Review

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Biomimetic nanopores: learning from and about nature

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Through recent advances in nanotechnology and molecular engineering, biomimetics – the development of synthetic systems that imitate biological structures and processes – is now emerging at the nanoscale. In this review, we explore biomimetic nanopores and nanochannels. Biological systems are full of nano-scale channels and pores that inspire us to devise artificial pores that demonstrate molecular selectivity or other functional advantages. Moreover, with a biomimetic approach, we can also study biological pores, through bottom-up engineering approaches whereby constituent components can be investigated outside the complex cellular environment.

Biological nanopores

The biological cell is filled with many different types of pores and channels that control the exchange of ions and molecules between subcellular compartments. These passageways are of vital importance to cellular function [1]. Examples include: ion channels at the cell surface that regulate the flow of ions; pores inserted into cell membranes upon viral infection that serve as conduits for genome transfer; the nuclear pore complex (NPC) that controls the transport of mRNA and proteins across the nuclear envelope of eukaryotic cells; and pores that are used for protein secretion into cell organelles.

Advances in nanotechnology now make it possible to study and shape matter at the nanometer scale, opening the way to imitate biological structures at the molecular level to both study and harness their ingenuity [2–6]. Indeed, since their first fabrication about a decade ago, nanometer-sized pores and channels in solid-state materials have served as a scaffold for a variety of biology-inspired applications (for recent reviews, see [7,8]). A major direction of these efforts has been to devise molecular separation methods that exhibit selectivity based on specific biochemical properties [9]. In other work, wild-type and genetically modified biological pores have been used as sensitive biosensors to detect molecules in solution (see [10] for an overview). Alternatively, biomimetic approaches have used a bottom-up engineering strategy, using both biological and synthetic components so that a complex biological system can be simplified for *in vitro* studies that are not otherwise possible. A related approach – similar but different to biomimetics – is the emerging field of synthetic biology, which aims to use and develop biology as an engineering-like science to achieve new biological structures and functions [11]. Typically, however, synthetic biology does not involve artificial (non-biological) materials, as do biomimetics.

In this work, we review the field of biomimetic pores and channels, focusing specifically on two different approaches (Figure 1) and their applications. First, we highlight engineering efforts inspired by the ingenuity and physical characteristics of natural biological systems. This entails both purely synthetic systems that seek to mimic a biological counterpart and systems that incorporate biomolecules or complexes to harness their specificity or function. Second, we discuss how biomimetic pores have been employed to study the mechanistic properties of their biological counterparts. Although this review focuses on experimental studies, it should not go unmentioned that theoretical approaches, such as computational modeling and simulations, have contributed to both our understanding of natural systems and to design principles for engineering approaches (for recent reviews see [12,13]).

Biology-inspired engineering

Fabrication of artificial pores and channels

Central to the construction of a biomimetic pore or channel is the fabrication of an artificial pore or channel that can serve as a scaffold for further modification. Note that the naming of pores/channels merely reflects the aspect ratio of the passageway: where a pore has a diameter larger than its depth and a channel has a depth much larger than its diameter. Using various fabrication technologies [7,14–17], nanopores or channels can be obtained in a variety of different shapes and structures. For example, nanochannels can be made by straightforward planar lithography but also by ion-track etching, which allows accurate control of the pore diameter. In the latter case, a single high-energy heavy-metal ion from a cyclotron is shot through a thick polymeric film, followed by chemical wet etching, which removes damaged material faster than undamaged material, resulting in a conical nanochannel with a diameter down to 2 nm [18] (Figure 2a,b). Similarly made membranes, with multiple channels ranging in size from 10 nm to 10 μm and density from 10^5 to 10^9 pores/cm^2, are commercially available (Poretics, http://www.sterlitech.com).

Nanopores can be made using two different approaches. First, ion beam sculpting [19] has been used to create single nanopores in thin free-standing silicon nitride (SiN) membranes. Here, an ion beam is focused at the membrane to open up a tiny hole with a diameter down to a few nanometers. Feedback from ion detectors below the

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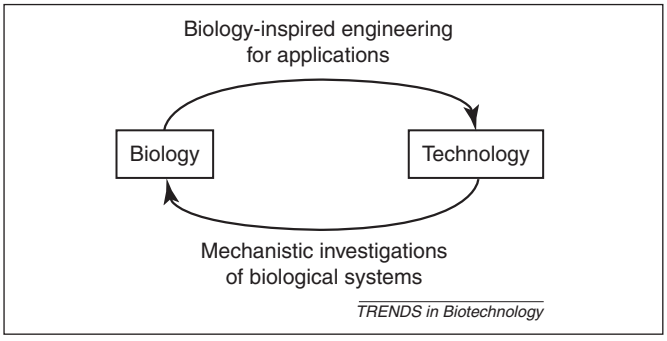

Figure 1. Engineering efforts can be inspired by the ingenuity and physical characteristics of natural biological systems. Conversely, biomimetic devices can be used to study the mechanistic properties of their biological counterparts.

membrane signals when to turn off the beam. Alternatively, an electron beam from a transmission electron microscope can be used to drill and shape pores down to sub-nanometer diameters [20] (Figure 2c,d). The latter method allows direct visual feedback as well as modes to enlarge (with a locally focused beam) or shrink (with wide-field illumination) the nanopore in a controlled manner.

