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Louise Wogelred, Fredrik Höök, Björn Agnarsson, et al.

Toward multiplexed quantification of biomolecules on surfaces using time-of-flight secondary ion mass spectrometry

Louise Wogelred
RISE Research Institutes of Sweden, P.O. Box 857, SE-50115 Borås, Sweden and Department of Physics, Chalmers University of Technology, SE-41296 Göteborg, Sweden

Fredrik Höök and Björn Agnarsson[a)]
Department of Physics, Chalmers University of Technology, SE-41296 Göteborg, Sweden

Peter Sjövall[a)]
RISE Research Institutes of Sweden, P.O. Box 857, SE-50115 Borås, Sweden and Department of Physics, Chalmers University of Technology, SE-41296 Göteborg, Sweden

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Accurate detection and quantification of individual molecules is important for the development of improved diagnostic methods as well as biochemical characterization of disease progression and treatments. Time-of-flight secondary ion mass spectrometry (ToF-SIMS) is a surface analysis technique capable of imaging the distribution of specific molecules on surfaces with a high spatial resolution ($<1\,\mu$m) and high sensitivity. ToF-SIMS is particularly suitable for detection of molecules up to $\sim$2 kDa, including lipids, whereas larger molecules, such as peptides and proteins, are fragmented during analysis, which makes them difficult to identify. In this study, an approach for extending the molecular detection capability of ToF-SIMS is presented, based on the specific binding of functionalized liposomes to molecular targets on the sample surface and subsequent detection of the liposomes by ToF-SIMS. Furthermore, by using different recognition elements conjugated to liposomes with different lipid compositions, simultaneous detection of different targets was accomplished. This multiplexing capability was investigated for two types of recognition elements (antibodies and cholera toxin) and for target molecules immobilized on surfaces using two frequently applied surface functionalization strategies: a supported lipid bilayer aimed to mimic a cell membrane and a polyethylene glycol modified surface, commonly employed in bioanalytical sensor applications. The efficacy of the conjugation protocols and the specificity of the recognition mechanism were confirmed using quartz crystal microbalance with dissipation monitoring, while fluorescence microscopy was used to validate the ToF-SIMS data and the reliability of the freeze-drying step required for ToF-SIMS analysis. The results demonstrated specific binding of the two types of liposomes to each target and showed a concentration-dependent binding to the targets on the different model surfaces. In particular, the possibility to use the contrasts in the mass spectra of SIMS to identify the concentration dependent coverage of different liposomes opens up new opportunities for multiplexed detection and quantification of molecules at biotechnology relevant interfaces. https://doi.org/10.1116/1.5019749

I. INTRODUCTION

Reliable and quantitative detection of biomolecules is essential for the development of new medical diagnostic methods and therapeutic treatments. As new disease markers are discovered, the demand for biomolecular detection methods with improved sensitivity, specificity, and quantitative accuracy is rapidly growing. In addition, increasing the capability to simultaneously monitor many different biomolecular species, so-called multiplexing, is important, e.g., to enable simultaneous screening for multiple diseases and to improve the capability for early and reliable diagnoses of serious diseases.[1] Simultaneous detection of many biomolecular species also improves the possibility to characterize the complex biomolecular processes involved in disease progression and the biochemical responses to therapeutic treatments.

Imaging mass spectrometry is a powerful approach for biomolecular detection that allows for label-free identification of many different molecular species in parallel.[2–8] However, the different techniques applied, such as matrix-assisted laser desorption/ionization or time-of-flight secondary ion mass spectrometry (ToF-SIMS), are typically limited with respect to the types of biomolecular species that can be detected at the same time, and in addition, the sensitivity is typically inferior to methods that rely on detection of single target molecules. A variety of methods, based on immuno-histochemical labeling of specific molecules using antibodies and subsequent detection using fluorescence microscopy, provide high sensitivity, but they are typically limited to simultaneous detection of only a few analytes (3–4) in parallel.[1,9,10]

[a)]Authors to whom correspondence should be addressed; electronic addresses: bjorn.agnarsson@chalmers.se; peter.sjovall@ri.se

In this work, a new methodology is evaluated that allows for parallel detection and quantification of multiple biomolecular species on surfaces using a combination of functionalized liposomes and imaging mass spectrometry. The approach is based on specific binding of liposomes to target molecules on the surface, using a recognition element (such as an antibody) that is conjugated to the liposome, and subsequent detection and imaging of the liposomes using ToF-SIMS. Potential advantages of this approach compared to conventional optical imaging include (1) high sensitivity since each binding event is monitored by the detection of a single liposome, (2) high spatial resolution in the 1–2 μm range, and (3) high multiplexing potential. The latter is achieved by using different types of liposomes in parallel, each with a different recognition element uniquely linked to a specific lipid composition of the liposome.

In order to investigate the feasibility of the approach, we previously studied the binding of biotin-functionalized liposomes to NeutrAvidin (a protein with four binding sites for biotin) on model surfaces using quartz crystal microbalance with dissipation monitoring (QCM-D), fluorescence microscopy, and ToF-SIMS.[11] By varying the concentration of NeutrAvidin on the sample surfaces, it was concluded that ToF-SIMS is capable of detecting single liposomes on the surface and that the approach can be used to quantify the NeutrAvidin concentration on the surface in a coverage interval from 0.1×10^9 to 5×10^9 molecules/cm^2. The biotinylated liposomes were also applied for imaging of a specific peptide, amyloid-β (Aβ), in mouse brain tissue sections by ToF-SIMS, using biotinylated antibodies directed to Aβ and liposome binding with NeutrAvidin as a linker.[12] However, to achieve multiplexed detection of several targets on the surface, it is necessary to use liposomes with different lipid compositions, each with a specific recognition element, so that the target molecule can be uniquely identified by the lipid composition of the detected liposome. In the present work, we therefore extend the protocol for conjugating recognition elements to different types of liposomes and evaluate the multiplexing potential by studying the parallel detection of two target molecules on the surface. Furthermore, the potential and general applicability of the approach are demonstrated by the parallel detection of target molecules on two commonly applied target-presenting surfaces: self-assembled monolayers of poly(L-lysine) (PLL)-g-polyethylene glycol (PEG) polymers and supported lipid bilayers (SLBs), respectively. These two interfaces were selected since they are frequently applied as biomimetic and/or biosensor interfaces and can either be used to suppress unspecific binding of both liposomes and proteins or be functionalized to incorporate target molecules of interest.

