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Caselli, Lucrezia; Nylander, Tommy; Malmsten, Martin

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Neutron reflectometry as a powerful tool to elucidate membrane interactions of drug delivery systems

Lucrezia Caselli a, Tommy Nylander a, Martin Malmsten a,b,*

a Physical Chemistry 1, Lund University, S-221 00 Lund, Sweden
b Department of Pharmacy, University of Copenhagen, DK-2100 Copenhagen, Denmark

1. Introduction

The last couple of decades have seen an explosion of novel colloidal drug delivery systems, which have been demonstrated to improve drug efficacy, reduce side-effects, and provide various other advantages for both small-molecule and biomacromolecular drugs. The interactions of delivery systems with biomembranes are increasingly recognized to play a key role for efficient eradication of pathogens and cancer cells, as well as for intracellular delivery of protein and nucleic acid drugs. In parallel, there has been a broadening of methodologies for investigating such systems. For example, advanced microscopy, mass-spectrometric “omic”-techniques, as well as small-angle X-ray and neutron scattering techniques, which only a few years ago were largely restricted to rather specialized areas within basic research, are currently seeing increased interest from researchers within wide application fields. In the present discussion, focus is placed on the use of neutron reflectometry to investigate membrane interactions of colloidal drug delivery systems. Although the technique is still less extensively employed for investigations of drug delivery systems than, e.g., X-ray scattering, such studies may provide key mechanistic information regarding membrane binding, re-modelling, translocation, and permeation, of key importance for efficacy and toxicity of antimicrobial, cancer, and other therapeutics. In the following, examples of this are discussed and gaps/opportunities in the research field identified.

A B S T R A C T

The last couple of decades have seen an explosion of novel colloidal drug delivery systems, which have been demonstrated to increase drug efficacy, reduce side-effects, and provide various other advantages for both small-molecule and biomacromolecular drugs. The interactions of delivery systems with biomembranes are increasingly recognized to play a key role for efficient eradication of pathogens and cancer cells, as well as for intracellular delivery of protein and nucleic acid drugs. In parallel, there has been a broadening of methodologies for investigating such systems. For example, advanced microscopy, mass-spectrometric “omic”-techniques, as well as small-angle X-ray and neutron scattering techniques, which only a few years ago were largely restricted to rather specialized areas within basic research, are currently seeing increased interest from researchers within wide application fields. In the present discussion, focus is placed on the use of neutron reflectometry to investigate membrane interactions of colloidal drug delivery systems. Although the technique is still less extensively employed for investigations of drug delivery systems than, e.g., X-ray scattering, such studies may provide key mechanistic information regarding membrane binding, re-modelling, translocation, and permeation, of key importance for efficacy and toxicity of antimicrobial, cancer, and other therapeutics. In the following, examples of this are discussed and gaps/opportunities in the research field identified.
applies particularly for synchrotron-based methods, by which detailed structural information can be obtained for wide ranges of systems in a high throughput manner, also neutron scattering methods are gaining increased recognition as powerful tools for investigating colloidal systems in drug delivery. Among different neutron-based methodologies, focus will here be placed on neutron reflectometry (NR) which is gaining increased appreciation for elucidating structural and compositional aspects of interfacial layers. In the broader context of biological membranes, NR may provide detailed information on key functions of biological membranes, including, e.g., signal transduction, cell adhesion, and membrane-localized processes in diseases such as atherosclerosis, Alzheimers disease, and Parkinsons disease, as described in excellent previous reviews, covering also methodological aspects of NR in some detail [7-10]. In addition, NR is increasingly recognized as a powerful tool for investigations into membrane interactions of drugs and drug delivery systems.

2. Methodological considerations

In NR, a collimated neutron beam is reflected at a macroscopic interface and the reflected beam is monitored as a function of wavelength or angle [11-13] (Fig. 1A). From such experiments, information can be obtained about the composition and structure of thin films. In contrast to light reflection, which depends on the refractive index

\[ \text{NR} = \text{incident wave} \times \text{reflected wave} \times \text{refractive index} \]

can be obtained about the composition and structure of thin films. In contrast to light reflection, which depends on the refractive index. In NR, the ratio between the reflected and the incident beam is monitored versus wavelength and/or angle. The data obtained can be modeled by standard optical approaches, where the neutron refractive index can be obtained from the sum of scattering lengths of elements i (b_i, available in literature for all stable isotopes) present in a volume (V).

Based on this, multilayer models are generally constructed assuming uniform thickness (sometimes allowing for roughness), structure, and composition with each slab. By simultaneously fitting reflectivity curves for the same system at different solvent contrasts (and/or using deuterated compounds), detailed structural information can be obtained regarding properties of the multilayer model. For supported lipid bilayers, information that may be obtained in this way include, e.g., the thickness of the entire bilayer or individual thicknesses of the two bilayer leaflets, the thickness of the core acyl chain and the headgroup.

![Fig. 1.](image-url)

(A) Schematic representation of a typical NR experiment for a lipid bilayer on a silicon substrate, ki and kr are the incident and reflected wave vectors, \( \vartheta \) the incident angle, and \( q_z \) is the component of the momentum transfer perpendicular to the interface. (B) Principle of contrast matching for hydrogenated and deuterated lipid bilayers adsorbed on a silicon substrate in three different solvents, i.e., 100% H\(_2\)O (SLD = -0.56·10\(^{-6}\) Å\(^{-2}\)), Contrast-Match Silicon (CMSi, SLD = 2.05·10\(^{-6}\) Å\(^{-2}\)) and 100% D\(_2\)O (SLD = 6.35·10\(^{-6}\) Å\(^{-2}\)). (C) Simulated NR profiles and corresponding SLD for a hydrogenated phospholipid membrane adsorbed on a silicon substrate in different solvents (100% D\(_2\)O, 68.3% D\(_2\)O (CMSi), 37.5% D\(_2\)O (Contrast-Match SiO\(_2\)), CMSiO\(_2\)) and 100% D\(_2\)O. The interfacial structure was modeled with a multilayer model, accounting for the following layers (each of them with uniform thickness and SLD, and roughness = 4 Å): (1) Si (SLD = 2.05·10\(^{-6}\) Å\(^{-2}\)), (2) SiO\(_2\) (SLD = 4.19·10\(^{-6}\) Å\(^{-2}\), thickness = 4 Å), (3) intermediate solvent layer (SLD of 6.35·10\(^{-6}\) Å\(^{-2}\) (100% D\(_2\)O), 4.19·10\(^{-6}\) Å\(^{-2}\) (68.3% D\(_2\)O), 2.05·10\(^{-6}\) Å\(^{-2}\) (37.5% D\(_2\)O) and -0.56·10\(^{-6}\) Å\(^{-2}\) (H\(_2\)O), thickness = 2 Å), (4) lipid polar headgroups inner leaflet (SLD = 2.17·10\(^{-6}\) Å\(^{-2}\), thickness = 7.5 Å), (5) lipid hydrophobic chains (SLD = -0.56·10\(^{-6}\) Å\(^{-2}\), thickness = 32 Å), (6) lipid polar headgroups outer leaflet (SLD = 2.17·10\(^{-6}\) Å\(^{-2}\), thickness = 7.5 Å) and (7) solvent solution (same SLD as above).
regions, and the thickness of any solvent layer between the bilayer and the underlying surface. In addition, since interfacial excess of a compound can be calculated from the scattering length density \( SLD = \Sigma b_i/\nabla \) obtained from such models, information on the composition can be obtained as well [13] (Fig. 1C). This, in turn, provides information of, e.g., bilayer defects, headgroup hydration, or binding of solutes such as drugs and drug delivery system components.

Another advantage of NR is that neutrons readily penetrate most materials of interest in biological systems, allowing buried interfaces to be investigated. This is a key feature for investigations of multilayer systems, such as biological membranes [14,15]. Related to this, various substrates offering the smoothness necessary for NR investigations (e.g., silicon and quartz) are effectively transparent to neutrons and can be used as substrates onto which supported bi- or multilayers can be formed. Furthermore, neutron beam damage is generally much smaller than radiation damage and heating induced by X-rays, making neutron-based techniques suitable to studies of sensitive biological systems, including membranes.

In the analysis of NR results for supported bilayers, the simplest level of analysis assumes that the layer can be described by two parameters only, i.e., the total (uniform) thickness and the (average) SLD (Fig. 1). A roughness (smaller than the thickness of each layer) is frequently introduced at each interface as the next level of complexity. For multicomponent systems, however, this level of analysis is not sufficient. For such systems, simultaneous fitting of multiple reflectivity profiles, obtained at different contrast for one or several of the components, are generally done [15] assuming isotope effects to be small as depicted in Fig. 1. Depending on the system investigated, additional constraints can be imposed, e.g., requiring the number of headgroups and tails to be the same, the two leaflets of bilayers to have the same thickness and SLD, the area of headgroups to be the same as that of the corresponding acyl chains, or other restrictions that make physical sense in the specific system. Further restrictions in the fitting procedure may include, e.g., fixing parameters to values obtained from other experimental methods. In yet other cases, restrictions may be imposed based on either theoretical models or results from numerical simulations of the system at hand.

3. Membrane models for NR studies

The first step in investigations of biological membranes by NR involves the formation of mono- or bilayers supported on a smooth interface/surface. Most frequently, NR investigations of membrane models involve phospholipid bilayers supported by a solid substrate. During the last couple of decades, a wide range of phospholipid bilayer model membranes have been reported, covering different: (i) bilayer geometry, (ii) number of bilayers, and (iii) bilayer order and phase. Composition-wise, the simplest models include phospholipids only, for which acyl chain and headgroup properties are varied. Acyl chain lengths frequently investigated are C16 and C18 with various degrees of unsaturation to mimic cell membranes, or C14 for convenient bilayer formation. Acyl chains shorter than this are more rarely investigated since the finite solubility of such phospholipids as well as packing considerations preclude formation of stable bilayers. Acyl chains longer than C16-C18 generally form a gel phase under conditions relevant for biological systems. For such systems, single-component composition and saturated chains favor the formation of ordered structures, whereas lipid mixtures and/or unsaturated acyl chains favor bilayer fluidity. With regards to polar headgroups, phosphatidylcholine (PC) is commonly used among zwitterionic phospholipids, as is phosphatidylethanolamine (PE). Among anionic phospholipids, phosphatidylserine (PS) is frequently investigated, although phosphatidic acid (PA), phosphatidylinositol, and others have been used as well, generally as a minor component in mixtures with zwitterionic phospholipids. In contrast to zwitterionic and anionic phospholipids, cationic phospholipids are more rarely investigated in membrane research. For all these systems, cholesterol is a frequent addition, while studies involving ergosterol and other sterols are less frequent [7–10,16].

