Impact of Tether Length and Flexibility on the Efficiency of Analyte Capture by Tethered Receptors

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Abstract

Structure and functionality of molecular layers play a crucial role in determining the outcome of analyte-receptor interactions on affinity biosensors. The control over the structure of these molecular layers gives an independent means to enhance the sensor performance. Here we study the impact of the length and flexibility of molecular tethers on analyte capture by tethered receptors on QCM and SPR sensors. Our results show clear enhancement of analyte-receptor interactions when receptors are bound to the sensor via flexible, and longer tethers. The findings further reveal a qualitative similarity of the impact of tether length on widely different type of binding interactions, viz. gold nanoparticle binding to tethered amine layers, and neutravidin binding to tethered biotin layers. By independent determination of tether densities, our investigations decouple the impact of receptor densities, and the tether conformations, and confirm the role of tether length on adsorption densities and kinetics. The results agree with theoretical reports in literature that predict enhanced analyte capture by receptors anchored to surface via long, flexible tethers, owing to enhanced freedom of movement and thereby its ability to “seek” the analyte in solution. These findings highlight the significance of factoring in the structure of the molecular tether to enhance analyte capture by tethered receptors, and thereby the performance of affinity biosensors.
KEYWORDS: Tether flexibility, Tethered receptors, Poly (ethylene glycol), Analyte capture efficiency, Affinity Biosensor.

1. Introduction

Receptor-analyte interactions on biosensing surface is determined by different factors, including physicochemical attributes at the interface in relation to the medium, and the nature and distribution of receptors on the surface. Different receptors, e.g. nucleic acids,[1] antibodies,[2] peptides,[3] or enzymes[4] have been used in varied contexts to exercise specific interaction with targeted analyte (or ligands). The receptors can be bound to the surface by physisorption or chemical linkage. Physisorbed receptors have often been used associated with enzyme-linked immunoassays (ELISA) where the simplicity of the coating protocol outweighs the disadvantage that may arise due to low binding efficiency of the analytes.[5] This could result from multiple factors, viz. denaturation or lack of appropriate orientation of receptors, or low receptor densities. Attempts to improve such efficiency, especially for on-chip devices have progressed in the direction of oriented immobilization of proteins at high density through molecular linkers.[6] Different linkers have been used in the past, including rigid organic molecules,[7] protein G or A,[8] alkane-based self-assembled monolayers (SAMs),[9] nucleic acids,[10] neutral[11] or charged[12] polymers. Chemical binding is normally preferred due to the lower degree of molecular denaturation and higher stability associated but it implies also lengthier and more complex protocols.[13] The characteristics of such linkers (or tethers) play an important role in the outcome of the biochemical events on different device surfaces, e.g., non-specific binding, electrostatic interaction or ability to regenerate the surface. Neutral hydrophilic polymers, such as polyethylene glycol (PEG), have been widely investigated in literature and they show particularly interesting properties in terms of protein-resistant layers,[14,15] while also enabling end-functional groups that can be used to tether receptors to the sensor surface.[16] Studies have also shown that the antifouling capabilities are strongly dependent upon polymer conformation[17,18] and chain length.[19] Unlike in case of rigid molecules, the PEG monolayers have shown to result in different conformations, mushroom or brush owing to the flexibility that can be attributed to the polymeric backbone. In particular, mushroom-like conformation was attributed to low polymer chain density with limited possibilities of lateral interaction. At high polymer chain density a different conformation can be observed namely brush-like which represents a condition where the tether chain are highly stretched inside the medium due to the presence of other polymeric chain in close
proximity.[20] PEG tether density and dimensions was reported to have an impact on the binding of nanocarriers to biological cells.[21] In an early report from Anne et al. PEG chain has been predicted to move according to elastic models while the chemically reactive tail dynamic is regulated by the diffusion coefficient.[22] Later, Moore et al. has described PEG chain as a spring with a characteristic constant which decreases with the increased length of the tether with the consequence that a small tether behaves as a spring and a long one closer to a loose chain confirming de facto previous hypothesis from Maaloum et al. which first considered long PEGs as “Gaussian chains”. [23,24] They also mathematically predicted the probability of capturing a specific target at a given distance from the surface. Using a combination of Monte Carlo simulations, diffusion reaction theory and surface probe measurements, Jeppesen et al. analysed the impact of PEG chain dynamics on the spatial range of tethered receptor-analyte interactions, for the specific case of streptavidin-biotin interaction.[25] Their study showed and concluded that the capabilities of a tethered receptor to sample the space, both radially and vertically, is not random but biased by the tether dynamic. Moreira et al. have also proposed a more detailed formalism to describe the polymeric linker dynamics and especially the end-grafted receptor in the context of polymer reaction-diffusion theory.[26] The theoretical treatment by Reeves et al made a convincing case for how the structure of molecular tethers impact analyte capture efficiencies.[27] This work is particularly interesting as it relates the diffusive motion of the flexible tether to the rates of analyte capture by tethered receptors. These theoretical predictions have however not been experimentally tested or leveraged in literature to enhance analyte capture efficiency on biosensing interfaces. Here we investigate the impact of the chain length of PEG tethers on the adsorption occurring onto tethered receptors on gold surfaces, under two vastly different scenarios, viz. electrostatic attachment of citrate-gold nanoparticles onto NH2-(EG)n-SH (APT) and biospecific binding of neutravidin (NAv) to biotin-(EG)n-SH (BPT). Despite the difference in the nature of interaction that drives the binding, the similarity in size with NAv makes the Au NPs interesting as a simplistic model of a bioanalyte to study biomolecular adsorption.[28]