Monolayers of PLL-g-PEG can be formed on various solid supports, and the PEG chains can be functionalized with various molecules, such as biotin, making them widely used for biomedical applications such as sensing. Here, biotin-conjugated target molecules, Aβ (0-40) and Tau, were immobilized at different ratios on a PLL-g-PEG/PLL-g-PEG-biotin monolayer mixture using NeutrAvidin as a linker. Aβ and Tau are the main constituents of amyloid plaques and neurofibrillary tangles, respectively, which are formed in the brains of Alzheimer's disease patients. Furthermore, their concentration ratio in bodily fluids has been identified as a biomarker for early stages of the disease,[13] making the parallel detection of these two target molecules highly relevant. Liposomes made of two different types of lipids were thus conjugated with anti-Aβ or anti-Tau antibodies, respectively, and used for simultaneous detection of the two molecules on the surfaces [see Fig. 1(a)].

SLBs provide an alternative strategy for immobilizing either recognition elements or target molecules on surfaces. Since many relevant proteins are represented in biological membranes, SLBs provide a natural environment for many membrane or transmembrane proteins. Furthermore, SLBs are fluid, which allows the target molecules to diffuse rather freely along the SLB surface. Here, SLBs containing biotin and the glycosphingolipid GM1 at different ratios were prepared and

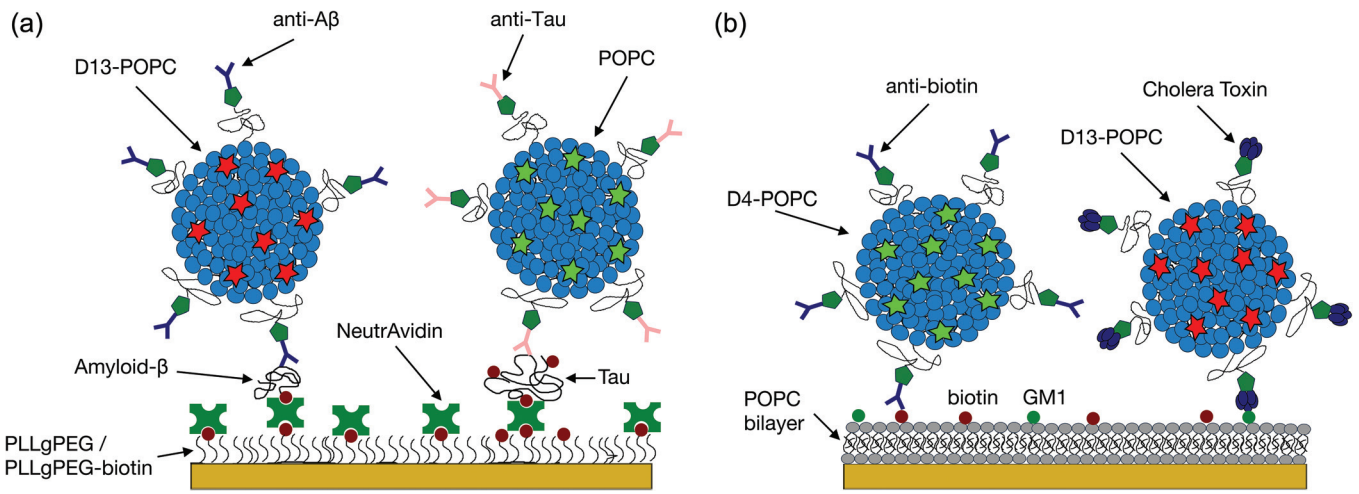

FIG. 1. Schematic illustrations of the two target-liposome systems used to study multiplexed molecule detection. (a) 100–200 nm diameter immuno-liposome binding to biotinylated Aβ or Tau molecules attached to a PLL-g-PEG-biotin model surface through biotin-NeutrAvidin binding. (b) Liposomes conjugated with either cholera toxin or antibiotin antibodies, binding to GM1 or biotin in a supported lipid bilayer.

investigated with respect to specific binding of liposomes conjugated with either antibiotin antibodies or protein cholera toxin subunit B (Chtx), which is known to bind specifically to GM1 (Ref. 14) [see Fig. 1(b)].

II. EXPERIMENT

A. Preparation of protein-conjugated liposomes

Liposomes were created by mixing 2 mg of the desired lipids in a round bottom flask followed by drying of the solvent (methanol/chloroform) in a flow of N_2 gas and subsequent incubation in vacuum for 1 h. The lipid mixture included 89 wt. % of deuterated (D4 or D13) 1-palmitoyl-2-oleoyl-sn-*glycero*-3-phosphocholine (POPC, Avanti Polar Lipids, Alabaster, AL), 10 wt. % of 1,2-distearoyl-sn-*glycero*-3-phosphoethanolamine-*N*-[maleimide(poly-ethylene glycol)-2000] (Avanti Polar Lipids), and 1 wt. % of one of the three types of fluorescently labeled lipids: (1) lissamine rhodamine B 1,2-dihexadecanoyl-sn-*glycero*-3-phosphoethanolamine (DHPE-Rhodamine, Invitrogen) or (2) 1,2-dioleoyl-sn-*glycero*-3-phosphoethanolamine ATTO 488 (DOPE-ATTO 488, ATTO-TEC GmbH) or (3) *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-*glycero*-3-phosphoethanolamine (NBD-PE, Invitrogen, Life Technologies). After drying, the resulting lipid film was hydrated in a 1 ml buffer solution containing 10 mM Sodium Acetate (NaAc, *p*H 5, Sigma-Aldrich) and vortexed for 5 min, followed by extrusion 11 times through a polycarbonate filter (pore size 100 or 200 nm), resulting in the formation of unilamellar liposomes at a concentration of 2 mg/ml. The size distribution of the liposomes was measured using nanoparticle tracking analysis (NTA, NanoSight, Malvern Instruments Ltd, UK) to diameters of 120 (±45) nm and 160 (±50) nm, dependent on the size of the filter (100 and 200 nm, respectively).

