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Membrane Fluidity Sensing on the Single Virus Particle Level with Plasmonic Nanoparticle Transducers

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Abstract

Viral membranes are nanomaterials whose fluidity depends on their composition, in particular the cholesterol (chol) content. As differences in the membrane composition of individual virus particles can lead to different intracellular fates, biophysical tools capable of sensing the membrane fluidity on the single-virus level are required. In this manuscript, we demonstrate that fluctuations in the polarization of light scattered off gold or silver nanoparticle (NP)-labeled virus-like-particles (VLPs) encode information about the membrane fluidity of individual VLPs. We developed plasmonic polarization fluctuation tracking microscopy (PFTM) which facilitated the investigation of the effect of chol content on the membrane fluidity and its dependence on temperature, for the first time on the single-VLP level. Chol extraction studies with different methyl- β -cyclodextrin (M β CD) concentrations yielded a gradual decrease in polarization fluctuations as function of time. The rate of chol extraction for individual VLPs showed a broad

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Supporting Information

The supporting information is available free of charge on the ACS Publications Website at DOI: ...

Seven supporting sections, including (A) Supporting Materials and Methods, (B) Scattering intensities of 40 nm gold and silver NPs, (C) Single silver NP labels' experimental and *in-silico* PFT behavior, (D) MD simulation for calculating the membrane fluidity, (E) PFTs histograms and their changes as function of chol extraction from VLPs, (F) Liposomes as standard models for validating the MD simulations and experimental VLP results and (G) Cumulative probability plots of Var(P) for different chol extraction time points, and corresponding Figures S1-S9.

Movie S1: SNPs diffusing laterally on a planar lipid membrane

Movie S2: Image processing and obtaining the PFTs.

Movie S3: 20 ns movie (30 fps) of the MD simulations of DOPS lipid diffusion in the membrane at different chol concentrations and temperatures.

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Author Contributions

A.F. performed experiments, FDTD simulations and image/signal processing in Matlab. D.S. and T.K. performed MD simulations and analyses of diffusion coefficients. C.W. collaborated with the experiments. H.A. generated the HIV-1 VLPs used in the experiments. S.G., T.K. and B.M.R. contributed expertise and supervision. A.F. and B.M.R. developed the idea and wrote the manuscript. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

spread, presumably due to differences in the membrane composition for the individual VLPs, and this heterogeneity increased with decreasing M β CD concentration.

TOC image

Keywords

Membrane Fluidity; Virus; Cholesterol; Nanoparticle diffusion; Polarization fluctuation tracking microscopy; Silver nanoparticles

The human immunodeficiency virus (HIV) is an enveloped virus that contains a capsid wrapped in a lipid bilayer derived from the host cell. The virus preferentially buds from cholesterol (chol) enriched regions, so-called lipid rafts, whose composition determines viral membrane properties¹. In a series of classic experiments Aloia *et al.* quantified the lipid composition and fluidity of HIV-1 and HIV-2 membranes and found that the chol to phospholipid ratio determines the phase of the viral membrane^{2,3}. Subsequent studies with different methodologies also confirmed that the HIV membrane fluidity strongly depends on its chol content^{4,5}. Importantly, extraction of chol from the membrane and the associated change in membrane fluidity result in a reduction of viral infectivity^{4–6}. Given this functional relationship, the ability to measure the viral chol content, its impact on membrane fluidity, ideally on the single-virus level, and its ties with other virological properties such as particle trafficking, Env protein density, maturation and dynamics^{7–9} are of great current interest in virology¹⁰.

The effect of chol on fluidity has been shown to be temperature-dependent^{11,12} and, on the ensemble level, this has been extensively studied in liposome and virus systems using several different techniques^{13–15}. Nuclear Magnetic Resonance (NMR), Electron Spin Resonance (ESR) and fluorescence techniques are common methods for studying the fluidity of cellular and viral membranes^{16,17}. NMR provides membrane information in a label-free fashion^{18–20}, while ESR relies on spin probes covalently linked to specific target lipids^{21,22}. Despite their versatility and accuracy, both NMR and ESR suffer from the need for large sample quantities, which typically lie in the milligram range^{19,21}. These sample requirements are cumbersome for viral membrane studies where sample amounts are often limited, as in the case for patient-derived samples²³. Considering that HIV is known to lead to abnormal cellular membrane compositions in patients²⁴, the ability to obtain information

about viral membrane chol content and fluidity in patient-derived samples is necessary to investigate their importance in the clinical setting and design personalized medicine.

Several optical tools have been developed to probe membrane fluidity spatially resolved. These methods were mostly based on fluorescence and included Förster Resonance Energy Transfer (FRET)²⁵, Fluorescence Correlation Spectroscopy (FCS)²⁶, Fluorescence Recovery after Photobleaching (FRAP)^{27,28}, Fluorescence Anisotropy²⁹ and Generalized Polarization (GP) of the Laurdan probe³⁰. These studies have included investigations of lipid behavior in nanoparticle-modified lipid bilayer systems^{28,31,32}. Some of these techniques can – in principle – be implemented on the single-cell or even -virus level. However, utilizing them on a single virus particle whose dimensions are significantly below the diffraction limit is difficult due to a variety of reasons including the limited number of dyes, their limited photostability which results in blinking and bleaching, their relatively low intrinsic intensities and, for some techniques, the inability to resolve individual moving dyes.

