studied directly or used as part of a (bio)sensor, assay, or binding study.\(^3\)\(^\text{−}\)\(^5\) The high association constant between biotin and SAv provides an easy method of generating a saturated sensing surface. Biosensors are increasingly utilizing multivalent binding as a means to enhance affinity and selectivity.\(^2\)\(^\text{−}\)\(^6\)\(^\text{−}\)\(^8\) Because the affinity in multivalent binding is strongly dependent on density,\(^9\)\(^\text{−}\)\(^10\) a fully saturated sensing surface is not always desired.\(^5\)\(^\text{−}\)\(^7\)

The SAv density on a surface can be varied easily, but the predictability is dependent on the method by which the SAv is immobilized. Many substrates are coated by physisorption, with limited control over the resulting SAv density. Better control is achieved when using a self-assembled monolayer (SAM) or a supported lipid bilayer (SLB). SAv-functionalized surfaces are typically formed by adding a fraction of a biotinylated compound to the SAM or SLB. The fraction of biotinylated compound determines the density of the SAv layer, while the underlying SAM or SLB provides an antifouling background.\(^11\)

Even when using biotin to control the SAv adsorption, predicting the resulting SAv density is not straightforward. SAv is a tetrameric protein and is capable of binding multiple biotin moieties, meaning that the SAv density is not simply proportional to the biotin density in the SAM or SLB.\(^11\) Furthermore, SAv is much larger than biotin and may sterically block biotin from binding at higher biotin fractions.

Other methods to vary SAv density include the variation of loading time and concentration of SAv.\(^12\) These will leave a fraction of the exposed biotin moieties unbound, which can be difficult to control, depending on the type of application and geometry. Immobilization on SAv can also be controlled by varying the loading time\(^2\) and concentration\(^7\) of the biotinylated compound or by mixing in dummy molecules,\(^6\) with similar downsides. Each of these methods requires the
SAv coverage to be measured directly and obtain the desired density by iteration. In this study we show a method to predict the streptavidin coverage on biotinylated surfaces. To vary the SAv coverage, biotinylated SLBs with a range of biotin contents were prepared. The SAv coverage on the SLBs was measured by using localized surface plasmon resonance (LSPR) for the dry mass and quartz crystal microbalance (QCM) for the wet mass. We explore a predictive model of the biotin-dependent SAv coverage without any fit parameters. We then show that this model works for any biotinylated SAM/SLB if the size of the thiol or lipid is known.

2. RESULTS AND DISCUSSION

2.1. SAv Coverage Control by Tuning SLB Biotin Content. SLBs are used to create platforms in which the biotin density can be varied at will, thus allowing control over the adsorbed SAv density, as shown schematically in Figure 1. This method provides an effective means of producing easily functionalizable, antifouling surfaces with predictability and control over the surface density, especially useful in multivalent binding applications. Further functionalization and residual valence of SAv have been addressed by Dubacheva et al. and will not be covered here.

Unilamellar vesicles with a diameter of 100 nm consisting of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and a varying percentage of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(biotinyl) (DOPE-biotin) were used to form supported lipid bilayers (SLBs) in situ by the vesicle fusion method. A solution of SAv is flowed over the substrate once a stable SLB has been obtained, and the substrate is rinsed after the SAv adsorption step. Analysis of the QCM frequency shift and LSPR peak position change yield the biotin-dependent SAv coverage on the SLB.

We employ a combined quartz crystal microbalance (QCM)/localized surface plasmon resonance (LSPR) setup to detect both the wet and dry mass of the adsorbed SAv as a function of the biotin density. Figure 2a schematically shows a QCM sensor chip containing gold nanodiscs for LSPR that are covered with silicon nitride. The nanodiscs have a thickness of ∼20 nm; the silicon nitride top layer is about 10 nm and covers the substrate conformally.

