

Mechanisms of diffusion in nuclear pore complex mimics

by

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Mechanisms of diffusion in nuclear pore complex mimics

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Placeholder text for abstract.

Dedication

Nice dedication goes here.

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There are lots of people to acknowledge.

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Chapter 1

Introduction

1.1 Intrinsically disordered proteins are essential to cellular function

For decades, it was conventional wisdom among biologists that a protein's folded shape determined 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, overturning 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 [?]. 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 [?].

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 disease, Parkinsons, and prion diseases. Often, normally-disordered proteins aggregate into amyloid fibrils, a stable structure based on parallel beta-sheets.

IDPs are commonly involved in cell signaling and regulation [?]. 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. 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.

1.2 Major components of nucleocytoplasmic transport

The nuclear pore complex (NPC) resides in the nuclear envelope of eukaryotes and regulates all macromolecular traffic between the nucleus and cytoplasm. The NPC is one of the largest protein complexes in the cell, at about 60 MDa in yeast and 120 MDa in humans [5]. As the regulator of nucleocytoplasmic transport, the NPC must rapidly and specifically pass a wide array of macromolecules: transcription factors into the nucleus, and RNA into the cytoplasm. Moreover, it must be robust to problems and able to accomodate mechanical strain as the nuclear envelope changes shape, and to passage large cargo.

A typical yeast cell has xx nuclear pores, each with a dimension of xx. Human cells have about xx pores with xx dimensions.

There are about 30 different types of Nups, all present in multiple copy numbers.

These functions are accomplished through a structure with two main parts, both made of proteins known as nucleoporins, 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. Aside from the NPC itself, transport factors (TFs) are a class of proteins essential for selective transport. The energetic cost of selectivity is captured in the Ran GTP/GDP cycle.

1.2.1 Scaffold nucleoporins are ordered and form ringlike complexes.

The nuclear pore itself is formed of scaffold Nups, which are ordered proteins that form ringlike complexes with eightfold symmetry [5]. There is an inner ring and two outer rings, the nuclear and cytoplasmic rings. The outer rings are slightly larger. The nuclear ring is on the side of the nucleus and includes the nuclear basket. The cytoplasmic ring includes the cytoplasmic filaments, which are (probably?) disordered proteins extending out into the cytoplasm.

The eightfold symmetry of the pore arises from its modular nature. Scaffold Nups form various stable subcomplexes, of which one of the most important is the Y-complex. The Y-complex forms the inner ring; there are 32 copies of the complex per pore [5]. The rings themselves are relatively flexible, as they need to be in order to accommodate deformations of the nuclear envelope. This flexibility is achieved in part by through short linear motifs (SLMs) which connect the subcomplexes to each other.

Recent cryo-EM studies have achieved unprecedented resolution of the scaffold Nups [?, ?].

1.2.2 FG nucleoporins are disordered and fill the central channel of the pore.

The central channel of the pore is filled with disordered FG nucleoporins (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 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 x-x FG repeats, leading to an extremely high density of FG repeats within the pore.

Since the FG Nups are disordered, most conventional visualization techniques do not work. When imaged over time or when several pores are imaged, the averaged results do not show the disordered portion of the FG Nups. Cryo-EM and x-ray crystallography don't work. Techniques such as NMR and very fast AFM can help gain insight into their conformational ensembles [?, ?]. Early research suggested that the FG Nups formed a central plug or "transporter", but more recent work suggests that there is no central structure, just disordered proteins (the AFM study from Lim or Lemke group). 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 [?, ?]. This may contribute to selective transport, although the pore still functioned with all of the asymmetric FG Nups removed. 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 [5].

1.2.3 Energy for selective transport is provided by the Ran cycle.

Selective transport requires an energy source, which in the case of the NPC is provided by the Ran cycle. When a TF-cargo complex passes from the cytoplasm into the nucleus (nuclear import), it encounters a RanGTP on the nuclear side which binds to the TF and displaces the cargo. Then the TF-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 [6].

From the perspective of transport, this means that the process of passing through the pore is itself passive and does not consume energy. The selectivity arises from concentration gradients maintained by the Ran cycle.

1.2.4 Transport factors

Transport factors (TFs) 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. In fact, many TFs have several binding pockets. Likewise, the binding affinity between TFs and FG Nups remains unknown for most TFs. Estimates of dissociation constant K_D vary from nanomolar to millimolar, depending on the environment (cellular, buffer, etc.) in which the measurement is made [?]. There are many types of TF, of which some of the most important are the importins and exportins (karyopherins), NTF2, (and mRNA exporters? CRM? mex67?).

The karyopherins (Kaps) are the most-studied family of TFs. They are also known (in human cells?) as importins and exportins, or collectively as the importin β superfamily [7]. The twenty or so different Kaps are responsible for most nucleocytoplasmic transport [8]. Kaps typically consist of multiple HEAT repeats, a helical motif which conveys structural flexibility [9]. 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 5-7 amino acid tags found on cargo []. However, Kap95? (importin β) uses the adaptor protein Kap60? (importin α) to bind its cargo. In general, Kaps are on the order of 100 kDa in size, well above the passive permeability limit []. Kaps may contribute to the selectivity barrier.

Unlike the karyopherins, nuclear transport factor 2 (NTF2) does not transport a wide variety of cargo across the NPC. Instead, NFT2 is focused on maintaining the Ran gradient needed for transport. It transports RanGDP across the pore - why does this help maintain a gradient? If it transports in both directions, wouldn't it help wash out the gradient? NTF2 is a homodimer whose components are 14 kDa and have one FG binding site. 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 30-150 that of similarly-sized proteins that do not bind to FG Nups.

There are other TFs such as Mex67 and CRM, but I don't really know what they do. Is RNA exported using Kaps or are there other TFs for that?

1.3 Observed features of nucleocytoplasmic transport

The nuclear pore has been the subject of investigation since the 1950s [?]. During that time, many different experimental techniques have been used, from in vivo studies to single molecule fluorescencence to SPR to NMR. Likewise, simulations have attempted to explain the selectivity properties of the NPC using a broad array of techniques, such as more or less coarse-grained molecular dynamics simulations. These studies together shed light on the mechanisms of selective transport, but are also all hindered by the small size and rapid timescale of transport. Many of the studies produce results that seem, at least at first glance, to contradict those of other studies. A comprehensive picture of the facts of nuclear transport has yet to emerge, although there are some universally accepted features.

1.3.1 Flux through the NPC is very high.

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 roughly 1000 transport events per pore per second [10]. Experiments focusing particularly on NTF2 report fluxes between 50 and 250 molecules per pore per second [10–12]. Fluxes this high mean a continuously high occupancy of the NPC, estimated at around 100 karyopherins at once [13]. It is unclear whether the effect of crowding with TFs in the pore has an impact on selectivity.

1.3.2 TFs show millisecond dwell times.

One reason that individual NPCs can accommodate such high flux is the rapidity with which molecules transit the pore. A wide range of molecules, such as NTF2, Importin β , and GFP-NLS cargoes, have a dwell time of less than 10 ms in the pore [?, ?, 7, 14]. Typically, this is determined using single-molecule tracking with superresolution microscopy [7]. There is some evidence that a subpopulation of karyopherins resides in the pore over long timescales, potentially contributing to the selectivity barrier [8].

The flux through an NPC is determined not only by the transit time, but also by the success rate of transit attempts. Single-molecule microscopy suggests that the nuclear import efficiency of Importin β ranges from 50% to 80%, depending on concentration [14]. Modeling supports these numbers [15].

1.3.3 TF-FG Nup binding kinetics are ultrafast.

The short dwell time of transport factors and cargo at the pore contributes to a source of confusion in the NPC community: the binding kinetics of TFs and FG Nups. Measurements of dissociation constant K_D and its components k_{on} and k_{off} ($K_D = k_{\text{off}}/k_{\text{on}}$) span several orders of magnitude, depending on the measurement conditions. Original in vitro measurements, performed in buffer, show low K_D values in the nanomolar range, indicating tight binding between TFs and FG Nups. However, these values were not compatible with a transit time around 10 ms, as a typical binding event as calculated from the off-rate would last much longer than 10 ms.

More recent measurements are converging on a consensus that TF-FG Nup binding kinetics are in fact much faster than previously estimated. Diffusion-limited on-rates and K_D values around 1 mM would permit rapid transit through the pore.

1.3.4 Passive permeability barrier

There is a cutoff around 30 kDa where molecules stop being able to passively transit the pore at any appreciable rate. Work from the Timney lab suggests that the passive permeability barrier

is not a sharp cutoff, but broad.

Dwell times for various TFs and TF-cargo complexes has been studied in many cases, giving a typical dwell time of 5-10 ms, with a small minority of complexes spending much longer in the pore [7].

1.3.5 Effect of transport factors

The presence of transport factors may make the selectivity barrier more robust. SPR measurements as discussed above give estimates of layer compaction or extension, and populations of tight- or weak-binding TFs. Other studies (?) suggest that crowding with TFs might help reduce non-specific interactions and increase the selectivity of transport.

1.3.6 Permeability barrier/flux studies

People have measured the flux through the pore in vitro and in vivo in various ways. One group developed OSTR, where they seal an NPC to the pore of a membrane and measure fluorescence flux of TFs through that pore. The Gorlich group (Ribbeck) have permeabilized cells and injected fluorescent transport factors and watched how long it took them to localize to the nucleus. These measurements show selectivities of 20-150 (ish) fold flux of TFs as compared to inert proteins.

1.4 Theoretical models of the NPC

Many theoretical models exist, both qualitative and quantitative.

1.4.1 Entropic barrier model

The entropic barrier model postulates that Nups remain disordered within the pore (should talk about experimental evidence for and against). In this model, inert proteins are kept out of the pore through an entropic barrier, because their entry into the pore would restrict the possible conformations of the disordered Nups. TFs can get into the pore, in contrast, because the binding

energy offsets the entropic penalty. This is one of the two main qualitative models of the pore, the other being the hydrogel or selective phase model. The Rout lab supports this model. I think most labs other than the Gorlich lab are beginning to support this model, but I'm not sure.

1.4.2 Hydrogel model

The hydrogel model is the other main qualitative NPC model. The Gorlich group supports this model. This model postulates that the Nups interact via their FG motifs (discuss evidence in previous section, maybe?) and form a hydrogel. A dynamic hydrogel. Inert proteins are kept from passing through because they can't get through the gel mesh, but transport factors disrupt the crosslinks by binding to FGs and "melt" through.

1.4.3 Forest/intermediate models

This model proposes that a mix of the entropic barrier model and selective phase model are at play.

1.4.4 Effect of crowding

Some people (Zilman paper, etc) have modeled the effect of crowding on the selectivity barrier. A lot of overlap with effect of transport factors.

1.4.5 Effect of transport factors

Same as previous section sort of. Kap-centric models of the NPC (short for karyopherin-centric, for the class of TFs called karyopherins) propose that a permanent population of Kaps lives within the NPC. This strengthens the selectivity barrier.

1.4.6 Energy landscape models

Most quantitative models of the nuclear pore are energy landscape models. These are typically computational studies that require a fair amount of detail and assumptions about the pore. They

incorporate effects of charge, hydrophobicity, specific binding interactions, etc. Generally the result is a picture of the free energy landscape encountered by TFs and by inert proteins as they travel along the axis of the pore.

1.5 Synthetic NPCs have seen limited success.

Many different groups have attempted to make synthetic nuclear pore complexes, but they are generally not very successful.

1.5.1 Gold nanopores (Rout group)

One of Loren's colleagues in New York grafted FG Nups onto a gold-coated nanopore and monitored flux through the pore. She saw low (less than 10-fold) selectivity. I'm not sure whether other nanopore-based approaches have been tried.

1.5.2 Hydrogels (Gorlich)

The Gorlich group keeps making hydrogels out of Nups and testing the entry of various proteins. They take Nsp1 or fragments of it or other Nups that spontaneously form hydrogels in buffer, and let them form a gel. Then they introduce fluorescently-tagged TFs and inert proteins and monitor the progression of the fluorescent front into the gel. They see very high (100 or more) partition coefficients, indicating that the TFs really bind very strongly to the gel. They do not see rapid exit from the gel as would be required for rapid transport.

1.5.3 Other models (peptide hydrogels, DNA origami, etc)

There are a grab-bag of other models that I need to learn more about. Some groups have done similar things to what we want to do, making hydrogels out of non-aggregating Nup peptides and a pentameric crosslinking domain. There was a group that made an NPC ring out of DNA origami and attached Nups at particular points, though I'm not sure how that can be used to test selectivity because I can't see how to anchor the rings into a membrane.

1.6 Conclusions of introduction?

NPCs are interesting and important.