In this manuscript, we set out to avoid some of the limitations associated with monitoring membrane fluidities of single virus particles with fluorescence by replacing the fluorescent labels of membrane lipids with non-blinking and non-bleaching noble metal nanoparticles (NPs)^{33,34}. The rationale for our approach is that a polarizable metal NP interacts with an image charge in the VLP to which it is bound and that this interaction results in a net polarization of the scattered light³⁵. Furthermore, the nanoscale confinement of two or more NPs on an individual virus particle with a typical diameter of 150 nm induces distance-dependent plasmon coupling between the NP labels^{36–39}, which can further enhance the light polarization⁴⁰. The effect is schematically illustrated in Figure 1. If the near-fields associated with the resonant surface charge density oscillations (plasmons) of two NPs diffusing on the virus surface penetrate each other, the charge density oscillations are no longer independent. The plasmon resonance wavelength shifts and the scattered light becomes polarized in an interparticle separation dependent fashion as consequence of the coupling of the plasmons. These effects have been extensively utilized for nanoscale distance measurements. For instance, Yang *et al.*⁴¹ showed that the strong plasmon coupling between silver NPs can be used for developing molecular rulers for DNA length measurements. The light polarizing effect of plasmon coupling provides additional information about the orientation of the NP dimer⁴⁰. Rong *et. al.* demonstrated the feasibility of detecting both changes in interparticle separation as well as orientation of NP dimers diffusing on cellular plasma membranes^{38,42}. In this manuscript we utilize modulations of the far-field response associated with the relative motion of one or more NPs on the virus particle surface as transducer for detecting changes in the fluidity of the viral membrane. Different from previous approaches that used NPs as labels for cell membrane imaging and tracking^{43–47}, we set out to characterize these fluctuations as a measure to probe the fluidity of viral membranes on sub-diffraction limit length scales. We introduce PFTM to quantify fluctuations in the scattering signal of NP-labeled HIV-1 virus-like-particles (VLPs) before and after chol extraction at different temperatures to probe the effect of chol on the membrane fluidity of individual VLPs.

EXPERIMENTAL SECTION

DOPS labeling on VLPs and liposomes with SNPs and GNPs

40 nm silver and gold NPs (from TedPella Inc.) were functionalized with single stranded DNA oligonucleotides using the method by Liu *et al*⁴⁸. The oligonucleotides HS-AAAAAAAAAACTCACGCTAC-GACTGACACC and HS-AAAAAAAAAAGACCTACTAAGACTACTACACAACCAGAGA-Biotin oligonucleotide were mixed in a 4:1 ratio. The biotinylated oligonucleotides were 10 nucleotides longer than the passivating nucleotides to ensure accessibility of the biotin groups on the surface of the NPs. This mix was added to nanoparticle colloid in DDI water to yield a final concentration of 25 μ M DNA and 1.5 nM nanoparticles in a total volume of 10 μ L and incubated for 30 min at room temperature. The pH was then lowered to pH = 3 by adding 10 mM sodium citrate buffer in a negligible volume. The mix was incubated for another 30 min. After that, the nanoparticles were washed by repeated centrifugation (2,400 $\times$ g for gold and 5,000 $\times$ g for silver NPs, 10 min) and resuspension in 1.5 mL in DDI water. After the third washing step, the NPs were passed through a 0.22 μ m syringe filter to remove any agglomerates or impurities. The particles were then collected by a final round of centrifugation. The resulting DNA-conjugated NP pellet was diluted with DDI water before 10 $\times$ PBS was added to reach a final concentration of 0.15 nM NPs in 1 $\times$ PBS. The volume of added PBS was adjusted in experiments that required higher/lower concentrations. The concentration and size of the NPs were measured in 1 $\times$ PBS buffer by UV-Vis spectrometer and Malvern ZetaSizer right before use. The NPs consistently showed an average diameter of $\sim$ 44 nm, indicating no significant agglomeration.

PS in the VLP/Liposome membrane was functionalized with biotinylated Annexin V (Abcam), using a 10000:1 ligand:VLP ratio. For this purpose, 10⁷ VLPs/liposomes in 0.5-2 μ L of stock were added to 10 μ L of 1 $\times$ Annexin Binding Buffer (ABB: 10 mM HEPES pH = 7.4, 137 mM NaCl, 2.5 mM CaCl₂) and 0.5 μ g of AnxV was added and incubated for 30 min at room temperature. Then, an 8-fold excess of neutravidin (NTV, Thermoscientific Inc.) in PBS and 1% Bovine Serum Albumin (BSA) were added and incubated for 30 min at room temperature. Subsequently, the functionalized VLPs were purified from excess proteins in solution through ultracentrifugation (Beckman-Coulter Optima TLX) on 60% sucrose cushion (100k $\times$ g, 30 min, 4°C). Liposomes were purified by 24-48 h of dialysis against 1 $\times$ ABB using a 100 nm pore size polycarbonate (PC) membrane in a micro-dialyzer (Harvard Apparatus Inc.) in an ice bath. The functionalized VLPs/liposomes were diluted to 150 μ L in ABB, and stored at 4°C for experiments during the following week. This amount of functionalized VLPs/liposomes was enough for at least 3 optical measurements or 5-10 electron microscopy samples.

