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Design and Characterization of Bio-inspired Antimicrobial Nanomaterials

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Abstract: Colloidal structures are crucial components in biological systems and provide a vivid and seemingly infinite source of inspiration for the design of functional bio-inspired materials. They form multi-dimensional confinements and shape living matter, and transport and protect bioactive molecules in harsh biological environments such as the stomach. Recently, colloidal nanostructures based on natural antimicrobial peptides have emerged as promising alternatives to conventional antibiotics. This contribution summarizes the recent progress in the understanding and design of these bio-inspired antimicrobial nanomaterials, and discusses their advances in the form of dispersions and as surface coatings. Their potential for applications in future food and healthcare materials is also highlighted. Further, it discusses challenges in the characterization of structure and dynamics in these materials.

Keywords: Antimicrobial peptide · Liquid crystals · Nanocarriers · Nanostructured coatings · Self-assembly

Mahsa Zabara holds a PhD in Materials Chemistry from the Royal Melbourne Institute of Technology, Australia (2016) on designing photo-tunable antimicrobial nanomaterials; and a second PhD in Chemical Engineering from TMU University in Tehran, Iran (2012) on developing hybrid nanoparticles as contrast agents. She moved to Switzerland and joined the group of Prof. Salentinig at Empa (2017), and later at the University of Fribourg as postdoctoral fellow, working on antimicrobial nanomaterials. She has experience in both academic and industrial settings with a constant focus on material science and nanotechnology.

Linda Hong received her PhD from Monash University (Parkville, Australia) in 2019 studying stimuli-responsive liquid crystalline nanoparticles using microfluidic approaches and advanced synchrotron-based scattering techniques. She has since moved to Switzerland to undertake a postdoctorate at the University of Fribourg under the supervision of Prof. Salentinig. Her research now focuses on investigating the interactions between bacterial membrane components, antimicrobial peptides, and lipid-based nanocarriers. She is also collaborating with industry to formulate drug delivery systems.

Stefan Salentinig is a professor of physical chemistry at the University of Fribourg, where he has established the Biocolloids lab, researching bio-inspired materials for health applications. He holds a PhD in physical chemistry focusing on food colloids and scattering methods from the University of Graz, Austria, 2010. He then moved to Australia to take on a scientist position on separation technology at the CSIRO, and then became lecturer at Monash University in Melbourne working on nanoscale drug delivery systems. In 2015, he moved to Switzerland and joined Empa as a group leader on functional materials, before starting his current role in 2019.

1. Introduction

Bacterial contamination is one of the major challenges for maintaining food quality and safety during processing and storage.[1] Further, the attachment and colonization of pathogenic bacteria on the surface of medical implants or open wounds may result in severe infections.[1a,2] These infections are particularly concerning if multi-resistant bacteria or difficult to treat microorganisms are involved. Hence, the design of suitable and efficient antimicrobial materials is crucial to maintain or even improve the health of our society. The search for alternative materials to conventional antibiotics has attracted great interest. Ribosomal synthesized, natural, antimicrobial peptides (AMPs) based materials have been emerging as promising alternative candidates.[3] AMPs are a native part of the human innate immune system, occur as native host-defence molecules in virtually all species of life, and have a broad spectrum of activities against pathogenic bacteria and can modulate the immune response of the host.[4] AMPs were also discovered in human breast milk and may provide a selective advantage through evolution by protecting both the mother's mammary gland and her nursing offspring from infection.[5] One such AMP is lactoferricin B, an AMP obtained from the digestion of lactoferrin. It is an iron binding multifunctional protein existing in high concentrations in body secretions including colostrum, milk, tear, nasal secretion, saliva, bile, urine, and genital secretions.[6]

Selected food-grade AMPs such as Nisin (E 234) are also widely used as food preservatives, for instance in unripen cheese and in heat-treated meat products.[7] It is interesting to mention that although these AMPs have been used in food for more than fifty years, no significant bacterial resistance has been reported yet.[8] However, the major challenge to overcome for the broader clinical application of AMP-based antimicrobials is their lack of stabil-