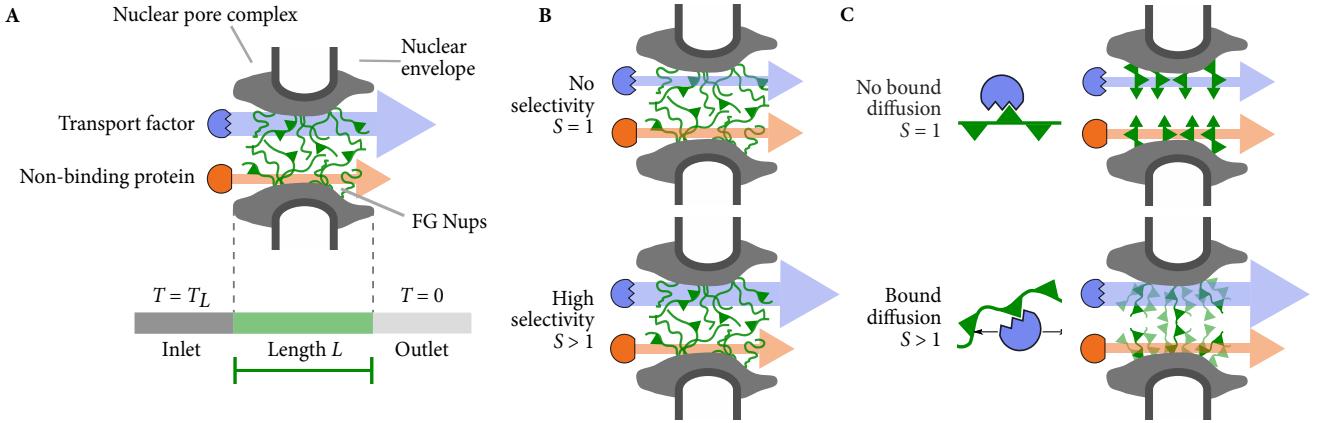


Figure 2.1: Schematics of the nuclear-pore complex and model. (A) The nuclear pore complex (gray) is filled with FG Nups (green polymers) that selectively passageway transport factors that bind to FG Nups (blue) while blocking non-binding proteins (red). The central channel of the pore has length L . Protein concentration is high on the left (inlet) and low on the right (outlet). (B) Selectivity quantifies the degree of selective transport through the pore. A non-selective pore with $S = 1$ has the same flux for a transport factor as for a non-binding protein (top). A selective pore with $S > 1$ has a larger flux for a transport factor than a non-binding protein (lower). (C) The bound diffusion coefficient quantifies the mobility of a bound transport factor. A transport factor may be immobile (top) or mobile (lower) when bound.

Chapter 2

Modeling

Models of the NPC selective barrier have proposed that the FG Nups may form an entropic brush [16], a dynamic hydrogel [10,17], an intermediate state between a brush and gel [18], or liquid droplets [19]. These mechanisms may be modulated by spatial organization [?,20] and binding of TFs to multiple FG repeats [21,22]. Attempts to distinguish these models have been hindered by the pore's small size, the redundancy and multiple copies of FG Nups, and contradictory experimental results on FG Nups and TF binding [18]. Some FG Nup fragments form less-dynamic hydrogels *in vitro* [17], but remain highly dynamic within cells [23]. Molecular dynamics simulations find

highly dynamic FG Nups, though the degree and extent of motion depends on the affinity of FG repeats for each other and for TFs [18, 24]. Crowding and competition modulate affinity [?] and may contribute to selective transport [15]. However, the connection between the amino-acid level behavior of the FG-TF interaction and macroscopic transport selectivity remains unclear. Here we address the central contradiction of selective transport through the NPC: how does binding of TFs to FG Nups within the pore increase the flux rather than decreasing it [25, 26]?

Using a biophysical model, we demonstrate that TF diffusion and binding are sufficient for selective transport, as long as binding only partially immobilizes TFs. Binding increases the local concentration, and these molecules contribute to the flux if mobile. Thermally-driven diffusion of TFs bound to flexible tethers gives sufficient particle mobility to produce selectivity similar to experimental measurements. Tether flexibility also allows bound TFs to hop between tethers, further enhancing selectivity.

2.1 Biophysical model of transport through the NPC

We consider a minimal model of the central channel of the NPC containing FG Nups homogeneously anchored Figure 2.1. This model is sufficiently general to describe the common features of a range of biopolymer filters. The NPC, unlike most other biopolymer filters, has a wide capture area that may increase transport rates [27]. In order to focus on basic principles of transport, we neglect this effect. A varying free energy landscape along the axis of the NPC may play a role in selective transport [15, 28–30]. However, the NPC is robust to deletion of all asymmetric Nups and many Nup combinations, indicating that spatial variation in pore properties is not necessary [31, 32]. Experiments *in vitro* with simplified, homogeneous Nup composition produced selective transport [?, 33].

Rapid transport requires TF-FG Nup binding, while a protein similar to a TF but unable to bind FGs is excluded. Therefore, in our model we compare two proteins that are identical, except that one binds FG Nups and the other does not. As a model TF, we consider nuclear transport factor 2 (NTF2) [34]. NTF2 is small (~ 5 nm) relative to the diameter (~ 50 nm) and length

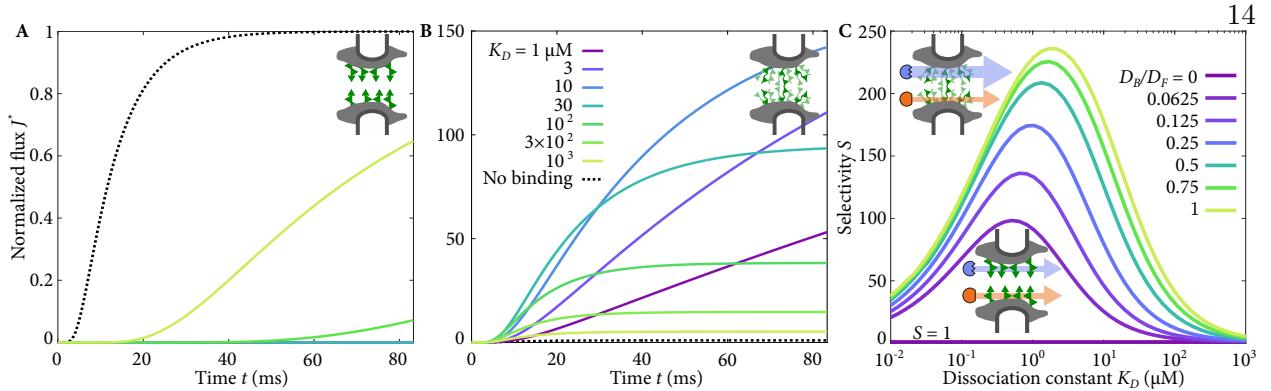


Figure 2.2: Flux through the pore and selectivity for TFs with varying bound mobility. (A) Flux as a function of time when TFs are immobile while bound, with varying binding affinity as in (B). (B) Flux as a function of time when TFs are mobile while bound with $D_B = D_F$, with varying binding affinity. (C) Selectivity as a function of dissociation constant with varying bound diffusion coefficient.

of the pore (~ 100 nm), suggesting that passage of NTF2 does not require large-scale molecular rearrangements that have been proposed for larger molecules [?, 21]. Because of the small size of NTF2 we neglect effects of steric crowding, which can enhance selectivity in a transport model [15]. NTF2 appears not to be actively released from the pore, suggesting that selective transport is an intrinsic property of the NPC [15,35], and in contrast to actively released karyopherins [?, 21,35,36].

Transport through the NPC requires entry into the pore, passage, and exit. In single-molecule measurements, most of the transport time is spent in a random walk within the central channel [?, 29]. We therefore assume that entry and exit rates are determined by binding kinetics (see Supporting Information, section ?? for the model when entry and exit are rate-limiting.) The directional bias in TF transport is controlled outside the NPC through a concentration difference between the nucleus and cytoplasm generated by the Ran-GTP system [?]. In our model, we impose a fixed concentration difference across the pore.

We consider a channel of length L filled homogeneously with Nups that separates two reservoirs Figure 2.1A. Within the channel are free transport factor (concentration T), free FG Nups (N), and bound TF-FG complex (C), with total Nup concentration $N_t = N + C$. TF diffusion

within the channel ($0 < x < L$) is described by the reaction-diffusion equations

$$\frac{\partial T}{\partial t} = -k_{\text{on}}TN + k_{\text{off}}C + D_F \frac{\partial^2 T}{\partial x^2}, \quad (2.1)$$

$$\frac{\partial C}{\partial t} = k_{\text{on}}TN - k_{\text{off}}C + D_B \frac{\partial^2 C}{\partial x^2}. \quad (2.2)$$

TF-FG interaction has on-rate constant k_{on} , off-rate k_{off} , and dissociation constant $K_D = k_{\text{off}}/k_{\text{on}}$. We include competition between TFs for FG binding sites [30]. The diffusion constants of free (D_F) and bound (D_B) TFs are spatially constant. The fixed reservoir TF concentrations are T_L (inlet, left) and 0 (outlet, right).

The flux of transport factor out of the pore $J = -D_F \partial T / \partial x|_{x=L}$. We numerically integrated the full equations. Because flux measured in experiments is typically linearly proportional to TF concentration [19, 37], TF concentration likely remains below binding saturation in the NPC. Therefore, we also solved eqns. (2.1, 2.2) analytically in the low binding limit. We define the transport selectivity S as the ratio of steady-state flux of a binding versus a non-binding species

Figure 2.1B

$$S = \frac{J_{\text{binding}}(t \rightarrow \infty)}{J_{\text{non-binding}}(t \rightarrow \infty)}. \quad (2.3)$$

2.1.1 No selective transport occurs if bound TFs are immobile

If TF-FG Nup binding immobilizes the TF, the bound-state diffusion coefficient $D_B = 0$. For immobile bound TFs, transport is not selective: the steady-state flux $J = D_F T_L / L$ for both binding and non-binding proteins, so $S = 1$ (figs. 2.2, ??). The binding TF accumulates within the pore, but its immobility means it does not enhance transport compared to the non-binding case. Notably, this effect is independent of binding kinetics. Prior to steady state, binding slows transport Figure 2.2A. In systems such as airway mucus, immobilization may increase the time available for degradation or active clearance, consistent with the observation that binding tends to inhibit selective transport in those systems [?, ?, ?]. This effect is related to the binding-site barrier seen in antibody delivery to tumors [?], and observations that non-binding nanoparticles are often more effective in drug delivery to tumors than binding particles [26].

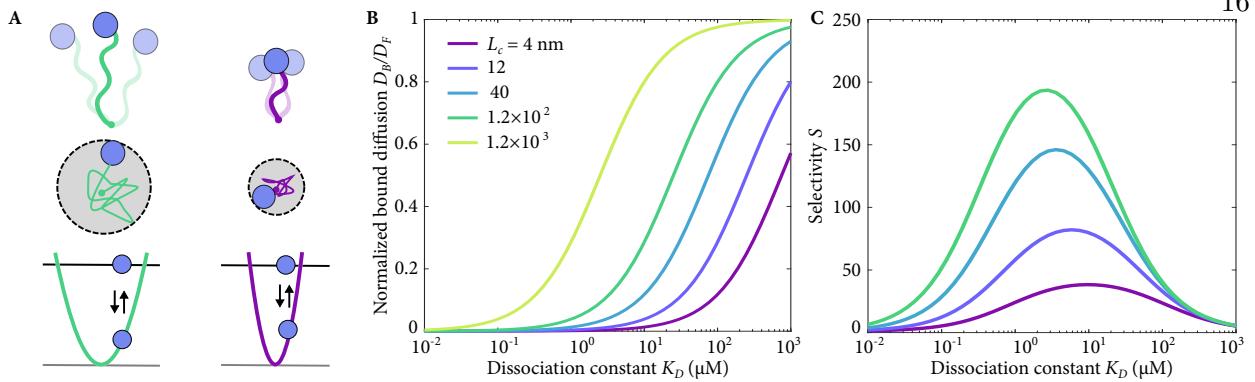


Figure 2.3: (A) Schematic of the flexible tether model of bound-state diffusion. FG Nups are treated as entropic springs that constrain the motion of TFs more (top and center left, longer FG Nup) or less (top and center right, shorter Nup), which corresponds to changing width of the harmonic potential well (lower). (B) Ratio of bound to free diffusion coefficient as a function of dissociation constant, with varying polymer length in the tethered-diffusion model. (C) Selectivity as a function of K_D , with varying polymer length in the tethered-diffusion model.

Our model is related to the classic problem of molecular transport through an oil membrane separating two aqueous reservoirs [?]. The relative concentration of a species just inside the oil barrier to the concentration in water is called the partition coefficient. The steady-state flux through the membrane is directly proportional to the partition coefficient (Supporting Information, section ??, fig. ??). By analogy, one might expect the TF-FG binding affinity to determine the flux across the pore. However, binding is different from partitioning. In systems where the increase in intra-pore concentration arises from binding, the effective diffusion coefficient is typically inversely proportional to the partition coefficient, making the flux independent of binding affinity [25]. This result led us to consider whether TFs may be mobile while bound to FG Nups.

2.1.2 Bound-state diffusion allows selective transport

When bound TFs are mobile, selective transport occurs with a selectivity up to 240 for a conservative set of parameters (figs. 2.2B,C, ??, Supporting Information, section ??). Remarkably, this selectivity is comparable to experimental measurements of NTF2 versus GFP flux (Table 2.1). The interplay between binding kinetics and diffusion leads to an optimal dissociation constant $\sim 1 \mu\text{M}$ for maximum selectivity Figure 2.2C. Selectivity decreases for high K_D because binding is too

weak to significantly increase TF concentration in the pore. For low K_D , tight binding causes the concentration of bound complexes to become approximately constant across the pore. Because diffusive flux is driven by a concentration gradient, this washing out of the gradient by tight binding decreases flux and selectivity.

