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Conformational control of antimicrobial peptide amphiphility: consequences for boosting membrane interactions and antimicrobial effects of photocatalytic TiO$_2$ nanoparticles

Lucrezia Caselli, Sebastian Köhler, Davide Schirone, Ben Humphreys and Martin Malmsten

This study reports on the effects of conformationally controlled amphihellicity of antimicrobial peptides (AMPs) on their ability to coat TiO$_2$ nanoparticles (NPs) and boost the photocatalytic antimicrobial effects of such NPs. For this, TiO$_2$ NPs were combined with AMP EFK17 (EFKRIVQRIKDFLRLNV), displaying a disordered conformation in aqueous solution but helix formation on interaction with bacterial membranes. The membrane-bound helix is amphiphilic, with all polar and charged amino acid residues located at one side and all non-polar and hydrophobic residues on the other. In contrast, the d-enantioter variant EFK17-d (EdFKRdIVQRdKdDFdFLRLNV) is unable to form the amphiphilic helix on bacterial membrane interaction, whereas the W-residues in EFK17-W (EWKRWQRWKDFLRLNV) boost hydrophobic interactions of the amphiphilic helix. Circular dichroism results showed the effects displayed for the free peptide, to also be present for peptide-coated TiO$_2$ NPs, causing peptide binding to decrease in the order EFK17-W > EFK17 > EFK17-d. Notably, the formation of reactive oxygen species (ROS) by the TiO$_2$ NPs was essentially unaffected by the presence of peptide coating, for all the peptides investigated, and the coatings stabilized over hours of UV exposure. Photocatalytic membrane degradation from TiO$_2$ NPs coated with EFK17-W and EFK17 was promoted for bacteria-like model bilayers containing anionic phosphatidylglycerol but suppressed in mammalian-like bilayers formed by zwitterionic phosphatidylycholine and cholesterol. Structural aspects of these effects were further investigated by neutron reflectometry with clear variations observed between the bacteria- and mammalian-like model bilayers for the three peptides. Mirroring these results in bacteria-like model membranes, combining TiO$_2$ NPs with EFK17-W and EFK17, but not with non-adsorbing EFK17-d, resulted in boosted antimicrobial effects of the resulting cationic composite NPs already in darkness, effects enhanced further on UV illumination.

Introduction

Photocatalytic nanoparticles (NPs) attract intense current interest as antimicrobial agents and therapeutics due to their potent and wide-spectrum effects, particularly against bacteria resistant to antibiotics. The origin of such effects is the excitation of electrons in photocatalytic NPs by light, resulting in free electrons and positively charged holes able to react with water, dissolved oxygen, and other solutes at the NP surface. This, in turn, results in the production of reactive oxygen species (ROS), which are able to degrade essential components in bacteria, including membrane lipids, essential proteins, or DNA. Despite numerous reports in the literature demonstrating potent antimicrobial effects by photocatalytic NPs, some key issues prevent them from being further developed for antimicrobial therapeutics. Among these, the selectivity between bacteria...
and human cells remains a key challenge, as photocatalytic NPs may also degrade human cell membranes and other essential components, resulting in toxicity effects.\(^3\) Addressing this, we previously demonstrated that photocatalytic TiO\(_2\) NPs coated with antimicrobial peptides (AMPs\(^4\)–\(^6\)) may be used to enhance NP binding to bacterial membranes, thereby increasing the probability of ROS generated during illumination reaching the bacterial membrane during their short lifetime.\(^7\)

Although previous studies demonstrate that AMP coatings can be used to selectively boost the antimicrobial effects of photocatalytic NPs, it is unclear how the properties of peptides influence membrane interactions and antimicrobial effects. This is unfortunate since much is known about how AMP properties such as charge, amphiphilicity, length, and secondary structure affect membrane interactions and antimicrobial effects for free peptides in solution. This knowledge could potentially be used to optimize the antimicrobial effects and selectivity of AMP-coated photocatalytic NPs.\(^4\)–\(^6\) Attachment to a particle surface is, however, expected to reduce conformational and translational freedom of AMPs. Since membrane destabilization and antimicrobial effects of AMPs depend critically on their ability to insert into the lipid membranes, it thus remains unclear if knowledge of peptide properties for free AMPs in solution can be used for optimizing membrane interactions and antimicrobial effects of AMP-coated photocatalytic interactions. In a first investigation into this, we reported on the effect of hydrophobic W-tags for TiO\(_2\) NPs coated with the AMP KYE21 and its variant KYE21WWW.\(^8\) Mirroring the results obtained for the free peptides in solution,\(^9\) hydrophobic W-tagging resulted in AMP-coated TiO\(_2\) NPs displaying denser and more highly cationic peptide coatings, promoting binding of the peptide-coated NPs to anionic bacteria-like phospholipid membranes. Since the W-tagging did not detrimentally influence either ROS formation or the susceptibility of the peptide coating to oxidative degradation, the higher membrane binding by KYE21WWW-TiO\(_2\) resulted in promoted membrane degradation and antimicrobial effects. However, some saturation in photocatalytic effects was observed at very high binding densities, inferred to be due to diffuse light scattering effects.