The proteins {antibiotin (1D4-C5 IgG monoclonal antibody, 0.5 mg/ml, BioLegend, San Diego, CA), cholera toxin subunit B (1 mg/ml, Sigma-Aldrich, St. Louis, MO), anti-Aβ [6E10, beta amyloid (1-16) monoclonal antibody, 1 mg/ml, Covance], and anti-Human Tau (HT7 monoclonal antibody, 0.2 mg/ml, Thermo Scientific)} were conjugated to the liposomes using the maleimide-thiol reaction, as described previously,[15] using a thiolation agent called Traut's reagent (2-iminothiolane hydrochloride, Sigma-Aldrich). The proteins were preincubated with Traut's reagent [0.04 mg/ml in 0.01 M phosphate buffered saline (PBS, *p*H 7.4, Sigma-Aldrich) + 5 mM ethylenediaminetetraacetic acid (*p*H 8, Sigma-Aldrich)] at a molar ratio of 1:35 for 15 min at room temperature to form thiol groups on the proteins. The thiolated proteins were then added to the liposomes at a concentration ratio corresponding to roughly 50:1 protein molecules per liposome and incubated for 1 h at room temperature, to induce the conjugation reaction between the thiol-groups on the protein and the maleimide groups on the liposomes. The mixture was then filtrated by gel chromatography, using a gel column packed with Sepharose 4B (GE Healthcare Life Sciences, Sweden), to remove the unreacted proteins from the liposome solution. The liposome/protein mixture was eluted in the column with PBS, allowing separation of the components according to size, and collected into different fractions containing 250 μl of PBS each. The fractions containing liposomes, detected using the fluorescently labeled lipids, were pooled together and diluted to 0.5 mg/ml in PBS and kept at 4 °C until use.

B. Preparation of model surfaces and liposome binding

Model surfaces comprised of functionalized self-assembled polymers or SLBs were used as target-presenting surfaces. Polymer surfaces with controlled concentrations of biotin were prepared by adsorbing a polymer layer consisting of a mixture of poly(L-lysine)-g-poly(ethylene glycol) {PLL(20)-g[3.5]-PEG(5), SuSoS AG, Switzerland} and PLL(20)-g[3.5]-PEG(5)-biotin (45%–65%) (SuSoS AG, Switzerland) on a sputter-coated silica surface. Each PLL-g-PEG graft polymer molecule consists of a PLL backbone and 28 PEG chains, and in PLL-g-PEG-biotin, approximately 50% of the PEG chains are functionalized with a biotin group.[16] Different biotin concentrations on the model surface were obtained by using different ratios of PLL-g-PEG-biotin and PLL-g-PEG in the adsorbed layer. The PLL-g-PEG model surfaces were prepared by incubating the substrates with the PLL-g-PEG/PLL-g-PEG-biotin mixtures (10 μg/ml total concentration) for 20 min followed by rinsing with Milli-Q water.

The targets (Aβ and Tau) were attached to the polymer model surface using biotin-NeutrAvidin coupling chemistry. Prebiotinylated Aβ (1-40) peptides were purchased from Bachem, Switzerland, but the Tau proteins [Tau-352 (0N3R), rPeptide] had to be conjugated with biotins in-house by mixing *N*-Hydroxysulfosuccinimide-PEG-biotin (Sigma-Aldrich) with Tau in 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, *p*H 6.8, at a ratio of 5:1 for 1 h before being diluted in MES and filtered using Amicon Ultra 10 kDa filter, three times for 15 min at 10 000 × g.

After forming a PLL-g-PEG layer with 10% PLL-g-PEG-biotin (corresponding to a PLL-g-PEG-biotin concentration of ~1000 molecules/μm^2, assuming 100 nm^2 area per adsorbed PLL-g-PEG molecule[11]) and rinsing, the surface was preincubated with NeutrAvidin (10 μg/ml) for 30 min before being rinsed thoroughly, first with Milli-Q water and then PBS buffer. The biotinylated targets were then introduced to the surface at different ratios, keeping the total target concentration fixed at 400 nM, and allowed to incubate for 20 min. After incubation, which is expected to result in protein binding to NeutrAvidin on nearly all adsorbed PLL-g-PEG-biotin molecules, the surface was rinsed with PBS buffer.

The SLB model surfaces were formed by adsorption of small unilamellar vesicles containing 99% POPC and 1% of either 1,2-distearoyl-sn-*glycero*-3-phosphoethanolamine-*N*-[biotinyl(polyethylene glycol)-2000] (Avanti Polar Lipids) or monosialoganglioside GM1 (from bovine brain, Sigma-Aldrich). The vesicles were produced according to the method described above by dissolving the dried lipids (1 mg) in 1 ml of PBS followed by vortexing and extrusion through a 30 nm polycarbonate filter 11 times. The diameter of the

liposomes was estimated by NTA to be 120 (±40) nm. The SLBs were produced by incubating the substrates with the POPC-biotin/GM1 vesicles (0.1 mg/ml diluted to 0.01% biotin/GM1 mixture in pure POPC vesicles, resulting in an estimated target concentration on the SLB of ∼150 molecules/μm^2) for ∼30–45 min, at different biotin/GM1 ratios (0%–100%) to produce model surfaces with different relative concentrations of the two targets, while keeping the total target concentration constant at 0.01%, followed by rinsing with PBS and Milli-Q water.

In the final step, the model surfaces were incubated with the protein-conjugated liposomes (0.1 mg/ml and 10 μg/ml for the PLL-g-PEG/PLL-g-PEG-biotin and SLB model surfaces, respectively) for 1.5–2 h and then rinsed with PBS buffer. All the incubation steps were conducted under stagnant conditions by placing a droplet (with 5–100 μl of sample for each model surface, respectively) on the sample area for the specified time. Occasional stirring (every 15 min) of the droplet solution was conducted using a pipette.

The efficiency and specificity of the functionalized surfaces for liposome binding were confirmed and evaluated using QCM-D (see Figs. S1–S3 in the supplementary material).[21]

C. Analysis methods

Analysis of liposome binding was first conducted using fluorescence microcopy (Olympus BX 63), with the different model surfaces still in the liquid state, prior to plunge-freezing and freeze-drying. The liposomes attached to the model surfaces were detected using the incorporated fluorescent markers in the liposomes, which were excited and selected using matching filter cubes [ET-CY3/TRITC and ET6-EGFP (FITC/Cy2), Chroma Technology]. Fluorescence images were acquired using a 40×, NA 0.8, water immersion objective and an Olympus DP72 camera by sequentially exciting each of the fluorophores at the same location on the model surfaces.

