Biologically-inspired selective filters

by

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Biologically-inspired selective filters

Thesis directed by Assistant Professor Loren Hough

Selective biofilters are essential to life, controlling the transport of proteins, nucleic acids, and other macromolecules. Of particular interest are filters which require rapid motion or high flux of proteins that must still bind targets with high specificity. Despite the apparent competition between these two attributes, many selective materials exist which leverage binding interactions to fulfill both. In this work, we investigate the mechanisms by which such filters function through both modeling and experiment, using the nuclear pore, a well-studied example of selective transport, as inspiration. The nuclear pore, a channel lined with intrinsically disordered FG nucleoporins, permits a high flux of transport factor proteins and their cargos while suppressing transport of proteins which cannot bind to the FG nucleoporins. We developed a minimal model of nuclear transport which relies on the bound-state mobility of the Nup-transport factor complex for selectivity. This model reproduces the experimentally-observed properties of the nuclear pore and demonstrates that bound-state diffusion can arise from transient, multivalent binding and binding to flexible, dynamic tethers. We then designed tunable hydrogel mimics of the nuclear pore for use in measuring bound-state diffusion and testing the predictions of our model. Fluorescence microscopy demonstrated that our mimics display non-zero bound diffusion. Both the model and experimental system are sufficiently general that their principles can be applied to a wide variety of selective biomaterials.

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Introduction

Selective biofilters are found in all manner of living systems and control the motion of proteins, nucleic acids, and other macromolecules. While many operate by size exclusion, permitting only the passage of particles below a size cutoff, more sophisticated filters make use of binding interactions. Of particular interest are filters which require rapid motion or high flux of proteins that must still bind targets with high specificity. The two requirements would seem to be in direct competition, yet there are many examples of these systems. In this work, we investigate the mechanisms by which such filters function through both modeling and experiment, using the nuclear pore, a canonical example of selective transport, as inspiration.

1.1 The nuclear pore complex is a unique selective filter

The nuclear pore complex (NPC) is the filter that regulates all transport between the cell's nucleus and its cytoplasm (Fig. 1.1). It shows unusual selective properties. The nuclear pore prevents significant flux of macromolecules larger than about 30 kDa ($\sim$ 5 nm) [1]. However, a class of proteins known as transport factors carry cargo molecules quite rapidy across the pore, although the complex of transport factor and cargo can be up to 40 nm in size. Far from being a typical size-exclusion filter, therefore, the nuclear pore complex is a highly specific selective barrier which the cell uses to tightly control passage in and out of the nucleus. While the important biochemical components of nuclear transport have been identified, the mechanism of selectivity is

still not well understood. Even though the precise mechanism is under debate, it is clear that the NPC is a fascinating example of the cell leveraging intrinsically disordered proteins to accomplish a unique task.

1.1.1 Intrinsically disordered proteins

It has been long-standing conventional wisdom among biologists that a protein's folded shape determines its function. Most enzymes and other proteins that were studied had a stable folded configuration, the lowest point on a well-defined folding energy landscape. A protein's conformation provided specific docking points through which it could interact with ligands or other proteins in a "lock-and-key" model.

However, a few decades ago, it began to become clear that not all proteins have a well-defined ternary or even secondary structure, but rather exist as extended polymer chains. These intrinsically disordered proteins (IDPs) were initially dismissed as nonfunctional, but evidence began to accumulate that they were in fact essential for cellular function, modifying the structure-function paradigm. Their roles and importance are still being understood, as are the unusual mechanisms by which they accomplish their functions without a well-defined structure.

Today, it is estimated that 30% of eukaryotic proteins are disordered or contain significant disordered regions [2]. While there is significant sequence heterogeneity among IDPs, they tend to contain a large proportion of hydrophilic residues, and often have long stretches of low-complexity regions where only a few amino acids are represented. They also often have high net charge.

Some IDPs fold (or partially fold) upon binding with an ordered partner, while others form "fuzzy" complex that remains disordered. Their advantages over folded proteins may include their plasticity, which enables them to bind many different binding partners. Multivalency, either as one-to-many or many-to-one binding, may also play a role. They may act as hubs that bring together larger complexes. Similarly, IDPs are often known for having high specificity at relatively weak binding strengths [3, 4].

While the normal functioning of IDPs is very important to the cell, IDPs are also prone

to aggregation and are at the root of pathologies such as Alzheimer's, Parkinson's, and prion diseases [5]. Often, normally-disordered proteins aggregate into amyloid fibrils, a stable structure consisting of stacked β-sheets.

IDPs are commonly involved in cell signaling and regulation [3]. Their disordered nature makes them useful as hubs that bring together many other proteins, and as scaffolds that many proteins can bind to at once. IDPs appear to be prevalent in transcriptional regulation, and they are playing increasingly apparent roles in liquid-liquid phase separation within cells [6]. One of the most fascinating examples of IDP function is in the nuclear pore complex (NPC), a unique selective barrier that regulates all transport between the nucleus and the cytoplasm. The link between disorder and selectivity is not well understood in this case.

1.1.2 Basics of nuclear transport

The nuclear pore complex (NPC) resides in the nuclear envelope of eukaryotes and regulates all macromolecular traffic between the nucleus and cytoplasm (Fig. 1.1). The NPC is one of the largest protein complexes in the cell, at about 60 MDa in yeast and 120 MDa in humans [7]. As the regulator of nucleocytoplasmic transport, the NPC must rapidly and specifically allow a wide array of macromolecules to pass: transcription factors into the nucleus, and RNA into the cytoplasm. It must also be robust to problems and able to accomodate mechanical strain as the nuclear envelope changes shape, as well as accomodating large cargo. These functions are accomplished through a structure with two main parts, both made of proteins known as nucloporins, or Nups: the scaffold Nups, which form a ringlike complex, and the FG Nups, which are disordered and fill the central channel created by the scaffold Nups (Fig. 1.1).