Nanoparticles assemblies have been widely investigated due to their highly interesting electronic,[29,30] optical[31] and catalytic[32,33] properties which are very different from their bulk counterpart. Specific applications in molecular diagnostic, in-vivo imaging and stealth drug delivery have drawn value from the characteristics of AuNPs/receptor complex. Parameters related to the AuNPs adsorption, such as their density, distribution, and distance from the underlying substrate, can be used to engineer electromagnetic field enhancements,
which is an aspect of high importance for localized surface plasmon resonance (LSPR) based sensors. The nanoparticle attachment to the surface can be controlled by using molecular functionality and driven by electrostatic, chemisorption or biospecific interactions.[34] Electrostatic interactions are easy and quick to realize, and in some cases provide possibility to regenerate the surface through desorption of nanoparticles.[35] AuNP attachment to oppositely charged surfaces has been studied extensively in literature, mediated by different charged molecular layers, including amine SAMs,[36] plasma polymers[37] and polyelectrolyte multilayers.[38] Onses et al. have shown that many parameters, including the tether molar mass, to affect the nanoparticles adsorption outcome.[39] They have observed that an increase of tether molar mass would correspond to an increase in density of NP adsorbed. The work attributes the effect to a reduced grafting density, while this assumption has not been confirmed through an independent determination of the PEG chain densities on surface.[40] Confirmation of this fact is important to ascertain if the grafting density is indeed the parameter to target, in order to enhance nanoparticle (or analyte) binding to the surface. Moreover, the authors have not considered if tethers at low molar mass would behave differently, while this is of particular interest for sensors where the nanoparticle’s proximity to the surface is highly desired.[41] In this report, we show the impact of the tether length on the electrostatic adsorption of AuNPs at a distance which is smaller or in the range of the NPs themselves (less than 10nm). This corresponds to the range of tether dimensions where the spring constant has been hypothesized to scale the most in relation to the PEG length. In fact, spring constant was measured to not follow the PEG length proportionally but rather be higher (in relative percentage) for smaller polymers thus making them more similar to rigid sticks. [23] Furthermore, the impact of the tether length on nanoparticle adsorption is compared with adsorption of neutravidin to tethered biotin receptors on gold surface. Neutravidin is a 60 kDa homo-tetrameric protein derived from the glycoprotein avidin in which the carbohydrate was removed achieving an almost neutral pI[42] while maintaining intact its capacity to bind up to four biotin molecules. NAv/biotin complex is the strongest non-covalent interaction known in nature (K_d = 10^{-14} mol/L) and has been used in a wide variety of applications including: bioassays,[43] drug delivery[44] and tissue engineering.[45] As such, neutravidin (and the streptavidin companion) are used in immuno sensing applications because of their reduced nonspecific binding.[46] It has been demonstrated that passive surface adsorption of Neutravidin leads to protein alteration in terms of conformation and aggregation (multilayers) which may alter the interface outcome, and that can be overcome by the use of PEG tethers.[47,48] Teramura et al. have pointed to the possibility of enhancing avidin density by increasing molar mass of PEG tethers, when the
density of tethered biotin is conserved.[49] However, the study does not take into account the viscoelastic effects during the QCM measurements that could result in overestimation in PEG mass per surface area at molar mass above 5000 g/mol.[50] There is no study to date that investigates the role of tether length and flexibility on the analyte capture efficiencies, while factoring in the potential influence of other contributing variables including the tether densities and the nature of interactions that drives analyte adsorption. Our findings here show potential to enhance analyte capture efficiencies thereby the means to improve sensitivity and response times of affinity biosensors, by employing receptors tethered via longer and flexible molecular tethers.

