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Interfacing the Cell with "Biomimetic Membrane Proteins"

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Integral membrane proteins mediate a myriad of cellular processes and are the target of many therapeutic drugs. Enhancement and extension of the functional scope of membrane proteins can be realized by membrane incorporation of engineered nanoparticles designed for specific diagnostic and therapeutic applications. In contrast to hydrophobic insertion of small amphiphilic molecules, delivery and membrane incorporation of particles on the nanometric scale poses a crucial barrier for technological development. In this perspective, the transformative potential of biomimetic membrane proteins (BMPs), current state of the art, and the barriers that need to be overcome in order to advance the field are discussed.

1. Introduction

Cellular processes that maintain cell division, differentiation, growth, chemotaxis, and programmed cell death, rely on cellular signaling involving cascades of input/output (I/O) signals. These signals, in the form of modulated ion fluxes, molecular recognition and binding, protein conformational changes, or transport of molecules across the membrane, are mediated by membrane proteins.[1,2] Due to their central role in the regulation of key cellular processes, membrane proteins (MPs) are an important class of therapeutic targets. While MPs make up only ≈23% of the proteome,[3] they are the targets for over 60% of protein targeted drugs.[4–6]

In recent years, the rich repertoire of native (wild type) membrane proteins has been expanded by the introduction of genetically engineered MPs. A major achievement in that area was the genetic manipulation of light sensitive ion channels and opsins which allows for the precise optical activation or inhibition of neuronal signals with exquisite spatiotemporal control, leading to the emergence of optogenetics.[7,8] Other examples are the reprogramming of cellular functions by the empiric modular design strategy of single-pass transmembrane proteins for immunotherapeutic applications[9] and the development of genetically encoded membrane voltage indicators (GEVIs) by fusion of fluorescent proteins to ion channels.[10]

De novo design of membrane proteins by implementation of computational design principles has been also demonstrated. For example, a redox-active membrane protein was designed for facilitation of electron transfer across bilayers,[11] and recently, a successful expression of de novo multipass membrane proteins was achieved,[12] which holds promise for new functionalities in the form of ion channels, sensors, and as activators/suppressors in cell signaling.[13]

An entirely different class of materials, which have the potential to act as integral membrane proteins with an expanded set of properties, are inorganic nanoparticles (NPs).

Progress in synthesis methodologies of inorganic colloidal materials have allowed the assembly of functional NPs with ever-increasing control over chemical composition, shape and size, enabling fabrication of sophisticated heterostructures that display uncommon magnetic, photophysical, electronic, and chemical properties.[14–17] These capabilities allowed for precise control on the engineering of excited-state wavefunctions,[18–20] charge confinement, spatiotemporal control of charge-separated states,[21] spin states, and manipulation of Fermi levels and redox potentials. As a result, NPs have proved to be very useful in numerous applications in optoelectronics,[22,23]

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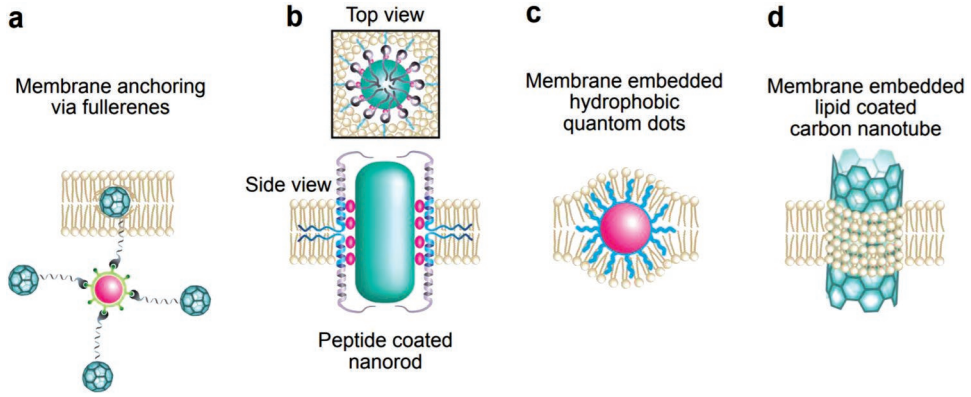

Figure 1. Published functionalization schemes for membrane insertion. a) Fullerene assisted membrane anchoring.[40] b) Peptide coated NR.[41] c) Hydrophobic QD membrane fusion.[78] d) Lipid coated CNT.[36]

biological imaging[24] and sensing,[25–27] catalysis,[28] energy harvesting,[29] biomedicine, and cell surface engineering.[30–33]

While integrating inorganic nanomaterials with biological machineries already provided highly sophisticated hybrid nanobiomaterials, current approaches typically target NPs to interact with the membrane surface or undergo cellular uptake, and do not address the potential of incorporating and retaining NPs in the membrane bilayer core itself, functioning as integral "biomimetic membrane proteins" (BMPs). This type of membrane association has its own unique properties and potential to drastically expand the repertoire of tools for cell membrane engineering. Such materials could be engineered to stably self-insert into the membrane using biomolecular recognition principles while maintaining the superior properties of inorganic materials.[34,35] Serving as molecular-level interfaces, BMPs with unique properties could interact and expand the function of native (and/or biologically modified) integral membrane proteins, and allow for unprecedented level of cellular manipulation and control.

2. Examples for Possible BMP Applications

Several "early bird" demonstrations of BMPs have already been reported (**Figure 1**). A few examples are membrane ion channels formed by lipid coated carbon nanotubes (CNTs),[36] ion channels,[37,38] and membrane sculpting materials[39] fabricated from DNA origami, and membrane potential sensors using quantum dots (QDs) conjugated to fullerene via peptides[40] and amphiphilic peptide coated nanorods (NRs).[41]

A concerted effort for developing BMPs will allow to customize the cellular interface, introduce new (man made) I/O modalities, and directly modulate and add new membrane functions.