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ity in the biological environments.[6,9] Self-assembled structures, including micelles, microemulsions and inverse lyotropic non-lamellar liquid crystalline phases, have attracted increasing attention for their potential applications in peptide/drug delivery.[10] Encapsulating AMPs in such colloidal structures can preserve them from degradation and improve their antimicrobial properties.[10a,11] A wide range of carrier systems for encapsulating AMPs has been developed.[10d,12]

The mechanisms underlying the self-assembly of surfactants, and formation of the various liquid-like 'soft' structures was reviewed recently.[13] This work focuses on advances of these materials as antimicrobial nanocarriers and coatings. It sheds light on the functional association of colloids formed by the co-assembly of selected surfactants with AMPs. It discusses advances in (i) low-viscosity, solution-based systems that may be injectable and drinkable; and (ii) high-viscosity films that can be used for the functionalization of surfaces. Related to this, our team recently demonstrated functional lipid-based nanocarriers that can protect AMPs from degradation, and improve their antimicrobial activity.[10a,11a,b,14] Further, we demonstrated the design of multifunctional nano-biointerfaces based on the human cathelicidin-derived AMP LL-37 that are antimicrobially active, while simultaneously promoting cell proliferation.[11a] We also pioneered stimuli-responsive antimicrobial materials that can switch their activity 'on' and 'off' through external triggers such as pH, humidity or temperature.[14]

Overall, this new class of antimicrobial nanomaterials was inspired by nature's own food and nutrient delivery systems. Milk, for instance, contains nanocarriers such as vesicles to protect labile nutrients or metabolic messages from degradation in the harsh environment of the stomach and targets their delivery.[15] The recently discovered *in situ* formation of nanostructures during milk digestion may also help maintain the uptake of co-delivered nutrients and drugs even under compromised digestion conditions such as a fat maldigestion, a low bile-salt concentration, or a limited lipase action (Fig. 1).[16]

2. Self-assembled Nanocarriers for AMPs

2.1 *Micelles and Vesicles*

Micelles have been studied for the encapsulation of a variety of bioactive molecules, including antifungal drugs,[17] and immunosuppressant peptides.[18] Recently, mixed AMP/lipid micelles in various shapes and forms were discovered to be highly active nanocarriers for AMPs.[11a,14,19] In this context, functional nano-biointerfaces in the form of micelles with pH-triggered biological activity for the site-specific pH-guided delivery of AMPs were developed. The oleic acid (OA)/LL-37 micelles can switch between antimicrobially active and inactive states through pH-triggered structural transformations from cylindrical micelles at approximately pH 7 to aggregates of branched micelles at approximately pH 5 (Fig. 2). The antimicrobially active OA/LL-37 aggregates at approximately pH 5 were highly effective against *Escherichia coli* (*E. coli*) bacteria, whereas the cylindrical micelles at pH 7 were mostly inactive. The geometry of the aggregates together with electrostatic interactions between OA and LL-37 molecules as well as the negatively charged bacteria membrane were discussed to contribute to the underlying mechanism. Such systems may be interesting, for example, in the treatment of infections of the digestive tract, killing bacteria at desired locations such as the stomach, and keeping pro-biotic bacteria in other locations intact.[14]

Lamellar structures (*i.e.* dispersed in the form of vesicles in solution) are among the most abundant lipid structures in nature. Vesicles can also encapsulate hydrophilic molecules in their hydrophilic core, hydrophobic molecules within their hydrophobic compartment of the bilayer, and amphiphilic molecules including AMPs in the lipid–water interface of the bilayer.[10h,20] The integration of the honeybee-venom AMP, melittin and the AMP, alameticin, from the fungus *Trichoderma viride*, into the bilayer membrane of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) vesicles has been investigated, observing that these AMPs have a membrane perturbing effect and prefer to interact with (locally) curved lipid surfaces.[21] In addition, it has been demonstrated that the integration of the 13-residue cathelicidin AMP, indolicidin, into the lipid bilayer membrane of vesicles resulted in the enhancement of its antifungal activity.[22] More recently, the encapsulation of the LL-37 into PEGylated liposomal formulations was shown to significantly reduce its cytotoxicity, and the antiviral activity profile including in the 3D epidermis model was significantly improved while expanding its therapeutic window.[23]

