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Membrane interactions and antimicrobial effects of layered double hydroxide nanoparticles

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Abstract

Membrane interactions are critical for the successful use of inorganic nanoparticles as antimicrobial agents and as carriers of, or co-actives with, antimicrobial peptides (AMPs). In order to contribute to [an increased understanding of these](#), we here investigate effects of particle size (42-208 nm) on layered double hydroxide (LDH) interactions with both bacteria-mimicking and mammalian-mimicking lipid membranes. LDH binding to bacteria-mimicking membranes, extraction of anionic lipids, as well as resulting membrane destabilization, was found to increase with decreasing particle size, also translating into size-dependent synergistic effects with the antimicrobial peptide LL-37. Due to strong interactions with anionic lipopolysaccharide and peptidoglycan layers, direct membrane disruption of both Gram-negative and Gram-positive bacteria is suppressed. However, LDH nanoparticles cause size-dependent charge reversal and resulting flocculation of both liposomes and bacteria, which may provide a mechanism for bacterial confinement or clearance. Taken together, these findings demonstrate a set of previously unknown behaviors, including synergistic membrane destabilization and dual confinement/killing of bacteria through combined LDH/AMP exposure, of potential therapeutic interest.

Introduction

Driven by increasing challenges to reach efficacy and safety for “difficult” drugs, such as proteins and peptides, the scope of drug delivery research has broadened considerably during the last decade or so, to include not only “traditional” drug delivery systems, such as lipids, surfactants, and polymers, but also a wide range of inorganic nanomaterials (1-4). In addition, such nanomaterials are attracting considerable current interest as novel antimicrobial agents, not the least due to increasing resistance against conventional antibiotics, such as tetracycline, β -lactam, aminoglycoside, macrolide, and quinolone antibiotics (5-10). Apart from scalability and low cost, such materials offer advantages related to presently undeveloped bacterial resistance, as well as possibilities of responsiveness of antimicrobial and other effects, controlled by a range of triggering factors (e.g., light and magnetic fields).

Numerous types of inorganic nanoparticles are of potential interest in this context, including metal nanoparticles, metal oxides and quantum dots, iron oxides and other magnetic nanoparticles, mesoporous silica, nanoclays, and carbon-based nanomaterials (7-10). Of these, the present investigation focuses on layered double hydroxide (LDH) nanoparticles, which are nanoclays formed by positively charged layers with charge-balancing anions between them (11-13). Due to dissociation under acidic conditions, as well as a considerable anionic exchange capacity, LDH nanoparticles are interesting for pH-sensitive delivery of both biomacromolecular and low-Mw antimicrobial agents. An interesting question in this context is what factors determine membrane interactions of LDH nanoparticles, as well as the performance of such nanoparticles (and other nanoclays) as drug carriers of, or co-actives with, antimicrobial peptides (AMPs) (14,15), the latter causing bacterial membrane destabilization, resulting in fast and broad-spectrum antimicrobial effects (16). Combination therapies based on AMPs and inorganic nanoparticles may be of interest, e.g., in infections characterized by high proteolytic activity, mediated both by bacterial proteases and proteases of human inflammatory cells (17,18), either through AMP incorporation into the nanoparticles, as previously demonstrated for mesoporous silica nanoparticles (19,20), or through nanoparticle-facilitated membrane activity of partially degraded AMPs. Co-administration with nanoparticulate delivery systems may also reduce AMP binding to serum proteins (21,22), thus contributing to increased bloodstream circulation, or facilitate intracellular uptake, of interest, e.g., for tuberculosis (23).

Although there has been little work done on membrane interactions and antimicrobial effects of LDH nanoparticles, a few relevant studies on related systems and research issues can be noted. Thus, addressing the role of surface modification of nanoclays on their antimicrobial properties, Wu et al. investigated the antimicrobial properties of anionic clay minerals intercalated by cationic tetradecyl tributyl phosphonium bromide (TDTB). The antimicrobial activities of these materials against *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) were found to depend on several factors, including the amount TDTB released and the size and z-potential of the loaded nanoparticles, higher antimicrobial activity observed at higher amount TDTB released and higher positive z-potential (24). Similarly, Wang et al. modified anionic montmorillonite with cationic C18 tallow alkyl amine surfactants and found the modified nanoclays to display good antimicrobial activities against *S. aureus* and *E. coli*, as well as low cytotoxicity (25). Furthermore, Hesse et al. investigated LDH as carrier for ciprofloxacin for the treatment of chronic otitis caused by *Pseudomonas aeruginosa* (*P. aeruginosa*) (26). Male New Zealand White rabbits were implanted with middle ear prostheses coated with ciprofloxacin-loaded LDH. Clinical outcome, blood counts, histological analyses, and microbiological examination showed excellent antimicrobial activity for the group microbially challenged directly after implantation. Although the mechanisms underlying these effects were not investigated in these previous studies, these prior results seem to suggest that the antimicrobial activity observed is due to released cationic antimicrobial guest molecules, rather than to the loaded clay nanoparticles themselves. Furthermore, demonstrating the strength of the LDH interactions with bacterial lipopolysaccharides, Halma et al. investigated layer-by-layer films formed by sequential adsorption of LDH nanoparticles and *P. aeruginosa*, and found the attraction between these components to be sufficiently strong to form extended and stable multilayers (27).