Even without any additional modifications, these pores have proven to be useful as single-molecule biosensors, for example for the detection of DNA [21–24], RNA [25], proteins [26,27], carbon nanotubes [28], or local protein structures along DNA [29,30]. However, engineering efforts inspired by biological systems can enhance the functionality of bare nanopores. Typically this starts with chemical modification and coating of bare nanopores that enables their chemical properties to be tuned [31]. A chemical scheme that is often used in the case of polymeric membranes consists of coating the membrane with a gold layer through electroless deposition, followed by functionalization via thiol chemistry [32]. Although this approach is also applicable to silica or alumina membranes, polymeric membranes may also be directly reacted with functionalized silanes [33], thereby reducing the number of chemical treatments needed from typically two or more to a single one.

Mimicking naturally occurring pores and channels

Several types of biomimetic pores have been developed to demonstrate selectivity for specific molecular species, as inspired by naturally occurring pores and channels. Recently a single solid-state nanopore was coated with a fluid lipid bilayer (Figure 3a) [34]. This approach was inspired by the olfactory systems of insects, which have lipid-coated nanochannels in their external skeleton. The lipid coatings bind and preconcentrate odorant molecules before transporting them to the olfactory neurons in the antennae. The biomimetic lipid-coated nanopores showed several advantages over bare nanopores, such as: (i) the possibility of fine tuning the translocation speed of proteins by regulating the lipid-bilayer viscosity; (ii) the prevention of non-specific adsorption of proteins to the membrane; and (iii) the possibility to bind streptavidin-functionalized molecules selectively to a lipid bilayer that contains biotin groups.

Another artificial system has been designed to mimic all major components of the receptor-mediated transport of the nuclear pore complex (Figure 3b) [35]. Here, nanoporous membrane filters functionalized with polyisopropylacrylamide (pNIPAM) allow faster translocation of a