Since ToF-SIMS measurements are conducted under vacuum conditions, the samples had to be subjected to a freeze-drying procedure prior to ToF-SIMS analysis, including the following steps: (1) thorough rinsing of the sample surfaces with Milli-Q water, (2) rapid freezing (plunge-freezing) of the samples in liquid propane at −185 °C, and (3) freeze drying of the samples by placing the frozen samples in a vacuum chamber and slowly allowing them to warm up to room temperature overnight at a pressure $<10^{-6}$ mbar. In order to generate a thin film of water that is stable on the sample surface during plunge-freezing, a specially designed sample-substrate was used.[11] The substrates consist of optically flat Si chips with a 50 nm thick sputter-coated SiO_2 layer, onto which a 60 μm thick layer of epoxy (SU-8, MicroChem Corp.) is spin coated, excluding a 3 mm diameter circular area in the center of the chip. The substrate thus provides a clean SiO_2 sample area in the center of the chip, surrounded by a polymer film that acts to retain a water film on the SiO_2 area also when the substrate is held in the vertical direction. Before use, the samples were cleaned for 20 min by UV/ozone treatment, rinsed with Milli-Q water, and dried using a flow of nitrogen gas.

ToF-SIMS analyses were conducted under static SIMS conditions using a TOFSIMS IV instrument (IONTOF GmbH, Germany) using 25 keV Bi_3^+ as primary ions. High mass-resolution images of $500 \times 500\ \mu m^2$ with 256×256 pixels were acquired in the high-mass-resolution mode ($m/\Delta m \sim 7000$, beam diameter 3–5 μm) with a pulsed current of 0.10 pA and a cycle time of 150 μs. Images of $100 \times 100\ \mu m^2$ with 256×256 pixels were acquired in the high-resolution imaging mode ($m/\Delta m \sim 300$, beam diameter 350–450 nm) with a pulsed current of 0.05 pA and a cycle time of 110 μs (maximum primary ion dose density $6.8 \times 10^{11}\ cm^{-2}$). The protein-conjugated liposomes were detected in the positive ion mode by monitoring molecular fragment ions from POPC at m/z 58, 86, 104, 166, 184, and 224, D4-POPC at m/z 62, 90, 108, 170, and 188, and D13-POPC at m/z 66, 98, 117, 179, 197, and 237 (see supplementary material Table S1 for molecular structures and monitored fragment ions of the different POPC lipids).

III. RESULTS AND DISCUSSION

A. Single target analysis

In order to investigate the capacity of the liposome-binding approach for quantitative biomolecular analysis, the specific binding and detection of liposomes to a single target on a substrate surface were studied in detail. Figures 2(a) and 2(b) show fluorescence microscopy and ToF-SIMS images of anti-biotin-conjugated liposomes bound to a PLL-g-PEG/PLL-g-PEG-biotin model surfaces with different concentrations of biotin targets, respectively. The images show an increased liposome concentration with the increasing biotin concentration, thus demonstrating that the liposomes are specifically bound to the biotin targets on the surface. Furthermore, the liposome concentrations are similar in the fluorescence micrographs (obtained with the sample still in buffer solution) and the ToF-SIMS images at the same target concentration, suggesting that the localization of the bound liposome lipids is preserved during the freeze-drying process of the sample prior to ToF-SIMS analysis.

Individual liposomes are clearly distinguishable at the lower biotin concentrations in both the fluorescence and ToF-SIMS images [the assignment to individual liposomes is mainly based on the lack of overlap between liposomes in the cases where mixtures of two different types of liposomes have been used, see Figs. 3(a) and 3(b)]. This suggests that the target concentration can be quantified simply by counting the number of liposomes within a given surface area, assuming that each detected liposome is bound to a single biotin target on the model surface and that all biotin targets are tagged by a detected liposome. At higher biotin concentrations (around 0.2% and above), individual immuno-liposomes become increasingly difficult to resolve, making accurate counting of the liposomes challenging. In this target concentration regime, quantification of the liposome concentration, and thereby the target concentration, must instead be

