On Modeling Protein Diffusion: Mobility of Colloidal Particles on Supported Lipid Bilayers

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Abstract

Theoretical results have shown that in cells and other biological systems the geometry of the membrane controls diffusion of membrane associated proteins. Our aim is to develop an artificial system using colloidal particles on SLB to study this phenomenon. In quantitatively analyzing the linking mechanism of the Avidin-Biotin bond with respect to particle mobility we have conducted a series of experiments dedicated to optimizing the conditions of our system.

Keywords: cell membrane, lipid bilayer, membrane protein, colloidal particles, geometry-influenced diffusion.
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Life on earth comes in an immense variety of forms, shapes and sizes. Underneath this huge diversity of life forms lies an astonishingly similar machinery. In particular all known living organisms are made up of cells. (See figure 1.1 for an example of a cellular structure.) Cells are the smallest units of an organism that are able to replicate oneself. From a single cell the whole organism can be generated via cell division and the cell is widely seen as the fundamental biological unit for life [1].

*Figure 1.1: Shown here are mouse intestinal epithelial cells made by in vivo imaging. Various proteins within the cell are fluorescently labelled [2].*

One fundamental structure of the cell is the plasma membrane (figure 1.2), which forms the boundary between the inside of the cell and its surroundings. It is involved in various essential cell processes such as
Introduction

communication between the cell’s interior and environment, transport of specific molecules in and out of the cell, and altering the shape of the cell. The membrane consists of a thin film (around 4 nm thick) called the lipid bilayer with proteins residing in it (integral membrane proteins) or proteins that are loosely bound to the bilayer (peripheral proteins) [1, 3, 4].

Figure 1.2: Sketch of a plasma membrane of an eukaryotic cell. The integral membrane proteins reside within the lipid bilayer, while peripheral membrane proteins lie outside the lipid bilayer. Some peripheral proteins are bound to the bilayer through an integral membrane protein, others are anchored to the bilayer by an attachment to a phospholipid or a glycolipid (lipid chemically attached to sugar chains) [3].

The bilayer is made up of two layers of lipid molecules. These molecules are amphipathic, i.e. they have a hydrophobic (“water-hating”) and a hydrophilic (“water-loving”) end. The most common membrane lipid; a phospholipid (see figure 1.3) consists of one hydrophilic head and two hydrophobic tails. Due to a combination of the amphipathic nature and their shape, the phospholipids spontaneously self-assemble when immersed in an aqueous environment.

In particular, phospholipids are configured in such a way as to minimize the exposure of the hydrophobic tails to water, which results in forming a bilayer (see figure 1.2) with the tails pointing inward and the heads
As first proposed by the fluid mosaic model from Singer and Nicolson in 1972 [6], the lipid bilayer is recognized as a two-dimensional fluid where the individual lipids can move laterally within the bilayer and are subject to diffusion. Furthermore the model regards the integral membrane proteins as embeddings within the fluid bilayer also experiencing lateral diffusive motion. Though still relevant, the model has undergone many alterations and revisions. For example, the membrane proteins are described to be able to move freely laterally along the bilayer, however it was found that they are less mobile than expected and in some cases their motion is strongly constrained [4].

Therefore, different theories have been proposed after the model of Singer and Nicolson in order to explain reduced mobility of membrane proteins [4]. Some of the theories that have been considered are for example: crowding effects due to high concentration of proteins, hydrodynamic effects which occur when a fraction of the proteins are immobile or direct binding of the proteins with immobile structures [7]. However recent studies indicate that among all previous geometry plays a fundamental role in membrane protein mobility.

In particular, theoretical analysis has shown that diffusion of objects on a curved surface is significantly affected by the geometry of the surface [8–10]. Moreover there are several biological systems in which curvature influences diffusion [8]. For example, we can consider the dendritic spines of neuron cells that have a specific shape consisting of a bulbous

\[ \text{Figure 1.3: 3D structure of a phospholipid molecule. We can see a hydrophylic head and two hydrophobic tails [5].} \]
spine head and a spine neck (figure 1.4). At the top of the spine head a contact site with another cell is located (synapse), which allows for chemical signalling to another neuron [1]. Theoretical calculations show that the dendritic spine’s shape confines certain membrane proteins at the site of the synapse [8, 10].

**Figure 1.4:** Shown here is a dendrite (part of a neuron cell) with a small protusion, i.e. the dendritic spine. The dendritic spine has a bulbous spine head attached to a spine neck. The head is part of the synapse which allows for chemical signalling between neurons. It has been shown that the shape of the dendritic spine might influence diffusion of particles in the spine [10]. The image was made by a two photon laser scanning microscope after enhanced green fluorescent protein were used as reporters for expression in neurons. Figure obtained from [11].

Inspired by the theoretical results of geometry-influenced diffusion we would like to observe it experimentally and study it in a quantitative way. However, since cell membranes are difficult to study directly due to their complexity and their interactions with networks inside and outside the cell [12] we aim to create an artificial model. To do this, we have designed a state of the art artificial system mimicking the diffusion of a protein in a membrane with a curved geometry. Our artificial system consists of colloidal particles anchored by linker molecules to a fluid solid supported lipid bilayer with a specific geometry (figure 1.5). By using confocal microscopy, we track the colloidal particles and quantify their motion along the bilayer by calculating the diffusion constant.

