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ABSTRACT

Optical trapping of plasmonic nanoparticles for controlled nanoscopic damage of cellular plasma membranes can be used to gain deeper insight into the role of plasma membrane repair proteins. Here we present a synthetic platform of giant unilamellar vesicles (GUVs) in the vicinity of trapped nanoplasmonic particles as a proposed model assay to characterize the permeability of a damaged GUV membrane, i.e. size of an inflicted hole. Water soluble fluorescent molecules with different sizes are used to characterize the extent of the membrane lesion since their differential permeability will provide information about the size of the rupture. We find that trapped gold nanoparticles can create substantial holes, observed via the discriminating influx of various sized molecules across the membrane. The technique, yet unrefined, provides groundwork for future investigations of annexin repair proteins, using nanoscopic heating of plasmonic particles to create quantifiable membrane damage.

Keywords: optical trapping, plasmonic heating, artificial membranes, annexins

1. INTRODUCTION

The cellular plasma membrane is essentially a semipermeable barrier that is not simply a vessel protecting the cell from the extracellular environment, but also regulates a variety of key cellular processes vital for its survival. While small uncharged molecules can pass the membrane by diffusion, large ions (e.g. $\text{Ca}^{2+}$) or , however, particles are tightly regulated by specific proteins and dedicated pathways.

Plasmonic nanoparticles (NPs) irradiated with light also affect the permeability of plasma membrane due to their heat generation capacity. In\textsuperscript{1,2} it was shown how using an optically trapped gold nanoparticles (AuNP) as a local heat source, a local lipid phase transition can be achieved. At the transition temperature the permeability for certain molecules was observed. Complementary, we can use phase transition of lipids to characterize the heating capabilities of plasmonic particles.\textsuperscript{3,4}

Beyond the heating capabilities of trapped NPs, there are also other associated effects, such as the thermophoretic effect and convection, to be taken into account. These effects are proportional to the heat gradient, which can be extremely high near a NP but quickly levels off at few 100nm away from the NP. If the NPs are irradiated using a focused laser beam, they will also experience gradient forces (see Section 2.2) coupled to the plasmonic effects.\textsuperscript{5} Recently, a new technique called opto-injection has been explored for injecting NPs into cells;\textsuperscript{6} it relies on the optical pressure ($F_{\text{abs}} + F_{\text{scat}}$) exerted on the NP and the combined heating used to disrupt the plasma membrane. This technique has been used for a number of applications like injecting signaling molecules for focal adhesion activation,\textsuperscript{7} as a molecular release mechanism in red blood cells,\textsuperscript{8} delivery of therapeutic nano-structures\textsuperscript{9} and even cell transfection.\textsuperscript{10}

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However, so far the nature of the membrane puncture occurring in membranes upon artificially induced rupture, by thermoplasmonics or other techniques like pulsed laser puncture, remains poorly understood. In\textsuperscript{1} it was shown that membranes exposed to localized heating exhibited transient leakage near the phase transition of the membrane. This may also be the case for fluid phase membranes since transient passive leakage has also been measured for fluid phase membranes by patch clamp techniques.\textsuperscript{12,13} The characterization of the physical hole induced by thermoplasmonics is of great interest since this technique has a great potential to be used for investigating the role of membrane repair proteins in healing membrane ruptures. Here, we explicitly address the size of the induced rupture in fluid membranes by including water soluble fluorophores of different hydrodynamic radii and monitor their influx upon local heating. We show that optical trapping of different plasmonic nanoparticles can be used for locally heating the membranes while confocal microscopy is used to visualize the influx of the reported molecules. Furthermore we addressed the theoretical uncertainty of the heating based on shell thickness of different NPs.

2. EXPERIMENTAL METHODS

2.1 Reagents

Dextran, Alexa Flour 647 (dextran) and Alexa Flour 488 Hydrazide (alexa488), and Alexa Flour 633 Hydrazide (alexa633) from ThermoFisher (D22914,A10436,A30634), are added to 500 µL observation buffer to get a 5 nM concentration of both. 2.5 µL dextran from a stock solution containing 10 g/L dextran are added to the 500 µL observation buffer and 1.45 µL Alexa488 from a stock solution containing 1 g/L Alexa488 are added to the same 500 µL observation buffer as well. A gold nanoparticle colloid suspension is created by adding 1 µL AuNPs (100 nm or 200nm) from stock solution to 100 µL observation buffer and sonicating for 2-5 min before use. The AuNP are coated with Poly(ethylene glycol) (PEG) in order to prevent aggregation of the particles and stabilize them in solution. Gold nanoshells (AuNS) were also used, their core consisting of a 120 ± 4nm silica sphere upon which a thin gold shell (20 ± 7nm) has been grown giving a total diameter of the complex of ∼160nm (Nanocomposix, CA USA). To generate C-terminal GFP-tagged recombinant Annexin A5 (ANXA5) protein, ANXA5 cDNA was subcloned into pETM11SUMO3sfGFP.\textsuperscript{14} Proteins were produced using Immobilized Metal-Affinity Chromatography (IMAC) followed by fast protein liquid chromatography (FPLC).