Sample Preparation for Optical Measurements

The functionalized VLPs, in 50 μ L ABB, were flushed into rectangular borosilicate capillaries (100 $\times$ 2 $\times$ 0.1 mm³, from Vitrocom), where they stuck non-specifically to the fused silica surface. Under typical experimental conditions, the VLPs were incubated until approx. 50 VLPs were bound in the field of view to avoid interference between the individual VLPs (this step took normally around 30 min at 4°C). We used VLP samples with

a concentration of 6×10^4 VLPs/ μ L and consumed a total of $\sim 3 \times 10^6$ VLPs per experiment. After that, excess VLPs were removed by flushing the chamber with $1 \times$ ABB. The surface was then blocked by 10% BSA in $1 \times$ ABB for 2 hr at 4°C . After washing the samples with 1% BSA in $1 \times$ PBS, the samples were incubated with 0.1 mg/mL polyinosinic acid (PI) in 1% BSA/ $1 \times$ PBS for 30 min at room temperature. PI was subsequently removed by flushing the flow chamber with 1% BSA in $1 \times$ PBS. In the next step, the immobilized VLPs were incubated with a 1.5 nM solution of biotin-functionalized silver or gold nanoparticle labels in $1 \times$ PBS for 15 min at room temperature. Unbound nanoparticles were flushed out with $1 \times$ PBS containing only 20 mM NaCl.

Dark-field microscopy

All imaging experiments were performed with an inverted microscope (Olympus IX71) equipped with an oil immersion dark-field condenser and $60\times$ objective. The sample was illuminated with a 100 W Tungsten lamp through a high numerical aperture ($\text{NA} = 1.20$) dark-field condenser. The scattered light was split according to polarization using a polarizing beam splitter and two images of the entire field of view (128×128 pixels = $62 \times 62 \mu\text{m}^2$) with orthogonal polarizations were recorded on two separate electron multiplying charged coupled device (EMCCD) cameras with a frame rate of 500 fps. Each field of view contained approximately 50 individual VLPs under typical experimental conditions. The experimental $I_{\parallel}$ and $I_{\perp}$ values were determined by fitting the point-spread-functions of the labeled VLPs to 3D-Gaussians and integrating the volumes under the surfaces. In addition to the scattering channel, the microscope was equipped with an epi-fluorescence channel to record a fluorescence image (400/530 nm excitation/emission filters and a 470 nm dichroic) of the same field of view. Images were all recorded with Andor iXon EMCCD cameras. To obtain scattering movies, we recorded 2000 frames each with 2 ms exposure time (500 fps) per experiment with an electron multiplier gain of 50. For fluorescence measurements, we used an exposure time of 1 sec and an electron multiplier gain of 100. The $60\times$ objective had a variable numerical aperture (NA), so the scattering images were recorded with $\text{NA} = 0.65$ and the fluorescence images were recorded with $\text{NA} = 1.25$. To account for the spectral profile of the Tungsten lamp used as excitation source the intensities were corrected by dividing through the corresponding average scattering data obtained from 200 nm polystyrene nanoparticles.

Movie data processing and obtaining PFTs and $\text{Var}(P)$

All image processing and data analysis was performed using a home-written Matlab code. The point-spread-function (PSF) of every VLP in each frame of the movie in both orthogonal polarization CCD images were fitted with Gaussian functions of the form:

$$G(x, y) = A e^{-\left[\frac{(x-x_o)^2}{2\sigma_x^2} + \frac{(y-y_o)^2}{2\sigma_y^2}\right]} + BG \quad (1)$$

where x_o and y_o are the Gaussian peak position, σ_x and σ_y are the full width at half maximum of the Gaussian along x and y directions, A is the Gaussian amplitude, and BG is the background. After subtracting the background (BG), the integrated intensity of the PSF

was taken as the scattering intensity of the VLP/liposome at that specific 2 ms frame. We implemented the Matlab code in the Boston University shared computing cluster (SCC) system to have unlimited access to many CPUs at the same time. This enabled us to process a relatively large amount of data in a short time. After calculating the scattering intensities in orthogonal polarizations, $I_{\parallel}$ and $I_{\perp}$, we calculated P for each frame to obtain the polarization fluctuation trajectory (PFT), its variance over the 2000 frames, $\text{Var}(P)$, the total intensity per each frame, $I_t = I_{\parallel} + I_{\perp}$, and the average total intensity over the full trajectory,