Before studying curved surfaces however we would like to study the system on flat substrates. This thesis describes a series of experiments dedicated to optimizing the conditions to study diffusion of colloidal particles
Figure 1.5: Representation of the artificial model we use to mimic diffusion of proteins on a curved lipid bilayer. A colloidal particle of about 1 µm in diameter is linked to the lipid bilayer which is supported by the substrate. The substrate shown here resembles the shape of a dendritic spine [13].

on a supported lipid bilayer created on a flat glass surface. In doing so, we have quantitatively analyzed the linking mechanism between the particles and the supported bilayer.
Chapter 2

Materials & Methods

2.1 Materials: Chemicals, Substances and Substrates

In this table we have listed chemicals, substances and substrates used in the experiments.

<table>
<thead>
<tr>
<th>Description</th>
<th>Short Name</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid 1,2-dioleoyl-sn-glycero-3-phosphocholine</td>
<td>DOPC</td>
<td>Avanti® Polar Lipids</td>
</tr>
<tr>
<td>Lipid 1,2-dioleoylsn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000]</td>
<td>DOPE-PEG2000</td>
<td>Avanti® Polar Lipids</td>
</tr>
<tr>
<td>Lipid 1,2-dioleoylsn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)</td>
<td>DOPE-Rhod</td>
<td>Avanti® Polar Lipids</td>
</tr>
<tr>
<td>Lipid 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[biotin(polyethylene glycol)-2000]</td>
<td>DSPE-PEG2000-Biotin</td>
<td>Avanti® Polar Lipids</td>
</tr>
<tr>
<td>Solvent 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
<td>HEPES</td>
<td>Sigma-Aldrich®</td>
</tr>
<tr>
<td>Solvent calcium chloride</td>
<td>CaCl₂</td>
<td>Sigma-Aldrich®</td>
</tr>
<tr>
<td>Substrate Circular D 263 M colorless borosilicate glass coverslips.</td>
<td>glass coverslip</td>
<td>ThermoScientific™</td>
</tr>
<tr>
<td>Cleaning Solution Milli-Q water</td>
<td>MQ</td>
<td>Millipore</td>
</tr>
<tr>
<td>Cleaning Solution ethanol</td>
<td>EtOH</td>
<td>Sigma-Aldrich®</td>
</tr>
<tr>
<td>Cleaning Solution Hellmanex III</td>
<td>Hellmanex</td>
<td>Hellman® Analytics</td>
</tr>
</tbody>
</table>
2.2 Experimental Setup

This section describes how we create a supported lipid bilayer and which mechanism we used to link colloidal particles to it.

2.2.1 Supported Lipid Bilayer (SLB) Formation

Creating SLB’s is a very popular means to model the cell membrane and investigate its properties [12, 14, 15]. The substrate provides mechanical stability to the bilayer and control of the bilayer’s geometry.

With the creation of a SLB it is fundamental to preserve a crucial property of the bilayer, its mobility. Mobility of the bilayer means that the lipids are all continuously moving and this property depends on the interactions (electrostatic, hydration, van der Waals and steric forces [16]) between the bilayer and the surface of the support. The substrate material and bilayer properties are some of the main parameters that affect mobility.

In this experiment, we have used glass substrates as a support. Glass is the most commonly used material in supporting lipid bilayers [14] and previous efforts within our research group succeeded in obtaining fluid lipid bilayers supported by glass substrates [13].

To coat a substrate with a lipid bilayer we have used the process of automatic SLB formation by small unilamellar lipid vesicles (SUV’s, figure 2.1.) The formation process is still an active research topic and involves vesicle adsorption, rupture of the vesicles into bilayer patches upon contact with the surface and the fusion or spreading of the bilayer patches along the surface (figure 2.2) [14]. There are many methods to create SUV’s. In our experiment we have used the technique of extrusion, which provides a high quantity of vesicles of uniform diameter [19]. The extrusion technique is described in further detail in section 2.6.1.

Figure 2.1: Representation of a small unilamellar lipid vesicle [17].

Figure 2.2: Schematic of the SLB formation by lipid vesicles on contact with a substrate surface [18].
2.2.2 On Linking Colloids to a SLB

A schematic of our setup is shown in figure 2.3. We use homemade micronmeter-sized polystyrene colloids [20]. In order to prevent aggregation, the colloids have been coated with a steric stabilizer polymer, polyethylene glycol 5000 (PEG5000). The same polymer but with a shorter length, polyethylene glycol 2000 (PEG2000) has been used to tether the bilayer for the prevention of aspecific binding.

In order to link the colloids to the bilayer we use the avidin-biotin complex. These molecules have a very strong and specific bond [21] between the protein avidin (found in egg-white) and the cofactor biotin (non-protein chemical compound present in all living cells). Lipids in the bilayer were functionalyzed with biotinylated PEG2000 (DSPE-PEG2000-Biotin) which provides for Biotin binding sites on the bilayer. The particles were coated with NeutrAvidin (electrically neutral modified form of Avidin) together with PEG5000, following a procedure described in [22]. We have used two different batches of particles that are coated with dif-
Materials & Methods

Different quantities of NeutrAvidin designed to obtain particles with 1% and 0.1% NeutrAvidin surface coverage.

