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Membrane interactions of mesoporous silica nanoparticles as carriers of antimicrobial peptides

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Abstract

Membrane interactions are critical for the successful use of mesoporous silica nanoparticles as delivery systems for antimicrobial peptides (AMPs). In order to elucidate these, we here investigate effects of nanoparticle charge and porosity on AMP loading and release, as well as consequences of this for membrane interactions and antimicrobial effects. Anionic mesoporous silica particles were found to incorporate considerable amounts of the cationic AMP LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES (LL-37), whereas loading is much lower for non-porous or positively charged silica nanoparticles. Due to preferential pore localization, anionic mesoporous particles, but not the other particles, protect LL-37 from degradation by infection-related proteases. For anionic mesoporous nanoparticles, membrane disruption is mediated almost exclusively by peptide release. In contrast, non-porous silica particles build up a resilient LL-37 surface coating due to their higher negative surface charge, and display largely particle-mediated membrane interactions and antimicrobial effects. For positively charged mesoporous silica nanoparticles, LL-37 incorporation promotes the membrane binding and disruption displayed by the particles in the absence of peptide, but also causes toxicity against human erythrocytes. Thus, the use of mesoporous silica nanoparticles as AMP delivery systems requires consideration of membrane interactions and selectivity of both free peptide and the peptide-loaded nanoparticles, the latter critically dependent on nanoparticle properties.

Introduction

Due to increasing problems with resistance development against conventional antibiotics (1), antimicrobial peptides (AMPs) are currently receiving considerable attention as potential therapeutics (2,3). Through lysis of bacterial membranes, AMPs provide fast and broad-spectrum antimicrobial effects. Key for the successful use of AMPs as therapeutics is their selective lysis of bacterial membranes, without simultaneous rupture of mammalian cells and resulting toxicity. In order to identify selective AMPs, several approaches have been used, including quantitative structure-activity relationship studies, identification of endogenous peptides derived from infection-related proteolysis, and end-tagging with short tryptophan or phenylalanine stretches (4). In addition to their antimicrobial effects, some AMPs also display other host defense properties, including anti-inflammatory and anticancer effects (5,6), both of which depend on membrane interactions of AMPs.

While there have been considerable efforts to identify potent and selective AMPs, drug delivery aspects of such compounds have been rarely investigated in literature. This is somewhat surprising, considering numerous potential hurdles related to efficient and safe delivery of AMPs. For example, infected tissue is often characterized by high proteolytic activity, mediated both by bacterial proteases and proteases of human defense cells. Thus, unless the peptide has been designed to be proteolytically stable (7,8), administration of AMPs to chronic wounds, infected eyes, or cystic fibrosis lungs is likely to result in rapid degradation of the peptide and in corresponding activity loss. Secondly, since AMPs are amphiphilic and positively charged, they bind to serum proteins, and are rapidly cleared from bloodstream circulation (9,10), which risks translating into reduced efficacy and toxicity effects related to accumulation in the reticuloendothelial system. In addition, some infections, such as tuberculosis, are characterized by intracellular bacteria localization, which poses a challenge in how to reach the intracellular bacteria without lysing and killing the host macrophages (11). Yet other challenges relate to need for sustained or triggered AMP release, e.g., for implants and recurring infection. In these and other contexts, it would therefore be advantageous to combine AMPs with delivery systems designed for the application at hand.

Apart from more traditional drug delivery systems, such as polymer, lipid, and surfactant systems (12), inorganic nanomaterials (e.g., gold and iron oxide nanoparticles, mesoporous

silica, layered double hydroxides, hydroxyapatite, graphene, carbon nanodots and nanotubes, quantum dots, and up-conversion particles) have attracted considerable interest during the last few years as delivery systems for biomacromolecular drugs, such as peptides, proteins, siRNA, and DNA (13). Such nanomaterials may provide key functional advantages, e.g., protection from chemical and enzymatic degradation, conformational stabilization, control of drug release rate, reduction of toxicity, and increased bioavailability. In addition, they offer opportunities related to their interaction with external fields (light, NIR, magnetic fields), e.g., as triggers for drug release, or for theranostic applications combining drug delivery and diagnostics. Among the inorganic nanoparticles, mesoporous silica nanoparticles (MSN) are of primary concern for the present investigation (14). Due to well-defined pores in the nm range, drug loading and release kinetics is widely controllable, e.g., through surface area, as well as pore size, form, and surface chemistry. Through this, functional advantages can be obtained, e.g., related to increased drug load, sustained drug release, or reduction of burst release. In addition, MSN display good physical and chemical stability, and have been found to be relatively biocompatible, although depending on MSN specifics, dose, and administration route (15,16).

The controlled use of MSN as delivery systems for AMPs requires a basic understanding of the factors determining loading and release of such peptides. In the present study, we therefore address this by investigating the effects of particle porosity and surface charge on the loading and release of the benchmark AMP LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES) (17), as well as consequences of this for particle interactions with lipid membranes, as well as for antimicrobial effect, cell toxicity, and proteolytic stabilization.