In the present study, we focus on the role of AMP secondary structures on peptide performance as surface coatings of photocatalytic NPs for boosting bacterial membrane destabilization and antimicrobial effects. In doing so, we investigate AMP EFK17 (EFKRIVQRKDFLRLNV), displaying a disordered structure in aqueous solution but helix formation on binding to bacterial membranes.\(^10\)–\(^11\) Importantly, the helix formed on membrane interaction is amphiphilic, with all charged/polar residues on one side, and all non-polar/hydrophobic residues on the other (Scheme 1). The conformational change induced on peptide binding to bacterial membranes is thus strongly interlinked to the amphiphilicity of the peptide. Given this, it is also possible to suppress conformationally induced peptide amphiphilicity by replacing selected amino acids in EFK17 with the corresponding \(\nu\)-amino acid enantiomers (EFK17-\(d\); E(dF)KR(dF)VQR(dF)KD(dF)LRNLNV). Alternatively, the overall peptide amphiphilicity can be strengthened by replacing the same residues by more hydrophobic W residues (EFK17-W; EFWKRWVQWRKDFLRLNV), boosting the amphiphilicity of the helix. We previously demonstrated that the \(\nu\)-amino acid substitutions in EFK17 efficiently suppressed amphiphilic helix formation, thereby suppressing peptide binding to, and destabilization of, bacterial membranes. W-substitutions, on the other hand, promoted membrane binding and antimicrobial effects.\(^10\)–\(^12\) Considering this, we selected EFK17, EFK17-\(d\), and EFK17-W as surface coatings of photocatalytic TiO\(_2\) NPs to elucidate if conformational amphiphilicity strongly influencing bacterial membrane interactions and antimicrobial effects for the free peptides, translates to similar effects for peptides bound to the NP surface. Regarding photocatalytic NPs, we chose to work with TiO\(_2\) NPs. Although the wide band gap for TiO\(_2\) NPs requires UV illumination for photocatalytic effects,\(^13\) which may be a disadvantage in some applications, it also allows excellent experimental control. Thus, normal laboratory light conditions effectively correspond to “in darkness” conditions, allowing particle–membrane interactions to be investigated in detail, whereafter oxidation effects can be conveniently “turned on” by UV illumination. Benefiting from such experimental control, detailed investigations were performed regarding NP and coating properties (peptide conformation, coating interference with ROS generation, and coating susceptibility to ROS-induced degradation), as well as how these properties influence NP binding to, and destabilization of, bacteria-like and human cell-like lipid model membranes.\(^14\)–\(^17\) We also investigated to what extent results obtained in such model studies translated into antimicrobial effects. In doing so, all experiments were
performed at pH 5.4, chosen to represent conditions relevant to skin infections.18,19

Materials and methods

Materials

TiO2 nanoparticles (anatase, 4–8 nm) were purchased from PlasmaChem GmHb (Berlin, Germany), while EFK17 (EFKRIV QRIKDFLRNLV), EFK17-d (EFKRIVQRIKDFLRNLV) and EFK17-W (EWKRWQVRWKDLRWNLV) (all >95%) were from Thermo Fisher Scientific (US). Palmitoyloleoylphosphocholine (POPC), palmitoylarachidonoyl phosphocholine (PAPC), palmitoyloleylophosphoglycerol (POPG), and cholesterol (bovine, Chol) (all >99% purity) were purchased from Avanti Polar Lipids (Alabaster, US), while C11-BODIPY 581/591 was from Molecular Probes/Thermo Fisher Scientific (US). MilliQ water (MQ, 18.2 MΩ cm) and D2O (99% deuterated, Sigma Aldrich) were used throughout the experiments. All other chemicals were of analytical grade.

Liposome preparation

Liposomes were prepared as described previously.20 In brief, 10 mg mL−1 lipid solutions in chloroform (for POPC and PAPC) or chloroform/methanol (3/1 vol%) (for POPG) were prepared in dark glass vials to 75/25 mol% POPC/PAPC (‘PC’), 50/25/25 mol% POPC/PAPC/POPG (‘+PG’), or 65/25/10 mol% POPC/PAPC/Chol (‘+Chol’) (for ROS generation and neutron reflectometry (NR) studies), or to corresponding systems with PAPC replaced by POPC (for quartz crystal microbalance with dissipation monitoring (QCM-d) and circular dichroism (CD) studies). The solvent was evaporated under N2 and then under vacuum. The resulting films were hydrated with MilliQ water (to form supported lipid bilayers), or 10 mM acetate buffer, at pH 5.4 (for ROS and CD studies). The resulting multilamellar vesicles were tip sonicated (UP50H, Hielscher Ultrasonic GmbH, Germany) for 15 minutes in an ice bath to obtain small unilamellar vesicles (SUV; for supported bilayers), or extruded 30 times through polycarbonate filters (Ø 100 nm, LipoFast miniextruder (Avestin, Ottawa, Canada)) to obtain large unilamellar vesicles (LUV; for ROS and CD studies).