We describe below three possible examples where BMPs could afford disruptive applications in biomedicine (neuronal recording and vision restoration by signal actuation) and biology (the "artificial leaf").

2.1. High Resolution Mapping of Neuronal Networks

One of the major goals of neuroscience is to unravel how the brain functions in its entirety and how it generates behavior. In order to understand the brain, tools need to be developed to allow the investigation of interactions between individual neurons.[42] Multielectrode recordings have provided important insights but have limited performance when dense local circuits need to be analyzed or when signals from specific types of near-by neurons need to be distinguished. For this reason, considerable efforts have been invested in developing new architectures of microelectrodes[43] and optical detection methods[44] such as voltage-sensitive dyes (VSDs)[45] and genetically encoded voltage indicators (GEVI).[46–49] VSDs, however, suffer from some shortcomings. They could alter membrane capacitance, be phototoxic, suffer from photobleaching, have a short retention time in the membrane, and generally stain the cell membrane in a nonspecific manner, resulting in nonspecific background labeling. GEVIs offer many advantages over VSDs, but are quite dim (compared to inorganic nanoparticles) and require protein over-expression which may alter normal cell function.

The potential of applying voltage sensing nanoparticles (vsNPs) in neurobiology have recently been demonstrated for asymmetric type-II seeded NRs.[41] These particles show large quantum confined Stark effect (QCSE) at room temperature on the single-particle level.[50] The surface of these particles was functionalized by transmembrane α-helixes peptides designed to impart the voltage sensing NRs (vsNRs) with membrane-protein-like properties, i.e., with hydrophobic side surface and hydrophilic tips (top and bottom) that extrude on both sides of the membrane, and thereby promote insertion of the vsNRs into the membrane in a vertical orientation. Once inserted, it was possible to optically record, noninvasively, membrane potentials at the single particle level (**Figure 2**). Another example of membrane potential visualization using NPs was recently demonstrated by electron transfer from a QD to its conjugated membrane embedded fullerene nanoparticle in cultured cell lines and in live mice.[40,51]

Further development of high sensitivity vsNPs could afford unprecedented ways for studying electrical activities in neuronal, neuromuscular, and visual systems, offering super-resolution voltage sensing on the nanoscale (such as across a single synapse), or the ability to record large number of signals from a large-field of view (high throughput recording). A wealth

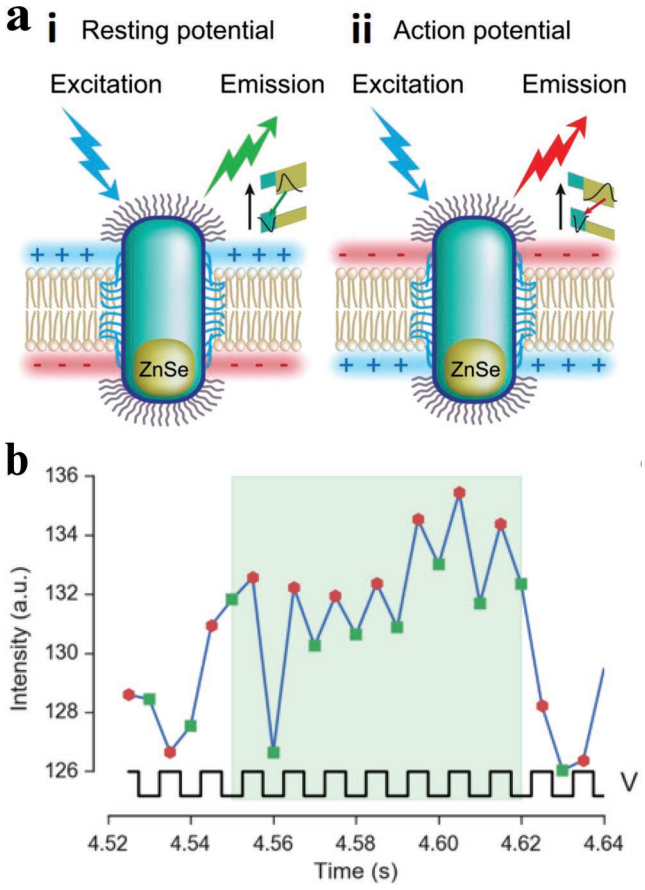

Figure 2. Single particle membrane potential sensing. a) A membrane embedded type-II semiconductor seeded nanorod is excited by absorption of UV/blue light. The excitonic emission (S1-S0 transition) experiences quantum confined Stark effect (QCSE) under external electrical field, which modulates the energy levels and hence the emitted photon energy. As compared to i) resting potential, the emitted photon during an ii) action potential could be either red- or blue-shifted. b) Fluorescence intensity trace of a single peptide coated NR embedded into cultured wild-type HEK293 cells, by simultaneous patch-clamp and fluorescence measurements. Each marker represents an average intensity during the voltage-on (green squares) and voltage-off (red dots). Adapted with permission.[41] Copyright 2018, The Authors, Published by American Association for the Advancement of Science.

of high-resolution data is expected to benefit many research areas. From a pharmaceutical perspective, high-resolution neuronal activity mapping has the potential to allow for drug design and therapies for neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, and depression. In a broader sense, data from high-resolution spatiotemporal recordings of neuronal circuits will enhance our understanding of neural networks and will fuel emerging fields like neuroinformatics, medical informatics, neuromorphic computing, and neurorobotics.