2.2 *Non-Lamellar Liquid-crystalline Nanocarriers*

Certain polar lipids, for instance from the phospholipid family, or selected oil-digestion products such as glycerol-monooleate (GMO) and OA, can self-assemble into highly organized non-lamellar liquid crystalline structures such as inverse (oil-continuous) cubic and hexagonal phases.[24] These non-lamellar liquid crystalline materials contain intricate multi-dimensional networks of hydrophilic nano-channels in their interior. The inverse (oil-continuous) bi-continuous cubic, the inverse hexagonal and the inverse micellar cubic structure are the most famous ones for lipid-based delivery systems. Depending on composition, the inverse bicontinuous cubic bulk phases are observed to occur in three major geometries: diamond (*Pn3m*), gyroid (*Ia3d*) and primitive (*Im3m*) types. The complex bicontinuous network of confined water channels with large lipid–water interfacial area, of up to 600 m^2 per gram of material, allows them to encapsulate a large capacity of cargo of various polarities.[25]

By incorporating a stimuli-responsive element, controlled and on-demand release of the cargo may be possible through

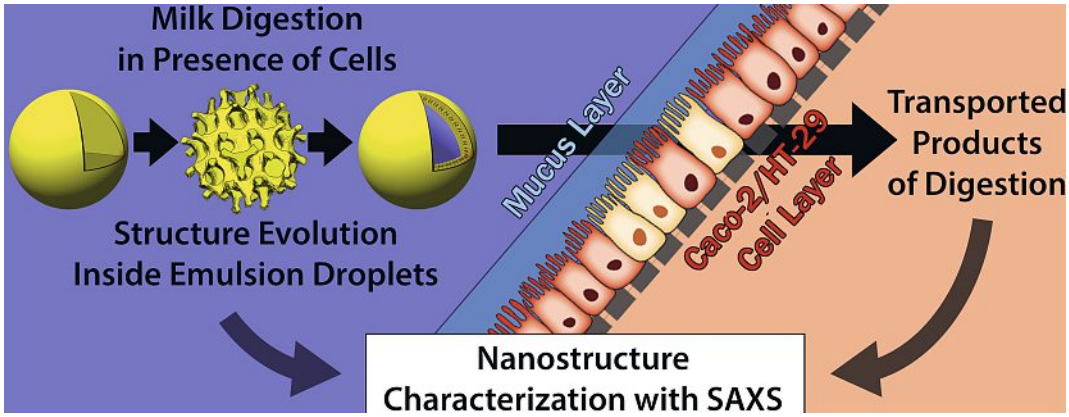

Fig. 1. An integrated online small angle X-ray scattering (SAXS) – *in vitro* cell co-culture model was developed and applied to study the digestion of a naturally occurring emulsion, milk. The impact of the *in vitro* cell culture on the digestion-triggered formation and evolution of highly ordered nanostructures in milk was demonstrated in this study.[16a] Reprinted from C. Hempt, M. Gontsarik, T. Buerki-Thurnherr, C. Hirsch, S. Salentinig, *J. Colloid Interface Sci.* **2020**, *574*, 430, https://doi.org/10.1016/j.jcis.2020.04.059, under Creative Commons CC-BY license.