As will be exemplified below, such model systems have been frequently found to be able to provide information of membrane interactions of molecules and nanoparticles that are relevant also for real cell membranes. That said, simple systems are unable to capture more delicate aspects of membrane function, such as those depending on asymmetry between membrane leaflets or on local membrane curvature (e.g., the function of membrane proteins and protein pores). Considering this, efforts have been undertaken for the design of membranes more closely mimicking the full cell membrane, e.g., by utilizing mixtures of lipids extracted from cells. Furthermore, attempting to address limitations of simpler models regarding compositional asymmetry of bilayers, as well as effects of the underlying substrate on supported bilayers, so-called floating bilayers [17] have attracted interest for NR studies of lipid membranes. In such membranes, a bilayer is “floating” a few nm away from the substrate surface and is thus in contact with water on both sides. This, in turn, allows bilayer hydration and structural fluctuations. It also allows the composition of each bilayer leaflet to be selected separately. Particularly for studies of transport over lipid membranes enabled by membrane proteins, floating bilayers offer opportunities for studies not provided by bilayers supported directly by an underlying substrate. Since the pioneering work of Sackmann et al. [18], several approaches have been devised for designing floating bilayers, including grated layers [19], polymer [20] and polyelectrolyte cushions [21], as well as tethered bilayers [22]. An interesting approach to spontaneously form cushioned model membrane was presented Gerelli et al. [23]. By using NR these authors showed that exposing a POPC/POPS supported lipid bilayer for a solution the intrinsically disordered protein histatin 5 leads to the formation of a protein cushion underneath the bilayer. It should be noted, however, that floating bilayers are generally less robust than bilayers supported directly on solid substrates, meaning that they can only form in some lipid systems, and that their use is more restricted for studies involving, e.g., changes in ionic strength or the presence of surface-active compounds and nanomaterials (Fig. 2).

Irrespective of the degree of complexity chosen for the membrane model, the aim is to mimic key aspects of the full biological membrane. Composition-wise, human cell membranes are dominated by zwitterionic phospholipids (e.g., PC, PE, and sphingomyelin (SM), and cholesterol, but contain also anionic PA [24,25] (Fig. 3A). In contrast, bacteria membranes of bacteria are rich in anionic phospholipids such as phosphatidylglycerol (PG) [25–27], and do not contain cholesterol. Fungal membranes, in turn, contain ergosterol instead of cholesterol. Furthermore, membrane structure differs between human cells and bacteria, as well as between different bacteria. Thus, Gram-negative bacterial walls consist of two phospholipid membranes with a peptidoglycan layer in between. The outer membrane (OM) is dominated by anionic lipopolysaccharide (LPS) (Fig. 3B). In contrast, Gram-positive bacterial walls consist of a single phospholipid membrane surrounded by a thicker peptidoglycan layer and contain no LPS (Fig. 3C). Viruses, in turn, contain a nucleic acid core, coated by a protein capsid and frequently surrounded by a lipid coat shed from membranes of the host cells on virus passage through these membranes (Fig. 3D).

Considering the stratified nature of bacterial membranes, and the importance of the OM in Gram-negative bacteria for protecting these bacteria against antimicrobials, more complex models of bacterial membranes have been devised and investigated by NR. The OM of Gram-negative bacteria is characterized by a pronounced asymmetry, where the outer leaflet is dominated by negatively charged LPS molecules, the packing of which is promoted by Ca2+ and Mg2+ cations. As a result of this, the OM is much less permeable to hydrophobic compounds than phospholipid bilayers [28–30]. Considering the asymmetry of the OM, as well as its importance for the biological function of bacteria and for antimicrobial resistance, efforts have been directed to better capture these features in membrane models of increasing complexity. Starting with the simplest
system of the OM, i.e., LPS monolayers, NR has been employed for studies of such systems to monitor the structuring of its hydrophobic lipid A domain and of its core oligosaccharide region, as well as of the conformation of the outer O-antigen moieties of smooth LPS [31]. A next level of complexity can be obtained by initial Langmuir-Blodgett deposition of a deuterated phospholipid monolayer, and subsequent Schaefer deposition of hydrogenous LPS for an isotopically asymmetric bilayer. Employing NR, retained asymmetry has been demonstrated for both lipid A and LPS [32], as has the loss of structural integrity caused removal of divalent cations [33].

Asymmetric model OM bilayers have been employed in NR in interactions of such systems with antibiotics of the polymyxin family. For example, Han et al. employed phospholipid/lipid A bilayers to study effects of lipid A acylation on membrane interactions of Polymyxin B [34]. Subsequently, the same group compared the mode-of-action of the antimicrobial drugs Polymyxin B and octapeptin A3 [35]. Octapeptin A3, but not Polymyxin B, was found to be able to penetrate the OM model, in agreement with findings on in vivo efficacy of the former peptide against Gram-negative pathogens resistant to Polymyxin B. Furthermore, Paracini et al. investigated the origin of the temperature-dependent bactericidal activity of Polymyxin B [36]. By combining NR with infrared spectroscopy, the membranolytic effect of Polymyxin B was demonstrated to relate to the liquid-gel transition of the LPS leaflet, demonstrating the biological relevance of the LPS phase transition. Extending OM membrane complexity even further, Clifton et al. reported on the deposition of such asymmetric phospholipid/LPS bilayers on top of a thin water cushion outside a thiolipid monolayer chemically bound to a gold substrate [37].

Given the extensive use of deuterated compounds in NR for achieving selective or multiple contrasts, access to deuterated material is of major importance. Importantly, therefore, a wide range of phospholipids are now commercially available or can be obtained from organic synthesis providers, allowing deuteriation of both acyl chains and headgroups [38]. In addition, an increasing range of deuterated lipids are becoming available through efforts by deuteration laboratories at the large-scale neutron facilities, as well as by independent research groups, to set up bacteria-, cell-, or yeast-based production of deuterated lipids and their hydrogenous equivalents. As discussed further below, the field has also seen increased efforts towards the development of more complex lipid mixtures extracted from cell, yeast, and bacteria membranes [39].

4. Membrane interactions of drugs

Before discussing membrane interactions of drug delivery systems, it is useful to consider membrane interactions of drugs in the absence of drug carrier. Since drug delivery systems release their drug cargo over time, membrane interactions of drug delivery systems generally depend on membrane interactions of both the drug carrier and of released drug, which vary over time. To decipher such effects, studies of membrane interaction of drug delivery systems should ideally be performed over time. When possible, control experiments are also needed on membrane interactions of the empty drug carriers, as well as of the free drug. Unfortunately, there are so far only a small number of such studies available in literature. Nevertheless, studies of membrane interactions of drugs in the absence of drug carrier represents a natural starting point for more extensive studies of drug delivery systems. For drugs gaining their biological effects from membrane interactions (e.g., some classes of antimicrobials), or for which the biological effects rely on membrane effects (e.g., RNA or other drugs having intracellular targets), such
studies may furthermore provide information of importance for understanding the pharmacological effects of the drug.

Exemplifying NR studies of membrane interactions of drugs in the absence of carrier, Foglia et al. combined NR with Langmuir film balance studies and Brewster angle microscopy for investigations of membrane interactions of the antifungal drug Amphotericin B (AmB) [40]. In doing so, a monolayer model of palmitoyloleoylphosphatidylcholine (POPC) in the presence of cholesterol (abundant in human membranes but not in fungal ones) or ergosterol (abundant in fungal membranes but not in human ones) was used. Langmuir monolayer studies showed AmB to cause faster and larger changes in surface pressure for fungi-mimicking POPC/ergosterol than for POPC or human cell-mimicking POPC/cholesterol membranes. Despite this, AmB insertion similarly for the three systems, with its macrocyclic ring located between the lipid acyl chains. However, membrane insertion was much faster for POPC/ergosterol than for POPC and POPC/cholesterol, and the effects on the surface pressure were larger. The latter were related to different in-plane structures, inferred to be key for AmB selectivity towards fungal cell membranes. Similarly, de Gellinck et al. used supported lipid bilayers to investigate AmB interaction with membranes derived from Pichia pastoris yeast and the influence of ergosterol, known to be important for AmB activity [41] (Fig. 4). In the absence of ergosterol, AmB was found to insert in yeast membranes and to form an outer layer of 4–5 nm thickness. In the presence of ergosterol, on the other hand, AmB insertion was accompanied by ergosterol extraction, resulting in membrane thinning, the extent of which depended on the degree of lipid unsaturation. As membrane thinning, and resulting hydrophobic mismatch, may interfere with the function of membrane proteins, these results were inferred to suggest that AmB potency is boosted by the presence of polyunsaturated lipids, and that low degrees of unsaturation could protect fungal cells from AmB.

Within the area of cancer therapeutics, Matyszewska et al. employed NR, Langmuir monolayer studies, and Brewster microscopy to investigate interactions of the two anticancer drugs doxorubicin (Dox) and idarubicin (IDA) with monolayers of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylserine (DMPS), as well as a mixture of these [42]. DMPC was selected as a model for healthy human cell membranes, whereas DMPS was included due to the higher presence of anionic phospholipids in the membranes of cancer cells. It was found that Dox displayed considerably higher affinity for anionic DMPS than for zwitterionic DMPC monolayers. Furthermore, smaller amounts of drug could be retained within DMPC monolayers at increasing surface...
pressure, compared to DMPS, before drug-lipid aggregates were expelled. The structures induced on drug incorporation into the membranes were furthermore found to differ between the two drugs, where Dox bound to the interfacial headgroup region of the monolayer and induced aggregation, while the more hydrophobic IDA located in the monolayer acyl region.

