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Artificial nanopores that mimic the transport selectivity of the nuclear pore complex

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Abstract

Nuclear pore complexes (NPCs) act as effective and robust gateways between the nucleus and the cytoplasm, selecting for the passage of particular macromolecules across the nuclear envelope. NPCs are comprised of an elaborate scaffold that defines a ~30nm diameter passageway connecting the nucleus and the cytoplasm. This scaffold anchors proteins termed ‘phenylalanine-glycine’ (FG)-nucleoporins, the natively disordered domains of which line the passageway and extend into its lumen¹. Passive diffusion through this lined passageway is hindered in a size-dependent manner. However, transport factors and their cargo-bound complexes overcome this restriction by transient binding to the FG-nucleoporins^{2–10}. To test whether a simple passageway and a lining of transport-factor-binding FG-nucleoporins are sufficient for selective transport, we designed a functionalized membrane that incorporates just these two elements. Here we demonstrate that this membrane functions as a nanoselective filter, efficiently passing transport factors and transport-factor–cargo complexes that specifically bind FG-nucleoporins, while significantly inhibiting the passage of proteins that do not. This inhibition is greatly enhanced when transport factor is present. Determinants of selectivity include the passageway diameter, the length of the nanopore region coated with FG-nucleoporins, the binding strength to FG-nucleoporins, and the antagonistic effect of transport factors on the passage of proteins that do not specifically bind FG-nucleoporins. We show that this artificial system faithfully reproduces key features of trafficking through the NPC, including transport-factor-mediated cargo import.

Several groups have used functionalized nanoporous membranes to selectively enrich between molecules such as chiral enantiomers^{11,12}, compounds of differing hydrophilicities¹³ or single-base mismatched oligonucleotides¹⁴; or more recently to selectively transport single-stranded DNA bound to polymer carrier molecules¹⁵. Similarly, the central element of our artificial system is a polycarbonate membrane perforated by ~30-nm-diameter cylindrical nanopores (Fig. 1a). The membrane is coated on one face with a thin layer of gold, to which is conjugated

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a single layer of a natively disordered FG-repeat domain from an FG-nucleoporins; these domains are characterized by the presence of multiple repeats containing degenerate 'Phe-Gly' (FG) motifs separated by low complexity hydrophilic spacers of approximately 5–30 amino acids in length. FG-repeat domains of similar size were selected from either of the nucleoporins Nsp1 or Nup100 from budding yeast. These two domains, referred to as Nsp1FG and Nup100FG, were chosen because they represent two major classes of FG-repeat motif composition (FxFG- and GLFG-repeats, respectively)¹⁶ (Supplementary Fig. 1). Our functionalized membrane is designed to mimic the following essential properties of NPCs: it has pores of similar diameter to those in NPCs; each pore is coated by a monolayer of FG-nucleoporins; these FG-nucleoporins are properly oriented, surrounding and partially occluding the pore opening as in the NPC; they are at approximately the same density as found in the NPC (~70 molecules per pore, Supplementary Methods); and transport takes place across a thin (~15 nm) barrier (Fig. 1a), akin to the nuclear envelope, with the transported material entering and (crucially) exiting the NPC mimic.

We explored the transport properties of our functionalized membranes by either changing the material coating the membrane and pores or changing the proteins passing across the pores. The flux of fluorescently labelled proteins through the functionalized membranes was measured using the device illustrated in Fig. 1a, a setup inspired by that used for optical single transporter recording of NPCs¹⁷. In each case, the proteins to be measured were allowed to reach equilibrium between the small lower chamber and the much larger upper chamber. The solution in the upper chamber was then rapidly diluted ~50-fold and the flux of protein diffusing from the lower to upper chamber was measured by quantifying the decrease in fluorescent material in the lower chamber (Fig. 1c). Before investigating the properties of the FG-nucleoporin functionalized membranes, we measured protein fluxes through a control membrane, coated with a layer of an inert low molecular weight (356 daltons (Da)) compound (small PEG). As expected, the resulting fluxes were largely dependent on protein size (Supplementary Fig. 5 and Supplementary Tables 2–5).