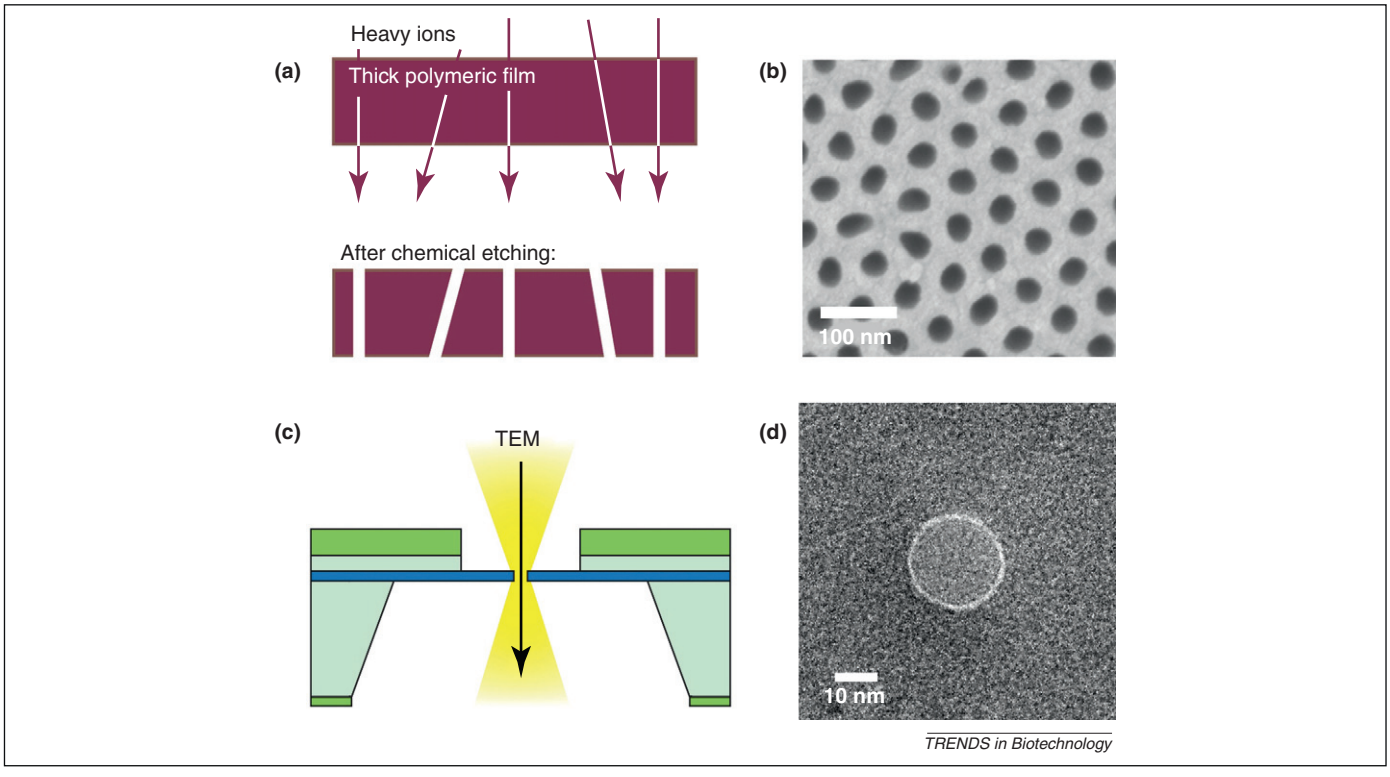

Figure 2. Fabrication of artificial nanochannels/nanopores. **(a)** Schematic showing heavy ions that penetrate a thick polymer membrane. The resulting damaged zones are then selectively etched with a chemical etchant and transformed into hollow nanochannels. **(b)** Scanning electron micrograph of the surface of an alumina membrane with a thickness of 40 μm with channels of 35 nm in diameter. Reprinted from [42] with permission from AAAS. **(c)** Side-view schematic showing a device consisting of a 20-nm thin free-standing SiN window (blue layer) embedded in a silicon wafer (light green). A single nanopore is drilled using a highly focused electron beam (yellow). **(d)** Transmission electron micrograph of a nanopore with a diameter of 20 nm.

