Experimental report

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Proposal:	**9-13-924**	**Council:**	4/2020
Title:	Combined membrane action of UV-triggerable TiO2 nanoparticles and antimicrobial peptides against bacteria-mimicking bilayers		
Research area:	Soft condensed matter		

This proposal is a resubmission of 9-12-586

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Samples: Quartz crystals
hydrogenated phospholipids
Antimicrobial peptides
TiO2 nanoparticles

Instrument	Requested days	Allocated days	From	To
D17	2	2	07/07/2021	09/07/2021

Abstract:

One of the main biomedical challenges nowadays is the development of new and optimised strategies to enhance bacterial killing and overcome antibiotic resistance. In this context, both photocatalytic TiO2 nanoparticles (NP), combined with UV irradiation, and antimicrobial peptides (AMP) are potent antimicrobial agents, but the use of each of them independently present some drawbacks: TiO2 NPs display low selectivity between bacteria and human cells, and AMPs present high sensitivity to proteolytic degradation and low bioavailability. Thus, promising combinations can arise from the use of TiO2 NPs as AMP delivery systems, while AMPs can act as a NP targeting moiety to enhance selectivity against bacteria. Based on our previous studies regarding the effects of different AMPs, UV, and TiO2 NPs on bacterial and mammalian cell membrane models, here we aim to characterise the changes induced in model bacterial membranes by AMP-loaded TiO2 NPs, in combination with UV, by neutron reflectometry. This study is expected to provide unique structural and kinetic information on their joint membrane interactions, disruption, and lipid degradation required for efficient bacterial killing.

Experimental Report 9-13-924

Combined membrane action of UV-triggerable TiO_2 nanoparticles and antimicrobial peptides against bacteria-mimicking bilayers

Abstract

Photocatalytic nanoparticles offer potent antimicrobial effects under illumination, due to the formation of reactive oxygen species, capable of degrading bacterial membrane components. In the context of the fight against antimicrobial resistance, powerful combinations for bacterial killing could arise from the use of photocatalytic TiO_2 nanoparticles (NPs), loaded with antimicrobial peptides (AMPs). These peptides are broad-spectrum antimicrobial and anti-inflammatory innate defense components, positively charged but also containing a significant fraction of hydrophobic residues.[1] AMPs can be designed to display exquisite selectivity for bacterial membranes, which are rich in negatively charged phospholipids and lipopolysaccharides, but lacking sterols.[1] Important drawbacks of the adoption of AMPs for infection or sepsis treatments include sensitivity to proteolytic degradation and low bioavailability, challenges that can be overcome by the use of NPs-based delivery systems.[2] Conversely, AMPs can effectively increase the selectivity of NPs by acting as a 'targeting' moiety. Hence, the combination of antimicrobial TiO_2 NPs with highly selective AMPs represents a promising strategy to enhance selectivity and efficiency against bacteria and to potentially achieve synergistic effects. Based on this, the present experiment aimed to characterize by neutron reflectometry (NR) the structural changes induced in model bacterial membranes by AMP-coated TiO_2 NPs, particularly the benchmark AMP LL-37, and photoactivated by UV irradiation. This builds on our previous knowledge of lipid membrane oxidation induced by the combined action of UV and TiO_2 NPs.[3-5]

Methodology

Structural features of supported POPC/POPG/PAPC bilayers before, during, and after exposure to 2 hours of UV were measured in the presence of TiO_2 NPs coated with LL-37, as well as bare TiO_2 nanoparticles (chosen as control sample), in 10 mM acetate buffer at pH 5.4, following a previously established method[4,5] with some modifications.

Experiments were performed on the vertical reflectometer D17 (Institute Laue-Langevin, Grenoble, France) to mitigate potential bubble formation. Using two incident angles (0.8° and 4.0°), the whole Q-region of interest (~0.01 to 0.4 $Å^{-1}$) was covered. For kinetic measurements, an intermediate angle of 1.8° was used to capture the Q-region where the main changes were expected to occur, and a divergent beam geometry was set to achieve improved statistics and shorter acquisition times, allowing high time resolution by 60 s acquisitions. Solid-liquid flow cells, the top plate of which was modified with a 30 mm diameter circular opening, were used together with UV-transparent quartz blocks (80x50x15 mm, 1 face polished, RMS < 4.5 Å; PI-KEM Ltd., Tamworth, UK) to allow *in situ* UV irradiation (Spectroline UV lamp ENF-260C, 6 W, 254 nm; 3 mW/cm^2). 3 quartz blocks were used and cleaned by Piranha acid cleaning (5:4:1 MilliQ/H_2SO_4/30% H_2O_2 by volume) before the experiments.