2. Materials and Methods

2.1 Materials.
Gold-coated quartz crystal microbalance sensor (5 MHz, AT-cut) were obtained from QuartzPRO (Jarfalla, Sweden). Gold-coated surface plasmon resonance sensors were obtained from Technex B.V. (Wormerveer, Netherlands). Phosphate Buffer Saline (PBS) and Bovine Serum Albumin (BSA) solution (10x concentration) were purchased from R&D System (Abingdon, UK). Water was filtered through a purification system (Milli-Q, Merck) and used immediately after. Hetero-functional thiols were obtained from Nanocs Inc (Boston, MA) and Genochem (Valencia, Spain). 4-aminothiophenol (4-ATP) was obtained from Sigma-Aldrich (Overijse, Belgium).

2.2 Measurements of AuNP binding to Amine Thiol SAMs.
Quartz crystal microbalance (QCM) and surface plasmon resonance (SPR) sensors with gold surface were washed with isopropanol, blow dried with N₂ and then exposed to oxygen plasma in a reactive ion etching chamber (Plasmatherm 790 RIE, St. Petersburg, FL, USA) for 5 minutes to ensure complete removal of carbon residues naturally present on the substrate. Cleaned substrates were then immersed in the designated aminated tethers solution for 2h at 1mM concentration in milli-Q water. The attachment of aminated receptors on QCM substrate is formed due to the presence of thiol head groups: thiol and gold form a relatively stable bond within a short period of time.[51] After the functionalization is complete the surface is thoroughly washed with milli-Q water to remove any loosely bound receptor. The grafting density of the tethered receptors was measured using MP-SPR NaviTM 220A Naali system (Bionavis, Tampere, Finland). A gold coated SPR sensor is incubated in the same solution and for the same time as the QCM sensor and the SPR shift is measured before and after functionalization. Relying on the SPR to quantify the tethers surface coverage removes the
uncertainties in play when QCM is used to measure such highly hydrophilic polymers.[52] Nevertheless, due to the interference with the optical signal of SPR, the Au nanoparticle adsorption was carried out only with QCM. QSense Explorer E4 (Bolin Scientific, Sweden) system was employed to measure mass per surface area of AuNPs adsorbed on different tether length. Functionalized QCM sensors were mounted in a QCM open module which allows the injection of liquids directly on the sensor. The solution with gold nanoparticles was pipetted on top of the QCM sensor after a stable baseline was reached. The gold nanoparticles were prepared according to Turkevich’s method[53] to achieve AuNPs with a mean diameter of 11.4 nm +/- 0.75nm and with a medium pH = 6.8. The adsorption was left to occur for 2h after which the substrate was washed repeatedly with water and dried in-situ, at the end of the process the adsorbed mass was considered. The conversion from frequency shift to bound mass is obtained through the Sauerbrey’s equation: \[ \Delta m = -c \frac{\Delta f}{n} \] where \( \Delta m \) is the shift in mass per surface area thus directly connected to the amount of nanoparticle adsorbed, \( \Delta f \) is the shift in resonance frequency, \( c = 17.7 \text{ngHz}^{-1}\text{cm}^{-2} \) for a 5 MHz crystal and \( n \) is the odd overtone number. The applicability of the Sauerbrey’s equation lies on the hypothesis that the bound mass does not give rise to any viscoelastic movement, the layer is, thus, rigid and sufficiently thin. PEG is a known hydrophilic biopolymer which tends to accumulate water molecules close to it, this behaviour could lead to an overestimation of the adsorbed mass in QCM. Overcoming the possible overestimation was performed by measuring the adsorbed AuNPs after washing and drying the surface. In this way, the measurement would not be impacted by the presence of water and the PEGs molecules would be mostly flat on the surface.