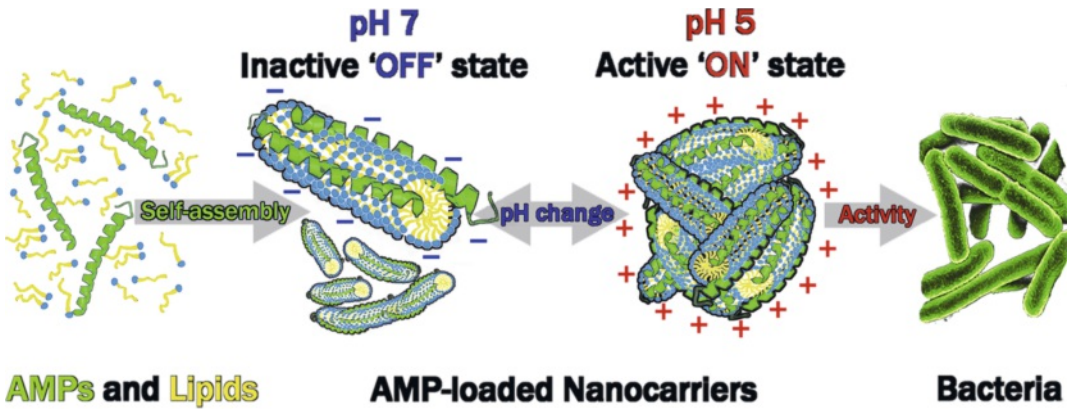

Fig. 2. Stimuli-responsive nanocarriers formed through the self-assembly of OA with LL-37. Colloidal transformations from core–shell cylindrical micelles at pH 7.0 to aggregates of branched thread-like micelles at pH 5.0 were detected. Antimicrobial *in vitro* assays using an *E. coli* bacterial strain showed high antimicrobial activity of the positively charged LL-37/OA aggregates at pH 5.0, which was not caused by the pH conditions themselves. In contrast, negligible antimicrobial activity was observed at pH 7.0 for the negatively charged cylindrical micelles.[14] Reprinted with permission from M. Gontsarik, A. Yaghmur, Q. Ren, K. Maniura-Weber, S. Salentinig, *ACS Appl. Mater. Interface* **2019**, *11*, 2821, https://doi.org/10.1021/acsami.8b18618. Copyright (2019) American Chemical Society.

phase transformations. For example, transforming from a slow releasing structure (inverse hexagonal phase) to the fast releasing cubic structure *via* a change in temperature,[26] or by modulating the pH to induce the electrostatic release of cargo.[27] In particular for the inverse hexagonal phase, the ends of the rod-like water channels in the lipid phase are capped, which impedes the release of polar cargo.[10g] Another unique characteristic of hexagonal phases is their ability to adhere strongly to mucosal linings, thus facilitating sustained absorption despite their slow release profile.[28] Controlling the phase behaviour of the system can subsequently restrict the release behaviour to specific conditions, which can thus improve the efficiency of the therapeutic effect and reduce the toxicity profile of the encapsulated content.

The highly-viscous bulk phases that are stable in excess solvent can be dispersed as low-viscosity particles, even at high concentrations.[29] This leads to so-called hierarchically organized systems, where the thermodynamically stable self-assembled phases are dispersed as particles that are kinetically stabilized in the excess solvent.[30] Using recent advances in *in situ* SAXS coupled to *in vitro* digestion models, these hierarchically organized particles were recently discovered to form naturally during the digestion of oil-in-water emulsions, including milk and mayonnaise.[16a–e] They possess a range of unique properties such as biocompatibility, ability to modulate release, and capacity to load different types of cargo, including hydrophobic and amphiphilic molecules in their internal water-channel structure.[20,31] In addition, based on the selection of the components and environmental conditions, these structures can be designed to respond to external stimuli such as pH[10c,11b,14,24d] and temperature.[30,32] They have been investigated as carriers for the delivery of several peptides and proteins such as insulin,[33] somatostatin,[34] and cyclosporine.[35] The nanostructured dispersions have also been shown to effectively deliver and transport drugs to and through the skin with no observed skin irritation.[36] Non-lamellar liquid crystalline nanoparticles have been used for the encapsulation of various AMPs, including LL-37.[10a,11,14] Intriguingly, LL-37 was observed to spontaneously integrate into the internal nanostructure of GMO- and OA-based nanoparticles when added to the aqueous phase of the dispersions (Fig. 3a).[10a,11a,b,14] The GMO/LL-37 micelles that resulted from the integration of LL-37 into GMO-based cubosomes were discovered to exhibit significant antibacterial activity, eliminating Gram-negative *E. coli* faster and more efficiently than free LL-37 (Fig. 3b).[10a]