$$\overline{I_t} = \frac{\sum_1^{2000} (I_{\parallel} + I_{\perp})}{2000}.$$

Noise threshold determination based on the PFTs of immobilized polystyrene NPs

Carboxyl-terminated Polystyrene NPs 200 nm in diameter were dispersed in PBS buffer similar to that used for VLPs and then flushed in the capillary tubes while being monitored in the dark-field microscope. As soon as nearly 100 of the NPs were observed to be electrostatically attached to the quartz substrate, the remaining free particles were washed out by PBS. Movies of these immobilized NPs at the two orthogonal polarization channels were recorded at different intensities of the incident light by starting from the highest intensity of the tungsten lamp and repeating the measurement after serially turning down the intensity manually. The PFT of each NP was calculated as described above at the different recorded intensities down to the lowest intensity with which still a reasonable goodness of fit could be achieved in the Gaussian fitting (r^2 above 0.7). Based on these data, the scatter plot of $\text{Var}(P)$ vs. $\overline{I_t}$ for each NP at each intensity was plotted in order to find the noise threshold in different intensity intervals. In this plot, for each 0.2 interval on the intensity axis, the 3rd standard deviation from the mean of the population in that interval was calculated as the threshold below which the noise in the PFT signal becomes dominant over the actual polarization fluctuations. Therefore, if the $\text{Var}(P)$ for a particle with a particular total scattering intensity, $\overline{I_t}$, is above this calculated noise threshold, its PFT was regarded as dominated by the actual polarization fluctuations coming from changes in the particle or labels' spatial configuration and used for further analyses.

Further details of the experimental and computational methods used in this work can be found in the Supporting Information.

RESULTS AND DISCUSSION

In-silico validation and experimental implementation of PFTM

To validate our hypothesis that the random diffusion of NPs tethered to a VLP generates a measurable optical signature, we simulated the polarization, P , of the light scattered off a VLP containing two randomly diffusing 40 nm silver NPs (SNPs) with the finite-difference time-domain (FDTD) method. We chose a VLP size of 150 nm as this corresponds to the average size of HIV-1 particles and we treated the virus as a dielectric particle of refractive index $n = 1.5^{49}$. P was calculated from the scattered light intensities with orthogonal polarizations, $I_{\parallel}$ and $I_{\perp}$, according to^{40,50,51}:

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \quad (2)$$

Assuming a typical diffusion coefficient of $4 \mu\text{m}^2/\text{s}$ for the SNP⁴³, its root mean square displacement (RMSD) is approximately 200 nm within 2 ms. Given this RMSD, each NP can reach almost any point on the virus surface between two subsequent 2 ms long frames, in the case of random diffusion. A representative simulated polarization fluctuation trajectory (PFT) for this random diffusion is included in Figure 2a (blue line). The simulation predicts a rich P dynamics with P values in the range between -0.6 and 0.6 . The P distribution is well fit by a single Gaussian.

In our experiments, SNPs were attached to phosphatidylserine (PS) lipids in the membrane of VLPs *via* annexin V (AnxV) and neutravidin (NTV) chemistry (Figure 2b,c). We tested the mobility of our particular SNPs on a flat supported lipid membrane with a lipid composition similar to that of HIV-1 (40% chol, 55% DPPC and 5% DOPS). We observed that only 8% of the SNPs are immobile and the average diffusion coefficient of the mobile SNPs is $7.6 \mu\text{m}^2/\text{s}$, consistent with previous findings⁴³. A video demonstrating the lateral diffusion of the SNPs on the lipid membrane is included in Movie S1, and the MSD plots and histogram of the calculated diffusion coefficients is shown in Figure S3. Our experimental set-up to monitor P was based on a dark-field microscope that records the light scattered from the NPs on two orthogonal polarization channels (Figure 2d). The procedure for calculating intensities and PFTs from raw EMCCD images is demonstrated in Movie S2 (for further details see Methods). Intriguingly, while the simulations show a normal distribution within a P -range defined by the polarization anisotropy of a touching dimer, the experimental PFTs often show a behavior in which the polarization switches between only a few distinct P values. An exemplary experimental trajectory is shown in Figure 2e. The higher probability for a limited number of specific P values is indicative of the existence of favored geometric NP configurations. The behavior can be emulated in the simulation by applying an adequate P probability distribution (red lines in Figure 2a), and it reveals that the NP labeled lipids do not diffuse freely across the membrane but that their motion is hindered. The latter is not entirely unexpected since the viral membrane derives from mammalian host cells, which are known to be compartmentalized⁵². Overall, our combined experimental and theoretical analysis confirms that P and its variance, $\text{Var}(P)$ are useful quantities for characterizing the NP motion on the membrane.