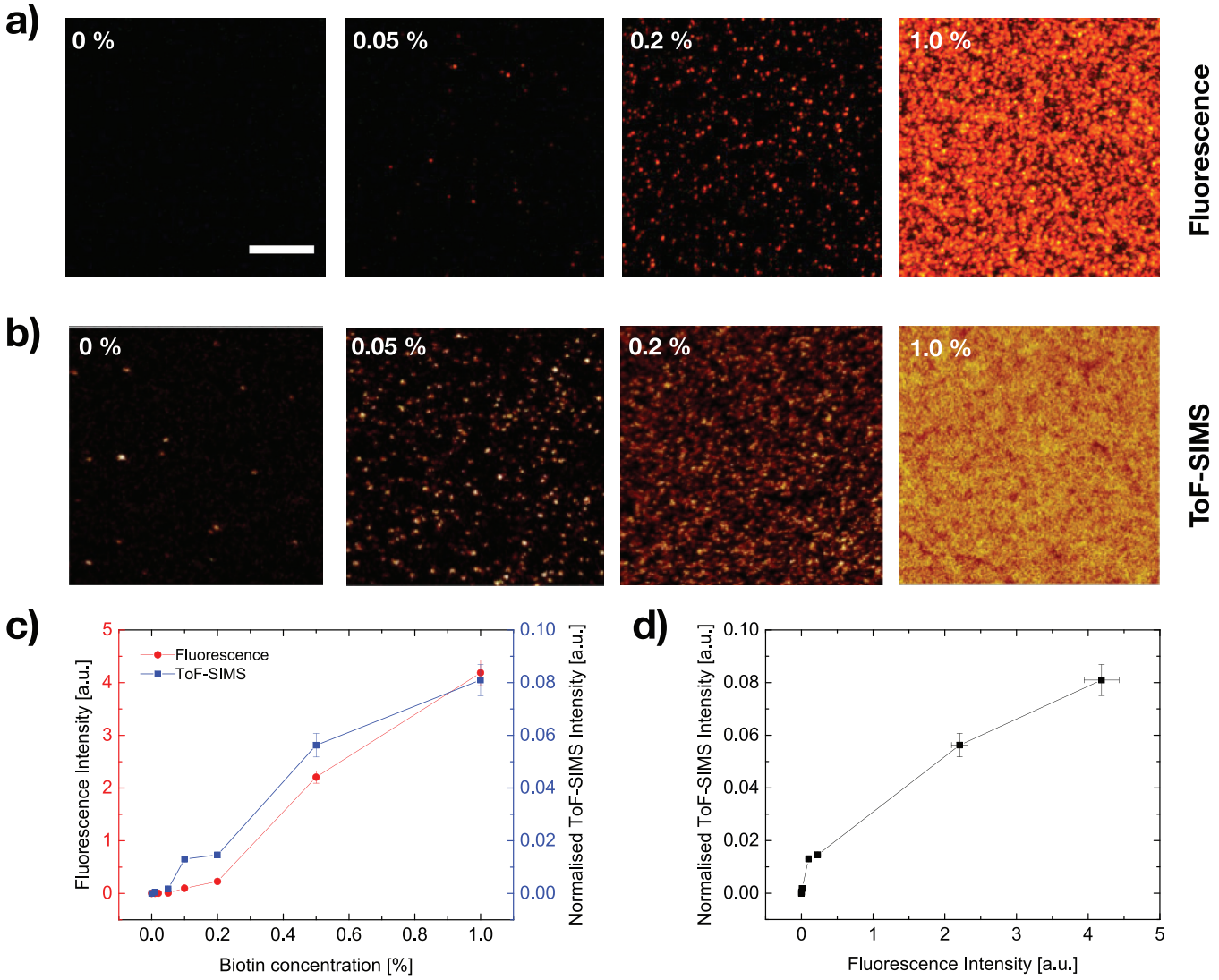

FIG. 2. Single-target detection using antibiotin immuno-liposomes bound to PLL-g-PEG-biotin surfaces. (a) Fluorescence microscopy and (b) ToF-SIMS images for increasing relative concentrations (0%–1%) of PLL-g-PEG-biotin on the surface. The scale bar is 25 μm^2. (c) Quantitative analysis of biotin on surfaces for different ratios of PLL-g-PEG-biotin. The fluorescence intensity corresponds to the collective intensity from all antibiotin immuno-liposomes in the field of view, and the error bars correspond to the standard deviation of six measurement areas (n = 6). The normalized ToF-SIMS intensity corresponds to the added secondary ion yields of fragment ions generated by the antibiotin immuno-liposomes (POPC, m/z 166, 184 and 224) normalized to the total ion signal, obtained for the same surfaces as in the fluorescence measurements. The ToF-SIMS data were collected in the high-resolution imaging mode (100 × 100 μm), with the error bars representing the standard deviation based on two to four spectra (n = 2–4). (d) The same data as in (c) with the fluorescence intensity and normalized ToF-SIMS intensities plotted against each other. Lines in plots (c) and (d) are guides for the eye.

based on the total signal intensity from the liposomes in the specified surface area.

The total signal intensity was found to be reasonably linear with respect to the relative biotin concentration in the PLL-g-PEG surface layer, however, with a few deviations [see Fig. 2(c)]. At low biotin concentrations (<0.2%), the observed fluorescence signals were lower than what would be consistent with a linear dependence, while the ToF-SIMS signals instead leveled off from a linear dependence at the higher biotin concentrations (>0.4%). Whereas the effect on the ToF-SIMS signal can be explained by matrix effects and/or the increased surface area taken up by the liposome as it collapses on the surface during freeze drying (resulting in a saturated lipid layer at lower liposome concentrations),[17,18] the effect on the fluorescence signal is more difficult to explain. One possible explanation may be that fluorescence microscopy is unable to detect the smallest liposomes, which may be more selectively bound at the lower target concentrations.[19]

In order to quantify the detected liposome concentration and correlate it with the biotin target concentration on the surface, the signal intensity per detected liposome was determined from the images at low liposome concentrations (both fluorescence microscopy and ToF-SIMS) and then used to estimate the number of liposomes in the images at the higher concentrations, i.e., assuming a linear dependence of the signal intensity versus liposome concentration (which, according to the above discussion, will underestimate the number of liposomes detected by ToF-SIMS). The resulting surface concentration of liposomes