In contrast to limited prior work on their antimicrobial effects, LDH nanoparticles have attracted interest as delivery systems for DNA and siRNA (11). For example, Xu et al. investigated LDH-mediated DNA delivery, and found transfection efficiency in HEK 293T cells to increase with increased DNA loading. Quantitatively, however, delivery efficiency was low, 7%–15% of FuGENE[®], which was speculated to be due to LDH aggregation caused by the long-chain plasmid DNA (28). Furthermore, Wong et al. found LDH-mediated delivery of siRNA into cortical neurons and NIH 3T3 cells to vary widely (6-80% and 2–11%, respectively), inferred to be at least partly due to variability of the degree of oligonucleotide intercalation (29). Addressing the issue of limited intercalation, Chen et al. prepared smaller

LDHs (≈ 45 nm) and found both dsDNA and siRNA to be more effectively intercalated into these small LDH nanoparticles (12).

The latter findings led us to speculate that membrane interactions and antimicrobial effects of LDH nanoparticles may be enhanced by decreasing their size, both on their own and in the simultaneous presence of AMPs. Addressing this issue, we here employ a battery of physicochemical methodologies to investigate the effects of LDH nanoparticle size on their interaction with both bacteria-mimicking and mammalian-mimicking membranes, as well as consequences of this for antimicrobial effects and cell toxicity. Furthermore, membrane interactions of mixed systems of LDH with the benchmark AMP LL-37 were addressed, and correlated to peptide-LDH interactions.

Experimental

Chemicals. LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) was synthesized by Biopeptide Co. (San Diego, USA), and was of >95% purity, as evidenced by mass spectral analysis (MALDI-TOF Voyager). DOPG (1,2-dioleoyl-*sn*-Glycero-3-phosphoglycerol, monosodium salt), DOPE (1,2-dioleoyl-*sn*-Glycero-3-phosphoethanolamine), DOPC (1,2-dioleoyl-*sn*-Glycero-3-phosphocholine), and NBD-DOPG (1-oleoyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl}-*sn*-glycero-3-[phospho-*rac*-(1 glycerol)] (ammonium salt), were from Avanti Polar Lipids (Alabaster, USA) and of >99% purity, while cholesterol (>99%) was from Sigma (St. Louis, USA). All other chemicals used were of analytical grade.

Microorganisms. The bacterial isolates *Escherichia coli* (*E. coli*) ATCC 25299 and *Staphylococcus aureus* (*S. aureus*) ATCC 29213 were obtained from the American Type Culture Collection.

LDH nanoparticle synthesis. Well-dispersed LDH nanoparticles were synthesized according to the method published by Xu et al. (30,31). Briefly, a mixed salt solution (10 mL) containing 0.3 M MgCl₂ and 0.1 M AlCl₃ (Sigma-Aldrich, ≥ 99.0 %) was quickly added to 30 mL of 0.20 M NaOH solution under vigorous stirring. After stirring for 10 min, LDH precipitate was collected and washed twice via centrifugation. Then, the collected LDH precipitate was re-suspended in 40 mL of water, which was transferred to a stainless steel

autoclave with a Teflon lining. Heating in an oven at 100°C for 6 and 48 h resulted in a homogeneous LDH nanoparticle suspension with the average size of ≈ 104 (LDH 104) and 208 nm (LDH 208), respectively. To prepare even smaller LDH nanoparticles (≈ 42 nm; LDH 42), a non-aqueous precipitation method was used (32). Briefly, 10 mL methanol (Fluka, $\geq 99.0\%$) solution containing 0.6 M $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ and 0.2 M $\text{Al}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ (Fluka, $\geq 99.0\%$) was added drop-wise to a 40 mL of methanol solution containing 0.4 M NaOH (Fluka, $\geq 97.0\%$, pellets) under vigorous stirring. The precipitate slurry was collected via centrifugation, then re-dispersed in 40 mL fresh methanol for heat-treatment at 100 °C for 4 h. The further collected LDH slurry was washed twice with water and then manually dispersed in 40 mL of water, resulting in a homogeneous LDH suspension after 4-6 days. Throughout, LDH nanoparticle concentrations used are given in mass-based parts-per-million (ppm).

LL-37 binding. Peptide binding to LDH nanoparticles was measured using a solution depletion method, as previously described (33). In brief, peptide solutions of varying concentration in 10 mM Tris, pH 7.4, were mixed with 25 μL of LDH suspension (40 or 200 ppm) and allowed to equilibrate overnight. The complexes thus formed were separated by centrifugation at 16500 rpm for 25 min. The amount of peptides remaining in solution was determined by bicinchoninic acid assay (34), performing absorbance measurements on a Sapphire plate reader (Tecan, Männedorf, Switzerland) at $\lambda = 562$ nm, and compared to the peptide concentration in a solution without LDH nanoparticles. From this, the amount of bound peptide (g peptide/g LDH) at various equilibrium concentrations was determined.

