Lipid mediated colloidal interactions

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Chapter 4

Surfactant-free colloidal particles with specific binding affinity

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Abstract

Highly specific coatings are essential for techniques such as in vivo and in vitro biosensing, targeted drug delivery, and micrometer-scale self-assembly. Key to these techniques are typically colloidal particles with a coating that provides a high affinity to a specific molecule. Current particle coating methods require adsorbed surfactants, which typically influence lipid membranes. To address this issue and employ these particles for targeting molecules in lipid membranes, we have developed a completely surfactant-free coating method that provides target-specific colloidal particles. After activating charge-stabilized polystyrene microparticles with EDC/Sulfo-NHS, we first coat the particles with a specific protein and subsequently with a dense layer of poly(ethylene) glycol. This polymer layer provides colloidal stability at physiological conditions as well as anti-adhesive properties, while the protein coating provides the specific affinity to a targeted molecule. We show that NeutrAvidin coated particles bind specifically to biotinylated membranes, and that Concanavalin A coated particles bind specifically to the glycocortex of *Dictyostelium discoideum* cells. The affinity of the particles changes with the protein density, which can be tuned during the coating procedure. The here reported generic and surfactant-free coating method transfers the high affinity and specificity of a protein onto colloidal polystyrene microparticles.

4.1 Introduction

Surfaces with a high affinity to specific molecules are crucial in biological applications such as biosensing[109,110] and drug targeting.[111,112] Central to these applications is a particle coating that provides a high affinity and specificity to certain target molecules of interest. For example, colloidal particles can be functionalized with an affinity to particular proteins that are over-expressed on the cellular membrane of a tumour cell,[113] which enables local drug release at targeted cells. We here focus on polystyrene microparticles that especially target molecules that are embedded in lipid membranes. Such particles are required in fundamental membrane studies such as membrane tether pulling,[114,115] membrane viscosity measurements,[116,117] and the quantification of membrane mediated forces.[21,104,118] Also, it has recently been shown that a specific linkage between particles and lipid membranes can be adopted for the self-assembly of particles into mobile structures such as colloidal clusters, polymers, and dynamic networks.[44,46,119,120]

A controllable affinity and specificity of colloidal particles requires a careful design of the surface coating process.[47,109,110,121–123] Next to moieties that provide the actual affinity, hydrophilic polymers are typically attached to the particle surface, which are necessary to prevent the undesirable aggregation of particles at physiological salt concentrations. These seemingly contradicting requirements—specifically attracting moieties next to generically repelling polymers—are usually met by adsorbing a polymer surfactant onto the colloidal particles before or during the coating process. These polymers provide steric stabilisation against aggregation, while the functional binding sites of the particles are still accessible. However, amphiphilic polymers are known to interact with lipid membranes.[124–127] If these colloidal particles are used in lipid membrane studies, desorbing surfactants may disrupt the lipid membranes and compromise the accuracy of the corresponding measurements. Therefore, a surfactant-free coating is required for studies involving lipid membranes.

To meet this requirement, we have developed a two-step method yielding stable and specific polystyrene microparticles that can be applied to lipid membrane studies. First, we created colloidal particles with a high affinity through immobilization of proteins that specifically bind to the desired target molecules.[128–130] We focus mostly on the use of NeutrAvidin, as its affinity to biotin is used in many practical applications. This protocol can readily be used to prepare particles with other target-specificity, as we demonstrate by also employing the sugar-binding Concanavalin A. Second, we do not stabilize the colloidal particles by using adsorbed surfactants, but instead by covalent grafting of methoxylpoly(ethylene) glycol (mPEG) to the particle surface, in such a way that it preserves the affinity and specificity of the tested proteins. At sufficiently high surface densities, mPEG is known to suppress particle aggregation.[131] Also, a dense mPEG coating has been proven to suppress non-specific protein adsorption on the particles, thus providing "stealth" properties necessary for drug targeting in living organisms.[109,111] These properties make mPEG an ideal choice as a stabilizer for colloidal particles at physiological conditions.

In this chapter, we will first describe the synthesis method and study the effect of

various parameters on the resulting surface density of avidin. Then we will assess the binding affinity of avidin-functionalized colloidal particles on biotinylated Giant Unilamellar Vesicles (GUVs). Finally, we show that avidin-functionalized particles bind specifically to biotinylated lipid membranes, and Concanavalin A-functionalized particles to the cellular membrane of *Dictyostelium discoideum* cells.