A fluorescence assay as done in [22] shows the linker density distribution (figure 2.4) of the two particle samples, that from now on we will name sample A and sample B. We see peaks around 100 and 10 linkers/µm² for sample A and B respectively, which means given the particle size most sample A particles have around 300 linkers and most sample B particles have around 30 linkers.

Figure 2.4: Normalized particle NeutrAvidin linker distribution shown on semilog plot and obtained from a fluorescence assay as done in [22]. To samples A and B different amounts of NeutrAvidin were added during the coating procedure (5 µg and 0.5 µg, see [22]). The amounts were added to 15 mg particles. Figure made with the courtesy of Casper van der Wel.
2.3 Principles of Fluorescence and Confocal Laser Scanning Microscopy (CLSM)

CLSM is a special type of fluorescence microscopy. In fluorescence microscopy, specialized fluorescent molecules (fluophores) are used to label objects of interest allowing the identification of almost any given facet of biological systems. Different cellular, subcellular or even molecular components can be simultaneously imaged by using different fluophores making fluorescence microscopy a very popular imaging technique within cell and molecular biology [23].

Fluorescence is the absorption and almost simultaneous emission (in the order of nanoseconds) of light due to the excitation and relaxation of molecules between different energy states. The energy transitions corresponding to the band of the emitted light is longer than the one corresponding to the excitation light. This difference due to non-radiative relaxation of the excited molecule known as Stokes shift (figure 2.5) is crucial in order for fluorescence microscopy to work [23, 24].

![Figure 2.5: A typical absorption and emission spectrum. The band maximum of the emission spectrum is at a longer wavelength than the band maximum of the absorption spectrum, a difference known as Stokes shift [25].](image)

The cycle of excitation and relaxation by fluorescent molecules can usually be looped only a limited number of times (between 10000 and 40000 cycles are typical). Thus fluophores are susceptible to bleaching, i.e. permanent loss of their fluorescent property [23]. We will exploit this process to quantify the mobility of the lipid membrane (section 2.4).

A fluorescence microscope illuminates the sample with specific wavelengths (excitation wavelengths) and collects the subsequent fluorescent
Materials & Methods

Radiation characterized by the emission wavelengths. The most favored approach to this is by means of epi-illumination, a microscope configuration where the objective not only serves to image the specimen but also to act as a condenser [23]. In figure 2.6 the principle of the epi-illumination microscope is explained.

![Figure 2.6: Schematic showing the basic principle of a fluorescence microscope. Light coming from the source passes the excitation filter and is reflected by the dichroic mirror subsequently illuminating the specimen with light only having the desired excitation wavelengths (blue line in diagram). The specimen radiates the fluorescent emission (green in the diagram) in all directions and a fraction is collected by the objective. The dichroic mirror is designed to reflect the light with the excitation wavelengths and transmit light with the emission wavelengths. Thus most of the excitation scattered off the specimen and collected by the objective is reflected back to the source, while the emission is transmitted toward the detector. An emission filter serves to block residual excitation light leaving the detector to receive only the light coming from fluorescence [26].](image)

With CLSM two apertures (pinholes) are added to the setup of the fluorescence microscope and lasers are used as light source. The apertures are placed at confocal positions to block out of focus excitation light of reaching the specimen and prevent out of focus emission light of arriving at the detector. In this way only the small in focus point of the sample is imaged at a time. By moving either the objective or the sample a 2D scan can be made at a given height. 3D images can be constructed by making 2D scans at different heights (Z-stack).
2.4 Fluorescence Recovery After Photobleaching (FRAP)

FRAP is a well known technique to check if the bilayer is mobile. Moreover, quantitative analysis of a FRAP experiment allows the calculation of the lateral diffusion constant of individual lipids.

In a FRAP experiment a region of interest (ROI, figure 2.7 A) is bleached by the short exposure to an intense focused laser beam. The result is a loss of fluorescent signal in the ROI (figure 2.7 B). If the bilayer is mobile the

\[\text{Normalized Fluorescence Intensity} \]

\[\text{Time (s)}\]
fluorescent signal from the ROI should recover due to diffusion and subsequent mixing of the lipids in the ROI and the surrounding unbleached area (figure 2.7 C and D).

From the recovery signal of the ROI the lateral diffusion constant can be calculated.

2.4.1 Quantitative Analysis of FRAP experiments

During a FRAP experiment we measure the mean signal intensity of a ROI (defined as $F_1(t)$) and the mean signal of a background area (defined as $F_2(t)$) (Figure 2.8 and 2.9).

Figure 2.8: ROI and the background area in a typical FRAP experiment.

From the normalized recovery curve defined as $F_R(t) = F_1(t)/F_2(t)$ (figure 2.9), we calculate the diffusion constant of the individual lipids using an approach based on the analysis [28] presented by Axelrod et al. In this approach the normalized recovery curve is described with the following equation:

$$F_R(t) = A(1 - e^{t/\tau}) + B$$  \hspace{1cm} (2.1)
2.4 Fluorescence Recovery After Photobleaching (FRAP)

Figure 2.9: Mean intensity signal from the ROI and the background area, $F_1(t)$ and $F_2(t)$ respectively. Blue points and scale show the normalized recovery curve $F_R(t) = \frac{F_1(t)}{F_2(t)}$. Intensity is measured in counts of photons detected by the detection camera on the microscope.