Relating to therapeutics to combat bacterial infections, considerable attention has been directed to antimicrobial peptides (AMPs) during the last few decades. AMPs are amphiphilic molecules, typically consisting of 10–40 amino acid residues, being net cationic and containing a sizeable fraction of hydrophobic amino acids. While AMPs may reach antimicrobial effects through different modes-of-action, a key effect is that of destabilization of bacterial membranes [43]. Therefore, membrane interactions of AMPs have attracted considerable attention [44]. For example, Gong et al. combined small-angle neutron scattering (SANS) with stochastic optical reconstruction microscopy (STORM) and molecular dynamics (MD) simulations to explore membrane interaction of the AMP G(IIKK)3-I-NH2 (G3) and a less hydrophobic variant of G3, KI(KKII)2-I-NH2 (KI) [45]. As expected, KI was found to display minimum inhibition concentrations (MICs) much higher than those of G3. Furthermore, a more hydrophobic peptide variant, G(ILKK)3-L-NH2 (GL), displayed higher toxicity than G3 but without further MIC suppression. In parallel, studies of model membranes consisting of lipid monolayers using NR and Brewster angle microscopy, as well as STORM for real bacterial membranes, demonstrated weaker membrane interaction of KI, differences in membrane distribution between G3 and GL, as well as the formation of membrane-inserted peptide aggregates for G3. (Fig. 5).

Together, these results elegantly demonstrate how investigation in model lipid mono- and bilayers can be combined with those on full bacterial membranes to allow for a mechanistic understanding of membrane destabilization and antimicrobial effects of AMPs.

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Fig. 5. Effects of AMP sequence on interactions with DPPG membranes for the AMP G3 and a derivative of this peptide with weaker hydrophobicity (K1). (A) Bacterial killing ability of G3 as compared to control antibiotics, indicating high G3 killing efficiency against different bacterial strains. (B) Hemolytic activity of G3 and K1, showing that K1 exhibited considerably lower membranolytic activity than G3. (C) MD simulations showing weak affinity of G3 for DPPC membranes, but high electrostatically-driven interaction with DPPG bilayers. (D) MD results reporting on G3 insertion into DPPG bilayers, showing preferential localization between the outer polar headgroups and acyl tails. (E) NR binding results showing a condensed monolayer. Mirroring this, no binding was observed by NR, Langmuir balance studies, and fluorescence microscopy. Langmuir film balance results indicated association of short single-stranded (ss) RNA with liquid expanded DPPC monolayers, whereas no such association was detected for double-stranded (ds) RNA and dsDNA. NMR in turn, showed that ssRNA adsorbed to and penetrated liquid expanded monolayers, whereas no RNA penetration was observed for the liquid condensed monolayer. Mirroring this, no binding was observed by NR for dsDNA to zwitterionic monolayers, suggesting such binding to be driven largely by hydrophobic interactions (Fig. 6).

Addressing membrane investigations of NA-loaded LNPs, Michanek et al. investigated NA association with zwitterionic phospholipids [62]. Among NAs, particular focus on LNP composition are emerging. Among NAs, particular interest lies on RNA (ranging from small siRNA to large messenger RNA (mRNA)) rather than DNA, since RNA “only” needs to reach the cytosol for therapeutic effect, whereas DNA for traditional gene therapy requires its uptake into the cell nucleus. Apart from the RNA cargo, LNPs generally consist of cationic ionizable lipids, helper lipids, cholesterol, phospholipids, and poly(ethylene glycol)-conjugated (PEGylated) lipids. A key feature of LNP composition is that of the level of encapsulated RNA, since even the most efficient LNPs currently delivers only about a couple of percent of NA to the site-of-action. Furthermore, to allow for uptake through the external cell membrane, it is key that the NA-loaded LNPs are small and with the NA fully encapsulated. Due to the gradual acidification of the endosomes, as well as the requirement of RNA molecules reaching the cytosol for a biological effect, it is also key that the LNPs are able to escape from the endosomes.

A key feature of endosomal trafficking is that of a gradual acidification, which can be employed by including ionizable lipids which are neutral at blood pH (advantageous for suppressing toxicity prolonging bloodstream circulation time) but cationic at acidic pH. Such lipids promote binding to the endosomal membrane and trigger their rupture at decreasing pH, thus facilitating endosomal escape. Furthermore, PEGylated lipids are included to allow formation of small LNP particles, to improve their colloidal stability, and to reduce serum protein adsorption, macrophage uptake, and clearance from bloodstream circulation. While PEGylated lipids provide advantageous functions to LNPs by preventing unwanted serum protein adsorption and opsonization, they may also interfere with payload delivery. For example, the binding of ApoE, a key part of the uptake of LNPs to hepatocytes, is suppressed by PEG-lipids on the LNP surface [53]. Furthermore, endosomal escape is suppressed by the PEG corona or LNPs precluding LNP binding to the endosomal membrane [54]. To address these shortcomings, as well as to suppress the immunogenicity related to permanently bound PEG-lipids [55,56], the latter can be designed to detach from the particle controlled, e.g., by the size of their hydrophobic anchor domain [57,58]. Even with detachable PEG-lipids, however, endosomal escape remains a major challenge in NA delivery. For example, it has been found that even if uptake through the external cell membrane is very efficient, such as in the case of CM3:DSPC:Cholesterol:DMPE-PE (showing an endocytosis efficiency of 95% after 6 h), ~2-3% of the RNA is able to escape the endosome to reach the cytosol [59]. Here, the choice of the cationic ionizable lipid used has been found to strongly influence the extent of endosomal escape and subsequent RNA release into the cytosol payload, e.g., by influencing the stability of complex salts formed together with RNA at acidic pH [60,61].

Addressing membrane investigations of NA-loaded LNPs, Michanek et al. investigated NA association with zwitterionic phospholipids [62]. In particular, effects of NA length and base pairing, as well as challenges to escape the acidifying endosomes during endocytosis. Consequently, delivery systems for siRNA, as well as for other types of NAs, have attracted interest during the last couple of decades. From this, LNPs have emerged as a particularly powerful type of delivery system for such drugs. Illustrating this, the first LNP-based siRNA product (ONPATTRO™) has already been approved for the treatment of amyloidosis. Following this success, siRNA-loaded LNP systems have been investigated for a range of indications including, e.g., cancer, viral infections, and neurological disorders [52].

From investigations of LNPs as NA delivery systems in both therapeutic and vaccines (including COVID-19 vaccines), some general features on LNP composition are emerging. Among NAs, particular interest lies on RNA (ranging from small siRNA to large messenger RNA (mRNA)) rather than DNA, since RNA “only” needs to reach the cytosol for therapeutic effect, whereas DNA for traditional gene therapy requires its uptake into the cell nucleus. Apart from the RNA cargo, LNPs generally consist of cationic ionizable lipids, helper lipids, cholesterol, phospholipids, and poly(ethylene glycol)-conjugated (PEGylated) lipids. A key feature of LNP composition is that of the level of encapsulated RNA, since even the most efficient LNPs currently delivers only about a couple of percent of NA to the site-of-action. Furthermore, to allow for uptake through the external cell membrane, it is key that the NA-loaded LNPs are small and with the NA fully encapsulated. Due to the gradual acidification of the endosomes, as well as the requirement of RNA molecules reaching the cytosol for biological effect, it is also key that the LNPs are able to escape from the endosomes.

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Moreover, Gilbert et al. employed NR and QCM-d to investigate the interaction between NA and supported DOPC layers containing the cationic ionisable lipid (CIL) DLin-MC3-DMA (MC3) as a function of pH,
MC3 concentration and type of NA [63]. Lower pH and increased MC3 bilayer content were shown to result in an increased adsorption of erythropoietin mRNA, polyadenylic acid (polyA), and polyuridylic acid (polyU). The effect on the lipid layer structure, as revealed by NR, could be linked to the NA secondary structure. Thus, under conditions where polyA was double stranded, it adsorbed to the bilayer surface. For other conditions and NAs, NA penetration into the bilayer and even formation of multilayers was observed. MD simulations of the effect of the NA primary structure revealed a stronger interaction between polyA and the bilayer surface and therefore a more tightly bound and condensed structure in the charged lipid systems, leading to dehydration of DOPC. A weaker interaction was observed for the NAs and the neutral form of MC3, resulting in a less compact layer.

Furthermore, Spada et al. employed reflectometry-based techniques to investigate the interactions of NA-loaded LNPs with model endosomal monolayers [64]. Lipid transfer from the LNPs to the endosomal membrane was found to be most pronounced at pH 5.5–6.5. While LNPs bound to these membranes also at higher pH, lipid insertion was suppressed, indicating pH to be important for LNP fusion during endosomal escape (Fig. 7). Independent of pH, however, early- and late-stage endosomal membranes showed similar results, suggesting the increased fluidity and anionic content of late-stage endosomes to be less important for LNP interaction. Furthermore, delivery was found to be more efficient from mRNA-loaded LNPs than from Poly(A)-loaded ones, demonstrating that the NA sequence affects such membrane interactions. Also addressing membrane interactions in endosomal escape, Sebastiani et al. employed SANS for investigations into the structure and distribution of LNPs in the presence of apolipoprotein E (ApoE), a key component of blood [65]. It was found that di-tearoylphosphatidylcholine (DSPC) and cholesterol were both enriched at the LNP surface in buffer solution, but that binding of ApoE to the LNPs induced lipid re-distribution. The latter was suggested to trigger a change in the internal LNP structure, causing release of mRNA.