4.2 Methods

Materials Styrene (99%), itaconic acid (99%), 4,4'-azobis(4-cyanovaleric acid) (98%, ACVA), hydrochloric acid (HCl), D-glucose (99%), sodium phosphate (99%, Na_2HPO_4), deuterium oxide (70%, D_2O), Pluronic F-127, N-hydroxysulfosuccinimide sodium salt (98%, Sulfo-NHS), 1,3,5,7-tetramethyl-8-phenyl-4,4-difluoroboradiazaindacene (97%, BODIPY), and Newborn Bovine Serum (NBS) were acquired from Sigma-Aldrich; methoxypoly(ethylene) glycol amine (mPEG, M_w = 5000) from Alfa Aesar; sodium hydroxide (NaOH, 98.5%), sodium chloride (NaCl, 99%) and sodium azide (NaN_3, 99%) from Acros Organics; 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (99%, EDC) from Carl Roth; NeutrAvidin (avidin) and biotin-4-fluorescein from Molecular Probes; biotin-5'-TTTAATATTA-3'-Cy3 DNA oligonucleotides (btn-DNA-Cy3) from Integrated DNA Technologies; Δ9-cis 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-[biotinyl-(polyethylene glycol)2000] (DOPE-PEG-biotin), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)2000] (DOPE-PEG), and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamineN-(lissaminerhodamine B sulfonyl) (DOPE-rhodamine) from Avanti Polar Lipids; OptiMEM (without phenol red), Dulbecco's modified minimal essential media (without phenol red, DMEM), Geneticin, Blasticidin-S HCl, GlutaMAX, and Trypsin from Life Technologies; Fetal Calf Serum (FCS) from Biowest; Penicillin-Streptomycin (P/S) from Duchefa Biochemie. All chemicals were used as received. Deionized water with 18.2 MΩcm resistivity was used, obtained using a Millipore Filtration System (Milli-Q Gradient A10). HL5 medium consisted of 5 g L^{-1} protease peptone, 5 g L^{-1} thiotone E peptone, 10 g L^{-1} glucose, 5 g L^{-1} yeast extract, 0.35 g L^{-1} $Na_2HPO_4 \cdot 7\,H_2O$, 0.35 g L^{-1} KH_2PO_4, and 0.05 g L^{-1} dihydrostreptomycin sulphate, with a pH of 6.4-6.7. A549 cell imaging medium consisted of OptiMEM, 2.5 vol% FCS, 0.2 g L^{-1} P/S, and 1.8 mM Glutamine-S. 3T3 cell imaging medium consisted of DMEM, 10 vol% NBS, 0.2 g L^{-1} P/S, and 2 mM GlutaMAX.

Particle preparation Carboxylic acid functionalized polystyrene spheres were prepared using a surfactant-free dispersion polymerization protocol.[132] To a solution of 500 mg itaconic acid in 125 mL water, 25 g styrene was added in a magnetically stirred round bottom flask. The mixture was purged four times with nitrogen and heated to 85 °C in an oil bath. Separately, 250 mg ACVA was dissolved in 9 mL of 0.2 M NaOH. This initiator was injected into the reaction mixture while stirring at 500 rpm. The reaction was allowed to proceed overnight, after which the dispersion was cooled down to

room temperature. Macroscopic polystyrene aggregates were filtered out through glass wool, after which the sample was washed three times with water. In order to obtain fluorescent particles, the synthesis procedure was repeated with additionally dissolving 10 mg of BODIPY (ex. 488 nm, em. 515 nm) in the styrene before addition to the reaction mixture. The size distribution was determined using a FEI nanoSEM 200 scanning electron microscope (see for example Fig. 4.1e). The non-fluorescent particles employed in this chapter were 1.06 ± 0.02 µm in diameter, and the fluorescent particles 0.98 ± 0.03 µm. Their respective ζ-potentials in water (pH 5.7) were determined to be −54 ± 4 mV and −55 ± 4 mV using a Zetasizer Nano ZS from Malvern Instruments. These strongly negative values reflect the high density of carboxylic acid groups (1–10 nm^{-2}) on the particle surface.[132]

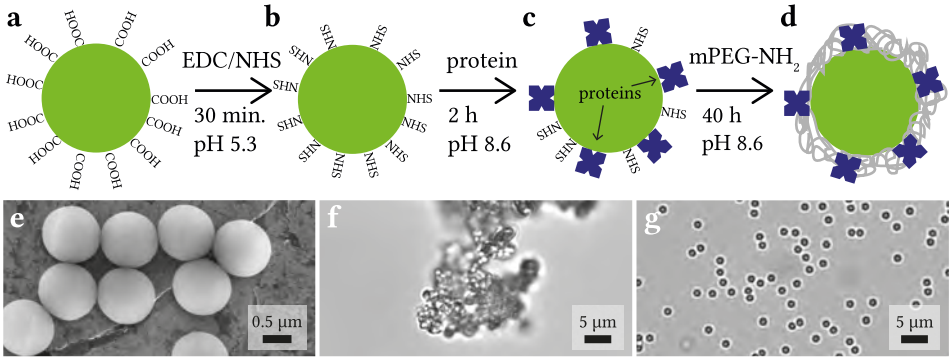

Figure 4.1. Illustration of the particle coating procedure. (a) Highly carboxylated (COOH) polystyrene particles were first activated using EDC/NHS so that the carboxylic acid groups form NHS esters. (b) The activated particles were then mixed with a precise quantity of protein at pH 8.6. (c) After a delay time of typically 2 hours, an excess of amine-functionalized methoxypoly(ethylene) glycol (mPEG-NH_2) was added to stabilize the particles. (d) The resulting particle coating contains the added proteins as well as a dense PEG layer that provides colloidal stability. (e) A Scanning Electron Micrograph of the uncoated particles. Light microscopy images after 1 h in 1 M NaCl show that (f) the uncoated polystyrene particles aggregated strongly and (g) the mPEG coated particles remained dispersed.