We then measured the flux of proteins across membranes when both a transport factor and a control protein were present at the same time. In each case, the transport factor can specifically bind the FG repeats with relatively high affinity, whereas the control can at most form weak, nonspecific interactions (Supplementary Fig. 10c, d). Each protein was labelled using a different fluorophore (i.e., two-colour experiment). We measured the flux of the dimeric transport factor human nuclear transport factor 2–glutathione *S*-transferase (NTF2–GST) (82 kDa) versus the control bovine serum albumin (BSA; a protein of similar mass (66 kDa), Stokes radius and isoelectric point to the NTF2–GST dimer) through Nsp1FG-coated membranes (Fig. 1d). The flux of BSA across the Nsp1FG membrane was on average threefold lower than that of NTF2–GST (Supplementary Table 3). In contrast, in an assembly containing a small-PEG-coated membrane, the same mixture of NTF2–GST and BSA showed equally rapid efflux of both proteins out of the lower chamber, as expected from two such comparably sized proteins through essentially open holes (Fig. 1d). Thus, the Nsp1FG-coated pores were as permeable to NTF2–GST as the small-PEG-coated pores, but simultaneously much less permeable to BSA. Figure 2a shows 'flux ratios' (a measure of transport efficiency) for several control proteins of various sizes, in the presence of NTF2–GST. We found that the fluxes of these control proteins are significantly impeded by the Nsp1FG membrane in a size-dependent manner, with fluxes of larger proteins being impeded more than those of smaller ones. At the same time, the flux of NTF2–GST in these paired mixtures exhibited no discernable reduction (Supplementary Table 3), showing that membranes functionalized with Nsp1FG behave as a selective filter with a strong preference for a transport factor (NTF2).

We next asked whether the Nsp1FG-functionalized membranes had selectivity for other transport factors. Whereas NTF2 is the major transport factor for the Ras-related nuclear protein

Ran, most transport factors belong to a different structurally related family that are collectively termed karyopherins (Kaps). Karyopherins that mediate import bind to specific 'nuclear localization signals' in their cargos and, by virtue of their ability to bind FG-nucleoporins, facilitate passage of these cargos through the NPC into the nucleus. We studied two budding yeast karyopherins in our device, Kap95 and Kap121 (also known as Pse1). Both karyopherins have been used as standard reporters for nuclear import, and their specific interactions with Nsp1 are well documented^{18,19}. Similar to NTF2–GST, we found that these karyopherins pass essentially unimpeded through the Nsp1FG membranes compared with the control membranes. At the same time, BSA is again significantly impeded to the same degree as in the NTF2–GST experiments (Fig. 2b and Supplementary Table 3). Hence, the selective gating of the Nsp1FG-functionalized membranes seems to be general for FG-repeat-binding transport factors.

We also tested whether transport factors can carry their cargo molecules efficiently through the FG-nucleoporin-coated membranes, as they do through the NPC *in vivo*. To make a fluorescent cargo, we attached the Kap95-binding nuclear localization signals (the 'Ibb' (importin- β binding domain of Kap60) sequence) from Kap60 onto green fluorescent protein (Ibb–GFP), with GFP acting as a control. We used a twofold excess of unlabelled Kap95 over the cargo; as expected, all Ibb–GFP was bound to Kap95 under these conditions, whereas GFP did not bind to Kap95 (Supplementary Fig. 9)^{20,21}. Like Kap95 alone, the Kap95/Ibb–GFP complex was efficiently transported across the membranes with a flux ratio of ~ 1.0 . In contrast, GFP without the Ibb domain and Ibb–GFP (in the presence of NTF2–GST but without Kap95, Supplementary Fig. 9c) had a markedly lower flux ratio of ~ 0.55 and 0.44 , respectively, even though they have significantly smaller Stokes radii than the transport-factor–cargo complex (Fig. 2c). Similar results were obtained with NTF2–GST and its cargo, RanGDP (Supplementary Table 3). We have thus reconstituted the transport-factor-mediated import step of nuclear transport in our device.

Reversible binding to FG-nucleoporins is an essential feature of nuclear transport, allowing transport factors to transiently interact with multiple docking sites as they traverse the NPC⁵. This feature is recapitulated in the present functionalized membrane, because we observed a specific and reversible accumulation of transport factors at the FG layers (e.g., Fig. 1d and Supplementary Fig. 10a).