Experimental

Chemicals. LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES) was synthesized by Biopeptide Co. (San Diego, USA), and was of >95% purity, as evidenced by mass spectral analysis (MALDI-TOF Voyager). Tetramethylorthosilicate (TMOS, purum, $\geq 98\%$, Fluka Analytical/Sigma Aldrich), tetraethylorthosilicate (TEOS, purum, $\geq 99\%$, Sigma Aldrich), cetyltrimethylammonium bromide (CTAB; Sigma-Aldrich), (3-aminopropyl)trimethoxysilane (APTMS, 97%, Aldrich Chemistry), methanol (technical,

VWR), ethanol (99.5 % denatured with 1 % MEK, VWR), NaOH ($\geq 98\%$, Sigma Aldrich), and ammonium hydroxide solution (28 %, VWR) were used without further purification. All other chemicals used were of analytical quality.

Microorganisms. The bacterial isolate *Escherichia coli* (*E. coli*) ATCC 25922 was obtained from the American Type Culture Collection.

Silica nanoparticle synthesis. Mesoporous silica particles were synthesized according to the synthesis published by Rosenholm et al. (18). Briefly, in order to obtain amino-functionalized silica nanoparticles (MSNa) the structure-directing agent CTAB was dissolved in a methanol–water solution under basic conditions. Additionally, tetramethyl orthosilicate, TMOS, and aminopropyltrimethoxysilane, APTMS, were mixed under an inert atmosphere and added rapidly to the alkaline surfactant solution, with a final sol composition of TMOS:APTMS:NaOH:CTAB:methanol:water 1:0.1:1.24:1.36:1.3 $\times 10^3$:3 $\times 10^3$ (molar ratios). The CTAB was subsequently extracted with ammonium nitrate in ethanol. Particles were ultrasonicated in ammonium nitrate solution three times and washed twice in ethanol for purification. The particles were then dried under vacuum. To obtain calcined mesoporous silica particles (MSNc), MSNa particles were calcined at 550 °C for 4 h with a heating rate of 1 °C/min. Finally, a modified synthesis method leading to non-porous (Stöber-type) silica nanoparticles (NSN) was used as described by Sato-Berrú et al. (19). Ethanol, water, 3 mL of ammonium hydroxide solution (28%), and 1.5 mL of TEOS, corresponding to a molar ratio of TEOS:NH₃:etanol:water 1:4.74:93.05:89.92, were mixed and stirred for 1h at room temperature. The as obtained particles were washed twice in ethanol and dried under vacuum.

Nanoparticle characterization. Morphologies and dimensions of the silica nanoparticles were obtained using a Hitachi S-5200 Scanning electron microscope (SEM) (Hitachi, Tokyo, Japan) operated at 20 kV. Microtomed particles were also studied by TEM using a Jeol 1400 transmission electron microscope operated at 80 kV. Nitrogen adsorption and desorption isotherms were measured at -196°C using a Quadrasorb-SI (Quantachrome Instruments, Boynton Beach, USA), and the pore size distributions were calculated using an NLDFT equilibrium kernel developed for silica (Quantachrome). Particle dissolution was studied by ICP-OES using an Ultima 2 inductively coupled plasma optical emission spectrometer (ICP-OES) from Horiba Jobin Yvon S.A.S. (Longjumeau, France). For these measurements, particles were dispersed (5 mg/mL) and incubated in 10 mM Tris buffered solution (pH 7.4)

at 37 °C. At given time-points, particles were separated from the solution through centrifugation and the Si concentration in the supernatant was measured after 1:20 dilution with Milli-Q water (20). Thermogravimetric analysis (TGA) measurements were performed on a STA 449C Jupiter (Netzsch Gerätebau GmbH, Selb, Germany) with a heating rate of 10 °C/min to a temperature of 1000 °C. The hydrodynamic size of the particles and the corresponding z-potentials in the absence and presence of peptide (in 10 mM Tris, pH 7.4) were determined by dynamic light scattering at a scattering angle of 173° using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) setup. Measurements were performed in triplicate at 25 °C. Finally, XPS spectra and atomic concentrations of N, Si, O, and C were determined using a PHI 5800 setup (Physical Electronics, Chanhassen, USA). For the calibration of the binding energy, C (1s) was set to 284.4 eV.

Peptide adsorption. For peptide adsorption, 5 mg of particles were dispersed in 500 µL of 10 mM Tris buffer, pH 7.4, and incubated for 1h with additional 500 µL of peptide solution with equivalent concentration at room temperature. After incubation, the particles were separated via centrifugation and the amount of adsorbed peptide determined by measuring the amount of peptide remaining in the supernatant after adsorption via fluorescamine assay (21). For peptide quantification, 180 µL of the sample were added to 20 µL of fluorescamine solution (5 mg/mL in acetone) and fluorescence intensity measured at 465 nm after excitation at 360 nm.