Coating and stabilization The particle coating procedure was extended from a covalent mPEG grafting protocol from literature[131] (see Figure 4.1). All reactions were performed on a vortex mixer at 4 °C. The poly(styrene) particles with carboxylic acid groups were activated by adding 8 mM EDC and 2.5 mM Sulfo-NHS to the particles, which were suspended at 20 g/L. The EDC and Sulfo-NHS were dissolved immediately before use. At this concentration, the hydrochloric acid in the EDC ensures a pH of 5.3, which was confirmed using a calibrated Hach PH17-SS ISFET pH probe. After 30 min of mixing, the pH was brought to 8.6 using 0.2 M NaOH. At this pH, NHS hydrolysis starts, which

is reduced by performing the reactions at 4 °C. Then 5–500 µg of protein (NeutrAvidin or Concanavalin A) was added to 750 µL (15 mg) of the activated particles. After a delay time of 2 h, 4.0 mg mPEG5000-NH_2 dissolved in 200 µL water was added. The reaction was allowed to proceed for at least 40 h. To remove the remaining NHS groups, the pH was increased to 12 for 5 min using 1 M NaOH. For some proteins this high pH is undesirable, in which case pH 9 may be used for a longer time period to hydrolyse the NHS esters at room temperature.[133] The particles were washed one time with 10 mM HCl to neutralize the pH and three times with water. Finally, sodium azide was added to a concentration of 3 mM to prevent bacterial growth. The colloidal stability of each sample was assessed as follows: 50 µg particles were dispersed in 10 µL 1 M NaCl. After 1 h of mixing, the sample was diluted 20 times with 1 M NaCl and imaged using bright field microscopy (Fig. 4.1g). Resulting particle suspensions can be stored for at least two months at 4 °C. For longer shelf life, the particle suspensions should be frozen at −20 °C.

Avidin titration assay The number of biotin binding sites on the particles was measured using a titration assay, based on the binding of biotin-4-fluorescein to the particle-attached avidin. Because of the strong scattering of the particles, we could not measure the fluorescence of the avidin-bound biotin-4-fluorescein directly as described elsewhere.[134] Instead, we determined the remaining fluorescence of the supernatant for different amounts of added biotin-4-fluorescein. From a single particle batch, precisely known aliquots of 0.3–0.6 mg particles were dispersed into 1.00 mL PBS buffer (12.5 mM Na_2HPO_4, 50 mM NaCl, 3 mM NaN_3, with a pH of 7.5). Subsequently, quantities of 0–10 µL of 1.8–7.3 µM biotin-4-fluorescein were incubated with the particles for 20 min at 55 °C. After this, the particles were removed by centrifugation. Then, 750 µL supernatant was diluted with 750 µL PBS and the fluorescence of each aliquot was determined using a Varian Cary Eclipse fluorescence spectrophotometer (ex. 494 nm, em. 524 nm). From the intersection of the baseline fluorescence and the final slope of the titration curve, the amount of biotin binding sites in the sample was determined. Together with the separately determined dry weight and diameter of the particles, the amount of biotin binding sites per particle was computed.

Avidin fluorescence assay In order to measure the distribution of biotin binding sites on avidin-functionalized particles within a single batch, we added a fluorescent marker for use in fluorescence microscopy. The employed fluorescent marker was a biotin- and Cy3-functionalized DNA oligonucleotide, as DNA is well soluble in water and shows no aspecific adsorption to the particle surface. To stain the particle-attached avidin, we incubated 50 µg particles with 60 pmol btn-DNA-Cy3 in 310 µL PBS buffer for 30 min at 55 °C in a nuclease-free plastic microtube. To be able to use the same protocol for unstable particles, we added 0.5 wt% of the steric stabilizer Pluronic F-127 to the PBS buffer (see previous subsection for the contents). After cooling down to room temperature, the samples were washed three times with water and one time with PBS. Finally, the samples were inserted into a rectangular capillary and imaged on a Nikon

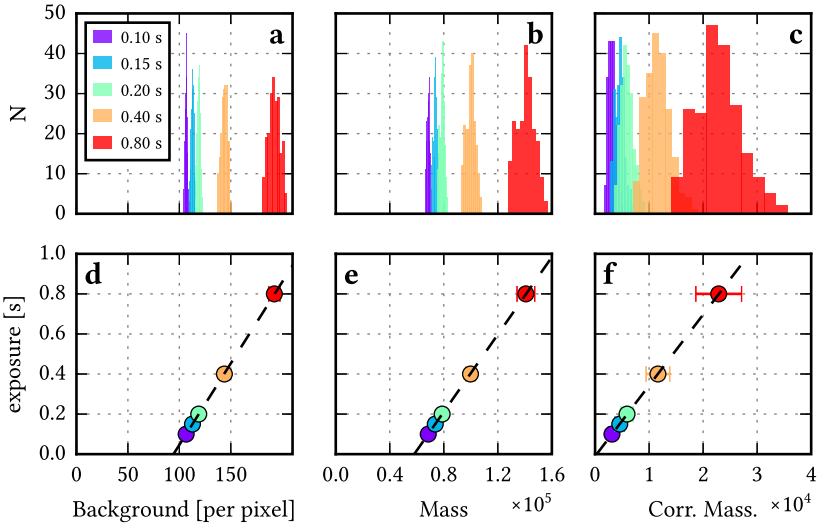

Figure 4.2. Emission intensity of the same particle batch at different camera exposure times. (a) Histograms of the background emission around each particle for different exposure times. (d) The corresponding fit shows a linear relation, but nonzero intercept. (b) Histograms of the integrated intensity of each particle. (e) The corresponding fit shows a linear relation. (c, f) The background-corrected mass is proportional to the camera exposure time.