The nuclear pore itself is formed of scaffold Nups, which are ordered proteins that form ringlike complexes with eightfold symmetry [7, 9]. The central channel of the pore is filled with disordered FG Nups. FG Nups typically consist of an ordered domain that anchors them to the wall of the channel, and an entirely disordered domain that extends into the channel. As with all Nups, FG Nups have eightfold symmetry in the pore, and some of them are present in much

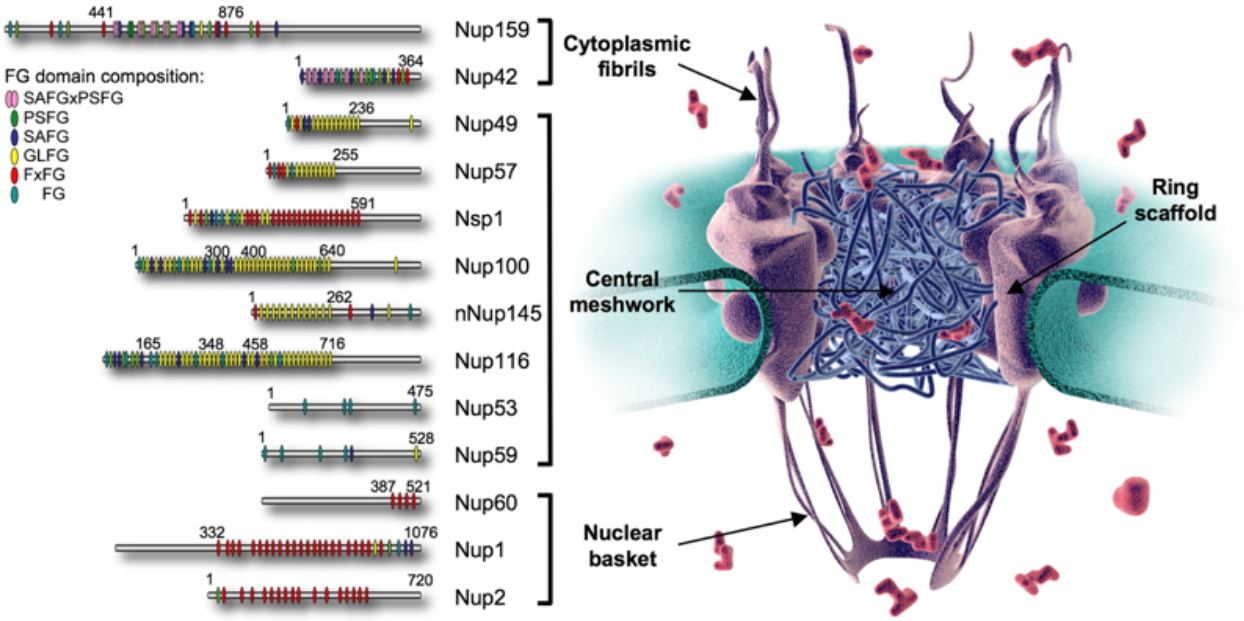

Figure 1.1: Nuclear pore complex and FG Nups. The cartoon shows the role of the FG Nups filling the central channel. The left panel shows a schematic of the sequence of important FG Nups and the locations of their FG motifs. Figure adapted from [8].

higher copy number. The disordered portion of every FG Nup contains phenylalanine- glycine (FG) motifs which bind to the hydrophobic binding pockets of transport factors. While there are multiple binding motifs, all are short sequences which incorporate an FG repeat; for instance, FSFG, GLFG, and others. Each FG Nup contains tens of FG repeats, leading to a high density of FG repeats within the pore [9, 10].

Since the FG Nups are disordered, most conventional visualization techniques, such as cryogenic electron microscopy and x-ray crystallography, are ineffective. When imaged over time or when several pores are imaged, the averaged results do not show the disordered portion of the FG Nups. Techniques such as NMR and atomic force microscopy (AFM) can help gain insight into their conformational ensembles, as can some superresolution microscopy techniques, though the time and length scales of transport are often prohibitive for any microscopy [11–13]. Early research suggested that the FG Nups formed a central plug or "transporter", but more recent work suggests that there is no central structure and the central channel is filled instead with highly dynamic disordered proteins [12, 14, 15]. There is some evidence from simulations that the density of the FG Nups, as well as their charge density and hydrophobic properties, are not uniform along either the radial or axial directions [16–18]. This may contribute to selective transport, although the pore still functions without the asymmetric FG Nups [19]. Indeed, the NPC is remarkably robust to FG Nup deletion. Over half of the mass of FG Nups can be removed without eliminating the selectivity barrier [19–22].

Transport factors are ordered proteins that carry cargo through the NPC. While there are various types, they share several features in common, most notably the fact that all known transport factors have more than one hydrophobic binding pocket which binds to FG repeats. Binding affinities between transport factors and FG Nups are surprisingly difficult to measure accurately. Values of the dissociation constant K_D measured outside of the cellular context are often in the low nanomolar range, implying a binding lifetime which is inconsistent with the experimentally-observed rapid translocation through the nuclear pore [23, 24]. Recent consensus is that binding is much weaker in the cellular environment, with K_D values between hundreds of micromolar and

millimolar, and some transport factors additionally being actively released from the pore [25–27]. The extreme binding multivalency of Nup - transport factor interactions adds a further layer of complexity to interpreting affinity data. It is increasingly accepted that the highly transient and multivalent binding of transport factors to FG Nups is key to the combination of high specificity and rapid transport exhibited by the NPC [28].

The karyopherins (Kaps), also known as importins and exportins, are largest family of transport factors. The approximately twenty different Kaps are responsible for most nucleocytoplasmic transport [29]. Kaps typically consist of multiple HEAT repeats, a helical motif which conveys structural flexibility [30]. Most Kaps bind their cargo directly via a nuclear localization signal (NLS, for nuclear import) or nuclear export signal (NES, for nuclear export). NLS and NES are relatively short amino acid tags found on cargo [31]. However, sometimes the adaptor protein importin α is also needed. In general, Kaps are on the order of 100 kDa in size, well above the passive permeability limit [1]. As discussed below, there is evidence that the presence of Kaps contributes to the selectivity barrier [29, 32–34]. Many Kaps must be actively released from the nuclear pore and from their cargo after transit [35, 36].

Unlike the karyopherins, nuclear transport factor 2 (NTF2) does not transport a wide variety of cargo across the NPC. Instead, NFT2 maintains the Ran gradient needed for transport by carrying RanGDP across the pore [37, 38]. NTF2 is a homodimer whose monomers are 14 kDa and contain at least one FG binding site apiece. Although its small size of 28 kDa is near the 30 kDa cutoff for passive transit through the pore, its flux through the pore is still at least 30 times that of similarly-sized proteins that do not bind to FG Nups [39–41]. NTF2 does not require adaptor proteins or active release from the pore. We predominantly use NTF2 as a model transport factor in both the theoretical and experimental work discussed here, because of its simplicity as well as its ease of expression and purification from bacterial cells.