Liposome preparation and leakage assay. Model liposomes investigated were anionic (DOPE/DOPG 75/25 mol/mol), frequently used as bacteria membrane models (22). DOPG (1,2-dioleoyl-*sn*-Glycero-3-phosphoglycerol, monosodium salt) and DOPE (1,2-dioleoyl-*sn*-Glycero-3-phosphoethanolamine) were from Avanti Polar Lipids (Alabaster, USA) and of >99% purity. The lipid mixture was 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 Ø140 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 µM 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 (23). 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°) 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 (24). 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 µM, and subsequently by rinsing

with buffer (5 ml/min for 15 minutes) when liposome adsorption had stabilised. 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 is 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 three subsequent peptide additions to 10, 50, and 100 ppm, in all cases monitoring the adsorption for one hour. All measurements were made in at least duplicate at 25°C.

Proteolysis. Peptides (2 µg) were incubated at 37 °C with *Pseudomonas aeruginosa* elastase (0.2 µg, 25000 units/mg, BioCol □ GmbH (Potsdam, Germany)), or human neutrophil elastase (0.8 µg, 29 units/mg; Calbiochem (La Jolla, USA)) 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 were grown to mid-exponential phase in Todd-Hewitt (TH). Bacteria were then washed and diluted in 10 mM Tris, pH 7.4, containing 0.15 M NaCl. 2×10^6 cfu/mL bacteria were incubated in 50 µL, 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=3).

Hemolysis. EDTA-blood was centrifuged at 800 g for 10 min, and plasma and buffy coat removed. Erythrocytes were washed three times and re-suspended in 5% PBS, pH 7.4. The cells were then incubated with end-over-end rotation for 1 h at 37°C in the presence of nanoparticles with or without peptide at the indicated concentrations. For comparison, 2% Triton X-100 (Sigma-Aldrich, St. Louis, USA) served as positive control. The samples were then centrifuged at 800 g for 10 min. The hemoglobin release was measured by absorbance at 550 nm and is expressed as % of Triton X-100-induced hemolysis (n=3).

Results

In order to investigate effects of particle charge on LL-37 loading and release from MSN, as well as consequences for membrane interactions and antimicrobial effects, silanol-rich calcined MSN (MSNc) was compared with the corresponding aminated nanoparticles (MSNa). Furthermore, in order to elucidate effects of porosity and specific surface area, MSNc was also compared to non-porous (Stöber; NSN) silica nanoparticles. As shown in Figure 1 a, SEM micrographs show that these nanoparticles all display a relatively narrow particle size distribution, with mean diameters of 294.6 ± 20.9 , 307.9 ± 39.1 , and 306.9 ± 17.3 nm for MSNc, NSN, and MSNa, respectively. TEM results on microtomed particles support this (Figure S1). Furthermore, BET-analyses of N_2 isotherms point out the expected surface of 700-1000 m^2/g for MSNc and MSNa, respectively, whereas that for NSN is much smaller (10 m^2/g) (Figure 1b, left), demonstrating that only an outer particle surface of the spherical particles is accessible for the latter. The pore diameter was determined by DFT-method and points out a centre at 3.1-3.5 nm, which is typical for the CTAB templates used for the MSN synthesis (Figure 1b, right). As seen in Figure 1c, XPS demonstrates abundant presence of nitrogen at the MSNa surface, but not at MSNc. From TGA, the amine surface density of MSNa was calculated to be $0.098 \mu mol/m^2$. The presence of the surface amine functionalities in MSNa is seen also from the difference in z-potential, being negative (≈ -35 mV) for MSNc, but positive ($\approx +2$ -10 mV) for MSNa. Like MSNc, non-porous NSN is highly negatively charged (≈ -55 mV).

LL-37 adsorbs extensively at MSNc as a result of the highly negative charge and the large surface area of MSNc (Figure 2a), as well as of the net positive charge (+6) of LL-37 at these conditions. For the non-porous NSN, saturation adsorption is reached at $1.8 \cdot 10^{-13}$ $\mu mol/particle$, corresponding to a specific surface area of $286 \pm 64 \text{ \AA}^2$ per peptide. Interestingly, LL-37 adsorbs also at the net positively charged MSNa, although at a very low saturation adsorption density (≈ 1 molecule/ $9200 \text{ \AA}^2$). The latter can be taken to indicate that also non-electrostatic interactions contribute to the adsorption driving force for LL-37. In addition, LL-37 adsorption at MSNa may be due to the presence of residual silanol groups, not uncommon for silane treatments (25), or partial re-arrangement of the amine groups to carbamate residues, again previously observed for amine-functionalization of silica (26), both resulting in an effective reduction of the positive surface charge, as indicated also by the relatively modest positive charge of MSNa. On binding of LL-37 (net charge +6 at pH 7.4),

the z-potential of non-porous NSN increases strongly and finally ends up strongly positively charged, clearly reflecting LL-37 adsorption to be confined to the external surface. For mesoporous MSNc on the other hand, z-potential is essentially unaffected by peptide adsorption at low peptide concentrations (Figure 2b), whereas also MSNc becomes positively charged at high peptide concentrations (Figure S2). These results show that initially added LL-37 is bound internally in the MSNc nanoparticles, and that the outer surface is occupied by the peptide only at full coverage.