Ti-E microscope equipped with a Nikon 100 × CFI Apo TIRF oil-immersion objective (NA = 1.49), Intensilight fluorescent lamp (ex. 540 ± 25 nm, em. 605 ± 55 nm), and a DS-Qi1 monochrome CCD camera with an exposure time that was set such that all particles had a fluorescence signal in between the camera background and saturation levels (100–800 ms). In a separate experiment, the background-corrected emission intensity per feature was confirmed to be proportional to the exposure time (see Fig. 4.2). The resulting images were ensured to be in the same focal plane and the integrated intensity per particle was computed using Trackpy.[86]

Giant unilamellar vesicles GUVs of 10–100 µm diameter were prepared by electroformation.[135] Lipid mixtures consisting of 97.5 wt% DOPC, 0.5 wt% DOPE- rhodamine and 2 wt% DOPE-PEG-biotin (or DOPE-PEG) were prepared in chloroform at 2 g L^{-1}. 10 µL of this lipid solution was dried on each of two 6 cm^2 ITO-coated glass slides (15–25 Ω/sq, Sigma-Aldrich). The two electrodes were placed in 1.8 mL of 100 mM glucose solution (with 0.3 mM NaN_3) and subjected to 1.1 V (rms) at 10 Hz for 2 h, with a gradual increase during the first 2 min. The vesicles were stored in a BSA-coated vial at room temperature.

Dictyostelium discoideum cell preparation *Dictyostelium discoideum* SadA-GFP Lim-RFP cells were cultured in HL5 medium at 21 °C and supplemented with 10 mg L^{-1} Geneticin and 10 mg L^{-1} Blasticidin-S. For the experiments the cells were harvested and washed three times with phosphate buffer (PB; 2.00 g L^{-1} KH_2PO_4 with 0.36 g L^{-1} $Na_2HPO_4 \cdot H_2O$, with a pH of 6.0) by centrifugation at 400 rcf for 5 min.

Imaging Samples were prepared on a hydrophobic coverslip that was incubated for 15 min in Pluronic F-127 (5 wt% in water). Subsequently, the excess Pluronic F-127 was removed by washing three times with the appropriate buffer. GUV samples were prepared by consecutive addition of 50 µL phosphate buffered saline solution (with a total osmotic content of 100 mM), 0.5 µL 3 wt% particles, and 2 µL GUVs. *D. discoideum* samples were prepared by subsequent addition of 50 µL PB (see previous subsection), 0.5 µL 1.5 wt% particles, and 10 µL cells. All microscopy images were taken 30–45 min after mixing. Microscopy images were acquired with a Nikon Ti-E microscope equipped with a A1R confocal scanhead and a 60× water immersion objective (NA = 1.2). The sample was mounted on a MCL Nanodrive to enable fast z-stack acquisition.

4.3 Results and Discussion

We here describe a surfactant-free preparation method of colloidal particles that specifically bind to target molecules. In this method, polystyrene microparticles are first coated with a protein that provides the specific affinity, and subsequently with a layer of mPEG that provides stability against aggregation under physiological conditions (see Fig. 4.1). As protein we primarily use avidin, which has a high affinity to biotin, and further show that the method is readily transferable to the sugar-binding Concanavalin A. In the following, we will characterize the particle coating and show its affinity and specificity on vesicles as well as on living cells. Firstly, we study the colloidal stability of the particles at various physiological conditions. Secondly, we quantify how the number of biotin binding sites on the avidin-coated particles depends on the synthesis parameters. Thirdly, we measure the binding affinity of the avidin-coated particles to biotinylated membranes. Finally, we demonstrate that avidin-coated particles bind specifically to biotinylated membranes, and that Concanavalin A-coated particles bind specifically to the glycocortex of *D. discoideum* cells.

4.3.1 Colloidal stability

One of the most important requirements of a colloidal dispersion is its stability. Without a polymer coating, colloidal stability relies on Coulomb repulsion: the surface charge of the particles prevents aggregation in deionized water. At a physiological salt concentration, however, the Coulomb force is screened by counter-ions so that the short ranged Van der Waals force induces permanent particle aggregation (see Fig. 4.1e). Therefore, steric stabilization is necessary to use colloidal particles at physiological conditions. This involves adsorbing or grafting a layer of well-soluble polymers to the particle surface. When particles approach each other, the layers on neighbouring particles will interpenetrate, which costs free energy as the polymers can occupy less volume.[136] For a sufficiently dense and thick polymer layer, this yields a repulsive force that counteracts the Van der Waals attraction and protects the particles from aggregating at arbitrary salt concentrations.[137]

We here achieve this steric stabilization by covalently grafting methoxypoly(ethylene) glycol (mPEG5000) to the particles by an NHS/Sulfo-NHS linking procedure.[131] mPEG is an excellent candidate for stabilizing colloidal particles as it is uncharged and hydrophilic. Furthermore, mPEG coatings have been shown to reduce the non-specific adhesion of particles to proteins, cells, and tissues.[122] As these properties make the particles less visible to the immune system, mPEG coatings are particularly interesting for drug delivery applications.[111]

Indeed, we found that after 1 h in 1 M NaCl, the here employed mPEG5000 coated particles showed excellent stability against aggregation. We observed that performing the mPEG coating at 4 °C for a minimum of 24 h is essential for obtaining the required colloidal stability, which is in agreement with earlier reports on mPEG-coated particles.[131] The ζ-potential before particle coating (-55 ± 4 mV) clearly becomes less negative after

the mPEG coating (see Table 4.1), which additional further evidence for the presence of a dense PEG layer.[131] The remaining negative charge is likely caused by unreacted carboxylic acid groups.