Selective transport requires an energy source, which in the case of the NPC is provided by the Ran cycle. When a transport factor-cargo complex passes from the cytoplasm into the nucleus, it encounters a RanGTP on the nuclear side which binds to the transport factor and displaces the

cargo, actively releasing it. Then the transport factor-RanGTP complex can collect a cargo destined for nuclear export, and this ternary complex can diffuse back through the NPC to the cytoplasm. The protein RanGAP then hydrolyzes the RanGTP to RanGDP, disrupting the complex into its three original pieces. Ultimately, the energy source for selective nuclear transport comes from the RanGTP-RanGDP gradient from the cytoplasm to the nucleus, a gradient which is maintained partially by NTF2, which carries RanGDP through the pore. In the nucleus, RanGDP is returned to its RanGTP state by RanGEF, a protein which is localized to the nucleus [28,37,42]. Therefore, even though there is no obvious directionality to NTF2-RanGDP transport, the effect is to replenish the pool of RanGTP in the nucleus. From the perspective of transport, this means that, for many molecules, the process of passing through the pore is itself passive and does not consume energy. The selectivity ultimately arises from concentration gradients maintained by the Ran cycle, but the selective mechanism is not itself active.

One surprising feature of nuclear transport is its sheer speed and volume. The high macromolecular traffic between nucleus and cytoplasm requires high flux through each NPC. Experiments with permeabilized cells estimate that the total molecular flow through the NPC could be as high as 10-20 MDa per pore per second, corresponding to 100-1000 transport events per pore per second [39]. Experiments focusing particularly on NTF2 report fluxes between 50 and 250 molecules per pore per second [39–41]. Fluxes this high mean a continuously high occupancy of the NPC, estimated at up to 100 karyopherins at once [43]. One reason that individual NPCs can accomodate such high flux is the rapidity with which molecules transit the pore. A wide range of transport factors and cargo have a dwell time of less than 10 ms in the pore [13, 44–46]. Typically, this is determined using single-molecule tracking with superresolution microscopy [13].

1.1.3 Models of nuclear transport

While the components of nuclear transport are well-understood, questions remain regarding the mechanism behind its unusual selective properties. Broadly speaking, two of the most important theoretical frameworks are the hydrogel model and the entropic barrier model (Fig. 1.2). Both are

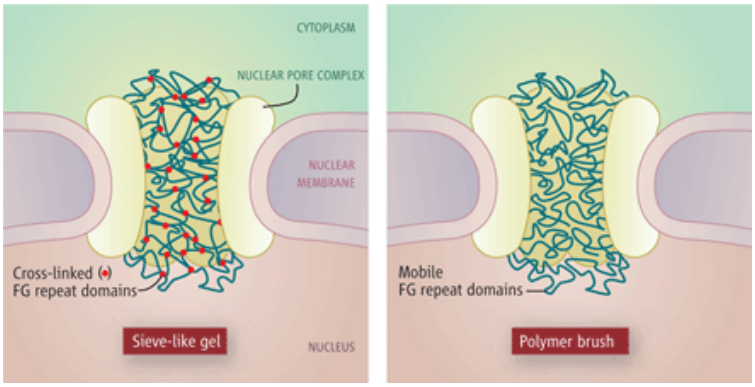

Figure 1.2: The two major models of the nuclear pore: the hydrogel (sieve-like gel) and entroptic barrier (polymer brush) models. Figure from [47].

supported by some experimental results and challenged by others, and they are not mutually exclusive. More recent work suggests that the environment of the nuclear pore is somewhere between these two extremes.

The hydrogel model posits that the FG Nups within the nuclear pore are transiently crosslinked at their FG repeats into a hydrogel-like structure. Inert proteins are prevented from passing through the pore due to the small mesh size, while transport factors carrying cargo can also bind to the FG repeats, disrupting the hydrogel and moving through the pore. This view of the nuclear pore is supported by experiments showing that the disordered domain of the essential FG Nup Nsp1 aggregates into a hydrogel when purified into buffer [48]. These hydrogels show strong selectivity for import of transport factors and their cargo over inert proteins, demonstrating binding between the aggregated FG Nups and the transport factors [49–51]. Theoretical models of diffusion through a transiently-crosslinked hydrogel show selective properties [39, 52, 53]. However, while nuclear pore mimics which consist of aggregrated FG Nups display highly selective entry of transport factors, they do not permit the exit of transport factors over timescales consistent with transport [49, 50]. Furthermore, some Nups which aggregate in buffer remain disordered in the cellular environment [11].

Conversely, the entropic barrier model supposes that FG Nups instead act as polymer brushes within the pore. In this view, inert proteins are prevented from entering the pore due to the entropic penalty they would incur by restricting the possible conformations of the Nups. However, the decrease in free energy upon a transport factor binding to a Nup offsets the entropic penalty

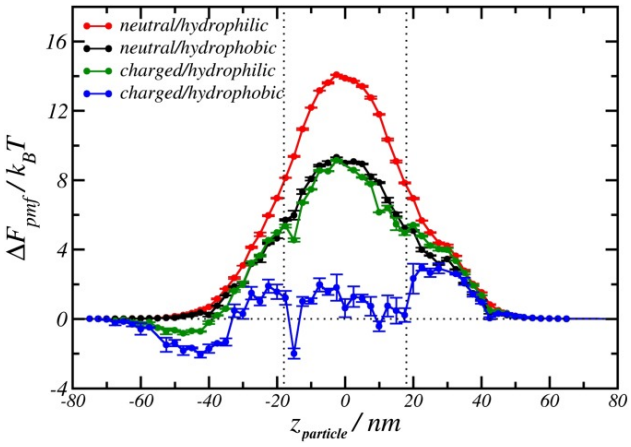

Figure 1.3: Example of effective-potential model of nuclear transport. Figure shows simulated free-energy landscape for a variety of possible transport factors as a function of position within the nuclear pore. Figure from [18].

and allows transport factors and their cargo to pass [54, 55]. Evidence for this view comes from a number of studies in which single layers of FG Nups are grafted onto a surface and their extension monitored as transport factors are titrated on and off the surface [56–58]. Layer height tends to change non-monotonically as transport factors are added, suggesting that the presence of transport factors affects the selectivity of the nuclear pore.

More generally, FG Nups exist on a continuum of "cohesiveness," ranging from Nups (or portions of Nups) which do not aggregate under any physiological conditions to ones which do so readily [11]. Nup cohesiveness depends on their charge, length, and hydrophobicity. Many simulations of the nuclear pore aim to understand the role of Nup cohesiveness in transport [18, 53, 59, 60]. There may be distinct regions of differing Nup properties within the nuclear pore. A relatively common method of modeling transport is to computationally reduce the problem to that of a transport factor diffusing in a one-dimensional effective potential representing its interactions with Nups (Fig 1.3) [1, 18, 61, 62]. These models predict selective transport while being highly dependent on the precise details of Nup composition and location within the nuclear pore. These models also do not presuppose that either the hydrogel or entropic brush model is fully correct, but allow for a mixture of cohesive and non-cohesive Nups.