2.2. Vision Restoration

The emerging fields of "bioelectronics" and "electroceutical therapies"[52,53] intend to treat diseases by corrective stimulation of neural pathways. Recording aberrant neuronal signals from peripheral neurons would present the opportunity to correct them, through an external feedback circuit.[54] Advances in fabrication and miniaturization of (micrometer-scale) devices like microelectromechanical (MEMs) systems,[43] together with the growing understanding of nervous system diseases, has paved the way for neuromodulation in therapeutic applications. These advances have led to innovative treatments for inflammatory diseases such as Crohn's Disease and rheumatoid arthritis.[55] However, micrometer-scale MEMS devices are huge compared to membrane proteins, their nanometer-sized biological I/O counterparts. A specific bioelectronics implementation of BMPs could be used in vision restoration. In cases of outer retinal degenerative diseases, such as retinitis pigmentosa (RP) and age-related macular degeneration (AMD), there is a loss of photoreceptors while the inner retinal neurons that process the visual signals and relay them to the brain are left relatively well preserved.[56,57] Patterned activation of the remaining inner retinal neurons, while bypassing the diseased photoreceptors, has the potential to restore vision. Electrical stimulation of the retina by an implanted retinal prosthesis, the current leading technology for visual restoration, shows some restoration of useful sight, but the obtained visual acuity is poor with most patients, seeing low contrast and low-resolution vision. Light sensitive BMPs however, can be targeted to either nonfunctioning photoreceptors, or to the bipolar or ganglion cells in cases where the photoreceptors are degenerated. Another potential approach which is suitable for cases where the entire eye is diseased, or in cases of blindness from inner retinal diseases (e.g., glaucoma), where there is a loss of ganglion cells, is to optically activate the visual cortex. Indeed, Ma et al. have recently demonstrated the first implementation of this concept, facilitating enhanced near-infrared (NIR) vision in mice by ocular injection of retinal photoreceptor binding nanoparticles that upconvert NIR light into the visible spectrum. Patterned activation of the retina, by NIR light would have the benefit of avoiding photophobic reaction and phototoxicity.[58]

2.3. Light Harvesting: The "Artificial Leaf"

Due to semiconductor NPs broad absorption spectrum, tunability of emission spectrum, and control over nonradiative energy transfer, NP-membrane integration could be employed for energy harvesting purposes. In particular, the utilization of semiconductor QDs and NRs as nanoantennas that funnel energy into natural light harvesting complexes, or as artificial photosynthetic reaction centers (RCs), could expand the spectral range of natural light harvesting and the efficiency of proton pumping, and ultimately increase energy conversion efficiency. However, to this date, most hybrid light harvesting bio-nanomaterials have been synthesized from purified components and assembled in solution (in vitro) or in films. In contrast, most biological machineries involved in light harvesting and photosynthesis are embedded in membranes (as for example, light harvesting complexes, reaction centers, proton pumps, ATPases) as they require compartmentalization for their energy transaction reactions.

In *Halobacterium salinarum*, an archaeon which grows in salt ponds under low-oxygen conditions, conversion of light into high-energy molecules consists of bacteriorhodopsin (bR) which directly transfers a proton across the membrane upon light absorption. The resulting proton gradient is then used by ATPase to convert ADP into ATP.[59] The efficiency of this energy conversion process is, however, quite low (only about ≈5% in plants). One of the reasons for the low efficiency is due to photosystems not utilizing high-energy UV photons (since that might destroy the light harvesting chromophore).

Inorganic NPs could act as robust UV chromophores and therefore overcome the above- mentioned limitation. They can provide higher absorption cross-section and wider spectral range for light harvesting than their organic counterparts. It was recently theoretically suggested that inorganic QDs could achieve significantly greater absorption than a natural photosynthetic system.[60] Following that suggestion, QDs were coupled to purple bacteria photosynthetic RC via förster (fluorescence) resonance energy transfer (FRET) and enhancement energy transfer factors were indeed observed. QDs were also coupled to purple membrane (PM) patches containing bR trimers (arranged in a closely packed 2D hexagonal array) by electrostatic interaction,[61,62] molecular recognition,[63] and covalent conjugation[62] and efficient FRET from QDs to bR was demonstrated.[62,63] Moreover, enhanced proton pumping was demonstrated for QDs coupled to bR that was incorporated into proteoliposomes.[62] Very recently, Milano and coworkers inserted small (>≈3 nm) QDs into membranes of liposomes containing RC from *Rhodobacter sphaeroides*,[14] and Woodbury and coworkers conjugated dyes as artificial antennas to RC from *R. sphaeroides* and measured higher charge separated state formation efficiency due to FRET.[64]

Another important advance has been the recent utilization of NPs as artificial reaction centers for the photocatalytic production of hydrogen. Alivisatos and coworkers demonstrated a long-lived charge separated state in a platinum-tipped CdSe-seeded CdS nanorod, supporting efficient water reduction and hydrogen production at the platinum (Pt) tip.[65] CdSe/CdS core/shell QDs in a Na_2S/Na_2SO_3 solution have also exhibited efficient photocatalytic water splitting activities.[66] Han et al. reported efficient light-driven water splitting using CdSe QDs capped with dihydrolipoic acid (DHLA) as the light absorber and a soluble Ni^{2+}-DHLA catalyst for proton reduction with ascorbic acid as an electron donor at pH 4.5.[67] Zhu et al. reported near-unity quantum yield light-driven reduction of methylviologen (MV2+), a common redox mediator, using colloidal quasi-type II CdSe/CdS seeded NRs as a light absorber and charge separator and mercaptopropionic acid as a sacrificial electron donor. In the presence of Pt nanoparticles, their system efficiently converted sunlight into hydrogen.[28] In all of these cases semiconductor NPs were homogeneously distributed in solutions.