Figure 2.10 shows a graphical interpretation of the fitting parameters $A$ and $B$. We can understand $A+B$ as the asymptotic value of recovery (some-

Figure 2.10: A three-point exponential fit is made of the FRAP recovery curve and a graphical interpretation of the parameters $A$, $B$ and $\tau_1/2$ is given.

times smaller than 1 due to an immobile fraction of lipids in the ROI) and
B as the offset in recovery (caused by incomplete bleaching and/or lag-time between start of recovery and imaging). $\tau_{\frac{1}{2}}$ can be seen as the half life of the process (figure 2.10). Under the assumptions of a circular bleaching spot, we can calculate the diffusion constant $D$ from $\tau_{\frac{1}{2}}$ by using the following equation:

$$D = 0.22 \frac{w^2}{\tau_{\frac{1}{2}}}$$  

(2.2)

where $w$ is the radius of the bleached spot. [28] We obtain $\tau_{\frac{1}{2}}$ from our fitting parameter $\tau$ as $\tau_{\frac{1}{2}} = \tau \ln(2)$.

We estimated the error in $D$ to be 30% following the approach of [13]. The error in $\tau_{\frac{1}{2}}$ was assumed to be negligible when compared to the error in $w$. In all our experiments $w$ was set using the microscope’s software to 3 $\mu$m. We estimated an error of 15% in $w$. The propagation of error method then gives:

$$\frac{\sigma_D}{D} = \frac{1}{D} \sqrt{\left( \frac{\partial D}{\partial \tau_{\frac{1}{2}}} \right)^2 \sigma_{\tau_{\frac{1}{2}}}^2 + \left( \frac{\partial D}{\partial w} \right)^2 \sigma_w^2}$$

$$\approx \frac{1}{D} \left| \frac{\partial D}{\partial w} \right| \sigma_w$$

So

$$\frac{\sigma_D}{D} = \frac{1}{D} * 2 * 0.15 * D$$

$$= 0.3$$
2.5 Two-Dimensional Diffusion and Particle Tracking

Mobile particles coupled to the lipid bilayer experience two-dimensional diffusion. We can quantify their motion using Einstein’s theory of Brownian motion. Following [29] the probability density \( P(r, t) \) of the two-dimensional displacement \( r \) by a diffusing particle is given by:

\[
P(r, t) = \frac{1}{4\pi Dt} \exp \left( -\frac{r^2}{4Dt} \right)
\]  

(2.3)

where \( D \) is the diffusion constant. By taking the second moment we obtain an equation relating the observable mean squared displacement (msd) of a particle to the diffusion constant:

\[
\langle r^2 \rangle = 4Dt
\]  

(2.4)

We track our colloidal particles using Trackpy [30], a python package implementing the Crocker and Grier algorithm [31]. With the program we analyzed the trajectories by computing their msd.
2.6 Experimental Details

2.6.1 SUV Preparation

SUV’s are prepared through extrusion. First, a lipid mixture of 500 µg dissolved in chloroform is left to dry for two hours in a vacuum dessicator at 200 mBar. Then 250 µl of HEPES is added bringing the lipid concentration to 2 g/l. HEPES with a pH of 7.4 was used mimicking the physiological environment. To disperse the lipids, the solution is vortexed for 10 minutes. The Avanti Mini-Extruder® (figure 2.11) is used to create SUV’s from the mixture.

Figure 2.11 shows an exploded view of the Mini-Extruder. Two poly-

![Image of the Mini-Extruder](image_url)

**Figure 2.11:** Figure showing the fully assembled extruder (top) and an exploded view of the extruder cell (bottom) [5].

...carbonate membranes with 30 nm pore size are held between filter supports adhered onto the orifices of the internal membrane supports. The extruder outer casting and the retainer nut are finger tightened whereafter two gas thight 250 µl syringes are mounted opposite to each other in the extruder supported by the extruder stand. To clean the syringes, we wash them 3 times with EtoH, MQ and HEPES. To reduce the dead volume, the
2.7 Substrate Treatment

extruder is pre-wetted by flushing a full syringe of HEPES into the extruder. The HEPES solution flows through the extruder cell into the other syringe, filling it automatically. The HEPES in the receiving syringe is discarded and the flushing is repeated for another two times.

After pre-wetting, we fill one syringe with a 2 g/l lipid solution and carefully transfer the mixture through the membrane into the other syringe by pushing gently on the filled syringe’s plunger. We repeat this operation for 21 times. By using this method, SUV solutions with a volume between 100 and 250 µl can be obtained.

2.7 Substrate Treatment

Before use circular glass coverslips are cleaned in the following manner: rinsed with a 2% Hellmanex solution (three times), then rinsed with MQ (three times), then rinsed with EtoH (two times), immersed in EtoH for 30 minutes under gentle stirring, rinsed again with MQ (two times) and finally immersed in MQ for 30 minutes under gentle stirring. Finally the coverslips are dried in an oven (100 ° C) for one hour.