The QCM signal is sensitive to the total mass coupled to the sensor surface. Monitoring the dissipation of the signal provides information about the rigidity of this mass. The formation of SLBs can easily be verified by looking for typical changes in absorbed mass and rigidity. When performing experiments in aqueous medium, water tends to acoustically couple to the substrate. In QCM, this coupled water is indistinguishable from the substrate, which complicates interpretation of mass values obtained from the commonly used Sauerbrey equation. LSPR is sensitive to a change in refractive index close to the sensor surface. Quantification requires knowledge of the optical properties of the substrate and the thickness, but the technique is insensitive to coupled medium. In conjunction, QCM and LSPR allow the quantification of bound mass along with the degree of hydration.

The gold nanodiscs determine the shape of the LSPR field. The LSPR field is most enhanced around sharp features; in our case of thin nanodiscs we expect a cylindrical symmetrical field which is strongest at the outer rim. The outer rim is the main contributor to the total signal even before considering field strength. The combined effect implies the LSPR signal is mostly representative for the SLB and SAv coverage on the outer rim of the nanodisc.

The presence of gold nanodiscs on the sensor surface does not affect SAv formation. SLBs can form on strongly curved surfaces such as 110 nm diameter silica nanoparticles and inside 75 nm diameter cylindrical pores. Mornet et al. have shown that the curved SLB follows the shape of the surface faithfully, even in the case of strong curvature. Overall, we assume here that we cover the substrates homogeneously with an SLB without effects of the nanodiscs on the local variation of the biotin density.

2.2. Bound Mass Quantification. A representative experiment is shown in Figure 2c with schematic side views at important times indicated in Figure 2b. First, a stable baseline in water is obtained for both QCM and LSPR. Upon addition of DOPC vesicles containing 2% biotin–DOPE, the frequency drops and the dissipation increases strongly (not shown), indicating forming a layer of intact vesicles. As the concentration of vesicles on the surface reaches a critical limit, the vesicles burst and form an SLB. Excess mass is removed during the SLB formation, and the QCM frequency increases slightly. The dissipation drops strongly as the SLB forms a rigid layer.

The shape of the QCM sensogram of the vesicle binding and rupture as well as the frequency shift (23.8 Hz, corrected for 0.3 Hz/min drift) is comparable to literature and previous work in our group (24 ± 1 Hz). The frequency shift corresponds to mass of 431 ng/cm². DOPC weighs 734 g/mol...
and has a lipid footprint of 0.72 nm$^2$ resulting in an expected mass of 339 ng/cm$^2$ for a double layer. Below the SLB, a small hydration layer of $\sim 1$ nm is normally present.\textsuperscript{26,27} In this case 92 ng/cm$^2$ of water was found, indicating a water layer of 0.92 nm below the SLB. This is assuming the water layer above the SLB is similar to the water layer above the bare sensor. In this SLB formation process, the LSPR signal only increases during vesicle binding, as the total mass does not change much during the subsequent vesicle rupture.

When the solution is changed from water to PBS, needed to absorb SAv, the signal increases in both QCM and LSPR. The resonance frequency of QCM is extremely sensitive to medium density and viscosity changes, which means this QCM shift does not need to indicate a change in mass, though it is likely some ions from the PBS enter the layer of bound water near the SLB.

A solution of 2 $\mu$g/mL SAv is then flowed over the surface which results in clear SAv binding steps, in both QCM and LSPR, in which the size of the step is indicative of the SAv coverage. Afterward, the solution is switched back to PBS, showing no desorption of the SAv, and also back to water, showing the inverse step compared to the water to PBS switch (data not shown). For quantification, the SAv step size, in both the QCM frequency change and the LSPR wavelength change, is always taken as the difference between the plateau values of the PBS steps before and after SAv incubation.

Wet mass was calculated from QCM frequency changes by using the Sauerbrey equation (eq 1). With a Sauerbrey constant ($C$) of 17.9 ng/cm$^2$/Hz, the molar mass of SAv ($M_w$)
taken at 60 kDa and $f_5$ the normalized fifth overtone. More details in section 5.5.

$$\theta_{\text{SAv}} = -\frac{C}{M_w} \Delta f_5$$

(1)

Dry mass was calculated from LSPR wavelength change ($\Delta \lambda_{\text{NPS}}$) based on a method described by Jonsson and Höök et al.\textsuperscript{20} Details and derivation can be found in section 5.4. In addition to two sensor-dependent parameters, the sensitivity ($S_0$) and decay length ($L_z$), it requires knowledge of the surface layer thickness ($d$) and composition ($d_n/d_c$). We assumed a water layer of 0.92 nm below the SLB, an SLB thickness of 4.08 nm, and a SAv thickness of 4 nm.

