Nanomaterial interactions with biomembranes: bridging the gap between soft matter models and biological context

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Synthetic polymers, nanoparticles, and carbon-based materials have great potential in altering biological functions, drug delivery, gene transfection, *in vitro* and *in vivo* imaging. Nature and humans use different design strategies to create nanomaterials: biological objects have emerged from billions of years of evolution and from adaptation to their environment resulting in high levels of structural complexity. In contrast, synthetic nanomaterials result from minimalistic but controlled design options limited by our current understanding of the biological world. This conceptual mismatch makes it challenging to create synthetic nanomaterials possessing desired functions in biological media. An essential transport barrier is the cell protecting plasma membrane and hence the understanding of its interaction with nanomaterials is a fundamental task in biotechnology. We present open questions in the field of interaction of nanomaterials with biological membranes, including: how physical mechanisms and molecular forces acting at the nanoscale restrict or inspire design options; which levels of complexity to include next in computational and experimental models to describe nanomaterials crossing barriers via passive or active processes; and how the biological media and protein corona interfere with the functionality of nanomaterials. In this perspective article, we address these questions with the aim to offer guidelines for the development of next-generation nanomaterials to function in biological media.

I. INTRODUCTION

Functional nanomaterials are used in many products of our daily life, from sunscreens to toothpastes[1], but bring uncontrolled risks such as nanotoxicity, and environmental pollution[2,3]. The proper design of “smart” or “intelligent” nanomaterials that perform a desired function in living organisms is an appealing but challenging task: the complexity of living organisms results from their adaptation to the environment during billions of years of evolution, whereas fabrication of synthetic nanomaterials is usually based on the optimization of a relatively small number of parameters. By offering precise control of design parameters, robustness and simplicity of construction, synthetic nanomaterials can promise new functions that do not yet exist in the biological world. However, the changes that they induce in complex biological media and their lack of adaptability may compromise the design goals due to degradation or limited biocompatibility. The design of biologically active nanomaterials therefore requires a clear definition of the design goals, the conception and implementation of the material as well as its testing. While essential parameters – size, shape, elasticity, composition and surface properties – of nanomaterials have been identified,[4,5] and the chemical properties can be precisely controlled, the major challenges in nanomaterial design arise in monitoring, understanding, and controlling their interaction with biological media,[6] ranging from specific biological barriers to the immune system.

Using the prototypical example of transport of nano-objects into eukaryotic cells, we map out the difficulties of nanomaterial design, and elaborate our opinion on how

design obstacles are linked to fundamental questions in understanding transport into living cells. We also highlight starting points for extending experimental and theoretical models for the prediction of a nanomaterials' functionality in biological environments: what are the next degrees of increased complexity that are most important to consider? In particular, in section 2 we describe where we see the major obstacles for an optimal design flow that integrates all necessary design steps. In section 3 we focus on challenges in understanding and exploiting already known mechanisms of nanomaterial transport across plasma membranes, and in section 4 we give examples on how the presence of biological media challenges theoretical and experimental approaches but also inspires new conceptions.

II. MISSING LINKS TO BIOLOGICAL CONTEXT

When coming from a physical and chemical background one often focuses on microscopic mechanisms of nanomaterial interaction with model environments such as single component lipid membranes, although the biological context is essential for formulating critical design goals and testing their functionality.

Modern chemistry allows us to synthetize a large variety nanomaterials with a broad range of architectures (e.g. quantum dots, polymers, nanostars, nanorods, nanodisks, nanocages), chemical composition (organic/inorganic, liquid/solid), and surface properties (e.g. decoration with ligands and charges)[7]. A good illustration on recent progress in advanced synthesis is the possibility to dynamically control the number of ligands on 23-gold-atom nanoparticle within so-called molecular surgery.[8] With fairly high precision, one can control the chemical composition of nanomaterials, the length and order of synthetic peptide sequences, and the architecture, chemistry and length of