2.2 Optical trapping and imaging

Confocal imaging was performed on a Leica SP5 confocal microscope into which an optical trap, based on a 1064 nm laser (Spectra Physics J201-BL-106C), was implemented [\textsuperscript{REF 14}]. Optical trapping was done at the focal plane of the microscope and by using a Leica PL APO, NA=1.2, 63X water immersion objective to tightly focus the laser light. The optical trap was stationary, but the trap could be moved relative to the GUVs by translating the sample which was mounted on a piezoelectric stage (PI 731.20, Physik Instrumente, Germany) allowing lateral movements with nanometer precision. A glass bottom open chamber, containing the GUVs, molecular fluorescence probes and gold nanoparticles, was mounted on the microscope and kept at room temperature during the experiment. The 488 nm argon laser line was used to excite the Dextran and GfP fluorophores. A 613 nm laser line was used to excite the Alexa 613 hydrasize fluorophore. To detect the scattered and reflected light from the AuNPs, a 476 nm argon laser line was used in reflection mode.

2.3 Plasmonic heating

The temperature profile around an irradiated NP can be found by solving the heat transfer equation\textsuperscript{15} where the local heat intensity comes from light dissipation inside the irradiated NP,\textsuperscript{16} the temperature increase \( \Delta T(r) \) can be written as a function of distance to the source \( r \) as: \textsuperscript{17}

\[
\Delta T(r) = \frac{VQ}{4\pi kr}, \quad r > R, \tag{1}
\]

where \( R \) is the radius of the spherical NP, \( k \) is the thermal conductivity of water (0.58 W/mK in water at room temperature), \( V \) is the volume of the particle and \( Q \) is the local heat dissipation.\textsuperscript{18} From\textsuperscript{19} equation (1) can be re-written to relate the increase of temperature to the cross section absorption \( C_{abs} \) as
\[ \Delta T(r) = \frac{IC_{abs}}{4\pi kr} , \]  

(2)

where \( \rho \) and \( C_p \) are the density and the specific heat capacity at constant pressure, respectively. \( \Delta T(r) \) is the relative temperature increase relative to ambient temperature and \( \kappa \) is the thermal conductivity. \( Q = IC_{abs} \) is a measure of the generated heat (the amount of heat produced per unit time and volume inside the NP), largely contributed by Joule heating inside the NP. \( I \) is the intensity of the laser irradiation at the sample plane. The temperature profile around an irradiated NP is governed by eq. 1, which reaches a steady state within tens of nanoseconds, satisfying Laplace’s equation. The solution for the temperature gradient will be a simple function of distance, \( r \), to the surface of the NP with radius of \( R \).

Through Mie based calculations, \(^{20}\) we can calculate the absorption cross section, \( C_{abs} \), from which the temperature profiles of irradiated strongly absorbing nanoparticles \(^{21}\) can be obtained. Figures 2.C/D shows equation 2 plotted as a function of distance to the particle center.

### 2.4 Silica-gold nanoshells (AuNS)

We mostly used the AuNSs conjugated with a polymer (polyethylene glycol PEG). The voluminous PEG chains can reduce the adhesion of the particles through steric repulsion, thus preventing them from attaching to the cell surfaces and from agglomerating. Unlike non-PEGylated NPs, they will remain longer in suspension, minimizing the number of single particles sedimenting at the bottom of the chamber during the experiments. All these factors increase the probabilities of a particle being trapped while trying to study permeability of membranes.