2.8 Sample Preparation

Following [13], samples were prepared by mixing 50 µl of the prepared SUV solution together with 600 µl HEPES on the cleaned glass coverslips, bringing the lipid concentration to 0.15 g/l. The sample is left for about one hour at room temperature, to allow the formation of the SLB. Then, the sample is washed for 3 times to eliminate the excess SUV’s in order to prevent them from coating the particles.

After washing colloids in HEPES solution are added. The HEPES particle solution was sonicated beforehand in order to disperse the particles. Finally we wait for at least one hour to allow the particles to bind to the SLB, whereafter we observe the sample.

2.8.1 Imaging

Imaging was done with confocal microscopy. The microscope used is an inverted Nikon Eclipse TiE microscope with a Nikon A1R confocal scanhead. The scanhead allows both ultrafast imaging (7.8 kHz resonance frequency) and high resolution imaging in slow mode (4096x4096 pixels).
A 60x PLAN APO VC water immersion objective and a MCL NanoDrive stage were used.

We fluorescently labeled the bilayer by using a fluorescent lipid (Rhodamine Liss. B). The colloidal particles were labeled by adding 1, 3, 5, 7-Tetramethyl-8-phenyl-4, 4-difluoroboradiazaindacene (BODIPY) during the synthesis, see [22]. In the table below the excitation and emission maxima of the dyes, the wavelengths of the excitation lasers and the wavelength range of the emission filters used are listed. Furthermore we used a laser with a wavelength of 405 nm together with the lasers mentioned above for stimulation (FRAP) experiments.

**Table 2.2:** Dyes used, their excitation and emission maxima, color, corresponding laser wavelength used for excitation and emission filter range are listed.

<table>
<thead>
<tr>
<th>Dye</th>
<th>Excitation</th>
<th>Emission</th>
<th>Lasers</th>
<th>Emission Filters</th>
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</thead>
<tbody>
<tr>
<td>Rhodamine</td>
<td>560 nm</td>
<td>583 nm</td>
<td>561 nm</td>
<td>565-625 nm</td>
</tr>
<tr>
<td>Bodipy</td>
<td>326 nm</td>
<td>515 nm</td>
<td>488 nm</td>
<td>500-550 nm</td>
</tr>
</tbody>
</table>
Chapter 3

Results and Discussion

Experiments were conducted with two experimental goals in mind: measure the mobility of the bilayer and track colloidal particles attached to the bilayer. We present the results of measurements on the mobility of the SLB and the particles in section 3.1 and 3.2 respectively.

3.1 Lipid Bilayer Mobility

The first step in building our artificial system modeling protein diffusion is to create a mobile lipid bilayer. The mobility of SLB’s, prepared as explained in section 2.6.1, 2.7 and 2.8 was studied by doing FRAP experiments, as explained in section 2.4.1. In the following sections a representative FRAP experiment with a tethered lipid bilayer (section 3.1.1) is described and then the results of other FRAP experiments on SLB’s are schematically presented (section 3.1.2).

3.1.1 FRAP Experiment with a Tethered Lipid Bilayer

Acknowledging the results found in [13] the following lipid composition was used:

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Mass %</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOPC</td>
<td>89.8</td>
</tr>
<tr>
<td>DOPE-PEG2000</td>
<td>10</td>
</tr>
<tr>
<td>DOPE-Rhod</td>
<td>0.2</td>
</tr>
</tbody>
</table>
Images of the FRAP experiment are shown in figure 3.1. A typical example of recovery analysis is shown in figure 3.2 and 3.3.

![Figure 3.1: Sequence of images from the FRAP experiment with DOPC:DOPE-PEG2000:DOPE-Rhod = 89.8:10:0.2 mass % showing the bilayer before and after bleaching.](image)

(a) $t=0$ sec  (b) $t=1.70$ sec  (c) $t=3.40$ sec
(d) $t=5.10$ sec  (e) $t=7.14$ sec  (f) $t=8.84$ sec
(g) $t=10.54$ sec  (h) $t=12.24$ sec  (i) $t=13.94$ sec
3.1 Lipid Bilayer Mobility

Figure 3.2: Mean intensity signals $F_1(t)$ (ROI) and $F_2(t)$ (background area) measured during the FRAP experiment. Intensity is measured in counts of photons detected by the detection camera on the microscope.

![Graph showing mean intensity signals](image)

Figure 3.3: Analyzed recovery curve and the calculated diffusion constant $D$ are shown. The error is estimated as described in section 2.4.1

![Graph showing normalized intensity](image)

$D = 0.7 \, \mu m^2/s$

Error Estimate = 30%

The diffusion constant found is comparable (same order of magnitude) to results obtained in [13].
3.1.2 Results Adding CaCl₂ and Biotin

In different studies the presence of calcium ions was found to enhance SLB formation [33–35] and in anticipation of increased mobility samples prepared with SUV’s mixed in a 0.1 mM CaCl₂ HEPES solution were compared to samples with the same SUV solution mixed in plain HEPES. Also we examined samples with DSPE-PEG2000-Biotin added to the lipid composition in order to see if it has an effect on the bilayer mobility. The results are shown below.