synthetic polymers. Yet it is often not clear how chemical properties translate into physical control parameters when embedded in highly complex biological media. Beside the extensively discussed protein corona around nanoparticles,[9] more emphasize should be put on the question of how the protein-crowded environment, co-solvent properties, ionic strength and ion complexation, or pH modify the conformation and function of soft objects such as polymers or nano-gels. In turn, the impact of nanomaterials on the biological environment can be subtle. For example, one has observed that the band structure of metal oxide-based nanomaterials is an important factor for their toxicity. Depending on the band gap, these materials may interfere with the level of oxidative stress and can thereby be toxic [[Use of Metal Oxide Nanoparticle Band Gap To Develop a Predictive Paradigm for Oxidative Stress and Acute Pulmonary Inflammation]].

While theoretical and simulation approaches often investigate populations of identical nano-objects with idealised properties such as perfectly smooth spherical nanoparticles or monodisperse polymers, real nano-objects are not so pristine and, for instance, exhibit variations in surface roughness, polydispersity, and heterogeneity within a sample. Since small differences between nano-objects can be critical for their interaction with biological media, different fates are expected already from small variations in their properties, including decomposition into sub-populations due to the complex nature of the interactions.

Due to the Abbe diffraction limit, it is in aqueous solutions challenging to obtain insights at the molecular scale. However, optical imaging can reveal significant insights into the impact of non-objects on membrane properties, such as membrane morphology,[10] dynamics[11] and permeability.[12] Furthermore structural insights can be gained from

spectroscopic methods.[13] Towards imaging of individual nanoparticles, alternative approaches, such as Stimulated Emission Depletion Microscopy (STED)[14,15] and electron microscopy[16] are applied. Microfluidic and electrochemistry methods can be applied together to monitor individual translocation events of single and clustered nano particles across model lipid membranes.[17]

III. CHALLENGES FOR TRANSPORT ACROSS LIPID-BILAYER MEMBRANES

Whereas there is evidence for insertion and translocation of nano-objects such as cell-penetrating peptides, polymers, or coated nanoparticles across biological and lipid membranes from experiment and simulation, the thermodynamic driving forces and the molecular mechanisms for translocation remain hotly debated.[18–21] In analogy to other topology-altering (Fig. 1) membrane processes, such as fusion, fission and pore formation,[22] the passage across a membrane can be roughly subdivided into an initial recognition or docking stage and the subsequent penetration, as well as the separation of the object from the membrane. Generally, one may distinguish between active, assisted and passive transport across a membrane. Furthermore, one shall distinguish **translocation** mechanisms by direct penetration of the membrane's core or pore formation from **endocytic pathways** involving the wrapping of nano-objects into an invagination. For the design of a nano-object it is crucial to consider that the translocation and endocytosis lead to fundamentally different topological situations.

Active Transport

Active transport refers to mechanisms that are enabled by an expenditure of chemical energy. *Endocytotic pathways* are widely associated with active processes,[23] since in biological environments dynamin catalyses the separation step of an invagination.[24,25] Grafting of lipoproteins and other ligands onto nano-objects will make it possible to exploit active endocytic and phagocytic machineries of cells by binding to specific membrane receptors in the docking step.