The effect of plasmon coupling in polarization fluctuations

Some SNP-labeled VLPs (SNP-VLPs), despite containing multiple SNP labels, did not show significant P fluctuations. These VLPs contained NP labels that did not change their orientation and interparticle separation. To identify VLPs with mobile NP labels, which are of primary interest for characterizing membrane fluidity and how it is affected by chol, we compared the $\text{Var}(P)$ of individual VLPs to that of immobilized isotropic polystyrene NPs (200 nm) with identical scattering intensities. Figure 3a shows the $\text{Var}(P)$ of 250 individual polystyrene NPs at different incident light intensities as red markers. Figure 3a also contains

the $\text{Var}(P)$ for 500 NP-labeled VLPs as black circles. The number of SNPs per VLP was derived by comparison with the intensity of single SNPs immobilized on a substrate assuming a linear relationship (Figure S2). The VLPs carried up to $l = 13$ SNP labels leading to a broad spread of the data along the intensity axis. The contribution of each NP label number to the total ensemble is included as gray histogram in Figure 3a. In the following data analysis, we only included measurements with average $\text{Var}(P)$ values that were at least three standard deviations above the noise threshold defined by the mean of $\text{Var}(P)$ for polystyrene NPs at the same intensity. This fraction, which is included as solid black lines on the histogram in Figure 3a, increases through $l = 1 - 3$, and then gradually decreases with growing l . The non-negligible fraction of VLPs above the threshold for $l = 1$ is caused by electromagnetic interactions between the SNP and a mirror dipole in the supporting dielectric VLP and, in addition, some asymmetry of the individual SNPs which are often not ideal spheres (Figure S4). Importantly, as shown in Figures 2b,c (blue data) VLPs with a single SNP show only weak fluctuations in the total intensity, $I_t = I_{\parallel} + I_{\perp}$, and the fluctuations in P for individual SNPs were usually not accompanied by changes in I_t . Anisotropic SNPs have a preferred polarization axis and spherical NPs can acquire one through interaction with the dielectric support^{35,53}, but importantly a rotation of their polarization axis only changes the distribution of the scattered light on the two detectors but not the intrinsic anisotropy itself. In contrast, for an SNP dimer we typically observed correlated fluctuations in P and I_t (see orange data in Figures 2b,c). The difference between monomers and dimers arises from the fact that in dimers (and larger clusters) changes in the inter-particle separation modulate the polarization anisotropy as well as the total scattering cross-section due to the distance dependence of plasmon coupling^{40,41}. The total scattering cross-section increases with increasing plasmon coupling. These effects cause a nearly 2-fold increase in the fraction of the VLPs above $\text{Var}(P)$ noise threshold as we move from $l = 1$ to 2 or 3. This difference in behavior was also observed in our *in-silico* results simulating one and two spherical SNPs diffusing on a VLP surface as shown in Figure S5 (for further discussion and details refer to Supporting Information Section B). A systematic comparison of the Pearson's correlation coefficient, ρ , between P and I_t for nearly 40 monomers and small clusters (Figure 3d) confirms that the dimers and trimers show a significantly stronger ($p < 0.001$) correlation between P and I_t than the monomers. The decrease in $\text{Var}(P)$ for $l > 3$ is due to a decrease in polarization anisotropy of the SNP cluster going from dimers and trimers to larger clusters. Therefore, we conclude that there are various factors contributing to the P fluctuations and $\text{Var}(P)$, including SNP shape, metal-dielectric interaction and vectorial addition of the scattering along the longitudinal axis of SNP label(s) and VLP, in addition to the plasmon coupling between SNP labels.

Comparison of gold and silver as NP labels for PFTM

In the next step, we compared the performance of SNPs used in Figure 3 with that of gold NPs (GNPs) of identical size. To obtain GNP-labeled VLPs (GNP-VLPs), the GNPs were attached to the membrane using the same AnxV-NTV chemistry used for SNPs. Intriguingly, we found that under identical experimental conditions only 10% of the GNP-VLPs lie above the $\text{Var}(P)$ threshold defined by the polystyrene beads (Figure 4), which compares to approximately 70% for the SNPs. The difference between GNPs and SNPs can be attributed to three main effects. The scattering cross-section of the GNPs (and their clusters) is smaller

than that of SNPs (and their clusters) as shown in the Figure S2, GNPs have smaller polarizabilities and, therefore, weaker coupling to the mirror dipole in the VLPs, and the distance dependence of plasmon coupling has a steeper slope for SNPs than for GNPs⁴¹. At a given separation, the polarization anisotropy for SNPs is generally higher than that for GNPs and small changes in separation lead to larger signal fluctuations in the case of SNPs. Our analysis confirms that SNPs are better suited for characterizing NP diffusion through PFT analysis. We mention in passing that we found no significant differences in the shape and size uniformity between SNPs and GNPs. In addition, both types of NPs are equally stable in PBS after DNA conjugation and do not show any sign of agglomeration over time. Supporting data for these aspects are summarized in Figure S1.