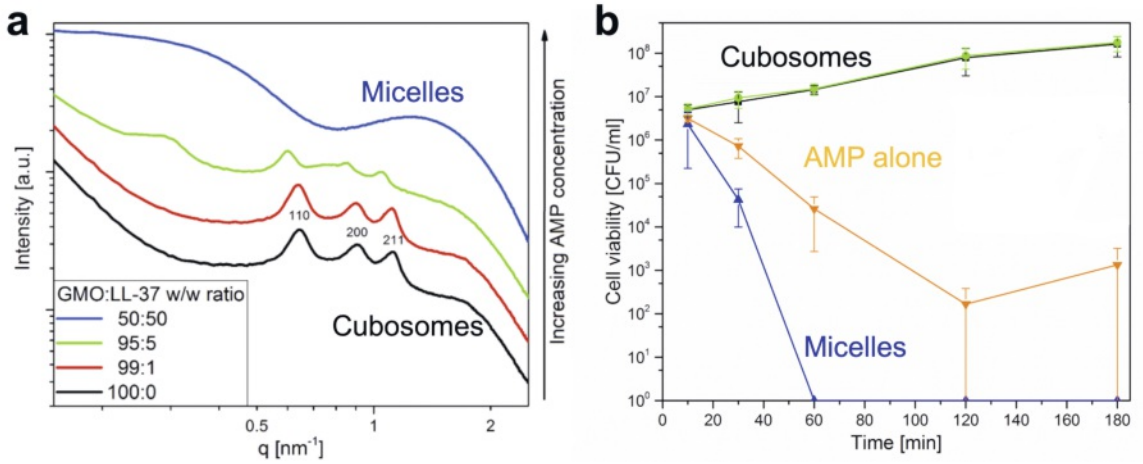

Fig. 3. a) SAXS patterns of the GMO-based samples with varying GMO/LL-37 w/w ratios at 25 °C. The composition-dependent change in lattice dimensions and transition from cubic (*Im3m*) structure to sponge and lamellar structures as well as normal micelles and small vesicles was observed;[10a] b) antibacterial activity against *E. coli* of different LL-37 nanocarrier formulations. The GMO/LL-37 (50/50) micelles showed significant antibacterial properties and killed more bacteria after 30 and 60 min compared with free LL-37. LL-37 formulated in cubosomes (at 95/5) showed no significant effect (ANOVA, $p < 0.05$). Also, unloaded cubosomes did not exhibit any antibacterial properties and were used as negative control.[10a] Reprinted with permission from M. Gontsarik, M. T. Buhmann, A. Yaghmur, Q. Ren, K. Maniura-Weber, S. Salentinig, *J. Phys. Chem. Lett.* **2016**, *7*, 3482, https://doi.org/10.1021/acs.jpclett.6b01622. Copyright (2016) American Chemical Society.

Usually, stabilizers in the form of amphiphilic proteins or triblock-copolymers such as the Pluronic F127 are added to kinetically stabilize the self-assembled nanoparticles in water. However, potential concentration-dependent cytotoxicity, hemolytic properties, and poor biodegradability of F127 may limit the clinical application of the stabilized nanoparticles.[37] We recently designed novel stabilizer-free antimicrobial nanocarriers based on GMO and LL-37 that are cytocompatible and promote cell proliferation for improved wound healing.[11a] Interestingly, antibacterial assays showed that these stabilizer-free systems exhibited higher antibacterial activity compared to the GMO/LL-37/F127 system. Also, the cell viability studies on human dermal fibroblasts (HDF) and THP-1 cells demonstrated that the stabilizer-free cubosomes and nanocarriers are nontoxic, contrary to the commonly used F127-stabilized systems that were found cytotoxic. In addition, cell proliferation assays on HDF cells showed that the GMO/LL-37 nanocarriers increase the cell proliferation by enhancing the cellular uptake of the LL-37.[11a]