2.3 Measurements of NAv binding to BPT

MP-SPR Navi\textsuperscript{TM} 220A Naali SPR was used to measure the effect of tether length on NAv/BPT interaction. SPR sensors with gold surface are cleaned in the same way as explained for AuNPs/APT experiment and then mounted in the SPR. The sensor is then equilibrated in water and a solution of BPT (1mM in milli-Q water) is flowed to reach the desired tether surface coverage which is achieved by manually interrupting the solution flowing above the sensor. After surface functionalization the substrate is washed with the same buffer and restabilized in PBS. BSA (1% in PBS) is then injected to fill pockets left unfunctionalized by the BPT, the sensor is washed with PBS afterwards. Neutravidin (10µg/mL in PBS) is flown for 20 minutes and the surface is again washed with PBS. A constant flow rate of 10 µL/min was maintained all throughout the experiment. All SPR measurements in this work were carried out with the 670 nm laser wavelength which has a spot size of 0.5 mm in diameter (from which the angular
shift is averaged). The surface coverage of the organic molecules immobilized on the sensor is proportional to the angular shift at a given wavelength and, as a generic rule of thumb, the conversion from angle shift to mass per surface area is obtained using the equivalent units: $1\text{mdeg} = 0.8 \text{ng/cm}^2$ at 670 nm.[54]

3. Results and Discussion

The impact of flexible long tethers over rigid, shorter counterparts towards efficiency of analyte capture was investigated using receptors tethered to polymer self-assembled monolayers on gold substrates. Two scenarios were considered, namely, adsorption of gold nanoparticles (as model analyte) to tethered amine receptors, and, the adsorption of neutravidin to tethered biotin receptors on gold sensor surface. The tethered receptor densities and their interaction with analyte was quantified using surface plasmon resonance (SPR) and quartz crystal microbalance sensors (QCM-D). The gold surface of these sensors allows use of simple thiol chemistry to attach the molecular tethers. Thiolated polyethylene glycol (PEG) was chosen as a representative polymer to tether the desired receptors to the gold surface. PEGs with desired head and tail functionality and desired length (or, the number of EG units) are easy to obtain commercially. In this work several PEG thiols of the type $Y-(\text{EG})_n\text{-SH}$ were used as a flexible tethered receptor, where ‘$Y$’ denotes the receptor that mediates the binding of the target analyte. Amine PEG thiols (NH$_2$-(EG)$_n$-SH or APT) were used to attach negatively charged citrate-stabilized AuNPs to positively-charged -NH$_2$ groups. A more rigid tethered receptor, namely, 4-aminothiophenol (4-ATP), a short molecule, was used for the sake of comparison. Biotin PEG thiols (SH-(EG)$_n$-biotin or, BPT) was used to study the tether length dependence of Neutravidin/Biotin interactions.

Table 1 Characteristics of amine containing tethers used for binding Au NPs. Length of the tether, Flory radius and distance between tethers are calculated as described in the section 3.1.

<table>
<thead>
<tr>
<th>Tethered receptor</th>
<th>Molar mass, $M_w$ (g/mol)</th>
<th>Length of the tether (nm)</th>
<th>Flory radius, $R_f$ (nm)</th>
<th>Distance between tethers (nm)</th>
<th>Number of EG monomers</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-ATP</td>
<td>125.2</td>
<td>0.5</td>
<td>0.47</td>
<td>0.19</td>
<td>NA</td>
</tr>
<tr>
<td>APT-400</td>
<td>400</td>
<td>3.2</td>
<td>1.27</td>
<td>0.53</td>
<td>8</td>
</tr>
<tr>
<td>APT-600</td>
<td>600</td>
<td>4.5</td>
<td>1.62</td>
<td>0.50</td>
<td>13</td>
</tr>
<tr>
<td>APT-1k</td>
<td>1000</td>
<td>7.8</td>
<td>2.20</td>
<td>0.50</td>
<td>21</td>
</tr>
<tr>
<td>APT-10k</td>
<td>10000</td>
<td>79.5</td>
<td>8.76</td>
<td>4.56</td>
<td>214</td>
</tr>
</tbody>
</table>
Fig. 1. Increase in AuNP adsorption on flexible and longer tethers. Molecular structure of (A) 4-ATP employed as a rigid tether and (B) APT employed as flexible tether is shown along with illustration of the respective tether dimensions. (C) plot of mass (left-Y) and density of AuNP (right-Y) adsorbed onto 4-ATP or NH$_2$-(EG)$_n$-SH (APT) monolayers on gold QCM sensors, as function of tether length. X-axis is not to scale with L$_{tether}$ values expressed as labels. AuNPs density is calculated by normalising the mass obtained by QCM with the mass of a single NP of 11.4 nm in diameter. Dashed line indicates jamming limit of 54.7% for AuNP monolayer predicted by RSA model.

