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Streptavidin-biotin binding in the presence of a polymer spacer: a theoretical description

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Abstract

The binding of streptavidin to biotin located at the terminal ends of poly(ethylene oxide) tethered to a planar surface is studied using molecular theory. The theoretical model is applied to mimic experiments (Langmuir 2008, 24, 2472) performed using drop-shape analysis to study receptor-ligand binding at the oil/water interface. Our theoretical predictions show very good agreements with the experimental results. Furthermore, the theory enables us to study the thermodynamic and structural behavior of the PEO-biotin+streptavidin layer. The interfacial structure, shown by the volume fraction profiles of bound proteins and polymers, indicates that the proteins form a thick layer supported by stretched polymers, where the distribution of bound proteins is greater than the thickness of the height of one layer of proteins. When the polymer spacer is composed of PEO (3000), a thick layer with multi-layers of proteins is formed, supported by the stretched polymer chains. It was found that thick multi-layers of proteins are formed when long spacers are present or at very high protein surface coverages on short spacers. This shows that the flexibility of the polymer spacer plays an important role in determining the structure of the bound proteins due to their ability to accommodate highly distorted conformations to optimize binding and protein interactions. Protein domains are predicted when the amount of bound proteins is small due to the existence of streptavidin-streptavidin attractive interactions. As the number of proteins is increased, the competition between attractive interactions and steric repulsions determines the stability and structure of the bound layer. The theory predicts that the competition between these two forces leads to a phase separation at higher protein concentrations. The point where this transition happens depends on both spacer length and protein surface coverage and is an important consideration for practical applications of these and other similar systems. If the goal is to maximize protein binding, it is favorable to be above the layer transition, as multiple layers can accommodate greater bound protein densities. On the other hand, if the goal is to use these bound proteins as a linker group to build more complex structures, such as when avidin or streptavidin serves as a linker between two biotinylated polymers or proteins, the optimum is to be below the layer transition such that all bound linker proteins are available for further binding.

II. INTRODUCTION

Ligand-receptor interactions are an important control mechanism in many biological systems. For example, the binding of extracellular matrix proteins to specific receptors on the cell surface triggers signal transduction pathways allowing the cell to sense and react to its environment in

ways such as aggregating with other cells, rearrange its actin cytoskeleton or undergoing cell division.^{1,2} At its core, ligand-receptor binding is basically a chemical equilibrium process that can be shifted into the direction of dissociated species or a bound pair, in which the intrinsic binding constant plays the dominating role. The binding constants of biologically relevant ligand-receptor pairs have a very wide range of values. One of the strongest known binding constants is that of biotin-avidin/streptavidin that can be of the order 10^{15}mol^{-1} .³⁻⁵ Due to the high affinity between biotin and the tetrameric protein streptavidin, or its homolog avidin the binding is almost irreversible. On the other hand, the binding constant for the interaction between nitrilotriacetic acid (NTA) and histidine is much lower on the order of $K_b = 10^6 \text{mol}^{-1}$.⁶ Many important applications take advantage of the wide range of interaction strengths between ligand-receptor pairs to control the binding of different species.⁷⁻¹¹ For example, in the field of targeted drug delivery, functionalization of polymer end groups can be used for specific targeting¹²⁻²⁰ of cells. In this case, liposomes were coated with a protective layer of end-grafted poly(ethylene glycol) (PEG), which serves a dual purpose: reducing nonspecific adsorption of proteins to the liposome surface due to the presence of polymer brushes which act as a steric barrier to the proteins²¹⁻²⁴ and improving the binding between ligands found at the terminal ends of the polymer and cell-bound receptors. Grafting polymers²⁵⁻³⁰ to modify protein-surface interactions have been widely used in the design of biocompatible materials, biosensors, and bioactive surfaces. In a similar train of thought, a recent experiment³¹ studies the binding of streptavidin to biotin at the oil/water interface using both hydroxyl-terminated and biotin terminated amphiphiles (C18- poly(ethylene oxide) (C18-PEO). The PEO spacer also allows the biotin at its end to sample an extended space and interact efficiently with streptavidin.

From the above examples, such as ligand-receptor binding at the liposome surface or biotin-streptavidin binding at an oil/water interface, we can see that many binding events happen in confined environments, where the existence of surfaces or interfaces breaks the symmetry of the system and leads to dramatic deviations from the ideal case. It is known that³² for ideal solutions, the binding constant is proportional to the exponent of the difference in species' standard chemical potentials before and after the formation of a bound pair. This relationship between the binding constant and the standard chemical potentials needs to be generalized by the presence of a surface or interface.³³ The question then becomes, what are the new binding constants? In previous papers we have demonstrated that the new binding constant should be found by minimization of a free energy that explicitly includes the fact that the system is highly inhomogeneous and takes into account the packing of the spacers and bound proteins.^{33,34} Other theoretical approaches and computer simulations have reached similar conclusions.^{35,36} The application of the theoretical approach developed in refs.^{33,34} show very good agreement with experimental observations for the binding of proteins^{37,38} as well as for the interactions between fibrinogen coated surfaces and surfaces with biotinated PEG.³⁹ Furthermore, the theory is capable of predicting both the structural and thermodynamic properties of the system. The good agreement with experimental observations provides confidence of the validity of the predictions for quantities not directly measured.

In the present work, we model a specific system involving the binding of streptavidin to biotins located at the terminal ends of a PEO spacer mimicking the experimental conditions.³¹ Because the binding constant between biotin and streptavidin is so large ($\sim 10^{15} \text{mol}^{-1}$), the binding of the streptavidin is irreversible within the time scale of the experiments. This was shown to be the case experimentally and therefore, the binding constant of streptavidin is not an important issue because the bound amount of streptavidin is constant during the experiment. What is explored in this paper are the important factors determining and controlling the binding and the changes in structure of the polymer layer when streptavidin binding occurs.