3. Antimicrobial Surface Functionalization with Lipid-based Coatings

Lipid-based self-assembled films have been studied for substrate-mediated gene delivery,[38] as templates for *in situ* metal growth into periodic nanostructures,[39] and as host materials to incorporate nanoparticles with macroscopic alignment.[39a] Recently, our team demonstrated the design and application of lamellar and non-lamellar liquid crystalline coatings for the antimicrobial functionalization of surfaces.[40] A facile method was established to fabricate stable GMO-based coatings on surfaces by depositing GMO/ethanol mixtures using methods such as spin-coating or drop-casting; followed by solvent evaporation.[40] Synchrotron grazing-incidence small-angle X-ray scattering (GISAXS) and atomic force microscopy (AFM) showed that the resulting GMO-based coatings self-assemble into lamellar structures at low hydration with transformations to a fluid isotropic (sponge) phase followed by a second lamellar and the bicontinuous cubic structures of *Ia3d* and *Pn3m* type upon increasing hydration (see Fig. 4).[40b] The fully hydrated *Pn3m* structure with a lattice constant of around 9.6 nm could coexist in excess water, in thermodynamic equilibrium with the surrounding aqueous medium, which is important for biological applications such as targeted drug delivery from implant surfaces or wound pad materials. Kang *et al*. recently prepared GMO/1, 2-dioleoyl-3-trimethylammonium-propane (DOTAP) films on coverslip substrate. These films could adopt three different structures including lamellar, hexagonal and *Ia3d* structures with proper control of lipid compositions, temperature, and relative air humidity.[38,39] The films have been used for siRNA delivery and slight structural changes were observed compared to lipid films upon adding siRNA molecules into the lipid films *via* electrostatic interactions.[38,39,41]

Nylander *et al*. investigated the dynamics of the layers in the cubic (*Fd3m*) phase and inverse hexagonal phase on a silicon substrate surface.[42] The *Fd3m* layers exhibited more rigidity at the substrate interface compared to the inverse hexagonal phase layers. The mobility at the interface depended on both the distance from the supporting surface and the type of liquid crystalline phase. The finding has relevance for drug delivery and biomedical formulations, for example, in topical formulations the interfacial behaviour influences the degree of the bio-adhesion and retention of the particles at the intraoral mucosal surfaces.[42,43]

Recently, antimicrobial coatings were designed in our group through the integration of LL-37 into nanostructured GMO-based films (Fig. 5).[40a] Upon LL-37 integration, nanostructural transformations were discovered, similar to the solution-based system discussed above. This conceptual correlation between solution and surface-based self-assembled structures agrees with previous reports: The formation of the various liquid crystalline film structures such as inverse bicontinuous cubic (*Pn3m* and *Ia3d*),[40b,44] inverse hexagonal,[38,42] and inverse micellar cubic (*Fd3m*)[42] were reported that correlate well with the structures in the corresponding bulk or dispersed phase system.