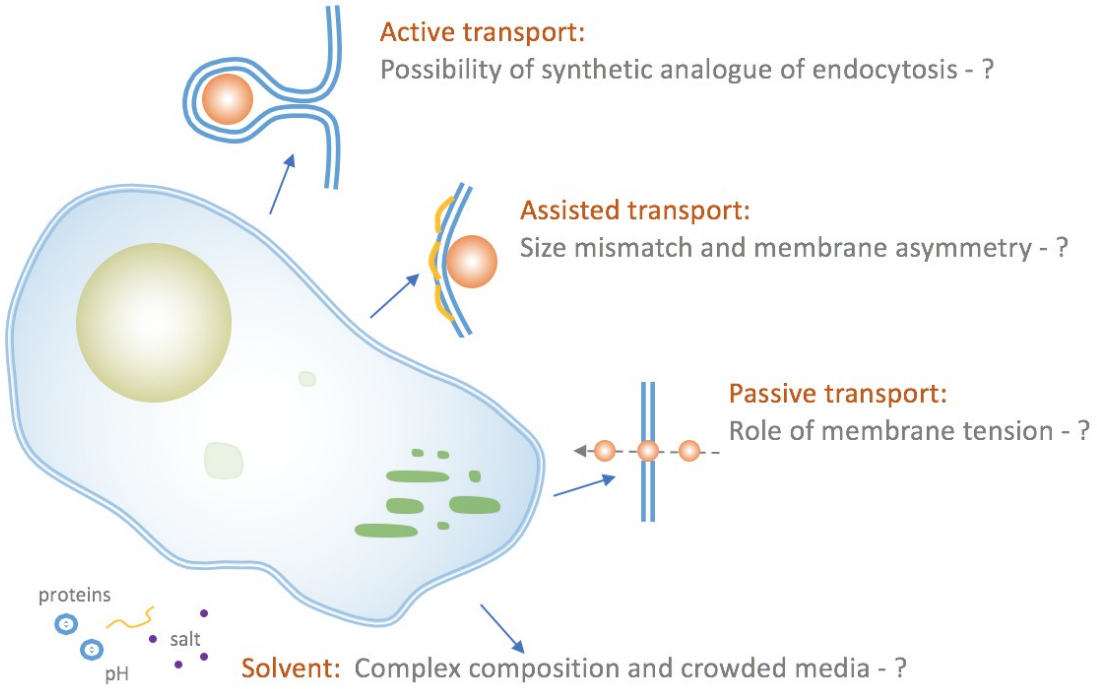

Fig. 1 Challenges and open questions in transport across a cell membrane.

Ion and glucose transporters are other common examples of protein machineries that facilitate active translocation across a membrane. Protein machineries that specifically transport also synthetic nano-objects across a bilayer are missing. Developing such a machinery will be particularly worthwhile because it has the potential to impart high selectivity onto translocation. Existing concepts on passive polymer translocation

through nano-pores[26] as well as voltage driven DNA translocation through biological pores[27] can be a starting points to develop translocation machineries for nano-objects driven by local chemical energy (ATP). It is this crucial to study more deeply the mechanisms of the existing trans-membrane transporters and active lipid flip-flop catalysing proteins. An interesting avenue of research could be aimed at finding minimal synthetic analogues or modifications of those proteins, so that they bind to nano-objects and subsequently catalyse their translocation. Accurately predicting the catalytic role of active proteins interacting with nano-structures is an open field for molecular simulation techniques. Intervening in active and regulatory transport systems can, however, easily show the fate of over-ambition: A nanomaterial that tempers into active machineries such as glucose- or ion transporters [[10.1016/j.tox.2009.08.005, 10.1098/rsif.2010.0158.focus, 10.1186/s12951-017-0327-9]], or active lipid exchangers between leaflets, may cause unpredictable regulatory failure and toxic effects.

Assisted Transport

Assisted *translocation* exploits global non-equilibrium processes or local response of the membrane that facilitate translocation processes, but that are not directly related to the translocation mechanism. A prototypical illustration of global non-equilibrium aspects is a translocation process that exploits the actively maintained lipid or protein asymmetry between the inner and outer monolayers. An interesting challenge is the possible transport of nano-objects driven by chemical potential differences – for instance, by developing analogues of secondary transporters. Another example of assisted translocation is the

enhanced permeability at boundaries between lateral lipid domains[28][[10.1039/C7NR08351C]] and the potential role of near-critical composition fluctuations or raft-like domains,[29] as well as interfaces between lipids and membrane-inserted nano-objects with critical hydrophobicity.[30] The adsorption of nano-objects at the membrane may locally alter the composition of the membrane in contact with the nano-object, and, in turn, facilitate translocation of the object.[31]