6.1. Cubosomes

Larsson & Buchheim first reported how to prepare partially-stabilized fragmented non-lamellar, cubic phase particles from monacylglycerols with bile salts or caseins [66,67]. It was later shown by Larsson et al. that the dispersions of these non-lamellar lipid crystalline lipid phases, named Cubosomes, could be refined by adding amphiphilic polymers [68–70]. It is important to note, that unlike the LNPs, these particles are in equilibrium with water and retain the internal structure of the bulk-phase in excess water and the dispersant add to their colloidal stability. As a result of these earlier studies, cubosomes and related structures have attracted growing interest for cargo transport in drug delivery [71,72]. The lipid bilayer in the inverse bicontinuous phases curves to form water channels in a three-dimensional network [66]. The systems of water channels allow water-soluble substances to be encapsulated with the possibility to be transported in and out of the structure, while the continuous lipid bilayer allows solubilisation of hydrophobic compounds. The large internal surface area allows high loading of amphiphilic drugs or loading of protein drugs requiring the presence of an oil/water interface for retained activity. Addressing membrane interactions of empty cubosomes, Vandoolaege et al. used a range of surface sensitive techniques such as ellipsometry, QCM-d, and
NR to provide insight into the mode of interaction as well as the kinetics of the process mechanism of glycerol monooleate (GMO) based cubosomes with a DOPC supported bilayer [73,74]. The use of QCM-d and ellipsometry allowed capturing the initial kinetics of the process, characterized by an initial rapid increase in the adsorbed amount, i.e., strong affinity of the cubosomes to lipid bilayer, followed by a decrease after about an hour, signifying a net release of material (i.e., cubosomes), from the surface. NR measurements revealed the composition of the initially deuterated phospholipid bilayers versus time after LCNP addition. The lipid bilayer is composed of about 72% of the cubosome components at steady state with very few residual intact cubosomes at the interface. The results could be related to phase behavior of the DOPC/GMO/aqueous system. Similarly, Shen et al. reported on the interaction of phytantriol-based cubosomes with POPC bilayers [75]. NR and QCM-d results indicated that the cubosomes bound to the POPC bilayer. Furthermore, NR results showed the cubosomes to cause a significant increase in hydrogenous cubosome-originating material within the POPC bilayer. Analysis of the NR results demonstrated cubosome penetration into the POPC bilayers to cause surfactant–lipid exchange and bilayer thinning. Additionally, a Bragg peak was observed after cubosomes addition, similar to that observed for the same cubosomes in the dispersion [76].

Employing cubosomes as drug delivery systems, Boge et al. investigated membrane interactions of glyceromonooleate (GMO) based cubosomes with Pluronic F127 block copolymer and loaded with the AMP LL-37 (LLGDFRKRKSFKEQFRKIVQKDFLRLNLPRTES) [77]. In doing so, NR and QCM-d were employed for investigations of the interaction of such cubosomes with dimyristoylphosphatidylcholine (DMPC)/...
dimyristoylphosphatidylglycerol (DMPG) bilayers, mimicking bacterial membranes. QCM-d showed that while empty cubosomes caused only minor lipid removal from the DMPC/DMPG bilayers, peptide-loaded ones caused pronounced bilayer destabilization. NR, in turn showed free LL-37 to localize within the outer leaflet of the DMPC/DMPG bilayer, whereas both empty and peptide-loaded cubosomes resided on top of the initial bilayer. For the peptide-loaded cubosomes, an additional Bragg peak was observed, signifying large-scale structural changes. In parallel to model membrane studies, super-resolution laser scanning microscopy showed that the peptide-loaded cubosomes, but neither the empty ones nor free LL-37 at the corresponding concentration, caused significant bacterial membrane lysis. In addition, cryogenic

Fig. 8. Interaction of empty and LL-37-loaded glyceromonooleate cubosomes stabilized by Pluronic F127 with bacteria-mimicking DMPC/DMPG bilayers. (A) QCM-d measurements showing that empty cubosomes caused only minor lipid removal from the DMPC/DMPG bilayer, while LL-37-loaded cubosomes display pronounced adsorption and caused subsequent bilayer destabilization. (B) NR data showing free LL-37 to reside in the outer leaflet of the DMPC/DMPG bilayer inducing pore formation, whereas either empty or peptide-loaded cubosomes formed an outer layer on top of the initial bilayer. For empty cubosomes a minor interaction was detected, associated with an outer layer mainly composed of water, while an additional Bragg peak was observed for LL-37-loaded cubosomes, signifying structural changes. (C) Representative fluorescence microscopy images showing bacterial membrane lysis induced by LL-37-loaded cubosomes, but not by bare LL-37 and bare cubosomes. (D) Cryogenic electron tomography images, demonstrating LL-37-loaded cubosomes to fuse and induce defects in bacterial membranes of E. coli. Adapted with permission from [77]. Copyright (2019) American Chemical Society.
electron tomography demonstrated the peptide-loaded cubosomes to be able to fuse and induce defects of both membranes of *E. coli* bacteria. Together, these results demonstrate that the primary antimicrobial entity in this system is the peptide-loaded cubosomes rather than the free peptide, presumably due to incomplete peptide release over the time-scale investigated (Fig. 8).

While the above example illustrates how drug-loaded cubosomes may provide boosted membrane activity translating to antimicrobial effects, the relative importance of the drug and the drug-loaded cubosomes in membrane interactions is complex and likely depends on properties of both the drug and the cubosomes. Illustrating this, Lai et al. investigated effects of phytantriol-based cubosomes loaded with Polymyxin B on asymmetric d-DPPC/Lipid A bilayers, mimicking the OM of Gram-negative bacteria [78]. Considering that Polymyxin B targets primarily LPS, whereas cubosomes may destabilize the inner phospholipid membranes in these bacteria, it was hypothesized that a combination of these might be advantageous. Indeed, it was found that a polytherapy, consisting of treatment first with Polymyxin B and subsequently with cubosomes, resulted in substantially increased antimicrobial effects compared to Polymyxin B-loaded cubosomes or to Polymyxin and empty cubosomes alone. NR showed the cubosomes to form a diffuse layer on top of the asymmetric d-DPPC/Lipid A bilayer. For bilayers pre-treated with Polymyxin B, but not for those treated with cubosomes first and Polymyxin B after that, a small fraction of phytantriol was subsequently observed in both membrane leaflets, showing that cubosome lipids can transfer into the bacteria-mimicking bilayer. Mirroring this, confocal microscopy showed that subsequent treatments of *E. coli* bacteria with Polymyxin B and cubosomes resulted in pronounced bacterial membrane lysis. In contrast, the Polymyxin B-loaded cubosomes were unable to penetrate the bacterial membranes.

Since the studies by Boge et al. and Lai et al. were performed with different cubosomes, compositional effects may be a reason for the seemingly conflicting results of the ability of peptide-loaded cubosomes to disrupt bacterial membranes. In addition, the peptides in the two examples discussed above are different. Thus, Polymyxin B is relatively small (Mw = 1203 Da) and contains an aliphatic hydrophobic tail, both factors facilitating cubosome incorporation. In comparison, LL-37 is much larger (Mw = 4492 Da) and contains no pronounced hydrophobic domain, potentially restricting the peptide to the outer part of the cubosome particle. Addressing the role of peptide properties for cubosome incorporation and bacterial membrane interactions, Boge et al. investigated cubosomes as carriers for the AMPs LL-37, AP114 (GFGCNPWEDDLRCHNHCCKSKGYGCGKCGFVCK), and DPK-060 (GKHNNKGGKNKHGKWVV) [79]. Effects of peptide incorporation on cubosome structure was monitored by SAXS and cryogenic transmission electron microscopy (cryoTEM). Among the peptides, LL-37 showed the highest degree of incorporation in the cubosome and AP114 the lowest. Mirroring this, AP114-loaded cubosomes displayed similar antimicrobial effect as the free peptide, whereas the antimicrobial effect of LL-37 was reduced after cubosome incorporation. Investigating effects of the method used for drug loading, the same authors reported on cubosome incorporation of LL-37 by: (i) peptide incorporation into a liquid crystalline gel before its dispersion (pre-loading), (ii) peptide loading into pre-formed cubosomes (post-loading), and (iii) peptide incorporation into cubosomes in the presence of ethanol/glycerol monoooleate (hydrotrope loading) [80]. No LL-37 release was observed from the pre-loaded cubosomes, showing that the peptide was kinetically trapped within the lipid nanoparticles. In line with this, pre-loaded LL-37 was protected against degradation when the cubosome formulation was challenged by proteases. Despite this, pre-loaded LL-37 effectively killed *Staphylococcus aureus* (S. aureus) bacteria in a wound infection model, in line with the finding of the LL-37-coated cubosomes being the primary antimicrobial unit. In summary, further work is clearly needed on this, including different mixing order, to better understand how cubosome and peptide properties influence cubosome interactions and membrane interactions of the loaded cubosomes.

6.2. Lipoproteins

Lipoproteins form a heterogeneous class of nanoparticles produced by the liver and the intestine. Lipoproteins are generally divided into different sub-classes, characterized by different proteins (apolipoproteins) and lipid content (notably triglycerides and cholesterol), resulting in different size and density of the lipid nanoparticles. The composition and structure of lipoproteins change throughout their life cycle, resulting in dynamic transitions between sub-classes [81]. The most important lipoproteins are low-density lipoprotein (LDL) and high-density lipoprotein (HDL), which both interact with cells involved in lipid metabolism through different receptors [82,83]. Importantly, some of these receptors are overexpressed in atherosclerotic plaque, as well as in cancer cells, which makes both LDL and HDL interesting as drug delivery vehicles to target these diseases [84,85]. Contributing to this, the structure of HDL gives them an ability to incorporate lipophilic drugs [86]. Similarly, LDL and LDL-like nanoparticles have been found to be good carriers of cancer drugs in tumor cells [87,88]. Besides cancer applications, LDH lipoproteins carrying acetylated lysine residues have been employed as carriers of anti-infectious drugs, where selective accumulation in infected macrophages was reached by receptor-mediated assimilation [89]. In yet another indication area, Tan-shinone IIA loaded reconstituted HDL (TA-rHDL) lipoproteins have been found to be interesting for targeting atherosclerotic plaque in a foam cells [90].