$$\theta_{\text{SAv}} = \frac{d_i - d_{i-1}}{M_w} \frac{\Delta \lambda_{\text{NPS}}}{S_0(e^{-2d_c/L_z} - e^{-2d_n/L_z}) \frac{dn}{dc}}$$

(2)

2.3. Biotin-Dependent Streptavidin Coverage and Associated Water. The experiment as shown in Figure 2c was performed for biotin fractions in the SLB, in the range of 0–5%. In addition, separate QCM measurements using 1-myristoyl-2-palmitoyl-sn-glycero-3-phosphocholine (MPPC) lipids were performed. MPPC forms a gel-state SLB at room temperature whereas DOPC forms a fluid-state SLB at room temperature. Any difference in SAv binding between DOPC and MPPC SLBs could be related to rearrangement of the SLB on the surface. In addition, MPPC lipids have a significantly smaller footprint. The obtained masses from separate QCM and coupled QCM/LSPR are shown in Figure 3a.

The dry mass shows an initial linear regime (indicated with a dashed line through the LSPR data in Figure 3a) and then transitions to a plateau. An initial slope of 1.14 was determined by fitting the data points for biotin coverages less than 2 pmol/cm² using a linear fit and keeping the intercept at 0. Between 2 and 5 pmol/cm² biotin, the SAv coverage levels off, reaching a maximum value of 3.7 pmol/cm² SAv at 8 pmol/cm² biotin.

The slope represents the stoichiometry of the SAv:biotin interaction. Biotin is incapable of binding multiple SAv; thus, the initial slope of 1.14 SAv per biotin must represent a 1:1 initial stoichiometry. As the biotin coverage increases, SAv starts to bind multiple biotins if they are in range. At the plateau value, the SAv coverage becomes independent from biotin availability as the bound SAv sterically blocks additional SAv from binding.

Dubacheva et al. showed SAv binds to biotinylated surfaces using up to three interactions with up to two interactions available for further binding.\textsuperscript{11} At 2 pmol/cm² biotin, the average distance between biotin is more than twice the size of SAv. Therefore, it is expected that biotin and SAv exhibit 1:1 binding in the limit of low biotin coverage. The maximum SAv coverage of 3.7 pmol/cm² is 20% below the reported crystalline coverage on SLBs by SAv.\textsuperscript{28,29}

The trend of the wet mass is different from the dry mass, but there is no clear distinction between DOPC and MPPC in terms of the SAv coverage. The wet mass initially increases much faster with biotin coverage than the dry mass, after which it levels off more sharply.

The overlapping trends of DOPC and MPPC indicate that the SAv coverage is not affected by surface mobility of the biotin. MPPC forms a gel-state SLB and DOPC forms a fluid-state SLB. In case rearrangement of biotin on the surface would be required to accommodate (additional) SAv at high densities, the plateau of SAv coverage would have been different between DOPC and MPPC. Interestingly, the SAv:biotin stoichiometry is identical for MPPC and DOPC at low SAv coverages, which indicates that already bound SAv does not recruit additional biotin and thereby prevents other SAv from binding, even though surface diffusion of biotin occurs at a faster time scale than SAv adsorption. This is discussed in more detail in section 3.3.

At low biotin coverage, SAv binds while being surrounded by a shell of associated water, which is detected by QCM but is invisible to LSPR. As the density of SAv increases, these water
shells begin to overlap and the amount of water associated with each additional SAv lowers. This results in a decreasing fraction of associated water measured with the QCM frequency and thus in the much sharper leveling off observed in the wet mass compared to the dry mass.