Our model predicts that selectivity is increased by increasing binding on-rate constant k_{on} Figure ???. Consistent with this, the on-rate constants of TF-FG Nup interactions have been measured to be diffusion limited [23, 38]. Large k_{on} makes transport more selective because fast binding kinetics relative to diffusive motion are necessary to maintain steep concentration gradients within the pore. High FG Nup concentration (as measured experimentally) leads to large N_t and low D_F , both of which increase selectivity. Decreasing D_F or increasing the length of the pore both reduce the magnitude of the flux and increase selectivity (figs. ??, ??). Therefore, varying TF free diffusion coefficient and pore length involves a trade-off between transit time and selectivity.

2.2 Mechanisms of bound transport factor mobility

Our result that bound-state diffusion is required for selective transport raises a mechanistic question: how can TFs move while bound to FG Nups? Here we consider two experimentally based mechanisms: movement of the bound TF due to the intrinsic flexibility of the FG Nups [?] and

Table 2.1: Comparison between experimental results for NTF2 and GFP (a similarly-sized non-binding protein) and model predictions. Flux measured in units of molecules per pore per second.

Method	Cell type	Species	Flux	Selectivity	Notes
OSTR	<i>Xenopus</i>	NTF2	91–123	24–37	[11]
		GFP	3.3–3.8		
OSTR	<i>Xenopus</i>	NTF2	47.3	43	[12]
		GFP	1.1		
Permeabilized cells	HeLa	NTF2	250	125	[10]
		GFP	2		
Model	–	Binding	2–480	1–240	This work
		Non-binding	2		

multivalent binding that allows hopping of TFs between neighboring Nups [39].

2.2.1 FG Nup flexibility allows tethered diffusion

Previous measurements have found that FG Nups are flexible and dynamic [23, 40, 41]. One end of an FG Nup is attached to the inner ring of the NPC scaffold. However, the Nup's flexibility means that a TF which binds far from the scaffold-attachment point is mobile. Tethered diffusion therefore provides a mechanism of bound-state diffusion and selectivity.

2.2.1.1 Bound-state diffusion depends on polymer and kinetic properties.

Flexible polymers behave as entropic springs [42] if they are not highly stretched. Therefore, a bound TF diffuses while attached to a spring-like tether, which can be represented as diffusion in a harmonic potential well Figure 2.3A. The width of the harmonic well is related to the length of the flexible domain. The effective length is the full FG Nup length if the FG Nups are not crosslinked, while the effective length is reduced if they are crosslinked or entangled [10].

In order to calculate the bound diffusion coefficient of the TFs, an averaging procedure is followed. First, the diffusion is assumed to be Fickian, which is a reasonably good though not perfect assumption (see section on Fickian diffusion). In the Fickian diffusion case, the diffusion coefficient is proportional to a mean-squared displacement (MSD) divided by time. We calculate the mean binding lifetime τ and the MSD corresponding to this “typical” binding event and divide them.

To begin, note that the duration of a binding event follows the exponential distribution

$$\rho(t) = \exp(-t/\tau)/\tau, \quad (2.4)$$

where $\tau = 1/k_{\text{off}}$ is the mean binding lifetime.

Next, the positional probability density of a bound TF is

$$P(x,t) = e^{-\frac{x^2}{2\alpha(t)}} / \sqrt{2\pi\alpha(t)}, \quad (2.5)$$

$$\alpha(t) = (1 - e^{-2kD_F\beta t})/(k\beta) \quad (2.6)$$

where k is the spring constant of FG Nup tethering and $1/\beta = k_B T$ is the thermal energy [?]. The center of the well is set at $x = 0$.

The mean-squared displacement (MSD) of the TF as a function of time is calculated, as any expected value, with the integral

$$\langle x^2(t) \rangle = \int_{-\infty}^{\infty} P(x, t) x^2 dx = \alpha(t). \quad (2.7)$$

Finally, the typical TF MSD during a binding event can be determined by evaluating

$$\overline{\langle x^2 \rangle} = \int_0^{\infty} \rho(t') \langle x^2(t') \rangle dt' = \frac{2D_F L_c \ell_p}{L_c \ell_p k_{\text{off}} + 3D_F}. \quad (2.8)$$

Here we assume that the spring constant is that of a worm-like chain polymer $k = 3/(2\beta L_c \ell_p)$, where L_c is the contour length and ℓ_p the persistence length [42].

Combining these results, the one-dimensional bound diffusion coefficient is

$$D_B \approx \frac{\overline{\langle x^2 \rangle}}{2\tau} = \frac{D_F L_c \ell_p k_{\text{off}}}{L_c \ell_p k_{\text{off}} + 3D_F} = \frac{D_F}{1 + 3\frac{D_F}{D_P}}. \quad (2.9)$$

Here $D_P = L_c \ell_p k_{\text{off}}$ controls the bound-state diffusion coefficient: higher D_P corresponds to a lower constraint of the TF by the tether and greater bound mobility. Bound mobility increases with increasing chain length and persistence length, or decreasing binding lifetime. When D_P is large ($D_F/D_P \ll 1$), D_B approaches D_F , since the long chains barely affect TF motion during the short binding event. For small D_P ($D_F/D_P \gg 1$), TF motion is inhibited by a short tether, giving $D_B \approx D_P/3 \ll D_F$. This result highlights that the kinetics of TF-FG Nup interaction are a primary determinant of the bound mobility: the faster the binding kinetics, the higher the bound diffusion constant.

Flexible disordered proteins typically have low persistence lengths [43], so we estimate $\ell_p \approx 1$ nm. If the on-rate constant is diffusion limited, $k_{\text{on}} = 10^{-3} \mu\text{M}^{-1} \mu\text{s}^{-1}$ [23, 38], the binding affinity determines the off rate. Disordered FG Nups have $L_c \approx 100\text{--}280$ nm (250–700 amino acids long [?] with a contour length per amino acid ≈ 0.4 nm). For our conservative parameters, tethered diffusion alone predicts selectivity $S \approx 200$, a large flux enhancement for TFs over nonbinding proteins.

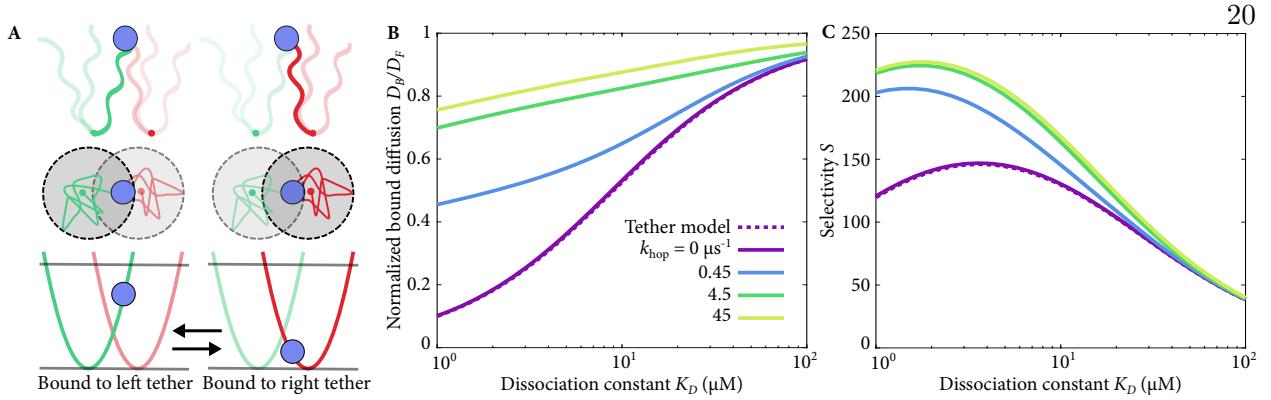


Figure 2.4: (A) Schematic of the inter-chain hopping model of bound-state diffusion. FG Nups are treated as entropic springs that constrain the motion of TFs, and inter-chain hopping allows a TF to move from one FG Nup (top and center left, green Nup) to another (top and center right, red Nup) without unbinding, which corresponds to switching from one harmonic potential well to another (lower). (B) Ratio of bound to free diffusion coefficient as a function of dissociation constant, with varying hopping rate in the inter-chain hopping model. (C) Selectivity as a function of K_D with varying hopping rate. FG Nup contour length $L_c = 40 \text{ nm}$ in (B, C).

2.2.2 Inter-chain hopping increases selectivity

The tethered diffusion mechanism is constrained by a trade-off: tighter binding increases the TF concentration in the pore, but hinders motion. Multivalent TF-FG interactions can relax this constraint, because a TF can bind simultaneously to more than one FG Nup, moving hand-over-hand while remaining bound [44]. Consistent with this, TFs may slide between nearby FG sites rather than fully unbinding and re-binding [39]. If the newly-bound FG repeat is on a neighboring chain, the FG tether site that constrains TF motion moves while the TF remains bound. We model inter-chain hopping with a TF that undergoes tethered diffusion when bound to an FG Nup and hops between neighboring, randomly distributed tethers Figure 2.4. Numerical simulations of this model determined the bound diffusion coefficient (fig. 2.5, Supporting Information, section ??). We note that intra-chain hopping does not change the flux, since the anchor point of the tethering chain is not changed; therefore we neglect it.

2.2.3 Detailed explanation of hopping simulation

In our simulation of TF motion with hopping between FG Nups while bound, we represented each FG Nup as an entropic spring (i.e. as a harmonic potential well). Well positions were randomly chosen from a uniform distribution, with the exception that we always placed one well at the starting position of the TF. The particle (the TF) started the simulation bound to this FG Nup, and remained bound throughout the simulation. While bound to one FG Nup, the TF diffused within the harmonic well representing that FG Nup. We recorded the position and mean-squared displacement of the TF from its starting location, which we then used to determine a bound diffusion coefficient, as described in more detail below. The TF could hop between tethers by changing which well it moved in.

2.2.3.1 Diffusion in a potential well

The TF moved in the harmonic potential of the FG Nup according to Brownian dynamics. At each timestep, the TF position was updated using a force-dependent diffusive step [45].

$$x(t + \delta t) = x(t) + \frac{F}{\Gamma} \delta t + \delta x, \quad (2.10)$$

where F is the force acting on the particle, Γ is the drag coefficient, δt is the timestep, and δx is a random Brownian step drawn from a Gaussian distribution with variance $\sigma^2 = 2D\delta t$. The drag coefficient of a spherical particle at low Reynolds number is given by Stokes' Law as $\Gamma = 6\pi\eta r$, where η is the fluid's viscosity and r is the sphere's radius. This result can be combined with the Einstein relation $D = k_B T / (6\pi\eta r)$ to give

$$\Gamma = \frac{k_B T}{D}. \quad (2.11)$$

The force $F = -k\Delta x$, where k is the spring constant of the FG Nup and Δx is the displacement of the particle from the Nup attachment point. We model the FG Nup as a worm-like-chain at small extension, so that $k = 3k_B T / (2\ell_p L_c)$, where ℓ_p is the tether persistence length and L_c is

the contour length. Then

$$x(t + \delta t) = x(t) - \frac{3D\Delta x\delta t}{2\ell_p L_c} + \delta x = x(t) - DK\Delta x\delta t + \delta x, \quad (2.12)$$

where K is the normalized spring constant $K = k/k_B T = 3/(2\ell_p L_c)$.

2.2.3.2 Hopping probability

We designed the hopping probability P_{hop} in order to satisfy the principle of detailed balance. During every iteration of the simulation, we picked an FG Nup at random from a list of the M Nups near enough to have a reasonable probability of hopping. TF hopping to the new FG Nup was attempted with success probability

$$P_{\text{hop}} = r_{\text{hop}} M \delta t e^{-\Delta G/2}. \quad (2.13)$$

Here the base hopping rate r_{hop} is a dimensionless input parameter, and the change in free energy (in units of $k_B T$) between the current Nup and the proposed new Nup is

$$\Delta G = \frac{1}{2} K(x - x_{\text{new}})^2 - \frac{1}{2} K(x - x_{\text{cur}})^2, \quad (2.14)$$

where K is the normalized spring constant, x is the particle's current position, x_{cur} is the anchor location of the Nup to which the particle is currently bound, and x_{new} is the anchor location of the proposed new Nup. Note that when a hop succeeds, the energy landscape changes to that of the new Nup, but the TF's position does not change during the hop. There is no upper bound on P_{hop} , but we adjusted the timestep to ensure that P_{hop} was greater than unity no more than 0.5% of the time that a hop was attempted.

2.2.3.3 Mean-squared displacement and diffusion coefficient calculation

We ran each simulation for 10^7 time steps with $\delta t = 0.01 \mu\text{s}$, and recorded the particle's position every 100 time steps. We calculated the mean-squared displacement $\langle x^2 \rangle$ (MSD) of the TF and averaged it over 100 runs Figure 2.5A. We then computed

$$\rho_{\text{MSD}}(t) = \langle x^2(t) \rangle \rho(k_{\text{off}}, t) = k_{\text{off}} \langle x^2(t) \rangle e^{-k_{\text{off}} t}, \quad (2.15)$$

as shown in fig. 2.5B, and numerically integrated the distribution in time. We determined the bound diffusion coefficient from the typical MSD-per-binding-event $\overline{\langle x^2 \rangle}$ using

$$D_B = \frac{k_{\text{off}} \overline{\langle x^2 \rangle}}{2}. \quad (2.16)$$

Here, the factor of 1/2 is appropriate because we consider a one-dimensional random walk.