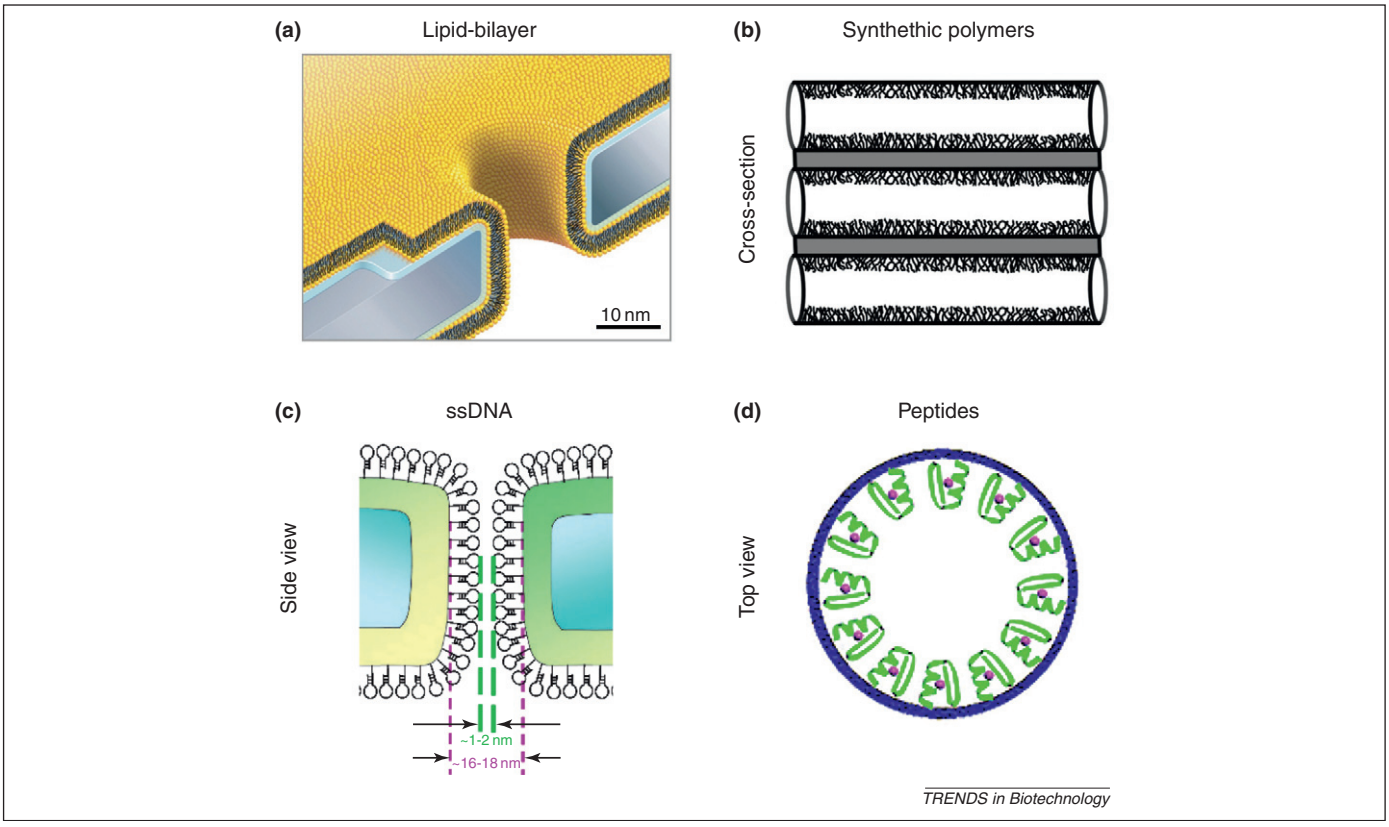

Figure 3. Biology-inspired nanopore functionalization. **(a)** A lipid-coated (yellow) synthetic nanopore in an SiN substrate (grey). Reprinted from [34] with permission from Macmillan Publishers. **(b)** Cross-section of a membrane with an array of nanopores grafted with pNIPAM. Reprinted from [35] with permission from the American Chemical Society. **(c)** Side-view of a solid-state nanopore functionalized with hairpin-DNA molecules (not drawn to scale). Reprinted from [46] with permission from Macmillan Publishers. **(d)** Top view of zinc fingers (green) immobilized into a nanochannel: after Zn^{2+} binding (pink), the zinc fingers fold to finger-like conformations, yielding an increase of the effective channel diameter. Adapted from [38].

single-stranded DNA (ssDNA)–pNIPAM complex compared to that of the smaller ssDNA alone. This is similar to mediated transport through the NPC, in which a transporter (pNIPAM in this case) ferries a cargo (ssDNA) through the pore.

Ion channels have also inspired a number of mimics [36]. These membrane protein complexes facilitate the transport of ions across membranes, often gated by allosteric binding or transmembrane voltage. Many types of ion channels exist and they are well documented. Atomic-resolution structures have been determined for ion channels that are selective for the four most abundant ions in biology: sodium, potassium, calcium, and chloride [1]. Recently, a biomimetic potassium-responsive nanochannel has been demonstrated [37]. Here, a nanochannel was lined with bound G-quadruplex DNA, which, upon addition of potassium, undergoes a conformational change that alters the effective channel size. Similarly, a biomimetic zinc-activated ion channel has been demonstrated by incorporating zinc finger peptides into a polymeric nanochannel [38] (Figure 3d). Artificial proton-reactive channels with transport properties that depend on the surrounding proton concentration have also been shown [39]. They mimic biological proton-gated ion channels by binding pH-responsive poly(4-vinyl pyridine) brushes onto solid-state nanopores. Consequently, the channels switch between an 'off' to an 'on' state in response to a pH change. Conical nanopores in polymeric membranes have also been designed. Their asymmetric shape together with the negative carboxylate groups that are left over from the chemical etching lead to ion current rectification, mimicking an effect found in voltage-gated ion-channels [40]. Additionally, a nanopore-based photoelectric conversion system has been inspired by the light-driven cross-membrane proton pump rhodopsin. In this case, photosensitive molecules are grafted to a nanopore [41].

Realizing selectivity and biosensing

Several approaches are not directly aimed at mimicking biological pores, but use biomolecules to confer selectivity. An early study has shown that antibodies attached to the inner walls of silica nanochannels can select for enantiomers [42]. Recently, enantioselective recognition has also been achieved in a single β-cyclodextrin-modified nanochannel [43]. Similarly, apoenzymes (enzymes lacking a required cofactor) bound to a porous membrane select for transport of its substrate molecule [44]. The same approach works for cDNA pieces: by attaching short ssDNA hairpin 'probes' to either a membrane with nanochannels [45] or a single nanopore [46,47], complementary ssDNA molecules transiently bind, unzip, and work their way through the pore, whereas mismatched DNA exhibits a lower translocation probability, attributed to electrostatic and mechanical friction (Figure 3c). This is a remarkably sensitive technique: translocation events for short DNA oligomers (15 bases) with a single base mismatch occur 30 times less frequently than that for perfectly complementary DNA [46]. Other examples of bio-inspired artificial pores

have successfully demonstrated the separation of proteins [9,48,49], fluorophores [9,49], and other small molecules [29,50,51]. In a different approach, functional polymers can be used as the bases for separation filters. For example, poly(acrylic acid) is bound to tin (Sn^{2+}) binding sites in nanochannels of commercial polycarbonate membranes that are pretreated with a solution of Sn^{2+} ions [9]. Here, multiple polymers, dyes, and proteins are separated based on size, charge, and hydrophobicity.