The coupled QCM/LSPR experiments allow the associated water to be quantified. Figure 3b,c shows the water content of the adsorbed mass from the QCM/LSPR experiments, with the water content taken as $m_{\text{LSPR}} - m_{\text{QCM}}$. Figure 3b reports the water content as a function of biotin coverage, while Figure 3c provides the water content vs SAv coverage and has a linear fit indicated. The decrease in water content is linear with SAv coverage, starting at 79.9% ± 2.5% and dropping with a slope of −5.8% ± 1.2% per pmol/cm² SAv to 56% at maximum SAv coverage. The linear decrease in water content is consistent with independent binding of SAv; the fraction of associated water shell overlap is proportional to the fraction of the surface covered with associated water shells.

The obtained values compare well to the literature, which reports 83% water at negligible SAv coverage to 49%–55% at maximum SAv coverage. Reference 30 discusses the size and shape of the associated water shell in more depth.

3. MODELING

3.1. Predicting the SAv Coverage as a Function of Biotin Coverage. We now set out to develop a model to describe the SAv coverage as a function of biotin coverage. The goal is to use physics-based equations and assumptions without needing to resort to fitted parameters. This way, the model is applicable to a wide variety of surfaces and opens the use of nonsaturated SAv-functionalized surfaces in applications where density control is required but direct measurement is unavailable.

We will look at SAv binding as an independent process and ignore dissociation and surface mobility of SAv and/or biotin. The biotin-SAv bond is essentially irreversible on experimental time scales, and therefore we do not need to distinguish singly, doubly, or triply bound SAv for the sake of dissociation. Lipid diffusion in fluid-state SLBs happens on time scales much faster than SAv adsorption, while gel-state SLBs again have very slow diffusion. The initial biotin densities of mobile and immobile, nonuniform surfaces are identical. This behavior is
perturbed as soon as SAv starts binding, though we observed no difference in behavior between fluid-state (DOPC) and gel-state (MPPC) SLBs in Figure 3a. We will therefore treat mobile and immobile surfaces identically and discuss the limitations of this assumption in section 3.3.

There are approaches to control the SAv density through loading time.\(^1\) In this approach, the relation between SAv coverage and loading time will depend on the system and geometry, thus requiring calibration. It is therefore not suitable for applications where direct measurement of SAv density is unavailable. Our approach is exact as long as reaction times are long enough that diffusion and mass transport limitations can be ignored.

Predicting the SAv surface coverage equates to predicting the density of SAv binding sites. A binding site in this sense is any place with enough room for a SAv to bind and at least one biotin available for binding. Under thermodynamic equilibrium, monolayer formation is given by a Langmuir isotherm, which takes the number of binding sites, their stoichiometry, and a binding constant into account. The high equilibrium constant between biotin and SAv leads to SAv binding to all available binding sites at any experimentally relevant SAv concentration. The SAv coverage control exerted through controlling the biotin coverage does not influence the equilibrium; instead, it defines the number of sites.

The minimum room SAv needs to bind is given by its crystalline packing limit. SAv can crystallize on SLBs in several crystal structures with footprints ranging from 25.3 to 33.6 nm\(^2\), as visualized by AFM.\(^25,35\) Above pH 7 a square-lattice crystal with sides of 5.8 nm is favored,\(^30\) and thus 33.6 nm\(^2\) was taken as the size of a binding site.

We then need to know the number of biotins per binding site. SAv can bind up to three biotins,\(^11\) though more biotin may be effectively blocked from binding if there is no room for an additional SAv. This effect is shown in Figure 4d, with the dark-red biotin bound to SAv and light-red biotin free but blocked from binding other SAv. This has two consequences: (1) We do not explicitly have to account for SAv stoichiometry; any biotin in a binding site is either bound to a SAv or blocked from binding other SAv. (2) We assume a Poisson distribution to determine the number of biotins per binding site.

A Poisson distribution gives the probability for finding \(k\) biotins in an area, given an average of \(\lambda\) biotins in that area:

\[
\text{prob}(k|\lambda) = \frac{\lambda^k e^{-\lambda}}{k!}
\]  

(3)

With the binding site size and biotin distribution known, all the ingredients required to predict SAv coverage are available. Equation 4 gives the SAv coverage, \(\theta_{SAv}\) in #/nm\(^2\), based on the average biotin coverage in #/nm\(^2\) and the SAv footprint area \(A_{SAv}\) in nm\(^2\). Effectively, this divides the surface into patches of size of SAv, each of which either are uncovered (0 biotin) or fully covered (>0 biotin) with SAv. This model does not use any fitting parameters; it instead uses biotin coverage and physical size as the only inputs.