Liposome preparation and leakage assay. Model liposomes investigated were either anionic (DOPE/DOPG 75/25 mol/mol) or zwitterionic (DOPC/cholesterol 60/40 mol/mol), frequently used as bacteria and mammalian membrane models, respectively (16). The lipid mixtures were dissolved in chloroform, after which solvent was removed by evaporation under vacuum overnight. Subsequently, 10 mM Tris buffer, pH 7.4, was added together with 0.1 M carboxyfluorescein (CF) (Sigma, St. Louis, USA). After hydration, the lipid mixture was subjected to eight freeze-thaw cycles, consisting of freezing in liquid nitrogen and heating to 60°C. Unilamellar liposomes of about $\varnothing 140$ nm were generated by multiple extrusions (30 passages) through polycarbonate filters (pore size 100 nm) mounted in a LipoFast miniextruder (Avestin, Ottawa, Canada) at 22°C. Untrapped CF was removed by two subsequent gel filtrations (Sephadex G-50, GE Healthcare, Uppsala, Sweden) at 22°C,

with Tris buffer as eluent. CF release from the liposomes was determined by monitoring the emitted fluorescence at 520 nm from a liposome dispersion (10 μ M lipid in 10 mM Tris, pH 7.4). An absolute leakage scale was obtained by disrupting the liposomes at the end of each experiment through addition of 0.8 mM Triton X-100 (Sigma-Aldrich, St. Louis, USA). A SPEX-fluorolog 1650 0.22-m double spectrometer (SPEX Industries, Edison, USA) was used for the liposome leakage assay. Measurements were performed in triplicate at 37 °C.

Ellipsometry. Nanoparticle adsorption to supported lipid bilayers was studied *in situ* by null ellipsometry, using an Optrel Multiskop (Optrel, Kleinmachnow, Germany) equipped with a 100 mW Nd:YAG laser (JDS Uniphase, Milpitas, USA). All measurements were carried out at 532 nm and an angle of incidence of 67.66° in a 5 mL cuvette under stirring (300 rpm). Both the principles of null ellipsometry and the procedures used have been described before (35,36). In brief, by monitoring the change in the state of polarization of light reflected at a surface in the absence and presence of an adsorbed layer, the mean refractive index (n) and layer thickness (d) of the adsorbed layer can be obtained. From the thickness and refractive index the adsorbed amount (Γ) was calculated according to:

$$G = \frac{(n - n_0)}{dn/dc} d \quad (1)$$

where n_0 is the refractive index of the bulk solution (1.3347), and dn/dc the refractive index increment (taken to be 0.154 cm³/g). Corrections were routinely done for changes in bulk refractive index caused by changes in temperature and excess electrolyte concentration.

Supported lipid bilayers were generated by liposome adsorption. DOPE/DOPG (75/25 mol/mol) liposomes were prepared as described above, but the dried lipid films re-suspended in Tris buffer only with no CF present. In order to avoid adsorption of peptide directly at the silica substrate (surface potential -40 mV, contact angle <10° (37)) through any defects of the supported lipid layer, poly-L-lysine (M_w = 170 kDa, Sigma-Aldrich, St. Louis, USA) was pre-adsorbed from water prior to lipid addition to an amount of 0.045 ± 0.01 mg/m², followed by removal of non-adsorbed poly-L-lysine by rinsing with water at 5 ml/min for 20 minutes (38). Water in the cuvette was then replaced by buffer containing also 150 mM NaCl, followed by addition of liposomes in buffer at a lipid concentration of 20 μ M, and subsequently by rinsing with buffer (5 ml/min for 15 minutes) when liposome adsorption had stabilized. The final

layer formed had structural characteristics (thickness 4 ± 1 nm, mean refractive index 1.47 ± 0.03), suggesting that a layer fairly close to a complete bilayer was formed. After lipid bilayer formation, the cuvette content was replaced by 10 mM Tris buffer at a rate of 5 ml/min over a period of 30 minutes. After stabilization for 40 minutes, nanoparticles were added to a concentration of 1 ppm, followed by subsequent particle additions to 2, 5, 10, 30, 50, and 100 ppm, in all cases monitoring the adsorption for one hour. All measurements were made in at least duplicate at 25°C.

Size and zeta potential measurements. Mean hydrodynamic diameter of LDH nanoparticles, DOPE/DOPG liposomes, and LDH-liposome aggregates were determined by dynamic light scattering at a scattering angle of 173° , using a Zetasizer Nano ZSP (Malvern Instruments, Malvern, UK). Measurements were performed as a function of LDH concentration in the absence or presence of a fixed liposome concentration of 10 μ M in 10 mM Tris, pH 7.4. [In the absence of liposomes, LDH nanoparticles undergo slow aggregation in 10 mM Tris, pH 7.4, but primarily so after more than 4-8 hours, during which the experiments were performed \(Figure SM1\).](#) [In the case of LDH/liposome mixtures, these](#) were incubated for 45 min before measurements were initiated. Using the same experimental setup, z-potential of LDH and LDH-liposome aggregates was measured as a function of LDH concentration, and z-potential calculated using the Smulochowski approximation. In both cases, measurements were performed in duplicate at 25 °C.

CD spectroscopy. CD spectra were measured by a Jasco J-810 Spectropolarimeter (Jasco, Easton, USA). Measurements were performed [in 10 mM Tris, pH 7.4](#) in duplicate at 37°C in a 10 mm quartz cuvette under stirring with a peptide concentration of 10 μ M. The effect on peptide secondary structure of LDH at a nanoparticle concentration of 40 ppm was monitored in the range 200-260 nm. To account for instrumental differences between measurements, background correction was performed routinely by subtraction of spectra for buffer (with or without LDH) from spectra of the corresponding samples in the presence of peptide.