Preparation of peptide-coated TiO2 NPs

To prepare peptide-coated TiO2 NPs, EFK17, EFK17-d and EFK17-W peptides were dissolved in 1 mL of 10 mM acetate buffer, pH 5.4, at the desired concentration (from 5 to 100 μM), from 1 mM peptide stock solutions in MilliQ. 20 μL of bare TiO2 NPs were subsequently added from a concentrated stock dispersion (5000 ppm TiO2 in 10 mM acetate buffer, pH 5.4) under continuous stirring to obtain a 100 ppm TiO2 NP dispersion (for live/dead bacterial viability assay, 40 μL of bare TiO2 NPs were used instead to obtain a final TiO2 concentration of 200 ppm in the mixed peptide/NP dispersion, to be diluted down to 100 ppm upon mixing with bacteria). Following this, pH of the dispersion was adjusted to 9.4, where TiO2 NPs display a negative surface charge,7 to promote binding with positively charged peptides. The dispersions were subsequently tip-sonicated (UP50H, Hielscher Ultrasonics GmbH, Germany (50 W, 30 kHz) in an intermittent-pulse mode (5 s), at 100% amplitude) for 15 minutes in an ice bath. After that, pH was lowered back to 5.4, and peptide-coated TiO2 NPs were further tip sonicated for 15 minutes as described above. The so-obtained NP dispersions (100 ppm) were used without further modifications for dynamic light scattering and ζ-potential measurements, as well as for ROS generation assays and circular dichroism experiments. In contrast, NPs were diluted down to 20 ppm for NR measurements, or 5 ppm for QCM-d.

Size and ζ-potential measurements

Dynamic and electrophoretic light scattering (DLS and ELS; 173° back-scattering angle) were carried out with a Zetasizer Nano ZSP (Malvern Pananalytical Ltd, Malvern, UK) to obtain particle size and ζ-potential. Measurements were performed in triplicate at 25 °C using automatic attenuation. The results are reported as number-average effective particle diameters. Samples were measured within 2 hours of preparation. Within this timeframe, stable size and ζ-potential values were recorded for TiO2 NPs coated with EFK17, EFK17-d and EFK17-W. For longer times from sample preparation and room temperature storage, aggregation of particles coated with EFK17, EFK17-d (but not with EFK17-W) starts to be observed. Such longer-term aggregation can be suppressed by storing samples at 4 °C, for longer usage. Alternatively, tip-sonication (UP50H, Hielscher Ultrasonics GmbH, Germany (50 W, 30 kHz) used in an intermittent-pulse mode (5 s), at 100% amplitude) allows for recovering efficient dispersion.

C11-BODIPY 581/591 oxidation assay

ROS generation under UV illumination was investigated as described previously.20 C11-BODIPY 581/591 was incorporated into PC, +Chol and +PG LUV by adding 0.5 mol% of the probe prior to lipid film drying, keeping it under an Ar atmosphere. After vesicle formation, LUVs were illuminated by UV (Spectroline ENF-260C, 254 nm; 3 mW cm−2, sample-lamp distance ~6 cm) in the presence peptide-coated TiO2 NPs. Fluorescence spectra (λex = 485 nm; λem = 500–700 nm) were acquired using a Cary Eclipse fluorescence spectrophotometer with a Xe pulse lamp (Agilent Technologies, USA). Oxidation was quantified from the spectral shift of the probe emission. All measurements were performed in triplicate at 25 °C.

Quartz crystal microbalance with dissipation monitoring (QCM-d)

QCM-d measurements were performed using a QSense analyzer with UV-transparent sapphire window modules (Biolin Scientific, Sweden). Cells and tubing were cleaned by bath sonication in a 2% Hellmanex solution, followed by MilliQ water and ethanol. SiO2 surfaces (QSense QSX 303 SiO2, 4.95 ± 0.05 MHz) were washed in Hellmanex 2%, followed by MilliQ water and ethanol, and dried with N2. Then they were plasma cleaned for 10 minutes (Model PDC-32G, Harrick Plasma, USA) and were
mounted into the measurement cells without further modification. A liquid flow of 0.1 mL min\(^{-1}\) was employed throughout the experiments. Lipid bilayers were prepared by SUV deposition on SiO\(_2\) surfaces as previously described.\(^{20}\) In brief, SUVs (0.1 mg mL\(^{-1}\) in MQ) were injected using a peristaltic pump, followed by rinsing with MilliQ to remove excess non-adsorbed vesicles. Full vesicle rupture and bilayer formation were confirmed by changes in frequency (\(\Delta f\)) of \(-23\) Hz and dissipation (\(\Delta \delta\)) of \(-0.2 \times 10^{-6}\). Then, the bilayer was rinsed with buffer (10 mM Acetate, pH 5.4) and peptide-coated TiO\(_2\) NPs were subsequently added. Right after, the buffer was flushed for 10 minutes to remove NP excess. Finally, buffer flow was stopped and samples were illuminated by UV light (Spectroline lamp ENF-260C, 6 W, 254 nm; 3 mW cm\(^{-2}\), placed at \(-2\) cm from the QCM-d cell) for 2 h. Measurements were performed at 25 °C in triplicate.