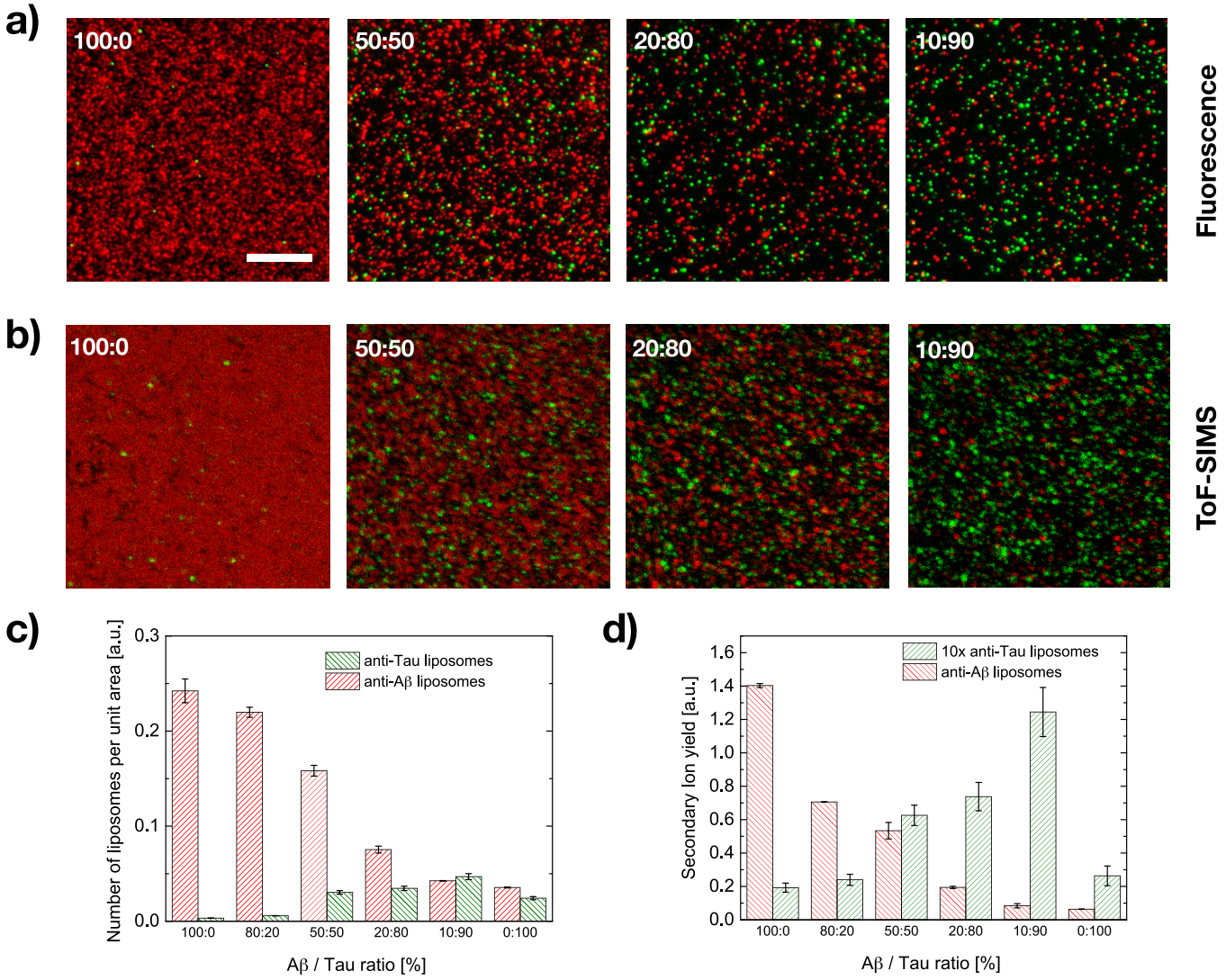

FIG. 3. (a) Fluorescence and (b) ToF-SIMS images of multiplexed detection of Aβ and Tau in the ratios [100:0], [50:50], [20:80], and [10:90] on a PLL-g-PEG/PLL-g-PEG-biotin (10:1) surface using anti-Aβ-liposomes (rhodamine labeled, red) and anti-Tau-liposomes (NBD labeled, green). (See supplementary material Figs. S5–S6 for individual liposome images.) Scale bar = 25 μm. (c) Quantitative detection of Tau (green) and Aβ (red) mixed at different ratios (0%–100% of Aβ and Tau, respectively), based on fluorescence images as in (a). (d) Added secondary ion yields of fragment ions corresponding to anti-Aβ liposomes (D13-POPC, m/z 179, 197, and 237) and anti-Tau-liposomes (POPC, m/z 166, 184, and 224) normalized to the total ion signal, at surfaces with different ratios (0%–100%) of Aβ and Tau. For clarity, the measured signal intensities of the anti-Tau liposomes have been multiplied by 10×. The data in (d) were collected in the high-resolution imaging mode (100 × 100 μm), and the standard deviation is based on two samples (n = 2).

at 1% biotin in the PLL-g-PEG layer was approximately 13 000 and 7000 per 100 × 100 μm^2 in the fluorescence microscopy and ToF-SIMS images, respectively. Considering that the surface area occupied by each PLL-g-PEG and PLL-g-PEG-biotin molecule is approximately 100 nm^2,[11] the number of PLL-g-PEG-biotin molecules (i.e., biotin binding sites) in a 100 × 100 μm^2 area at a mixing ratio of 1% should be around 1×10^6, which is fairly similar to that expected for a close-packed layer of liposomes with a diameter of 120 nm (8×10^5 liposomes on 100 × 100 μm^2). Even at a packing density of 50% or less, this is at least 1 order of magnitude larger than the estimated number of liposomes based on the fluorescence and ToF-SIMS results, i.e., the observed liposome density between 7000 and 13 000 is considerably lower than expected. This indicates that only a fraction of the biotin targets are available for liposome binding, e.g., due to inhomogeneous distribution of the biotin targets or due to the fact that some of the biotin groups are not sufficiently exposed on the PEG branches. Other possible reasons could be that the liposome solution contains a small fraction of free antibiotin antibodies, which due to their small size compared to the liposomes will quickly bind to biotin targets and thus block the liposome binding at this target site or due to the fact that some of the bound liposomes are detached from the biotin following detachment of the lipid molecule conjugated to the antibiotin.

The effect of reducing the concentration of immunoliposomes in the incubation solution was studied in order to investigate possible depletion effects and if the concentration is sufficiently high to maintain a high fraction of equilibrium target binding. Using PLL-g-PEG surfaces with

0.1% PLL-g-PEG-biotin, the results show that reducing the liposome concentration from 0.1 to 0.01 mg/ml results in a moderate signal reduction of approximately 50%, whereas the signal at 0.001 mg/ml drops to only 11% (fluorescence microscopy) and 16% (ToF-SIMS) of the signal intensities at 0.1 mg/ml (see Fig. S3 in the supplementary material).

The observed effect of reducing the liposome concentration can be partly rationalized by relating the number of liposomes in solution to the number of binding sites on the surface. At a lipid concentration of 0.1 mg/ml, the number of liposomes in the 5 μl droplet applied during the incubation can be calculated to be 3×10^9, whereas the number of liposomes bound to the 3 mm diameter sample surface at a concentration corresponding to 10% of a close-packed layer can be calculated to be 6×10^7. This means that at 0.1 mg/ml, the liposome concentration is not significantly reduced by the loss of liposomes caused by binding to the surface. At 0.01 mg/ml, the relative number of liposomes engaged in binding to the surface starts to be significant, and finally, at 0.001 mg/ml, the number of liposomes in solution (3×10^7) is similar or even lower than the number of liposomes required to obtain 10% of a close-packed liposome layer on the surface (6×10^7). Furthermore, due to limitations in the diffusion of liposomes to the sample surface, the binding rate will be dramatically reduced at the lowest concentrations, suggesting that the incubation time (2 h) may not be sufficient to allow for binding of all liposomes in the 5 μl solution droplet at stagnant conditions.