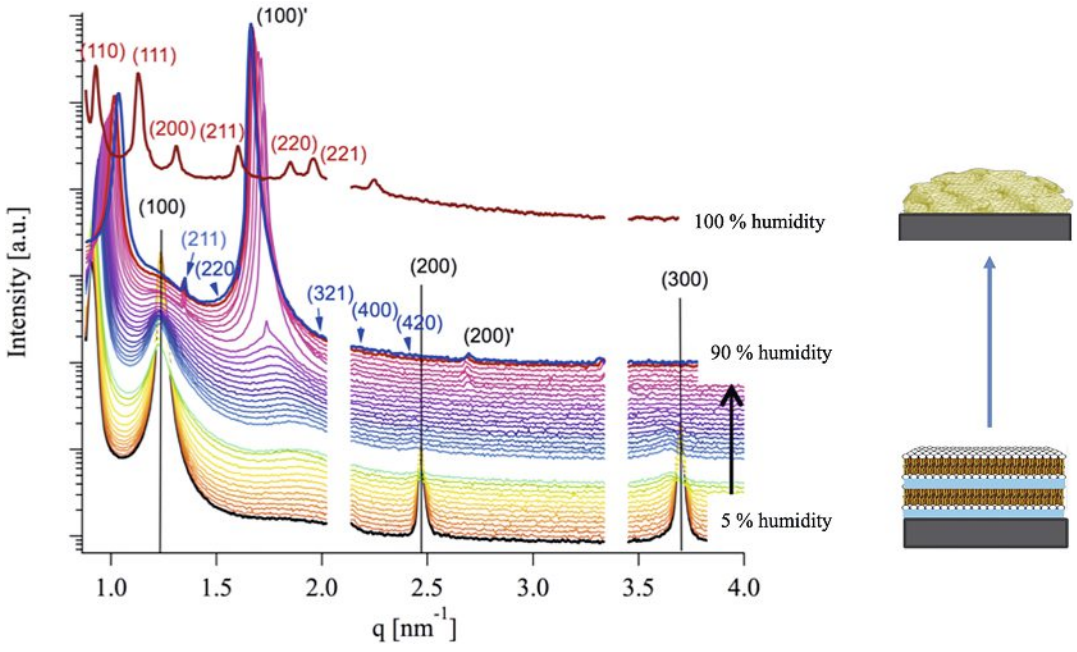

Fig. 4. Hydration-induced transformations in the internal nanostructure of the GMO coating on silicon surface studied using *in situ* GISAXS. Upon hydration of the GMO thin-film, a variety of liquid crystalline nanostructures ranging from fluid isotropic (sponge-) to lamellar and bicontinuous cubic phases were observed between 5 and 100% relative humidity. The identifiable Bragg peaks and further calculated theoretical peak positions are indexed with the corresponding Miller indices for the lamellar structure in black, corresponding to a d-spacing of approximately 3.7 nm; and the *Ia3d* and *Pn3m* type cubic structures in blue and red corresponded to lattice constants of 11.5 and 9.6 nm, respectively. The scheme presents an artistic view of the nanostructural transitions from lamellar to inverse bicontinuous cubic structure, in agreement with the GISAXS data. Adapted from S. Salentinig, M. Zabara, P. Parisse, H. Amenitsch, *Phys. Chem. Chem. Phys.* **2018**, *20*, 21903, https://doi.org/10.1039/C8CP03205J with permission from the PCCP Owner Societies.

The design of AMP-loaded liquid crystalline surface coatings that mimic colloidal structures from natural systems, as discussed for solution systems above, appears to be a convenient way for surface modification. Depending on the selection of molecules, the coatings can be food grade and biocompatible, as well as stimuli-responsive (Fig. 5). Stimuli-induced phase changes in these coatings, similar to the ones discussed in the solution part above, can be associated with distinct differences in the properties of these materials, making them a unique platform for various applications, including biosensing, nano-templating, and drug delivery.[39a,41]

For the rational design of such bio-inspired functional coatings, a detailed understanding of the interactions of the colloidal structures with the substrate as well as the correlation between structure and (biological) activity is key. However, so far, very little research has been published on these systems that require highly contemporary experimental methods for characterisation, as discussed below.

4. Methods for Nanomaterial Characterization

The characterization of self-assembled nanostructures in solution and on surfaces in their 'native' state can be a challenging task. As these systems are sensitive to environmental conditions such as temperature and hydration, methods need to be carefully selected to minimize interference of the experiment with the structure. Usually, these systems are studied using scattering methods (light, X-ray and neutron scattering) combined with imaging techniques such as cryogenic electron microscopy.[45] To study colloidal structures on surfaces, synchrotron GISAXS and bio-AFM are often applied to study the sample under native (hydrated) conditions or even under water using suitable sample environments.[40b]