Regarding NR studies of membrane interactions of lipoproteins, work done so far has concerned lipoproteins in the absence of drug loading, and no studies have so far been reported on NR studies of membrane interactions of drug-loaded lipoproteins. The studies reported on membrane interactions of empty lipoproteins do, however, provide valuable information of key aspects of such membrane interactions, providing a basis for future studies for drug-loaded lipoproteins. For example, Browning et al. developed a protocol for investigating lipoprotein interactions with model DMPC/DMPS membranes using NR and QCM-d [91]. From NR, it was found that both LDL and HDL removed lipids from the bilayer, at the same time depositing hydrophobic material into the lipid bilayer. The relative importance of these opposing processes was found to depend on lipoprotein type, in line with findings in literature of HDL (commonly referred to as ‘good’ cholesterol) being able to extract lipid away from cells, whereas LDL (commonly referred to as ‘bad’ cholesterol), deposits lipid material into the vascular wall, resulting in atherosclerotic plaque formation. Analogously, Browning et al. employed NR and QCM-d to investigate effects of membrane charge on lipoprotein-membrane interactions by spiking DMPC bilayers with anionic DMPS [92]. The amount of material transferred to the bilayer from LDL or HDL was found to depend only weakly on the membrane charge, whereas increased membrane charge promoted lipoprotein-induced lipid removal from the bilayer, particularly so for LDL. Addressing effects of lipid composition in further detail, Waldie et al. reported on effect of phospholipid saturation and the presence of cholesterol [93]. In line with known risk factors for atherosclerosis, lower exchange was observed in the presence of cholesterol. In subsequent studies, Correa et al. investigated the relationship between SARS-CoV-2 and lipoproteins, motivated by reports of the severity of COVID-19 being inversely related to HDL plasma levels, and of the SARS-CoV-2 spike protein binding to HDL particles [94,95]. In doing so, spike protein exposure was found to interfere with lipid exchange between HDL and DMPC/cholesterol bilayers.

7. Membrane interactions of polymer-based delivery systems

A wide range of polymer-based nanomaterials are interesting as drug delivery systems, including microgels, dendrimers, and block copolymer micelles. While there is an abundant literature demonstrating the pharmaceutical performance of such systems, as well as on physicochemical properties of these carriers, much less is known about their
membrane interactions, and how such interactions contribute to pharmacological efficacy and adverse side effects. Here, NR offers opportunities for elucidating how the drug carrier and released drug combine to induce structural and compositional effects in cell membranes, and how such effects may be connected to pharmaceutical and biological effects.

7.1. Microgels

Microgels (nanogels) are particle equivalents of macroscopic hydrogels [96]. Such systems display responsiveness to various stimuli, biocompatibility, good colloidal stability, and a structure allowing incorporation of hydrophilic biomacromolecular drugs. Consequently, microgels have attracted attention as delivery systems for a range of drugs and in various indication areas. For example, microgel-based drug carriers have been demonstrated to be attractive for cancer immunotherapies, as well as for the delivery of cancer drugs, e.g., since pH-responsive microgels can be designed to respond to pH differences between tumor and healthy tissue, allowing targeted delivery of antitumor drugs [97,98]. While microgels are particularly promising carriers for hydrophilic drugs [96–100], the stimuli responsiveness, colloidal stability, and favorable biocompatibility of microgels/nanogels make these systems relevant also for hydrophobic drugs [101]. Due to the high degree of water swelling displayed by microgels, however, the delivery of hydrophobic drugs requires incorporation of hydrophobic domains within microgels/nanogels. This may be achieved, e.g., by introducing host-guest pairs (e.g., cyclodextrins able to incorporate hydrophobic drugs) or core-shell structures (hydrophobic core), or by designing microgels/nanogels formed by polymers of switchable hydrophobicity (e.g., polyamides or polyethers displaying reversed temperature dependence) [101]. Furthermore, hybrid lipid (glycerol monooleate and diglycerol monooleate) and poly(N-isopropylacrylamide) microgel films can be formed [102]. NR together with FTIR and SAXS results for such systems show that the hydration of the cubic phase lipid films, as well as the lateral organization of the lipid domains in the interfacial layer of the formed reverse cubic phases, respond to temperature stimulus. By combining the possibility to include hydrophobic drugs in the lipid phase with the micrnel thermal response, new biomedical applications can be envisaged.

Another area where microgels offer opportunities is as delivery systems for AMPs [100]. Due to bacterial resistance development, an increasing number of bacteria are becoming unresponsive to antibiotics treatment. In this context, AMPs are attracting intense current interest for combating severe infections. While focus has primarily been placed on the identification of potent and selective peptides, as well as on elucidating their mode(s)-of-action [43], it has become increasingly recognized that a key bottleneck for the translation of AMPs into therapeutics is the difficulty of efficiently delivering AMPs to their site of action due to the relative large size, proteolytic susceptibility, net positive charge, and amphiphilicity of such peptides [103]. Consequently, the development of AMP to therapeutics requires careful consideration of AMP delivery systems for the application at hand, exemplified by recent reviews on AMP delivery aspects for pulmonary and cutaneous [104,105].

The ability of AMPs to destabilize bacterial membranes is key for their antimicrobial effects. Consequently, a considerable body of studies focuses on membrane interactions of AMPs, investigated with various techniques, including NR. In contrast, very few studies have so far addressed the interplay between AMPs and drug carriers in membrane interactions. Exemplifying such studies, Nordström et al., investigated anionic poly(ethyl acrylate-co-methacrylic acid) (MAA) microgels as carriers for the cationic AMP LL-37 [106]. NR results showed that the free peptide bound to DMPC/DMPG bilayers in a concentration-dependent manner. At low peptide concentration, the peptide inserted into the outer leaflet only, whereas the peptide was present in both bilayer leaflets at higher peptide concentrations, causing membrane defects. Microgel-loaded LL-37 displayed qualitatively similar concentration dependence, while the empty microgel particles displayed very limited membrane binding. Furthermore, FTIR-ATR and circular dichroism (CD) results showed LL-37 to form an α-helix on membrane insertion, the kinetics of which was suppressed for microgel-loaded LL-37. Together, these results show that membrane interactions for the peptide-loaded microgels were dominated by released peptide, and that the slow release of microgel-loaded LL-37 contributed to kinetic effects on both bilayer structure and peptide localization within the bilayer (Fig. 9). In line with this, Nöstöm et al. found loading into MAA microgels to protect LL-37 from proteolytic degradation [107]. As a result of their net negative z-potential also after peptide loading, the microgels did not bind to supported bacteria-mimicking dioleoylphosphatidylethanolamine (DOPE)/dioleoylphosphatidylcholine (DOPG) membranes. Instead, membrane destabilization relied on peptide release. Analogously, antimicrobial effects against methicillin-resistant S. aureus (MRSA), Pseudomonas aeruginosa, and E. coli were promoted by factors facilitating peptide release, such as decreased microgel charge density and peptide length. Mirroring the effects of the free peptides, the loaded microgels displayed low toxicity towards erythrocytes at the conditions investigated. Also along the same line, Nystrom et al. reported on surface-bound MAA microgels loaded with the AMP KYE28 (KYEITTTINHNLRLTHFLFRNFGYTLR), as well as the PEGylated peptide, KYE28PEG [108]. Again, microgel-peptide interactions were facilitated at higher microgel charge density, while PEGylation suppressed peptide binding. Furthermore, peptide release was facilitated at increasing ionic strength, particularly so for KYE28-PEG. In line with this, contact killing was responsible for antimicrobial effects at low ionic strength, whereas released peptide caused antimicrobial effects in bulk solution at physiological salt concentration.

As other colloidal drug delivery systems, microgels may interact with serum proteins in blood. Serum protein adsorption at the micrnel surface may promote macrophage uptake and shorten blood stream circulation time. In turn, this may cause suppressed potency of the drug, as well as dose-limiting local toxicity. Addressing this, Traldi et al. investigated protein corona formation on neutral, anionic and cationic acrylamide-based microgels, using serum albumin (BSA), fibrinogen, and immunoglobulin G (IgG) as a simplified serum model [109]. Surface tension results showed complex formation to occur between positively charged nanogels and BSA. Furthermore, NR results showed the interfacial structure to consist of three regions, i.e., a dense top layer, a solvated intermediate layer, and a bottom layer with polymer chains reaching into bulk solution. At increasing concentrations of BSA, more material adsorbed, extending into the aqueous subphase.

7.2. Dendrimers

Dendrimers are attracting increasing interest as materials for life science applications, including drug delivery [110]. Dendrimers differ from linear polymers by their low degree of polydispersity, high degree of symmetry, and surface polyvalency. During synthesis, repeated growth reactions to higher generations dendrimers lead to increased branching and functional group density, eventually resulting in a 3D spherical structure. Through their stepwise synthesis, dendrimers can also be designed to display well-defined core–shell structures. Dendrimers have been widely investigated as agents for gene regulation [111] as well as drug carriers. For example, dendrimers containing a hydrophobic core and a hydrophilic shell have been used to entrap hydrophobic drugs in the central dendrimer cavity. Furthermore, cationic dendrimers have been widely investigated as agents for gene regulation [111] as well as drug carriers. For example, dendrimers containing a hydrophobic core and a hydrophilic shell have been used to entrap hydrophobic drugs in the central dendrimer cavity. Furthermore, cationic dendrimers offer opportunities as carriers for NAs. The versatility of dendrimers as delivery systems can be further enhanced by polymer conjugation to their surface groups. For example, conjugating dendrimers with PEG, polysaccharides, and polypeptides can be used to enhance drug solubility and stability, as well as for improved biocompatibility [110,112,113].