The plasmonic resonance for these particles can be tuned over the NIR range by changing the core/shell ratio, with smaller shell thickness shifting the plasmon resonance towards higher wavelengths. Smaller cores give plasmon resonances in the visible region but by increasing the size of the core and decreasing the thickness of the shell the plasmon resonance can be shifted to wavelengths deep into the infrared. This tunability does however come with some drawbacks: during the fabrication of these NPs there is always a tolerance for error. The specifications of the AuNSs that we normally use, report an indicated tolerance of \( \pm 5 \text{ nm} \) for the core and \( \pm 7 \text{ nm} \) for the shell. Even though these tolerances are of the same order for the AuNP (i.e \( \pm 5 \text{ nm} \)), the impact on the plasmonic properties of the NPs is vastly different. A change of a few nanometers in the core size of a solid gold NP has little effect on the plasmonic properties (i.e. the \( C_{abs} \)). However, for the AuNSs the effect of a small change of a few nanometers (specially in the shell thickness) has a dramatic impact on the plasmon resonance. Therefore, when utilizing AuNSs for experiments one needs to realize the heterogeneity in plasmonic heating that they entail compared to AuNPs.

This effect is clearly demonstrated by the calculations in Figure 1, where we compare the \( C_{abs} \) for different AuNS with the 200 nm AuNP (in dashed black). Small changes in the Core-Shell configuration can lead to difference in the absorbance cross section of one order of magnitude - see green and purple curves in Figure 1. It is worth keeping in mind that the temperature increase is directly proportional to \( C_{abs} \) (see eq.(2)), meaning that the increase on temperature will be so large that the particle will probably instantly disintegrate under plasmonic resonance. The lack of thermal stability of the nanoscale gold layer was observed in \(^{22}\) by using SEM on irradiated gold nanoshells which had similar dimensions as here.

The silica-gold nanoshells are very strong scatters. In fact, the ratio between scattering and absorption efficiency is about seven. This is not only the case for AuNSs; in general, the larger the particle, the more the tendency to scatter dominates over absorption of light. Unfortunately, metallic NPs have relatively high extinction cross sections and hence experience significant radiation pressure (which scales linearly with \( C_{ext} \)), in an optical trap. \(^{23}\) These large scattering forces complicate stable trapping and the NP tends to fall into local intensity maxima outside of the main focus of the optical tweezers, \(^{24}\) where heating is much reduced due to the lower intensity.

Another important factor we need to be aware of, in fact, is that AuNSs will be hotter at their surface. Since they are smaller than a 200 nm AuNP, therefore by looking again at eq.2, one can see that at equal \( C_{abs} \) and laser intensity smaller particles will get significantly hotter.
Despite their drawbacks, AuNSs (specially when PEGylated) are a more efficient tool than other NPs of same size to perform membrane fusions and membrane rupturing studies\textsuperscript{25} and some “unpublished results” where the temperature at the particle surface is most critical.

![Absorption cross sections for 200 nm (dashed black) AuNPs and a collection of AuNS with different sizes of core and shells, calculated with NanoComposix Mie Calculator.](#)

The plasmon resonance shifts when the gold shell is made thinner. The absorption cross section for the wavelength of interest (1064 nm) is highlighted in blue.

2.5 Vesicle swelling

Polyvinyl alcohol gel (PVA) (5%, 50 mM sucrose, 25 mM NaCl/Tris) was heated in a 60 degrees oven for 20 min. Glass slides was first cleaned in ethanol and dried with ultra spray, followed by heating in an air Plasma Cleaner model Harrick Plasma cleaner PDC-002 in the high mode setting with pressure in between 200-400 turns for 3 to 5 min. 90 µL of warm PVA gel was applied on the clean glass slides, after which they were heated in 50 C oven for 50 min. Subsequently The vesicles were grown on top of the prepared PVA glass slides. A 50 µL glass Hamilton syringe was cleaned in chloroform to ensure minimum contamination. 30 µL of prepared (95% DOPC, 5% DOPS) was added on top of the PVA gel layer on the glass slides. The applied lipidmix was dried with nitrogen in 30 sec followed by vacuum drying for 2 hours. A clean vesicle chamber was used to grow the vesicles. 350 µL growing buffer (NaCl 70mM, Tris (pH 7.4) 25 mM, Sucrose 80 mM) was added to the chamber on top of the PVA-coated glass slide and the vesicles were left to form for 1 hour and 5 minutes. 340 µL of vesicle and buffer solution was collected from the chamber and deposited in an Eppendorf tube. 300 µL observation buffer (NaCl 70 mM, Tris (pH 7.4) 50 mM, Glucose 55 mM) where added for the vesicles to sink to the bottom.