However, in order to construct energy harvesting hybrid devices inside the membrane, NPs need to be stably inserted and positioned in the membrane with minimal size constraints that could jeopardize their capabilities to i) sustain a long-lived charge separated state, ii) act as light harvesting antennas that nonradiatively transfer energy to a RC, iii) catalyze reactions on both sides of the membrane, or iv) sense the voltage across the membrane. A robust and size-independent approach is therefore needed for their membrane insertion. **Figure 3** illustrates possible configurations for efficient membrane embedded light harvesting hybrid constructs.

3. The BMPs' Membrane Insertion and Retention Challenge

The important role of NPs in biomedical applications requires to understand the key factors and mechanisms that affect the interactions between NPs and cells. Model cell membrane systems studied by computer simulations and physicochemical experiments have elucidated many of the factors and mechanisms involved in NP-membrane interaction.

These studies have revealed the intricate interplay between diverse properties of NPs (geometry,[68] size,[69] ligand surface charge and density,[70] ligand type, and patterning[71]) and membranes (curvature,[72] fluidity, surface charge, lipid composition,[73,74] and lipid lateral diffusion[75]) that modulates the energetics of various phases of NP-membrane interaction, i.e., adsorption, fusion, permeation, pore formation, and aggregation. The case for NP interaction with biological membranes in a cellular environment is more complicated due to additional cellular uptake mechanisms as has been demonstrated by in vitro and in vivo studies.

The properties of NPs employed in biomedical applications are typically modified in order to meet the specific requirements for a particular application. Numerous functionalization and bioconjugation methods have been developed for the integration of inorganic-biological hybrid nanomaterials that are water-soluble and biologically active.[76,77] The overwhelming majority of them are aimed towards either NP adsorption to the cell membrane surface, or gaining efficient cellular uptake.

Compared to published literature studies on cellular entry of NPs, only few studies investigated the integration and retention of hydrophobic NPs into the membrane bilayer, and to a lesser extent, the integration of NPs with diameters exceeding the thickness of biological membranes.

The main reason relates to the challenge associated with the unique requirements of NP surface functionalization for this purpose. The second reason relates to the apparent lack of obvious applications—which will be solved if the challenge is solved.

3.1. Why Membrane Insertion and Retention is a Challenge?

In many cases, the surfaces of as-synthesized NPs are decorated with a mixture of highly hydrophobic ligands and are insoluble in biologically relevant media. Therefore, without any surface modification, only small, hydrophobic NPs can be efficiently incorporated into synthetic vesicle membranes in between the two leaflets. For example, very small QDs (<3 nm diameter) were incorporated into the lipid bilayer of fusogenic vesicles that were subsequently delivered into membranes of human embryonic kidney (HEK) 293 cells via vesicle fusion in QDs.[78] In another study, small QDs coated by membranes of

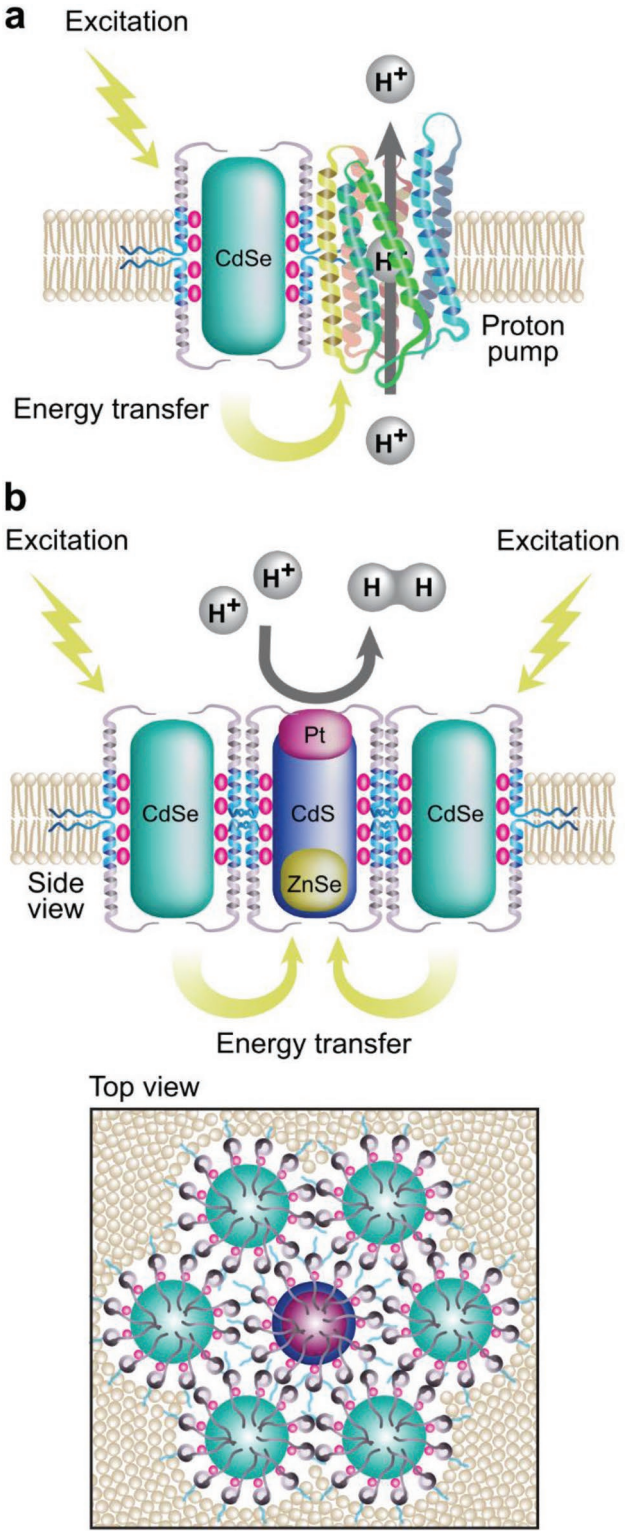

Figure 3. Light harvesting by membrane embedded nanoparticles. a) A membrane-embedded nanoparticle is spatially coupled to a protein proton pump. Conversion of the absorbed high-energy photons to emitted low energy photons which are absorbed by the pump increases pumping efficiency. b) Light harvesting NR antennas are coupled to a ZnSe/CdS/Pt nanoparticle and provide the required energy for photocatalytic hydrogen production.