Confocal microscopy. *E. coli* were grown to mid-exponential phase in 3% Tryptic Soy Broth (TSB), before washed and diluted in 10 mM Tris, pH 7.4, to a density of 10^8 cfu/mL. Detection of live and dead bacteria was done by SYTO 9 and propidium iodide dye, staining intact and compromised bacterial membranes, respectively. For this, 1.5 μ L of a 1:1 mixture of SYTO 9 and propidium iodide dye (LIVE/DEAD® BacLight™ Bacterial Viability Kits

L7012, ThermoFischer Scientific, Waltham, USA) was added to 500 μ L of sample. After incubation at room temperature for 15 minutes in 10 mM Tris, pH 7.4, the bacteria suspension was plated onto a coverslip and samples imaged with a 100x/1.25 oil objective using a Confocal Leica DM IRE2 laser scanning microscope (Leica Microsystems, Wetzlar, Germany). The images were collected using Leica TCS SL software (Leica Microsystem, Wetzlar, Germany) and further analyzed using ImageJ Software.

CryoTEM. Cryogenic Transmission Electron Microscopy (cryoTEM) investigations were performed with a Zeiss EM 902A Transmission Electron Microscope (Carl Zeiss NTS, Oberkochen, Germany), operating at 80 kV and in zero loss bright-field mode. Digital images were recorded under low dose conditions with a BioVision Pro-SM Slow Scan CCD camera (Proscan GmbH, Scheuring, Germany) and analySIS software (Soft Imaging System GmbH, Münster, Germany). In order to visualize as many details as possible, an underfocus of 1-2 μ m was used to enhance the image contrast. The method for sample preparation has been thoroughly described elsewhere (39). In short, samples (in 10 mM Tris, pH 7.4) were equilibrated at 25°C and at close to 100% atmospheric humidity within a climate chamber. A small drop ($\sim$ 1 μ l) of sample was deposited on a copper grid covered with a perforated polymer film and with thin evaporated carbon layers on both sides. Excess liquid was thereafter removed by blotting with a filter paper, leaving a thin film of solution on the grid. Immediately after blotting, the sample was vitrified in liquid ethane, held just above its freezing point. Samples were kept below -165°C in a protected atmosphere during transfer and examination.

Lipid exchange. Lipid exchange from liposomes to LDH nanoparticles was investigated by contacting LDH nanoparticles with DOPE/DOPG liposomes, in which a fraction of the anionic DOPG lipids were fluorescently labeled. By monitoring the decrease in the fluorescence intensity in the liposome solution after LDH interaction, an estimate was obtained for anionic lipid bound to, or intercalated in, the LDH nanoparticles, and how this was dependent on LDH particle size. In doing so, DOPE/DOPG/NDB-DOPG liposomes (75/15/10 mol/mol/mol) were prepared as described above, but with 10% DOPG replaced by NBD-DOPG. The LDH concentration was held constant (100 ppm) while the liposome concentration was varied between 0.5-60 μ M in 10 mM Tris-HCl. Samples were left to equilibrate in at 5-8°C overnight. Thereafter, samples were centrifuged at 14000 rfc for 10 minutes at room temperature to separate LDH nanoparticles from liposomes. Fluorescence

emitted from the supernatant was determined at 534 nm using a Fluorolog-3 (Horiba Scientific, Japan). All measurements were performed with 10 mM Tris, pH 7.4, in duplicates at room temperature.

Proteolysis. Peptides (2 µg dissolved in 10 mM Tris, pH 7.4) were incubated at 37 °C with *P. aeruginosa* elastase (1.0 µg, 25000 units/mg, BioCol GmbH (Potsdam, Germany)) in a total volume of 15 µl for 16 h. The materials were analyzed on 10-20 % precast Tris-Tricine sodium dodecyl sulfate polyacrylamide (SDS-PAGE) Tris-Tricine gels (Invitrogen) and analyzed after staining with Coomassie Brilliant Blue. Quantification of band intensities, from triplicate measurements, was performed by Molecular Imager Gel DOC with Image Lab Software (BioRad, Hercules, USA).

Viable count analysis (VCA). Antimicrobial activity was assessed by viable count assay. *E. coli* ATCC 25299 or *S. aureus* ATCC 29213 were grown to mid-exponential phase in Todd-Hewitt (TH). Bacteria were then washed and diluted in 10 mM Tris, pH 7.4. Bacteria (2×10^6 cfu/mL) were incubated, at 37°C for 2 h, with peptides and particles at the indicated concentrations. Serial dilutions of the incubation mixture were plated on TH agar, followed by incubation at 37°C overnight and cfu determination (n=4-6).

Hemolysis. EDTA-blood was centrifuged at 800 g for 10 minutes, whereafter plasma and buffy coat were removed. The erythrocytes were washed three times and re-suspended in PBS, pH 7.4, to a 5% suspension. The cells were then incubated with end-over-end rotation for 1 h at 37°C in the presence of LDH (0-200 ppm). Triton X-100 (2%, Sigma-Aldrich) served as positive control. In another experiment, whole citrated blood was diluted 1:1 in PBS and incubated with the LDH nanoparticles at indicated concentrations for 1 h at 37°C. In all cases, the absorbance of hemoglobin release was measured at λ 540 nm and is expressed as % of Triton X-100-induced hemolysis (n=3).