Biological pores can also be used as sensitive biosensors, which is an extension of the above approach where biomolecules are used to functionalize a pore or channel. We first discuss applications using biological pores in their natural environments, and then continue to discuss how it can be advantageous to put them in a synthetic environment.

The archetypical protein nanopore for biosensing is α-hemolysin [52–54], a toxin protein released by the bacterium *Staphylococcus aureus* that spontaneously inserts itself into lipid bilayers. It contains a heptameric transmembrane channel with a width of about 1.4 nm at its narrowest constriction, making it just large enough to allow the passage of ssDNA, but not that of double-stranded DNA (dsDNA). Transport of ssDNA and ssRNA through α-hemolysin has been extensively studied [for a review, see [54] and references therein]. Although these biological pores have the disadvantage of being inserted into relatively fragile free-standing lipid bilayers (no solid-state support), they can be engineered with molecular biology techniques, such as mutagenesis [55–58], to make very specific local changes in their structure that can influence transport properties. For example, the internal charge can be manipulated [57] in such a way that the speed of DNA translocation can be strongly reduced [58]. The α-hemolysin pore has been used to detect proteins [59], organic molecules [60], and (enantiomers of) drug molecules [61] (for an extensive overview see [10]). Other examples of biological pores that have been used as biosensors include anthrax spores, which have a structure very similar to α-hemolysin [62], and the OmpF porin, which interacts with antibiotics [63]. Recently, a mutant of the MspA porin, with an inner diameter of ~1.2 nm, was used to detect single-molecule translocation events of ssDNA [64] and even to distinguish all four DNA nucleotides [65]. One of the main advantages of the MspA porin over α-hemolysin is that the inner constriction of MspA is very short in its longitudinal direction, whereas α-hemolysin has a narrow barrel that is relatively long (Figure 4a,b). As a result, MspA has a higher specificity for individual bases, and is therefore potentially more suitable for DNA sequencing, similar to the advantage of using atomically thin graphene nanopores [66–68] versus thicker SiN nanopores. Another biological pore that has been used for bioengineering is the mechanosensitive MscL channel of *Escherichia coli*, which has been chemically modified into a light-activated nanovalve and utilized for triggered delivery in synthetic liposomal vesicles [69,70]. For a comprehensive review of applications of biological pores in nanomedicine, sensing, and nanoelectronics see [71].

Recently, hybrid biological/artificial pores have been demonstrated. The biological ion channel gramacidin-A