Scattering methods are powerful tools for the characterization of nanostructures in solution and on surfaces as they are non-invasive and provide statistical information on the structures in the scattering volume. However, they are indirect methods and the interpretation of the data in real space can be a complex task. In this context, the indirect Fourier transformation method (IFT) provides model-free information on the form factor describing the shape, size and morphology of the particles.[46] The generalized IFT (GIFT) method simultaneously considers a potential structure factor contribution to the signal, resulting from concentration-dependent interparticle interactions.[47] Model-dependent fitting of the form factor scattering are also commonly applied. The theoretical scattering curve for a specific geometry is calculated, and parameters such as size, electron densities and morphology can be optimized to obtain the best possible fit to the experimental data.[48] Static light scattering is a technique similar to SAXS and neutrons, however, it is best suited for the characterization of larger (micron-sized) objects such as emulsions due to the much longer wavelength used.[49] Dynamic light scattering (DLS) has no such size restriction and can be used throughout the entire colloidal regime. It measures the collective diffusion of the colloidal system and is mostly used for particle sizing. The diffusion behaviour is also influenced by particle interactions and gives independent access to these effects.[47a] The study of dynamic self-assembly processes is only possible through recent advances in experimental techniques and sample manipulations that provide access to the mesoscopic length scale with a high spatio-temporal resolution. Examples of these processes include the response of self-assembled systems to temperature or pressure jumps or the study of enzyme-triggered nanostructure formation such as during digestion. Additionally, online time-resolved SAXS at high intensity synchrotron sources, as well as combining the technique with microfluidics, can be used for the real-time monitoring of dynamic structural alterations within fractions of seconds.[50] *In situ* GISAXS, potentially combined with custom-made sample environments such as solvent cells, is a crucial technique to gain insights into the nanostructure formation and transformation on surfaces.[40b,51] However, scattering techniques are statistical methods providing an average over all structures in the scattering volume.

The determination of coexisting structures requires the application of techniques that can resolve the characteristics of individual nanoparticles in solution. Here, electron microscopy techniques such as cryogenic transmission or scanning electron microscopy (cryo-TEM/cryo-SEM) are integral approaches to gaining further insight into the morphology and can distinguish the possible coexistence different colloidal structures in solution. The application of cryo-TEM to study lipid nanoparticles has been reviewed recently.[52] The coupling of these biophysical methods with biochemical assays and advanced *in vitro* models biological assays is crucial to bridge the colloidal structure to the biological activity. In our team , we combined the *in vitro* intestinal digestion model with integrated cell co-culture model and also online SAXS and we could demonstrate the *in situ* formation of the nanostructures during the digestion of food emulsions.[16a] We also used the combination of the biophysical evaluation studies using methods such as SAXS, cryo-TEM, ellipsometry and AFM with antimicrobial assays on clinically relevant bacterial strains which led to the design and development of novel antimicrobial nanocarriers and coatings discussed in this contribution.

Acknowledgments

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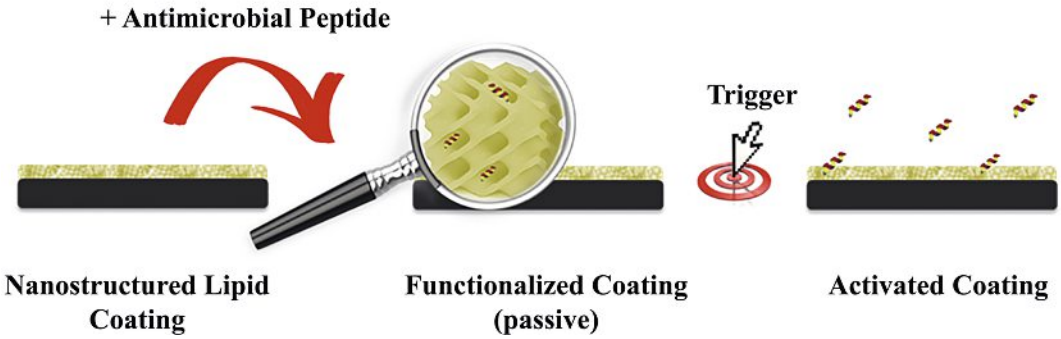

Fig. 5. Antibacterial coatings based on the co-assembly of selected lipids and antimicrobial peptides. These nanostructured coatings can be functionalized with an 'on/off' switch for their antimicrobial activity.[40a] Stimuli such as temperature, pH, humidity or bacterial metabolites can trigger the activity.

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