We next assayed what happens to the flux of the control proteins across Nsp1FG-coated membranes in the absence of transport factors (Fig. 3), measuring the flux of fluorescently labelled BSA in either the absence or the presence of NTF2–GST. In the absence of NTF2–GST the flux of BSA was only modestly impeded by the Nsp1FG-coated membrane when compared to the small-PEG-coated membrane. Notably, the addition of NTF2–GST caused a substantial reduction in the flux of BSA (Fig. 3a); similar results were obtained for transferrin (Supplementary Tables 2 and 3). To test whether it was the binding of NTF2–GST to the Nsp1FG layer that caused this marked reduction in BSA flux, we repeated the experiment using membranes functionalized with mPEG thiol of ~ 30 kDa (30 kDa PEG), which binds neither BSA nor NTF2–GST but is similar to Nsp1FG in both its size and its behaviour as an unstructured polymer^{9,22,23}. Both NTF2–GST and BSA showed only moderately reduced fluxes through the 30 kDa PEG membrane as compared to the control; moreover, the presence or absence of NTF2–GST had no effect on the flux of BSA (Fig. 3b). We conclude that the strong flux reduction of control proteins occurs only in the presence of FG-nucleoporin-binding transport factors—that is, addition of transport factors markedly changes the transport properties of the functionalized membrane. Possible explanations for this effect include direct competition between transport factors and other macromolecules for space and binding sites inside the NPC²⁴ and changes in the configurations of the FG-nucleoporin barrier on transport factor binding (see for example ^{ref 8}). These effects are not necessarily mutually exclusive.

We predicted that an increase in pore size should reduce the concentration of FG-nucleoporins in the pore, thus reducing the selectivity of the functionalized membrane. Indeed, we found that the larger the pore size, the weaker the selectivity of the membrane (Fig. 4a). We also predicted that an increase in the thickness of the gold layer should allow more FG-nucleoporins to bind to the internal gold surface at the entrance to the polycarbonate pores, while leaving the flat gold surface unchanged (Fig. 1a). We observed that a ~2-fold increase in gold layer thickness yielded a ~2-fold increase in selectivity (Fig. 4b), without changing the effective pore size (Supplementary Table 6). This result also shows how future carefully tuned alterations in the design of our device could significantly enhance its selectivity.

Because binding of transport factors to FG-nucleoporins is a central feature of transport selectivity, we assessed the effect of mutations that alter the binding strength of NTF2 to Nsp1. We used mutant NTF2 (NTF2(W7A)), which has a reduced interaction with FxFG nucleoporins due to an alteration in the main phenylalanine binding site of NTF2 (ref. 25). As with NTF2–GST, the NTF2(W7A)–GST mutant is a dimer (Supplementary Fig. 1), but has reduced binding capacity for Nsp1 (Fig. 4c; Supplementary Fig. 11a). In our device, NTF2–GST and NTF2(W7A)–GST were combined in equimolar ratios, and their transport through Nsp1FG and small PEG membranes was measured. As expected because of its reduced binding, the mutant showed a significantly reduced flux ratio compared to the wild type (Fig. 4d). Another way of changing binding is by changing the FG-repeat motif, because different kinds of motif have differing affinities for a given transport factor. For example, Nup100FG binds strongly to Kap95 but more weakly to NTF2 (refs 18, 26, 27; Supplementary Fig. 10). We therefore compared the flux ratios of Kap95 and NTF2–YFP through membranes functionalized with either Nsp1FG or Nup100FG. As expected, Kap95, which binds strongly to both Nsp1FG and Nup100FG, efficiently transits these membranes and impedes the transit of a control protein (Fig. 4e). Importantly, this result also demonstrates that other FG-nucleoporins behave in a similar manner to Nsp1 and can support selective transport in our device. In contrast, the flux of a control protein was reduced only slightly through the Nup100FG membranes when NTF2–YFP was present (Fig. 4e), again emphasizing that efficient binding is required for selective transport. It has been shown that treatment by hexanediol abolishes the NPC barrier *in vivo*^{4,28} and collapses FG-nucleoporins *in vitro*⁸. Consistent with these results, we observe that addition of the 5% hexanediol largely eliminates the selectivity for the transmission of NTF2–GST over BSA through an Nsp1FG membrane (Supplementary Fig. 8).

We have built a minimal assembly that encapsulates the essential functional features of NPC architecture as they are currently understood—namely, a simple pore of the correct dimensions coated with FG-nucleoporins. Notably, this assembly recapitulates key features of nucleocytoplasmic transport, selectively discriminating against control proteins in favour of transport factors and transport-factor/cargo complexes. Our NPC mimic also provides insight into the role of transport factors in the mechanism of selectivity. In a sense, transport factors may be considered transient components of the NPC that help to discriminate against the passage of nonspecific material across the nuclear envelope. A feature of our device is that it incorporates properties of nuclear transport that may prove useful for purification techniques in general, including an ability to extract macromolecules of interest most efficiently from crude mixtures. Such NPC-inspired molecular sorters could have many practical analytical and preparative applications.