In our previous paper,³¹ we showed that the theoretical results for the surface tension of the PEO-biotin+streptavidin layer agree with the experimental data very well as the drop is compressed, which encouraged us to explore the system in greater detail. One major aim of this work is to elucidate the distribution of proteins and polymers by examining volume fraction profiles for bound proteins, polymers bound to proteins and polymers not bound to proteins. Moreover, we analyzed the stability of the polymer-protein layer, and predict the presence of protein domains, which have been observed in previous experiments,^{40–43} and phase separation of protein layers. As will be demonstrated, the competition between protein-protein attractive and repulsive interactions plays an important role in determining the structure and stability of the bound layer. Another important finding is the significant effect that the length of the polymer spacer has on the structure of the PEO-biotin+streptavidin layer. These types of theoretical predictions provide, in addition to the fundamental insights, a powerful tool for the design of interfaces for practical applications.

The paper is organized as follows. First, we describe the molecular theory. Then, we present relevant results, including comparisons with experimental results, and explore molecular factors affecting the structure and stability of the polymer-protein layer. In the last section, we draw conclusions on the biotin-streptavidin binding and discuss how to extend our theory to other ligand-receptor systems while presenting the limitations of our model.

III. MOLECULAR THEORETICAL APPROACH

We use a mean-field molecular theory^{33,34} that considers the size, shape, and conformation of every molecular type explicitly. The system of interest here is the binding of streptavidin to biotin localized at the oil/water interface as presented in the Chao et. al experiment.³¹ Streptavidin is basically a rectangular box with the dimensions $5.8 \times 5.4 \times 4.8 \text{ nm}^3$ and four biotin binding sites, two on each of its largest opposing faces. The distance between two binding sites on the same face is about 2.0 nm .⁴⁴ Being amphiphilic in nature, C18-PEO(3000)-biotin molecules cover the oil/water interface presenting biotin on the water side of the interface, making it accessible for binding. We consider the possibility of the bound proteins to have different orientations with respect to the surface, and denote the angle between the protein and the surface by θ . Each streptavidin can bind up to two biotins. The biotins can bind on the same face or one in each face of the protein. We explicitly consider two types of tethered polymer chains: bound polymers, (b), with their terminal biotins bound to a streptavidin, and free tethered polymers, (f), those that are not bound to streptavidin. The polymers can be found in many types of conformations, which are described in detail in the Appendix. For the purpose of this paper, a streptavidin molecule with two polymer molecules bound to it, will be referred to as a bound pair.

The experimental system uses a curved drop, however, the curvature of the interface is so small in terms of the polymers and the proteins that for all practical purposes it can be considered planar in terms of molecular scales. Thus, we model the interface as planar. Furthermore, we do not include specifically the effects of the C18 hydrophobic tails since their size is much smaller than that of the polymer and therefore they do not strongly interact with each other. This is justified also by the experimental density of tethered polymers on the surface which is low enough for the C_{18} chains not to see each other, see Figure 2 and discussion thereafter. Based on our previous work in determining the pressure area isotherms of PEO at water-hydrophobic interfaces, which are in very good agreement with experimental observations,⁴⁵ we include attractions between the interface and PEO segments.

The system that we consider, see Fig.1, is composed of a surface of area A that spans the x - y plane at the origin of the z axis. Tethered polymer chains, either bound or free, are only allowed in the $z \geq 0$ half-space. The coverage of bound pairs is expressed as: $\sigma_b = N_b/A$, that is, as a

surface coverage of bound proteins, where N_b is the number of bound pair. The surface coverage of free tethered chains is given by $\sigma_f = N_f/A$, where N_f is the number of free tethered polymers. Both bound and free tethered polymers have the same chain length N and each EO segment has a volume v_p . The aqueous environment has N_w water molecules with a volume v_w each, which is the unit of volume used throughout the paper. The existence of an interface induces an inhomogeneous distribution of all the molecular species involved. We consider that the only inhomogeneous direction is the one perpendicular to the interface, i.e., the z -direction.

The total free energy per unit area of the streptavidin-biotin binding system is given by

$$\frac{\beta F}{A} = -\frac{S}{k_B A} - \frac{S_w}{k_B A} + \frac{\beta U_{surf}}{A} + \frac{\beta U_{inter}}{A} + \frac{\beta U_{rep}}{A} \quad (1)$$

The first term in Eq. (1) denotes the entropy per unit area of bound/free tethered chains, which is given by

$$\begin{aligned} \frac{-S}{k_B A} = & \sigma_b \sum_{\alpha_1} P_b(\alpha_1) \ln P_b(\alpha_1) + \sigma_f \sum_{\alpha_2} P_f(\alpha_2) \ln P_f(\alpha_2) \\ & + \sigma_b \ln \sigma_b + \sigma_f \ln \sigma_f \end{aligned} \quad (2)$$

where the first and second terms account for the conformational entropy of bound/free tethered chains. The third and forth terms correspond to the translational entropy of these two types of chains. $P_b(\alpha_1)$ and $P_f(\alpha_2)$ are the probability distribution functions (pdf) of finding a pair of bound chains in conformation α_1 and a free tethered chain in conformation α_2 , respectively. Given the pdf we can calculate any thermodynamical and structural quantity of bound/free tethered polymer and protein. For example, the bound/free tethered polymer and bound protein volume fraction can be expressed as follows

$$\langle \phi_b(z) \rangle = \sigma_b \sum_{\alpha_1} P_b(\alpha_1) v_p(\alpha_1; z) = \sigma_b \sum_{\alpha_1} P_b(\alpha_1) n(\alpha_1; z) v_p \quad (3)$$

$$\langle \phi_f(z) \rangle = \sigma_f \sum_{\alpha_2} P_f(\alpha_2) v_p(\alpha_2; z) = \sigma_f \sum_{\alpha_2} P_f(\alpha_2) n(\alpha_2; z) v_p \quad (4)$$

$$\langle \phi_{pro}(z) \rangle = \sigma_b \sum_{\alpha_1} P_b(\alpha_1) v_{pro}(\alpha_1; z) \quad (5)$$

Here $v_p(\alpha_1; z)dz$ ($v_p(\alpha_2; z)dz$) denotes the volume of PEO segments in conformation α_1 (α_2) that contribute to the layer between z and $z+dz$. This volume is equal to the number of segments $n(\alpha_1; z)dz$ ($n(\alpha_2; z)dz$) multiplied by the volume of each EO segment v_p . $v_{pro}(\alpha_1; z)dz$ is the volume of protein bound to the pair of chains in conformation α_1 in the layer between z and $z + dz$.