\[
\theta_{SAv} = \left(1 - \text{prob}(0)_{\text{biotin}A_{SAv}}\right) \frac{1}{A_{SAv}}
\]

(4)

The predicted biotin-dependent SAv coverage on a DOPC SLB is shown in Figure 4c. In the low biotin coverage limit, the SAv coverage scales 1:1 with the biotin coverage, exactly like the dry mass in Figure 3a. Above 10 pmol/cm\(^2\) the model does not reach a true plateau value like the experimental data in Figure 3 showed, but instead slowly increases until a full crystalline coverage in the maximum biotin coverage limit. On the right-hand side axis the average number of biotins available per SAv is given; this is simply the biotin coverage times the SAv footprint.

We can clearly distinguish the three regimes shown in Figure 1. In the initial linear regime the biotins are spaced far apart, and each biotin constitutes a binding site. The average number of biotins per SAv is lower than 1; therefore, a full monolayer cannot be formed. As the number of biotins per SAv approaches 1, adsorbing SAv will start binding or blocking multiple biotins, and the SAv coverage levels off. At biotin coverages beyond 10 pmol/cm\(^2\), adding more biotins hardly generates any additional sites as there is a lack of room for more SAv.

The model does not explicitly define the shape of a site, only its size and the presence of a biotin. As soon as the average distance between SAv molecules becomes smaller than the size of SAv, there will be areas that fit a SAv in size but not shape, requiring rearrangement of SAv in neighboring sites. At high SAv coverages, this may lead to differences between mobile and immobile surfaces for which the model does not account. Experimentally this occurs at SAv coverages close to the plateau value, and thus any meaningful variation of the SAv coverage occurs in the range where the model is valid.

SAv readily forms domains, which further reduces the impact of surface mobility. The formation of domains, and 2D crystals, reveals there are attractive SAv–SAv interactions.\(^12,34,37,38\) These interactions influence the free energy of SAv binding, but not in a way that changes the coverage, as SAv still binds to all available binding sites at any experimentally relevant SAv concentration. It does, however, imply that the SAv footprint, taken as the crystalline limit, is valid before close-to-crystalline coverages are reached and explains why there is no clear transition from a more disordered to a more crystalline surface coverage.

### 3.2. Comparing Model Predictions with Experimental Data

The model presented in the previous section can easily be extended to various surface chemistries. The assumption of mobility or immobile nonuniformity of the surface holds for self-assembled monolayers (SAMs), gel-state SLBs, and fluid-state SLBs alike. The model does not take any fitting parameters; it only uses the size of SAv and the biotin coverage. It does, however, not apply to surfaces where a significant fraction of SAv is nonspecifically adsorbed, as reported for a number of disordered, biotinylated SAMs.\(^34,39,40\) Biotin coverages are generally reported as percentages of the total amount of lipids or thiols. Lipids can vary significantly in footprint, and thus it is important to quantify the biotin fraction into a coverage per unit area instead.

Figure 5 provides a comparison between the data obtained in this study, the model, and literature data from multiple sources. The SAv coverage was calculated from the source data by using the footprint of the bulk thiol or lipid. The surface type, assumed lipid/thiol footprint size, technique, and source are tabulated in Table 1. Where applicable, the SAv coverage reported as mass/area was recalculated by using a \(M_w\) of 60 kDa. Both SAv coverage based on dry mass and apparent SAv coverage based on wet mass are shown. The predicted wet mass was included by using the linear fit for the water
LSPR data, especially when considering that one biotin can be available for low SAv coverages. The measurements by our QCM data implies predicting the SAv coverage based on surface biotin content is more accurate than measuring the wet mass and calculating the dry mass based on that.

Overall, both wet and dry mass data agree well between the various sources and the model presented here. This confirms that the SAv coverage is solely governed by the exposed biotin density, regardless of the substrate type. The match between model, data, and literature is close enough that the model can be used in applications where the biotin content of the surface is known, but the SAv coverage cannot be measured.