The diffusion of NP labels on VLPs is non-Brownian

We hypothesized that PFTs of SNP-VLPs provide insight into the membrane fluidity and its change as function of chol depletion on the single-VLP level. As a first step towards a systematic prediction of the membrane behavior under different lipid compositions and temperature conditions, we performed MD simulations for the lateral diffusion of PS lipids in a model virus membrane. The latter comprised a ternary mixture of the main components of the HIV-1 membrane: dipalmitoylphosphatidylcholine (DPPC), chol, and dioleoyl-sn-glycero-3-phospho-L-serine (DOPS). The DOPS concentration was kept constant at 10 mol %, while the DPPC and chol concentrations were varied. The simulations (Figure 5a and Figure S6 and Movie S3) reveal that the slope of change in diffusion coefficient, D , as a function of chol concentration is temperature dependent. Figure 5b shows D for 10 and 40 mol% chol at 25 °C, 40 °C and 50 °C (based on the simulated mean squared displacement (MSD) plots shown in Figure S7). These temperatures were chosen as they frame the S_0 to L_d transition temperature, 42 °C, in the DPPC-chol binary system (no DOPS)⁵⁴. 40 and 10 mol% are the chol concentrations expected for HIV-1 particles before and after treatment with M β CD^{1,13}. D increases with increasing chol content at 25 °C and 40 °C, but the trend reverses (D decreases with increasing chol content) at 50 °C. Similar trends in D for different temperatures have been reported before with different membrane compositions¹². The different temperature dependencies from Figure 5b can be understood by considering the fluidities of the different phases in the DPPC-chol binary phase diagram⁵⁴ (see Supporting Information Section C). It should be mentioned that our MD simulations predict the diffusion coefficient of an individual PS lipid, while in our experimental conditions each AnxV protein (the linker between PS and each SNP) binds to several PS lipids⁵⁵.

The connection between the behavior of individual lipids and lipid clusters is provided by a diffusion model proposed by Knight *et al.*⁵⁶, who refined the conventional Saffman-Delbrück model⁵⁷, predicting that D of the lipid cluster shows a size dependence that scales with $1/N$ where N is the number of the lipids in the cluster. Although multivalent attachment of SNPs to the viral membrane decreases the absolute value of D for SNPs,⁴³ we expect the effect to be systematic so that differences in the membrane fluidity for a set of conditions should persist and lead to relative differences in the diffusion coefficients. Another point of potential concern relates to phase changes induced by multivalent binding to curved surfaces.⁵⁸ The curvature of the VLPs of around $1/150 \text{ nm}^{-1}$ is, however, sufficiently low to exclude curvature effects on the membrane behavior.

As discussed above, the experimentally-recorded PFTs do not support the model of a random diffusion within a confinement defined by the VLP. Instead, fluctuations of the polarization between preferred P values indicate a hindered diffusion in which the NPs commute between different discrete trap sites. Similar behavior has been previously observed in the studies of the NPs on planar lipid bilayer and liposome systems^{59–61}. In the presence of chol, frequent rapid transitions between these different preferred configurations are observed at both 25 °C and 45 °C (two temperatures below and above the phase transition temperature of the viral membrane⁶²), as shown with the blue PFTs and their corresponding histograms in Figures 4c,d (more PFT histograms are shown in Supporting Information Section D and Figure S8). Energetic differences between the geometric configurations (S_j) result in different occupation probabilities, which are quantifiable through the measured P distributions. P values with the highest probability indicate energetically-favored or “low energy” configurations while P values with lower probability indicate “high energy” configurations. At both 25 °C and 45 °C, extraction of chol with 5 mM M β CD reduces the frequency of interconversion between the different NP configurations, increasing the contribution from one configuration at the expense of the others. This behavior is illustrated with the orange PFTs recorded after chol extraction and their corresponding histograms in Figures 4c,d.

We calculated the Pearson’s correlation coefficient (ρ) between the most probable or “peak” polarization before ($P_{p. bef.}$) and the peak polarization after ($P_{p. aft.}$) chol extraction for eight individual SNP-VLPs at the two different temperatures (Figure 5e). We find a strong correlation with $\rho = 0.97$ at 45 °C, while at 25 °C the correlation is weaker with $\rho = 0.76$. Interestingly, we are experimentally observing that at 45 °C chol depletion often leads the system to preferentially take the lowest energy configuration observed before chol extraction ($P_{p. aft.}$ often gets associated with $P_{p. bef.}$), while at 25 °C there is a non-negligible probability for the system to get trapped in the high energy configuration. Consequently, we propose the following model to account for the experimental observations, as demonstrated in Figure 5f. For a set of SNP configurations, S_j , associated with light polarizations P_j , the activation barrier for an interconversion between S_j ’s increases with decreasing membrane fluidity. At 45 °C, chol removal increases the membrane fluidity (Figure 5b) resulting in sufficiently shallow barriers, which allows the system to localize to the energetic minimum, S_1 . But at 25 °C, chol removal decreases the membrane fluidity and increases the energetic barriers between the different configurations. In this condition, the system can get trapped and “freeze” in a high energy configuration, S_2 . Although we are proposing our model based on a two-“state” system, the SNP labels can acquire any number of preferred configurations (and local P maxima in PFT histograms), as shown in the examples of Figures S6b,c.