The second term in Eq. (1) is the z -dependent translational (mixing) entropy of the water molecules, which is given by

$$\frac{-S_w}{k_B A} = \int dz \rho_w(z) [\ln \rho_w(z) v_w - 1] \quad (6)$$

where $\rho_w(z)$ corresponds to the number density of water molecules at z . The volume fraction of water is given by $\phi_w(z) = \rho_w(z) v_w$.

The third term in the free energy, Eq. (1), describes the attraction between the interface and polymer segments. We model these interactions by a square well potential of range δ and thus the total attraction of the polymer segments with the surface is given by

$$\frac{\beta U_{\text{surf}}}{A} = \sigma_b \beta \varepsilon \sum_{\alpha_1} P_b(\alpha_1) n(\alpha_1; 0) + \sigma_f \beta \varepsilon \sum_{\alpha_2} P_f(\alpha_2) n(\alpha_2; 0) \quad (7)$$

where $n(\alpha_1; 0)$ and $n(\alpha_2; 0)$ denote the number of bound and free tethered polymer segments within a distance δ from the surface and ε is the depth of the square well potential which is chosen as $-1.0 k_B T$. This value is taken from previous work, where the predictions of the theory are in very good agreement with experimental observations.⁴⁵

The forth term in the free energy expression, Eq. (1), describes the effective intermolecular attractions between proteins. The reason for including protein-protein attractions is that there is experimental evidence that streptavidin on lipid layers tends to form two dimensional crystals.⁴⁰⁻⁴³

$$\frac{\beta U_{\text{inter}}}{A} = \frac{\beta \chi}{2 v_w} \int dz \sigma_b \sum_{\alpha_1} P_b(\alpha_1) v_{\text{pro}}(\alpha_1; z) \langle \phi_{\text{pro}}(z) \rangle \quad (8)$$

where the χ parameter is a measure of the strength of the attractions between the proteins. We use χ is $-1.07 k_B T$, which was obtained from the case where the theoretical model achieves accurate experimental values for the interfacial tension of the bound layer during drop compression.

The last term in the total free energy expression, Eq. (1), represents the repulsive interactions of the system. These are modeled as hard-core repulsions and can be written in the form

$$\frac{\beta U_{\text{rep}}}{A} = \beta \int dz \pi(z) [\langle \phi_b(z) \rangle + \langle \phi_f(z) \rangle + \langle \phi_{\text{pro}}(z) \rangle + \phi_w(z)] \quad (9)$$

where $\pi(z)$ represents the position dependent repulsive interaction field. This field is determined by the requirement that the total volume at each distance z is filled with either polymers, proteins or solvent. In other words, we impose packing constraints associated with the excluded volume interactions, which are z -dependent and have the form

$$\langle \phi_b(z) \rangle + \langle \phi_f(z) \rangle + \langle \phi_{\text{pro}}(z) \rangle + \phi_w(z) = 1; (z \geq 0) \quad (10)$$

The free energy per unit area now becomes:

$$\begin{aligned}
\frac{\beta F}{A} = & \sigma_b \left[\sum_{\alpha_1} P_b(\alpha_1) (\ln P_b(\alpha_1) + \beta \varepsilon n(\alpha_1, 0)) \right] \\
& + \sigma_f \left[\sum_{\alpha_2} P_f(\alpha_2) (\ln P_f(\alpha_2) + \beta \varepsilon n(\alpha_2, 0)) \right] \\
& + \sigma_b \ln \sigma_b + \sigma_f \ln \sigma_f \\
& + \int dz \rho_w(z) [\ln \rho_w(z) v_w - 1] \\
& + \frac{\beta \chi}{2v_w} \int dz \sigma_b \sum_{\alpha_1} P_b(\alpha_1) v_{\text{pro}}(\alpha_1; z) \langle \phi_{\text{pro}}(z) \rangle \\
& + \int dz \beta \pi(z) [\langle \phi_b(z) \rangle + \langle \phi_f(z) \rangle + \langle \phi_{\text{pro}}(z) \rangle + \phi_w(z) - 1]
\end{aligned} \tag{11}$$

After the binding of streptavidin to the biotin functionlized PEO spacer, the number of bound polymers and bound proteins is fixed. However, the free tethered polymers are in contact with the chloroform drop solution, and thus may exchange with those dissolved in the drop solution. Therefore, the calculations have to be performed in the semigrand canonical ensemble. The semigrand potential density is

$$\frac{\beta W}{A} = \frac{\beta F}{A} - \beta \mu_f \sigma_f \tag{12}$$

Here the chemical potential of free tethered polymers μ_f is same as that for polymers dissolved in the chloroform drop solution. In other words, the free tethered polymers remain at a constant chemical potential during the whole experiment.

Minimizing of Eq. (12) with respect to $P_b(\alpha_1)$ and $P_f(\alpha_2)$, we get:

$$\begin{aligned}
P_b(\alpha_1) = & \frac{1}{q_b} \exp \left[-\varepsilon n(\alpha_1, 0) - \int dz \beta \pi(z) (n(\alpha_1, z) v_p + v_{\text{pro}}(\alpha_1; z)) \right. \\
& \left. - \frac{\beta \chi}{v_w} \int dz v_{\text{pro}}(\alpha_1; z) \langle \phi_{\text{pro}}(z) \rangle \right]
\end{aligned} \tag{13}$$

and

$$P_f(\alpha_2) = \frac{1}{q_f} \exp \left[-\varepsilon n(\alpha_2, 0) - \int dz \beta \pi(z) n(\alpha_2, z) v_p \right] \tag{14}$$

Here q_b and q_f are normalization constants ensuring that $\sum_{\alpha_1} P_b(\alpha_1) = 1$ and $\sum_{\alpha_2} P_f(\alpha_2) = 1$. Note that the excluded volume interactions, the surface interaction and interactions between proteins enter into the pdf via the expected Boltzmann factor. This implies that the probability of conformations chains adopt is determined by interactions arising from the environment, which leads to the difference in the results between inhomogeneous confined systems and homogeneous bulk solutions.