red blood cells were fused into human hepatoma cells as cell imaging agents.[79]

Since phospholipid bilayers are typically 4–5 nm thick,[80,81] the inclusion of hydrophobic NPs within the bilayer is size limited to ≈5 nm in diameter, including the thickness of the organic coat (Figure 1c). Inclusion of larger hydrophobic NPs is thermodynamically unfavorable due to the energetic penalty associated with protrusion of hydrophobic ligands into the polar solvent.[79] This limitation practically excludes higher order structures and functionalities that could be beneficial in terms of signal or actuation strength. To overcome this limitation, the surface properties of NPs must be modified to be amphiphilic in order to maintain colloidal stability in biological media, while having the capacity to favor interactions with the hydrophobic bilayer core following adsorption to the membrane surface.

3.2. The NP-Membrane Insertion Process: Lessons from Heterofunctional Monolayer Coated Gold NPs

A systematic study on the insertion mechanism of monolayer-protected amphiphilic gold nanoparticles into single component membrane bilayers was performed by Van Lehn, Alexander-Katz, and colleagues.[72,82–91] In their work, particles coated with different surfactant monolayers, composed of mixtures of hydrophilic and hydrophobic ligands (resulting in amphiphilic surface properties), were modeled by either thermodynamic calculations,[85,86] or atomistic[72,87–90] and coarse-grained[83,84,91] molecular dynamics simulations. They showed that fusion capacity emerges from a delicate interplay between ligand type, ligand composition, ligand surface distribution, and NP size.

More specifically, membrane insertion energetics was simulated for gold nanoparticles (AuNPs) coated by monolayers composed of mixtures of an anionic ligand, 11-mercaptoundecane sulfonate (MUS) and a hydrophobic ligand (OT) at various MUS:OT compositions.[82] The simulation was applied for AuNPs in the size range of 1–10 nm in diameter. The simulations yielded free energy curves which showed that AuNP insertion into the membrane was favorable 1) with increasing content of hydrophobic ligands in the monolayer and 2) for an AuNP diameter at some intermediate value along the curve obtained for each composition. The free energy curve for insertion above and below that value followed a sharp increase in the free energy until the overall change was positive, indicating a maximum cutoff diameter for stable insertion.

The simulation results were interpreted as a consequence of the conformational flexibility of the anionic ligands which allows the ligands to deform and snorkel charged end groups to the nearest aqueous interface, thereby minimizing the unfavorable insertion of charges into the bilayer core, and simultaneously increasing the amount of hydrophobic surface area exposed to the bilayer core. A clear dependence was observed for the degree of ligand deformation on NP diameter. For particles of smaller diameters, the large amount of free volume contained in the monolayer, is maximizing ligand fluctuations and minimizing the barrier to snorkeling. For AuNPs of large

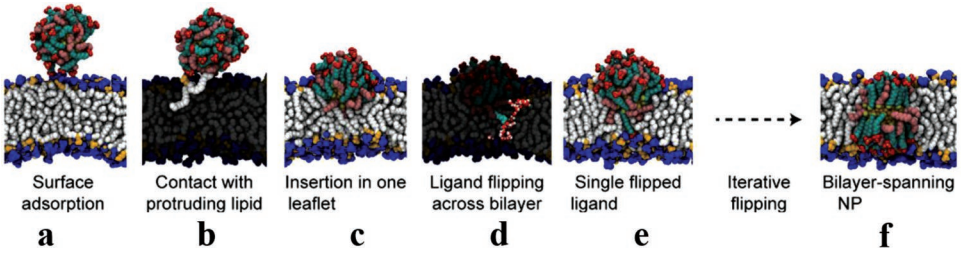

Figure 4. A membrane fusion model for AuNPs: a) An NP adsorbs to the membrane surface by electrostatic interactions, diffuses on the surface, and at some point b) contacts a lipid tail protruding from the bilayer. c) This interaction promotes insertion of the NP into the upper leaflet. d) Charged ligands cross the bilayer (flipping) e) positioning the head group solvent exposed at the inner leaflet. f) After a series of iterating flipping motions, the NP is fully embedded within the bilayer with the charged groups distributed evenly on both sides of the membrane. Adapted with permission.[90] Copyright 2019, Public Library of Science.

diameter, however, the radius of the NP curvature is smaller, which leads to a decreased volume accessibility for each ligand.

The validity of the results was tested by capacitance measurements of black lipid membranes in the presence of AuNPs synthesized to have the same size and monolayer composition used in the simulations. The observed increase in membrane capacitance in the presence of AuNPs was interpreted as resulting from integration of the AuNP into the bilayer, confirming the simulations results. When the same particles were applied to tissue cultured cells however, they were internalized into the cell and did not retain in the cell membrane.