PFTM for tracking the kinetics of chol extraction on an ensemble and a single-virus level

To corroborate that the $\text{Var}(P)$ associated with the confined diffusion of the NP labels is a useful measure to detect chol-mediated changes in the membrane fluidity, we analyzed P trajectories from 130 individual VLPs before and after chol extraction with 5 mM M β CD at 40 °C. The resulting $\text{Var}(P)$ data are shown in scatter plot and histograms in Figure 6a, with $\text{Var}(P)_{\text{bef}}$ and $\text{Var}(P)_{\text{aft}}$ showing the $\text{Var}(P)$ values before and after chol extraction, respectively. In this analysis, we used VLPs with $\text{Var}(P)$ values above 0.005 (a threshold

required for a properly high sensitivity of the assay) and found that the $\text{Var}(P)$ distribution shows a significant ($p = 0.01$) shift to smaller values after chol extraction. Positive controls (Figure S9) performed with two liposome samples (unilamellar liposomes ~ 120 nm in diameter) containing 10 and 40 mol% chol contents verified a systematic difference in the membrane fluidity as a consequence of chol content difference (for further details refer to Supporting Information Section E). Importantly, negative control in which VLPs were treated with pure buffer (0 mM M β CD) did not exhibit a significant change in $\text{Var}(P)$ distribution (Figure S10).

In the next step, we recorded the PFTs of a population ($5 \times \sim 50$) of SNP-VLPs at six consecutive time points (0, 0.5, 1, 2, 5 and 10 min) after incubating them with two different concentrations of M β CD (2 mM and 10 mM) at 40 °C and quantified their $\text{Var}(P)$. Cumulative $\text{Var}(P)$ distributions for the VLP population show a continuous shift to smaller values with increasing incubation time (Figure S11). This trend is illustrated in Figure 6b by a plot of $V_{0.5}$, which is the $\text{Var}(P)$ value corresponding to a 50% probability in the cumulative distribution, as function of time. The plots show a continuous decrease that is well described by a fit of the form:

$$V_{0.5}(t) = V_{0.5}^0 + (V_{0.5}^{\min} - V_{0.5}^0) * (1 - e^{-\tau t}) \quad (3)$$

where $V_{0.5}^0$ is the initial value of $V_{0.5}$ (at time 0 min), $V_{0.5}^{\min}$ is the minimum value that $V_{0.5}$ acquires, τ is the characteristic extraction time and t is the time of chol depletion. Based on this model, the characteristic time of chol depletion with 2 mM M β CD, τ_{2mM} , was found to be 1.0 min, while with 10 mM M β CD we obtain $\tau_{10mM} = 0.5$ min.

Since the PFT approach provides P information of individual VLPs, we can determine τ values for individual VLPs also by fitting the change in $\text{Var}(P)$ of each VLP at different time points (0, 0.5, 1, 2, 5 and 10 min) with the above model, and calculating the characteristic time of chol depletion. Figure 6c compares the single-VLP τ values for the two investigated conditions (we have included only the cases with a goodness of fit above 0.9). The distribution of chol depletion times is broader for 2 mM M β CD, indicating a more heterogeneous extraction behavior in this case. We attribute the higher heterogeneity to the fact that in the case of a lower M β CD concentration, characteristic parameters of the individual VLPs, such as size, curvature, membrane composition, density, and nature of surface proteins have more leeway in perturbing the chol depletion. In general, the heterogeneity observed in chol extraction kinetics indicates a variability in the VLP membrane that could lead to different efficacies in early steps of virus infection that include host cell binding and infection.

CONCLUSION

We have demonstrated that the polarization fluctuations of SNPs bound to individual VLPs depend on the fluidity of the membrane, which we utilized for sensing chol extraction on the single VLP level. The diffusion of SNPs resulted in measurable polarization fluctuations, which were enhanced through co-confinement of multiple NPs due to distance dependent

plasmon coupling. We detected systematic changes in the polarization fluctuations as response to chol extraction through M β CD at temperatures below and above the phase transition temperature. At both temperatures the PFTs were indicative of confined, non-Brownian diffusion in which the NPs switched between different geometric configurations. Whereas chol extraction at low temperatures “froze” the NPs in the configuration that they were presently occupying, at high temperatures the NPs relaxed preferentially into the low energy configuration. A characterization of the impact of chol extraction on Var(P) for the VLP ensemble at 40°C revealed a loss of lateral mobility of the NPs and a sharpening of the Var(P) distribution. The broad spread of Var(P) and extraction rates, in particular at low M β CD concentrations for individual VLPs, indicates some heterogeneity of chol content in the membrane among individual VLPs. Given the relevance of chol for viral function^{5,6}, virion-to-virion differences in chol content is expected to create variability in the infectivity. PFTM now provides an optical sensing tool to quantify this heterogeneity and, thus, paves the path to future studies in which the impact of membrane heterogeneity on specific virologically-relevant outcomes, such as host cell binding affinity, fusion with the host cell plasma membrane, Env protein maturation, and others can be established. These studies will help to elucidate the role that the host-derived membrane plays in shaping virus-host cell interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic representation of fluctuations in the resonance wavelength (“color”) and polarization (P) due to rotational and translational diffusion of two silver NP labels on the surface of a VLP.

Figure 2.