For all the nanoparticles, peptide desorption is quite slow in 10 mM Tris, pH 7.4, reaching $\approx$ 4% release after 24 hours for NSN, and $\approx$ 17% release for MSNc and MSNa. For both MSNc and MSNa release increases after 48 h, most likely reflecting the similar pore size distributions of these nanoparticles, as well as dissolution-related effects on pore size and connectivity (Figure 3a). The smaller release observed for NSN is most likely due to the higher charge density of the latter (-55 mV) than that of MSNc (-35 mV). As shown in Figure 3b, particle dissolution is quite slow under these conditions (above the solubility limit), reaching only 3-4% over the time frame investigated. Hence, peptide release is determined by detachment rate rather than by particle dissolution rate. As a result of the surface localization in the case of NSN, as well as the slow peptide release displayed by these nanoparticles, peptide-loaded NSN retains its positive z-potential for an extended period even after extensive dilution in 10 mM Tris buffer (Figure 3c). For MSNc, on the other hand, release of only a small fraction of loaded peptide results in fast charge reversal, since peptide release is initiated from the outer surface of the loaded particles, even though the pores remain loaded with peptide for longer time.

The surface properties of MSN affect their interaction with (bacteria-mimicking) lipid bilayers. Thus, as shown in Figure 4a, positively charged MSNa binds to negatively charged ($z \approx -35$ mV (22)) DOPE/DOPG bilayers in the absence of LL-37, whereas the negatively charged MSNc and NSN do not. Reflecting the transition in z-potential of NSN after LL-37 incorporation (1 μ M/100 ppm nanoparticle), peptide-loaded NSN displays high binding to DOPE/DOPG bilayers, while MSNc, remaining highly negatively charged after (partial) LL-37 loading, continues to display non-adsorption (Figure 4b). Reflecting nanoparticle membrane binding, Figure 5a shows MSNa, but not MSNc and NSN, to disrupt DOPE/DOPG liposomes in the absence of LL-37, while Figures 5b and c show that LL-37 loading to MSNc and NSN provides these with a potent membrane-disrupting capacity once fully loaded with

LL-37, although much more peptide is needed to saturate MSNc ($\approx 7.5 \mu\text{M}$) than for NSN ($\approx 1 \mu\text{M}$). For MSNa, finally, the presence of LL-37 results in a small additive effect between particle and peptide, most clearly seen at low LL-37 concentrations.

In order to address the relative importance of membrane rupturing effects due to the peptide-loaded particles and that of free peptide present in solution, MSNc and NSN were first fully loaded with LL-37 (at the onset of the plateau of the adsorption isotherm to minimize free peptide), followed by removal of free peptide by centrifugation, replacement of supernatant with 10 mM Tris buffer, and re-suspension. As shown in Figure 6, particle-induced membrane lysis for NSN after this procedure remained at the same level as before centrifugation and re-suspension, indicating free peptide not to have any important effect for membrane disruption in this system, in agreement with the slow peptide release rate (Figure 3a) and subsequent reduction in positive z-potential (Figure 3c). For MSNc, on the other hand, z-potential after dilution turns negative after release of only a small fraction of LL-37 loaded, indicating peptide release to start from the surface of the loaded nanoparticles (Figure 3c). Mirroring this, MSNc does not retain its membrane-rupturing capacity after centrifugation and re-suspension (Figure 6). Together, these results show that for MSNc, membrane disruption is dominated by that of released peptide during most of the release process, whereas for NSN, the peptide-loaded particles provide most of the membrane destabilization.

Finally, we note that antimicrobial effects of MSN with and without LL-37 loading correlate to both lysis of model DOPE/DOPG liposomes and particle binding to lipid membranes. Thus, as shown in Figure 7a and b, MSNa is efficient in lysing *E. coli* bacteria also in the absence of peptide, whereas MSNc and NSN are not. After loading with LL-37, the antimicrobial effect of MSNc increases in a dose-depending manner, reflecting the peptide-induced z-potential transition, as well as resulting particle binding to, and disruption of, anionic (bacteria) membranes. Importantly, MSNc only displays antimicrobial activity once the particles are fully loaded with peptide, i.e., $\approx 10 \mu\text{M}$, whereas NSN displays initial antimicrobial effects already at about $0.5 \mu\text{M}$ LL-37. Regarding toxicity, we note that hemolysis is low for MSNc, NSN, and also for MSNa in the absence of LL-37, then increasing with peptide concentration, as is the case also for the peptide in the absence of nanoparticles (Figure 8a). In the presence of LL-37, however, MSNa displays substantially higher hemolysis than either LL-37 alone or LL-37 incorporated in MSNc and NSN,

reflecting the membrane-destabilizing effect of MSNa observed also in the liposome leakage experiments. Hence, MSNa provides some toxicity to human erythrocytes in combination with LL-37. Finally, we note that MSNc, in contrast to NSN, and MSNa, offer (partial) protection of LL-37 against proteolytic degradation by infection-related enzymes from either bacteria (*P. aeruginosa* elastase, PE) or human defense cells (leukocyte elastase, HNE) (Figure 8b). This is in line with the preferential localization of LL-37 within the pores of MSNc, demonstrated above, and limited access for the relatively large HNE and PE enzymes into the small pores.