**Circular dichroism (CD)**

CD spectra of 100 ppm peptide-coated TiO\(_2\) NPs (at 50 µM peptide concentration) in 10 mM acetate (pH 5.4) were recorded in the absence and in the presence of PC, +Chol and +PG unilamellar vesicles (100 µM lipid). The CD spectra for such samples contain contributions from both free peptides and peptides bound to the TiO\(_2\) NPs. To reduce contributions from free peptide, compositions were selected to correspond to contain the lowest possible amounts of peptide that display efficient NP dispersion. Spectra of free peptides in the same buffer were also collected as control samples. The CD signal of each sample was recorded in 1 mm path length quartz cuvettes (110-QS; Hellma, Mullheim, Germany) using a JASCO (Tokyo, Japan) J-715 CD instrument, working with 20 nm min\(^{-1}\) scanning speed, 2 s response time, and 5 mm bandwidth. The signal was collected in the 200–260 nm wavelength range as an average of 10 accumulations. The measurements were performed at 20 °C in triplicate. The mean residual ellipticity (MRE) for each sample was then obtained after subtracting the background (10 mM acetate, pH 5.4). CD spectra were subsequently analyzed using software BESStSel\(^{21}\) for quantification of \(\alpha\)-helix content.

**Neutron reflectometry (NR)**

NR experiments on supported +PG and PC bilayers interacting with EFK17-W-coated TiO\(_2\) NPs were performed on the D17 vertical reflectometer (Institute Laue-Langevin, Grenoble, France), operating in the time-of-flight mode.\(^{22,23}\) The Q-region of interest (0.007 to 0.3 Å\(^{-1}\)) was accessed using two incident angles (0.8° and 3.0°), using the wavelength range between 2 and 30 Å and wavelength resolution between 1 and 4%. The reflected intensity was acquired as a function of the momentum transfer \(q_s = (4\pi/\lambda) \sin(\delta)\), where \(\lambda\) is the wavelength and \(\delta\) is the incident angle. Solid-liquid flow cells with a top plate characterized by a 40 mm diameter circular opening were employed in combination with UV-transparent quartz blocks (80 × 50 × 15 mm, 1 face polished, RMS < 4.5 Å, PI-KEM Ltd, Tamworth, UK) to allow in situ UV irradiation. HPLC tubing, PEEK troughs, and O-rings were cleaned with a 2% Hellmanex (Hellma Analytics, UK) solution in MilliQ under bath sonication, then thoroughly rinsed and sonicated in MilliQ. Cells were connected to a circulating water bath to keep them at a constant temperature of 25 °C during the measurements. Before mounting them into the liquid flow cells, NR blocks were cleaned in piranha solution (5/4 H\(_2\)O/H\(_2\)SO\(_4\)/H\(_2\)O) at 80 °C for 15 minutes (Caution: Piranha solution is highly corrosive), rinsed in MilliQ and cleaned through plasma cleaning for 15 minutes (Harrick Plasma PDC-002, USA). After that, blocks were mounted in the solid-liquid flow cells and characterized in D\(_2\)O and MilliQ H\(_2\)O. Then, PC or +PG SUV dispersions (0.1 mg mL\(^{-1}\)) were injected manually (right after tip-sonication) and allowed to deposit for 20 min. Excess SUV was subsequently rinsed off with 20 mL MilliQ water at 2 mL min\(^{-1}\), followed by buffer (10 mM acetate, pH 5.4) rinsing at 2 mL min\(^{-1}\). The bilayers thus formed were characterized in three contrasts, i.e., d-, qm- and h-buffer, corresponding to 10 mM acetate buffer in D\(_2\)O, 68.6/31.4% v/v D\(_2\)O/H\(_2\)O mixture (with the same scattering length density (SLD) as quartz), and H\(_2\)O, respectively. Contrasts were exchanged by pumping 20 mL of the desired buffer at 2 mL min\(^{-1}\).

Subsequently, EFK17-W-TiO\(_2\) NPs (20 ppm) in h-buffer were injected manually and incubated for 10 minutes with the bilayer. Following this, h-buffer was flushed to remove excess NPs and the reflectivity was acquired in h-, qm- and d-buffers. The bilayers were then subjected to in situ UV irradiation (Spectroline lamp ENF-260C, 6 W, 254 nm; 3 mW cm\(^{-2}\)) for 2 h, with the UV lamp placed at \(-2\) cm from the NR cell. After UV exposure, reflectivity curves were measured in d, qm- and h-buffers. Quantitative information about the scattering length density profile of the sample normal to the interface can be obtained through data fitting. Experimental NR profiles were fitted by using the genetic optimization method using the analysis package Motofit, available within the software IGOR Pro.\(^{24,25}\) A series of parallel layers were used to model the interfacial structure, each of these described by thickness, roughness, hydration, and SLD. Monte Carlo error analysis allowing for refitting data 100 times was employed to quantify the uncertainty associated with data fitting.\(^{26}\)