Results

In order to investigate effects of particle size on LDH-membrane interactions, three different LDH fractions were synthesized, all displaying similar flake-like structures, similar z-potential, and similar D-spacing between the layers, but varying in average size (Figure 1, Table SM1). As seen from both cryoTEM images directly, and the relatively good agreement

between these and the results obtained from PCS, the LDH suspensions were well dispersed in aqueous solution (Figure 1, Table SM1).

Monitoring the adsorption of these LDH fractions at supported bacteria-mimicking DOPE/DOPG bilayers by ellipsometry shows that the adsorption affinity of the LDH nanoparticles depends strongly on nanoparticle size. More precisely, although the saturation adsorption is higher for LDH 104 and 208 than for LDH 42 (7.2 ± 1.7 , 6.0 ± 1.1 and 3.6 ± 0.4 mg/m^2 , respectively), adsorption affinity for LDH 42 is substantially higher than that of the larger LDH nanoparticles. Thus, as shown in Figure 2a, the smallest LDH nanoparticles display adsorption onset already at a particle concentration of about 1-2 ppm, while the adsorption of LDH 104 display pronounced adsorption onset at 5-10 ppm, and that of LDH 208 only above 50 ppm. In analogy to their ability to bind and intercalate anionic surfactants and other anionic compounds (discussed below), LDH nanoparticles are also able to extract anionic NDB-DOPG lipids from DOPE/DOPG membranes, to an extent increasing with decreasing particle size (Figure 2b). Mirroring this (although with some minor quantitative differences due to potential effects of leaflet asymmetry in the supported bilayers, packing density and curvature differences between supported bilayers and liposomes), LDH-induced leakage of DOPE/DOPG liposomes is initiated at much lower concentration for LDH 42, followed by LDH 104 and LDH 208 at increasing particle concentrations (Figure 3a). The latter was observed also for weakly negatively charged ($z \approx -10$ mV) DOPC/cholesterol liposomes (Figure SM2). Taken together, these data show that smaller LDH nanoparticles display a higher affinity for anionic lipid membranes and membrane components, translating into an increased capacity for membrane disruption.

Considering the membrane activity of both LDH and AMPs, we next investigated the properties mixed LDH/AMP system for the benchmark antimicrobial peptide LL-37 (Figure 3b). As shown in Figure 4a, solution depletion measurements indicated some, *although not very large*, adsorption of the net positively charged LL-37 to the similarly positively charged LDH nanoparticles, *suggesting binding primarily to the outer LDH nanoparticle surface. This is in line with* the lack of adsorption-induced helix induction, frequently observed for LL-37 (16) (Figure 4b), as well as from the lack of protection by LDH of LL-37 from proteolytic degradation (Figure 4c). *For the higher peptide concentrations investigated, a fraction of* LL-37 remains unbound in mixtures with the LDH nanoparticles. *As shown in* Figure 3b, the membrane disruption caused by the LL-37/LDH mixtures is substantially higher than that of

the individual components, and again dependent of LDH particle size. Thus, while the effects of LDH and LL-37 are only additive for LDH 208, pronounced synergistic membrane disruption is observed for LDH 42.

Investigating the role of electrostatics for the particle size dependence observed, z-potential measurements were performed. However, as shown in Figure 5a (left), LDH 42, 104, and 208 all display comparable positive z-potential. Clearly, therefore, the size-dependent effects observed above are not due to particle net charge differences directly, but potentially instead to particle “dipolarity” and the role of anionic lipid [extraction](#). In the presence of anionic DOPE/DOPG liposomes, the net z-potential remains low at low nanoparticle concentrations, similar to the ≈ -35 mV observed for the liposomes in the absence of LDH nanoparticles. Again mirroring nanoparticle adsorption to supported lipid bilayers, increasing nanoparticle concentrations results in charge reversal, observed at about 8, 18, and 30 ppm for LDH 42, 104, and 208, respectively (Figure 5a (right)). As shown in Figure 5b, liposome flocculation occurs as a result of the decreased z-potential of the liposome/LDH complexes. This explains the unusual concentration dependence observed in the liposome leakage experiments (Figure SM2), with pronounced maxima in fluorescence intensity. Below these maxima, leakage is induced by particle binding, anionic lipid extraction, and membrane destabilization, whereas above, LDH-induced liposome flocculation causes fluorescence intensity to decrease.

Investigating to what extent the effects observed on LDH binding to bacteria-mimicking DOPE/DOPG liposomes, as well as nanoparticle-induced liposome aggregation, translated into bacteria, a series of experiments are performed with the different size LDH nanoparticles were added to *E. coli* and *S. aureus* suspensions. For both these bacteria, the presence of 100 ppm of LDH 42, 104, or 208 had limited effect on the concentration-dependent antimicrobial effect of LL-37. Furthermore, LDH particles alone, i.e., without LL-37, displayed limited antimicrobial effect (Figure 6). The contrast between the VCA results on *E. coli* and *S. aureus* and those obtained with the DOPE/DOPG membranes, previously demonstrated in numerous studies to mirror antimicrobial effects of antimicrobial peptides well (16), indicates that the LDH nanoparticles, in contrast to AMPs, are either unable to penetrate the outer lipopolysaccharide (LPS)-rich membrane and the peptidoglycan layer of Gram-negative and Gram-positive bacteria, respectively, or that the LDH nanoparticles are passified by adsorption of released negatively charged LPS/peptidoglycan molecules/residues, in analogy

to corona formation by serum proteins from serum, and resulting suppressed hemolysis (Figure SM3).