3.3. Surface Mobility. The model as derived is technically valid for immobile, nonuniform surfaces only. In such a case, the biotins are Poisson-distributed over the surface. Initially, biotin on a mobile surface will also be Poisson-distributed, but biotin mobility during SAv binding may influence this.

Biotin diffusion is fast compared to SAv adsorption; however, biotin binding to already adsorbed SAv is slow. Figure 2c shows SAv binding to an SLB, which happens on the time scale of minutes. The biotin diffusion coefficient is 10 μm²/s⁴² for DOPC, which means a biotin can probe an area equivalent to a billion SAv in a few minutes. Based on this argument, it would be expected that SAv binding to a mobile surface binds with the maximum valence. At low biotin coverages, this would lead to a clear distinction in SAv coverage between mobile and immobile surfaces, but this has not been observed experimentally (see Figure 3a). The explanation may come from the SAv crystal structure (see PDB entry 3RY243). The biotin binding sites on SAv are toward the center on the bottom and top sides of a rectangular cuboid. We interpret the observed binding behavior to indicate that these sites are easily accessible when SAv is binding from solution but not easily accessible to laterally diffusing biotin. The process of biotin binding to SAv from solution is thus fast compared to binding already absorbed SAv under most experimental conditions. As a result, the biotin mobility during SAv binding can be ignored, and a Poisson distribution can be assumed for biotin in both the mobile and immobile, nonuniform cases.

Mobile and immobile surfaces may behave differently in the high biotin coverage limit combined with long experimental time scales. At high biotin coverages, not all biotins are bound by SAv, but some are only blocked from binding other SAv. This is shown in Figure 4d and has been discussed in the accompanying section. The probability of finding >3 biotin in a 33.6 nm² area is only 1.9% at 5 pmol/cm² biotin, a coverage at

Table 1. Source of the Data from Figure 5

| Surface | Footprint | Technique | Marker | Ref. |
|---------|-----------|-----------|--------|------|
| SLB     | 0.72 nm²  | LSPR      |        |      |
| SLB     | 0.48 nm²  | QCM-D (w) |        | 19   |
| SLB     | 0.62 nm²  | SPR       | QCM-D (w) | 32   |
| SLB     | 0.68 nm²  | SPR       | QCM-D (w) | 30   |
| SLB     | 0.72 nm²  | SE        | QCM-D  | 33   |
| SLB     | 0.68 nm²  | R         | QCM-D (w) | 41   |
| SLB     | 0.72 nm²  | SE        | R      | 11   |
| SAM     | 0.28 nm²  | SE        | R      |      |

*Diamonds (DOPC) and triangle (MPPC) are from this study; from other studies, circles are SAMs and squares are SLBs. Black borders and (w) indicate wet mass. Techniques: QCM-D = quartz crystal microbalance with dissipation monitoring, SE = spectroscopic ellipsometry, SPR = surface plasmon resonance, XPS = X-ray photoelectron spectroscopy, R = reflectometry, SPM = scanning probe microscopy. DOPC and POPC lipid footprints are from ref 25; MPPC and EggPC footprints are from ref 24.

associated with SAv (Figure 3c) with the SAv coverage as given by eq 4.

In several of the cited references a full SAv coverage was desired. The biotin coverage at which the SAv coverage reaches its plateau is in good agreement between the various sources. The SAv coverage plateau value varies from 3.13 pmol/cm² to 4.55 pmol/cm², or 63% to 93% of the crystalline density. At these coverages, full thermodynamic equilibrium may not always be reached, depending on the concentration, adsorption times, and mobility of the surface. The model assumes equilibrium and therefore predicts a SAv coverage in line with the higher literature values. Limited data are available for low SAv coverages. The measurements by Dubacheva et al. seem to be in good agreement with our LSPR data, especially when considering that one biotin can never bind multiple SAv and any data point above the y = x line (not shown) must be an experimental error.

The experimental plateau value verifies the assumption of the SAv footprint area used in the model. The SAv footprint was taken as the crystalline limit and is inversely proportional to the plateau value predicted by the model. Depending on experimental conditions, denser crystal structures than the one assumed can be obtained, and this would increase the plateau value and widen the gap between model and experiment. Less dense crystal structures than the one assumed have not been reported; thus, using SAv footprints of more than 33.6 nm² implies some degree of random packing is assumed and contradicts the domain formation as discussed at the end of section 3.1.