Further studies enabled to compile a complete description of the incorporation process (**Figure 4**).[90] The initial step is NP adsorption to the bilayer surface due to electrostatic interactions between the charged ligand end groups and dipolar lipid head groups. Following adsorption, the NP diffuses along the bilayer surface until it encounters a protruding hydrophobic lipid tail. The encounter triggers partial insertion into the upper leaflet of the bilayer enhanced by ligand "snorkeling," and from this partially inserted configuration, charged end groups iteratively cross the bilayer, in a similar way to the well-known lipid "flip-flop" motion in cells, until a thermodynamically favorable membrane-spanning configuration is reached.

The model implies that if the ligands are not fixed to the nanoparticle surface by strong covalent bonds, but rather are free to diffuse and re-distribute along its surface, charge-free patches could be created, lifting the limitation posed by the accessible free volume for ligands to "snorkel" and possibly allow for larger particles to be inserted into the membrane.

This was recently demonstrated for AuNPs functionalized with hydrophilic (deprotonated mercaptoundecanoic acid, MUA) and hydrophobic (octadecanethiol, ODT) ligands, that are known to dynamically redistribute on the surface of AuNPs in response to changes in the local environment. In the presence of surfactant vesicles having membrane thickness of 2.5 nm, AuNPs of 6 nm core diameter were incorporated into the bilayer.[92]

These studies highlight the unique surface properties required for membrane incorporation: for an efficient membrane insertion and retention, the NP surface should be amphiphilic, with the capacity for dynamic modulation of surface properties in response to changes in the molecular environment, through spatial redistribution of surface ligands.

For the case of nonspherical particles, the situation is even more complicated. For some applications, nonspherical NPs must be inserted in a specific orientation relative to the membrane surface. That means that specific facets of the NPs must be selectively functionalized to drive integration into the membrane at a specific orientation. While recent computational and experimental studies have provided important insights into the interaction process between NPs and lipid membranes, the development of the theoretical and experimental framework dedicated to meet the unique requirements for NP integration membranes is still in its early stages.

4. Chemical Solutions for the Membrane Insertion and Retention Challenge

While heterofunctional monolayer surface coatings proved to be an efficient functionalization strategy for spherical AuNPs, the repertoire of NP types, materials and shape can benefit from other surface functionalization approaches.

4.1. Facet Selective Surface Functionalization

Integration of nonspherical particles, such as NRs, into the membrane, may pose even more stringent conditions on surface functionalization than those for spherical NPs. This is because for some applications (discussed in Section 2), the NR orientation inside the membrane relative to the membrane surface needs to be controlled. To achieve directional insertion, facet selective surface functionalization (stereoselective chemistry) schemes must be developed.

Facet selective surface functionalization can be designed based on i) NP properties (geometrical, chemical, etc.), ii) ligand properties (length, stiffness, structure, and chemical composition), or iii) the combination of the two.

An example for utilizing an intrinsic property of NPs for facet selective surface functionalizing was demonstrated by Banin and coworkers which used type-II CdSe/CdS NRs that produce large charge separation upon light illumination to selectively grow a gold sphere onto one tip of the NR.[93] Park et al. took a different approach by introducing peptides with sequences containing blocks of hydrophobic and hydrophilic amino acid segments that were designed for preferential binding to the long axis of CdSe/CdS NRs.[41] They showed that functionalized NRs insert into the membrane and can act as single molecule membrane potential sensor.

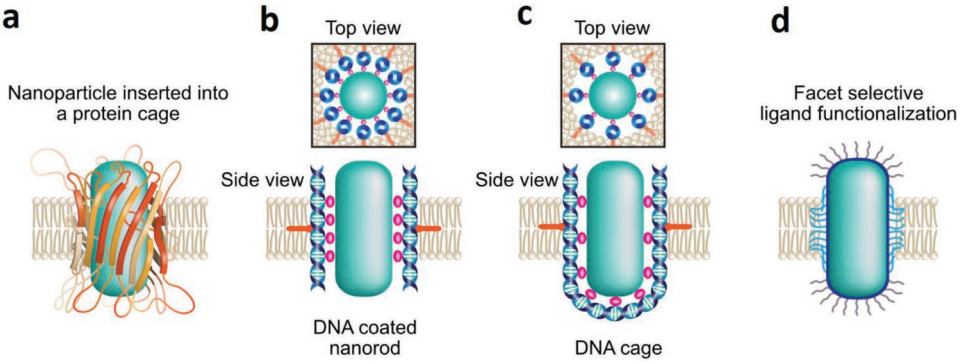

Figure 5. Depiction of potential functionalization schemes: a) protein cages for encapsulation and anchoring of NPs. b) dsDNA surface functionalization. c) DNA origami cages; d) facet selective functionalization with hydrophobic and hydrophilic ligands.

4.2. Geometry-Based Surface Functionalization

Another possibility is the utilization of NP geometry. For example, curvature dependent chemical reactivity was demonstrated for gold NRs coated by 11-mercaptoundecanoic acid (MUA). The curvature dependent ligand density resulted in variation of the pKa of the carboxy end group of MUA which in turn, yielded pH dependent chemical reactivity.[94,95] The effect was demonstrated by attachment of small, cationic gold NPs to different facets of gold NRs as a function of pH. In principle, the effect can also be used to covalently modify specific facets in the design process of BMPs to promote membrane insertion and retention (**Figure 5**d).

5. Bioinspired Solutions for the Membrane Insertion and Retention Challenge

Integrating NPs with biological scaffolds is an intriguing and sophisticated functionalization route. In the following section, we describe and suggest possible functionalization schemes based on functionalized DNA origami channels (Figure 5b), DNA origami cages (Figure 5c), and pore forming proteins (Figure 5a).