3.1 Impact of Tether Length on AuNPs adsorption to Tethered Amine Monolayers

QCM sensors functionalized with different aminated tethers were tested for the impact of tether length on the adsorption of gold nanoparticles. Table 1 summarizes the main characteristics of five different amine containing tethers used for the investigation, viz. a rigid, 4-aminothiophenol (4-ATP) chosen to represent a short (0.5 nm), rigid molecule, and 4 different APTs chosen to represent flexible tethers with different tether lengths in the range of 3.2-79.5
nm. The tether length was estimated using the Cambridge Scientific Chem3D 19.0 software to be the distance from the -SH terminal to the atom closest to the tail group functionality, viz. carbon attached to amine group in case 4-ATP and the oxygen atom attached to aminoethyl group in case of APT. (Fig. 1) The QCM sensors were exposed to an aqueous suspension of citrate stabilized AuNPs with a diameter of 11.4 +/- 0.75 nm, for a duration of 2 hours. The sensors were washed with water to remove any unadsorbed AuNPs and were subsequently dried prior to QCM measurements. The difference in frequency of the dried AuNP layers, with that of the tethered SAMs prior to exposure to nanoparticle suspension was used to estimate the total mass of the AuNP layers using Sauerbrey’s equation. (Fig. 1C) The use of dry mass overcomes the consideration of viscoelastic behavior due to solvated nanoparticle layers and ensures the validity of rigid layer approximation for use of Sauerbrey’s equation.[54] The QCM results show a clear increase in the mass of AuNPs with the increase in tether length (Fig. 1A). The adsorption of AuNPs onto the smallest and rigid tether, namely, 4-ATP results in an increase of mass of 1964 ng/cm², equivalent to 1309 NPs/µm², which corresponds to ~13% of the total surface. (Fig. 1B) While moving from rigid 4-ATP tether to flexible APT tethers, we observed an increase of AuNP density by 71.3%, 153.4% and 288.4% respectively for 3.2 nm (APT-400), 4.5 nm (APT-600) and 7.8 nm (APT-1K) tethers. For longest tether tested, with tether length of 79.5 nm (APT-10k), the AuNP density was found to increase by 861.8% over the rigid tethers. Control experiments show no AuNP adsorption occurred on PEG layers in the absence of amine groups.

Fig. 2. AuNP distribution as function of tether length. FESEM micrographs of AuNPs adsorbed on aminated tethers, with increasing tether length from left to right, with the corresponding labels indicated.
Fig. 3. Au NP densities on tethered amine monolayers, factoring in the density of tethers as function of tether length ($L_{tether}$) (A) Tether surface density and footprint as function of tether length as measured by SPR. (B) Evolution of normalized AuNP densities as function of tether length. X-axis is not to scale with $L_{tether}$ values expressed as labels.

The AuNP densities obtained on the amine tethers was compared with the theoretical limits of maximum density for monolayer coverage. The maximum density of gold nanoparticles that could result from electrostatic adsorption of AuNP onto amine monolayers is defined to be 54.7% by random sequential adsorption (RSA) models.[55] For the AuNPs used in these experiments, this would indicate a coverage of 5359 NPs/µm². The surface coverage of AuNPs were found to be 13%, 23%, 34%, 51%, 128% for 4-ATP, APT-400, APT-600, APT-1k and APT-10k respectively. The AuNP surface coverage on the APTs are higher than what has been typically observed for AuNP assemblies in literature.[56–59] In case of APT-10k, the surface coverage is equivalent to 200% of what has been predicted by the RSA model, which may potentially result from multi-layer formation. An independent analysis of the AuNP distribution on the tethered amine layers on the QCM sensors was performed using SEM. (Fig. 2) FESEM images shows a monolayer of reasonably well isolated gold nanoparticles on 4-ATP.
and APT-400, small aggregates is observed for APT-600 and APT-1k. (Fig. 2) In case of APT-10k, the gold nanoparticles were found to form multilayers, resulting in semi-continuous nanoparticulate films, which could explain the high values of surface coverage measured by the QCM.