Endocytic pathways are assisted by families of curvature-inducing proteins that attach to the membrane: clathrin and BAR proteins. Anisotropic and Janus nanoparticles can mimic curvature-inducing proteins[32,33], and promote the formation of invaginations[34]. *In-vitro* experiments indicate that so-called N-BAR proteins, by having a transmembrane domain, promote endocytosis in the absence of dynamin, while pure BAR-domains seem to restrict fission but support tubular shapes.[35] On one hand it is often discussed that specific assisting mechanisms are required for the final pinch-off to occur; on the other hand computer simulations indicate that spontaneous endocytosis of wrapped nanoparticles also occurs in cases where N-BAR or equivalent molecules are not present.[36–39] A key question here is how the barriers for altering membrane topology and concomitant time scales depend on the object enclosed. To this end, the prediction of the pinch-off dynamics and time scales can be seen as benchmark case for molecular simulation models. It is particularly challenging to map time scales and free energy barriers between atomistic and coarse-grained models – motivating the development of new simulation techniques bridging the gap. Beside computer simulation, it will be worthwhile to test existing theoretical models for the pinch-off[40,41] and the role of "universal membrane remodellers"[42] via focused experiments with model membranes.

An interesting question to address is the relation between nano-object size, and the spontaneous curvature induced by assisting proteins or synthetic analogues: can we predict matching sizes and shapes for selective transport?

An important aspect of nano-object transport attracting more attention is the role of cell membrane tension, which natively is in the order of 0.01mN/m.[43] In many cases the underlying actin cortex is also relevant by inducing a cortical tension in the order of 0.01 – 1 mN/m (see for example[44]). Experimental evidence shows that endocytosis efficiency typically decreases with increasing membrane tension, but for some cell types the response can be inverse.[45] Theoretically, it is expected that tension-induced restraining forces for particle wrapping appear for particle sizes larger than a characteristic length scale defined by bending rigidity and tension.[46] For larger particles, the degree of wrapping is controlled by the competition between tension and adhesion.[47] The release of membrane reservoirs[43] and membrane remodelling[48] upon increasing tension or areas consumed by wrapping, complicate the situation. Before disentangling all contributions in biological environments, however, it will be interesting to investigate wrapping and endocytosis as a function of tension in model experiments with reduced complexity. Tension of the membrane can play a crucial role also for translocation pathways across a membrane. The probability of transient pores induced, for instance, by cell-penetrating peptides is expected to be sensitive to the ratio between cell membrane tension and line tension of the pore.[49]

Passive transport

Passive *translocation* refers to diffusion of small (<10 nm) nano-objects across the membrane, which is chiefly dictated by the properties of the nano-objects and their

interactions with the membrane. By passive we mean processes that do not require any external forces or gradients of other components between both sides of the membrane. They are rather robust, fast and present a universal platform for developing translocation approaches. In particular, stimuli-responsive coatings with multicomponent brushes provide ample opportunities to tailor the passive translocation processes by environmental characteristics such as pH, salt concentration or temperature.[50] The ratio between nano-object size and the membrane thickness as well as its geometric shape are critical parameters.[51] Additionally, the mechanical or chemical responsiveness of the nano-object,[52] i.e., the deformability, and adaptability of the chemical surface composition and charge determine insertion and translocation. Flexible polymers in contact with a membrane may undergo conformational changes such as a coil-globule transitions.[53,54] It is suggested that some cell penetrating peptides switch to helical amphipathic structure in the presence of the membrane.[55,56] In addition, making synthetic analogues of these self-assembled peptides is a challenge. Surface properties of a nano-object can be controlled, e.g., by grafting polymers onto the surface of the nano-object.[57]

Beside passive translocation across the membrane also “passive *endocytosis*” was hypothesised[58] and debated over several decades.[59] The docking step and wrapping of nanoparticles has been described theoretically,[46,60] and explored numerically as a function of shape and adhesion strength of the particle at the membrane.[51,61] While the formation of an invagination can be driven by adhesion at the membrane as observed in model experiments,[11] it remains an interesting question to what extent assisted or active processes are essential for the final pinch-off.