In contrast to the suppressed membrane-disrupting antimicrobial effects, however, the liposome aggregation caused by the LDH nanoparticles at higher concentrations is maintained also for bacteria. Thus, as shown in Figure 7a and Figure SM4, *E. coli* bacteria are well dispersed in the absence of LDH particles. On addition of LDH, bacteria flock formation is observed already at a LDH concentration of 5 ppm for LDH 42, whereas no aggregation is observed for the larger LDH particles. At higher LDH concentrations (≥ 20 ppm) however, also LDH 104 and 208 induce bacteria flocculation. Therefore, corona formation by free LPS is not the reason for the limited LDH antimicrobial effects in the VCA assays, thus instead pointing to poor LDH nanoparticle penetration of the LPS layers of Gram-negative bacteria. This is supported also by viable count antimicrobial results obtained for *S. aureus*, showing the LDH/LL-37 samples to display similar antimicrobial effects as LL-37 alone (Figure 6b).

Interestingly, live-dead assay indicates that most of the bacteria (>95%) remain alive also after LDH-induced flocculation, up to a particle concentration of at least 400 ppm (Figure 7b (left) and Figure SM5), effects observed also when decreasing the bacteria concentration from 10^8 to 10^7 and 10^6 cfu/mL (results not shown). Thus, the effect of the LDH nanoparticles is mainly one of bacterial confinement. As shown in Figures 7b (right) and Figure SM5, such confinement effect by the LDH nanoparticles is readily combined with the potent membrane-destabilizing effects of LL-37, resulting in a drastic increase in the fraction of dead aggregated bacteria.

Discussion

Reports on the effect of particle size on membrane interactions, antimicrobial effects, and cell toxicity show divergent results, and seem to be strongly system-dependent. Moreover, surface properties of nanoparticles frequently depend on particle size, hence the two factors are not always separable. It is therefore difficult to draw any conclusive generalizations on the effects of particle size on membrane interactions [from present literature](#). For numerous systems, however, membrane interactions seem to become increasingly important with decreasing particle size. Thus, a wide range of studies indicate that smaller nanoparticles display more pronounced cytotoxic effects both *in vitro* and *in vivo* (40). Analogously, [bactericidal and](#)

antiviral effects of Ag nanoparticles has been observed to increase with decreasing particle size (41,42). The mechanisms underlying such biological effects remain, however, partly unclear due to the simultaneous presence of several cell internalization mechanism (e.g., passive membrane disruption and various active internalization pathways, addressing particles of specific sizes) and antimicrobial effects (e.g., membrane rupture versus ion release, oxidative stress, and other alternative pathways). However, for simpler model membrane systems, a similar increased membrane destabilization has been observed for smaller nanoparticles. For example, Bailey et al. investigated the interactions of 2, 5, 10, and 40 nm Au nanoparticles with supported lipid bilayer of L- α -phosphatidylcholine, using quartz crystal microbalance, and observed a net mass increase, but only in the case of smaller NPs (43). For the largest nanoparticles, a more complex behavior was observed, interpreted as being due to a combination of particle adsorption, lipid bilayer engulfment of the nanoparticles, and subsequent detachment from the crystal surface. Contrasting this, Alkhamash et al. investigated the interactions of silica nanospheres of different size and surface chemistry with PC membranes (44). From liposome leakage experiments, it was found that 200 and 500 nm nanoparticles caused significant membrane destabilization, while 50 nm diameter ones were considerably less efficient in doing so. It was inferred that for the smaller nanoparticles, strong silica–bilayer interactions are instead manifested as bilayer engulfment of membrane-bound particles, with localized lipid depletion, eventually leading to collapse of the vesicular membrane, but only at higher nanoparticle concentrations. Such qualitative differences in nanoparticle-liposome interactions depending on the liposome and the nanoparticles relative sizes have been discussed also by Michel et al. (45). Despite the spread and complexities outlined above, the present findings of more potent membrane destabilization of small LDH nanoparticles are in line with the broadly found higher degree of membrane interactions and cell internalization of smaller nanoparticles.

Similar to their ability to intercalate DNA and siRNA, LDH nanoparticles have been demonstrated to be able to intercalate various anionic surfactants, including alkyl carboxylates, alkyl sulfates, and alkyl sulfonates (46). In general, the hydrocarbon chains of such surfactants are closely packed either in a monolayer/interdigitated mode or in a bilayer mode, with the chains at a slanting angle with respect to the hydroxide layer. Although the bulk of work done on amphiphile uptake/intercalation by LDH has been done for single chain surfactants, LDH uptake of amphiphilic molecules is unlikely to be restricted to such compounds. Illustrating this, Filho et al. demonstrated considerable uptake of bulky cholic

acid anions into LDH nanoparticles (47). The capacity to bind anionic amphiphilic compounds both at their external surface and between layers may therefore provide LDH nanoparticles with an additional membrane destabilization mechanism, analogous to those displayed by cyclotide peptides (although for zwitterionic phosphatidylethanolamine lipids in that case) (48). Since Figure 5a shows that the z-potential of the LDH nanoparticles is essentially independent of particle size, the increased capacity of LDH nanoparticles to extract anionic NDB-DOPG lipids from DOPE/DOPG liposomes (Figure 2b) suggests that either the increased specific surface area or the increased fringe fraction with decreasing LDH particle size facilitates intercalation and binding of the anionic lipids, thus contributing to the enhanced membrane destabilization observed for LDH 42 in particular. [The relative importance of lipid extraction through binding to the external surface of these positively charged particles, and through binding between layers through intercalation, requires further investigation. For the purpose of the present investigation, however, the lipid extraction, as such, is the effect of primary importance.](#)