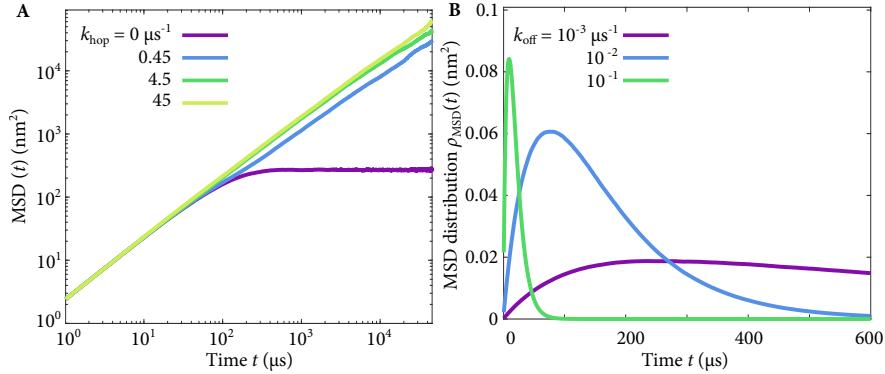


Figure 2.5: (A) Examples of mean-squared displacement (MSD) of a simulated TF in the inter-chain hopping model, with varying hopping rate. (B) Examples of MSD distributions $\rho_{\text{MSD}}(t)$ used in estimating the diffusion coefficient, with varying unbinding rate. Tethers have 40 nm contour length; other parameters are as discussed in the text.

2.2.4 Hopping simulation results

Inter-chain hopping increases selectivity most for tight binding and short chains, the parameter regime where tethered diffusion gives limited selectivity (figs. 2.4, 2.6, 2.7). Hopping may therefore be important for FGs that form transient crosslinks: if FG Nups are highly crosslinked, our model suggests that inter-chain hopping is the key mechanism of TF movement. For weaker binding and longer chains, inter-chain hopping leads to a modest increase in selectivity.

2.2.5 Fickian diffusion

Talk about how diffusion is almost but not quite Fickian, and show the anomalous diffusion plots (made with Mike's script) for various parameters. Talk about timescales and how this might affect the results.

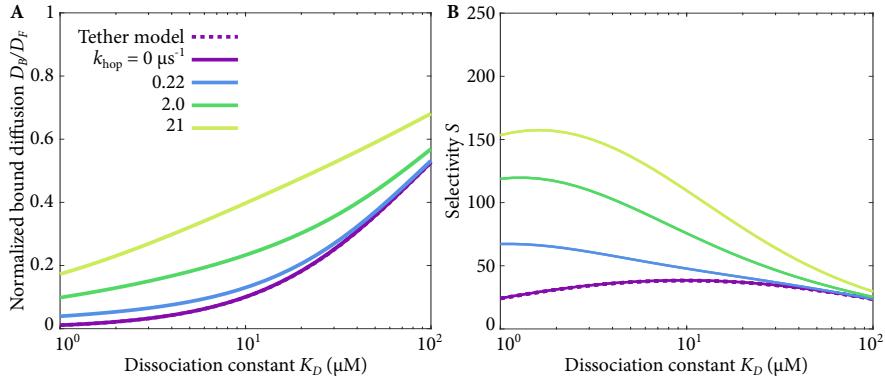


Figure 2.6: Bound diffusion and selectivity as a function of dissociation constant, with varying hopping rate for FG Nups with $L_c = 4$ nm.

2.3 Discussion

A key puzzle of the NPC is how transport-factor binding allows rapid transport through the pore. Binding typically immobilizes the bound particle, and so the increase in concentration resulting from binding does not, in general, result in increased flux. The biophysical theory we developed includes diffusion of TFs due to thermal fluctuations, binding to polymeric tethers, and the hopping of bound species between these tethers. Thus we identified principles of selective transport resulting from binding Figure 2.1, emphasizing that bound-state mobility is essential for selective transport Figure 2.2. Binding increases the local concentration, and any bound mobility increases the flux. We characterized two mechanisms to obtain bound-state mobility and found that thermally-driven diffusion of TFs bound to flexible tethers and rapid binding kinetics [23,38] allow TF mobility, leading to selectivity similar to that observed experimentally Figure 2.3. In addition, tether flexibility enables multivalent bound particles to hop between binding regions Figure 2.4 [21,22], further enhancing selectivity. Mobility of bound or partitioned molecules occurs in many biological contexts, suggesting that the mechanisms we study here may be broadly applicable [?,46].

Our model for selective transport by tethered diffusion generalizes to a range of FG-FG interactions [18], if we decrease the effective chain length L_c for cohesive FG Nups. For short chains the selectivity simply due to chain flexibility is modest, suggesting that other mechanisms, like hopping, may be important. Our model suggests that transient cross-linking of FG repeats

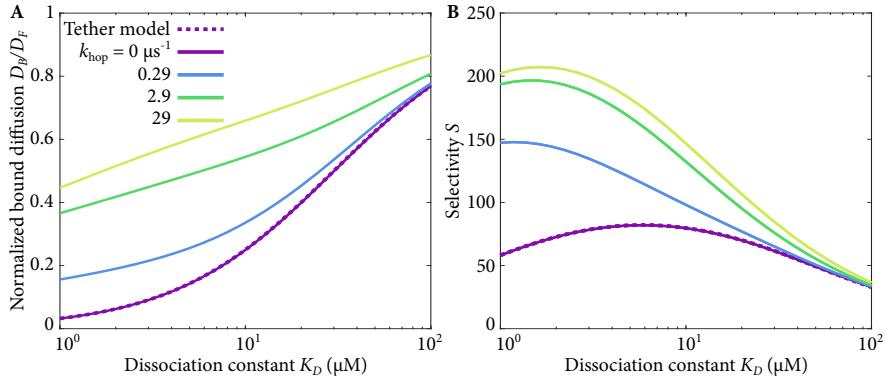


Figure 2.7: Bound diffusion and selectivity as a function of dissociation constant, with varying hopping rate for FG Nups with $L_c = 12$ nm.

proposed to occur within the pore may serve to increase the viscosity and therefore the selectivity.

Crosslinks need not be actively melted by TFs to enhance selectivity Figure ??.

Our model provides a quantitative tool to evaluate selective transport. Materials formed *in vitro* by spontaneous self-assembly of FG Nups [17] or transient crosslinking by alpha-helical peptides [47] show strong selective *entry*. Using published data, we predicted whether these gels also showed selective *transport* (table ??). Most synthetic gels are predicted to have $S < 10$, less than the selectivity of NTF2 in cells (table 2.1). The predicted selectivity of one hydrogel is $S \approx 200$, apparently the most selective synthetic gel to date [17].

2.3.1 Overcoming the limitations of binding

Binding, even in the presence of bound-state motion, limits selectivity. Biological systems appear to have developed strategies to avoid this, for example, by using true partitioning. Lipid domains in complex membranes partition proteins [?]. Membraneless organelles spontaneously assembled from low-complexity proteins and nucleic acids can localize a molecule without immobilizing it [?]. Because membraneless organelles are fluid, the constraints imposed in our NPC model by binding are released. Our work thereby suggests a benefit of phase-separated droplets to cells: they provide significantly higher selectivity than can occur with immobilizing binding. This may be especially important for spatially complex assemblies [?].

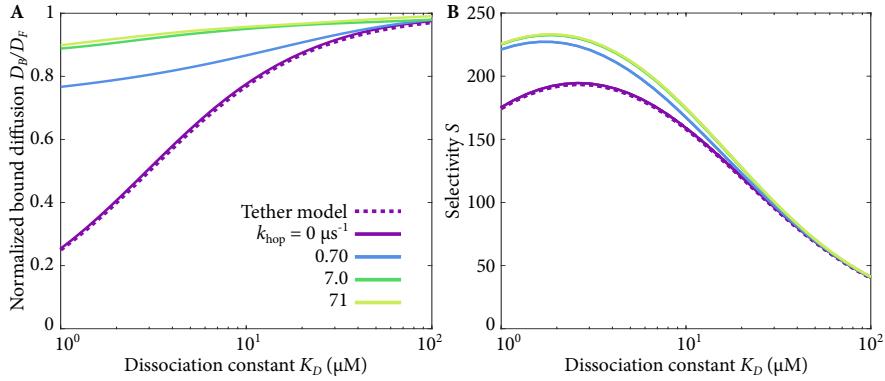


Figure 2.8: Bound diffusion and selectivity as a function of dissociation constant, with varying hopping rate for FG Nups with $L_c = 120$ nm.

Though we show it is not necessary, the active dissolution of polymeric biomaterials has been proposed to occur in the NPC [10]. This strategy is used by *Helicobacter pylori* to penetrate the gastric mucus [?]. Because the particularly dense extracellular matrix of solid tumors blocks the motion of particles, especially larger nanoparticles, ECM dissolution has been used to enhance drug delivery [48]. Unfortunately, this approach may not be universally applicable: breaking down the ECM surrounding tumors may promote cancer metastasis [?].

2.3.2 Design principles of selective transport by binding

Filtering by polymeric biomaterials occurs in many systems for particles of different sizes: for example, nutrients reach our intestinal walls while larger molecules are excluded. However, controlling the selective transport of similarly-sized molecules by tuning specific interactions has proven elusive. In drug delivery applications, inert nanoparticles are typically more effective at penetrating extracellular spaces and reaching their cellular targets [26]. Because biopolymer filters are the first point of contact of nanoparticles used for drug delivery, specific targeting of transport through mucus may enhance the effectiveness of drug delivery. If NPC-like bound mobility as described in our model could be achieved in these systems, it would increase the rates of transport and drug delivery.

Chapter 3

Hydrogel design for protein separation

In order to test the predictions of our bound-state diffusion model of selectivity, we needed to develop a biomaterial that could support bound diffusion and be used to test selectivity. There are several biological examples which show selectivity that could be explained by bound-state diffusion, including nucleocytoplasmic transport, liquid-liquid phase separated droplets, diffusion of DNA-damage-repair proteins in the nucleus, and passage through mucus [26,48–50]. We decided to focus on making a material that mimics the environment of the nuclear pore.

One of the attractions of the bound-state diffusion model is that it does not rely on the geometry of the selective material. As long as the dimensions of the material are significantly larger than the protein diffusing through it, there is no practical difference between a nanoscale cylindrical pore and a macroscale bulk material. For ease of fabrication and testing, we opted to design a bulk material for protein separation.

A hydrogel substrate was chosen as the basis of the nuclear pore mimic and protein fragments taken from FG Nups anchored into it. Hydrogels are versatile materials, many of which are biocompatible, many of whose properties can be easily tuned . We used a hydrogel substrate to provide an inert scaffold to which fragments of FG Nups were conjugated. These Nups were conjugated to the hydrogel at one end and free at the other, mimicking the arrangement found in the nuclear pore and providing a mechanism of tethered bound diffusion. The properties of these Nups, such as their length, number of transport factor binding motifs, and propensity towards aggregation, can be easily varied in order to test how these properties impact bound-state diffusion and thus

protein selectivity.

This hydrogel model is therefore the simplest way to make a material to explore the effects of bound diffusion on protein separation. While it is inspired by the nuclear pore, the goal is not to directly reproduce the mechanism of nucleocytoplasmic transport. Instead, we hoped to show that bound-state diffusion was measurable and controllable in a biomaterial, paving the way for molecular filters that might make use of this principle.

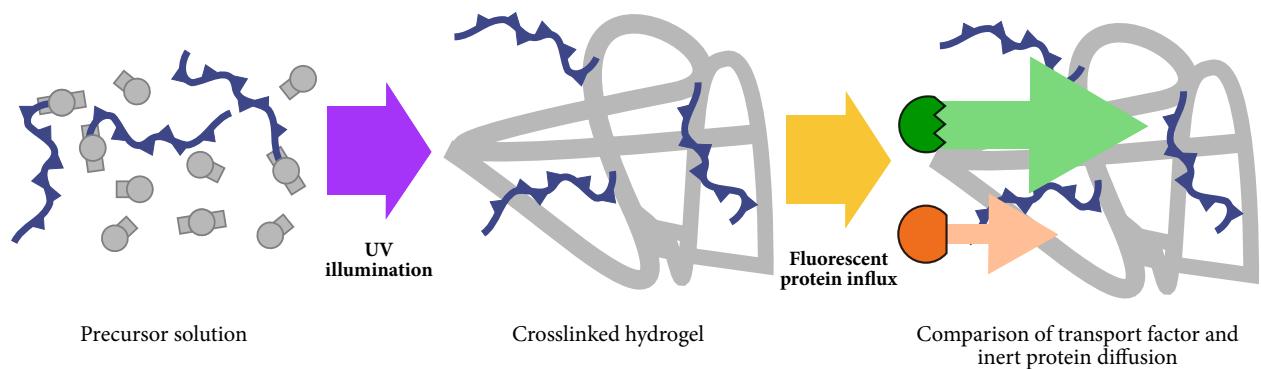
Over the course of making these materials, we ran into several significant roadblocks. Although hydrogels with a wide range of properties have been well-studied, gels suitable for protein separation are harder to come by. They require an intermediate pore size, large enough that the diffusion of a protein is not significantly hindered by the presence of the hydrogel meshwork, but small enough that the Nup peptides can easily reach the protein as it diffuses. This average pore size of 5-20 nm is difficult to find, though much smaller and much larger pores are easy to make. The problem of pore size is made more difficult by the need to create a well-sealed hydrogel barrier in order to separate proteins; such a barrier must necessarily be confined and therefore unable to swell to its equilibrium size. The creation of a hydrogel for selective protein separation poses many stringent requirements, often in competition with each other. This chapter describes the setbacks we faced and the progress we have made towards creating a hydrogel-based biomaterial suitable for selective separation of proteins.