2.6 Protein encapsulation

Annexin A5 (900nM) was added to the prepared cover glass. Followed by an addition of the prepared growing buffer at room temperature (total volume of 300 L) was then set to form encapsulated annexin A5 giant unilamellar vesicles for 1 hour and 5 minutes. The vesicle solution was extracted by pipetting directly from the cover glass into an Eppendorf tube and 1000 L observation buffer. The vesicles were set to sink for 5 minutes followed by centrifugation at 600 rcf for 10 minutes at 13C. Subsequently the added observation buffer was carefully removed from the top and down to ensure minimal loss of GUVs and to remove excess annexin A5 on the outside.

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2.7 Data analysis

All images were analyzed using Matlab (The MathWorks, Inc., Natick, Massachusetts, United States) as well as the temperatures calculated in Figure 2.C/D were plotted using Matlab and calculations were based on Mies equations.\textsuperscript{20}

3. RESULTS

3.1 Effect of local heating on artificial membranes

It is broadly understood that a strong increase of temperature on a nanoscopic area can in turn lead to an increase of membrane permeability\textsuperscript{26,27} especially near a phase transition.\textsuperscript{1,13} The mechanism behind leakage of fluid phase membranes is most likely thermal expansion of the membrane which increases the likelihood for transient opening of the bilayer. Fluid membranes expand around 0.5%/K\textsuperscript{28} and hence strong local heating can easily cause expansion of the lipid packing and eventually result in a mechanical disruption of the membrane and the formation of pores.\textsuperscript{29} Permeation near a phase transition has been suggested to occur by enhanced thermal fluctuations of membranes in the vicinity of the phase transition temperature which could lead to thermally activated transient pores.\textsuperscript{12,13} We have also just mentioned how this alteration of lipid ordering has been reported and used to quantify plasmonic heating of a range of NPs.\textsuperscript{1–4} There is still, however, a lack of experimental characterization of permeability effects in the fluid membranes which are studied here.

By controlling both the distance between the bilayer/vesicle and the particle and the trapping power, the particle surface temperature can rise by several hundred Kelvin even at moderate trapping laser powers - see Figure 2.C/D. It has also been shown how the particle temperature can be controlled with a precision of a few degrees by tuning particle size and laser power.\textsuperscript{25} Using this spatially/thermally controlled system we have developed a simple assay to quantify the size of permeation events induced by local heating of trapped NPs, depicted in 2.A. A 100 nm AuNP was trapped and placed in the vicinity of a GUV in the chamber with buffer solution containing fluorophore as a reporter for permeability and size.

Any distance larger than 1.5\(\mu\)m will result in temperature increases of less than 5 degrees. As mentioned above, the area and volume of membranes can be affected by temperature,\textsuperscript{30} so it is possible that the change in bilayer structure could result in porous membranes and thus cause diffusion across the membrane with an increase in temperature.

The pore size induced by plasmonic heating can be assessed by using fluorophores conjugated to molecules with different molecular weight and hence with different molecular radius. In Figure 3 results are shown for the same experiment depicted in Figure 2.A, but using two probes with very different sizes instead of one. The same Alexa488 hydrazide (small probe, MW:570) and a bigger sized Dextran-647 (bigger probe, MW:10000). The small Alexa488 probe leaked in faster than the bigger Dextran conjugated dye thus showing that the pores have a size comparable to the size of the probing molecules. The fact that both probes do leak at the same temperatures could indicate that the pores are fluctuating in size and therefore could allow passage of different sized molecules although with different size dependent probability for the two fluorophores. However, we cannot get conclusive results from this type of experiments. It is clear that we are dealing with nanoscopic pores when the hot nanoparticle gets close to the membrane. No microscopic hole was observed with 100nm AuNPs: these would be detectable with a confocal microscope. Also, holes of micron size would fill the vesicle much faster than measured here, since the diffusion of the utilized probes is ca. 300 \(\mu m^2/s\).\textsuperscript{31}