**Live/dead bacterial viability assay**

\(E.\ coli\) ATCC 25922 were stained using the live/dead BacLight Bacterial Viability Kit (Thermo Fisher Scientific Inc., Waltham, USA). Bacteria were grown to the stationary phase in 25 mL Lennox broth (LB Broth; Sigma Aldrich [St. Luis, USA]) under shaking at 180 rpm overnight at room temperature. After that, bacteria were pelleted and washed by centrifugation (10 000 \(g\), 10 min, twice) and finally re-suspended in 10 mM acetate, pH 5.4. Nanoparticle dispersions (500 µL), either bare or coated with EFK17, EFK17-d or EFK17-W were added to 500 µL of bacteria suspensions. NPs had a 100 ppm concentration in the final mixture, while the optical density OD600 of the bacterial dispersion was 1.2, corresponding to \(12 \times 10^8\) \(E.\ coli\) colony forming units (CFU) mL\(^{-1}\) (confirmed on subsequent dilution, plating, culture, and counting of colonies). Samples were transferred to quartz cuvettes and incubated for 1 h at 25 °C, either in darkness or under UV illumination (Spectroline ENF-260C, 254 nm; 3 mW cm\(^{-2}\)) applied at a cuvette-lamp distance of 6 cm. Samples were subsequently diluted in the buffer to obtain an OD600 of 0.6, corresponding to \(6 \times 10^8\) CFU mL\(^{-1}\)
E. coli. Right after, 200 µL of samples were mixed with 0.5 µL of a 1/1 (v/v) mixture of the fluorescent probes SYTO 9 (excitation/emission maxima 480/500 nm) and propidium iodide (excitation/emission maxima 490/635 nm). After 10 minutes incubation, samples were imaged by confocal microscopy. Bacteria in the absence or in the presence of NPs were plated onto a cover slide at 108 CFU mL⁻¹ and then imaged with a 100×/1.25× oil objective using a Leica DMi8 confocal microscope (Leica Microsystems, Washington, DC, USA). For each sample, 20 randomized, wide-field images (100 µm × 100 µm) were collected. Quantification of the fraction of live/dead bacteria was performed using the software ImageJ (National Institutes of Health, Bethesda, USA). Experiments were performed in triplicate at 25 °C.

Results

Properties of peptide-TiO₂ composite nanoparticles

With an isoelectric point of 6–6.5, TiO₂ NPs only carry a modest positive charge (+5 ± 1 mV) at pH 5.4 and are therefore, collooidally unstable at this pH, as seen from very large effective particle sizes (Fig. 1A). For EFK17-W, the concentration-dependent peptide binding to the TiO₂ NPs is clearly shown from the positive ζ-potential increase, reaching +24 ± 1 mV at the highest peptide concentration investigated. As a result of the build-up of the positive ζ-potential, the TiO₂ NPs are colloidal stabilized on the peptide coating, seen from a much smaller average particle size of 147 ± 16 nm at peptide concentrations of 50 mM and above. Keeping in mind that the primary particle size for the fully dispersed TiO₂ NPs is 2–8 nm, each composite NP contains multiple TiO₂ NPs which have an outer region dominated by the peptide. While a minor increase in positive ζ-potential and a minor decrease in effective particle diameter is observed as a function of EFK17-d and EFK17-d concentrations, the binding of these peptides to the TiO₂ NPs is too weak to allow colloidal stabilization of the aggregating TiO₂ NPs.

Since peptides will ultimately degrade when exposed to ROS,¹² the susceptibility of the systems to photocatalytic degradation was investigated by monitoring the ζ-potential as a function of UV exposure. As seen in Fig. 1B, all systems displayed a minor decrease in the positive ζ-potential with increasing UV exposure time, indicating that peptide oxidation occurred. Quantitatively, however, this decrease in ζ-potential was relatively minor, suggesting that the peptide coatings are relatively robust to photocatalytic degradation over the 2 h exposure time investigated. While the AMP coatings on the TiO₂ NPs are essential for enhanced and controlled NP binding to bacterial membranes, it is possible that these coatings prevent UV-induced free electrons and holes generated during UV illumination from reaching the aqueous solution for ROS generation.

Addressing this, we investigated how the peptide coating affected ROS generation.

As seen in Fig. 2 and Fig. S1 (ESI†), free TiO₂ displays slightly higher ROS generation than either EFK17-TiO₂, EFK17-d-TiO₂, or EFK17-W-TiO₂. These minor differences could be ascribed to ROS suppression caused by the peptide shell, as well as to...
differences in the size and surface charge of bare and peptide-coated TiO₂ (NPs). Quantitatively, however, the difference is very small, confirming that the peptide coatings have a minimal influence on ROS generation. Next, peptide conformational aspects were investigated by CD (Fig. 3A–D).