3.1 Hydrogel fabrication

A wide range of hydrogels systems have been well-studied. Many are not suitable for the addition of proteins, but both PEG and acrylamide hydrogels, among others, can be crosslinked in an aqueous solution that does not harm proteins. We used both PEG and acrylamide hydrogels in our diffusion experiments. Thanks to Stephanie Bryant, Sadhana Sharma, Christopher Bowman, Danielle Konetski, and Benjamin Fairbanks for their help as we learned to make these hydrogels.

Whether using PEG or acrylamide, the basic hydrogel fabrication procedure was the same (Fig. 3.1). First, a precursor solution was mixed, which contained the hydrogel monomer, a

Figure 3.1: Overview of hydrogel fabrication and fluorescent protein influx. A precursor solution is mixed, containing hydrogel monomers, crosslinkers, a photoinitiator, and the Nup fragment. Upon exposure to UV light, the precursor solution crosslinks, and one end of each Nup is conjugated to the hydrogel. The diffusion of a fluorescently-tagged transport factor can then be compared to that of a similarly-sized inert fluorescent protein.



crosslinker, a radical generator, and the Nup fragment, labeled with a reactive group at one end that would conjugate it to the hydrogel. The radical generator was almost always a photoinitiator, which caused the precursor solution to crosslink when exposed to UV illumination. In a few cases, a chemical initiator was used, in which case the precursor solution crosslinked 10-30 minutes after mixing. By the end of the crosslinking process, the hydrogel contained Nup fragments tethered to the gel at one end, mimicking the arrangement of Nups in the nuclear pore and providing a mechanism of tethered bound diffusion for transport factors but not for non-binding inert proteins. The diffusion of both types of protein within the hydrogel could then be quantified using fluorescence microscopy. Detailed protocols and precursor solution recipes are reported in Appendix D.

There were four major components to the precursor solution: monomers, crosslinkers, initiators, and Nup fragments. To an extent, these components can be chosen independently of each other. Bisacrylamide or PEG-diacrylate crosslinkers must be used with acrylamide monomers, and PEG-dithiol crosslinkers with PEG-norbornene monomers, but the initiators and Nup fragments can be varied. There are many variations on crosslinker length as well, leading to a wide variety of possible hydrogels even using a relatively small set of components.

The PEG hydrogels made use of 20-kD 8-armed PEG-norbornene monomers (synthesized by the Bryant lab and Nathan Crossette) and either a 1-kD or 8-kD PEG-dithiol crosslinker (Sigma) (Fig. 3.2). To conjugate the Nup fragment to the PEG hydrogels, a C-terminal cysteine was added to the peptide. Both the crosslinker and Nup fragment made use of Michael-thiol “click” chemistry [51] [52].

The acrylamide hydrogels used an acrylamide monomer and bisacrylamide crosslinker (Bio-Rad) (Fig. 3.2). An additional step was needed to prepare the Nup fragment for tethering to the hydrogel: either bisacrylamide or 700-Da PEG-diacrylate (Sigma) was conjugated to the terminal cysteine (see Appendix C).

Regardless of the monomers and crosslinkers used, a radical generator is needed to initiate polymerization. We used either a photoinitiator, activated by UV light, or a chemical initiator system which began polymerization upon its addition to the precursor solution. Both systems have

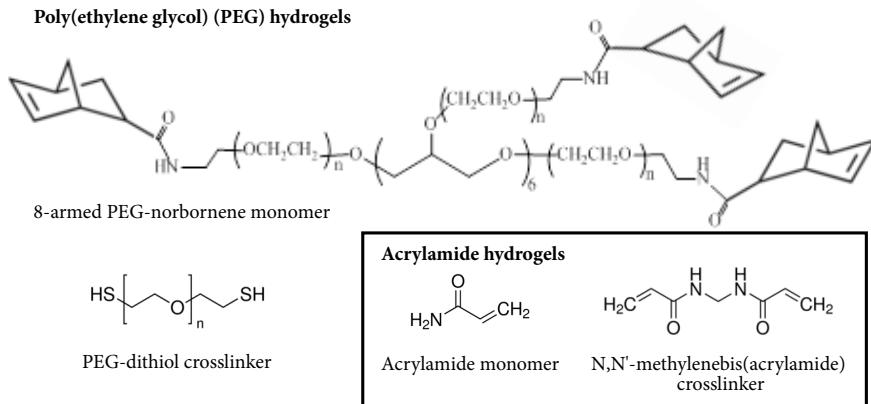


Figure 3.2: Chemical structures of monomers and crosslinkers for PEG and acrylamide hydrogels. The total molecular weight of the 8-armed PEG-norbornene was 20 kD, and either a 1-kD or 8-kD PEG dithiol crosslinker was used. Structures from Sigma and CreativePEG-Works.

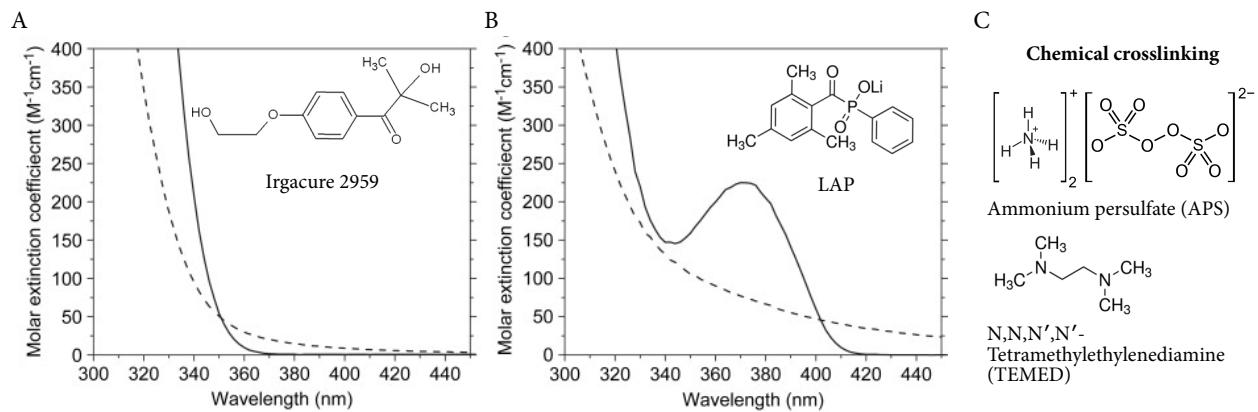
advantages: Photoinitiators are useful for patterned polymerization using photomasks and allow the precursor to be mixed prior to polymerization, while chemical crosslinkers do not require careful protection from light. We nearly always used a photoinitiator.

Two photoinitiators were tested: 1-[4-(2-hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propane-1-one (commercially known as Irgacure 2959) and lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP). Figure 3.3 (A) and (B) shows the absorption spectra of each photoinitiator and that of its cleavage products [1]. LAP has an absorbance peak in the near-IR / violet range, is highly water-soluble, and is more effective at crosslinking, so it was used most of the time. However, due to its absorbance into the visible range, care must be taken to protect solutions containing LAP from ambient light wherever possible. Our LAP was synthesized by the Bryant lab, but it is now commercially available from Sigma as well.

An APS/TEMED chemical crosslinking system was occasionally used in place of the photoinitiator. Large numbers of hydrogels can be polymerized at once in this way, without the need for a large area of uniform UV illumination. Figure 3.3 (C) gives the chemical structure of ammonium persulfate (APS) and N,N,N,N-tetramethylethylenediamine (TEMED). APS is the radical generator, and TEMED accelerates the radical formation. The time to polymerize can be controlled by adjusting the concentration of both components (Appendix D).

It should be noted that the presence of oxygen inhibits all the initiators discussed above. Precursor solutions were degassed for 10 minutes before use in a vacuum desiccator and polymerized

Figure 3.3: Absorption spectra of (A) Irgacure 2959 and (B) LAP (solid lines) and with that of their cleavage products (dotted lines) [1]. (C) Chemical structures of the APS/TEMED crosslinking system. Structures from Sigma.



no more than 10 minutes after degassing.

When a photoinitiator was used, the hydrogels were polymerized using UV illumination at either 365 nm (ThorLabs M365LP1 mounted LED) or 405 nm (laser on Nikon A1R confocal microscope) inside flow chambers as described in Sec. 3.2. The typical intensity of the LED was 220 mW/cm² and the typical crosslinking time 30 s. Depending on the situation, photomasks were used to selectively expose regions of precursor to UV, or entire droplets of precursor solution were polymerized in an otherwise-empty chamber (Fig. 3.4). After crosslinking, hydrogels were rinsed with 10-100 times their volume with buffer and allowed to soak in fresh buffer solution overnight at 4°C in order to approach swelling equilibrium and remove any remaining precursor solution.

Following the buffer soak, typically a fluorescent solution of proteins was added to the gels. Usually this consisted of a transport factor (typically NTF2) and a similarly-sized inert protein (typically the red fluorescent protein mCherry). A typical experiment consisted of a video at 4x or 10x magnification of the hydrogel, reservoir chamber, and (if applicable) an outlet/inner reservoir which slowly accumulated protein as it passed through the gel. Experiments ranged from 1-24 hours, with 2 hours being the most common. Typical data produced was a plot of accumulation in the inner reservoir or hydrogel over the course of the experiment, as well as a concentration profile through the gel and inner reservoir. Experiments are described in greater detail in Chapter 4.

3.2 Flow chamber fabrication

The hydrogels were usually crosslinked in microfluidic flow chambers as shown in Fig. 3.5 (A). The thin chambers ensured that the top and bottom of the gel were sealed, so that transport factors and inert proteins could enter the gel only by diffusing into it. The small chamber size also reduced the quantity of transport factor and inert protein solution needed. Finally, the chambers were designed to be easily mounted on a microscope stage for recording experiments.

The basic flow chamber design consisted of an acrylic slide, patterned spacer or gasket, and coverslip, stacked and sealed together. Ports were almost always drilled in the plastic slide before chamber assembly, in order to allow the chamber to be filled and emptied. Several methods of

Figure 3.4: Procedure for photopolymerization with (A) or without (B) a photomask. (A) A flow chamber is filled with precursor solution. The photomask is placed to hide all areas that should not be crosslinked, and the chamber is exposed to UV illumination. Excess precursor solution is removed with a buffer rinse. (B) Microliter droplets of precursor solution are pipetted onto the slide surface before the chamber is assembled. After assembly, the chamber is exposed to UV illumination. The chamber is then filled with buffer.