Furthermore, we assessed the stability of the mPEG-coated particles in a standard PBS buffer and in three typical cell culture media. After 1 h of mixing, we did not observe any aggregation of the particles inside any of these solvents. In HL5 medium, the ζ-potential was similar to that in the PBS buffer, while we observed a significant change in both the mammalian cell media A549 and 3T3 (see Table 4.1). While HL5 contains mostly salts, sugars, and amino acids, the A549 and 3T3 cell media contain significant amounts of proteins from bovine serum. Protein adsorption has been shown to be suppressed, but not completely prevented by a dense mPEG coating such as employed here.[138,139] Therefore, we attribute the less negative ζ-potential in the A549 and 3T3 cell media to non-specific protein adsorption.

Table 4.1. The ζ-potential of mPEG-coated particles after 1 h incubation in water, phosphate buffered saline (PBS), the *D. discoideum* medium HL5, and mammalian cell media A549 and 3T3 (see Materials section for the contents of the media).

Solvent	pH	ζ-potential [mV]
Water	5.7	-24 ± 4
PBS	7.3	-18 ± 4
HL5	6.6	-15 ± 5
A549	7.6	-7 ± 5
3T3	8.1	-7 ± 5

To summarize, the here described particles with a dense layer of mPEG on their surface do not show aggregation at high salt concentration, as well as in several cell culture media. To now provide these particles with an affinity to a specific molecule, we introduce an additional step in the procedure: we first coat the EDC/Sulfo-NHS activated particles with a functional protein before saturating the surface with an excess of mPEG (see Fig. 4.1). The resulting particles possess functional proteins as well as mPEG polymers on their surface. We still observed that these particles were stable in 1 M NaCl after 1 h, for protein densities of up to 67 µg per mg particles. Although the proteins occupy significant space on the particles (see next section), we conclude that the density and size of mPEG5000 is still sufficiently high to protect the particles from aggregating. This combination of covalent colloidal stability with a specific-binding protein is key to targeting specific molecules on lipid membranes.

4.3.2 Accessible binding sites

In general, a specific binding affinity of a colloidal particle is provided by a finite number of binding sites on the particle surface. The number of sites will directly influence the binding affinity of the particle and therefore we quantified this parameter for the here

described particles. In the coating method, we immobilized the protein via NHS groups on the particles, which are known to form a covalent amine bond with random lysine residues that are typically present in the protein exterior.[128,129] As an example, we coated particles with avidin, which is a protein that binds to biotin with high affinity (approx. 17 k_BT per bond[140]). Because of this high affinity, and the versatility of avidin, it has been widely applied as a connector in biochemistry, for instance as a binding site for attaching biotinylated monoclonal antibodies.[141,142] We here use NeutrAvidin, which is a neutral and non-glycosylated avidin, and quantify the number of biotin binding sites using a combined titration and fluorescence assay for several synthesis conditions.

To rule out non-covalent adsorption of the avidin on the particles during the preparation,[143] we performed the synthesis without the EDC/NHS activation (Fig. 4.1a). The absence of NHS esters resulted in particles without biotin binding sites, while at the same time the particles were unstable in 1 M NaCl. This shows that the NHS activation is necessary for avidin and mPEG binding: we conclude that all biotin binding sites that we observed in this section are caused by avidin that is covalently linked to particles via amine bonds through the EDC/NHS chemistry.

The number of biotin binding sites on the particles is controlled by the amount of avidin that is added during synthesis. To probe this relation, we changed the quantity of added avidin between 0–7 µg per mg particles and measured the resulting average number of biotin binding sites per particle with a titration assay (Fig. 4.3a). Clearly, the amount of added avidin allows for continuously changing the average number of biotin binding sites per particle up to at least 2.9×10^4 . Assuming a typical protein size of 5 nm, we estimate that at this value approximately 1% of the particle surface is covered with protein. The slope of the linear regression in Fig. 4.3a provides information on the synthesis yield: the biotin binding capacity corresponds to 0.71 accessible biotin binding site per added avidin protein, which has a total of 4 binding sites. Apart from the chemical yield of the avidin immobilization, we presume that part of the biotin binding sites are actually present on the particles, but not accessible because of the random orientations of the avidin proteins,[128] steric hindrance by the covalently grafted mPEG polymers, or unfolding of the proteins during the pH 12 synthesis step.

Additionally, we measured the distribution of the number of biotin binding sites per particle using quantitative fluorescence microscopy. By incubating the particles with btn-DNA-Cy3, we visualized the accessible biotin binding sites for individual particles. As can be seen in Fig. 4.3b, the resulting distributions are broad and skewed towards lower values of fluorescence. The relatively large spread is a consequence of the random immobilization of the avidin on the particle surfaces. To mitigate this effect, it is important that the added protein is well mixed with the particles during the coating procedure. Here, we achieved this by vortexing the mixture directly after the addition of protein to the particles.