In line with previous results, EFK17 (Fig. 3A) and EFK17-W (Fig. 3C) display disordered conformation in solution but pronounced helix formation after binding to anionic +PG LUVs. In contrast, EFK17-d (Fig. 3B) is unable to form the amphiphilic helix even in the presence of +PG LUVs. The latter applies also to EFK17-d in the presence of TiO₂ NPs and in the simultaneous presence of TiO₂ NPs and +PG LUVs. For both EFK17 and EFK17-W, on the other hand, the presence of either TiO₂ NPs or simultaneous presence of TiO₂ NP and +PG LUVs induce an increase in the helical conformation compared to the free peptide. Similar results were found for PC and +Chol LUVs (Fig. S2 and S3, ESI†).

UV-induced degradation of model membranes

The findings that relatively high peptide concentrations were needed to disperse TiO₂ NPs by EFK17-W, and that neither EFK17 nor EFK17-d was able to stabilize the TiO₂ NPs even at high peptide concentrations, suggest peptide-binding to the TiO₂ NPs was relatively weak. As a consequence, there is a risk of a fraction of the peptide added to TiO₂ NP dispersions being present as free peptides in solution. Considering this, the effects of free peptides on supported +PG bilayers were investigated (Fig. S4, ESI†). From QCM-d experiments, it was revealed that at a concentration <1.5 μM free peptides adsorb to the +PG bilayer (the extent of which depends on the peptide), without inducing any significant bilayer removal. Considering this, we selected an NP concentration of 5 ppm for the QCM-d experiments, corresponding to 2.5 μM peptide concentration, hence eliminating any effect of free peptide on membrane destabilization.

Results displayed in Fig. 4A–C show the photocatalytic effects of 5 ppm TiO₂ NPs coated with either EFK17 (A), EFK17-d (B), or EFK17-W (C), while corresponding QCM-d kinetics are reported in Fig. S3–S7 (ESI†). As shown here, EFK17-W-TiO₂ displays potent photocatalytic degradation of bacteria-like +PG bilayers, whereas that of mammalian-like +Chol bilayers were much less degraded. EFK17-TiO₂ displays a similar dependence on the bilayer composition, although quantitatively, photocatalytic effects are significantly smaller than those of EFK17-W-TiO₂.

EFK17-d-TiO₂, finally, displays the smallest photocatalytic degradation, with results for +PG being essentially at the same level as bilayers in the absence of NPs or in the presence of bare TiO₂ NPs (Fig. S9, ESI†). Furthermore, Fig. 4D shows concentration effects for EFK17-W-TiO₂, with corresponding kinetics shown in Fig. S8 (ESI†). Altogether, these results show that (i) bare TiO₂ NPs (i.e., zero peptide concentration) display very modest photocatalytic degradation (Fig. S9, ESI†), and (ii) photocatalytic degradation of +PG by EFK17-W-TiO₂ increases with the concentration of peptide-coated NPs at low particle concentrations, after which the effects saturate (Fig. 4D).
As discussed below, the latter is likely an effect of diffuse light scattering at high NP concentrations in the bilayer.

**Structural aspects of lipid membrane degradation**

To obtain structural information of UV-induced bilayer degradation, NR was performed for EFK17-W-TiO$_2$ NPs interacting with PC and +PG bilayers. Experiments were performed in 10 mM acetate buffer at pH 5.4, in three different contrasts, i.e., h-buffer, qm-buffer and d-buffer. NR profiles with best curve fits and corresponding SLD for bilayers before and after the addition of 20 ppm EFK17-W-TiO$_2$ NPs, as well as after 2 h of UV illumination, are shown in Fig. S10 (for the PC bilayer) and Fig. S11 (ESI†) (for the +PG bilayer). NR profiles acquired in different contrasts were fitted simultaneously to a 4-layer model (Scheme S1, ESI†), with input parameters summarized in Tables S1 and S2 (ESI†). To reduce the number of free parameters, the bilayer headgroup thickness was fixed at 7.5 Å.34 PC and +PG bilayers before EFK17-W-TiO$_2$ NP addition were characterized by >99% coverage and ~0% tail hydration (Table S2, ESI†). Values of area per molecule (APM), headgroup hydration, bilayer thickness and surface coverage ($f$) were calculated as described previously20 and are consistent with the previous literature35,36 (Table S2, ESI†). Key structural parameters obtained from NR fits are shown in Fig. 5 and listed in Table S2 (ESI†).

With the exception of a modest decrease in the bilayer thickness (from 43 ± 1 Å before NP addition, to 39 ± 1 Å after NP addition, and to 37 ± 1 Å after UV illumination), no significant structural modification was detected for the PC bilayer upon EFK17-W-TiO$_2$ NP addition and UV illumination. Similarly, small structural changes were observed for +PG upon incubation with EFK17-W-TiO$_2$ in the absence of UV illumination, mainly consisting of a decrease in the bilayer thickness from 44 ± 1 Å to 37 ± 1 Å. In contrast, pronounced +PG destabilization was detected upon UV illumination. Specifically, a significant increase in the hydration of the acyl chains was observed (from 0.30 ± 0.01% to 34.0 ± 0.1%), mirrored by an overall increase of the area per molecule from 72 ± 4 Å$^2$ to 96 ± 4 Å$^2$. Similar increases in the hydration of bilayer tails and headgroups were previously observed for phosphatidylcholine membranes interacting with polystyrene nanoparticles bearing a soft protein corona shell.37 In addition, the bilayer thickness decreased from 37 ± 1 Å (before UV) to 34 ± 1 Å (after UV), paralleled by a significant lipid removal ($f$ decreasing from 3.50 ± 0.05 mg m$^{-2}$ to 2.60 ± 0.05 mg m$^{-2}$).