Implementation of PFTM. (a) Simulated PFTs for two 40 nm SNPs on a 150 nm diameter virus particle with random motion (blue) and with two preferred geometric NP configurations (red) (right: geometric configurations corresponding to the two marked points in the PFT). (b) SNPs are tethered to phosphatidylserine in the VLP membrane through biotinylated single stranded DNAs bound to the NPs and NTV-AnxV chemistry. (c) Schematic illustration of SNP-labeled VLP structure with two different configurations. (d) The optical set-up used to record PFTs contains a tungsten lamp, dark-field condenser, 60 $\times$ oil objective, polarizing beam-splitter and two CCD cameras. (e) Left: Exemplary experimental PFT and corresponding P histogram. Right: CCD images (7 $\times$ 7 pixels per VLP) of an SNP-labeled VLP in the $I_{\parallel}$ and $I_{\perp}$ channels at two consecutive time points t_i and t_{i+1} and their corresponding Gaussian fits.

Figure 3.

Plasmon coupling between the SNPs enhances the P fluctuations. (a) $\text{Var}(P)$ for SNP-labelled VLPs (black circles, $n = 500$) and 200 nm diameter polystyrene NPs (red x, $n = 250$) as function of average total scattering intensity. The inset shows transmission electron micrographs of VLPs with different numbers of bound SNPs; the length of the scale bar is 100 nm. The number of SNPs bound per VLP was determined by dividing the measured scattering intensity of individual SNP-VLPs through the average SNP intensity. The gray histogram shows the number of SNP-VLPs carrying a specific number of SNP labels (top x-axis). The fraction of SNP-VLP with $\text{Var}(P)$ three standard deviations higher than the $\text{Var}(P)$ of the polystyrene NPs with identical intensity is included as solid black lines. (b) Fluctuations of the total intensity, I_t , as function of time for an individual SNP (blue) and for an SNP-VLP with two SNPs (orange). (c) P as function of I_t for the two SNP-VLPs shown in (b). Only the SNP-VLP with 2 labels (orange) shows a correlation between P and I_t . (d) Comparison of the absolute value of the Pearson correlation coefficient, ρ , for SNP-VLPs with 1 and 2-3 SNPs. *** indicates a Student's two-sided t -test p -value below 0.001.

Figure 4.

Silver NPs prove to be better than gold NPs as labels in this application of PFTM. Scatter plot of $\text{Var}(P)$ as function of trajectory-averaged total intensity, $\bar{I}_t$, for GNP- and SNP-labeled VLPs ($n = 170$ for each case). Inset: Fraction of NP-labeled VLPs with $\text{Var}(P)$ values that are at least three standard deviations higher than the $\text{Var}(P)$ of polystyrene NPs with the same intensity. Error bars show s.e.m for three independent samples measured. Background: Histogram of $\text{Var}(P)$ as function of $\bar{I}_t$ for GNP- and SNP-labeled VLPs.

Figure 5.

Chol content change affects the membrane fluidity and NP labels' behavior in different ways depending on temperature. (a) Snapshot of an MD simulation for the diffusion of an individual DOPS (orange) lipid in a membrane composed of DOPS (green), DPPC (gray), and chol (red). The trajectory of the orange DOPS molecule over a time frame of 250 ns is included in blue. (b) Simulated diffusion coefficient, D , for DOPS at various chol concentrations and two temperatures (25°C, 40°C and 50°C). Error bars show the s.e.m for D calculated from three different areas of the MSD plots. (c,d) Experimental PFTs for individual SNP-VLPs measured before and after chol depletion at 25°C and 45 °C, respectively. (e) $P_{P, aft.}$ as function of $P_{P, bef.}$ (see text) for individual SNP-VLPs ($n = 8$). Inset: Correlation coefficients, ρ , for $P_{P, bef.}$ and $P_{P, aft.}$ calculated for the two temperatures. (f) Model to account for the differences in correlation between $P_{P, bef.}$ and $P_{P, aft.}$ at low and high temperature (25°C and 45 °C). At 25°C, the system can get frozen in a high energy configuration while at 45 °C, the system can localize to the energetically favored configuration.

Figure 6.

Chol extraction kinetics can be tracked in HIV-1 VLPs on a single-virus level. (a) Chol extraction from the membrane of HIV-1 VLPs ($n = 130$) with MβCD at 40 °C significantly ($p = 0.01$) shifts the $\text{Var}(P)$ distribution and also would like to acknowledge the guidance of Xin Zhao with electromagnetic simulations, as shown in the histograms. Data points below the dashed line are the individual VLPs whose $\text{Var}(P)$ s have decreased. (b) $\text{Var}(P)$ corresponding to a 50% probability in the cumulative distribution, $V_{0.5}$, as function of time for treatment of two VLP populations from the same batch ($n = 250$ in each case) with 2 mM and 10 mM MβCD. Error bars show the s.e.m for 3 consecutive measurements of the sample at each time point. (c) Characteristic extraction time, τ , determined for individual VLPs upon chol extraction with 2 mM ($n = 27$ VLPs) and 10 mM ($n = 29$ VLPs) MβCD and their corresponding box plots. τ is the time until $\text{Var}(P)$ has dropped to $1/e$ of its initial value. * indicates a paired Student's t -test p -value below 0.05.