Methods Summary

Protein expression and purification

All proteins were expressed in *Escherichia coli*. His-tagged versions of Nsp1FG-Cys, Nup100FG-Cys and human NTF2–yellow fluorescent protein (YFP) were purified from

clarified lysate using Ni- or Co-affinity chromatography followed by gel filtration chromatography. Human wild-type NTF2–GST and NTF2(W7A)–GST were purified using glutathione sepharose resin and labelled with Cy dyes according to the manufacturer's instructions (GE Healthcare). Kap95 and Kap121 were obtained as described previously²⁹ and labelled with an Alexa 488 protein-labelling kit according to the manufacturer's instructions (Invitrogen). His tagged versions of Ibb–GFP and GFP were purified using Co-affinity resin.

Construction of the functionalized membrane

Gold-sputtered polycarbonate membranes (GE Osmonics) were cleaned with 25% HNO₃ and rinsed in water. Chemisorption of 6.5 μM Nsp1FG–Cys, 5.1 μM Nup100FG–Cys, 2mM mPEG-thiol (30 kDa) or 2mM mPEG-thiol (356 Da) was performed at room temperature (22 °C) for 1 h. Protein-coated membranes were subsequently incubated with 2mM mPEG-thiol (356 Da) in 8M urea for 1 h and all membranes were washed in TBT buffer (20mM HEPES, pH 7.4, 110mM KOAc, 2mM MgCl₂, 10 μM ZnCl₂, 10 μM CaCl₂, 0.1% Tween-20). Amino acid analysis on the protein-coated membranes confirmed specific chemisorptions of 0.05 and 0.04 molecules of Nsp1FG and Nup100FG per nm², respectively.

Fluorescence imaging

Fluorescence images were recorded on a Leica TCS SP spectral confocal microscope with a PL × 100/1.4 oil-immersion objective lens in XZT mode. Mean fluorescence values were obtained using ImageJ, normalized, and fit using a simple exponential decay (SigmaPlot) yielding the flux (molecules pore^{−1} s^{−1} μM^{−1}). Full Methods are provided in the Supplementary Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Design and operation of the NPC mimic

a, Far left: schematic of a single pore in the functionalized membrane. A 6- μ m-thick polycarbonate membrane perforated by ~ 30 -nm channels (8×10^8 pores per cm^2) was coated on one face with a ~ 15 -nm-thick gold layer to which we attached FG-nucleoporins (FG-nups) by a single carboxy-terminal cysteine; hence, all FG-nucleoporins were similarly oriented. Subsequent attachment of 356-Da PEG-thiol (small PEG) molecules blocked any remaining exposed gold surface^{8,9}. Centre left: exploded view of the device carrying the membrane. Centre right: sectional view of the device mounted on a confocal microscope and loaded with a fluorescently labelled protein solution (green). Lower chamber: height, ~ 25 μ m; diameter, ~ 1.6 mm. Upper chamber: height, ~ 1 mm; diameter, ~ 2 mm; overflow capacity, 100 μ l. Far right: top-view photograph of the device. **b**, Transmission electron micrograph of a single pore on Nsp1FG-functionalized membrane, incubated with gold-labelled NTF2-GST (pseudocoloured red). NTF2-GST is seen to bind the FG-nucleoporin layer projecting from the gold surface and transit into the 30-nm pore. **c**, Method for measurement of protein fluxes across the nano-selective filter. Left: confocal microscopy z-axis section through the lower chamber, membrane and upper chamber showing the fluorescence signal (blue) from a single protein in equilibrium between the two chambers. The measured volume is indicated by the dashed box. Right: plot showing the decrease in fluorescence signal in the lower chamber after

dilution of the upper chamber, resulting from an efflux of protein from the lower to upper chamber (Supplementary Information). **d**, Two-channel fluorescence measurements of simultaneous diffusion of fluorescein isothiocyanate (FITC)-labelled BSA (blue) and Cy5-labelled NTF2–GST (red) through either an Nsp1FG-coated membrane (top) or a control small-PEG-coated membrane (bottom). Left: time course of confocal images collected as in **c**. Colours were altered for clarity; the same data with unmodified colours are provided in Supplementary Fig. 12. Right: corresponding fluorescence decrease curves and fluxes (in molecules pore⁻¹ s⁻¹ μM⁻¹). The flux of NTF2–GST was similar through both membranes, whereas the flux of BSA was significantly lower through just the Nsp1FG-coated membrane.