**Antibacterial effects**

To investigate how results obtained in the bacteria-like model membranes correlated with antibacterial effects, we employed live/dead assay in combination with confocal microscopy. As presented in Fig. 6, both EFK17-TiO$_2$ and EFK17-W-TiO$_2$ killed bacteria already in darkness, mirroring the antimicrobial effect of free peptides.11 In contrast, EFK17-d-TiO$_2$ displayed limited antimicrobial effect in darkness, again mirroring the effects of the free peptide in solution.11 On UV exposure, some bacterial killing was observed under the conditions employed also for the bacterial suspension alone, reflecting the antimicrobial activity of UV illumination in the absence of TiO$_2$ NPs. In addition to this effect, however, the peptide-TiO$_2$ hybrid particles displayed boosted photocatalytic antimicrobial effects, particularly so for EFK17-W-TiO$_2$ and EFK17-TiO$_2$, whereas the antimicrobial effect under UV illumination for EFK17-d-TiO$_2$ was much smaller, and comparable to that in the absence of TiO$_2$ NPs.

**Discussion**

It has been well established in the literature that the affinity of various types of NPs for bacterial membranes can be increased by cationic surface modifications,32 a consequence of bacterial membranes being rich in anionic phospholipids and lipopolysaccharides.15,16 At the same time, however, cationic NPs are frequently toxic to human cells.33,38,39 Considering that AMPs can be employed to achieve cationic surface modification, while also displaying selectivity between bacteria and human cells,5,40 these have recently attracted interest as nanomaterial coatings in combatting infection.40–43 Although such coatings may potentially be interesting for photocatalytic NPs,3,41 the situation is more complex compared to non-photocatalytic NPs. For example, dense cationic coatings could prevent light-generated free electrons and holes from reaching the aqueous solution surrounding the NPs, therefore being unable to react with water, dissolved oxygen, and oxygen-containing solutes to form ROS. This should also apply to physisorbed peptide-based coatings, since the desorption of peptides is much slower than the ROS lifetime.41,45 The absence of suppressed ROS generation in the present study indicates, however, that the surface coatings in the presently investigated systems including the EFK17-W layers, are...
diffusion length required for generated ROS to reach their
with UV illumination. This effect is supported by the short
membrane disruption in darkness, which is strongly enhanced
charged bacterial membranes. In turn, this results in bacterial
2 h, they may bind extensively and preferentially to negatively
remain positively charged during UV illumination for at least
coated with the AMP LL-37. Since the peptide-coated TiO2 NPs
however, oxidative degradation is minor for at least 2 h of UV
exposure. As shown by the modest change in
peptide coating will ultimately degrade with extended ROS
peptide-based surface coatings of photocatalytic NPs is that the
oxidative degradation of organic toxins, as well as for photolytic antibacterial
effects have been reported previously in photocatalytic degra-
ation, it has been suggested that anionic phospholipids undergo
oxidative degradation differently as well. Irrespectively, the
dependence on lipid membrane composition was similar in that
previous study (controlling peptide amphiphilicity by hydrophobic
end-tags) to that observed in the present study (employing con-
formationally controlled peptide amphiphilicity).

An interesting feature of the present study is the dependence of
photocatalytic degradation on nanoparticle concentration,
showing a pronounced increase as a function of nanoparticle
concentration before reaching a plateau (Fig. 4D). A similar
effect was previously found for TiO2 NPs coated with AMP
WWWKYE21 and is likely due to diffuse light scattering by
membrane-bound NPs deflecting some of the light, therefore
resulting in less efficient ROS generation per particle at high
local particle concentrations in the lipid bilayer. Similar
effects have been reported previously in photocatalytic degra-
dation of organic toxins, as well as for photolytic antibacterial
effects. This concentration dependence indicates that there is
a limit for how far binding of peptide-coated photocatalytic
NPs can be used to boost photocatalytic degradation.