Table 3.1

<table>
<thead>
<tr>
<th>Lipid Composition (Mass %)</th>
<th>CaCl₂ Added?</th>
<th>$D$ (µm²/s)</th>
<th>Estimated Error $D$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOPC:DOPE-PEG2000:DOPE-Rhod 89.8:10:0.2</td>
<td>Yes</td>
<td>0.7</td>
<td>30%</td>
</tr>
<tr>
<td>DOPC:DOPE-PEG2000:DOPE-Rhod:DSPE-PEG2000-Biotin 84.8:10:0.2:5</td>
<td>No</td>
<td>0.4</td>
<td>30%</td>
</tr>
<tr>
<td>DOPC:DOPE-PEG2000:DOPE-Rhod:DSPE-PEG2000-Biotin 84.8:10:0.2:5</td>
<td>Yes</td>
<td>0.4</td>
<td>30%</td>
</tr>
</tbody>
</table>

We observe no effect of CaCl₂ on the mobility of the bilayer and as we want to keep our artificial system as simple as possible we decide to stop using CaCl₂. We suggest to increase the quantity of CaCl₂ for future experiments.

We do see a slight decrease of the diffusion constant when DSPE-PEG2000-Biotin was added to the lipid composition, but conclusions are hard to make from this experiment. In contrast to experiments with CaCl₂ we are now comparing bilayers created with different SUV solutions, moreover the difference in diffusion constant almost falls into the margin of error.
3.2 Colloidal Particles linked to the SLB

NeutrAvidin coated colloidal particles were linked to the SLB as explained in 2.2.2. The SLB was prepared as described in section 2.6.1, 2.7 and 2.8. Section 2.8 also describes how the particles were added to the sample and section 2.5 presents how the particles were tracked. First some representative images of the particles and the bilayer are shown (section 3.2.1), then a representative experiment with particle tracking is described (section 3.2.2), and finally results of varying the linker density are presented (section 3.2.3).

3.2.1 Overview of Particles and the SLB

Figure 3.4: 72x72 µm representative images of micronmeter-sized particles on SLB’s with different amounts of DSPE-PEG2000-Biotin. Different settings were used for the lasers and the microscope with each image. With all images respectively 10 mass % and 0.2 mass % DOPE-PEG2000 and DOPE-Rhod were used. For the remaining percentage of lipid composition DOPC was used. Particles with two different amounts of NeutrAvidin coating were added (sample A and B, see section 2.2.2).
Results and Discussion

It is important to point out that not all particles seen in the above images are attached to the bilayer. They come in and out of focus as they experience 3D diffusion. The 3D image below shows the non-attached particles above the bilayer.

![Image](image.png)

**Figure 3.5:** Volume view of a Z-stack (depth = 62 µm) showing the colloidal particles (Sample B) and the bilayer (DOPC:DOPE-PEG2000:DOPE-Rhod-DSPE-PEG2000-Biotin = 87.3 : 10 : 0.2 : 2.5 mass %). Field of view bilayer is 143x143 µm.

### 3.2.2 Analyzing Particle Mobility; An Example Experiment

We imaged a 143x143 µm field of view with sample A particles attached to a bilayer with lipid composition: DOPC:DOPE-PEG2000:DOPE-Rhod:DSPE-PEG2000-Biotin = 87.3 : 10 : 0.2 : 2.5 mass %. We followed their motion for half a minute taking images at 10 frames per second. The particles were tracked (figure 3.6a) and their mean squared displacement (msd) computed (figure 3.6b).

A linear relation between the msd and the lagtime indicates the particles experience Brownian motion (equation 2.4 in section 2.5) and from the slope of the msd vs lagtime plot we can determine the diffusion constant:

$$\langle r^2 \rangle = 4Dt \implies D = \frac{\langle r^2 \rangle}{4t} = \frac{1}{4} \ast slope$$  \hspace{1cm} (3.1)

To determine the slope we selected an interval starting from t=0 up to a cutoff time (judged by eye) where the slope of any particle’s msd starts to
3.2 Colloidal Particles linked to the SLB

(a) Superimposed image of the particles’ position in time. The axis units are given in pixel.

(b) Mean squared displacements from the trajectories of the particles in (a) vs lag time.

Figure 3.6: The trajectories of mobile particles (colored paths) and stuck particles (grey paths) are shown in figure (a). Figure (b) shows the corresponding mean squared displacements.

change and fitted our data (figure 3.7). The change in slope can be a result of changes in the particle’s diffusive motion resulting in several linear intervals with different slopes (diffusion constants).
Results and Discussion

We recommend improving the analysis by automating the process of finding an appropriate cutoff time. One can think of varying the endpoint of the interval used to fit the data. Then one selects the cutoff time as the endpoint where the errors in the fit parameters of the whole particle ensemble is at a minimum (e.g. lowest value found for the sum over each particle’s fit parameter error).

Following [13] we labeled the particles mobile if their diffusion constant was greater than 0.01 \( \mu m^2/s \). In order to make this threshold less arbitrary we suggest studying the particle distribution with respect to diffusion coefficient. One can then probably observe different diffusive regimes and decide which regimes are of most interest. We chose not to this as it requires a lot of data and more time to do the analysis.