NR was employed to study the interaction of poly(amide amine) (PAMAM) dendrimers of generations 2 (G2), 4 (G4), and 6 (G6)
supported palmitoyloleoylphosphatidylcholine (POPC) bilayers deposited on silica surfaces [114]. The results showed that all these three types of dendrimers were able to transverse the supported lipid bilayer. The smaller dendrimers left the bilayer intact, while G6 dendrimers led to partial bilayer destruction. Coarse-grained simulations of the interaction of generation 3 (G3) dendrimers and POPC bilayers showed that the dendrimers could directly penetrate the bilayer without endocytosis. Dendrimer and Luciferase plasmid DNA of 4331 bp complexes at dendrimer/DNA charge ratio of 0.5 was found to bind to the POPC bilayer for G2 and G4 dendrimers, and G6/DNA did not interact at all. Furthermore, Lind et al. used NR to study the interaction of a peptide dendrimer, BALY, with POPC and DPPC bilayers to elucidate how this compound interacts with fluid and gel phase bilayers [115]. In addition to the one component systems, a POPC/DPPC mixture was included as a model phase-separated membrane. For fluid phase POPC, BALY inserted in the outer membrane leaflet, resulting in membrane thinning and headgroup disordering. Furthermore, dendrimer insertion was observed through the entire acyl chain region for DPPC above its phase transition temperature, as well as for phase separated DPPC/POPC membranes. Together, these results show that fluidity affects membrane interactions with dendrimers and indicate that phase separation may promote antimicrobial effects of the peptide dendrimer.

Furthermore, Nordström et al. investigated PEGylated anionic dendritic nanogels (DNG) as AMP carriers [116]. In these systems, the anionic carboxyl acid groups of the dendritic moiety of the DNG bound cationic AMPs, while the PEG chains formed a shell for suppression of
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serum protein binding. In doing so, DNG charge density, cross-linking, and degradation were investigated and correlated to antimicrobial effects. For the smaller DPK-060, peptide loading increased with increasing DNG charge. In contrast, peptide loading was insensitive to nanogel charge for the larger LL-37, indicating that this peptide was unable to enter the core of the DNGs. Ellipsometry and liposome leakage results showed that both free and DNG-loaded peptides contributed to lysis of bacteria-mimicking DOPE/DOPG membranes, in line with findings on antimicrobial effects. Mirroring the properties of the free peptides, the loaded DNGs did not display any substantial toxicity towards erythrocytes even at high peptide concentration, in line with minor effects of the peptide-loaded DNGs on mammalian-like DOPC/cholesterol bilayers.

7.3. Polymeric micelles

Over the last couple of decades, polymeric micelles have become some of the most well-studied colloidal drug delivery systems [117–119]. Polymeric micelles are small (10–200 nm) polymeric particles formed on self-association of amphiphilic block copolymer, generally consisting of a hydrophobic core and a hydrophilic shell. Compared to surfactants, key advantages of polymeric micelles include their frequently much lower critical micelle concentration (cmc) and much higher kinetic stability [120–122]. Examples of widely investigated hydrophobic polymer (blocks) for micelles formed by block copolymer include poly(lactide), poly(caprolactone), poly(propylene oxide), and polyesters, while common hydrophilic polymer (blocks) include PEG, poly(oxazolines), poly(vinylpyrrolidone), and poly(acryloylmorpholine) [119]. Compared to many other types of delivery systems discussed here, polymeric micelles represent some of the more mature technologies in relation to pharmaceutical development. Thus, numerous polymeric micelle formulations are currently in clinical trial and some have already been approved for human use [118]. The small size and hydrophilic exterior of polymeric micelles reduce macrophage uptake, which results in prolonged bloodstream circulation and increased drug uptake in tumors. At the same time, drug accumulation in non-tumor tissues is suppressed, resulting in reduced adverse effects. Drug loading into the hydrophobic core of polymeric micelles further provides increased drug stability, of importance for drugs susceptible to chemical or biological degradation. While polymeric micelles containing a hydrophobic core are the ones most extensively investigated, it should be noted that core-shell structures can be obtained by any interactions that can be tuned to reach liquid-liquid phase separation, including, e.g., polymeric micelles formed by complexation between oppositely charged polyelectrolyte pairs, by selective hydrogen bonds, or host-guest interactions. This, in turn, means that incorporation into the micellar core is not restricted to hydrophobic drugs, but available also to, e.g., hydrophilic biomacromolecular drugs. Furthermore, while simple partitioning is most frequently employed for drug loading into polymeric micelles, drugs may also be covalently conjugated to reach specific pharmaceutical benefits, e.g., within combination drug delivery [119]. Similarly, while passive drug diffusion out from the micellar core into the surrounding solution is the most common way to control drug release kinetics, also other designs are possible, such as those based on stimuli-sensitive breakdown of either the micelles or of chemical links holding drugs attached to the polymeric micelles. In this context, focus has been placed on systems responsive to the tumor microenvironment, including pH, hypoxia, and upregulated enzyme levels. In other approaches, external stimuli can be applied for triggered drug release, including light, ultrasound, and temperature [119].

Despite the importance of polymeric as drug delivery systems, there are so far no studies employing NR for the interaction of such systems with lipid membranes. There are, however, a couple of publications reporting on polymeric micelles at model interfaces in the absence of drugs. Thus, Zarbakhsh et al. reported on NR investigations of an oil-water interface, obtained by placing a thin oil layer between a silicon substrate and the underlying aqueous solution [123]. Employing this approach, the interface structure between hexadecane and an aqueous solution of the triblock copolymer Pluronic L64 (EO13-PO47-EO13), EO and PO being ethylene oxide and propylene oxide, respectively. Pluronic L64 was found to occupy both the oil and the water side of the interface. Furthermore, Hayden et al. employed NR to investigate surface-supported DMPC bilayers on addition of Triton X-100, as well as effects of co-addition of Pluronic F98 (EO87-PO47-EO87) [124]. Addition of Triton X-100 was found to cause a slight bilayer thinning and result in an EO layer next to the supporting substrate. Addition of Pluronic F98 to the DMPC/Triton X-100 bilayer was found to broaden its phase transition, and to result in further membrane thinning and growth of the hydrophilic layer next to the substrate surface. The structural changes were found to be temperature dependent, with onset of block copolymer insertion at 37 °C [124]. Moreover, while referring to surfactant-based rather than polymeric micelles, Foglia et al. investigated how formulation of Amb with sodium cholesteryl sulfate (SCS) affected its membrane interactions [125]. In doing so, effects of equimolar Amb/SCS micelles were compared to those of free Amb for POPC mono- and bilayers formed in the absence and presence of cholesterol and ergosterol. NR results showed free Amb and Amb/SCS to induce similar final changes to these membranes. Stopped-flow SANS results, however, showed faster structural changes for Amb/SCS than for free Amb. For POPC, the structural effects induced by Amb/SCS reached saturation after ~30 min. The corresponding effects for sterol-loaded systems, however, much slower. Furthermore, faster kinetics was observed for POPC/ergosterol than for POPC/cholesterol, consistent with known differences in affinity of Amb for these two sterols. Similarly, Saaka et al. employed NR and surface tension measurements for investigations of the interaction between sodium dodecyl sulfate (SDS) monolayers and testosterone enanthate [126]. NR results showed the hydrophobic drug to locate in the hydrophobic tail region largely independent of drug concentration. These finding were supported by MD simulations. The surfactant/drug ratio at the air–water interface above the cmc of SDS was found to be ~3/1, comparable to that obtained for SDS micelles from bulk solubilisation studies. While some interesting observations were thus reported from these previous studies, which contained method development of value also to studies of membrane interactions of polymeric micelles as drug delivery systems, it is clear that further studies on the latter aspect are needed considering the importance of polymeric micelles in drug delivery.

8. Membrane interactions of ionic liquids and deep eutectic solvents

Ionic liquids (ILs) are compounds composed of ions only, which together form a liquid at low temperature. Compared to conventional organic solvents, ILs offer advantages related to low vapor pressure, high thermal stability, and structural and compositional diversity, which has attracted interest in diverse research fields, including, e.g., chemical synthesis, electrochemistry, and biotechnology. ILs are also extensively investigated within drug delivery where they offer advantages, e.g., for promoting drug dissolution and solubility, and for overcoming biological barriers such as the intestinal epithelium or the stratum corneum of skin [127,128]. ILs consist of one anion and one cation, where anions are typically small and inorganic, and cations relatively large and organic. The difference in size between the ions disfavours crystal formation, causing ILs to remain liquid at low temperature. The structure of ILs stems from their bulky cations, which are generally characterized by a low charge density, resulting in modest electrostatic repulsion and contributing to structural ordering characteristics of ILs. Due to the delicate nature of such ordering, ILs can also be designed to display responsivity to, e.g., pH, ultrasound, and thermal responsivity [129,130].

Exemplifying pharmaceutical advantages provided by ILs in drug delivery, choline-based ILs have been reported to increase transdermal

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permeability of the peptide SIINFEKL by a factor up to 28 compared to aqueous delivery systems [131]. For insulin, the permeability was increased even more impressive, 58-fold, at the same time as hypoglycemic effects were reduced by 56% [132]. In addition to boost drug permeability over biological barriers, ILs may provide increased drug stability. Exemplifying this for a model protein, imidazole-based ILs have been reported to enhance the stability of BSA [133]. In addition, ILs may have pharmaceutical effects on their own. For example, pyridine-based ILs display potent antimicrobial effects due to the combination of a cationic charge and the presence of an alkyl chain [134]. Regarding adverse effects, choline-based ILs have been reported to display favorable biodegradability and limited toxicity [135,136]. Together, these results show that ILs can be used as solubilizers, skin penetration enhancers, antibacterial agents, and stabilizers for proteins and sparingly soluble drugs. In this context, one should also mention deep eutectic solvents (DES) which are somewhat similar to ILs, typically formed by a combination of hydrogen bond donors (e.g., quaternary ammonium salts) and acceptors (e.g., amides, carboxylic acids, or polyols). As for ILs, it is possible to fine-tune a wide range of physicochemical properties of DES by changing the composition, molar ratio, or functional groups. Apart from being able to deliver both biomacromolecular and small-molecule drugs, the latter can be incorporated as a DES component. For example, amine-containing drugs can be converted to hydrogen bond acceptors and combined with, e.g., phenolics, sugars, or acids as hydrogen bond donors to form DESs [137,138].