IV. CHALLENGES IN BIOLOGICALLY COMPLEX MEDIA

Experimental and theoretical studies on the interactions between synthetic nanomaterials and membranes in biomimetic or in *in vitro* cellular systems often assume nano-objects of idealized shape, size, and surface in a simple fluid environment. Typical solvent environments considered are salt buffers such as phosphate buffered saline or water with a given concentration of monovalent ions respectively, while biological membranes are embedded in molecularly crowded aqueous environment, such as the cytoplasm and extracellular fluids.

Properties of the surrounding media

Ionic components, proteins or RNA do not only determine simple physical properties such as pH and screening of electrostatic interactions. Very recently it has been discovered that several types of proteins together with RNA give rise to spatially controlled intracellular phase separation into droplets, called RNA bodies or granules.[62] If foreign substances such as macromolecules, micelles, or nanoparticles are inserted into living systems, it is very likely that their properties and interactions with the cell membranes are different from those in simple aqueous solutions. For instance bare nanoparticles can adsorb proteins and thus change their surface properties,[63] but polymers also can change their properties by adsorbing and binding components of the biological fluid. For instance,[64] the puzzling phenomenon of passive translocation of positively charged arginine-rich peptides, and even of oligo-arginines, was explained by the binding of (counter-)anions from the buffer. Few theoretical and simulation studies have taken into account complex formation between nano-objects and other components typical for biological solutions including binding of counter-ions. The compensation of charge in

polymers such as polypeptides can switch the monomer solubility from hydrophilic to hydrophobic since in many cases the uncharged backbone is hydrophobic. If nano-objects are close to the membrane this binding process can be further influenced by the interaction with the membrane in particular by the charge and counterions located near the lipid head-groups. It must be noted that arginine itself is positively charged and strongly hydrophilic which should prevent any passive pathway of these polymers through lipid bilayer membranes. Arginine-rich peptides such as TAT or homeoprotein transcription factors appear in nature and thus are evolutionarily optimized in the presence of biological fluids. Recently it was demonstrated that also cube-octameric silsesquioxanes[65] with similar positively charged ligands efficiently translocate through cell membranes. Experimental evidence thus opens new possibilities for developing bio-inspired cell-penetrating nano-objects but also presents a challenge for theory and experiments using model membranes in artificial environments. A key question is how many and which components of extracellular fluids (if considering the insertion process into the cell) are essential in order to mimic a typical extracellular environment in a representative way? Is there a standard for such a biological medium that is elementary enough to retain the advantages of minimal model systems? Is there a better standard for a biological medium than the typically used phosphate-buffered saline suspensions (pH 7.4 and physiological salt levels) to study nano-object membrane interactions?

Nanomaterials represent length scales where molecular crowding of cellular as well as intracellular environments substantially influence diffusion dynamics, excluded volume effects, and inter-molecular association [[Ellis 2001]]. The new standard medium

therefore potentially contains crowding agents such as PEG or polysaccharides in order to simulate those effects [[10.1110/ps.03288104.]].

Another level of complexity arises when taking into account dynamically changing environments. As an example, during endocytic uptake of nanoparticles, the endosomal compartment is acidified, which can lead to protonation of functional groups on a particle's surface changing its net charge. In the endosome, this change in the pH of the environment is coupled with a change in the lipid composition of the interacting endosomal membrane, which can lead to significant changes in the nanomaterial's ability to disrupt or cross the membrane.[78]

Recent attention is attracted by the dynamic feedback that membranes may induce in biological media via the recruitment of curvature-sensing proteins: One has found that membrane curvature and cortical proteins both can take part in coupled oscillations of shape and concentration [[10.1073/pnas.1221538110 , 10.1038/s41467-017-02469-1]], which presumably contribute to cell signaling processes. How would a nanomaterial interfere with those dynamics?