In this experiment we found that with respect to our criterion 7 of the total 114 particles tracked were mobile. The slopes of the mobile particles are shown in figure 3.7 and their diffusion constants with errorbars (based on the standard deviation from the fit) in table 3.2.

![Figure 3.7](image)

**Figure 3.7:** Plot and fit of a selected interval of the mean squared displacements from the mobile particles. The interval starts from \( t=0 \) up to a certain cutoff time that is judged by eye to be the time where a change in slope of any particle’s msd starts. From the fit the slopes of the particles msd’s are obtained.

**Table 3.2:** Diffusion constants of mobile particles. Particle labels correspond to labels in figure 3.7 and the errors are calculated from the errors of the fit parameters.

<table>
<thead>
<tr>
<th>Particle</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>( D (\mu m^2/s) )</td>
<td>0.036</td>
<td>0.0711</td>
<td>0.349</td>
<td>0.589</td>
<td>0.61</td>
<td>0.137</td>
<td>0.138</td>
</tr>
<tr>
<td>( \text{Error} (\mu m^2/s) )</td>
<td>0.002</td>
<td>0.0004</td>
<td>0.003</td>
<td>0.009</td>
<td>0.02</td>
<td>0.004</td>
<td>0.005</td>
</tr>
</tbody>
</table>
3.2 Colloidal Particles linked to the SLB

The spread in diffusion constants could be due to the distribution of linkages between particle and bilayer, as we believe particles with low amount of links diffuse faster than particles with high amount of links.

3.2.3 On Varying Linker Density and Particle Mobility

We performed a series of experiments like the one described previously with many different amounts of linker density on the bilayer and two different linker densities on the the particles that we labeled as sample A and B, see section 2.2.2. Most sample A and B particles have around 300 and 30 NeutrAvidin linkers respectively.

The amounts of DOPE-PEG2000 and DOPE-Rhod were kept constant at 10 mass % and 0.2 mass % respectively and the amount of DSPE-PEG2000-Biotin was varied as listed in table 3.3 and 3.4. For the remaining percentage of lipid composition DOPC was always used. Images with different fields of view and framerates were made of the particles and they were labeled as mobile by the criteria described in the previous section 3.2.2. The minimal cutoff time used was 2 seconds.

Table 3.3: Table listing the experimental conditions used in the experiments with sample A (300 linkers/particle).

<table>
<thead>
<tr>
<th>DSPE-PEG2000-Biotin (Mass %)</th>
<th>0</th>
<th>10⁻⁴</th>
<th>10⁻³</th>
<th>10⁻²</th>
<th>0.1</th>
<th>0.5</th>
<th>1</th>
<th>2.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area Viewed (µm x µm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>162x162</td>
<td>240x240</td>
<td>450x450</td>
<td>197x197</td>
<td>426x426</td>
<td>169x169</td>
<td>163x163</td>
<td>237x237</td>
<td></td>
</tr>
<tr>
<td>Particles Tracked</td>
<td>74</td>
<td>104</td>
<td>387</td>
<td>83</td>
<td>670</td>
<td>106</td>
<td>90</td>
<td>345</td>
</tr>
</tbody>
</table>

Table 3.4: Table listing the experimental conditions used in the experiments with sample B (30 linkers/particle).

<table>
<thead>
<tr>
<th>DSPE-PEG2000-Biotin (Mass %)</th>
<th>0</th>
<th>10⁻⁴</th>
<th>10⁻³</th>
<th>0.1</th>
<th>2.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area Viewed (µm x µm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>414x414</td>
<td>592x592</td>
<td>526x526</td>
<td>412x412</td>
<td>502x502</td>
<td></td>
</tr>
<tr>
<td>Particles Tracked</td>
<td>20</td>
<td>234</td>
<td>30</td>
<td>263</td>
<td>166</td>
</tr>
</tbody>
</table>
Apart from mobile and stuck particles we also observed particles exhibiting a ‘wobbling motion’. They appeared to be confined to a small domain and their movements may indicate confined diffusion. We labeled immobile particles to be wobbling if their mean squared displacement (msd) was found to be greater than the msd corresponding to a diffusion constant of 0.01 $\mu m^2/s$ at the first timepoint (in most cases 0.09 s and always within 2 s).

The mobile and wobbling fraction of particles found are shown in figure 3.8. We see that in all experiments the majority of particles on the bilayer are not mobile. We hypothesize two competing processes that causes our particles to be stationary: 1) an excess of linkages between the particles and the bilayer and 2) aspecific binding (link other than the Avidin-Biotin bond) between the particles and the bilayer.

![Figure 3.8: Mobile and wobbling fraction of sample A (300 NeutrAvidin linkers) and sample B (30 NeutrAvidin linkers) particles found on bilayers with different amounts of DSPE-PEG2000-Biotin. The errorbars are calculated according to the binomial distribution as: $\delta = z\sqrt{\frac{p(1-p)}{n}}$, where $p$ is the number of mobile particles, $n$ the total number of particles and $z=1.96$ (corresponding to 95% confidence). The horizontal axis is in logarithmic scale.](image)

Figure 3.9 shows the particles/$\mu m^2$ found on the bilayer with varying linker densities. With sample A we observe a baseline of aspecifically bound particles, i.e. a fraction of particles that will always be bound aspecifically regardless of the amount of linkers on the bilayer. Starting from
10^{-1} mass % DSPE-PEG2000-Biotin up to higher linker densities on the bilayer we see an increase in particles/µm², indicating that the Avidin-Biotin bond starts to have an effect in this linker density regime. No increase of sample B particles/µm² was found with increasing linker density on the bilayer. This suggests that all sample B particles observed were bound aspecifically and the NeutrAvidin linker density range used with sample B is too low for our purposes.