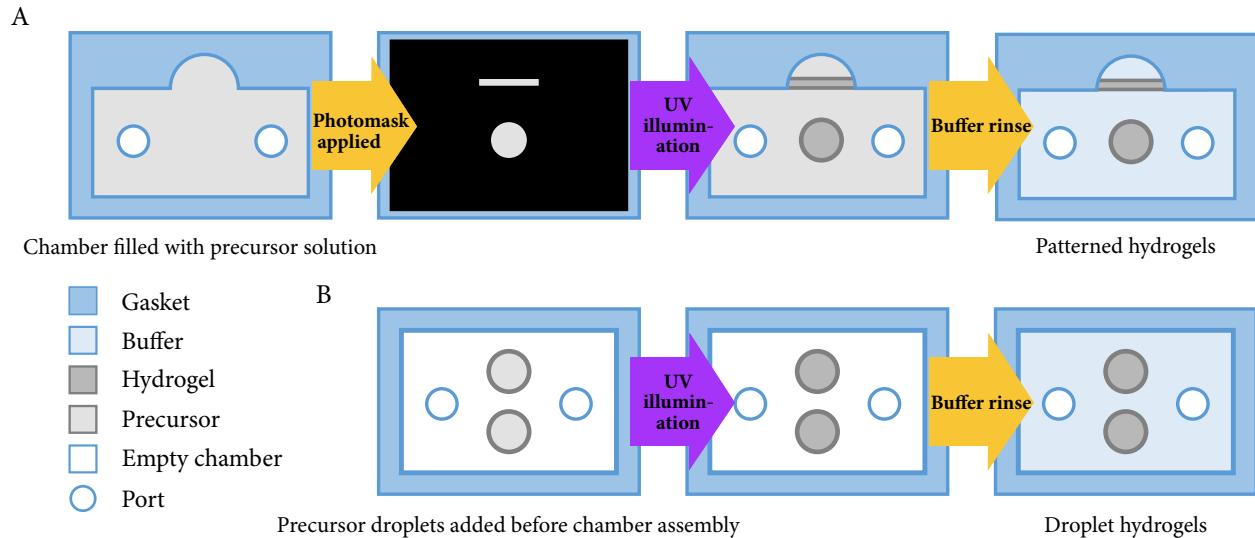
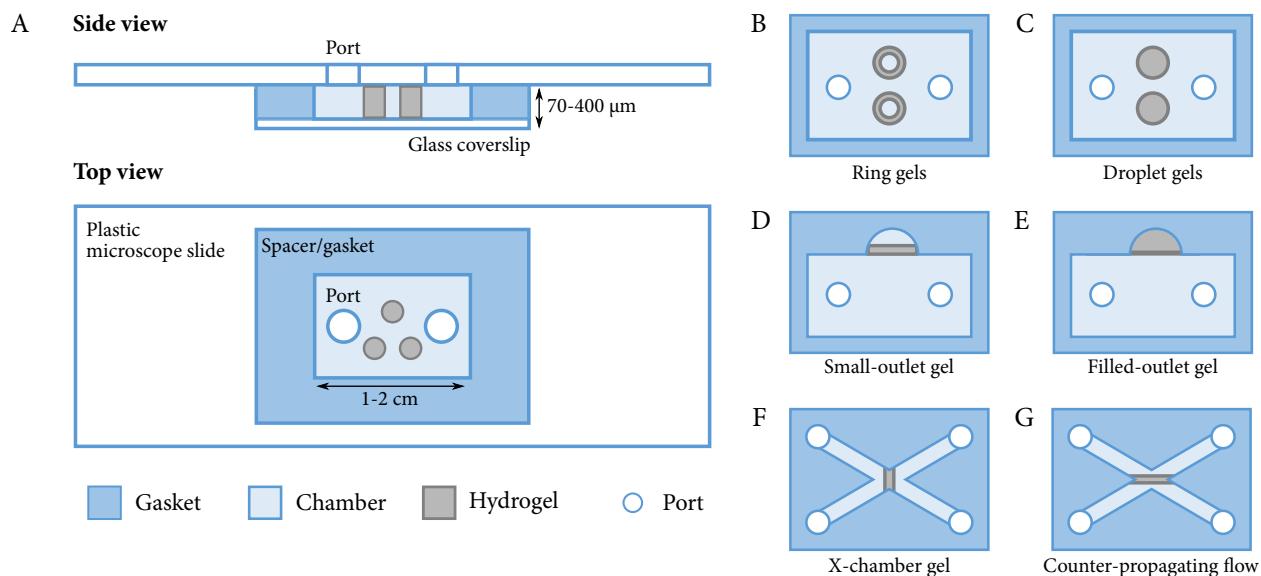


Figure 3.5: Flow chamber design and geometries. (A) Schematic of the most common flow chamber, showing slide, coverslip, gasket, ports, and hydrogels. (B)-(F) Common hydrogel geometries. Those in the left-hand column are suitable for selectivity measurements as well as diffusion measurements, while the right-hand column cannot be used to measure selectivity.



fabricating the gaskets and ports were tested in order to optimize the watertightness and longevity of the chambers as well as the ease of fabrication and re-use.

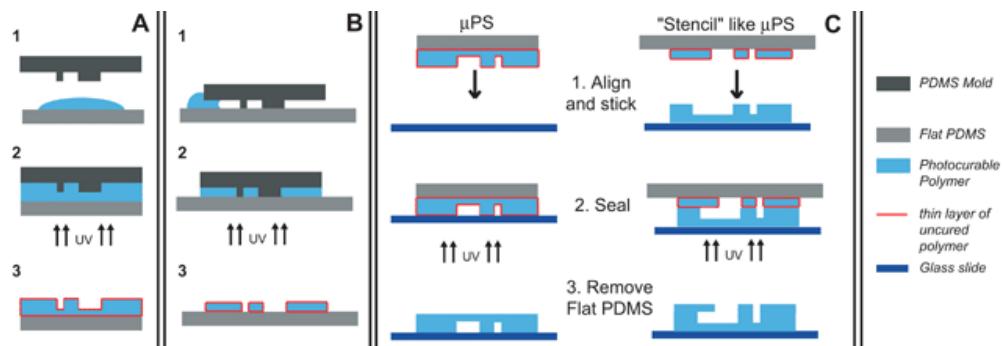
In particular, several materials were attempted for the gasket that determined the height and shape of the chamber. The simplest was double-stick tape, which could be used as a single width or cut into shape with a razor. The double-stick tape chambers were thin ($70\ \mu\text{m}$ per tape layer), of reliable thickness, and quick to make. However, they often leaked or dried over the course of several hours. Since many experiments take several days from preparation to finish, double-stick tape chambers were not usually not sufficient.

Norland Optical Adhesive (NOA), a liquid adhesive which cures upon exposure to UV, was used in another style of flow chamber. Thin semi-cured NOA layers can be molded and used as “stickers” to build flow chambers [2, 53]. This procedure is outlined in Fig. 3.6 and relies on the inhibition of curing by oxygen. A droplet of NOA is sandwiched between a glass coverslip and a PDMS mold, which is permeable to oxygen. The NOA is briefly (approximately 3 seconds at $220\ \text{mW/cm}^2$) cured, though a thin layer remains uncured due to the presence of oxygen at the adhesive’s surface. The PDMS mold is carefully peeled away, and the coverslip with NOA sticker is attached to the slide and sealed. The NOA is then fully cured and the chamber rinsed with ethanol to remove any uncured NOA remaining. Rather than applying NOA directly to the mold, as shown in Fig. 3.6 (A), it is also possible to seal the mold to the coverslip and wick NOA into the hollows (Fig. 3.6 (B)). This method is more error-prone and time-consuming but results in a flow chamber with glass on the top surface instead of NOA. Proteins are often less inclined to stick to the glass surface than to NOA.

PDMS molds for the NOA stickers were made with 0.5-mm resolution using a Silhouette Cameo craft cutter. After creating a template with the craft cutter’s software, the design was cut into a layer of packing tape that had been carefully applied to a large glass slide. The unwanted tape was peeled away using a razor and tweezers, leaving a depression where the chamber would eventually sit, and the slide used as the reverse-mold for a PDMS mold.

NOA-chambers are much more resistant to drying than double-stick-tape chambers, and can

Figure 3.6: Microfluidic sticker fabrication from [2]. (A) A PDMS mold is used to shape an NOA droplet on a flat slide. The NOA is briefly cured but retains a sticky surface. (B) NOA can also be wicked into a PDMS mold. (C) The NOA sticker is placed onto a permanent surface, sealed, and cured entirely.



withstand the largest pressure and most rapid flow. On the other hand, they are significantly more difficult to make and cannot be re-used. For most experiments, where rapid flow is not necessary, PDMS gaskets were the most useful. Chambers made with PDMS gaskets are thicker (100-400 μm) than the other varieties and their thickness is not as reproducible, but they are easy to reuse, often can last several days without drying out, and are quick to assemble.

PDMS gaskets were made by preparing the volume of PDMS mixture needed to create a layer of a given thickness in a standard Petri dish. Once the PDMS had been thoroughly mixed, degassed, and spread evenly across the dish, it was cured for an hour at 70° and cut into shape with a razor. Measurement with a micrometer indicated that the nominally 400- μm -thick PDMS film had an error of no more than 10%. This thickness proved to be optimal for sealing to both the acrylic slide and glass coverslip, as well as easily re-usable after thorough cleaning with ethanol.

While plasma-bonding the PDMS to the glass was tested, it proved unreliable. As long as the chamber was not subjected to high pressures, an adequate seal formed without additional treatment if all materials were cleaned with ethanol and dried with house air before use. Likewise, silanation of the glass was attempted, in order to bond the hydrogel more securely to the top and bottom chamber surfaces, but it was not needed. The gels sealed well to the chamber as long as they were in contact with the surfaces while crosslinking. However, crosslinking the hydrogels on PDMS and then transferring them to the chamber resulted in a poor seal. In consequence, almost

all experiments were run using gels that had been crosslinked inside a chamber.

The ports were another point of concern for the watertightness of the chambers. If liquid needed to be flowed through a chamber at an appreciable speed, short lengths of PEEK tubing were superglued into the ports and fitted with Tygon tubing, which was then attached to a blunt-tipped syringe. For sufficiently gentle flows, however, ports were left simply as holes in the plastic slide and the chamber filled by pipette. After the chamber was filled, PEEK tubing ports were sealed with parafilm, and holes were sealed with a flat slab of clean PDMS.

Finally, portless thin chambers were used occasionally, such as for attempting fluorescence recovery after photobleaching (FRAP) using a confocal microscope. To make these chambers, microliter or smaller droplets of precursor solution containing 6- μm glass spacer beads were placed on glass slides and covered with a coverslip. The hydrogel was crosslinked and fluorescent protein solution wicked into the chamber. The chamber was sealed with valap (a 1:1:1 ratio of vaseline, lanolin, and paraffin which easily melts over a burner and re-solidifies rapidly). Such chambers last several hours on the microscope without drying but should not be used for longer experiments.

3.3 Hydrogel geometries

Our ultimate goal was testing protein separation by monitoring their passage into and through a selective material. In order to truly measure selectivity, the accumulation of proteins in an outlet reservoir beyond the test material must be measured, not just influx into the material. Many hydrogel and flow chamber geometries were tested in search of a setup that would allow selectivity, as well as free and bound diffusion, to be directly observed.

The limiting factors were the resolution of patterned hydrogel features, the size and accessibility of the outlets, and the equilibrium swelling of the hydrogels. Attempts to improve one of these factors typically led to worse outcomes for the others. Two major classes of hydrogel geometry emerged: those with an outlet reservoir, and those without. The hydrogels without an outlet reservoir cannot be used to directly measure the gels' selectivity, but diffusion constants for the inert protein and transport factors can still be determined. Chapter 4 details the results of

bound-state-diffusion experiments using no-outlet hydrogels.

The hydrogel geometries that contained an outlet reservoir are shown in Fig. 3.5 (B), (D), and (F). Ring-shaped gels (B) provide a small outlet reservoir, which is quick to equilibrate. They were fabricated using a confocal microscope (Sec. 3.8) or using PDMS molds 1-5 mm in diameter. While these rings would have been ideal for selectivity measurements, we were never able to work out a procedure that would result in artifact-free, well-sealed rings.

Fig. 3.5 (D) features a portless outlet reservoir which is much smaller than the inlet. As with the ring gels, these outlets are quick to equilibrate and their precise volume can be calculated, as the entire outlet is in the field of view of a 4x objective. A thin hydrogel bar was polymerized using a photomask, separating the inlet and outlet reservoirs. Without ports in the outlet, the chamber was soaked in buffer for 24 hours to remove the remaining precursor solution from the outlet. The inlet could then be filled with a fluorescent protein solution and accumulation in the outlet measured over time. Unfortunately, it's likely that the outlet itself was lightly crosslinked, no matter how carefully we used the photomask. See Sec. 3.8 for more evidence of stray crosslinking. Additionally, the hydrogel bars were of irreproducible thickness (50-200 μm) and swelled or buckled unpredictably. Ultimately, the lack of reproducibility between replicates made this geometry unusable.

Finally, Fig. 3.5 (F) shows an x-shaped chamber with four ports. A thin bar of hydrogel was polymerized at the junction of the arms using a photomask. The inlet and outlet reservoirs are approximately the same size, leading to slow equilibration in the outlet. The precise volume of the outlet is impossible to determine, as the arms are usually partly filled. Additionally, these chambers must be made using NOA stickers, which are difficult to use, and they tend to dry rapidly. The x-chamber geometry is therefore not useful except in specialized situations. A slightly more helpful version is the counter-propagating flow chamber shown in Fig. 3.5 (G). In this version, the hydrogel bar is as long as possible, creating a "hydrogel window" that separates the two arms of the chamber. While this setup was never used to test protein selectivity, in principle a counter-propagating flow can be established using syringe pumps in order to simulate constant concentration in infinite inlet and outlet reservoirs while using a limited amount of material. A similar setup is demonstrated

in [53].

The no-outlet hydrogel geometries shown in Fig 3.5 (C) and (E) are much simpler to make but do not allow for measurement of protein selectivity. Panel (E) shows a variation on the small-outlet chamber in which the outlet is filled with hydrogel and crosslinked using a photomask. Finally, the droplet gels in panel (C) were added to the chamber before assembly and crosslinked without a mask (Fig. 3.4 (B)). These are the only hydrogels which were made without a mask, which greatly reduced the irreproducible edge effects (also seen in Sec. 3.8). Droplet gels have volumes of 0.5-2 μL and equilibrate 30-kDa proteins in 24-48 hours. While these gels cannot be used to monitor selective transport through and exit from the gel, we can use them to measure the diffusion constants of proteins within the gel and the selective influx of transport factors. Chapter 4 focuses entirely on droplet hydrogels made in 400- μm -thick PDMS gasket chambers with two PEEK-less ports.