5.1. DNA Origami Cages

DNA origami is a technique utilizing the self-assembly property of single-stranded DNA to create 2D and 3D folds by design. DNA origami, introduced by Paul Rothemund in 2006, is based on short oligonucleotides (staples) that bind to defined segments of a long single-stranded DNA (scaffold) molecule in a sequence-specific manner, and direct its folding into desired shapes in a predicted manner.[96] The technique is highly advantageous from an engineering point of view. Functionalization of the staple strands with chemically modified groups, enables the precise positioning of structural and functional elements like fluorescent dyes, nanoparticles, proteins, etc. Functionalization with amphipathic moieties allows DNA origami nanostructures to bind to lipid membranes. The control over biochemical functionalization, the sequence specificity, reproducibility, and ease of DNA design, its self-assembly and biocompatibility makes DNA origami a useful tool for biomimicking and investigating membrane proteins.[97]

Advances in DNA origami fabrication have already demonstrated synthetic transmembrane channels in lipid bilayers. These channel-like nanostructures, arranged as a bundle of six DNA duplexes with a nominal inner diameter of 2 nm, showed functional properties (e.g., gating, conductance, etc.) similar to natural ion channels anchored to lipid bilayer.[98–101] An alternative approach to the DNA origami nanopores has been described with amphipathic DNA tiles, creating a bundle of four duplexes with a 0.8 nm channel in lipid bilayers.[102] Recently, a large DNA membrane channel with a ≈4 nm diameter pore was reported. This channel has stable electrical properties and spontaneously inserts into flat lipid bilayer membranes.[103] Moreover, using cholesterol-based membrane anchoring, a large-conductance transmembrane funnel-shaped DNA origami porin was created having a nominal cross-section of 6 nm and high conductance.[37]

Another possible application of DNA origami is encapsulation of nanoparticles (Figure 5c). DNA origami nanocages were demonstrated to encapsulate gold nanoparticles of various sizes. The surface of the particles was covered with ssDNA that was designed to hybridize with complementary probes displayed on the inner surface of the origami cage.[104] Another significant advance is the formation of a "box-shaped" 3D DNA origami of ≈40 nm dimensions. This 3D DNA origami was used as a nanocontainer to encapsulate exactly one 10 nm gold nanoparticle. The particles were functionalized with thiol-modified DNA strands to attach complementary strands in the interior surface of the box.[105] Thus, there is a significant progress in creating DNA nanostructures functioning as biomimetic transmembrane channels and as nanoparticles vehicles. The combination of both design principles may be a useful approach for the encapsulation and membrane insertion of NPs (Figure 5c).

5.2. Pore Forming Proteins

Another possible solution to the problem of NP-membrane insertion would exploit existing biological machinery. Given that NPs are analogs of membrane proteins it is only logical to look at the machinery for insertion of native membrane proteins. In mammals, membrane insertion of native membrane

proteins is accomplished by a dedicated molecular machinery (translocons) that carefully threads nascent chains off the ribosome and into the endoplasmic reticulum (ER) membrane.[106] This is not an ideal mechanism as NPs are analogs of a folded protein and cannot be threaded into the membrane.

A much better solution might come from viral and bacterial pore forming proteins termed porins. These proteins are secreted as water-soluble proteins, and undergo a phase switch to hydrophobic proteins upon membrane interaction in a way that allows them to integrate into a host membrane, and form a pore at their center. Most of the known pore forming structures have an internal diameter of ≈1–2 nm which is too small for accommodating NPs of a minimal diameter of >3 nm. However, there are some exceptions to the rule. For example, the pore forming complex, Hcp1, derived from the type VI secretion system, has an inner diameter of ≈4 nm which is large enough to incorporate small NPs.[107] Another example of pore forming proteins that might be suitable for NP integration into the membrane are the twin-arginine translocation (Tat) system proteins which transports folded proteins across the bacterial cytoplasmic membrane and the thylakoid membrane of plant chloroplasts.[108,109] The system is composed of native membrane proteins forming pores with inner diameters of 3–7 nm. Protein transport across the membranes via the Tat system is initiated by binding of the signal peptide on the transported protein and the binding site of TatC (an essential membrane protein of the Tat system).

Inclusion of NPs in protein pores (Figure 5a) might be achieved by targeting an NP to the Tat system by conjugation of the Tat signaling peptide to the NP surface. Another way to insert an NP into a porin might be by conjugation of one of the pore forming subunits to an NP and allowing for self-assembly to proceed to form membrane embedded pore. This can be achieved only if the NP does not significantly alter the structural properties of the protein sub unit required for self-assembly.

Although with some underlying differences, this type of NP encapsulation was demonstrated for protein nanocages from viral capsids that have been applied for drug delivery of small molecules, DNA, organic dyes, and peptides, and encapsulation of nanoparticles.[110,111] In one case, viral capsids, self-assembled around a gold nanoparticle of over 8 nm in diameter.[112,113] We hypothesize that in analogy to encapsulated gold NP by viral capsids, in theory it should be possible to induce assembly of pore forming monomers around NPs.[107]

An alternative, highly attractive approach, is the utilization of de novo design of membrane proteins for the encapsulation and insertion of NPs. Breakthroughs in de novo design and expression of water soluble pore forming proteins and transmembrane proteins were recently demonstrated.[11–13] We argue that a dedicated effort to produce a pore forming membrane protein with precise geometries and tunable pore sizes would accelerate many aspects of the inorganic interface to biological systems.[12,114,115]

6. Probing Nanoparticle–Membrane Interactions

The interaction between nanoparticles and biological membranes has been an ongoing subject of study, as nanoparticles are getting more ubiquitous in pharmaceutics. The question of whether a nanoparticle will adhere, embed itself, or pass the cell membrane, is highly crucial in terms of its potential therapeutic/toxicity effects. Most studies have focused thus far on intracellular uptake and internalization[116–118] and on transient membrane poration.[119,120] Detailed work has also been done on nanoparticles that access the cell cytosol by spontaneously incorporating themselves into the lipid membrane.[121,122]