Membrane interactions are key for biological effects of ILs, including cell toxicity, antimicrobial effects, and permeation of biological barriers. An increasing number of studies addressing this are emerging in literature, demonstrating ILs to influence the structure, dynamics, and phase behavior of membranes. Such effects are strongly dependent on IL properties, notably hydrophobicity [139]. Illustrating this, Sharma et al. employed Langmuir balance experiments to investigate DMPC monolayers in the presence of ILs of different alkyl chain length [140]. The presence of ILs was found to cause isotherms to shift to higher areas per lipid molecule, indicating that ILs penetrated into the phospholipid monolayer. This effect was found to be more pronounced for DMIM [BF4] compared to BMIM[BF4], i.e., at longer acyl chain of the IL. Similarly, Jeong et al. investigated effects of alkyl chain length IL their interaction with phospholipid membranes [141]. For this, DPPC liposomes were used and their interaction of these with imidazolium-based ILs of different alkyl chain lengths. OMIM[BF4], having the longest

Fig. 10. NR investigations of the structure and stability of DMPC model bilayers hydrated by aqueous solutions of the IL [bmim][Cl]. (A) Representative NR profiles, together with best curve fits, for DMPC bilayers in three different contrasts, in the absence and presence of [BMIM][Cl], showing minor membrane thinning upon IL insertion. (B) IL distribution within the DMPC membrane, calculated from NR analysis, displaying a preferred location between the acyl chain and headgroup regions. (C) Representative NR profiles, together with best curve fits, for DMPC bilayers in three different contrasts in the absence of IL and after incubation with IL and subsequent rinsing with water, showing irreversible sorption of IL to the DMPC bilayer. (D) Schematic comparing results before and after rinsing with water, showing that a sizeable fraction of IL (~8% of bilayer volume) incorporated into the bilayers to be either irreversible bound or displaying slow kinetics. Adapted from with permission from [143]. Copyright (2014) American Chemical Society.
massive interest in ILs as drug delivery systems, as well as presently affecting their performance as drug delivery systems. Considering the incorporated into the bilayers to be either irreversibly bound or dis nanoparticles (MSNs) are receiving considerable interest as delivery uumes, and frequently favorable biocompatibility, mesoporous silica nanomaterials can similarly generate heat on interaction with metal nanoparticles interact with light to generate heat, whereas mag nanoparticles interact with light to generate heat, whereas magnetic nanoparticles can similarly generate heat on interaction with oscillating magnetic fields. Through this, metal and magnetic nanoparticles can be triggered to produce localized heating which has been found to offer therapeutic opportunities for combating both tumors and infections. For such nanoparticles carrying a drug, additive or even synergistic effects can be obtained. The same can be said for photocatalytic or chemonanoparticles, which are able to generate reactive oxygen species (ROS) on illumination and chemical reactions, respectively. As with other drug delivery systems, membrane interactions play an important but frequently overlooked role for the performance of such systems.

9. Membrane interactions of inorganic drug delivery systems

Inorganic nanomaterials are receiving considerable current interest in drug delivery. Reasons for this include their ability to act as drug carriers, but also to release drugs in stimuli-response manner and to provide functionalities beyond being a drug container. For example, metal nanoparticles interact with light to generate heat, whereas magnetic nanoparticles can similarly generate heat on interaction with oscillating magnetic fields. Through this, metal and magnetic nanoparticles can be triggered to produce localized heating which has been found to offer therapeutic opportunities for combating both tumors and infections. For such nanoparticles carrying a drug, additive or even synergistic effects can be obtained. The same can be said for photocatalytic or chemo-nanoparticles, which are able to generate reactive oxygen species (ROS) on illumination and chemical reactions, respectively. As with other drug delivery systems, membrane interactions play an important but frequently overlooked role for the performance of such systems.

9.1. Mesoporous silica nanoparticles

Due to their well-defined and tunable pores, considerable pore volumes, and frequently favorable biocompatibility, mesoporous silica nanoparticles (MSNs) are receiving considerable interest as delivery systems [145–147]. Since pore diameter, geometry, volume, and surface chemistry can be varied, drug release kinetics can be controlled. In addition, MSNs offer opportunities for co-delivery of compounds, e.g., drugs and therapeutically active ions for reaching osteogenesis, angiogenesis, antibacterial and anticancer properties [145]. Based on such properties, MSNs have been found to be promising for treatment of respiratory disease [146], as well as for oral delivery, including that of peptides and proteins [147].

In one of few studies addressing membrane interactions of MSNs as drug delivery systems, Malekhaïat Häfner et al. investigated silica nanoparticles as carriers for the AMP LL-37 [148]. In doing so, smooth MSNs were compared to “spiky” ones, as well as to non-porous silica nanoparticles, regarding their interaction with bacteria-like POPC/POPG bilayers. In the absence of peptide load, the spiky MSNs destabilized the bacteria-like bilayers, whereas the corresponding smooth ones did not. The effect of the surface spikes became even stronger after peptide loading. Thus, LL-37-loaded spiky MSNs displayed more severe membrane destabilization than both the peptide-loaded smooth MSNs and free peptide. NR demonstrated that the spiky MSNs caused incorporation of LL-37 throughout both bilayer leaflets. The relevance of these effects for bacteria lysis was also demonstrated for E. coli (Fig. 11).

The finding of minor membrane destabilizing effects of smooth MSNs containing LL-37 is in line with results reported by Braun et al. who found that membrane destabilization and antimicrobial effects to require peptide release based on ellipsometry, light and electroforetic light scattering, fluorescence spectroscopy and bacterial assays [149]. Interestingly, however, it was found that also smooth silica nanoparticles could be rendered membranolytic by increasing their negative charge (as in Stöber nanoparticles), to increase effectively irreversible surface loading of cationic LL-37, and the corresponding positive charge of the peptide-loaded nanoparticles.

9.2. Metal nanoparticles

Metal nanoparticles are interesting for drug delivery since their small particle size and corresponding large surface area allow high drug loading by physisorption or chemisorption at the nanoparticle surface [150-151]. Drug release from such nanoparticles occurs through simple desorption, which may be triggered, e.g., by changes in pH, or through breakage of chemical bonds (e.g., thiol-based ones) used for drug chemosorption. Among metal nanoparticles, gold nanoparticles (AuNPs) have received particular interest in drug delivery and diagnostics due to straightforward synthesis, stabilization, and functionalization, as well as due to a favorable biopharmaceutical profile and ease of detection. Regarding pharmacokinetics, it has been found that AuNPs are able to infiltrate blood vessels to reach the desired location (e.g., tumor tissues), but also enter inside cell organelles for more precise drug targeting. After reaching their target, site-specific drug release from gold nanoparticles can be achieved, e.g., in response to the lower pH and reducing conditions generally present within cancer cells, or upon an external stimulus (e.g., by light) [150]. These properties also make ultra-small (<10 nm) AuNPs interesting not only for drug delivery, but also for tumor visualization and bioimaging, either alone or in combination with other imaging methods [151].

In contrast to drug delivery applications of AuNPs and other metal nanoparticles, detailed investigations into membrane interactions of such systems are relatively rare, and there are no studies so far addressing AuNPs as drug delivery systems. Having said that, Anderson et al. investigated the combined effect of AuNPs and the antibiotic drug colistin sulfate on tethered DPhyPC/R-LPS lipid bilayers, the latter mimicking cell membranes of Gram-negative bacteria. Employing NR it was found that exposing the membrane to AuNPs prior to adding the colistin sulfate increased the effect of the antibiotic on the membrane [152]. In addition, a few studies have been published on membrane interactions of such nanoparticles in the absence of drug to provide a methodological foundation for further studies in the presence of drug loading. For example, Lolicato et al. combined NR studies with MD simulations to study the interaction between cationic AuNPs and bilayers formed by DSPC and diestearoylphosphatidylglycerol (DSPG) [153]. MD simulations showed that for DSPC bilayers an energy barrier suppressed AuNP binding at low temperature. At elevated temperature, this barrier was overcome, resulting in AuNP incorporation into the lipid bilayer, which was confirmed by NR. In contrast, AuNP binding was suppressed at elevated temperature for anionic DSPC/DSPG bilayers. Furthermore, NR results showed AuNPs to cause lipid extraction from DSPG bilayers, which was confirmed by MD simulations. Also addressing effects of electrostatics, Tatur et al. employed NR for studies of the interaction of floating DSPC lipid bilayers with AuNP modified with either cationic trimethylammonium or anionic carboxylate groups [154]. The cationic AuNPs were found to localize in the hydrophobic core of the lipid bilayers and to trigger membrane disruption. In
contrast, the anionic AuNPs did not penetrate into the DSPC bilayers. Furthermore, Caselli et al. investigated the interaction of AuNPs of different shapes (spheres and rods) with glycerol monooleate (GMO) and GMO/DOPC-based membranes, either flat (relevant for cell membranes), or cubic (relevant for non-lamellar membranes) [155]. NR and Grazing Incidence SANS (GISANS) results showed that membrane stability towards nanoparticle exposure depended on topological characteristic of both the nanoparticles and the lipid membrane, where higher stability was observed at higher symmetry.