Discussion

As with other nanomaterials, membrane interactions and cell internalization of mesoporous silica nanoparticles can be enhanced by cationic surface modification. Illustrating this, Botequim et al. investigated antimicrobial effects of silica nanoparticles coated with the cationic surfactant didodecyldimethylammonium bromide (DDAB) (27). At complete loading, z- potential measurements, thermogravimetry, and diffuse reflectance infrared analyses demonstrated DDAB to form a bilayer at the silica surface. Moreover, surfactant desorption was found to be limited or slow, the latter inferred from the very limited loss of antimicrobial activity over 60 days. Thus, antimicrobial activity does not require surfactant leaching, but is instead based on direct membrane destabilization by the modified silica particles. Similarly, a direct membrane-disruptive effect of MSN coated with cationic polypeptides was inferred also by Li et al. for lysozyme-coated mesoporous silica nanoparticles, who found these to display efficient antibacterial activity also in the absence of low-Mw antibiotics (28).

Alternatively, mesoporous silica can be rendered antimicrobial through inclusion of antibiotics or other antimicrobial compounds. For example, Molina-Manso et al. investigated mesoporous silica (SBA-15) as carrier for vancomycin, linezolid, and rifampicin, or combinations thereof (29). Loading of antibiotics into MSN has also been combined with antimicrobial proteins and polypeptides. Thus, Mas et al. investigated mesoporous MCM-41 nanoparticles loaded with vancomycin, the pores of which were capped by poly-l-lysine (pLys). As for the presently investigated MSNc and NSN, anionic MCM-41 nor did not show any detectable membrane activity or antimicrobial effects against *E. coli*. In contrast, a six-

fold decrease in the minimum inhibitory concentration was found for the vancomycin-loaded and pLys-capped nanoparticles, compared to those of the individual compounds (30). Although the mechanisms behind these observations were not addressed, the results suggest direct membrane interaction and disruption by the pLys-capped MSN, combined with improved antimicrobial effect of the incorporated antibiotic in the presence of particle-induced membrane defects, either due to increased diffusion to the cell interior or reduced efficiency of the efflux machinery of the bacteria.

In a rare example of studies directly dedicated to AMP-loaded mesoporous silica, Izquierdo-Barba et al. investigated the incorporation of LL-37, as well as the low Mw antimicrobial compound chlorhexidine, into monolithic mesoporous silica (31). Both LL-37 and chlorhexidine was released slowly (over several hundred hours) from the mesoporous silica monolith, the rate controlled, e.g., through incorporation of SH groups in the pore walls. Mesoporous silica containing either LL-37 or chlorhexidine displayed potent antimicrobial properties against *E. coli* and *S. aureus*. Furthermore, in contrast to the chlorhexidine-loaded material, that containing LL-37 displayed very low cell toxicity. From this, it was concluded that LL-37-loaded mesoporous silica holds potential as an implantable material (surface coating) for controlling implant-related infections.

Given the membrane-rupturing effects of MSNa and other cationic nanoparticles, a key issue is what additional advantage is provided by inclusion of an antimicrobial peptide, such as LL-37, in/on a silica nanoparticle. Clearly, cationic (silica) nanoparticles do not suffer from problems related to proteolytic degradation. They are, however, very rapidly covered by serum proteins and cleared from bloodstream circulation (32,33). Furthermore, provided that a selective AMP is used, cationic AMP-loaded particles may display potent antimicrobial effects but simultaneously lower toxicity than particles simply cationized by either covalent modification or surface coating with any cationic polyelectrolyte. For particles with large surface area, such as MSN, AMP loading will also enable a large dose of antimicrobial agents to be administered, where peptide release will also circumvent the problem of particle inactivation upon binding to bacteria. Such inactivation after attachment of the first bacteria has been observed to limit the efficiency of surfaces coated with cationic polyelectrolytes as antimicrobial surfaces (34), and may be avoided on relying instead on released AMP for antimicrobial action. Importantly, AMPs such as LL-37 also provide a spectrum of host defense functions, such as anti-inflammatory effect and immune modulation (17), providing

additional advantages. Considering these issues, mesoporous silica particles also have an advantage over non-porous ones related to much higher surface area and capabilities to carry a higher AMP load. In addition, pore localization of AMPs in MSN also provides protection against proteolytic degradation, not achieved for non-porous particles. However, as demonstrated in the present investigation, MSN become essentially inactive as antimicrobial entities after the release of the very first fraction of AMP, after which antimicrobial effects depend entirely on released AMPs. While this poses no particular concern for applications where long release is desired, such as in implants (31), this means that AMP release is a key issue to address for indications requiring faster release. This, in turn, can be done by tuning MSN surface charge, pore structure, and degradation rate.