Apart from the question of FG Nup conformation within the nuclear pore, the role of transport factors themselves in the permeability barrier has been under investigation [29, 32–34]. There is evidence that the presence of karyopherins in the nuclear pore increases its selectivity for transport

factors, possibily by establishing "tight-binding" and "weak-binding" subpopulations of Kaps which compete for binding sites on the Nups [56]. Even non-specific competition may make the nuclear pore's selectivity barrier more robust as well [63–66]. The precise role of crowding and competition in nuclear pore selectivity is fascinating but as yet unclear.

Relatively few non-computational, quantitative theories of nuclear transport exist. Reaction-diffusion models have been proposed with varying degrees of complexity and different underlying assumptions but they do not take both binding kinetics and binding site saturation into account [61, 67].

1.1.4 Nuclear pore mimics

Many people have tried to make artificial filters which show similar selectivity properties as the nuclear pore.

Some synthetic nuclear pores recreate the nanopore geometry of the NPC using a perforated membrane which is coated in FG Nups. One such solid-state nuclear pore mimic consisted of the FG domains of Nsp1, Nup98, or Nup153 grafted onto a porous silicon nitride membrane [21,68]. Transit events of transport factors or inert proteins through the bare or coated pores can be measured by monitoring the conductance of the pore, which spikes when a protein passes through. The flux of inert proteins drops significantly when the pore is coated with Nups, but that of transport factors remains roughly constant, suggesting selective transport [68]. Another nanopore NPC mimic used pores of varying diameter in 15-nm-thick polycarbonate membranes [22]. A portion of the pore was coated in gold to which FG domains of Nsp1 or Nup100 were conjugated. Measurements of flux through these pores showed a maximum of $\sim$ 3-fold selectivity for NTF2-GST as compared to the similarly-sized inert protein BSA. Similar selectivities were seen for Kap95 with and without cargo. The FG Nup concentration could be tuned by varying the extent of the gold coating; increasing the Nup concentration increased the selectivity.

One class of nuclear pore mimics consists of aggregated FG Nup domains. The FG repeat domain of Nsp1 will spontaneously aggregate into a hydrogel in buffer at physiological pH, as will

the GLFG domains of Nup49p and Nup57p [48, 69]. When these hydrogels are challenged with fluorescent transport factors or transport factor - cargo complexes, they show strongly selective influx as compared to inert proteins, with in-gel concentrations reaching 1000 times that of the reservoir surrounding the gel [49]. These hydrogels have amyloid-like characteristics and immobilize the FG Nups to a large degree, as shown by fluorescence recovery after photobleaching (FRAP) studies [48, 50]. In addition to transport factors partitioning into the gel, other FG Nup domains become incorporated into the gel when introduced to the surrounding reservoir. Gelation and selective influx are both destroyed by mutating the phenylalanines of the FG motifs to serines, indicating that interactions between FG repeats are necessary for these features [49]. While the selective influx is quite dramatic, direct measurements of diffusion constant or flux through an FG Nup hydrogel have not been made. Appendix A shows the estimated selectivity of several FG Nup hydrogels as predicted by our model described in Chapter **??**. Some gels reach predicted selectivities of ~ 200, within the range observed in the nuclear pore [39–41].

Other nuclear pore mimics also make use of hydrogels, but incorporate FG Nups as only one component of the gel rather than its entirety. Once such hydrogel was composed of a portion of the FG domain of Nsp1 fused to a domain that forms pentamers [51]. A fluorescent cargo targeted by Kap95 showed strong selective influx into these hydrogels, though equilibration was not achieved over the timescale of the experiments. The length of the FG Nup fragment used was varied so as to contain one, two, or six FSFG motifs, and the extent of binding of the transport factor-cargo complex was shown to depend on the number of binding motifs used. Predicted selectivities of these hydrogels are also shown in Appendix A.

Other hydrogel nuclear pore mimics used an inert hydrogel as a scaffold to which fragments of FG Nups or other disordered peptides were tethered. Selective influx of the transport factor fusion protein GFP-Kap95 was shown for an acrylamide hydrogel containing high concentrations (250-400 mM) of stand-alone FSFG motifs, not incorporated into a longer peptide [70]. Increasing the concentration of FSFG anchored to the hydrogel increased the partitioning of GFP-Kap95 into the gel, but the influx was relatively low given the high concentration of FSFG motifs in

the hydrogel. Other hydrogel mimics used engineered peptides with similar properties as those of FG Nups but not derived from them [67]. These were tethered to a PEG hydrogel and bound a fluorescent antibody (approximately 150 kDa) with varying affinity. The influx of antibody into the hydrogels showed a non-monotonic dependence on affinity, with an optimal dissociation constant $K_D \approx 30$ μM. FRAP measurements confirmed that binding antibodies diffused more slowly in the gel than did inert control proteins.

An interesting preliminary work demonstrated the use of DNA origami to mimic the scaffold of the nuclear pore by assembling DNA into a ring 46 nm in diameter and 14 nm in height, with up to 48 sites for FG Nup attachment [71]. When FG domains of Nsp1 or Nup100 were anchored to the attachment points, AFM confirmed that the Nups filled the center of the ring and remained highly dynamic.

While many of these mimics have shown selectivity to some extent, none have definitively shown a flux of transport factors into, through, and out of the mimic that matches that observed experimentally.

1.2 Other selective biofilters

Selective biofilters exist in many contexts outside of nuclear transport, and they frequently include a common set of elements that are exemplified by the nuclear pore. In particular, cells often need proteins to move rapidly within a cellular compartment but still possess high binding specificity. This is often accomplished with intrinsically disordered proteins that interact transiently and multivalently with their binding partners. In the crowded environment of the cell or extracellular matrix, nonspecific binding can immobilize proteins, hindering selective motion, unless those proteins are able to continue diffusing while bound, a feature which we argue is key to the selective transport of the NPC as well as the biofilters discussed here. The following section presents three particular examples of selectivity in biological systems, from widely disparate areas, which bear striking similarities to the selectivity of the nuclear pore. In the remainder of this work, we model selective transport using a minimal set of characteristics that, while inspired by the nuclear pore,

apply to all of the systems here as well.

1.2.1 Drug delivery through mucus barriers

One medically-important selective biological barrier is the mucus which lines organs such as the lungs, nose, and stomach. In particular, lung mucus presents a barrier to delivery of inhaled medication. If nanoparticles containing drugs for lung diseases such as asthma and lung cancer could be inhaled and taken up by lung cells, doses could be lower, as the uptake would be more targeted [72,73]. However, lungs are coated by a layer of mucus intended to prevent foreign objects from reaching the lung cells. In order to deliver nanoparticles to lung cells, they must be engineered to pass the selective mucus barrier.