Meanwhile, the volume fraction of water is given by:

$$\phi_w(z) = \rho_w(z) v_w = \exp[-\beta \pi(z) v_w] \tag{15}$$

In Eq. (15), the position dependent repulsive fields, $\beta \pi(z)$, are the z -dependent osmotic pressures, which are associated with the inhomogeneous distribution of solute and solvent. Thus, they are associated with the lateral repulsion created by having to pack all molecular species in an inhomogeneous environment due to the presence of the interface.

Substituting the expression for the pdf, Eq. (13) and Eq (14), and the water volume fraction, Eq. (15) into the free energy Eq. (11), gives the minimal free energy

$$\frac{\beta F_{\min}}{A} = -\sigma_b \ln q_b - \sigma_f \ln q_f + \sigma_b \ln \sigma_b + \sigma_f \ln \sigma_f - \int dz \rho_w(z) - \int dz \beta \pi(z) - \frac{\beta \chi}{2v_w} \int dz \sigma_b \sum_{\alpha_1} P_b(\alpha_1) v_{\text{pro}}(\alpha_1; z) \langle \phi_{\text{pro}}(z) \rangle \quad (16)$$

The minimal free energy, Eq. (16), represents the thermodynamic potential of the system, and therefore, all other thermodynamic quantities of interest can be obtained by taking the appropriate derivative. One of the most important quantities is the lateral pressure or surface pressure, Π , which is related to decreases in the interfacial tension; the quantity that was

measured experimentally.³¹ This is obtained by taking $\Pi = - \left(\frac{\partial F}{\partial A} \right)_{N_b, N_f, T}$. Using Eq. (16), the lateral pressure of PEO-biotin+streptavidin layer becomes:

$$\beta \Pi = \sigma_f + \sigma_b + \int dz \rho_w(z) + \int dz \beta \pi(z) + \frac{\beta \chi}{2v_w} \int dz \sigma_b \sum_{\alpha_1} P_b(\alpha_1) v_{\text{pro}}(\alpha_1; z) \langle \phi_{\text{pro}}(z) \rangle \quad (17)$$

The unknowns in the above equations are the position dependent repulsive fields, which are determined by substituting (Eq. 13–Eq. 15) into the packing constraints, Eq. (10). In practice, we discretize space and thereby convert the integral equations into a set of coupled nonlinear equations, which are solved numerically. The inputs necessary to solve those equations include two sets of conformations for bound and free tethered polymers, the surface coverage for both of them, the strength of the interaction between polymers and the surface, and the interaction parameter χ between proteins. Details concerning the discretization, the generation of the conformations of the polymers and numerical methodology used to solve the equations can be found in the Appendix.

Before turning to the results, it is important to emphasize that all bound proteins have two polymer chains attached to them in our model. The reason is that given that the typical individual biotin-streptavidin binding free energy is $35k_B T$, the probability of having only one chain bound is of the order of $\exp(-35)$ times lower than with two bound polymers. Therefore, it is assumed that the bound proteins always have two chains irreversibly attached to them.

IV. RESULTS AND DISCUSSIONS

As mentioned previously, the surface coverage of polymers is used as an input in our calculations. However, the experiment³¹ we are modeling did not measure the coverage of adsorbed C18-PEO-biotin molecules directly. Instead, DSA measures the surface tension, which is equivalent to measuring the rise in lateral pressure exerted by the adsorbed polymers. Thus, determining the full pressure-area isotherm from the theory and then using the value of the pressure that corresponds to the reduction of surface tension observed experimentally provides for the area per polymer surfactant adsorbed at the interface. To this end, we shown in Figure 2(a) the calculated pressure-area isotherm (full line) and a dashed line that represents the value of the surface tension reduction measured by DSA (from 32.8 (pure chloroform) down to 19.8 mN/m (chloroform + C18-PEO-biotin)). From the point where the two lines cross, we obtain the surface coverage of polymers for the DSA experiment which was found to be 0.217 nm^{-2} , i.e., the area per molecule is 4.61 nm^2 . We believe that this is a reliable result due to our previous quantitative prediction of the pressure area isotherm of related PEG systems.

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The next step is to consider the binding of proteins to the biotin ligand at the terminal end of the PEO chains. As we mentioned previously, the binding of the proteins is irreversible within the time scale of the experiments. Thus, in order to determine the amount of bound streptavidin we performed a calculation in which we varied the number of bound proteins, while the number of free tethered polymers is determined from the equilibrium condition that their chemical potential must be the same as that of the polymer before the addition of proteins, i.e. the μ_f of the polymer dissolved in the solution. Figure 2(b) presents the surface pressure as a function of the surface coverage of bound pairs, which is obtained from Eq.(17). The horizontal line marks the value of the pressure corresponding to a further reduction of 2.4 mN/m in the surface tension upon the addition of proteins that was found in the experiment. From this data we find that the surface coverage of bound proteins is $\sigma_b = 0.0128 \text{ nm}^{-2}$, or equivalently the area per protein is 78.1 nm^2 , which implies a small amount of PEO-biotin is actually binding to the proteins.

The experimental observations correspond to a particular point in phase space. However, the theoretical predictions cover a much wider range of variables, where some interesting behavior is predicted. For example, the inset in Figure 2(b) displays a portion of the curve where the pressure decreases with increasing σ_b , i.e. the interface shows a negative isothermal compressibility. This implies that the homogeneous layer is not stable at these surface coverages and in its place the formation of protein domains is expected. These domains are the result of attractive interactions between the proteins driving them to aggregate only when a small amount of bound proteins is present. As the number of bound proteins increases, the repulsive interaction become more important. Therefore, a competition between attractive and repulsive interactions determines the stability of the polymer-protein layer. In the results presented in Figure 2(b) the isotherm shows a van der Waals loop, where the pressure decreases with the increasing surface coverage of bound proteins. The loop indicates the bound layer will phase separate into two different phases. The phase separation is predicted in the regime of $(0.0101 \text{ nm}^{-2} < \sigma_b < 0.0117 \text{ nm}^{-2})$. Interestingly, the binding observed experimentally corresponds to a surface coverage where the system is stable in a one phase region of relatively high density of bound proteins and therefore the experiments do not indicate the presence of the phase transition. It is not clear whether changing the bulk concentration of streptavidin in the experimental systems will lead to the observation of the phase separation region. The reason is that, as mentioned above, the binding is effectively irreversible and therefore, the surface should be saturated with proteins even at infinitesimal bulk concentrations. Therefore, controlling the binding is more reasonable for systems where the ligand-receptor dissociation constants are larger than biotin-streptavidin.