Protein Corona

In physiological environments, a large number of proteins and other biomolecules are present. These molecules can rapidly bind in a temporally complex way to nano-objects, and form fluctuating coronas around nano-objects that may have a strong influence on their interactions with a biological environment.[67–70] In analogy with the Vroman effect,[71,72] the composition of coronas may vary dramatically over time.[73]

Nanoparticles that are immersed in human blood serum have coronas that consist of proteins such as albumin, immunoglobulins, fibrinogen, apolipoproteins as well as

proteins from the complement system.[9,74,75] There is a large class of proteins called opsonins that label foreign objects to be detected by immune system, and trigger the uptake by phagocytes and macrophages. In contrast, another class of proteins, dysopsonins, including albumins and apolipoproteins are known to inhibit phagocytic uptake.[67,76] The composition of both groups adsorbed at nanocarriers in blood serum controls their elimination by resident macrophages.[77,78] Recent experiments, for instance, seem to explain the so-called stealth effect of polyethylene glycol (PEG) coatings against phagocytosis by the selective adsorption of lipoproteins and apolipoproteins onto the PEG-coated nanocarriers.[79] However the hypothesis that PEGylation of particles increases the binding of dysopsonins that mask the particles was already put forward more than 15 years ago.[80] Since corona formation seems almost unavoidable, the central challenge is to control its composition and structure as a function of time.

Real Biomembranes

Lipid bilayers can be convenient model systems for nanoparticle-membrane interactions allowing detailed physical insights thanks to their relative structural simplicity and well characterised properties. However real biological membranes are far more complex in structure, containing a large amount of both integral and peripheral proteins[81,82] plus a high degree of glycosylation, which provides a complex coating with polymeric sugars. Further complexity is provided by the cell membrane's transmembrane asymmetry, lateral heterogeneities and underlying cytoskeleton, a dynamic network of semi-flexible to rigid polymers. Future theoretical and experimental model systems should start to take this increased membrane complexity into account in order to

understand the true extent to which a lipid bilayer can model nanoparticle interactions at a real biomembrane. For example, giant unilamellar vesicles can be fabricated directly from the plasma membranes of mammalian cells and are known as giant plasma membrane vesicles (GPMVs).[83] They contain most of the natural components of a real cell membrane but without the active processes of a real cell. Therefore, these materials are ideal experimental systems to bridge the gap between model lipid membranes and the whole cell. GPMVs not only allow to test the validity of more abstract theoretical and experimental models, but can be a starting point to study effects of protein and lipid sorting as well as more specific coupling of nanomaterials with biomolecular interaction networks. Although structurally impaired as compared to GPMVs, planar supported membranes made from native cell membranes serve as additional model systems, which allow for a large arsenal of sophisticated surface analytical tools.[84,85]

A further challenge arises in the design of nanoparticles that target a specific cell type. This is particularly important for nanomedicine applications, where drug loaded particles might be targeted to a specific sub-population of cells possessing particular disease pathology. In many disease states, e.g. cancers, it is known that cells upregulate specific cell surface receptors such that they are present in higher concentrations within the plasma membrane.[86] Among many others, well known examples include growth factor receptors,[87] vitamin receptors such as folate receptors[88] and the transferrin receptor.[89] In cancer, receptor overexpression is usually heterogeneous within different cells of a single tumour and also between different patients for a given type of cancer - posing a fundamental challenge when aiming for generalized descriptions of molecular and physical mechanisms of how nano-objects engage in receptor binding. Targeting

approaches have involved the attachment of high affinity ligands to the surface of a nanoparticle that targets these receptors. However, receptors that are overexpressed in disease state are also present in the membranes of healthy cells, albeit at lower concentrations, leaving significant chances for off-target binding to healthy cells. Therefore, we see a central challenge to clarify the effect of ligand density on nano-objects on receptor-mediated uptake. Complementary, the surface density of receptors needs attention as playing a role for nanoparticle targeting to diseased cells. An additional question for *in vitro* systems that are barely addressed in current mechanistic studies, but likely important to unravel the uptake process of nanoparticles, is the impact of hydrodynamic interactions in biological fluid flows on cell-specific adhesion.