However, it is important to consider that a change to tether length would also influence the density of tethers, which in turn could influence the resulting AuNP densities. [60] To factor this influence, the areal densities of the different tethers were independently determined for different tethered amine layers using SPR. The 4-ATP was found to exhibit highest molecular coverage of 5 molecules/nm² (green bar in Fig. 3A). This decreased to 1.8 molecules/nm² for the 3.2 nm long tethers, while continuing to remain the same for 4.5 nm and 7.8 nm long tethers (red, blue and dark green bars in Fig. 3A). The 79.5 nm long tether resulted in the least coverage of 0.3 molecule/nm² (cyan bar in Fig. 3A). Thus, the increase in AuNP density as a function of the tether length was achieved despite the decrease in tethers density. The grafting density of tethers is known to determine the conformation of PEG tethers, which in turn could influence the adsorption densities.[61] Thus, it is essential to check whether all the tethers are in the same conformation despite the different length and molar mass. In order to do so, we have calculated the Flory radius as following: $R_f = \alpha n^\nu$ where $\nu = 3/5$, $\alpha = 0.35 nm$ for an ethylene glycol monomer and $n$ indicates the number of monomers in the chain.[61,62] We estimated $n$ to be roughly equal to 8, 13, 21, 214, respectively for the 3.2 nm, 4.5 nm, 7.8 nm and 79.5 nm long tethers (Table 1). Flory radius as calculated above is to be compared with the average distance between individual tethered receptor estimated as below.

$$D = \frac{\sqrt{A}}{N}$$

where $N$ is the total number of tethered receptor on the surface ($N = S_c A$ where $S_c$ is the surface coverage shown in Fig. 3A and $A = 1 cm^2$).[63] The calculation of the Flory radius is possible only in presence of a polymer trapped in a good solvent which is not the case of the smallest tether employed. For this specific case we considered that the 4-ATP is a molecule whose main degree of freedom is the rotation around the vertical axis (virtually connecting SH and NH$_2$) which depicts an ellipsoid with the major axis measuring 0.58 nm and the minor 0.47 nm (the latter is considered in Table 1).[64] Regarding the APT, if the distance between tethers is less than the Flory radius, we can consider them to be immobilized at high density thus displaying a brush-like conformation. Comparing the figures, we determined that the tether density was high enough to stretch the tethers and make them assume a brush-like conformation.
for all the tethers analysed (APT-400 to APT-10k). Nevertheless, a mixed brush/mushroom conformation cannot be completely ruled out.[63]

Evolution of AuNP density as function of tether length for fixed receptor density is shown in Fig. 3B. This was obtained by normalizing the AuNP density shown in Fig. 1C, by the receptor densities (\( \delta_{\text{receptor}} \) in molecule/cm\(^2\)) shown in Fig. 3A, and following the equation,

\[
\frac{\Delta m}{m_{\text{AuNP}}} = \frac{\delta_{\text{receptor}}}{N_{\text{Ps per receptor}}}
\]

Where \( \Delta m \) is the total mass uptake of AuNPs in ng/cm\(^2\), \( m_{\text{AuNP}} = 1.5 \cdot 10^{-8} \text{ ng} \) is the estimated mass of a single AuNP with a diameter of 11.4 +/- 0.75 nm. Fig. 3B shows clear increase in the density of captured AuNP with increase in size of the tether, for the same number of receptors. As seen from Fig. 3B, the 0.7 nm long tether can possibly trap up to 0.2 NPs per 1000 molecules of amine receptors while this density can reach up to 2.3 NPs per 1000 molecules of receptor (for 7.8 nm long tether) before increasing by an order of magnitude due to the presence of multilayers and thus reaching 52 NPs per 1000 molecules of receptor in the case of APT-10k. Despite the similar the tether densities for tether lengths of 3.2, 4.5 and 7.8 nm (Fig. 3A), the AuNP/receptor ratios show a clear increase with increase in tether lengths (Fig. 3B).

3.2 Impact of Tether Length on Neutravidin Binding to Tethered Biotin

NAv/biotin interaction was determined using gold SPR sensor, starting with immobilization of biotin-PEG-SH (BPT), followed with BSA to fill any non-specific binding pockets and exposure to neutravidin (NAv). Control experiments were performed to rule out the non-specific binding of NAv on PEG-thiol layers in the absence of biotin functionality. BPTs with three different tether lengths was used, viz. 0.4nm, 1.4nm and 6nm which corresponds to BPT-200, BPT-400 and BPT-1k respectively (200 g/mol, 400 g/mol and 1000 g/mol). The length of the tether was calculated with the help of Cambridge Scientific Chem3D 19.0 software considering the distance between the sulfhydryl group and the last oxygen forming the EG monomer before the biotin complex. The 0.4 nm long tether is considered to have a comparatively rigid molecular structure given that 200 g/mol is around the molecular weight of a biotin molecule (see Table 2). In this case, we considered the Flory radius to be the radius of biotin (around 0.37 nm).[65]