Next we compare the predictions from theory and experimental results for drop compression, that is, reduction of the of drop volume, and hence decreasing the drop surface area, after binding of streptavidin proteins is allowed to reach a kinetic equilibrium. We consider the system to have a fixed amount of bound proteins and compress it reducing the area by roughly 30% following the experimental results. Because the free tethered polymers are in equilibrium with those dissolved in the bulk drop solution, they have a constant chemical potential, μ_f . We calculate the surface tension of the polymer-protein layer γ from the identity $\Pi = \gamma_0 - \gamma$, where γ_0 is the surface tension of the PEO-biotin layer without the presence of proteins. Since the amount of proteins bound to the PEO-biotin layer is fixed during the drop compression experiment, the surface coverage of proteins increases as the drop surface area decreases. The calculated results, shown in Fig. 3, are extracted from Fig. 2 (b) and match the compression performed in the actual DSA experiment. The agreement between the theory and the experiments is remarkable. These results have already been presented in Ref.[31]. The very good agreement between experimental results and the theoretical calculations provides confidence that we can use the theory to predict the structure of the interface and the arrangement of the polymers and proteins. Furthermore, we can use the theory in regimes where

the experiments have not been done and use the theory not only to explain experimental observations but also as a quantitative design tool.

There are three types of species that we need to describe to obtain a whole picture of how the polymer and proteins organize. They are the free tethered polymers, the bound proteins and the bound polymers. Figure 4 displays the surface coverage of free tethered polymers as a function of the surface coverage of bound pairs. The free tethered polymers are in equilibrium with those in the bulk drop chloroform solution. Therefore, their surface density is determined by the conditions at which the chemical potential of the molecules at the interface is the same as that of the molecules in solution or, equivalently, to the chemical potential of the tethered chains in the absence of proteins. It is clear that the presence of bound proteins results in the high pressure of the bound layer. Hence, the number of free tethered polymers decreases as the amount of bound proteins increases, in order to maintain equilibrium with the free polymers in the bulk solution. In Fig. 4, σ_b within the range of $[0.0128; 0.0183]nm^{-2}$ corresponds to the process of compression that was performed experimentally using the DSA. In this region the density of free tethered polymers continuously decreases. It shows a larger decrease in the phase separating regime ($0.0101nm^{-2} < \sigma_b < 0.0117nm^{-2}$), which implies there are big changes in the surface structure in this region.

The structure of the polymer layer is well described by the volume fraction profiles, that is, the variation of the local volume occupied by different species as a function of the distance from the interface. First, we explore how the the distribution of free tethered polymers changes during the different experimental conditions. Figure 5 provides the variation of polymer density of free tethered polymers as a function of the distance form the surface for three different conditions: the PEO-biotin layer in the absence of adsorbed proteins, the PEO-biotin +streptavidin before compression and PEO-biotin+streptavidin after drop compression and hence at a reduced surface area. A common feature of these three profiles is that a large amount of the PEO chains show many segments adsorbed to the interface due to attractive interactions between PEO and interface, followed by a region that shows the characteristics of a relatively extended polymer brush. This region serves as a barrier between the interface and large molecules like proteins and inhibits non-specific protein adsorption effectively. Comparing the three profiles, one can see that the polymer brush region shrinks with the presence of bound proteins, and further shrinks after compression when a larger surface coverage of bound proteins is present. These results imply that there is a decrease in the amount of free tethered polymers upon protein binding and then a further decrease upon drop compression. The results for the structure of the free tethered chains are another manifestation of the surface coverage variations presented in Fig. 4.

Figure 6(a) shows the volume fraction of the bound proteins both before and after drop compression. Due to the fixed number of proteins in the two cases, the surface coverage of bound proteins changes from $0.0128nm^{-2}$, before compression with the surface area $A = A_{max}$, to $0.0183nm^{-2}$ where the drop area is compressed with $A = 0.7A_{max}$. The presence of the polymer segments in the vicinity of the interface results in a depletion of the proteins $5nm$ from the interface. This is the result of the steric barrier that the PEO layer presents and is the mechanism also in many non-fouling surfaces^{22,23,25}. The smallest distance between the surface and bound proteins is about the thickness of the polymer brush.

In the compressed state the formation of the two-layer structure can be clearly seen with two distinct bound protein peaks. These two protein layers exist before compression but since they are very close to each other, they actually overlap somewhat giving rise to the single broad peak that appears in the volume fraction profile and indicates a relatively thick protein layer. After compression however, two protein layers are separated by a small distance giving rise

to the distinct peaks, because the steric repulsion between proteins increases with the increasing density of bound proteins.

Fig. 6(b) displays the distribution of orientations as a function of the angle between a bound protein and the surface. $P(\theta)$ is obtained as the sum of all the probabilities of bound proteins that have the same orientation, regardless of their separation from the interface. The distributions demonstrate that in the absence of compression, most of the proteins have their orientation parallel to the interface. When the layer is compressed the preferred orientation changes to the perpendicular one, even though the parallel orientation remains very important. The change in orientation with compression is because the bound proteins avoid large steric repulsions by reorienting, relaxing in that way some of the effect of the unfavorable compression.

Figure 6(c) shows the density profile for the PEO-biotin chains bound with proteins. There is a large qualitative difference between these profiles and those of the free tethered chains shown in Fig. 5. The bound chains are highly extended. The density of segments for these chains is somewhat higher in the region close to the interface than in the region far from the surface. According to the profile of bound proteins shown in Fig. 6(a), one can determine that polymers within the higher density regime are bound to the first layer of proteins, which are closer to the interface. The highly stretched polymer chains are those that are bound to proteins located in the second layer, which are further away from the interface. The stretching of the polymer chains is due to the need of the proteins to arrange themselves in an optimal structure which reduces repulsions, while still being bound to the terminal biotin on the PEO chains. The flexibility of the spacers enables the formation of a layer that is not strictly two dimensional, in contrast with what has been observed previously for the binding of streptavidin directly to the heads of lipids^{40–43}. The three dimensional layer seen in Fig. 6 optimizes maximal protein binding, without resulting in very large repulsions that arise from very high local densities of proteins. Furthermore, the disordered nature of the bound layer is favorable from the entropic point of view.