Finally, although not addressed in the present investigation, it should be noted that the presence of (antimicrobial) peptides at the outer surface of mesoporous silica nanoparticles may offer also other functionalities, depending on the peptide. For example, Li et al. incorporated the fusogenic KALA peptide in PEI-coated magnetic mesoporous silica (35), and found the resulting nanoparticles to be internalized into cells, to escape from the endosomes, and subsequently to release loaded siRNA into the cytoplasm, effects due to the peptide. Similarly, Pan et al. conjugated the membrane penetrating TAT peptide onto mesoporous silica nanoparticles, and noted peptide-mediated targeting to the nucleus, as well as anticancer effects (36). As with the direct antimicrobial effects investigated in the present study, however, these effects are expected to rely critically on the peptide being available at the outer surface of the mesoporous silica particles, thus displaying loading-dependent effects as in the present investigation, unless covalently grafted at the outer particle surface. In addition, since both phagocytosis and endosomal release depend on particle physicochemical properties (32,33), also the charge of the mesoporous silica particles, by itself, will influence cell uptake and how this is affected by peptide loading at the outer MSN surface.

Conclusions

Surface charge and surface area strongly influence loading and release of the antimicrobial peptide LL-37 onto/into mesoporous silica nanoparticles. Thus, considerable amounts of this net positively charged peptide can be incorporated into negatively charged calcined mesoporous silica nanoparticles (MSNc), whereas loading is much lower at positively

charged (MSNa) or non-porous (NSN) silica nanoparticles. Due to preferential peptide localization within the mesopores, MSNc offers protection against degradation by infection-related proteases. Although positively charged and directly antimicrobial at full peptide loading, MSNc regains its negative surface charge already after release of a minor part of its peptide load, after which it does not bind to, nor lyses, bacterial membranes. Instead, AMP function is mediated entirely by peptide release. In contrast, highly negatively charged NSN obtains a resilient LL-37 coating, displaying very slow peptide release and largely particle-mediated antimicrobial effects. For MSNa, incorporation of LL-37 results in a further increase in the membrane binding and disruption displayed by the particles in the absence of peptide, although at the expense of toxicity against human erythrocytes, not observed for LL-37-loaded MSNc and NSN. Taken together, the present study expands the knowledge from previous studies on mesoporous silica as delivery system of antimicrobial peptides (31) in that it demonstrates that control over antimicrobial effects in peptide-loaded mesoporous silica nanoparticle systems requires consideration of membrane interactions of both free peptide and the peptide-loaded nanoparticles, which depend critically on nanoparticle properties and consequences of these for peptide loading and release.

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Supporting material

Results from TEM imaging of the empty particles, as well as results on z-potential as a function of peptide load are available as supporting material.

References

- 1) Chambers, H.F.; Deleo, F.R. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. *Nature Rev. Microbiol.* **2009**, *7*, 629-641.
- 2) Brogden, K.A. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nature Rev. Microbiol.* **2005**, *3*, 238-250.
- 3) Hancock, R.E.; Sahl, H.G. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat. Biotechnol.* **2006**, *24*, 1551-1557.
- 4) Strömstedt, A.A.; Ringstad, L.; Schmidtchen, A.; Malmsten, M. Interaction between amphiphilic peptides and phospholipid membranes. *Curr. Opin. Colloid Interf. Sci.* **2010**, *15*, 467-478.
- 5) Schmidtchen, A.; Malmsten, M. Peptide interactions with bacterial lipopolysaccharides. *Curr. Opin. Colloid Interface Sci.* **2013**, *18*, 381-392.
- 6) Gaspar, D.; Veiga, A.S.; Castanho, M.A.R.B. From antimicrobial to anticancer peptides. A review. *Front. Microbiol.* **2013**, *4*, 1-16.
- 7) Schmidtchen, A.; Pasupuleti, M.; Mörgelin, M.; Davoudi, M.; Alenfall, J.; Chalupka, A.; Malmsten, M. Boosting antimicrobial peptides by hydrophobic oligopeptide end-tags. *J. Biol. Chem.* **2009**, *284*, 17584-17594.
- 8) Strömstedt, A.A.; Pasupuleti, M.; Schmidtchen, A.; Malmsten, M. Evaluation of strategies for improving proteolytic resistance of antimicrobial peptides by using variants of EFK17, and internal segment of LL-37. *Antimicrob. Agents Chemother.* **2009**, *53*, 593-602.
- 9) Singh, S.; Papareddy, P.; Mörgelin, M.; Schmidtchen, A.; Malmsten, M. Effects of PEGylation on membrane and lipopolysaccharide interactions of host defense peptides. *Biomacromolecules* **2014**, *15*, 1337-1345.
- 10) Veronese, F.M.; Mero, A. The impact of PEGylation on biological therapies. *Biodrugs* **2008**, *22*, 315-329.
- 11) van der Ven, B.C.; Fahey, R.J.; Lee, W.; Liu, Y.; Abramovitch, R.B.; Memmott, C.; Crowe, A.M.; Eltis, L.D.; Perola, E.; Deininger, D.D.; Wang, T.; Locher, C.P.; Russell, D.G. Novel inhibitors of cholesterol degradation in *Mycobacterium tuberculosis* reveal how the bacterium's metabolism is constrained by the intracellular environment. *PLoS Pathogens* **2015**, *11*, e1004679, 1-20.
- 12) Malmsten, M. Soft drug delivery systems. *Soft Matter* **2006**, *2*, 760-769.