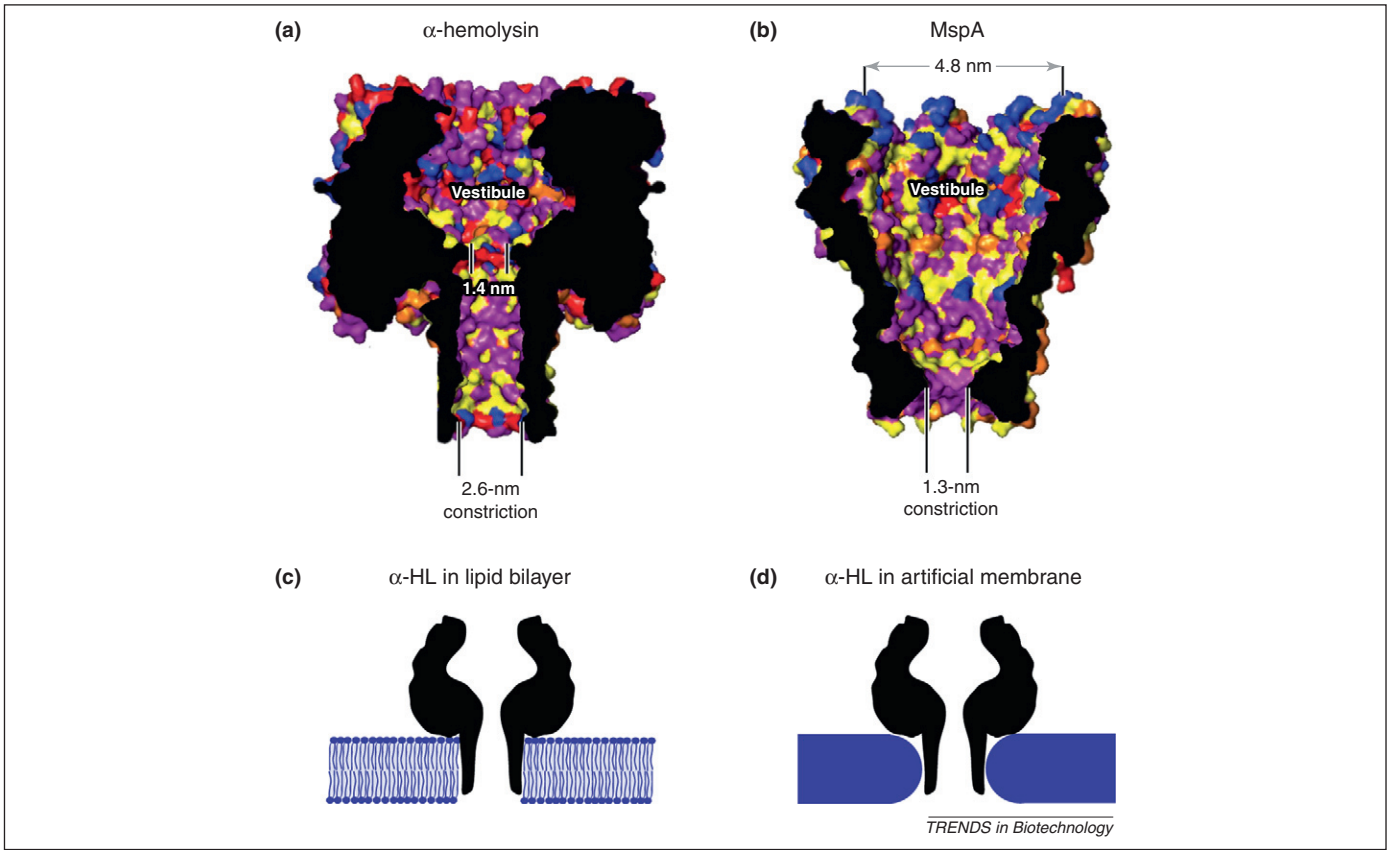

Figure 4. Biological nanopores. **(a,b)** Structural comparison of α-hemolysin and MspA nanopores [91]. Courtesy of J. Gundlach. **(c)** α-Hemolysin inserted into a lipid bilayer, its natural setting. **(d)** Hybrid nanopore: α-hemolysin inserted into a solid-state nanopore.

has been confined into a nanopore channel [72]. Also, individual α-hemolysin proteins have been inserted into a narrow solid-state nanopore, combining the two most experimentally studied nanopores [73]. Hybrid approaches have the great advantage of combining a biological, atomically precise, structure that can be genetically engineered with the robustness, sustainability and potential for parallelization and device-integration of solid-state nanopores (Figure 4c,d). Given the promising sequencing characteristics of both MspA [65], and α-hemolysin [74], a hybrid approach may turn out to be useful for genomic sequencing devices.

Mechanistic investigation of biological pores

Complementary to bio-inspired engineering, one can take advantage of the recent technological advances in nanoengineering to construct biomimetic pores for the purpose of studying the biological principles of their natural counterparts. Nature is highly organized in a hierarchical manner from the molecular to the macroscopic scale, starting with nano-architectures that together form a multitude of different macromolecular assemblies and interactions. Bottom-up engineering approaches, where complex biological systems are simplified to their constituent components, have been used to study the NPC at the fundamental level both in bulk [75] and single-molecule [76] investigations.

Bulk investigations of a biomimetic NPC

NPCs (Figure 5a) are the sole connection between the nucleus and the cytosol of eukaryotic cells, thus playing a key role in connecting the genetic material and the protein-synthesizing apparatus [77]. This remarkable cellular machine forms a pore with an ~40-nm inner diameter and controls all transport of proteins and RNA across the nuclear envelope. The NPC acts as a selective sieve: although permeable to ions and small solutes (up to ~40 kDa), transport through this gate is otherwise reserved for transport receptors (karyopherins) that ferry cargo across the complex. Vertebrate NPCs have a total mass of ~120 MDa and are composed of about 30 distinct types of proteins (nucleoporins) [78]. About one-third of these nucleoporins contain natively unfolded domains rich in phenylalanine–glycine repeat motifs (FG-domains) [79], which are believed to be crucial for selective transport of receptor–cargo complexes across the NPC channel [80].

By tethering FG-nucleoporins to commercial polycarbonate filters, NPC-like transport selectivity has been reconstituted in an artificial system (Figure 5b) [75]. Using track-etched polycarbonate membranes with cylindrical nanopores of 30 nm in diameter and 6 μm in length, a thin layer of gold was sputtered onto one side of the membrane. Next, thiol-modified yeast nucleoporins, Nsp1, were attached to the gold layer. The functionalized membranes were then mounted between two fluid chambers and the flux of fluorescently labeled karyopherin proteins through the pores was measured using confocal microscopy. Transport-receptor-bound cargo molecules translocated much faster (3–5 times) than molecules of similar size that did not bind FG-domains. The degree of selectivity was found to depend on the pore diameter and on the binding strength between nucleoporins and transport receptors. Interestingly, a single nuclear pore protein (Nup) is sufficient for