Mucus consists predominantly of disordered glyocoproteins known as mucins, though other components such as lipids are present as well [74]. These entangled mucins present multiple barriers to nanoparticles, as shown in Fig. 1.4. First, large particles are excluded due to the 150-350 nm average pore size of mucus gels [75,76]. Second, even particles which are small enough to enter the mucus layer often bind to mucins. In fact, mucoadhesive particles (MAPs) have been specifically engineered on the principle that increasing the nanoparticle's lifetime within the mucus will lead to more efficient drug delivery [77]. However, diffusion of MAPs is often so slow that they do not penetrate beyond the edge of the mucus layer. Furthermore, mucus gradually replaces itself on clearing timescales which vary depending on the type of mucus. Nanoparticles in the outermost region will be cleared more rapidly than those which diffuse deeply into the mucus layer [78].

Currently, the best nanoparticle delivery through the lung mucus barrier is by small, inert particles which minimize nonspecific interactions with mucins. These mucus-penetration particles (MPPs) are typically coated in PEG or another inert polymer [73, 80]. MPPs can reach much higher diffusion constants in mucus than can MAPs [76,81–83].

The selective barrier of lung mucus bears similarities to the selectivity of the nuclear pore in that it consists of disordered proteins which bind to particles impinging on the barrier. However, in the case of mucus, binding inhibits the flux of particles through the selective filter, while binding

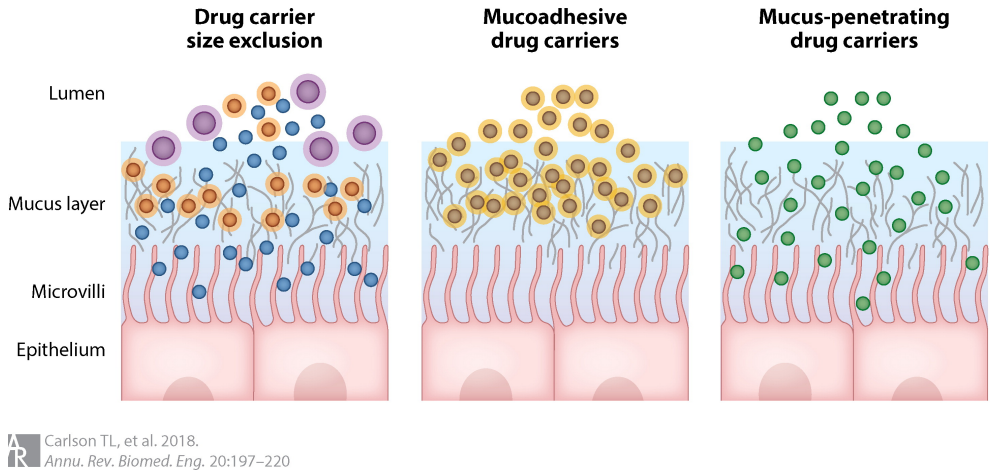

Figure 1.4: Nanoparticle drug delivery through a mucus barrier. Particles which are too large cannot pass the size-exclusion barrier. Mucoadhesive particles penetrate the mucus layer but are trapped by binding interactions. Inert mucus-penetrating particles have a much higher diffusion constant in mucus. Figure adapted from [72, 79]

enhances the flux of transport factors through the nuclear pore. A better understanding of the role of binding and bound diffusion to a selective filter such as the nuclear pore could suggest novel strategies for nanoparticle delivery through lung mucus. Perhaps binding can be leveraged in a way that enhances nanoparticle flux over that of inert particles, improving drug delivery to the lungs.

1.2.2 Diffusion of DNA-binding proteins in the nucleus

The principles of selective filtering in the nuclear pore appear in biological systems beyond straightforward filtering. In particular, transient binding and bound-state diffusion are important to DNA targeting in the nucleus by transcription factors and DNA damage repair proteins. A protein diffusing in the cell's nucleus has many of the same constraints and capabilities as a transport factor transiting the nuclear pore: it is surrounded by a high concentration of flexible tethers to which it binds transiently. Just as this situation leads, counterintuitively, to high flux of transport factors through the nuclear pore, DNA-targeting proteins find their specific targets much more rapidly than would be naively predicted [84].

Solutions to this "needle in the haystack" challenge resemble possible mechanisms of bound diffusion in the nuclear pore complex. In particular, it is widely accepted that the search for targets is made more rapid through facilitated diffusion, involving proteins sliding along strands of DNA. However, the optimal time spent in a one-dimensional search as opposed to a free three-dimensional search is unclear; as is the effectiveness of sliding as opposed to multiple, rapid binding and unbinding events [85]. This mechanism is reminiscent of both the sliding mechanism predicted in some transport factors and of the effect of diffusion while bound to a flexible tether [86]. Additionally, transfer of a multivalent transcription factor between two strands of chromatin, a mechanism known as intersegmental hopping, can increase the protein's search space similarly to the multivalent inter-Nup hopping that is available to transport factors [87, 88]. Finally, transient binding plays an important role in the search for DNA targets. This speed-stability paradox reflects the fact that, like in the nuclear pore, proteins must bind very weakly to their DNA tethers to avoid becoming immobile. Unlike nuclear transport, however, DNA-binding proteins must bind more

tightly to their targets upon reaching them [89, 90].

A case in point, further discussed in Sec. **??**, is that of poly(ADP-ribose) polymerase 1 (PARP1), a DNA damage repair protein which rapidly localizes to sites of DNA damage. PARP1 binds damaged DNA with low nanomolar affinities, and appears to bind undamaged DNA only a few orders of magnitude more weakly [91]. Given that there are up to 5 mM nonspecific DNA binding sites in the nucleus, the speed with which PARP1 diffuses ($\sim$ 10 times slower than in buffer) is remarkable [89]. PARP1 contains multiple DNA-binding sites, and an inter-strand hopping mechanism has recently been demonstrated, which may explain its rapid diffusion [92]. However, removal of that mechanism only slightly slows the recruitment of PARP1 to sites of DNA damage, suggesting that a further mechanism of bound-state diffusion is necessary [93]. The principles of selective nuclear transport may help to explain the rapid motion of PARP1, along with other DNA damage repair proteins and transcription factors.

1.2.3 Subcompartments in liquid droplets

Features of nuclear transport such as highly-concentrated disordered proteins and bound-state diffusion also appear in liquid-liquid phase separated droplets. These membraneless organelles are rapidly gaining prominance as it becomes clear that many cellular functions are regulated through the phase separation of mixtures into phase rich in various proteins and other cellular components. Membraneless organelles include nucleoli, which aid in processing ribosomal DNA genes within the nucleus, and RNA granules, which help sort mRNA [6, 95].