The variation of BPT tether densities as function of tether length was determined for two different BPT density regimes on surface, viz. denser (\( R/D \cong 1.4 \)) and sparser (\( R/D \cong 0.53 \)) tether density (where \( D \) is the inter-tether distance). (Fig. 4A) Assuring a stable \( R/D \) ratio
requires varying the tether surface coverage for the different tether lengths, viz. from 0.3 to 1.45 molecules/nm² for \( R_f/D \approx 0.53 \) and from 0.77 to 3.9 molecules/nm² for \( R_f/D \approx 1.4 \) (Fig. 4A). These two \( R_f/D \) ratios represent the density regimes that would result in \textit{mushroom-like} (\( R_f/D \approx 0.53 \)) or \textit{brush-like} (\( R_f/D \approx 1.4 \)) conformation, and thus carry potential to influence the adsorption density of NAv. [20] Therefore, the tether length dependence of NAv adsorption was investigated by following NAv/BPT ratios, for both the brush and mushroom conformations, to factor in the impact of the receptor densities and the tether conformations. (Fig. 4). The equilibrium mass density for NAv/BPT ratio to increase as function of tether length for both mushroom and brush conformations, while this increase was found be distinctly higher in case of the mushroom conformation. (Fig. 4B-D) Such increase in NAv/BPT ratios indicates that it is possible to obtain a greater density of NAv, for the same number of BPT receptors, just by increasing the length of the tethers.

Table 2. Characteristics of biotin-(EG)ₙ-SH tethers used for NAv binding.

<table>
<thead>
<tr>
<th>Tethered receptor</th>
<th>Molar mass, ( M_o ) (g/mol)</th>
<th>Length of the tether (nm)</th>
<th>Flory radius ( R_f ) (nm)</th>
<th>Distance between tethers, ( D ) (nm)</th>
<th>Number of EG units, ( n )</th>
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</thead>
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<tr>
<td></td>
<td>( R_f/D \approx 1.4 )</td>
<td>( R_f/D \approx 0.53 )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BPT-200</td>
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<td>0.4</td>
<td>0.37</td>
<td>0.26 nm</td>
<td>0</td>
</tr>
<tr>
<td>BPT-400</td>
<td>400</td>
<td>1.4</td>
<td>0.72</td>
<td>0.55 nm</td>
<td>3</td>
</tr>
<tr>
<td>BPT-1k</td>
<td>1000</td>
<td>6</td>
<td>1.86</td>
<td>1.30 nm</td>
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</tbody>
</table>

Previous instances in literature have demonstrated that reducing tether density would benefit the adsorption of the ligand owing to a reduced analyte-analyte hinderance.[66] The BPT in mushroom conformation is expected to present a lower steric hindrance for NAv adsorption, which could explain the higher observed NAv/BPT ratios for the mushroom (black curve) as compared with the brush conformation (red curve) as seen in Fig. 4B. The inter-tether distances measured experimentally varies between 0.26-1.3 nm (for brush) and 0.69-3.46 nm (mushroom) pointing to a decrease in receptor density at the brush and mushroom conformations. It is not yet possible to rule out the possible role of receptor densities towards the increasing trends observed in tether length dependence of NAv/BPT ratios. (Fig. 4B) In order to separate the role of the receptor densities, two points presenting virtually the same BPT surface coverages (within 6%) were chosen (indicated by dashed line in Fig. 4A), viz. \( L_{\text{tether}} \) of 1.5 nm and \( R_f/D \) of 0.53 (black square, Fig. 4A), and another at \( L_{\text{tether}} \) of 6 nm, and
Rf/D of 1.4 (red square, Fig. 4A). Comparison of the NAv/BPT ratios corresponding to these two points in Fig. 4B show an increase of 49% for the BPT in brush conformation at tether length of 6 nm (red dashed line, Fig. 4B) as compared to the BPT in mushroom conformation (black dashed line, Fig. 4B) at tether length of 1.5 nm. The higher NAv/BPT ratio observed for BPT with longer tethers, at constant BPT surface coverage, and despite being in brush conformation, clearly points to the role of tether length towards NAv adsorption densities. The NAv densities observed in our work ranges between ~200 – 400 ng/cm² agrees with the NAv densities previously reported on various surfaces including physisorption,[47] biotinylated supported lipid bilayers (b-SLBs),[47] PLL-g-PEG/biotin,[67] and by carbodiimide chemistry,[68] and is in agreement with geometric expectations based on NAv dimensions obtained from X-ray crystallography.[69]

Fig. 4. Surface coverage of BPT and NAv/BPT ratio as function of tether length, for brush and mushroom conformations. (A) Evolution in surface density of BPT tethers and (B) NAv bound to 100 BPT for both the Rf/D ratios are shown. Dashed lines in (A,B) show that (A) for the two tether lengths that show the same BPT densities, (B) an increase in NAv/BPT ratio is observed. Molecular structure of BPT, and the cartoon representation (not to scale) of the BPT and NAV bound to BPT layers are shown for reference. (C, D) Kinetics of adsorption of NAv to BPT tethers, for the different tether lengths shown for (C) brush and (D) mushroom configurations.