Key for the use of LDH nanoparticles in infection confinements and other therapeutic contexts is the issue of selectivity. For AMPs, considerable efforts have been directed to resolve this, resulting in very pronounced selectivity between bacteria and human cells in some cases (49). In comparison, membrane selectivity of bare LDH is poor, illustrated by their rupture of erythrocytes in buffer solution. In contact with blood and other biological fluids, however, positively charged nanoparticles generally bind considerable amounts of anionic proteins, effectively coating the nanoparticles and altering their biological performance (50). Also LDH nanoparticles display such protein adsorption capacity. For example, Gu et al. found saturation adsorption of bovine serum albumin by LDH to be considerable (0.7-1.27 g BSA/g LDH), thereby contributing to increased colloidal stability and improved functional performance (51). Analogously, Figure SM3 demonstrates that the presently investigated LDH nanoparticles change from being strongly positively charged (z-potential $\approx +25$ -30 mV) to being clearly negatively charged (z-potential ≈ -10 mV) on interaction with serum. As a result of this, hemolysis observed in 50% blood is considerable lower than that observed in buffer, approaching the negative control value. The latter is in line with the use of LDH nanoparticles as drug delivery systems, where limited toxicity has been observed (11).

Finally, we note that particle-mediated bacteria aggregation may provide antimicrobial effects

in ways other than by direct membrane destabilization. For example, bacteria viability in the particle-induced aggregates may be reduced after co-aggregation through restrictions in bacterial proliferation, e.g., related to mobility restrictions or to precluded nutrient/waste diffusion in compact aggregates. Furthermore, through LDH binding, the net negative charge of bacteria is reduced, which may facilitate endocytosis, in a similar way that cationic nanoparticles and other cationic compounds can facilitate endocytosis (and following transfection or gene silencing) for anionic macromolecules such as DNA and RNA (4,52). In addition to direct antimicrobial effects, endocytosis may result in reduced bloodstream circulation of bacteria through facilitated uptake in tissues related to the reticuloendothelial system, and in turn reduce also inflammatory effects related to bacteria in blood (53). While liposome flocculation in the presence of nanoparticles at intermediate coverage has been investigated previously (54), nanoparticle-induced bacteria flocculation as an antimicrobial clearing mechanism remains curiously unexplored in the literature of antimicrobial nanomaterials. This is in stark contrast to aggregation-triggered antimicrobial effects and bacterial clearance by AMPs, which has attracted considerable recent interest, and been demonstrated to represent a powerful antimicrobial mechanism for several classes of antimicrobial peptides and proteins (55-60). Clearly, therefore, and as demonstrated in the present investigation, further attention to bacterial flocculation by nanoparticles as an antimicrobial and anti-inflammatory mechanism is warranted.

Conclusions

Particle size plays a key role for membrane interactions of layered double hydroxide (LDH) nanoparticles. With decreasing LDH size, membrane binding and anionic lipid extraction is accentuated, making small LDH nanoparticles more potently membrane-disrupting than larger ones, translating into size-dependent synergistic effects with the antimicrobial peptide LL-37. Due to pronounced interactions with anionic lipopolysaccharide and peptidoglycan layers, direct membrane disruption of both Gram-negative and Gram-positive bacteria are suppressed. At higher concentrations, however, LDH nanoparticles cause charge reversal and result in flocculation of bacteria. Apart from providing some clues to the mechanisms underlying previous findings of size-dependent cell internalization of LDH-bound siRNA and resulting gene silencing (12), the present study demonstrates the interplay between membrane interactions and nanoparticle-induced bacterial flocculation, and also identifies an approach

for confinement of infection, with potential consequences also for increased phagocytotic clearance and suppression of anti-inflammatory effects.

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Supporting material

Results of fluorescence intensity for CF-loaded DOPE/DOPG and DOPC/cholesterol liposomes versus LDH particle concentration, [LDH nanoparticle size versus time in Tris buffer](#), hemolysis versus LDH particle concentration in 50% citrate blood and in 5% EDTA blood, z-potential of LDH particles before and after serum exposure, representative live-dead confocal microscopy images of *E. coli* in the presence of LDH and LL37, as well as data on the fraction of bacteria in aggregates after LDH exposure, are available as supporting material.

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Figure Captions

Figure 1. CryoTEM micrographs (a) and dynamic light scattering results (b) on LDH particle structure and size, respectively.

Figure 2. (a) LDH adsorption, normalized to saturation adsorption (Γ/Γ_0), at DOPE/DOPG bilayers from 10 mM Tris, pH 7.4. (b) Amount of anionic NDB-DOPE lipids extracted from DOPE/DOPG liposomes by LDH nanoparticles at the indicated (initial) lipid concentrations in 10 mM Tris, pH 7.4.