Streptavidin has four biotin binding sites, and with only two of them bound to the biotin at the terminal end of the PEO chains. The two other sites are thus free and suitable for binding other biotin functionalized molecules or proteins that can be used for assembly of more complex structures.^{46,47} However, due to the two-layered distribution of bound proteins, roughly one half of the bound streptavidin molecules are inaccessible for further binding as they reside in the inner layer that is closer to the interface and would be screened by the proteins in the outer layer. Therefore, from the interfacial design point of view, this arrangement of the proteins is optimal for maximal binding of the streptavidin to the surface, however it is very inefficient if the proteins are to be used as a template for further binding from solution.

The phase separation, see van der Waals loop in Fig. 2(b), was predicted in the same regime where a large decrease in the surface coverage of free tethered polymer chains is observed, see Fig. 4. The question to address is: what are the changes in the polymer-protein layer that drive the phase separation. Namely, is there a significant change in structure of the polymer-protein layer change in the two coexisting phases? To answer this question, Fig. 7(a) provides the volume fraction for bound proteins at different bound protein surface coverages. When the number of bound protein is small, $\sigma_b = 0.006\text{nm}^{-2}$, the proteins are located in a single monolayer. As the amount of bound proteins increases, the structure of the layer changes since the protein layer becomes thicker. This is the way that the bound proteins optimize the competition between the repulsions and attractive interactions. This can clearly be seen in Fig. 7(a) for $\sigma_b = 0.009\text{nm}^{-2}$. The thickness of the bound protein layer is larger than what would be expected for a monolayer of streptavidin, but it is smaller than the two-layered structure seen when $\sigma_b = 0.012\text{nm}^{-2}$. Since the phase separation happens when σ_b is within the range of

0.0101 to 0.0117 nm^{-2} , it is within this region that the distribution of bound proteins transitions from one layer to two layers in what we call a layer transition. The transition in structure is the result of the optimal balance between protein binding with attractive and repulsive interactions of the proteins. Fig. 7(b) shows the thickness of bound proteins as a function of the surface coverage of bound proteins. We define the thickness by $H = z_2 - z_1$, where

$\phi_{\text{pro}}(z_1) = \phi_{\text{pro}}(z_2) = \phi_{\text{pro}}^*/2$, where ϕ_{pro}^* is the maximum value of the volume fraction of bound proteins. This definition provides an appropriate measure for the thickness which is mostly covered by proteins. At low surface coverage of bound proteins there is a small increase in thickness with increases in σ_b . This increase is due to changes in the orientational distribution of the bound proteins. In the region where the van der Waals loop appears in Fig. 2b there is a sharp increase in the thickness that demonstrates that the predicted phase transition is clearly characterized by a change in structure of the bound proteins from one to two layers. At surface coverages of bound proteins higher than the transition there is a weak increase of thickness again due to changes in the orientation of the proteins in order to optimize their packing.

The results shown in Figure 6 and Figure 7 demonstrate that bound proteins can form two-layered structures due to the flexibility of PEO spacers. To further understand the role of polymer spacers on the structure of bound proteins, we studied the case of a short-chain spacer PEO(1100) at the same surface tension as the DSA experiment and compared it with the results from PEO(3000) case. Before the addition of streptavidin, the surface coverage of PEO(1100) is 0.38 nm^{-2} at same reduction in surface tension as the PEO(3000) experimental case where $\sigma = 0.217 \text{ mN/m}$. After the addition of proteins, the surface pressure changes with increasing amounts of bound proteins as shown in Fig. 8. When the density of bound proteins reaches 0.0086 nm^{-2} , the reduction of surface tension is 2.4 mN/m, which corresponds to the PEO(3000) experimental case where the density of bound proteins is $\sigma_b = 0.0128 \text{ nm}^{-2}$. This indicates that the efficiency of biotin-streptavidin binding decreases with shorter spacers. In addition, the pressure curve in Figure 8 shows a van der Waals loop, which implies the existence of phase separation. In this case the density of bound proteins needed to achieve phase separation is higher than the one needed in the longer spacer case.

It should be mentioned that we carry out the comparisons between the two chain lengths of PEO assuming the same reduction of surface tension for both the adsorption of polymers to the interface and the binding of the proteins. There is no particular reason for this choice, but we need to keep one of the variables constant to perform the comparison. We could expect the reduction of the surface tension upon binding of the protein to be the determining factor. However, we could have chosen to compare for the same amount of bound proteins. Since the aim is to obtain an understanding of the role of chain length, the results presented here provide for a clear picture of the effect.

Figure 9(a) shows the volume fraction profiles of bound proteins for the PEO(1100) case. These profiles are very different from the two-layer structure found with the longer PEO(3000) spacer that was shown in Fig. 6(a). The full line, at $\sigma_b = 0.0086 \text{ nm}^{-2}$, illustrates that most proteins reside within the same layer while a small amount of proteins distribute themselves either closer or further from the interface. The dotted line is the profile when $\sigma_b = 0.0123 \text{ nm}^{-2}$, and corresponds to the case where the surface area of the polymer-protein layer is compressed down to 70% of the original area. The compression causes an enlargement of the area of protein distribution similar to the case shown in Figure 7 with $\sigma_b = 0.009 \text{ nm}^{-2}$ that has a structure between one and two layers. Comparing Fig. 6(a) and Fig. 9(a), one can see that the aggregation of proteins is greater for the PEO(1100) spacer case, and that the region of bound proteins is larger for the PEO(3000) spacer case. Therefore, we conclude that the long chain polymers avoid the large repulsions between proteins at high protein densities by accommodating protein binding in multi-layer conformations through stretching of their PEO spacers. This is in line with systematic predictions of ligand-receptor binding in the presence of polymer spacer.³³

The shorter polymer is hard pressed to accommodate a two-layer structure, and thus the transition shifts to higher values of σ_b as shown in Fig. 8. Only when the surface coverage of bound proteins is large enough, such as when $\sigma_b = 0.0200 \text{ nm}^{-2}$, can the two-layer structure be formed. However, as seen in Figure 8, this state corresponds to very large pressures and therefore to very large reductions of the surface tension that may be hard to observe experimentally. The thickness of the bound proteins as a function of surface coverage is shown in Fig. 9(b). The transition from one to two layers occurs at very large surface coverages of bound proteins compared to the longer chain length polymers, compare Figures 7(b) and Figure 9(b). This result supports our conclusion that the flexibility of the longer chains enables the reduction of the large lateral repulsions by paying in conformational entropy, i.e. chain stretching.