3.2 Membrane repair proteins can be studied using thermoplasmonics

We continued the same approach of the previous experiments, where we investigated the permeability of GUV membranes. Using the same experimental set up, thermoplasmonics can additionally be used for studying how proteins react to perforation of membranes. We used the local heating of plasmonic nanoparticles near membranes to cause micron-size holes and the response of membrane repair annexins was investigated - See Figure 4. Unlike the previous experiments, however, here we encapsulated recombinant ANXA5-GFP using the PVA gel hydration method described in Section 2.6. The GUVs were composed of 95% DOPC lipids and 5% DOPS lipids. We used a 900 nM ANXA5-GFP growing buffer (70 mM of NaCl, 25 mM of Tris, 80 mM sucrose) solution for the hydration of the GUVs. Upon collection of the vesicles, they were spun down for 10 minutes at 10\(^{9}\)C at 900g diluted in
Figure 2. Quantification of permeability events in model membranes. (A) Schematic illustration of the experiment with GUV surrounded by free diffusive molecules which differ in size. Diffusion of the molecules across the GUV membrane depends on the size of the thermoplasmonic-created hole by the optical trapping of a gold nanoparticle. (B) Confocal image of an empty GUV composed of only DOPC lipids in a chamber full of Alexa633 dye (in green). (C) Heating capabilities of trapped metallic NP. 3D calculation of the heat profiles for gold nanoparticles (AuNPs) of 100 and 200 nm in diameter. (D) A 2D cross section of such heat profiles calculated earlier. All calculations are done considering water as the surrounding medium, and laser irradiation of 1064 nm wavelength with laser intensity of $I = 9 \times 10^{10}$ W/m².

an observation buffer (70 mM NaCl, 50 mM Tris and 55 mM Glucose) to wash the excess ANXA5-GFP. They were re-suspended in the observation chamber containing observation buffer with added 2 mM of CaCl₂, under the confocal microscope for the experiments. The presence of Ca²⁺ ions facilitates the binding of ANXA5-GFP to the membrane (See Figure 4). We chose AuNSs for this experiments instead of 100 nm AuNPs, because...
Figure 3. Quantification of permeability events in model membranes. Intensities of Dextran-Alexa647 (pink signal) and Alexa488 (green signal) inside a GUV, ambient temperature of T=23.1°C. Distances between optically trapped 100 nm particle (white dot) and the GUV (darker area) are imaged with the time-step and a 5 µm scale bar. (a) Plot and image of intensity inside GUV with a distance of 2.16 µm to the AuNP. (b) Plot and image of intensity inside GUV with a distance of 1.17 µm to the AuNP. (c) Plot and image of intensity inside GUV with the AuNP at the membrane, showing increase in intensity signal.
we need to generate sufficient heat to expand the membrane sufficiently to generate permeability events as the ones shown in Figure 3 large enough to generate a response from the encapsulated annexins. When trapping AuNSs with sufficient power and vicinity to the GUV with encapsulated Annexin 5 recombinant protein, we could successfully generate a local response of the protein to the area of injured membrane.

As shown in Figure 4.B, we observed up-concentration of ANXA5 in the vicinity of the membrane and the trapped nanoparticle. The up-concentration of protein increases gradually until vesicle collapse. This is in agreement with ANXA5 inducing the cooperative roll-up of supported membranes reported in Ref. In their work, authors added different recombinant annexin proteins to supported lipid bilayers, observing strong curvature and membrane rolling initiated in the free edges. Furthermore, it is also remarkable how the clustering behavior of ANXA5 upon membrane lesion is in agreement with recently published results which also resembles the proposed mechanisms of annexins wound repairing.

Figure 4. Plasmonic heating induces membrane permeability, allowing to study membrane repair proteins. (A) Schematic illustration of the experiment with ANXA5 encapsulated in a GUV. A trapped AuNs in the vicinity of the GUV will generate enough heat to provoke expansion of the membrane when sufficiently close. (B) Montage with the time evolution of up-concentration of ANXA5 in the vicinity of a trapped AuNS in GUV with ANXA5 bound to the membrane. Scale bar is 10µm.
4. CONCLUSION

Optical trapping works for a range of different AuNPs, inducing plasmonic heating in a nanoscopic range around the particle. Here, we have presented a model assay to quantify membrane lesions utilizing localized plasmonic heating of AuNPs. Our results demonstrate that this technique can be utilized to induce local membrane damage, monitored by the discrimination in influx of differently sized fluorophores across the membrane.

Thermoplasmonic driven membrane remodelling has proved to be extremely useful to study biological membranes. The heat generation capabilities combined with the large degree of localization and control is the key of this success. In this proceeding we theoretically evaluated a number of commercially available gold nanostructures in the context of thermoplasmonic heating in the near-infrared region with specific attention on the uncertainty in the fabrication of delicate plasmonic nanostructures. Novel methods that this technique allows include characterization of temperature dependent membrane permeability and membrane rupturing. Using optically controlled thermoplasmonics offers a way to trigger both small and larger pores in lipid membranes. Combined with encapsulation of proteins this strategy allows for the investigation of membrane repair effects from annexins or investigations of proteins on membrane free edges surrounding larger ruptures. In this manner it is possible to study curvature generation effects by proteins.

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