- 13) Malmsten, M. Inorganic nanomaterials as delivery systems for proteins, peptides, DNA, and siRNA. *Curr. Opin. Colloid Interface Sci.* **2013**, *18*, 468-480.
- 14) Vivero-Escoto, J.L.; Slowing, I.I.; Trewyn, B.G.; Lin, V.S.-Y. Mesoporous silica nanoparticles for intracellular controlled drug delivery. *Small* **2010**, *6*, 1952-1967.
- 15) Gultepe, E.; Nagesha, D.; Sridhar, S.; Amiji, M. Nanoporous inorganic membranes or coatings for sustained drug delivery in implantable devices. *Adv. Drug Delivery Rev.* **2010**, *62*, 305-315.
- 16) Fadeel, B.; Garcia-Bennett, A.-E. Better safe than sorry: understanding the toxicological properties of inorganic nanoparticles manufactured for biomedical applications. *Adv. Drug Delivery Rev.* **2010**, *62*, 362-374.
- 17) Durr, H.H.N.; Sudheendra, U.S.; Ramamoorthy, A. LL-37, the only human member of the cathelicidin family of antimicrobial peptides. *Biochim. Biophys. Acta* **2006**, *9*, 1408-1425.
- 18) Rosenholm, J.M.; Meinander, S.; Peuhu, E.; Niemi, R.; Eriksson, J.E.; Sahlgren, C.; Lindén, M. Targeting of porous hybrid silica nanoparticles to cancer cells. *ACS Nano* **2009**, *3*, 197-206.
- 19) Sato-Berru, R.; Saniger, J.M.; Flores-Flores, J.; Sanchez-Espindola, M. Simple method for the controlled growth of SiO₂ spheres. *J. Mater. Sci. Eng. A* **2013**, *3*, 237-242.
- 20) He, Q.; Shi, J.; Zhu, M.; Chen, Y.; Chen, F. The three-stage *in vitro* degradation behavior of mesoporous silica in simulated body fluid. *Micropor. Mesopor. Mater.* **2010**, *131*, 314-320.
- 21) Puddu, V.; Perry, C.C. Peptide adsorption on silica nanoparticles: evidence of hydrophobic interactions. *ACS Nano* **2012**, *6*, 6356-6363.
- 22) Schmidtchen, A.; Ringstad, L.; Kasetty, G.; Mizuno, H.; Rutland, M.W.; Malmsten, M. Membrane selectivity by W-tagging of antimicrobial peptides. *Biochim. Biophys. Acta* **2011**, *1808*, 1081-1091.
- 23) Malmsten, M. Ellipsometry studies of protein layers adsorbed at hydrophobic surfaces. *J. Colloid Interface Sci.* **1994**, *166*, 333-342.
- 24) Ringstad, L.; Schmidtchen, A.; Malmsten, M. Effect of peptide length on the interaction between consensus peptides and DOPC/DOPA bilayers. *Langmuir* **2006**, *22*, 5042-5050.
- 25) Sanz-Moral, L.M.; Rueda, M.; Nieto, A.; Novak, Z.; Knez, Z.; Martin, A. Gradual hydrophobic surface functionalization of dry silica aerogels by reaction with silane precursors dissolved in supercritical carbon dioxide. *J. Supercrit. Fluids* **2013**, *84*, 74-79.
- 26) Burns, N.L.; Holmberg, K.; Brink, C. Influence of surface charge on protein adsorption at an amphoteric surface. Effects of varying acid to base ratio. *J. Colloid Interface Sci.* **1996**, *178*, 116-122.