V. SUMMARY AND CONCLUSIONS

We presented a molecular theory that enables the study of the binding of streptavidin to biotin-functionalized PEO molecules at and oil/water interface. The molecular theory was used to model drop shape analysis experiment. Both in the model and the experiment, it was found that the surface pressure increases with the binding of proteins to the biotin located at the terminal ends of the PEO chains. Compression of polymer-protein layer caused a further increase in the surface pressure. These compression curves were successfully modeled showing very good agreement with the experimental results. This result supports the idea that the molecular theory is a good tool to describe polymers and proteins in confined environments. The microscopic structure of the layer is studied by looking at the volume fraction profiles of bound proteins, bound polymers and free tethered polymers. The calculations predict a thick two-layer structure of proteins supported by stretched PEO spacers. The structure of the polymer-protein layer largely depends on spacer length. The multi-layered structure of bound proteins is easier to form and happens at lower volume fractions of bound proteins with longer spacers than with shorter ones. These results are important as they serve in the design of experimental systems. Namely, if the need is to maximize protein binding, it is favorable to be above the layer transition, as multiple layers can accommodate greater bound protein densities and thus longer spacers would be preferred.³³ On the other hand, if the goal is to bind to and construct more complex structures, such as when avidin or streptavidin serve as a linker group between two biotinylated polymers or proteins, it is advisable to use shorter spacers and be below the layer transition such that all bound linker proteins are available for further binding. In this particular case, more proteins is not always better as part of the linker groups become inaccessible for further binding as molecules approach the layer from solution. This is the result of the steric barrier that the proteins facing the solution present to the molecules attempting to reach the inner bound proteins.

The stability of the polymer-protein layer was studied. With a small amount of bound proteins, the layer becomes unstable, and protein domains (aggregates) are predicted due to attractive protein-protein interactions. A competition between these attractive interactions and the steric repulsions between proteins arises with increasing amounts of bound proteins. As the number of bound proteins increases a transition happens where phase separation occurs and the bound proteins go from being located in one layer to a thick (multi-)layer structures.

It would be very interesting to run experiments with C18-PEO-Biotin molecules with shorter PEO spacers as the layer transition should be accessible through drop compression. One would expect that the interfacial tension would decrease during compression until the layer transition is reached. Inside the layer transition region interfacial tension should remain constant even as the drop area changes. This experiment would be a further test for the predictions of the theory presented in this paper.

It is important to note that the theoretical approach presented in this work is general, and can be applied to other ligand-receptor binding systems. For example, it could be used to model ligand-receptor binding on a lipid membrane.⁴⁰ The changes that need to be made to study other systems involve using the proper molecular model to generate the conformations for lipids, as has been done for mixtures of lipids and cholesterol.⁴⁸ For more complicated cases, such as cellular adhesion driven by specific binding,^{49,50} changes to take into account the different conformations of receptors, and possibly further intermolecular interactions are needed.

The theory not only provides good agreements to the experiment, but also gives a wealth of information on microscopic details, which helps understand the underlying mechanism of ligand-receptor binding in the presence of spacers and further can serve as design tool. It is important to emphasize that the theoretical approach is based on an average of the interactions; i.e. it is a mean field approach. The good predictive power of the theory is due to the fact that, in most cases that we have studied, the correlations that are not accounted for by the theory do not seem to be the most important contributions in determining the behavior of the system. In any case, there are several things that can be done in order to improve the theory. First, we need to develop a systematic way to build the molecular models for different types of molecules, including proteins. This can be achieved in a systematic way by using bulk molecular dynamic simulations and from them build the specific coarse-grained model, depending on their chemistry and their effective interaction parameters. Second, we need to solve the theory taking into account three-dimensional inhomogeneities, so that we can study domain formation, such as the protein domains predicted in the present work, and microphase segregation. The molecular theory has been extended to treat systems with more than one dimensional inhomogeneities,⁵¹ and further work with more complex systems is underway.

The molecular design of optimal interfaces or surfaces for binding depend upon the size of the binding moiety, the length of the polymer spacer, the ability to control the number of molecules at the surface, e.g. through chemical grafting, or the constraint of thermodynamic equilibrium like in the systems studied here, the specific details of the solution in contact with the surface/interface. The work presented here, together with previous calculations from our work^{33,34,52} as well as others^{35,36}, show that the use of theory and simulations enables the study and role of each and all of the mentioned parameters. Thus the combination of the theoretical predictions with experimental observations will enable the molecular design of interfaces and surfaces with optimal binding conditions for a large variety of applications.

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VI. APPENDIX

The free tethered polymer chains are generated as they were in previous work.^{33,34} The chain model is the three-state RIS model.⁵³ In this model, each bond has three different isoenergetic states. The conformations are generated by a simple sampling method and all the accepted conformations are self-avoiding and cannot penetrate into the surface/interface. We generated a set of 2×10^6 independent conformations for polymer chains which are not bound to proteins. The conformations of bound chains are generated by Molecular Dynamics simulations using Gromacs. This was necessary because of the highly stretched conformations that exist in the case of binding, which cannot be generated by simple sampling methods, see discussion in ref.³⁴. We use MD simulations to generate 10^5 (independent) conformations with a fixed end-to-end distance. The end-to-end distance is then varied in the range of $0 < z \leq z_{max}$, where $z = 0$

is the grafted surface/interface, and z_{max} is the fully stretched chain. We divide the range in 40 possible distances and thus for the bound polymer we generate 40×10^5 possible conformations, 10^5 corresponding to each distance. The justification for the use of the method and its consistency is discussed in ref.³⁴.