The wet mass is in good agreement between DOPC and MPPC, though generally higher than literature sources. The predicted wet mass is in good agreement with our QCM data. The difference between the wet mass from the literature and our QCM data implies predicting the SAv coverage based on surface biotin content is more accurate than measuring the wet mass and calculating the dry mass based on that.

The probability of finding >3 biotin in a 33.6 nm² area is only 1.9% at 5 pmol/cm² biotin, a coverage at
which SAv is already approaching a full monolayer. Even if these blocked biotins would diffuse away, they would most likely be bound by or blocked by an already adsorbed SAv. The additional number of SAv bound due to surface mobility is therefore expected to be small and outside the experimentally interesting regime.

4. CONCLUSIONS

In conclusion, we have experimentally assessed and theoretically modeled the SAv coverage as a function of biotin coverage on surfaces in a quantitative fashion. Dry mass coverage was measured by using LSPR on DOPC SLBs with varying biotin content. Wet mass was quantified by using QCM-D on DOPC and MPPC SLBs. Despite these lipids having significantly different properties and lipid size, their SLBs showed the same wet mass coverage trend. The obtained data correspond well to available literature data for both SLBs and SAMs, given that the correct lipid/thiol footprint is taken into account.

A model was presented that predicts the SAv coverage regardless of surface type, mobility, or composition. The predicted trend is in good, quantitative agreement with the dry mass obtained in LSPR measurements as well as literature data based on a variety of techniques.

With the work presented here it is possible to predict the SAv coverage based on the biotin coverage. This is of special importance in applications with multivalent binding where control over surface receptor density is required, but a direct measurement is not possible.

5. MATERIALS AND METHODS

5.1. Materials. PBS tablets were purchased from Merck. Milli-Q grade water was used where applicable. 1,2-Dioleoyl-sn-glycerol-3-phosphocholine (DOPC), 1-myristoyl-2-palmitoyl-sn-glycerol-3-phosphocholine (MPPC), and 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine-N-(biotinyl) (DOPE-biotin) were purchased from Avanti Polar Lipids, Inc. Streptavidin (SAv) from Streptomyces avidinii was purchased from Merck.

All materials were as received.

5.2. Vesicle Preparation. DOPC and MPPC were stored at −20 °C as a 10 mg/mL stock in chloroform. DOPE-biotin was stored at −20 °C as a 0.2 mg/mL stock in chloroform. Varying molar ratios of DOPC/DOPE-biotin and MPPC/DOPE-biotin were mixed in glass vials, dried under nitrogen stream, and kept under vacuum for at least 1 h. The dried lipids were resuspended in Milli-Q (DOPC) or PBS (MPPC) at 1 mg/mL by vortexing. The solution was extruded through a 100 nm polycarbonate membrane at room temperature for DOPC or a 50 nm polycarbonate membrane at 50 °C for MPPC. The vesicle solutions were stored at 3–7 °C and used within a week.

5.3. Surface Functionalization. Silica-coated surfaces from LOT-Quantum (MPPC experiments) or silicon nitride-coated sensors were activated by using O2 plasma for 30–45 min. The sensors were activated by using O2 plasma for 30–45 min. The sensors were mounted in a QCM chamber and rinsed with Milli-Q until a stable baseline was obtained. DOPC SLBs were formed at room temperature by using 0.3 mg/mL vesicle solutions in Milli-Q. MPPC SLBs were formed at room temperature by using 0.3 mg/mL vesicle solutions in PBS. After SLB formation, as evidenced by a frequency shift of 24 ± 1 Hz, the temperature was kept at room temperature for the duration of the experiment.