3.4 FG Nup peptides

The remaining components of the nuclear pore mimics are the FG Nup fragments which are tethered to the hydrogel scaffold. There are a wide range of FG Nups, many of which can be deleted without apparently impacting nuclear transport [31,32]. We chose to use Nsp1, an essential FG Nup for selective transport, as the basis for our Nup peptides. Like all FG Nups, Nsp1 has an ordered domain which anchors it to the channel wall, and a disordered region containing many FG motifs. As a whole, the disordered region aggregates when isolated in buffer [17,54].

As shown in Fig. 3.7, we make use of two distinct fragments of Nsp1, denoted FSFG and FG124 [23]. Each are entirely disordered, are approximately 120 amino acids (14 kDa) in length, and contain multiple FG motifs. FSFG is so called because it possesses six FSFG repeats. It is extremely stable and unlikely to aggregate, remaining unaggregated throughout a wide range of buffers, pH values, concentrations, and crowding conditions. As an additional advantage, FSFG can easily be expressed and purified. The other Nup fragment, FG124, contains 8 FG motifs. If not kept in 7M guanidine hydrochloride, FG124 will aggregate over the course of several hours. However, FG124 does not aggregate in the cellular environment, suggesting that Nsp1 itself may

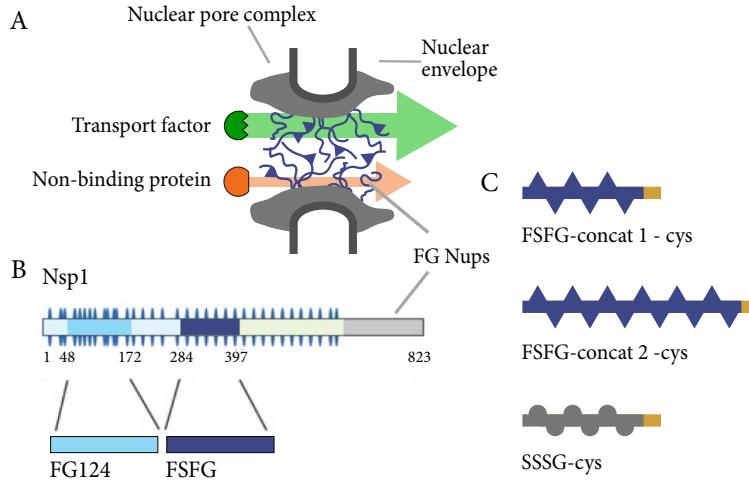


Figure 3.7: Nup fragments used in nuclear pore mimic experiments. (A) The nuclear pore with FG Nups filling the central channel. (B) The essential FG Nup Nsp1 shown as a schematic sequence, with FG124 and FSFG fragments noted. (C) Variants of FSFG: FSFG concat 1-cys with 6 FSFG motifs and a C-terminal cysteine; FSFG concat 2-cys, as above but twice as long; SSSG-cys, like FSFG concat 1-cys with the F's mutated to nonbinding S's.

not be aggregated in the nuclear pore [23].

Both FSFG and FG124 are expressed in Bl21 DE3 Gold cells in the pRSF plasmid, which is Kan resistant. Each has a C-terminus 6xhis tag and are purified using a metal affinity column as described in Appendix B. They are unharmed by lyophilization and resuspension.

We have access to a wide library of FSFG and FG124 variants, few of which I helped to create. Many originate in the Rout lab at Rockefeller University, and others were created by Andrea Egan, Nick Bax, Eric Verbeke, and myself. Appendix E provides the sequence of the most commonly-used Nup variants.

In order to test the effect of Nup length and number of FG motifs, FSFG also exists in concatenated versions: FSFG concat-2 (twice as long as FSFG) and FSFG concat-3 (three times as long). Although both have been expressed and purified, I was only able to express FSFG concat-2. All of these FSFG variants, as well as FG124 and the nonbinding negative control SSSG (Sec. 3.4.1), exist with one or both terminal cysteines as well. The C-terminal cysteine version is the most commonly used, as a cysteine is necessary to tether the Nup fragment to a hydrogel (Sec. 3.1 and Appendix C). It should be noted that ybbR FSFG cys is most commonly used in the nuclear pore mimics. The ybbR tag is intended for site-specific labeling with a fluorophore or other tag but almost always goes unused in the context of FSFG hydrogels [55].

The cysteines in all of the above Nup variants form disulfide bonds very rapidly (within

minutes) after being removed from reducing agents. In addition, the Bradford assay is unreliable for these Nup variants due to the relative lack of aromatic residues. A BCA assay should be used instead to quantify protein concentration.

In addition to the commonly-used Nup variants in Table E.1, we have access to several other Nup fragments and mutants. These include shortened versions of FSFG with two or four FSFG motifs, FSFG variants with most but not all binding motifs removed (FSFG mutated to FSYG), the full FG domain of Nsp1, and full-length Nsp1.

3.4.1 SSSG negative control

The obvious negative control for a Nup-filled hydrogel is a hydrogel containing no Nups. However, it is possible that the presence of FSFG in the precursor solution changes the final gel properties such as pore size, or that non-specific interactions between the Nup peptide and test proteins alter the behavior of the test proteins. To account for these possibilities, we designed a negative control peptide which is identical to FSFG except that the phenylalanine residues have been mutated to serine (i.e. FSFG goes to SSSG). This mutant does not bind NTF2, as demonstrated by the lack of NTF2 accumulation in hydrogels containing tethered SSSG. Figure 3.8 compares the intensity profile of a 10 wt% PEG hydrogel containing a nominal 10 mg/mL SSSG to that of a hydrogel with no nups. There is no dramatic difference between the two profiles, suggesting that the presence of peptide in the hydrogel does not itself alter the diffusion of NTF2 and mCherry. Following the initial tests, SSSG gels were used periodically to confirm that no-Nup gels served well as negative controls, but they were not used regularly as controls.

The SSSG peptide was prepared in an identical manner to FSFG: SSSG-cys was his-tagged, inserted into pRSF, and expressed in BL21-DE3 Gold cells. It was purified using a metal affinity column using the same procedure as FSFG variants and had a high yield. As with FSFG, SSSG is stable and non-aggregating over a wide range of conditions.

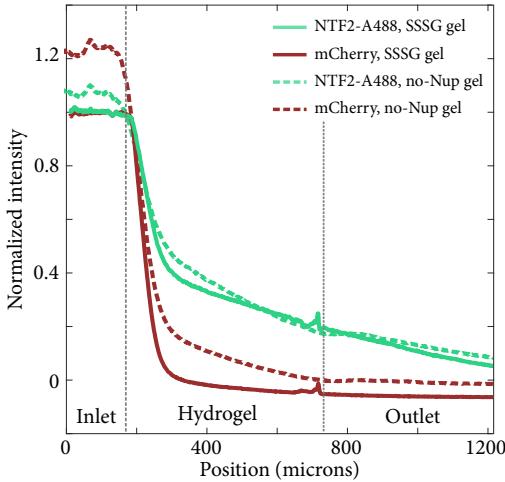


Figure 3.8: Comparison of intensity profiles for SSSG and no-nup (control) gels. Both hydrogels were 10% wt PEG with a 1-kDa PEG dithiol linker. The SSSG gel nominally contained 10 mg/mL SSSG. Inlet reservoir contained 20 μ M NTF2-A488 and mCherry in PTB. As expected, no binding is seen in either gel.

3.5 Transport factor constructs

A wide variety of transport factors exist, specialized for the import and export of particular cargo. Almost all protein separation experiments we performed made use of Nuclear Transport Factor 2 (NTF2). This transport factor is relatively small (28 kDa dimer) and, unlike most other transport factors, does not require active release of cargo after transport. NTF2 transports RanGDP as part of a mechanism which maintains a high RanGTP concentration in the nucleus, necessary for the active release of other protein cargos [34]. The choice to focus on NTF2 was made for a number of reasons: the lack of facilitated release makes it a simple first choice to study, it is small enough to move reasonably quickly through hydrogels, and it is readily expressed in bacteria and purified.

His-tagged yeast NTF2 in the Kan-resistant plasmid pRSF was expressed in BL21 DE3 Gold cells and purified using the procedure described in Appendix B. When necessary, NTF2 was covalently labeled with fluorescent dyes as described in Appendix A. In addition to wild-type yeast NTF2, several variants were created in order to address shortcomings of NTF2 in the experimental setup. In particular, efforts were made to prevent the presence of any NTF2 monomers and to eliminate the need for dye labeling. Finally, a point mutant of NTF2 was created with the intent of disrupting the binding pocket and providing another non-binding negative control protein to complement mCherry.

Beyond NTF2, we briefly tested the influx of Kap121, a member of the well-studied karyopherin family of transport factors, into hydrogels. Although the Kap121 and its fluorescent cargo protein clearly bound to the FSFG in the hydrogel, the pore size remained too small for significant import into the gel.

The remainder of this section provides an overview of the transport factor constructs we created. Much of the cloning work was done by Eric Verbeke and Scott Tilden.

3.5.1 Covalently-tethered NTF2 dimer

Despite the work done to increase the hydrogel's average pore size, we often observed that NTF2 and mCherry equilibrate to different extents and on different timescales even in hydrogels containing no Nups or nonbinding Nups. As these proteins are nominally the same size, this implies that they may be interacting differently with the hydrogel substrate, or that they may not be the same size after all. NTF2 is a 28 kDa homodimer whose monomers are 14 kDa. If there exists an appreciable population of NTF2 monomers within the hydrogel, these will equilibrate more rapidly and to a higher concentration than the larger mCherry. The dissociation constant K_D of mammalian NTF2 is about 1 μM [56]. Assuming this is approximately true for yeast NTF2 as well, there should be very little monomeric NTF2 present at our typical reservoir concentration of 20 μM .

However, we designed a covalently-tethered version of NTF2 to eliminate the possibility of monomerization. This consisted of two NTF2 monomers connected by a flexible amino-acid tether (Fig. 3.9 (A)). Several trial tethers with their length and composition are shown below:

10aa – linker :	TSGSGSGSPG
15aa – linker :	TSGSPRGSSGSGSPG
18aa – linker :	TSPGLVSRGSGSGSGSPG

Eric Verbeke and Scott Tilden successfully cloned covalently-tethered NTF2 with all three

tethers. Unfortunately, no binding was seen when the NTF2 was introduced to an FSFG hydrogel. It is likely that the tether interferes with the binding pockets or dimerization interface.

3.5.2 NTF2-GFP

In addition to the potential problem of NTF2 monomerization, the fluorophores that labeled NTF2 were prone to hydrolysis, leaving an unknown amount of free dye in the reservoir mixture. Free dye is indistinguishable from labeled NTF2 in the influx and selectivity experiments, and it is important that not be present in appreciable amounts. This problem is addressed through other means in Sec. 3.9, but we also approached it by engineering a GFP-NTF2 fusion protein. Such a protein would eliminate the need for dye-labeling the NTF2 and therefore eliminate free dye in the chamber entirely.

However, as GFP is also approximately 30 kDa, the fusion protein is much larger than NTF2 alone. To mitigate this problem, a GFP-NTF2-NTF2 version was also created (Fig. 3.9 (A)). While this is still larger than an NTF2 dimer, it contains only one copy of GFP. Both constructs are in the pET21 plasmid, his-tagged, and expressed in BL21-DE3 Gold cells. They were purified using a cobalt affinity column in PTB and 1:1000 PIC and eluted with 250 mM imidazole, yielding ample protein with moderate degradation products.

Figure 3.9 (B) and (C) show that neither construct is able to bind to FSFG hydrogels. Again, the binding pockets or dimerization interface may be disrupted by the tethers, or the fusion protein may simply be too large.

3.5.3 Non-binding NTF2 F5R

Although mCherry is a similar size as the NTF2 dimer and makes an acceptable negative-control inert protein, it may well interact differently with the hydrogel scaffold than NTF2 does. A better control would be a non-binding mutant form of NTF2. A W7A point mutant in mammalian NTF2 reduced its affinity for FG Nups [57, 58]. A corresponding mutant in yeast NTF2 is F5R. This mutant was cloned by myself and Scott Tilden, expressed, and purified. Further tests, such

Figure 3.9: Attempts to create functional NTF2 fusion proteins. (A) Cartoon of various NTF2 constructs: wild-type NTF2 dimer, covalently-tethered NTF2-NTF2, GFP-NTF2, GFP-NTF2-NTF2 (B) Intensity profile for GFP-NTF2 dimer showing no binding to FSFG hydrogel. Hydrogel contained a nominal 10 mg/mL FSFG; reservoir solution contained 20 μ M GFP-NTF2 and mCherry in PTB. (C) Similar experiment as (B) for GFP-NTF2-NTF2.