9.3. Magnetic nanoparticles

The most extensively investigated type of magnetic nanoparticles within drug delivery and nanomedicine are superparamagnetic iron oxide nanoparticles (SPIONs) which interact strongly with magnetic fields. Such nanoparticles therefore offer opportunities as contrast agents in magnetic resonance imaging (MRI) or in magnetically triggered drug delivery [156,157]. On exposure to an oscillating magnetic field, SPIONs may generate localized heat which is therapeutically interesting even in the absence of drugs, e.g., for providing antimicrobial and anticancer effects. To enhance the biological and pharmaceutical effects of SPIONs, such nanoparticles have been further modified by adsorption or covalent conjugation with a plethora of compounds, including drugs and targeting moieties (e.g., antibodies, aptamers, or folate) to allow for targeted functionalization for use in imaging, magnetic thermotherapy, and boosted drug delivery [158,159].

Regarding membrane interactions of SPIONs and other types of magnetic nanoparticles, Luchini et al. reported on SPION interactions with POPC/POPG bilayers (mimicking the plasma membrane) [160]. For this, oleic acid/oleylamine-functionalized SPIONs were coated in a second layer by the cationic surfactant cetyltrimethylammonium bromide (CTAB) or the zwitterionic phospholipid 18LPC. The interaction of such functionalized SPIONs was subsequently investigated at varying cholesterol content of the POPC/POPG bilayers. While none of the functionalized SPIONs were found to disrupt the lipid membranes, they bound to the outer part of the bilayers in a manner that was suppressed with increasing cholesterol content, potentially an effect of bilayer rigidity. In line with this, Montis et al. compared effects of

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Fig. 11. Effects of nanoparticle structure on the interaction of mesoporous silica nanoparticles with bacteria-like POPC/POPG bilayers. (A) Leakage of POPC/POPG liposomes induced by different types of SiO2 nanoparticles (left panel). In the absence of peptide load, spiky MSNs were able to destabilize the bacteria-like bilayers, whereas the corresponding smooth MSNs or non-porous nanoparticles were not (top left panel). The effect of the surface spikes became even stronger on LL-37 loading (bottom left panel), with LL-37-loaded spiky MSNs displaying more pronounced membrane disruption than peptide-loaded smooth MSNs and non-porous nanoparticles. (B) NR curves, together with SLDs profiles obtained from curve fitting, for smooth MSNs (top panel) and spiky MSNs (bottom panel) interacting with POPC/POPG bilayers. (C) Corresponding NR analysis demonstrating that the spiky MSNs induced trans-membrane defects and promote incorporation of LL-37 throughout both bilayer leaflets. (D) Confocal microscopy LIVE/DEAD assay images and analysis, showing bacteria killing ability of non-porous nanoparticles, smooth MSNs and spiky MSNs, empty or loaded with LL-37, for E. coli bacteria. Adapted from [148].
SPIIONS on supported lipid bilayers formed by deposition of extracellular vesicles with those on model POPC bilayers, employing XRR in conjunction QCM-d, AFM, and confocal microscopy \[161,162\]. Consistent with findings of Luchini et al., only minor structural deformation was observed for both bilayers, despite SPIIONS binding to the surface of the supported bilayers. While capturing the major findings of more complete lipid membranes derived from extracellular vesicles, the number of SPIIONS bound to supported POPC was found to be substantially lower, illustrating that the model captures some but not all properties of the complete biological membrane. Together, these studies form a nice basis, that can be built upon for studies employing NR or XRR for studies of membrane interactions of drug-loaded SPIIONS.

9.4. Photocatalytic nanoparticles

In photocatalysis, light excitation generates free electrons and positively charged holes. These may react with water, oxygen, or solutes to form ROS, which in turn can oxidize membranes and other essential components in bacteria \[163\], or in cancer cells \[164\]. For example, Sulek et al. found porphyrin-doped TiO$_2$ to cause a 7-log reduction of S. aureus \[165\], whereas Ahmed et al. reported potent antimicrobial effects of TiO$_2$ against antibiotics-resistant P. aeruginosa \[166\]. While TiO$_2$ was the first photocatalytic nanomaterial to be investigated in detail, a wide variety of materials are currently attracting attention, including, e.g., carbon-based photocatalysts characterized by facile synthesis, as well as by a wide and controllable band gap. Addressing membrane interactions of such systems, Malekhhaitat-Häffner et al. investigated effects of lipid bilayer charge and presence of cholesterol on membrane interactions of TiO$_2$ nanoparticles \[167\]. While nanoparticle binding to the supported bilayers was found to have some destabilizing effect alone, UV illumination boosted membrane destabilization due to ROS-mediated oxidation. Furthermore, bilayer composition strongly influenced membrane interaction and oxidative degradation of lipid bilayers. Thus, presence of POPG (mimicking bacterial membranes) rendered the bilayers more sensitive to oxidative destabilization, whereas membrane stabilization was provided by cholesterol (as in human cell membranes). Analogously, structural effects of photocatalytic degradation were particularly pronounced in the presence of POPG, including membrane thinning, increased hydration, and areas per molecule following UV exposure and resulting lipid oxidation.

Investigating membrane interactions of photocatalytic TiO$_2$ nanoparticles (NPs) as drug delivery systems, Caselli et al. reported on an approach for increasing the selectivity of photocatalytic nanoparticles between bacterial and human cells by coating them by AMPs \[168\]. ROS generation was found to be essentially unaffected after AMP coating, and peptide degradation to be sufficiently limited to allow peptide-mediated targeting. In line with this, QCM-d results showed that peptide coating promoted membrane binding of TiO$_2$ NPs, particularly so for anionic membranes lacking cholesterol. This caused oxidative membrane degradation during UV illumination to be strongly promoted for bacteria-like membranes, but not so for mammalian-like ones, effects demonstrated by NR to involve increased hydration, lipid removal, and membrane solubilisation. Analogously, peptide coating promoted antimicrobial effects of TiO$_2$ NPs for both E. coli and S. aureus bacteria, whereas toxicity against human monocytes remained low.

10. Outlook

As discussed above, NR offers powerful opportunities for investigating membrane interactions of colloidal drug delivery systems, as well as of the drugs themselves. In addition, NR can be used for investigating responsive membrane interactions, e.g., of nanoparticles responsive to light, magnetic field, ambient conditions, or presence of specific solutes. Such effects have already been reported for some types of nanomaterials, including photocatalytic nanoparticles, microgels and LNPs, and yet further systems have been investigated in the absence of such responsiveness. NR can provide unique and detailed information on how membrane structure and composition is affected by different types of drug delivery systems, including microgels, dendrimers, lipid nanoparticles, cubosomes, and different types of inorganic nanoparticles. Having said that, it is striking that key drug delivery systems attracting considerable interest and showing clear promise as therapeutics, including, e.g., polymeric micelles, ionic liquids, and various inorganic nanoparticles, have yet to benefit from detailed studies on their membrane interactions. In part, this may be related to the complexity of such studies for researchers not having NR as a core methodology in their research. Another challenge with NR is the requirement of nearly perfect and physiologically relevant (bi)layers to the full potential of such experiments to be unleashed. This, as well as the need for deuteration to maximally use the advantages related to contrast matching, puts stringent requirements on the lipid systems to be used. In consequence, most NR studies are based on simple models of complex cell membranes, which may or may not capture the biological effects aimed for. In this context, it is rewarding to see the development over the last few years regarding both deuteration and methodologies for controlled assembly of bilayers of a higher degree of complexity for closer mimicry of biological membranes. Exemplifying an approach for realizing NR studies of more complex and biologically relevant membranes, Kjellnerup Lind et al. investigated lipid bilayers formed by lipid extracts of E. coli \[169\]. Importantly, the head group composition of the deuterated and the hydrogenated lipid extracts was found to be similar. Furthermore, both lipid extracts could be used to form supported fluid lipid bilayers, opening the door for versatile use of such bilayers, e.g., for investigations of membrane interactions of drugs and drug delivery systems. Similarly, Tanaka et al. demonstrated almost 20 years ago that it is possible to deposit native biomembranes (human erythrocyte membranes and sarcoplasmic reticulum membranes) onto micro-templates while retaining transmembrane proteins in their native state and without affecting their distribution \[170\]. Although representing a very promising approach, this is by no means trivial and particularly challenging to do with large surface areas needed in today’s NR instrument (25–40 cm$^2$). Future facilities, such as ESS, will offer higher intensity and therefore also allow smaller substrate sizes (hopefully an order of magnitude), relaxing some of these requirements.

In addition to such compositional refinement, there have been developments regarding bilayer structure. Examples of such developments include, e.g., cushioned bilayers, asymmetric bilayers, as well as asymmetric bilayers containing non-lipid components (e.g., bacterial lipopolysaccharides), all discussed above. In addition, there has been recent progress to include curvature effects in studies of membrane interactions, which is important for studies mimicking highly curved membranes, such as those at caveola, extracellular vesicles, and intracellular membranes. Illustrating this, Parachini et al. reported on arrays of nanoparticle-supported lipid bilayers for studies of curved model biological membranes \[171\]. Fluorescence recovery after photobleaching showed a particle-covered silica substrate to allow formation of fluid lipid bilayers with high lateral mobility. NR results, in turn, indicated the formation lipid bilayers at high surface coverage. These findings demonstrate the approach taken to provide a promising approach for investigations of curvature-dependent effects in supported lipid bilayers. Thirdly, combination of NR with advanced simulation methods or theoretical models for NR data analysis offer opportunities for detailed analysis of cell membranes. For example, Armanious et al. employed NR and theoretical modelling, as well as DNA tethers, to obtain information on the distance separating nanoparticles from a supported lipid bilayer with sub-nanometer precision \[172\]. Together with a growing realization of the potential of NR studies of membrane interactions of colloidal drug delivery systems, as well the importance of nanomedicine for combatting diseases more broadly, such methodological developments are likely to contribute to NR playing an increasingly important role in providing mechanistic understanding on membrane interactions, thereby contributing to rational development of
new drug delivery system and engineering.

CRediT authorship contribution statement

Lucrezia Caselli: Writing – original draft, Writing – review & editing. Tommy Nylander: Writing – review & editing. Martin Malmlsten: Conceptualization, Funding acquisition, Resources, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors have no conflicts of interest to report.

Data availability

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