By combining the distributions from the fluorescence assay with the quantitative results from the titration assay, we obtained the most frequently occurring numbers of biotin binding sites, which are shown in Figure 4.3c together with the one-standard

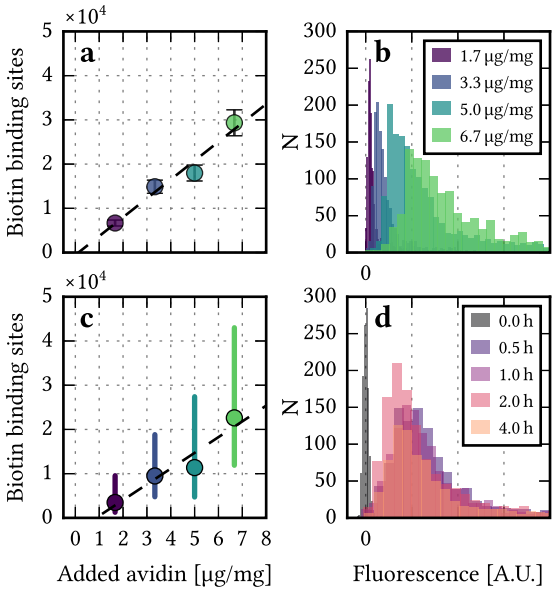

Figure 4.3. The amount of biotin binding sites on avidin-coated particles. (a) The average number of binding sites per particle, measured by titration. Four particle batches with varying amounts of avidin were analysed. Error bars denote the measurement precision. (b) Histograms of the btn-DNA-Cy3 fluorescence of same particle batches (see legend) show a skewed distribution for all samples. Combining the averages from the titration assay (a) with the relative spreads from the fluorescence assay (b), the spread in the number of binding sites within particles of a single batch could be recovered, which is displayed in (c). Here, error bars denote the one standard deviation spread of biotin binding sites per particle in a single batch, and the discs mark the most frequently occurring value. Lines in (a) and (c) are linear regressions. (d) Histograms of the btn-DNA-Cy3 fluorescence of five particle batches with varying delay time between avidin and mPEG addition (see legend), at a fixed avidin concentration of 3.3 µg/mg. As the distributions did not change after 0.5 h, we concluded that the avidin coating was completed within 0.5 h after avidin addition.

deviation spread. Due to the skewed distributions, the average number of biotin binding sites is higher than the most frequently occurring number of biotin binding sites. Still, this quantity is linear with the amount of added avidin and therefore it can be tuned readily by varying the avidin concentration.

After activating the carboxylic acid particles with NHS, avidin and mPEG are added sequentially. As the two reactions compete with one another for the NHS esters, we used a delay time between avidin and mPEG addition, so that the avidin immobilization is allowed to complete before mPEG starts to occupy particle surface area. The duration of this delay may affect the final avidin density on the particles. In order to find the

minimum delay time between the addition of avidin and mPEG, we varied the delay between 0–4 h at a fixed amount of avidin of 3.3 µg per mg particles. See Fig. 4.3d for the corresponding distributions of the btn-DNA-Cy3 fluorescence. As the distributions do not change significantly between 0.5–4 h, we conclude that the distribution of linker densities already reached a steady state within the first 0.5 h. Throughout this chapter, we fixed the delay time at 2 h to ensure the complete grafting of proteins.

To summarize, we have shown that the reported particle coating method allows for tuning the average number of accessible biotin binding sites up to 2.9×10^4 per particle, which is sufficient to achieve high binding affinity and specificity, as will be demonstrated in the next sections.

4.3.3 Binding affinity

In the previous section, we have shown that the avidin-coated particles bind fluorescently labelled biotin molecules. Next, we investigated whether this binding affinity also holds for lipid membranes that feature biotin target groups. As a model membrane, we used Giant Unilamellar Vesicles (10–50 µm in diameter) that contain biotinylated lipids. After 30 min incubation of these vesicles in a 0.02 vol% particle suspension, we imaged the vesicles with confocal microscopy. See Fig. 4.4a for a GUV that was incubated with

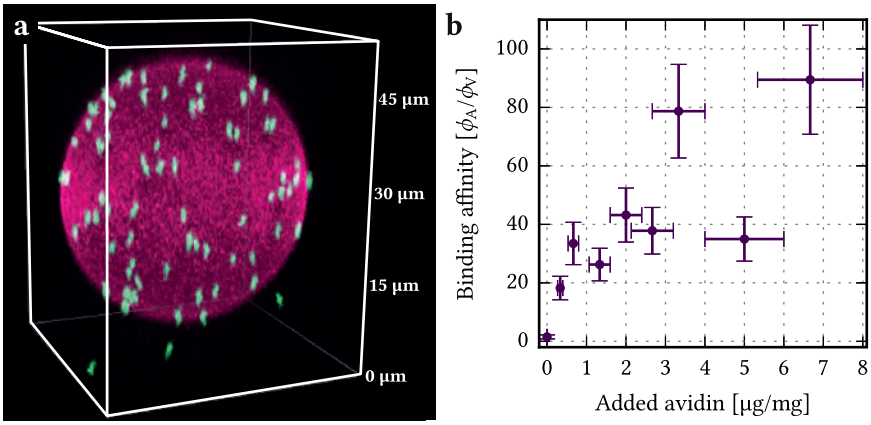

Figure 4.4. Binding affinity of avidin-coated particles on biotinylated lipid membranes. (a) A three-dimensional confocal image showing a biotinylated GUV (magenta) that has been incubated for 30 min with avidin-coated particles (green). (b) The membrane area coverage ϕ_A was measured and averaged over 10 randomly selected GUVs, for varying amount of avidin on the particles. The particle volume fraction ϕ_V was 0.021 ± 0.004 vol %. The vertical error bars denote the uncertainty in binding affinity due to the imprecision in particle volume fraction; the horizontal error bars denote the uncertainty in the amount of added avidin. The confocal image in (a) corresponds to the point at 3.3 µg/mg avidin.