B. Multiplexed detection of amyloid-β and Tau on a PLL-g-PEG model surface

The simultaneous detection of Aβ and Tau on a PLL-g-PEG/PLL-g-PEG-biotin model surface was investigated at different relative target ratios ([Aβ:Tau] = [100:0], [80:20], [50:50], [20:80], [10:90], or [0:100]), while keeping the total target concentration constant (at 10% biotin, for successive binding of NeutrAvidin and mixture of biotinylated targets). Liposomes made of D13-POPC (deuterated POPC with 13 D atoms in the phosphocholine head group) and (undeuterated) POPC were conjugated to antibodies for Aβ (6E10) and Tau (HT7), respectively, as well as combined with a red (rhodamine) and green (NBD) fluorescent lipid [Fig. 1(a)]. Fluorescence microscopy and ToF-SIMS images obtained at four different Aβ/Tau ratios are shown in Figs. 3(a) and 3(b), respectively (separate images of the two types of liposomes are presented in the supplementary material Figs. S5–S6), and diagrams of the total signal intensities at all measured target ratios are displayed in Figs. 3(c) and 3(d). For both methods, the two types of liposomes demonstrate a "spotty" distribution on the surfaces with little or no spatial overlap between the two types, which indicates the detection of individual liposomes (anti-Aβ liposomes in red and anti-Tau liposomes in green). It is interesting to note in the [100:0] ToF-SIMS image that although the high concentration of anti-Aβ liposomes (red) produces a nearly homogeneous lipid background, a few (nonspecifically bound) anti-Tau liposomes can be observed as sharp spots, suggesting that the lipids of the collapsed liposomes are not mixed on the surface during or after freeze drying and that the individual liposomes thus can be observed even at high total liposome concentrations.

The concentrations of the two types of liposomes increase and decrease in accordance with the corresponding target concentrations, thus demonstrating that the bound liposomes monitor independently the surface concentrations of the two targets. However, the concentrations of the anti-Tau liposomes (green) are in general considerably lower than the anti-Aβ liposome concentration, which indicates that the anti-Tau liposomes only bind to a fraction of the Tau targets on the sample surface (see below). The higher binding of the anti-Aβ liposomes was confirmed using QCM-D measurements on similar surfaces (see Fig. S2 in the supplementary material) and might indicate lower binding affinity of the anti-Tau antibody liposomes, inferior biotinylation of the Tau protein, less efficient conjugation of anti-Tau antibodies to liposomes, less effective removal of free anti-Tau antibodies after conjugation, or a combination of all these effects. Another striking effect seen in Figs. 3(c) and 3(d) is the very low binding of anti-Tau-conjugated liposomes to the surfaces containing only Tau ([0:100]), an observation that was not made in the QCM-D measurements (see supplementary material). We attribute this contradiction to differences in the sample handling using the two systems and the sensitive nature of Tau to undergo structural changes, which, in our hands, was manifested by the requirement to establish very precise protocols to obtain reproducible results.

C. Multiplexed detection of GM1 and biotin on a cell membrane mimic

Multiplexed target detection on a SLB model surface was demonstrated using biotin and the glycosphingolipid GM1 as targets and liposomes conjugated to antibiotin and cholera toxin (which binds specifically to GM1), respectively, for detection.

The fluorescence and ToF-SIMS results for the simultaneous detection of biotin and GM1 on SLB model surfaces are shown in Fig. 4. The data were obtained by varying the relative target concentrations ([biotin:GM1] = [100:0], [95:5], [90:10], [50:50], [10:90], [5:95], or [0:100]) while keeping the total target concentration constant at 0.01% in the POPC SLBs. The fluorescence images [Fig. 4(a)] show binding of both antibiotin- and Chtx-liposomes (green and red, respectively) on the surface with liposomes being clearly visible individually on the surface. The ToF-SIMS images [Fig. 4(b)], however, do not show as distinct spots as the fluorescence images or as was observed for the PLL-g-PEG surfaces [see Figs. 2(b) and 3(b)]. A likely explanation for this is that the lipids may spread out on the bilayer surface as the liposomes collapse during the freeze-drying procedure.

The quantitative analysis from the fluorescence and ToF-SIMS data for all the different surfaces is summarized in Figs. 4(c) and 4(d), demonstrating increasing/decreasing liposome binding in accordance with the concentration changes of the corresponding targets. However, the binding is significantly more efficient for the Chtx-liposomes, resulting in