The experimental evidence this far confirms the role of tether length on the adsorption of both AuNPs and NAv, which is in agreement with theoretical expectations of higher analyte capture
due to enhanced flexibility linked to longer tethers. Furthermore, the increase in the density of analyte on surface has important consequences for the response time of the sensor. This can be seen by comparing the time needed to attain areal densities of analyte (here, AuNPs or NAv) equivalent to that of the saturated density on the rigid tethers. The results show that the NAv densities on BPT in mushroom configurations, take 20 min to saturate (at 1.02 NAv per 100 BPT) on the 0.4 nm long tethers (BPT-200), while the equivalent densities are achieved in 8.3 min and 4.8 min in case of 1.4 nm (BPT-400) and 6 nm (BPT-1k) long tethers. (Fig. 4C) Similarly, NAv densities on BPT brushes, take 20 min to saturate (at 1.28 NAv per 100 BPT) on the 0.4 nm long tethers, while the equivalent densities are achieved in only 1.9 min and 1.7 min in case of longer tethers, viz. 1.4 nm and 6 nm respectively. (Fig. 4D) Similar trends are observed also in case of AuNP adsorption to APTs, where the saturated nanoparticle densities takes 100 min for the 0.5 nm long rigid tethers (4-ATP), while the equivalent densities are attained in much shorter durations for flexible tethers, viz. 8.2 min, 6.6 min 5.7 min and 1.8 min for increasing tether lengths of 3.2 nm (APT-400), 4.5 nm (APT-600), 7.8 nm (APT-1k) and 79.5 nm (APT-10k). (Fig. 1A)

The results confirm the role of tether length to have an independent role in determining the outcome of adsorption events. Furthermore, the qualitatively similar trends observed for the adsorption of AuNP and NAv onto APT and BPT respectively indicates the impact of the tether length on the adsorption events to be generic and independent of the nature of interactions that drive the binding events. However, the impact of tether length on the enhancement of adsorption of AuNPs onto NH2-(EG)n-SH was found to be more pronounced as compared to NAv adsorption to biotin-(EG)n-SH. This difference could potentially arise due to the limitation of 4 biotin binding sites per NAv as compared to the abundance of binding sites on surface of AuNPs. The latter has potential to be leveraged to achieve higher sensitivity in bioassays using detection reagents conjugated with AuNPs. The high surface densities of AuNPs achieved with longer tethers is independently useful in affinity sensors that can take benefit of the resulting optical and spectroscopic properties, e.g. surface-enhanced spectroscopic detection of molecular analytes [70] and colorimetric sensor for heavy metal detection.[71] Overall, the findings in this report strongly support the need to factor in the tether length and flexibility into the design of interface functionalization to enhance the performance of affinity biosensors.

4. Conclusions

Impact of tether length on analyte capture by receptors tethered to QCM and SPR based affinity sensors was investigated. Two different scenarios were investigated, namely, electrostatic
binding of gold nanoparticles to amine thiol monolayers and biospecific binding of neutravidin to biotinylated thiol monolayers. In both cases, the tether length and flexibility were found to have a strong impact on the densities and kinetics of adsorption. Adsorption of gold nanoparticles on NH2-(EG)n-SH SAMs was found to systematically increase with an increase in EG repeating units. The trend was found to be similar for NAv adsorption to biotin-(EG)n-SH SAMs with an increase of NAv density with increase in the number of EG repeating units. In both cases, higher adsorption kinetics on receptors on longer tethers reveal the opportunity to reduce response times of the sensor. Independent determination of the tether densities using SPR allowed decoupling the influence of the density and conformation of the tethers towards the adsorption outcomes. The matching trends in the impact of the length of the (EG)n tether on the adsorption of Au NPs and NAv, provides first experimental evidence in support of the theoretical predictions in literature of longer and flexible tether to have higher chances of capturing analyte due to greater conformational degrees of freedom. The findings reinforce the possibilities to enhance the design of biosensing interfaces by factoring in the flexibility and dimensions of the molecular tether to drive high analyte capture efficiency.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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**Appendix A. Supplementary Data**

Supplementary data to this article can be found online.

**References**