5.4. Quantifying the LSPR Signal. LSPR measurements were performed by using the Insplorion Acoulyte on QCM sensors modified with gold islands provided by Insplorion. LSPR is a surface-based technique that is sensitive to refractive index (RI) changes close to the surface. The sensitivity of the LSPR sensor falls off exponentially away from the surface. The LSPR dry mass was quantified by using a method described by Jonsson and Höök et al.20

The machine response (Δ(NPS)) is equal to a depth (z) integral of the sensitivity (Sθ) times RI chance (Δn), with the bounds given by the layer thickness.

\[ \Delta \lambda_{\text{NPS}} = \frac{2}{\theta_{\text{SAv}}} \int_{-L_z}^{d_{i}} S_{\theta}(\text{e}^{-2n_{z}} - \text{e}^{-2n_{i}}) \, \text{dn}/\text{dz} \]

(5)

in which \( d_{i} \) denotes the cumulative thickness up to and including the ith layer, \( S_{\theta} \) the sensor sensitivity, and \( L_z \) the probing depth. In integrated form and rewriting to get to the surface coverage (θSAv), we get

\[ \theta_{\text{SAv}} = \frac{d_{i} - d_{i-1}}{\text{M}} \cdot \frac{\Delta \lambda_{\text{NPS}}}{S_{\theta}(\text{e}^{-2n_{z}} - \text{e}^{-2n_{i}}) \, \text{dn}/\text{dz}} \]

(6)

The water layer below the SLB was assumed to be 0.92 nm thick (see section 2.2). The SAv thickness was assumed at 4.08 nm based on quantitative differential interference contrast microscopy work by Regan and Langbein et al.44 The height of the SAv layer was derived from its crystal structure and set at 4 nm, and the molecular weight (Mw) was set at 60 kDa. The σ/ρ0/dz was set at 0.185 cm3/g which is a typical value for proteins.

The machine parameters \( S_{\theta} \) and \( L_z \) were set by using a method described in depth in ref 20. The sensitivity constant \( S_{\theta} \) was measured by using a calibration experiment in which the medium was changed from water (RI = 1.33) to 15% glycerol (RI = 1.355) and back. The machine response is equal to the sensitivity constant times change in RI (Δn):

\[ \Delta \lambda_{\text{NPS}} = S_{\theta} \Delta n \]

(7)

A peak position shift of 2.63 nm was observed, resulting in an \( S_{\theta} \) of 105 nm/RI.

The depth dependence was determined by repeating the calibration experiment after a layer with known thickness (SLB, 4.08 nm) was deposited and comparing the shift. The \( L_z \) is given by

\[ L_z = \frac{2d \ln(\frac{\Delta \lambda_{\text{NPS}}}{\Delta \lambda_{\text{NPS}}})}{A \sqrt{\frac{\rho \mu}{2 \gamma^2}}} \]

(8)

in which \( \Delta \lambda_{\text{NPS}} \) is the signal after a layer with thickness \( d \) was deposited. An \( L_z \) of 56 nm was determined by this method.

5.5. QCM Measurements. QCM-D measurements were performed by using a Q-Sense E4 4-channel quartz crystal microbalance with a peristaltic pump (Biolin Scientific) at a flow rate of 30 μL/min. Measurements were started after obtaining a stable baseline with minimal drift. If the drift was nonlinear or more than 1 Hz/min the measurement was aborted.

The QCM wet mass was quantified via the Sauerbrey equation using the fifth overtone.14

\[ \Delta m = - \frac{\Delta f}{n} \cdot \frac{\sqrt{\rho \mu_{s}}}{2 \gamma^2} \]

(9)

in which \( \Delta f \) is the un-normalized shift, \( f_0 \) the fundamental frequency, \( n \) the overtone number (5), \( A \) the sensing area of the electrode (1.539 cm2), \( \rho_{s} \) the density of quartz, and \( \mu_{s} \) the shear modulus of AT-cut quartz.

The Sauerbrey equation assumes a rigidly coupled mass, which is a valid assumption as long as the dissipation shift, \( \Delta D \), is <0.05 × 10−6 per hertz of frequency shift, \( \Delta f \). In addition, it was verified that the calculated mass was overtone independent, another property of rigid masses.46 Masses obtained by fitting the data to a Voigt viscoelastic model47,48 were typically around 0–3% lower, which is another indication that the dissipation is negligible and can safely be ignored. Finally, the Sauerbrey method was preferred as the Voigt analysis is not well-suited for a protocol with changes in medium.
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