3.3 µg/mg avidin-coated particles, which clearly adhered to the biotin-containing membranes.

As the surrounding liquid is stationary, a diffusion-limited adsorption model seems applicable.[144] To explain the dependence of particle adsorption on avidin density, we presume that the avidin concentration on the particles determines a certain binding probability to biotin-containing membranes. For low membrane coverage, the covered membrane area fraction is proportional to the particle concentration in the surrounding liquid and therefore it is reasonable to define a (non-equilibrium) binding affinity with the membrane area that is covered by particles ϕ_A, divided by the bulk particle volume fraction ϕ_V, at fixed incubation time. We measured this membrane area fraction ϕ_A using tracking methods described in Chapter 5 on the three-dimensional confocal images.[86,145]

The observed binding affinities (ϕ_A/ϕ_V) after 30 min incubation are shown in Fig. 4.4b. The effect of avidin on the binding affinity to biotin-containing GUVs is clear: between 0 and 0.3 µg/mg, the binding affinity increased more than tenfold from 1.5 ± 0.7 to 18 ± 4. After that, binding affinity increased for increasing avidin densities, until a membrane area coverage of 1.9% after 30 min in a 0.02 vol % particle suspension was achieved. At amounts of added avidin larger than 3 µg/mg, we observed large fluctuations in the binding affinities. A possible reason for these is a large spread in the number of binding sites per particle: as mentioned before, it is important that the proteins are well mixed with the activated particles in order to obtain homogeneous coatings throughout the sample. From this we conclude that the binding probability of avidin-coated particles increases with increasing avidin density on the particles.

4.3.4 Binding specificity

We have shown that by immobilizing a protein on the surface of colloidal particles, the affinity to its target molecule is transferred from the protein to the particle surface. Now, we investigate whether also the binding specificity is conserved. For this, we assessed the binding affinity of three types of functionalized particles to different lipid membranes. As a control experiment, we first tested the binding affinity of particles with only mPEG on their surface. Then, in order to show the specific targeting to biotinylated surfaces, we used the avidin-coated particles that we investigated in detail in the previous sections (3.3 µg avidin per mg particles). Finally, to show the generality of the coating protocol, we also assessed particles functionalized with 33 µg/mg of the sugar-binding protein Concanavalin A (ConA).

The specific binding affinity of these three types of particles was tested first on model lipid membranes (GUVs) of known composition (see Method section). In Figures 4.5a–c, we show that none of the investigated particles bound to lipid membranes without exposed biotin molecules: the dense mPEG coating clearly suppressed aspecific binding to the GUVs, irrespective of the immobilized proteins that are also present on the particles. On biotinylated membranes, however, we observed binding of avidin-coated particles with high affinity, while the control and ConA coated particles did not show significant

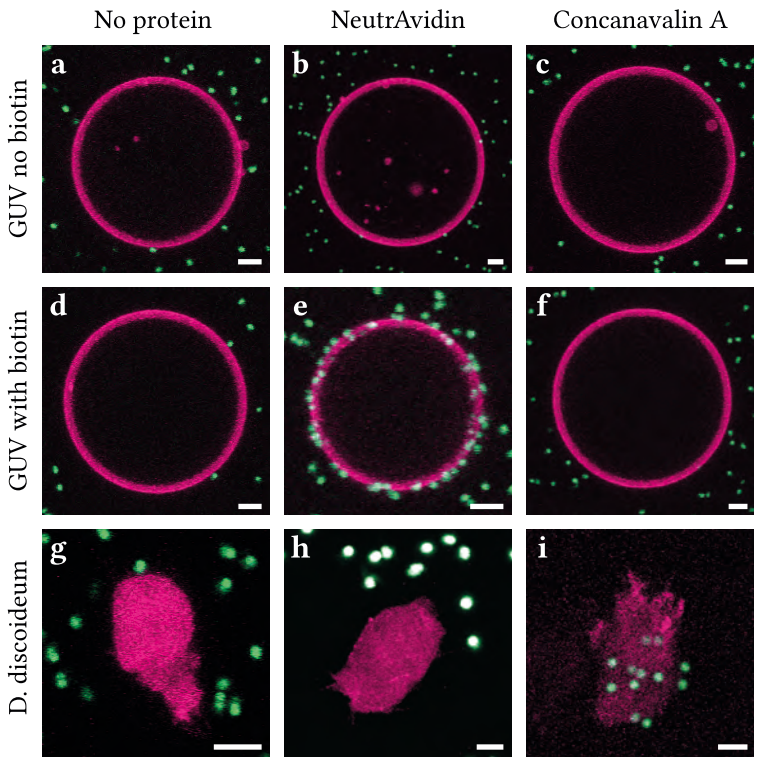

Figure 4.5. Binding specificity of non-functionalized particles, NeutrAvidin-coated particles, and Concanavalin A-coated particles. The specific adhesion is shown on (a)-(c) GUVs with only PEG on their surface, (d)-(f) GUVs with an excess of biotinylated PEG on their surface, and (g)-(i) *D. discoideum* cells. All images are maximum intensity projections of three-dimensional confocal images. For the GUVs in (a)-(f), the particles are displayed in green and the membrane in magenta. For the cells in (g)-(i), the magenta denotes the filamentous actin, which visualizes the cell contour. These pictures were chosen such that there were no particles floating in front or behind, otherwise the cells are representative of the samples. The scale bars denote 5 µm.

binding (Figs. 4.5d–f). Therefore, we conclude that the here described coating protocol successfully transfers the specificity of the avidin to colloidal particles.