Conclusions
Conformationally controlled amphiphilicity of antimicrobial
peptides affects membrane interactions and antimicrobial effects
of peptide-coated photocatalytic TiO2 NPs, as summarized in
Fig. 7. Specifically, we have demonstrated that coating TiO2 NPs
with the antimicrobial peptides EFK17, EFK17-d and EFK17-W
does not significantly suppress ROS generation (Fig. 7A, left panel).
Moreover, the peptide coatings display good stability towards UV-induced oxidative degradation (Fig. 7A, right panel).
Remarkably, TiO2 NPs coated with EFK17 and EFK17-W
toxicity against human cells low, it remains unclear to what
extent the properties of peptides in solution can be used as a
guide to optimize this performance. In a first investigation into
this, we recently studied the effects of hydrophobic end-tags
on membrane interactions and antimicrobial effects of AMP-
coated TiO2 NPs. By comparing the peptides KYE21 and
WWWKYE21, for which the W-tag was previously demonstrated
to strongly boost antimicrobial effects, it was found that
TiO2 NPs coated with these peptides behave similarly, although
WWWKYE21-TiO2 binds much more extensively than KYE21-TiO2
to bacteria-like +PG bilayers. In addition, WWWKYE21-TiO2
displayed similar membrane composition dependence as free
WWWKYE21, binding more extensively to bacteria-like +PG
bilayers than to mammalian-like +Chol bilayers. Since the select-
vity between bacterial and mammalian cell membranes for the
free peptides relates to effects of the membrane composition
(anionic lipid and cholesterol content), these results suggest
that the W-residues in WWWKYE21 remain sufficiently exposed
and mobile in WWWKYE21-TiO2 to be able to interact with the
lipid bilayer. Having said that, other mechanisms may contrib-
ute to membrane composition selectivity. For example, anionic
phospholipids generally pack less densely than zwitterionic
options, which may facilitate particle insertion. In addi-
Fig. 6 (A) Representative confocal microscopy images obtained using live/dead assay (red, green, and Differential Interference Contrast images
overlaid) for 10^7 CFU mL^-1 of E. coli bacteria in 10 mM acetate, pH 5.4,
without or with 1 h of incubation with bare TiO2, EFK17-TiO2, EFK17-d-
TiO2 or EFK17-W-TiO2 with or without in situ UV illumination. For each
system, live (green) and dead (red) bacteria are shown in separate images
(left images for alive bacteria and right images for dead ones). (B) Quantifi-
cation of confocal microscopy images, showing percentages of dead
E. coli bacteria with or without UV illumination. Results are shown for
bacteria alone and for bacteria exposed for 1 h to bare TiO2, EFK17-TiO2,
EFK17-d-TiO2 or EFK17-W-TiO2 in 10 mM acetate, pH 5.4, with or without
UV illumination (n = 3).
sufficiently “open” to allow free electrons and holes to form
ROS at the TiO2 NP surface. This is consistent with the high-
water content observed for adsorbed polypeptide and protein
layers, as well as our previous findings relating to TiO2 NPs
coated with the AMP LL-37. Another potential challenge with
peptide-based surface coatings of photocatalytic NPs is that the
peptide coating will ultimately degrade with extended ROS
exposure. As shown by the modest change in ζ-potential of the
peptide-coated NPs upon UV exposure in the present study,
however, oxidative degradation is minor for at least 2 h of UV
exposure. This is in line with our previous findings for TiO2 NPs
coated with the AMP LL-37. Since the peptide-coated TiO2 NPs
remain positively charged during UV illumination for at least
2 h, they may bind extensively and preferentially to negatively
charged bacterial membranes. In turn, this results in bacterial
membrane disruption in darkness, which is strongly enhanced
with UV illumination. This effect is supported by the short
diffusion length required for generated ROS to reach their
membrane target.

While we previously demonstrated that AMP-coating of TiO2
NPs provides a way to boost antimicrobial effects while keeping
peptides, both displaying amphiphilic helix formation on bacterial membrane interaction (Fig. 7B), promoted higher degradation of bacteria-mimicking +PG bilayers compared to bare TiO₂ NPs. These effects were further enhanced with UV illumination due to the production of ROS adjacent to the membrane surface. (Fig. 7C). In contrast, TiO₂ NPs coated with EFK17-d, which did not form an amphiphilic helix upon interaction with bacteria-like +PG vesicles, induced significantly smaller membrane degradation (Fig. 7C). Similar to the behaviour of the free peptides, effects of EFK17-TiO₂ and EFK17-W-TiO₂ were significantly smaller on mammalian-like PC bilayers compared to bacteria-like +PG bilayers (Fig. 7D). Specifically, NR results revealed negligible structural changes of PC bilayers upon interaction with EFK17-W-TiO₂ NPs and subsequent UV illumination. In contrast, massive membrane destabilization was observed for +PG bilayers exposed to the same conditions, involving decreased membrane thickness and increased area per molecule, as well as the hydration of both headgroup and acyl chain regions (Fig. 7D). Finally, the effects of the peptide-coated NPs on E. coli bacteria showed both EFK17-TiO₂ and EFK17-W-TiO₂ to display pronounced antimicrobial effects, which could be further enhanced through ROS formation via UV illumination (Fig. 7E). Significantly smaller antimicrobial effects were observed for EFK17-W-TiO₂ or bare TiO₂ NPs (Fig. 7E). Taken together, the results from the study demonstrates that conformationally controlled AMP amphiphilicity, which dictates membrane interactions and antimicrobial effects of free AMPs in solution, apply also to peptide-coated photocatalytic NPs. The results also define a limit for such effects at high local membrane concentrations of NPs.

Author contributions

LC and MM designed the study. All authors were involved in performing the experiments and the interpretation of the experimental results. LC and MM wrote the paper. All authors reviewed the paper.

Conflicts of interest

There are no conflicts to declare.

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