Liquid-liquid phase separation typically occurs for mixtures containing IDPs with "sticker" and "spacer" regions, similar to the hydrophobic FG repeats and hydrophilic linker regions in FG Nups [96]. These phases are highly concentrated, but the proteins that comprise them remain mobile. Interestingly, evidence is developing for subcompartments of varying composition within some liquid droplets. Nucleoli contain multiple subcompartments which are thought to perform distinct functions (Fig. 1.5) [94, 97, 98], while RNA granules have recently also been shown to have a core-shell structure [95, 99].

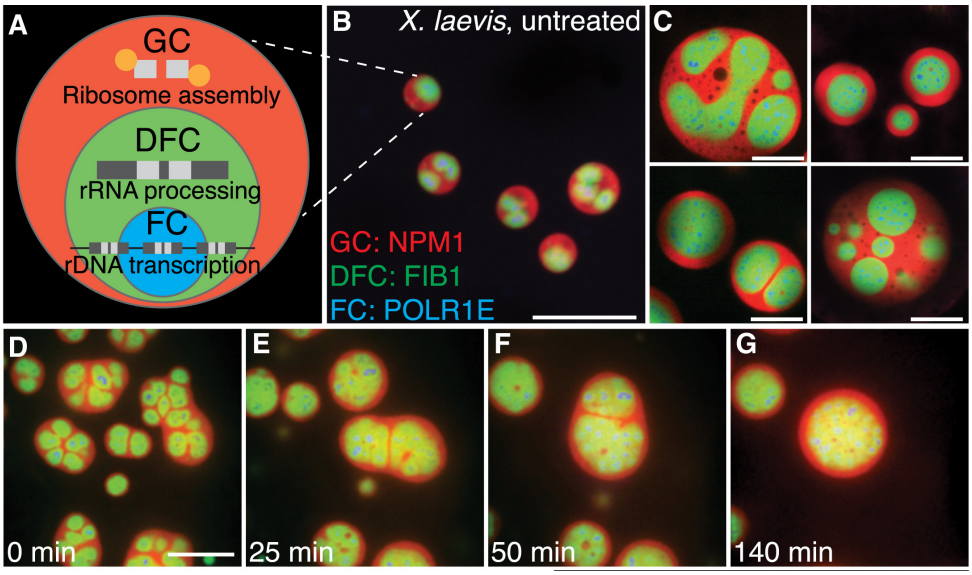

Figure 1.5: Phase-separated subcompartments of *Xenopus* nucleolus. (A) Schematic of subcompartments. (B)-(C) Fluorescence microscopy image of nucleoli with subcompartments in red, green, and blue. (D)-(G) Time-course showing dissolution of compartments after actin disruption. Figure adapted from [94].

The investigation of subcompartments in membraneless organelles is in its very early stages, with much of the current work going towards simply identifying the components of the compartments. Discussion of their purpose and mechanisms is still quite speculative. However, it seems plausible that one function of these compartments is to selectively concentrate enzymes or other proteins to increase reaction rates. RNA bodies in particular are known to sort and sequester mRNA [95]. As such, the presence of disordered, multivalent proteins may not simply be necessary for the formation of phase separated droplets, but could potentially assist in selectively filtering the components of the subcompartments. If nuclear transport is a guide, binding partners of the IDPs which form the outermost phase might have a higher flux into the inner compartments than nonbinding proteins. Additionally, in the context of bound-state diffusion, liquid droplets represent maximal bound diffusion, as binding to an IDP in solution will not appreciably slow the diffusion of the binding partner. While these possibilities will not be testable for some time, membraneless organelles may prove to be a fascinating application of selective biofiltering.

1.3 Project goals and motivation

All of the systems described above involve the rapid flux of proteins which still bind highly selectively to their environment. This apparent paradox further includes common features such as ultrafast, transient, multivalent binding and the presence of binding sites on flexible, dynamic tethers. The mechanism by which these features lead to such remarkable selective properties is not obvious. We approached the problem through both modeling and experiment and identified a common key parameter in the form of bound-state diffusion. While the theory and experimental setup were based on nuclear transport, both platforms are general enough to apply to a number of selective biofilters.

1.3.1 Model selective transport as simply as possible

Despite the complexity of nuclear transport, many components can be removed or approximated without eliminating its unusual selective properties. For example, Nups which are asym-

metrically distributed along the axis of the nuclear pore can be removed, as can a large fraction of Nups overall, without destroying selectivity [19–22]. And though many transport factors require active release from the pore, others, such as NTF2, do not; selectivity is not therefore dependent on active release. We decided to model the pore in order to answer the question: What are the minimum requirements for selective transport?

In the course of creating the model, we found that the minimum requirements are quite simple indeed. The model discussed in Chapter **??** not only dispenses with non-uniformly distributed Nups and facilitated release, but also with non-specific crowding, Kap-centric control of the nuclear pore, the Ran cycle, and even the nanopore geometry. Instead, the pore is treated as a bulk material of finite thickness with an artificially-imposed concentration gradient to drive flux across it. Competition for binding sites is retained in the form of binding site saturation, but even without this feature we see selective flux across the barrier. Our model fundamentally contains only diffusion and binding of transport factors to Nups - an extremely streamlined depiction of nuclear transport, and one that can even be treated semi-analytically.

The simplicity of our model made the key parameter clear: we could not reproduce the selectivity of the nuclear pore without allowing bound-state diffusion, that is, assuming that bound Nup - transport factor complexes were mobile within the pore. Bound diffusion provides a possible answer to the paradox of nuclear transport, resulting in a higher flux of transport factors, which bind to Nups, than inert proteins which do not. In our model, this straightforward mechanism alone gave rise to the decidedly unintuitive behavior of the nuclear pore.

Having identified bound-state diffusion as an important parameter for selective filters, we investigated possible methods of bound diffusion within the nuclear pore. Two possibilities arose from basic, well-accepted facts of nuclear transport: tethered diffusion arising from the disordered, flexible nature of the Nups, and inter-chain hopping of the transport factor due to its binding multivalency. Upon investigation, both mechanisms can plausibly provide the high bound diffusion constant needed to reproduce the selectivity of the nuclear pore.

While this model was inspired by nuclear transport, it ultimately relies on only a few key

properties of the system. With this model, we predict selective transport will occur where there is bound-state diffusion, which can be readily obtained if flexible IDPs are present for tethered diffusion, or if highly transient, multivalent binding allows for moving from one site to another while remaining bound. The systems described above in Sec. 1.2 fall into this category, as likely do many others. Furthermore, these principles might guide synthetic selective biofilters with novel rapid, highly-selective properties.