Supported lipid bilayers were formed on these quartz substrates, pre-coated in 2 mM $CaCl_2$, by fusion of POPC/PAPC/POPG (50/25/25 molar ratio) small unilamellar vesicles (0.1 mg/ml lipid concentration in 2 mM $CaCl_2$), rinsed and characterized in 2 contrasts (D_2O or H_2O acetate buffer at pH 5.4).

Anatase TiO_2 NPs in water were prepared by bath sonication of 50000 ppm commercial stocks, followed by centrifugation to remove the largest aggregates; the supernatants were then dispersed in D_2O to get a 5000 ppm NP working stock. 1 mM LL-37 stock solutions (MW= 4493.3 Da) were prepared in D_2O as well. To obtain LL-37-coated TiO_2 NPs, 250 μL of 1 mM LL-37 stock were diluted in 9.55 mL d-Tris buffer (10 mM, pH 9.4), then 200 μL of 5000 ppm TiO_2 stock were added dropwise under continuous mixing, to get final concentrations of 25 μM AMPs and 100 ppm TiO_2 NPs. Such a high pH, at which NPs and AMPs present opposite charges, was chosen to maximize the coating efficiency of TiO2 NPs by AMPs. AMP-coated NPs were further dispersed by 10 min bath sonication. The bare TiO_2 control was prepared similarly, by adding the corresponding component to the buffer in the absence of AMPs.

After bilayer formation and characterization, these LL-37-coated NPs (and corresponding bare NPs control) were injected manually into the cells at ~2 mL/min. 10 mL d-Tris buffer at pH 9.4 were then injected to rinse off the NPs excess, and the bilayer+NPs was characterized in d-Tris. After that, the bilayer+NPs were characterized in Acetate buffer (pH 5.4) in 3 contrasts:D-acetate buffer (D, 100% D_2O), quartz-matched buffer (QM, 68% D_2O + 32% H_2O) and H-acetate buffer (H, 100% H_2O), pumped into the cells for each buffer changing step at 2 mL/min for 10 min. Right after, the contrast was changed back to d-buffer and the UV lamp was remotely turned on, irradiating the samples for 2 hours while simultaneously acquiring reflectivity. The UV lamp was finally turned off and the bilayers were characterized in the whole Q-region in the same 3 buffer contrasts All the experiments were done at 37°C.

The experimental NR profiles were fitted by using the Genetic Optimization method the available on the Motofit software within the analysis package IGOR Pro. [6] A series of parallel layers were used to model the interfacial structure, each of these described by a set of physical parameters, i.e., thickness, roughness, hydration, and neutron scattering length density (SLD). A 4-layer model was employed to analyze the reflectivity from the bilayers and bilayers + bare TiO_2 NPs, including inner head groups, tails, and outer head groups, plus an extra water layer between the quartz surface and the inner head groups. In addition, a fixed head group thickness of 7.5 Å was assumed to reduce the number of free parameters, based on previous studies for PG containing bilayers.[4] To analyze the bilayers + LL37-TiO_2 NPs, a further rough outer layer was required to fit the experimental data[4], accounting for the adsorption of NPs and subsequent structural destabilization of the bilayer. The best fits of these parameters were converted into SLD profiles, which represent the density distribution in the direction perpendicular to the reflecting interface. A Monte Carlo error analysis allowing for refitting data 200 times, was employed to minimize the uncertainty associated to data fitting.[7]