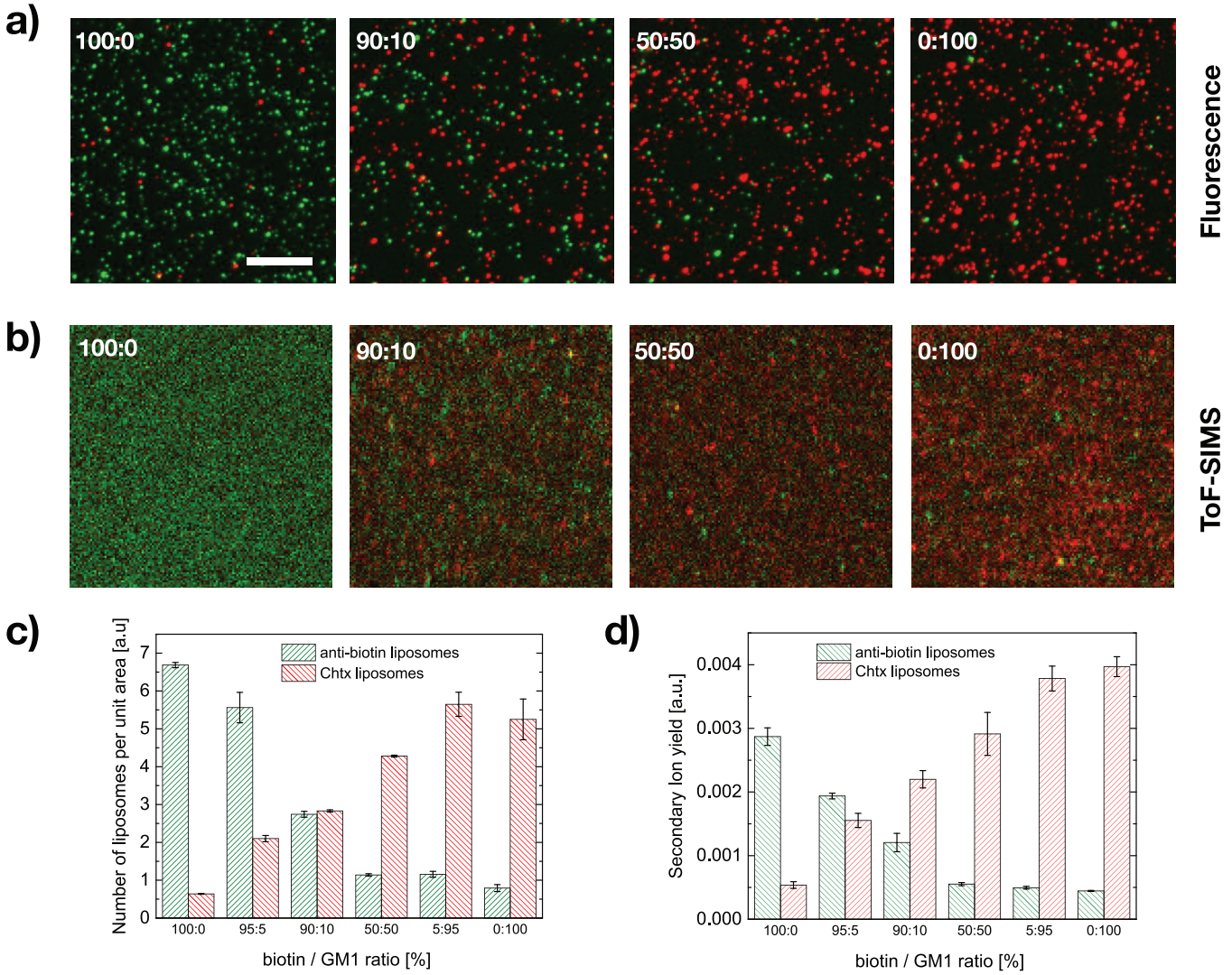

FIG. 4. (a) Fluorescence and (b) ToF-SIMS images of the two different liposomes, conjugated to antibiotin (ATTO-488 labeled, green) and Chtx (rhodamine labeled, red), binding to the target of interest (biotin and GM1, respectively) incorporated in a SLB at different ratios of biotin and GM1 of [100:0], [90:10], [50:50], and [0:100] (see supplementary material Figs. S7–S8 for individual liposome images). The scale bar is 25 μm^2. (c) Quantitative detection of biotin (green) and GM1 (red) mixed at different ratios (0%–100% of biotin and GM1, respectively), based on fluorescence images as in (a). The standard deviation is based on two samples (n = 2). (d) Added secondary ion yields of fragment ions corresponding to anti-biotin-liposomes (D4-POPC, *m*/*z* 62, 90, 108, 170, and 188) and Chtx-liposomes (D13-POPC, *m*/*z* 66, 98, 117, 179, and 197) normalized to the total ion signal, at surfaces with different ratios (0%–100%) of biotin and GM1. The ToF-SIMS data were collected in the high mass resolution mode from a larger surface area (500 × 500 μm). The standard deviation is based on two samples (n = 2).

equal concentrations of the two types of liposomes at a biotin/GM1 target ratio of [90:10]. In addition, significant liposome binding is also observed at very low or zero target concentrations, indicating that nonspecific binding is higher for this model system compared to the PLL-g-PEG model surfaces.

Considering the total target concentration of 0.01% that was used in the SLBs during these measurements and that each phospholipid contributes to a surface area of ∼0.7 nm^2, there should theoretically be enough binding sites for a fully covered liposome layer already at an effective target concentration of 40%, given a liposome with a diameter of ∼150 nm. Nevertheless, the total liposome concentration appears to be far from saturation at all biotin/GM1 target ratios, indicating that only a small fraction of the target molecules on the surface are active for specific liposome binding. In fact, it was found that the liposome binding was not saturated on the SLBs, even when increasing the target concentration (GM1 or biotin) in the SLB to 1%, which corresponds to 2 × 10^2 binding sites per liposome at a close-packed liposome layer. Besides possible differences in the accessibility of the targets on the SLBs and the PLL-g-PEG surfaces, a fundamental difference between the two model surfaces is that the biotin targets are fixed in the PLL-g-PEG layer, whereas the targets in the SLBs are linked to lipids in the bilayer, which are mobile and continuously diffusing within the bilayer structure. This diffusion may result in heterogeneous target distributions, thus decreasing the number of effective binding sites on the surface. It is also possible that several of the targets in these patches bind to the same liposome, creating a multivalent binding, especially considering

that each liposome contains about 9 proteins per liposome,[15] which can also move on the liposome bilayer surface.

IV. SUMMARY AND CONCLUSIONS

A novel approach was evaluated for multiplexed biomolecular detection based on specific binding of liposomes to target molecules on surfaces and subsequent liposome detection by ToF-SIMS. Simultaneous and concentration-dependent detection of two target molecules was demonstrated for Aβ and Tau on functionalized polymer surfaces (PLL-g-PEG) and for biotin and GM1 on SLB membrane mimic surfaces. Individual liposomes were detected and identified, and good correlation was found between liposome detection under aqueous conditions by fluorescence microscopy and subsequent detection by ToF-SIMS after freeze drying of the same samples. Although the results are promising for the possibility of quantitative multiplexed detection (and imaging) of molecular species that are not otherwise detectable by ToF-SIMS, unresolved challenges that need further studies include optimization of the liposome binding efficiency and minimization of unspecific liposome binding to the surface, both of which are required to obtain high sensitivity at low concentrations. Furthermore, the possibility of multivalent binding and limitations in the quantification capability at high concentrations, caused by the large size of the liposome compared to the target, need to be considered.

Advantages of the liposome approach include high sensitivity, here demonstrated by the detection of single liposomes, potentially representing single binding events, spatial resolution in the $\sim$1 μm range, and high multiplexing potential. The latter is achieved by using many different types of liposomes, each conjugated to a specific recognition element and produced by lipids that provide a strong and unique peak in the ToF-SIMS spectrum. Here, we have used liposomes made of standard POPC and different deuterated POPC lipids, which are well suited for making liposomes and also provide strong and well-characterized head group fragment ion peaks in ToF-SIMS. For increased multiplexing, POPC molecules with other deuterations of the head group may be used. Furthermore, alkylated POPC lipids, of which the ethylated POPC (POEPC) has been shown to produce strong head group fragment ion peaks,[20] may be possible to use in different deuterated versions to obtain high multiplexing capacity.

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[21]See supplementary material at https://doi.org/10.1116/1.5019749 for (i) specific liposome binding on mixed and single-target surfaces using QCM-D, (ii) effect of liposome concentration during incubation on target detection, (iii) molecular structures and fragment ions of lipids used for liposome detection by ToF-SIMS, and (iv) individual fluorescence microscopy and ToF-SIMS images for multiplexed detection on PLL-g-PEG and SLB's.