1.3.2 Experimentally test bound-state diffusion in biomaterials

After developing the minimal model of selectivity, we began developing a synthetic biofilter with which to experimentally probe the effect of bound-state diffusion on selectivity. The model itself is sufficiently general to apply to a wide range of filters, giving us some freedom in designing a material to test its predictions. We chose to use the nuclear pore as inspiration for this material because its key components are so well known, if not well understood. In order to capture the key features of selectivity, we designed hydrogels containing peptide tethers derived from FG Nups. The transport factor NTF2 served as a test protein whose diffusion we could compare with a similarly-size but nonbinding counterpart. These hydrogel nuclear pore mimics display the mechanisms that lead to selective transport in our model: flexible, dynamic tethers which can transiently bind to transport factors, along with multivalent transport factors which can "hop" between peptide chains without fully unbinding. We predict that these features will be sufficient for bound-state diffusion of the transport factor within the hydrogel.

As with the theoretical model, many of the NPC-specific details, such as the nanopore structure and Ran gradient, were omitted. A bulk material was used instead of nanopores because our model should apply equally well to both, and a macroscopic hydrogel is much easier to study. We quickly realized that hydrogels are a challenging system to use for protein separation. The nuclear pore mimics have a rigorous set of competing constraints: for example, the hydrogel should be in mechanical equlibrium and homogeneous, while still well-sealed to a flow chamber so that proteins cannot bypass the gel. Chapter **??** documents the design of the hydrogel nuclear pore

mimics. Many modifications intended to improve the diffusion properties and reproducibility of the hydrogels proved unsuitable for our needs. However, we eventually produced a biomaterial that can be used to measure bound diffusion, while also developing more general guidelines for designing hydrogels that are useful for protein separation.

The resulting bound-state diffusion measurements are presented in Chapter **??**. Fluorescence recovery after photobleaching (FRAP) was used to determine the effective diffusion constants of both NTF2 and an inert protein, from which the bound diffusion constant was calculated. The data analysis ultimately required a two-dimensional Fourier series solution to the diffusion equation, resulting in a set of data-processing scripts which can be applied to any circular material undergoing equilibration with a fluorophore. We measured a non-zero bound diffusion constant that is consistent with the predictions of our model, and tested the effect of varying Nup length on bound-state diffusion. Our results indicate that these nuclear-pore-inspired hydrogels can be used to probe the effect of our model's parameters on bound diffusion.

While bound diffusion is a key parameter in our selectivity model, the aggregation state of the Nups could also affect selectivity. Aggregated Nups will be less dynamic and effectively act as shorter tethers, limiting bound diffusion. In Chapter **??**, we probed the aggregation behavior of an FG-Nup-derived peptide in several crowded conditions. Using a fluorescent amyloid assay, we identified significant differences in the aggregation dynamics in the presence of different crowders, including between poly(ethylene glycol) (PEG) and polyvinylpyrrolidone (PVP), two inert polymers that are widely presumed to be interchangeable as crowders. We followed this aggregation assay with NMR and fluorimetry studies in order to investigate the nature of the changes. The results suggest that the presence of an aromatic ring in PVP may interact with the phenylalanines in the FG motifs of the peptide, changing its local chemical environment and therefore its aggregation behavior.

Although a true hydrogel-based selective biofilter proved challenging to design, our nuclear-pore-inspired material can be used to measure the bound-state diffusion of proteins. This parameter is likely important to a variety of problems involving rapid transit of highly-selective proteins. It is

my hope that the model and biomaterials developed here can be used to investigate these systems more generally in the future. This work suggests that bound-state diffusion, particularly when resulting from transient, multivalent binding, may explain a number of unusual biological filters.

1.4 Biophysics is beautiful

Even beyond the ample practical reasons to study nuclear transport, it is a fascinating process, full of counterintuitive results and apparent paradoxes. The nuclear pore is at once an incredibly intricate nano-machine and surprisingly robust to perturbation. Passage through the nuclear pore is carefully controlled by complex cellular processes, yet the mechanism of selectivity itself does not require energy input. Nuclear transport can enhance the flux of transport factors hundreds of times over that of inert proteins using a mechanism that conventionally *reduces* protein mobility.

Where the NPC is not apparently self-contradictory, it is a picture of extremes. FG Nups are not only disordered, many show virtually no signs of secondary structure whatsoever and do not order appreciably upon binding transport factors. Nup - transport factor binding is extreme on many axes: the affinity, likely in the millimolar range, is weak enough that most biochemists would characterize it as non-specific binding, yet it permits a high flux through the pore. At the same time, the on-rate is ultrafast, bounded by the physical diffusion limit rather than chemical considerations. Finally, with dozens of FG motifs along the length of each Nup, and multiple binding pockets on each transport factor, Nup - transport factor binding is dizzyingly multivalent. It is perhaps no wonder that measurements of the binding affinity have historically spanned six orders of magnitude; at this level of multivalency, even the notion of "binding affinity" itself becomes convoluted. In almost every respect, the binding interactions which underpin selective transport are as far removed as possible from textbook protein-protein interactions. The nuclear pore is a fundamentally *weird* system and deserves to be studied purely for the joy of discovering how something so unusual operates.

Furthermore, the details of nuclear transport are all but invisible even to the most cutting-edge biochemical techniques. The timescale of transport is too fast, the size too small, and the

disordered Nups too dynamic to permit direct visualization of transport. This is a system which practically demands to be studied using nontraditional, interdisciplinary methods.

There is value in approaching the nuclear pore specifically from the perspective of physics. Biophysics as a field experiences constant tension between the need to account for the incredible complexity of any living system and the drive to reduce it to its smallest, tidiest set of component parts. As a physicist, I take deep satisfaction from applying a broad and simple theory to a complicated system and still getting meaningful results. All of the messy intractable details are of course ultimately necessary for life to exist, but if a big, sweeping, absurdly simple, underlying principle can explain even 50% of a complex system, that's beautiful. Such opportunities abound in biophysics, from flies which right themselves after mid-flight perturbation, to the scaling of barbs in cat tongues, to how cucumber tendrils coil and overwind, to the nuclear pore complex and beyond [100–102]. Sometimes it's worthwhile to apply basic physical theories to ridiculously complicated things which they manifestly cannot fully explain - valuable practical advances frequently arise from surprisingly slender foundations.

Whether viewed through a practical, intellectual, or aesthetic lens, there is much to be gained from the study of nuclear transport and, more broadly, of binding and diffusion in selective biofilters. Certainly I have found that, despite the setbacks and disappointments which accompany all research, I have never been able to describe my work to others without becoming truly enthusiastic yet again.

Chapter 2

Conclusions and future directions

Living systems contain many examples of selective filters which control the transport of all manner of macromolecules. In this work, we studied biofilters which allow rapid transport of highly specific targets, counterintuitively relying on binding interactions to promote rather than hinder flux. These filters often contain similar features, such as transient, multivalent binding and binding to flexible, dynamic tethers. In order to understand the presence of these features, we approached the problem through both modeling and experiment. Bound-state diffusion proved to be a key parameter for selectivity. While the theory and experimental setup were based on nuclear transport, they were not designed to exactly reproduce the nuclear pore, but to be applicable to a wider variety of biofilters.