Figure 2. Selective trafficking of transport factors and cargo through the nanopores

A flux ratio was obtained for each protein by calculating the ratio of its flux through a functionalized membrane (in this case Nsp1FG) relative to its flux through the control small PEG membrane. A value of 1 indicates no reduction in flux through the functionalized membrane as compared with the control membrane, whereas a value of 0 indicates no flux through the functionalized membrane. **a**, Flux ratios of NTF2-GST versus control proteins. Flux ratios plotted against Stokes radius for variously sized proteins in the presence of NTF2-GST ($R_s = 3.6$ nm), namely: RNase A (1.75 nm), GFP (2.8 nm), BSA (3.5 nm), transferrin (3.6 nm), immunoglobulin G (IgG; 5.1 nm). The flux ratio of all proteins except NTF2-GST was reduced in a size-dependent manner. **b**, Flux ratios of two karyopherins versus control protein (BSA). Flux ratios for a mixture of BSA and Kap95 and a mixture of BSA and Kap121 showed BSA having a markedly lower flux ratio than either karyopherin (despite the smaller size of BSA) in a manner similar to a mixture of BSA and NTF2-GST. **c**, Karyopherin-mediated transport of cargo. Flux ratios for either GFP or Ibb-GFP in the presence of Kap95 (not fluorescently labelled) and IgG; Ibb-GFP forms a complex with Kap95, whereas GFP does not (see Supplementary Information). The flux ratio of Ibb-GFP in a complex with Kap95 was higher than that of either GFP alone (even though GFP has a much smaller Stokes radius than the Ibb-GFP/Kap95 complex) or IgG alone. Hence, the device mimics Kap-mediated cargo transport. Standard errors are shown.

Figure 3. Presence of transport factor enhances the selectivity of FG-nucleoporin-coated membranes

a, Left, time course of confocal images collected as in Fig. 1, comparing fluxes of BSA through the small PEG membrane (bottom), the Nsp1FG membrane (middle), and the Nsp1FG membrane in the presence of NTF2-GST (top); right, corresponding fluorescence decrease curves and fluxes (in molecules pore⁻¹ s⁻¹ μM⁻¹). **b**, Strong selectivity of NTF2-GST transport over BSA is observed for the Nsp1FG functionalized membrane and is not observed for the 'inert' 30 kDa PEG functionalized membrane. Flux ratios are plotted for BSA and NTF2-GST alone (1 species) or in combination with each other (2 species), through either Nsp1FG or 30 kDa PEG membranes versus small PEG membrane. Standard errors are shown.

Figure 4. The effect of pore geometry and FG-nucleoporin binding strength on transport

a, The selectivity of the Nsp1FG membranes decreases with increasing pore diameter. Flux ratio measurements of a mixture of BSA and NTF2-GST were performed as in Fig. 2a, for membranes having differing pore diameters. **b**, The selectivity of the Nsp1FG membrane was significantly improved when the thickness of the gold layer was increased. **c**, *In vitro*, NTF2 (WT)-GST (red) had a lower apparent K_d value (10 ± 3 nM) than mutant NTF2(W7A)-GST (pink, K_d of 21 ± 4 nM) for binding to Nsp1FG, and at the same time had about double the binding capacity compared to the mutant. **d**, The NTF2(W7A)-GST mutant had a reduced flux ratio compared to NTF2(WT)-GST (for flux through the Nsp1FG membrane, $P = 0.009$, single-tailed t -test), although no flux difference was seen on the control small-PEG-coated membranes ($P = 0.35$). **e**, Assessment of the effect on transport of binding strength to FG-nucleoporins by changing the FG-repeat motif. Flux ratios through either Nsp1FG or Nup100FG membranes are shown for the Kap95/Ibb-GFP complex with IgG and for NTF2-YFP with BSA. Because NTF2 binds poorly to Nup100FG compared to its binding strength to Nsp1FG, Nup100FG-coated membranes did not discriminate well between BSA and NTF2-YFP. Standard errors are shown in parts **a**, **b**, **d** and **e**, and standard deviations are shown in **c**.