Results

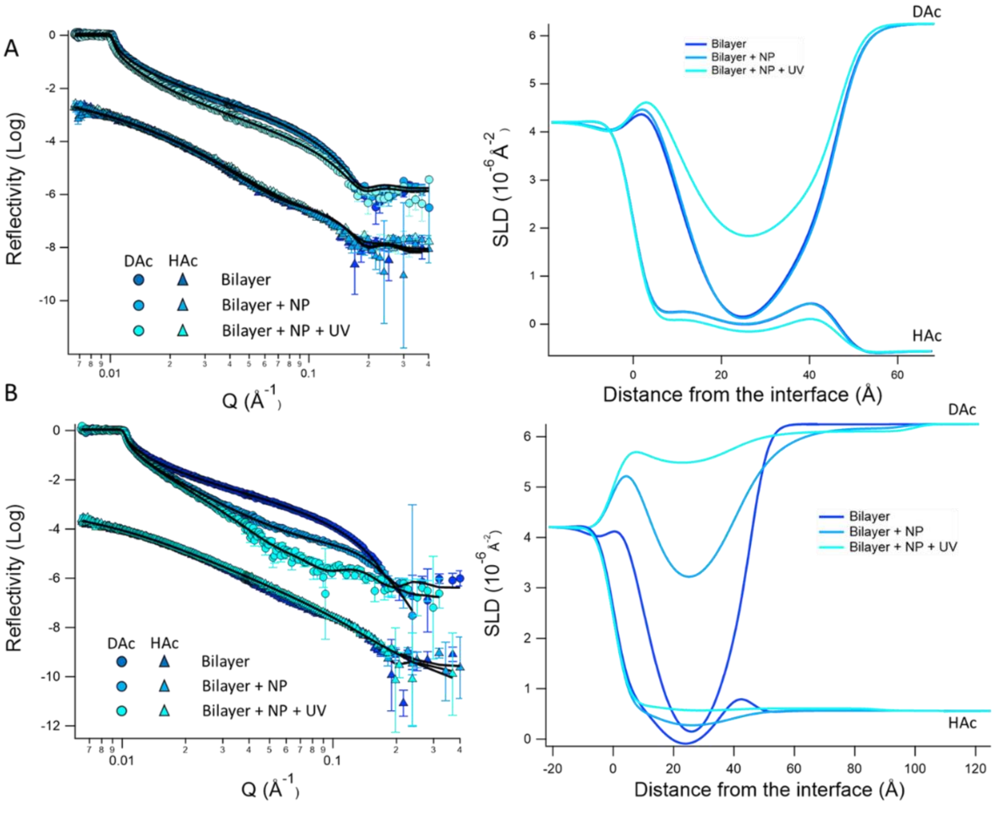

Figure 1 Neutron reflectivity profiles with best model fits (left) and SLD profiles (right) obtained for supported POPC/PAPC/POPG bilayers before, after incubation with TiO2 (A) and TiO2-LL37 NPs (B), as well as after 2 hours of in situ UV exposure, in 10 mM Acetate buffer pH 5.4. Curves are shown for two different buffer contrasts, dAcet and hAcet, with a proper offset applied to hAcet curves for clarity.

Figure 1 reports the reflectivity profiles with best model fits and corresponding SLD profiles for the bilayers before and after incubation with either TiO_2 (Figure 1A) or LL37-TiO2 NPs (Figure 1B), as well as after UV illumination, while full structural data calculated from the fits are shown in Table 1. The initial bilayers can be described with structural parameters consistent with previous literature, indicating full coverage[8,9].

Upon addition of bare TiO_2 NPs, only minor changes in the reflectivity and corresponding SLD were observed, while larger changes were obtained for the bilayers incubated with AMP-coated NPs. Such changes correspond to a significant increase in the hydration of the bilayer's head groups and tails, as well as a decrease in the surface coverage (Table 1). Further structural modifications were recorded after the 2 hours of UV irradiation, for both bare and LL37-coated NPs (Table 1). Remarkably, such modifications are much more pronounced for LL-37-TiO2 NPs than bare TiO2, leading to a massive increase in the area per molecule and an almost complete lipid removal. These results hints to a possible synergistic effect of LL-37 and TiO_2 NPs in promoting lipid oxidation and removal and other major structural rearrangements on the lipid bilayer under the exposure of UV illumination.

These results are part of a manuscript under preparation (expected to be submitted in November 2022) which investigates the membrane interactions of AMPs-TiO_2 NPs. The manuscript also includes Quartz crystal microbalance, fluorescence spectroscopy, and light scattering/ζ-potential measurements on AMPs-TiO_2 NPs interacting with bacterial model membranes, as well as biological tests to investigate the antimicrobial activity of AMPs-TiO_2 NPs against Gram-negative Escherichia coli and Gram-positive Staphylococcus aureus.

Bare TiO_2 NPs

	Bilayer Thickness (Å)	Γ (mgm^{-2})	APM (Å^{-2})	Heads Hydration (%)	Tails Hydration (%)
Bilayer	42.2 ± 0.2	4 ± 0.06	63 ± 13	31 ± 2	0.68 ± 0.2
Bilayer + NP	42.1 ± 0.2	4 ± 0.06	64 ± 13	31.6 ± 2	0.67 ± 0.2
Bilayer + NP + 2h UV	42.5 ± 0.3	2.8 ± 0.06	89 ± 17	50 ± 1	30 ± 2

LL-37-TiO_2 NPs

	Bilayer Thickness (Å)	Γ (mgm^{-2})	APM (Å^{-2})	Heads Hydration (%)	Tails Hydration (%)
Bilayer	42 ± 0.8	4 ± 0.1	63 ± 1.6	31 ± 2	0.5 ± 0.2
Bilayer + NP	42.9 ± 0.8	1.5 ± 0.7	173 ± 37	73 ± 15	49 ± 4
Bilayer + NP + 2h UV	41.9 ± 0.5	0.6 ± 0.06	419 ± 35	87 ± 2	88 ± 0.5

Table 1. Bilayer thickness, surface coverage (Γ), area per molecule (APM), heads and tails hydration obtained from the neutron reflectivity fits of POPC/PAPC/POPG bilayers before and after incubation with bare TiO_2 or LL-37-TiO_2 NPs, as well as after 2 hours of UV irradiation, in 10 mM Acetate buffer, pH 5.4.

References

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