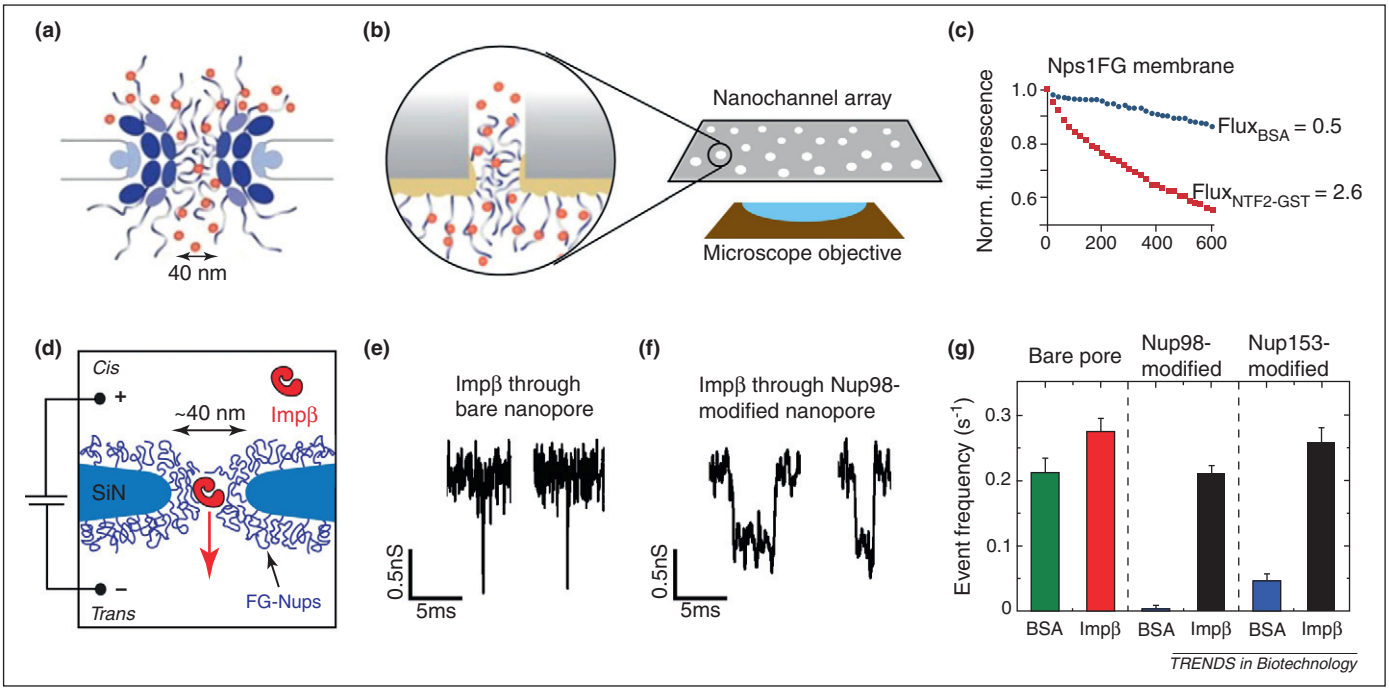

Figure 5. Biomimetic NPC. **(a)** Schematic representation of the cellular NPC. Reproduced with permission from http://newswire.rockefeller.edu/?page=engine&id=870. **(b)** Schematic of an artificial pore [75] in a functionalized membrane with many channels in parallel. The membrane was coated on one face with an ~15-nm-thick gold layer to which FG-nucleoporins were attached by a single carboxy-terminal cysteine. **(c)** Two-channel fluorescence measurements of simultaneous diffusion of fluorescently labeled BSA (blue) and transport receptor NTF2–GST (red) through an Nup(Nsp1)-coated membrane. Reprinted from [75] with permission from Macmillan Publishers. **(d)** A sketch showing the concept of the single-molecule biomimetic NPC study, which is engineered by attaching FG-Nups to a solid-state nanopore and transport of Impβ is measured by monitoring the trans-pore current [76]. **(e,f)** Single-molecule translocation events. Ion current after addition of Impβ in a bare pore **(e)** and a Nup98-modified pore **(f)**. Each spike is a single-molecule event. Event amplitudes are similar, whereas the dwell times differ by more than an order of magnitude [76]. **(g)** Event frequencies through bare and Nup-modified pores, showing NPC-like selectivity [76].

selectivity: when comparing the flux of bovine serum albumin (BSA; an inert protein) and nuclear transport factor 2 (NTF2; a transport receptor) through the functionalized membrane, the authors found that the transport receptors translocated (at best) five times more efficiently than the inert protein. Evidently, such a biomimetic NPC system might also be used for application in separation and bioanalytical devices. Although such an application would likely benefit from the parallelization derived from the large array of nanopores present in this experimental platform, such an array limits measurements of translocation properties to bulk, ensemble-averaged, behavior. Further investigation into the translocation properties of a single molecule requires the ability to measure translocation on an individual biomimetic NPC.