The difference between the sample A and B particles/µm² found with lower bilayer linker densities could indicate that sample B particles are more effectively sterically stabilized than the sample A particles, as the increase in NeutrAvidin coating leaves less room for the PEG molecules to coat the colloids.

In order to get a better insight into the mobile fraction of specifically bound sample A particles we took the average of the immobile particles/µm² in the lower linker density regime (0-10^{-2} mass % DSPE-PEG2000-Biotin, see figure 3.10) as the number of particles/µm² that are bound aspecifically. The difference between the immobile particles/µm² in the higher linker density regime (10^{-1}, 0.5, 1, 2.5 mass % DSPE-PEG2000-Biotin) and this average is then the amount of immobile particles/µm² that are bound specifically. We assume all mobile particles are bound specifically and the fraction with respect to the specifically immobile particles is shown in figure 3.11.
Figure 3.10: Graphical representation of our correction for aspecific binding with sample A. As baseline for immobile particles due to aspecific binding we take the average of immobile particles/$\mu m^2$ in the lower linker density regime (0-10$^{-2}$ mass % DSPE-PEG2000-Biotin): 0.0021 particles/$\mu m^2$. Then the remaining fractions of immobile particles in the higher linker density regime ($10^{-1}$, 0.5, 1, 2.5 mass % DSPE-PEG2000-Biotin) are bound specifically. We calculate the mobile fraction with respect to the specifically bound particles as:
\[
specific \ mobile = \frac{mobile}{mobile + specific \ stuck}.\]

Figure 3.11: Specific and the original mobile fraction of sample A particles found on the bilayer. The horizontal axis is in logarithmic scale.
3.2 Colloidal Particles linked to the SLB

3.2.4 Other Observations

To check if stationary particles were attached to immobile patches of the bilayer we conducted a FRAP experiment with the ROI surrounding a immobile particle (figure 3.12). Full recovery was observed and a diffusion constant of 1 $\mu m^2/s$ for the bilayer was found.

A stability assay of the sample A and B particles was done to see if the PEG coating provides sufficient steric stabilization. The particles were suspended in a salt solution and observed with a brightfield microscope. We observed very little aggregation indicating the particles are stable.

Figure 3.12: Sequence of images from a FRAP experiment surrounding an immobile particle. Lipid composition of DOPC:DOPE-PEG2000:DOPE-Rhod:DSPE-PEG2000-Biotin = 89.8 : 10 : 0.2 : 0.5 mass % was used.
Conclusions

In light of creating an artificial model mimicking membrane protein diffusion we have studied the mobility of colloids coupled to a lipid bilayer supported by a glass substrate.

The fluidity of SLB’s tethered with PEG molecules and functionalized with Biotin linkers was established by FRAP experiments. Diffusion constants up to $1 \mu m^2/s$ were found for the bilayer.

As a linking mechanism the Avidin-Biotin bond was used and quantitatively analyzed. The Biotin linker density on the bilayer was varied and particles with two different NeutrAvidin linker density ranges were used: sample A (around 300 NeutrAvidin linkers/particle) and sample B (around 30 NeutrAvidin linkers/particle).

We identified our main challenge in building a protein modeling system to be the aspecific binding. Our results indicate that all sample B particles observed were bound aspecifically, as we did not see an increase of particles attached to the bilayer with increasing bilayer linker densities. In sample A, we found that the Avidin-Biotin bond starts to have an effect with bilayers composed of at least $10^{-1}$ mass % DSPE-PEG2000-Biotin. After correcting for aspecific binding, we analyzed the mobile fraction of specifically bound sample A particles at this bilayer linker density to be around 40%.

In order to narrow down the optimal linker density range we suggest to study the mobile fraction of particles with linker density distributions close (same order of magnitude surface coverage) to that of sample A bound to bilayers with $10^{-1}$ or 1 mass % DSPE-PEG2000-Biotin. Regarding aspecific binding one can try to increase the PEG concentration on the bilayer or on the particles for more steric stabilization.
I would like to thank my direct supervisors Melissa Rinaldin and Casper van der Wel for their overall contribution to the project, their supreversion, guidance and readiness to answer all the questions I had at any moment during the project. Moreover I would like to especially thank Melissa for all her corrections and assistance in writing this thesis; Casper for all his help in programming. Ofcourse I would also like to thank my supervisor Daniela Kraft who oversaw the project, for her clear explanations and valuable guidance. I would like to acknowledge Ernst-Jan Vegter for his help and work that I so often used as a reference and Marcel Winter for the provision of colloidal particles. Finally I would like to thank the soft matter group in general for providing me with my first experiences as a researcher and giving me an inside view on how research within soft matter physics is conducted.
Bibliography