- 27) Botequim, D.; Maia, J.; Lino, M.M.F.; Lopes, L.M.F.; Simões, P.N.; Ilharco, L.M.; Ferreira, L. Nanoparticles and surfaces presenting antifungal, antibacterial, and antiviral properties. *Langmuir* **2012**, *28*, 7646-7656.
- 28) Li, L.L.; Wang, H. Enzyme-coated mesoporous nanoparticles as efficient antibacterial agents *in vivo*. *Adv. Healthcare Mater.* **2013**, *2*, 1351-1360.
- 29) Molina-Manso, D.; Manzano, M.; Doadrio, J.C.; Del Prado, G.; Ortiz-Perez, A.; Vallet-Regi, M.; Gomez-Barrena, E.; Esteban, J. Usefulness of SBA-15 mesoporous ceramics as a delivery system for vancomycin, rifampicin and linezolid: a preliminary report. *Int. J. Antimicrob. Agents* **2012**, *40*, 252-256.
- 30) Mas, N.; Galiana, I.; Mondragon, L.; Aznar, E.; Climent, E.; Cabedo, N.; Sancenón, F.; Muguia, J.R.; Martínez-Manez, R.; Marcos, M.D.; Amoros, P. Enhanced efficacy and broadening of antibacterial action of drugs via the use of capped mesoporous nanoparticles. *Eur. Chem. J.* **2013**, *19*, 11167-11171.
- 31) Izquierdo-Barba, I.; Vallet-Regi, M.; Kupferschmidt, N.; Terasaki, O.; Schmidtchen, A.; Malmsten, M. Incorporation of antimicrobial compounds in mesoporous silica film monolith. *Biomaterials* **2009**, *30*, 7729-7736.
- 32) Sahay, G.; Alakhova, D.Y.; Kabanov, A.V. Endocytosis of nanomedicines. *J. Controlled Release* **2010**, *145*, 182-195.
- 33) Tabata, Y.; Ikada, Y. Phagocytosis of microspheres by macrophages. *Adv. Polym. Sci.* **1990**, *94*, 107-141.
- 34) Lichter, J.A.; Van Vliet, K.J.; Rubner, M.F. Design of antibacterial surfaces and interfaces: polyelectrolyte multilayers as a multifunctional platform. *Macromolecules* **2009**, *42*, 8573-8586.
- 35) Li, X.; Chen, Y.; Wang, M.; Ma, Y.; Xia, W.; Gu, H. A mesoporous silica nanoparticle-PEI-fusogenic peptide system for siRNA delivery in cancer therapy. *Biomaterials* **2013**, *34*, 1391-1401.
- 36) Pan, L.; He, Q.; Liu, J.; Chen, Y.; Ma, M.; Zhang, L.; Shi, J. Nuclear-targeted drug delivery of TAT peptide-conjugated monodisperse mesoporous silica nanoparticles. *J. Am. Chem. Soc.* **2012**, *134*, 5722-5725.

Figure Captions

Figure 1. Characterization of the silica nanoparticles investigated. Shown in (a), (b), and (c) are SEM images and corresponding particle size distribution, surface area/pore size distribution, and XPS/TGA results, respectively. From the SEM histograms, particle diameters for MSNc, NSN, and MSNa were found to be 294.6 ± 20.9 , 307.9 ± 39.1 , and 306.9 ± 17.3 nm, respectively.

Figure 2. Peptide loading into/onto the nanoparticles investigated. (a) Adsorption isotherms of LL-37 to MSNc, NSN, and MSNa, and (b) z-potential of the nanoparticles after peptide loading at the indicated peptide concentrations (100 ppm particle concentration), both in 10 mM Tris, pH 7.4.

Figure 3. (a) Peptide release from the nanoparticles and (b) nanoparticle dissolution kinetics, both in 10 mM Tris, pH 7.4. Shown also in (c) is the release-dependent z-potential of MSNc and NSN after saturation LL-37 loading over night in 10 mM Tris, pH 7.4, followed (at $t=0$) by extensive dilution with 10 mM Tris, pH 7.4.

Figure 4. Nanoparticle adsorption to DOPE/DOPG supported bilayers without (wo; a) or with (w; b) pre-loading of LL-37 at 1 μ M/100 ppm nanoparticles over night. Measurements were performed in 10 mM Tris, pH 7.4.

Figure 5. Nanoparticle-induced leakage from DOPE/DOPG liposomes in the absence of LL-37 (a), and after peptide loading at the indicated concentrations, (b) and (c). Measurements were performed in 10 mM Tris, pH 7.4.

Figure 6. Test of the relative importance of free peptide and peptide-loaded particles for membrane disruption. MSNc and NSN nanoparticles at 100 ppm were loaded over night with LL-37 at a peptide concentration representing the onset of plateau adsorption (10 and 1 μ M for MSNc and NSN, respectively) in 10 mM Tris, pH 7.4, followed by centrifugation and re-suspension in peptide-free 10 mM Tris, pH 7.4. As demonstrated, LL-37-loaded NSN particles are membrane-lytic after this processing, whereas MSNc particles are not.

Figure 7. Antimicrobial effects against *E. coli* 25922. Shown are VCA results for MSNc, NSN, and MSNa at 100 ppm in the absence or presence of LL-37 at the indicated concentrations, shown in the low (a) and high (b) peptide concentration regime in 10 mM Tris, pH 7.4.

Figure 8. (a) Hemolysis results obtained for MSNc, NSN, and MSNa at 100 ppm in the absence or presence of LL-37 at the indicated concentrations. (b) Proteolytic degradation of LL-37 before (-) and after (+) particle loading. LL-37 (2 µg) was incubated at 37 °C with *Pseudomonas aeruginosa* elastase (PE; 0.2 µg, 25000 units/mg, left) or human neutrophil elastase (HNE; 0.8 µg, 29 units/mg, right) in a total volume of 15 µl for 16 hours, followed by SDS-PAGE analysis with Coomassie Brilliant Blue staining.

Figure 1.

(a)

MSNc

NSN

MSNa

MSNc

NSN

MSNa

(b)

(c)

Figure 2.

(a)

(b)

Figure 3.

(a)

(b)

(c)

Figure 4.

(a)

(b)

Figure 5.

(a)

(b)

(c)

Figure 6.

Figure 7.

(a)

(b)

Figure 8.

(a)

(b)