Single-molecule investigations of a biomimetic NPC

Recently, this approach has been realized wherein solid-state nanopores have been used for single-molecule transport studies on an individual biomimetic NPC [76] (Figure 5c). First, a nanopore was drilled into a 20-nm thin membrane with a focused transmission electron microscope beam and human nucleoporins (Nup98 or Nup153) were covalently tethered to it using maleimide chemistry. The membrane was then placed in a microfluidic flow cell where the nanopore formed the only connection between two fluidic compartments. Individual translocation events were monitored using sensitive ionic current measurements with sub-millisecond temporal resolution. The nucleoporins formed a very dense, low-conductivity network with pores up to ~25 nm in diameter, whereas larger pores formed a more open structure. Transport receptors (Impβ) proceeded with a dwell time of a few milliseconds, whereas the passage of nonspecific proteins (BSA) was strongly inhibited with selectivity factors of up to 60 (meaning that the average number of translocation events per second was 60-fold higher for Impβ than for BSA), with differing degrees of selectivity depending on the nucleoporin type. Also, one type of FG-nucleoporin was sufficient to form a selective permeability barrier. By reproducing key features of the NPC, this biomimetic approach provides a quantitative platform to study nucleocytoplasmic transport phenomena at the single-molecule level *in vitro*.

The transport through the NPC poses many interesting questions. How does the pore generate a diffusion barrier? How is this influenced by the composition of the nucleoporins, the sequence of their FG-repeats, and the number of repeats? Do the nucleoporin chains interact with one another? Do they interpenetrate to form a gel? Do they form a molecular brush? How exactly do receptors move through the pore? Do they change the local structure of the nucleoporin network? Do they bind to an individual Nup and are subsequently ferried through the NPC, or do they jump from one Nup to the next? Some of these questions may well be addressed using the biomimetic approach, which offers a new type of testing platform for mechanistic studies. Similar measurements on biomimetic NPCs may not only shed light on the fundamental workings of this important protein machine, but also provide new opportunities for, studying gene and drug delivery into the nucleus. Theoretical studies and molecular dynamics simulations may also shed light on these questions (for some very recent progress in these directions, see [81–84]).

Perspective

Biomimetic techniques can give insights into key molecular processes occurring in biology. Although there are multiple challenges that face biomimetics of pores, it has already proven a fruitful approach. The main challenge in biological systems is their complexity, therefore, complete assembly *in vitro* seems out of reach. Furthermore, for many systems, a lack of structural data limits our knowledge of the biological system and hence makes it challenging to mimic it accurately. Finally, even though technology has rapidly advanced, there are still obvious fabrication as well as surface chemistry limitations.

In this review we have given various examples of biomimetic systems, with an emphasis on nanopores. The field of biomimetics is of course still broader. For example, there has been much interest recently [85] in biomimetic artificial photosynthesis [86]. Earlier this year, an artificial leaf was reported that is 10 times more efficient than the real counterpart (http://www.technologyreview.com/energy/37310/).

The emergence of techniques to investigate transport through pores and channels in new ways can open up new avenues for research. For example, a new instrument that combines total internal reflection fluorescence microscopy with ionic current measurements has demonstrated synchronous optical and electrical detection of biomolecules traversing solid-state nanopores [87]. A similar instrument could be used for biomimetic nanopores to image diffusion of transport factors in nucleoporin-coated nanochannels. Alternatively, an integrated nanopore-optical tweezer setup [88] would allow measurement of the force on transport factors during the translocation process. These types of experiments may yield new information about the forces governing the translocation process, and accordingly shed new light on the mechanism of translocation.

An important question in the field of nucleocytoplasmic transport is which route the transporters take through the NPC. Despite a recent step toward monitoring interactions between nucleoporins and transporters using subdiffraction microscopy, the complete puzzle is yet unsolved because of instrumental limitations [89]. A comprehensive analysis of the biophysical properties of yeast FG-Nups has found that Nups organize as extended random coils and molten globules described as ‘trees’ and ‘shrubs’, depending upon whether the Nups are strongly charged or relatively uncharged [90]. Perhaps by tethering FG-Nups to nanopatterned slits, one could study through which pathway (i.e. tree or shrub) the transporter molecules diffuse through this ‘forest’. Such measurements would require nanometer-accuracy tracking, which appears to be possible using state-of-the-art single-molecule fluorescence methods.

New technological abilities to study and shape matter at the nanoscale have enabled the development and exploration of various types of biomimetic nanopores. Such pores can be used in an array of applications in biotechnology, notably as biosensors and separation filters. By constructing artificial systems that resemble biological pores from the

bottom up, the biomimetic approach has also yielded new knowledge about biological pores, in particular, the NPC. Biomimetic nanopores form an exciting emerging research field with ample opportunities for new applications and discoveries.

Acknowledgments

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