Figure 3. (a) LDH-induced leakage of DOPE/DOPG liposomes in 10 mM Tris, pH 7.4. (b) Additive and synergistic leakage of DOPE/DOPG liposomes of LDH and LL-37 at a concentration of 1 ppm and 0.1 μ M, respectively. LDH and peptide was pre-mixed in 10 mM Tris, pH 7.4, 45 minutes prior to addition to the liposome solution. Arrows indicate leakage assuming additive effects of LL-37 and LDH nanoparticles.

Figure 4. (a) Adsorption of LL-37 at LDH nanoparticles, as well as (b) CD spectra of LL-37 in the absence and presence of LDH, [suggesting peptide binding primarily to their outer surface](#). In parallel, LDH does not protect LL-37 from proteolytic degradation by *P. aeruginosa* elastase (c).

Figure 5. (a) z-potential of LDH nanoparticles (left) and of complexes between LDH and DOPE/DOPG liposomes (lipid concentration 10 μ M, respectively; right) as a function of LDH concentration in 10 mM Tris, pH 7.4. (b) Corresponding particle size of the LDH/liposome complexes. For (a, right) and (b), nanoparticles and liposomes were mixed 45 minutes before measurement.

Figure 6. Antimicrobial effect of LDH nanoparticles (100 ppm) in the simultaneous presence of LL-37 at the indicated concentrations against *E. coli* (a) and *S. aureus* (b) in 10 mM Tris, pH 7.4, as monitored by VCA analysis. "*" denotes zero cfu detected.

Figure 7. (a) Confocal microscopy images of *E. coli* bacteria (red, green, and DIC images overlaid) in the presence of the indicated concentrations of LDH in 10 mM Tris, pH 7.4. (b) Red/green (Dead/live) ratio as a function of LDH concentration in *E. coli* (10^8 cfu)

suspensions in 10 mM Tris, pH 7.4. (c) While *E. coli* bacteria are almost completely alive in LDH-induced bacterial aggregates (LDH 104 nm, particle concentration 200 ppm), simultaneous presence of LL-37 (50 μ M) causes dramatic suppression of the fraction of alive bacteria. Positive and negative controls, corresponding to 100% dead and 100% alive bacteria, respectively, are shown as well to demonstrate assay reliability.

Figure 1.

(a)

(b)

Figure 2.

(a)

(b)

Figure 3.

(a)

(b)

Figure 4.

(a)

(b)

(c)

Figure 5.

(a)

(b)

Figure 6.

(a)

(b)

Figure 7.

(a)

(b)

Supporting Material

Table SM1. Physicochemical properties of the three LDH samples used.

Sample	Average particle size (nm)	Polydispersity index (PDI)	Zeta potential ¹ (mV)	d-spacing ² (nm)	Estimated chemical formula
LDH 42	42	0.223	+42 (W) +25 (T)	0.79	Mg ₃ Al(OH) ₈ (NO ₃)·mH ₂ O
LDH 104	104	0.165	+35 (W) +29 (T)	0.76	Mg ₂ Al(OH) ₆ (Cl)·mH ₂ O
LDH 208	208	0.061	+38 (W) +26 (T)	0.76	Mg ₂ Al(OH) ₆ (Cl)·mH ₂ O

¹Measurements were made either in de-ionized water (W) or 10 mM Tris, pH 7.4 (T).

²X-ray diffraction patterns were collected at a scanning rate of 2° per minute from 2θ = 5° to 2θ = 80° with Co K_α radiation (λ=0.17902 nm) on a Rigaku Miniflex X-ray diffractometer with a variable slit width. The d-spacing was calculated using the formula: $d = (d_{003} + 2d_{006} + 3d_{009})/3$.

Figure SM1. Particle size of LDH nanoparticles as a function of time, after dilution to 50 ppm in 10 mM Tris, pH 7.4, at t=0 min.

Figure SM2. Fluorescence intensity for CF-loaded (a) DOPE/DOPG and (b) DOPC/cholesterol liposomes versus LDH particle concentration. Measurements were performed in 10 mM Tris, pH 7.4.

(a)

(b)

Figure SM3. (a) Anionic serum proteins bind extensively to LDH nanoparticles, resulting in charge reversal, demonstrated by z-potential measurements of LDH nanoparticles in 10 mM Tris, pH 7.4, before and after exposure for 60 minutes to 50% serum. (b) Hemolysis induced by LDH nanoparticles. Measurements were performed either in 5% EDTA blood (where serum proteins are largely absent) or in 50% citrate blood (in which serum proteins are abundant). As seen, the presence of serum proteins (essentially all of which are net anionic) results in dramatic suppression of LDH hemolysis through masking of the positive surface potential of the bare LDH nanoparticles.

(a)

(b)

Figure SM4. Fraction of *E. coli* in aggregates after mixing 10^8 cfu bacteria with 100 ppm LDH nanoparticles in 10 mM Tris, pH 7.4.

Figure SM5. Representative confocal microscopy images obtained by live-dead staining, showing 100% live (upper), 100% dead (middle) *E. coli* (10^8 cfu) in 10 mM Tris, pH 7.4. Shown also (bottom) are corresponding live-dead images obtained for LDH 104 nm at 200 ppm in the absence and presence of 50 μ M LL-37, demonstrating that LL-37, but not the LDH nanoparticles, displays membrane-disrupting antimicrobial effects.