V. CONCLUSIONS

Challenges	**Next levels of model complexity.**
I. Missing links to biological context	
The complex outcomes of modern chemical synthesis are often developed far beyond of being precisely trackable and having predictable interactions with biological media. On the other side, abstract theoretical approaches easily miss essential complications of the biological counterparts they try to describe.	Statistical nature of nano-object properties such as polydispersity, in-sample variations of surface shape and composition.
II. Challenges for transport across lipid bilayer membranes	
How to exploit protein machineries for specific nano-object transport?	Include active components in molecular models.
Can we rationalize dynamic barriers for topological transitions in membranes as a function of molecular composition and curvature-inducing nano-objects?	The role of membrane tension is often not investigated systematically in simulation studies and model experiments.
III. Challenges in biologically complex media	

How many and which components of biological fluids are essential in order to mimic a typical biological environment in a representative way? Is there a better standard for a biological medium than the typically used phosphate-buffered saline suspensions (pH 7.4 and physiological salt levels) to study nano-object membrane interactions?	- Diffusion in crowded environments including RNA-controlled granules - specific counter-ion condensation - dynamically changing solvent composition and pH. - binding and interaction of membranes with cytoskeleton and macromolecule-crowded media.
Can we control protein corona composition and structure as a function of time?	
Can we clarify the interplay between ligand density and surface density of receptors for receptor-mediated uptake of nano-objects?	

In this Perspective, we illustrate the progress and collect open questions in the design and function of nanomaterials interacting with lipid and biological membranes. The minimalistic but well controlled design approach used by scientists is conceptually different from biological adaptation and evolution. The mismatch between theoretical or experimental models with reduced complexity and the multitude of interactions concerted within rich biological environments makes it challenging to design functional materials. When focusing on nanomaterial transport through membrane one notices substantial progress in all related fields from theoretical and experimental models, synthesis, to *in vitro* testing involving biological complexity. Both endocytic pathways as well as translocation by penetration through a bilayer are extensively analysed via theoretical models, computer simulation, and experimental studies. On the other side, chemists are today able to synthesize highly advanced materials involving the dynamic control of attached ligands ("molecular surgery"[8]), and to monitor the transport of complex materials through biological membranes.[67] Even the molecular details of the immune response induced by polymer-based coatings and proteins have become more elucidated recently.[79] For further progress in the design flow between theory and in vitro testing we emphasize the potential to close missing links between model systems and the biological context. From one side, theoretical and experimental model systems may include more systematically the next levels of complexity: active components such as enzymes, solvent complexity and co-solvency, the nano-object´s interplay with proteins by means of protein corona and curvature-inducing proteins, the variation of membrane tension, coupling to the cytoskeleton, and the lateral structure of multi-component membranes.

From the other side, systematic model experiments may receive more emphasize before direct *in-vitro* testing of newly synthesized materials. For example, existing theoretical models for adhesion- and tension-dependent wrapping are not extensively tested yet in model experiments. An interesting phenomenon to understand on a physical molecular level will be the membrane fission event during endocytosis. We illustrate the importance of integrating the existing knowledge on membrane fusion, vesicle formation by membrane fission, and vesicle transport into a complete picture of the whole endocytic / exocytic cycle. To precisely determine topological pathway of a nano-object is crucial for knowing which sensitive parts of a cell, e.g. DNA, are exposed to the object for causing potentially toxic effects. ~~Nevertheless, it~~It is important not to over-define the targeted functionality, and to avoid aiming for multi-functionality. Instead, it would be advantageous to require the nanomaterial to be as minimally specific as necessary in order to act as delivery vector, nanosensor, or imaging agent. Finally, we close by throwing two challenging question: Can we create a synthetic analogue of a complete endocytic cycle? Can we adapt a synthetic analogue of active ion transporters for direct translocation of nano-objects?

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