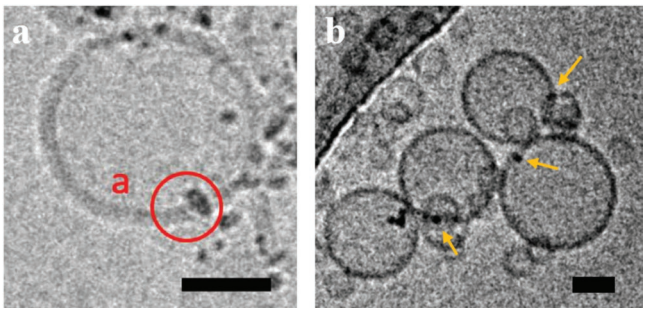

Figure 6. Examples of Cryo-EM images of nanoparticles fused into membranes: a) peptide-coated NRs. Scale bar 30 nm. Reproduced with permission.[23] Copyright 2007, Springer Nature. b) Lipid coated QDs embedded into SUVs. Scale bar 20 nm (unpublished data).

Cryogenic transmission electron microscopy (cryo-TEM) provides a direct evidence for the mode of NP-membrane interaction, and was used to demonstrate membrane insertion of nanoparticles[123,124] (**Figure 6**). However, cryo-TEM provides only a static image of the interaction state. It is also a low-throughput method and therefore less suited for large-scale studies with many varied parameters. Examples of experiments probing the interaction dynamics of nanoparticles with lipid membranes include: i) Leakage assays by fluorescence quenching,[125] in which the interior of small unilamellar vesicles (SUVs) contains high concentrations of quenched dyes (e.g., calcein). Nanoparticle-membrane interaction causes membrane disturbance, which releases the dye that becomes fluorescent and therefore detectable; ii) Quartz crystal microbalance assays,[126,127] in which a supported lipid bilayer is formed on a quartz resonator, and are highly sensitive to small mass changes of the bilayer upon nanoparticle adhesion or insertion; iii) Capacitance changes measurements with suspended lipid bilayers.[128] Nanoparticles can alter electrical properties of lipid bilayers by either integration into the membrane, thus altering its capacitance, and/or disturbing membrane integrity, thus creating transient ion conductance; iv) Atomic force microscopy (AFM).[129,130] AFM imaging of supported lipid bilayers exposed to NPs can provide a nanoscopic height map, by which the extent of NP embedding can be estimated.

Probing the interaction of nanoparticles with lipid membranes is a challenging task. However, considerable amount of research and relevant assays have been developed for probing peptide- and protein-membrane interactions. Such tools and insights could therefore be harnessed to study NPs-membrane interactions.

7. BMPs' Delivery and Targeting

Translating NP delivery under idealized conditions to a complex biological environment is one of the major challenges that

face precision medicine. This is because in order to deliver a nanoparticulate to a specific tissue or organ, the vehicle must be engineered to interact with nothing but the target. Modern approaches achieve that goal by conjugation of the carrier surface to a recognition molecule (antibody, aptamers, peptides, and small molecules[131,132]) or by biomimetic coatings such as cell membranes that camouflage the carrier.[133]

Typically, the delivery mechanism is tailored to meet the application requirements. In our view, precision delivery of BMPs in a complex biological media, can be achieved by application of methodologies developed in the field of liposome technology. Since BMPs function is associated to proper integration into a lipid bilayer, the selected surface functionalization method can be optimized for integration into the membrane of a fusogenic vesicle decorated with specific receptors for targeting, under controlled conditions. The two main advantages arising from this type of fabrication are 1) the decoupling of targeting capabilities from NP surface functionalization and 2) the capability to thoroughly characterize the construct with respect to integration efficiency, orientation, etc. This approach was demonstrated in the incorporation of small QDs into fusogenic vesicles and the subsequent membrane fusion to HEK293 cells in live cells experiments.[78]

Another advantage of liposome technology that is suitable for NP integration are the methodologies developed for the formulation of proteoliposomes. Initially developed for the reconstitution of native membrane proteins into model lipid bilayer vesicles for protein structure studies, the methodology involves denaturation of biological membranes using a mixture of lipid detergents and/or surfactants in the presence of excess lipids of choice, and the subsequent removal of the detergent which leads to folding and incorporation of the membrane proteins into the vesicles. This approach has yielded many examples of successful assembly of proteoliposomes of different types of membrane proteins and by various methods.[134] The basic principles of this technique was recently applied for membrane incorporation of small QDs.[14] An advantage of this technique to integration of NPs with lipid vesicles is that it opens up a different route for incorporation, that is, instead of designing an NP to penetrate into an assembled membrane, the NP is incorporated into the membrane during the self-assembly of the membrane. This route reduces the stringency in the requirement of the NP to have an amphiphilic surface that is capable of phase switching between hydrophilic and hydrophobic environments.

8. Conclusions

Further innovations along the lines described in this perspective will offer the merging of two mature nanotechnology subdisciplines: synthesis of functional NPs/nanodevices and their functionalization chemistries for such NPs. Functionalization chemistries will need to be further developed in order to achieve robust membrane insertion of NPs. This merger will afford the introduction of new I/O interfaces to cells, and open new vistas in biomedical and chemical engineering. Membrane embedded sensors, actuators, and catalysts will serve as novel tools for diagnostics, biomedical intervention, and industrial processes such as chemical synthesis and energy harvesting.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

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