Additionally, we also investigated the specificity of these particles to living cells. For *D. discoideum* cells, we found that the mPEG coating successfully prohibited adhesion to the cells: without mPEG coating, 50% of the particles were internalized by the cells, while with mPEG coating, we observed none out of over 200 observed particles to be stuck to the cellular membrane (see Fig. 4.5g). At the other hand, on two types of mammalian cells (A549 human lung carcinoma and 3T3 mouse fibroblasts), we found that the mPEG-

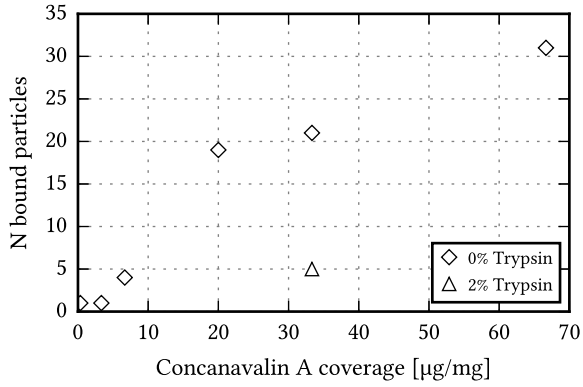

Figure 4.6. Number of ConA-coated particles that bound to the *D. discoideum* cells. We varied the amount of ConA per particle and observed the total number of particles that bound to two selected cells in the sample (diamonds). This provides a qualitative measure of the affinity of the particles to the cells. We also treated the cells with Trypsin (cells treated for 10 min in 2 wt% Trypsin at 30 °C), which removed glycoproteins, and indeed we observed a decrease in the number of adsorbed particles (triangle).

coated particles stuck non-specifically to the cellular membranes. As these cells are cultured in protein-rich media, we presume that the observed adhesion was caused by a protein corona that formed around the particles, which is supported by the ζ-potential measurements inside these media (see Table 4.1). Therefore, we conclude that protein-rich media may induce aspecific binding of mPEG-coated particles.

The particles that were functionalized with avidin also did not bind to the *D. discoideum* cells (Fig. 4.5h). These results are in line with the previous results on GUVs: the presence of the immobilized protein does not change the aspecific binding of the particles. The ConA-coated particles did however show significant binding (Fig. 4.5i). ConA binds selectively to glucose and mannose residues[146] which are present in the glycocortex of the *D. discoideum* cells. The ConA functionalization indeed resulted in particles that bound specifically to the *D. discoideum* cells, while the ConA did not cause any binding to the GUVs (Figs. 4.5c and f). As with the avidin-coated particles to biotinylated membranes, binding affinity of the ConA particles to the *D. discoideum* cells seems to depend on the amount of ConA on the particles (see Fig. 4.6). Also, we observed that after cleaving glycoproteins from the cellular membranes by addition of Trypsin,[147] the binding affinity decreased significantly.

These observations confirm that the here described surfactant-free coating protocol readily transfers the specificity of a certain protein to a colloidal particle. As long as non-specific binding is suppressed, which we showed on GUVs without biotin, GUVs with biotin, and on *D. discoideum* cells, our method provides a means to design colloidal

particles that 'target' specific molecules in lipid membranes, which has useful applications in fundamental membrane studies, micrometer-sized self-assembly, and directed drug release.

4.4 Conclusion

To direct colloidal particles to specific molecules in lipid membranes, we have developed a surfactant-free coating procedure that produces particles with a high affinity to specific molecules. A coating with proteins provides the binding affinity, while at the same time a dense layer of covalently coupled mPEG gives stability at physiological conditions. As the method is surfactant-free, the particles are especially useful for surfactant-sensitive applications, such as studying lipid membrane properties, also in living environments.

The protein density on the particles can be tuned in order to adapt the binding affinity, which we have shown for the biotin-binding protein NeutrAvidin using a combined titration and fluorescence assay. The yield of the coating method is such that 18 % of the biotin binding sites is accessible after the coating procedure, which is presumably caused by the random protein orientations and the high mPEG coverage that potentially obstructs the binding sites. The largest observed number of biotin binding sites was 2.9×10^4 per 1.06 µm diameter particle.

These avidin-coated particles spontaneously bind to biotinylated membranes. We have shown that the amount of NeutrAvidin on these particles directly influences the binding affinity. While particles without avidin did not exhibit any significant binding to these membranes, avidin coated particles reached up to 1.9% membrane coverage after 0.5 h in a 0.02 vol % particle suspension.

Additionally, we have shown that the coating procedure can be readily transferred to the sugar-binding protein Concanavalin A. Functionalized with this protein, particles become specific to the outside of *D. discoideum* cells, while they do not bind to biotinylated membranes. On the other hand, while the avidin-coated particles bind to biotinylated GUVs, they do not bind to these cells. This shows that the specificity of a protein can be transferred successfully to the surface of the colloidal particles.

Our surfactant-free coating method makes colloidal particles selectively adhesive to certain targeted molecules at physiological conditions. The fact that the coating procedure only applies covalently linked mPEG as stabilizer makes it applicable to cases in which surfactants are undesirable. The procedure is readily adjustable to include different proteins, which will provide custom colloidal particles for use in future lipid membrane measurements, micrometer-scale self-assembly, drug targeting, and biosensing applications.