We created a minimal model of the nuclear pore, containing very little beyond the diffusion of transport factors and their binding to FG Nups. Interestingly, even this highly simplified model was able to reproduce the selectivity properties of the nuclear pore, predicting that binding could increase the flux of a protein through this material up to 300-fold over the flux of an identical inert protein. The simplicity of our model made the key parameter clear: we could not reproduce the selectivity of the nuclear pore without allowing bound-state diffusion, that is, assuming that bound Nup - transport factor complexes were mobile within the pore. Plausible mechanisms of bound diffusion in the nuclea pore include ethered diffusion arising from the disordered, flexible nature of the Nups, and inter-chain hopping of the transport factor due to its binding multivalency.

We modeled both of these mechanisms and found that they allow for significant bound mobility and therefore high selectivity. These mechanisms may also be at work in other highly-specific, high-throughput biofilters.

After developing the minimal model of selectivity, we began developing a synthetic biofilter inspired by the nuclear pore with which to experimentally probe the effect of bound-state diffusion on selectivity. In order to capture the key features of selectivity, we designed hydrogels containing peptide tethers derived from FG Nups. The transport factor NTF2 served as a test protein whose diffusion we could compare with a similarly-size but nonbinding counterpart. Fluorescence recovery after photobleaching (FRAP) was used to determine the effective diffusion constants of both NTF2 and an inert protein, from which the bound diffusion constant was calculated. We measured a non-zero bound diffusion constant that is consistent with the predictions of our model, and tested the effect of varying Nup length on bound-state diffusion.

Additionally, we probed the aggregation behavior of an FG-Nup-derived peptide in several crowded conditions. We used a fluorescent aggregation assay as well as NMR and fluorimetry to investigate differences in the aggregated state between crowding conditions, and found that even inert crowders which are widely used interchangeably show differences in the local chemical environments of the peptide.

Although a true hydrogel-based selective biofilter proved challenging to design, our nuclear-pore-inspired material can be used to measure the bound-state diffusion of proteins. This parameter is likely important to a variety of problems involving rapid transport of highly-selective proteins. The model and biomaterials developed here could be used to investigate these systems more generally in the future. This work suggests that bound-state diffusion, particularly when resulting from transient, multivalent binding, may explain a number of unusual biological filters.

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Appendix A

Predicted selectivity of nuclear pore mimics

Nup fragment	Nup concentration	Molecule	MW	Partition coeff.	Diffusion coeff. in gel	Diffusion coeff. free	K_D	D_B	D_B/D_F	S	S with 10x partition coeff.	Notes
Nsp1 (2-601)	3 mM	IBB-MBP-mEGFP-ImpB MBP-mCherry	510 kD 70 kD	100 0.16	0.17 $\mu m^2/s$	4.03 $\mu m^2/s$	4.8 μM	0.17 $\mu m^2/s$	0.04	42	50	[69]
Nup57 (1-223)–Nup49 (1-246)	3.7 mM	IBB-MBP-mEGFP-ImpB MBP-mCherry	510 kD 70 kD	400 0.15	0.1 $\mu m^2/s$	2.7 $\mu m^2/s$	1.4 μM	0.1 $\mu m^2/s$	0.04	69	45	[69]
Nup57 (1-223) –Nsp1 (2-601)–Nup49 (1-246)	1.7 mM	IBB-MBP-mEGFP-ImpB MBP-mCherry	510 kD 70 kD	350 0.1	0.24 $\mu m^2/s$	4.03$\mu m^2/s$	0.48 μM	0.24 $\mu m^2/s$	0.06	38	16	[69]
Nsp1 (2-175)	3.0 mM	IBB-MBP-mEGFP-ImpB MBP-mCherry	510 kD 70 kD	100 3	0.04 $\mu m^2/s$	12.1$\mu m^2/s$	90 μM	0.04 $\mu m^2/s$	0.003	1.4	4.3	[50]
Nsp1 (2-601)	3.0 mM	IBB-MBP-mEGFP-ImpB MBP-mCherry	510 kD 70 kD	60 0.4	0.22 $\mu m^2/s$	6.94$\mu m^2/s$	20 μM	0.22 $\mu m^2/s$	0.03	15	40	[50]
Nsp1 (1-601)	2.2 mM	IBB-Redstar-ImpB IBB-Redstar	530 kD 150 kD	1000 0.3	0.1 $\mu m^2/s$	0.2$\mu m^2/s$	0.66 μM	0.1 $\mu m^2/s$	0.5	230	100	[49]
Nsp1 (1-601)	2.2 mM	GFP-ImpB IBB-Redstar	124 kD 150 kD	100 0.3	0.1-0.2 $\mu m^2/s$	0.2$\mu m^2/s$	6.6 μM	0.1-0.2 $\mu m^2/s$	0.5-1	210-250	230-240	[49]
Nsp1 (1-601)	2.2 mM	GFP-ImpB acRedStar	124 kD 117 kD	100 0.05	0.1-0.2 $\mu m^2/s$	0.2-1$\mu m^2/s$	1.1 μM	0.1-0.2 $\mu m^2/s$	0.1-1	94-260	53-130	[49]
P - Nsp1 (274-601) - P *	4.4 mM	IBB-MBP-mEGFP-ImpB IBB-MBP-mEGFP	510 kD 100 kD	7 0.9	2.78 $\mu m^2/s$	16.0 $\mu m^2/s$	560 μM	2.42 $\mu m^2/s$	0.15	5.3	25	[51]

Table A.1: Predicted selectivity of FG Nup hydrogels in previous work. We took partition and diffusion coefficients from tables in references or calculated them using concentration plots. We determined the dissociation constant K_D from the partition coefficient of the binding species (P_B) and non-binding species (P_N) and the Nup concentration N_t using $K_D \approx (P_I/P_B)N_t$. Note that the measured P_B is an underestimate of the true partition coefficient. We estimated the bound diffusion coefficient from the in-gel (effective) diffusion coefficient (D_{eff}) and the probability of the binding species being bound ($p_b \approx 1 - K_D/N_t$) using $D_B = p_b D_{\text{eff}}$ (Sec. **??**). We used the reaction-diffusion equations discussed in Sec. **??** to estimate the selectivity. Because partition coefficient estimates were lower bounds, we also calculated selectivity assuming that the reported partition coefficients were 10% of their actual value. P - Nsp1 (274-601) - P * refers to a fusion between Nsp1 (274-601) and a pentameric coiled-coil P which facilitates the aggregation of the Nsp1 domain into hydrogels. See [51].