The numerical solution for the position dependent osmotic pressure $\pi(z)$ is obtained by discretization of the packing constraints expressed by Eq. (10). This is done by dividing the z -axis into parallel layers of thickness δ . Functions are assumed to be constant within a layer, hence integrations can be replaced by summations. The i_{th} layer is defined as the region between $(i-1)\delta \leq z < i\delta$. The packing constraints, Eq. (10), for layer i in discrete form read

$$1 = \frac{\sigma_b}{\delta} \sum_{\alpha 1} P_b(\alpha_1) n(\alpha_1; i) v_{pol} + \frac{\sigma_f}{\delta} \sum_{\alpha 2} P_f(\alpha_2) n(\alpha_2; i) v_{pol} + \frac{\sigma_b}{\delta} \sum_{\alpha 1} P_b(\alpha_1) v_{pro}(\alpha_1; i) + \phi_w(i) \quad (18)$$

Eq. (13), Eq (14) and Eq (15) in discrete form are given by

$$P_b(\alpha_1) = \frac{1}{q_b} \exp[-\varepsilon n(\alpha_1, 1)] \exp[-\beta \sum_{i=1}^{i=i_{max}} \pi(i) (n(\alpha_1, i) v_{pol} + v_{pro}(\alpha_1; i)) - \frac{\beta \chi}{v_w} \sum_{i=1}^{i=i_{max}} v_{pro}(\alpha_1; i) \phi_{pro}(i)], \quad (19)$$

$$p_f(\alpha_2) = \frac{1}{q_f} \exp[-\varepsilon n(\alpha_2, 1)] \exp[-\beta \sum_{i=1}^{i=i_{max}} \pi(i) n(\alpha_2, i) v_{pol}] \quad (20)$$

and

$$\phi_w(i) = \exp[-\beta \pi(i) v_w]. \quad (21)$$

where $1 \leq i \leq i_{max}$ (i_{max} is the total number of layers considered in the system). Substituting Eq. (19), Eq. (20) and Eq. (21) into the discretized constraint equation (18), results in a set of coupled nonlinear equations that are solved by standard numerical methods.

Parameters used in the calculations are listed as follows: the degree of polymerization of PEO (3000) is $N = 75$, and PEO(1100) is $N = 25$, each segment of the polymers has a length $l_{seg} = 0.32nm$, and a volume $v_p = 0.06nm^3$ which was chosen according to the partial molar volume of PEO in water,⁵⁴ the volume of a water molecule is $v_w = 0.03nm^3$, and the layer thickness is $\delta = 0.48nm$, which is sufficient to obtain accurate solutions for the given set of equations. Changing the value of δ to smaller values did not change any of the quantitative results. Streptavidin is modeled as a rectangular box with the size of $5.8 \times 5.4 \times 4.8nm^3$.⁴⁴

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FIG. 1.

Schematic representation of the modeled system. Biotin (green) is chemically attached to the free ends of PEO chains (purple) tethered to a planar interface at the other end-group. Free tethered polymers (thin purple lines), are those that are not bound to streptavidin. Bound streptavidin proteins (light blue) are linked to two PEO chains (thick purple lines) through their biotins. The free tethered polymers are in equilibrium with free polymers in solution (not shown), while the density of bound proteins (and bound polymers) is fixed. The bound proteins may be found in a variety of different orientations with respect to the surface, see text. The direction perpendicular to the surface is denoted by z .

FIG. 2.

(a) The calculated lateral pressure, Π (full line), as a function of the area per PEO chain, a . The dashed horizontal line represents the lateral pressure corresponding to the reduction in the surface tension that was measured experimentally. (b) The lateral pressure (full line) as a function of the surface coverage of bound proteins. The dashed line corresponds to a further reduction in the surface tension of 2.4 mN/m. The insert shows the region of instability at small surface coverage of proteins where domain formation is expected, σ_b is within the range of $[0; 0.004] \text{ nm}^{-2}$.

FIG. 3. The surface tension as a function of the surface area. The area is scaled by the maximal area. The full line is the theoretical prediction. The dashed line, dotted line and dot-dashed line are the experiment observations. These results have already been shown in a previous paper.³¹ and are reproduced here for completeness.

FIG. 4. The surface coverage of free tethered (unbound) PEO-biotin chains, σ_f , as a function of the surface coverage of bound proteins, σ_b .

FIG. 5.

The volume fraction of the free tethered (unbound) PEO-biotin chains as a function of the distance from the surface. The dot-dashed line corresponds to the polymers in the absence of bound proteins, the full line corresponds to the free tethered polymers in the presence of bound proteins at the surface coverage derived in Figure 2. The dashed line is for the case in which the surface is compressed reducing the area down to $70\%A_{max}$, see Figure 3.

FIG. 6.

(a) The volume fraction of the bound proteins as a function of the distance from the surface; (b) the orientational distribution of bound protein and (c) the volume fraction of bound PEO chains as a function of the distance from the surface. The full line corresponds to the case before compression while the dashed line is for the maximal compression shown in Figure 3.

FIG. 7.

(a) The volume fraction of the bound proteins as a function of the distance from the surface for three different surface coverage of bound proteins. $\sigma_b = 0.006$ (full line), 0.009 (dotted line), and 0.012 nm^{-2} (dashed line). (b) The thickness of the protein layer as a function of the surface coverage of bound proteins. The circles correspond to the three surface coverages shown in (a).

FIG. 8.

The surface pressure (full line) as a function of the surface coverage of bound proteins with a short spacer, PEO (1100). The dashed line corresponds to a reduction of surface tension of 2.4 mN/m, equivalent to the experimental reduction shown in Figure 2.

FIG. 9.

(a) The volume fraction of bound proteins as a function of the distance from the interface for short spacers PEO(1100). The full line corresponds to the case before surface compression where $\sigma_b = 0.0086 \text{ nm}^{-2}$. The dotted line represents the case in which the surface is compressed down to $70\% A_{max}$, and σ_b is equal to 0.0123 nm^{-2} . The dashed line is the case with $\sigma_b = 0.0200 \text{ nm}^{-2}$. (b) The thickness of the protein layer as a function of the surface coverage of bound proteins. The circles correspond to the three surface coverages shown in (a).