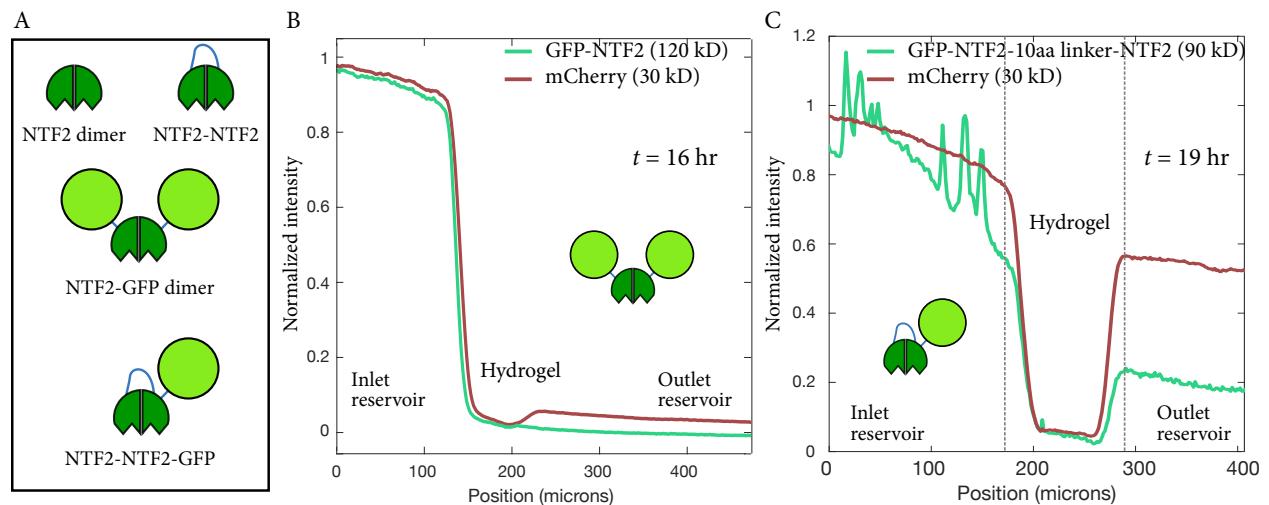
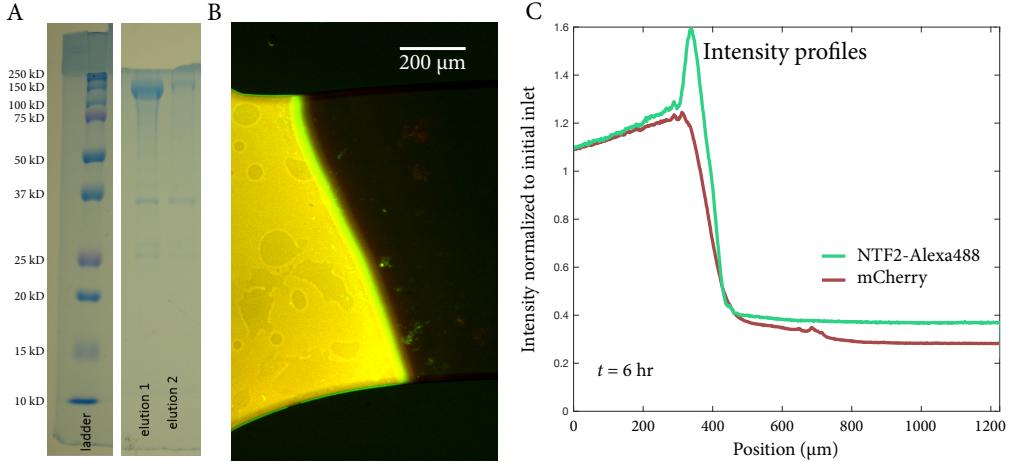


Figure 3.10: SDS-PAGE showing Kap121 purification (purified by Chris Lawton), picture of influx, intensity profiles



as NMR, are needed to verify its binding affinity for FSFG.

3.5.4 Kap121 and NLS-GFP

The karyopherins (Kaps) are a canonical class of transport factors. They transport proteins tagged with nuclear localization signals (NLS) through the nuclear pore. We were interested in testing Kaps because they are widely studied and much larger than NTF2, so selective transport would be more apparent. Eric Verbeke engineered two GFP-NLS constructs: GFP-Spo12 and GFP-Pho4 (Appendix E). These constructs were his-tagged, inserted into pET21b, expressed in BL21-DE3 Gold cells, and purified using a cobalt affinity column. Kap121-GST was expressed and purified following [44] and the GST subsequently cleaved with thrombin resin (Fig. 3.10 a).

Kap121 binding and influx was tested using a hydrogel made from precursor solution containing 0.05 wt% Irgacure2959, 110 mg/mL 20kD 8-armed PEG-norbornene, 11 mg/mL 1kD PEG-dithiol, and 1 mM TCEP in PTB, crosslinked for 2 minutes under the UV LED. After soaking in PTB overnight, 5 μM Kap121, 10 μM GFP-Pho4, and 100 μM mCherry in PTB were added to the inlet reservoir. Selective influx of the Kap121/GFP-Pho4 complex was evident, since the complex accumulated at the edge of the hydrogel (Fig. 3.10 b and c). The bright band demonstrates binding of FSFG to Kap121 as well as of Kap121 to GFP-Pho4. Over the course of several hours, the GFP

front remained localized at the edge of the gel, likely indicating that the pore size of the hydrogel was too small to accomodate the Kap121/GFP-Pho4 complex. Given the large size of this complex, the pore size was never increased sufficiently for influx into the hydrogels.

3.6 Pore size

The perennial difficulty with our hydrogel nuclear pore mimics has been the pore size of the hydrogels. While hydrogels with an average pore size between 10 and 100 μm are well-studied [59], as well as hydrogels with pores under 5 nm, the intermediate-porosity regime which would allow for separation of proteins in the size range 30-150 kDa (typically 5-10 nm) is difficult to reach. We wanted to be confident that any differences in the behavior of transport factor and inert protein were due to their interactions with the Nups, not slight differences in size or interactions with the hydrogel. At the same time, we wanted the anchored Nups to fill a significant portion of the pore. Much of our work on hydrogel design was with the aim of achieving a suitable pore size.

Hydrogel swelling in particular has been an obstacle in our search for protein-separating hydrogels. When polymerized from a precursor solution, hydrogels are almost never in mechanical equilibrium. They must be soaked in an aqueous solution until they have swelled and taken up enough water to reach equilibrium. Common methods of estimating pore size rely on the swelling ratio of the gel, i.e. the ratio of the wet to dry gel weight. However, our gels are small enough to make that method impractical, and it is unclear whether a Nup-filled hydrogel that is allowed to swell to equilibrium in buffer will have the same properties as one that is polymerized inside a confined flow chamber. In fact, repeated but unpredictable buckling and swelling of hydrogels within the flow chambers indicates that our hydrogels do not reach mechanical equilibrium. Despite efforts to improve the swelling ability of our hydrogels, the lack of equilibrium swelling likely contributes to the smaller-than-ideal pore size as well as inconsistency between replicate experiments.

Of the two hydrogel substrates tested, acrylamide gels appear to have a better pore size distribution than PEG gels. An order-of-magnitude calculation estimated 1 nm as the average mesh size of a 10 wt % PEG hydrogel, comparable to or smaller than the 5-nm size of NTF2

(Appendix F). We were not able to lower the weight percent of PEG and still achieve gelation. Overall, 6% w/v acrylamide gels are more mechanically stable and yield more reproducible results than 10% w/v PEG gels, the lowest stable percent weight PEG gel we were able to attain. Before settling on acrylamide hydrogels, we considered several alternative crosslinkers in hopes of creating more mechanically-stable PEG hydrogels that would support larger pores. Most notably, we moved from a 1-kDa PEG dithiol linker to an 8-kDa linker. This change did increase the pore size of the resulting PEG gel, but it was mechanically weak and required proportionately more swelling to reach equilibrium, an impossibility inside the flow chamber. DNA oligomers, coiled-coil rationally-designed proteins, and FSFG with both terminal cysteines were also considered as crosslinkers, but none were able to support gelation [60, 61]. Therefore, we turned our attention to acrylamide hydrogels.

Figure 3.11 shows the partition coefficient of equilibrated fluorescent macromolecules as a function of molecular weight for 6% acrylamide gels containing no Nups. The partition coefficient is the ratio of equilibrium concentration within the hydrogel to that in the reservoir outside; a partition coefficient of 1 indicates that the macromolecule's diffusion and equilibration is not impeded at all by the presence of the gel. Although 250 kDa dextran has a low partition coefficient, 30 kDa transport factors and fluorescent proteins have similar partition coefficients around $\gamma = 0.7$. The partition coefficient of inert proteins decreases when Nups are tethered to the hydrogel, as they further impede diffusion and equilibration.

The no-Nup hydrogel dataset used in Chapter 4, containing 17 experiments in total, provides another measurement of partition coefficient and diffusion. Both the NTF2 and mCherry partition coefficients are equal to 0.48 ± 0.03 , statistically indistinguishable. On the other hand, the mean diffusion constant of NTF2 was $59 \pm 5 \mu\text{m}^2/\text{s}$, as opposed to $44 \pm 4 \mu\text{m}^2/\text{s}$ for mCherry. The two values are significantly different at $p = 0.02$ using a two-tailed t-test. These results indicate that NTF2 and mCherry do not behave identically within the hydrogel, but that they equilibrate to similar values, suggesting overall a small interaction with the hydrogel scaffold. We deemed this to be acceptable for the NTF2-mCherry pair but were not comfortable comparing the diffusion of

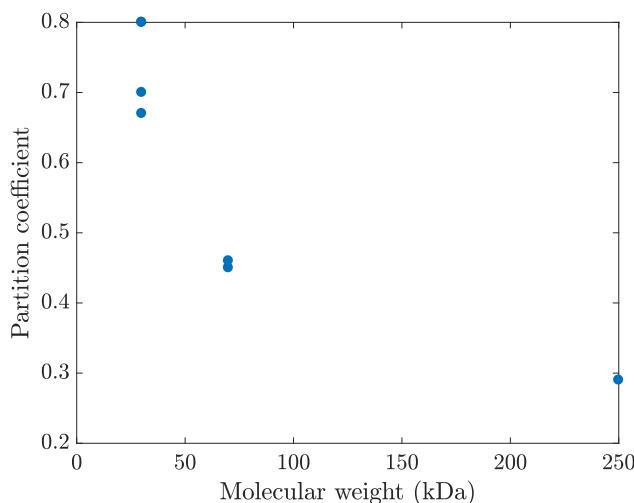


Figure 3.11: Equilibrium partition coefficient as a function of macromolecule molecular weight for 6% acrylamide no-Nup hydrogels. NTF2, mCherry and GFP were used as 30-kDa test proteins along with 70-kDa dextran-rhodamine and 250-kDa dextran-fluorescein.

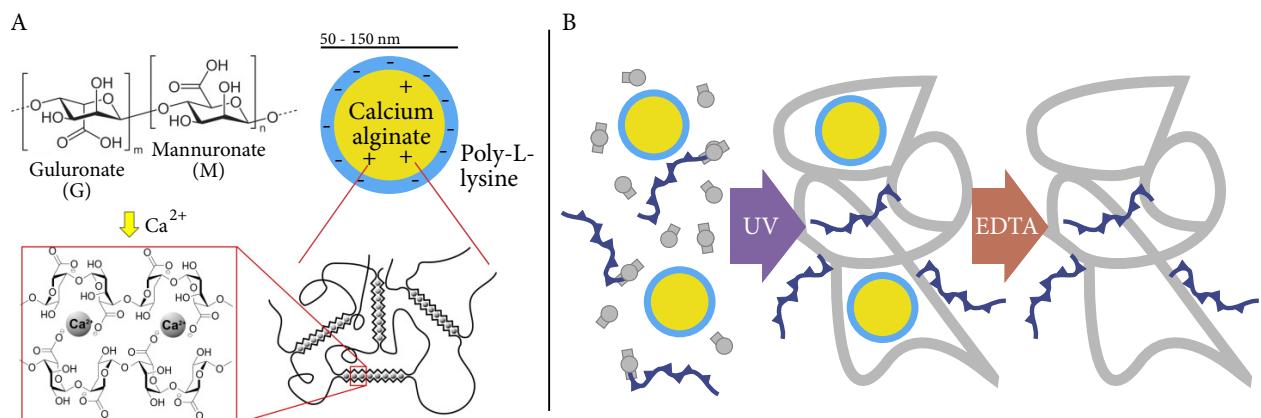
larger proteins using acrylamide gels.

3.7 Porogens

Given the difficulties of increasing the average pore size by changing the hydrogel composition, we investigated the possibility of introducing a porogen into the precursor solution. To be useful, the porogen would need to be a macromolecule or particle 10-100 nm in diameter that could be evenly distributed throughout the precursor solution and then hydrogel without disrupting polymerization. The porogen would then be digested, dissolved, or otherwise removed from the hydrogel, as it would be too large to passively diffuse from the gel on a reasonable timescale. The pores left behind would then increase the overall pore size of the gel as well as potentially allowing the hydrogel network to swell to equilibrium even while confined in a chamber.

Two possible porogens were tried: Alginate nanospheres and high-molecular-weight dextran. Alginate can be polymerized and depolymerized through the addition or removal of calcium ions, and dextran can be digested by dextranase. Although both showed promise, neither was ultimately well-suited to increasing a hydrogel's pore size.

Figure 3.12: Alginate crosslinking from [3]. (A) Alginate is composed of alternating guluronate (G) and mannuronate (M) blocks. Addition of calcium ions leads to an “egg-box” crosslinked structure. Calcium alginate nanospheres are coated with poly-L-lysine [4]. (B) Alginate nanospheres should act as a porogen when added to a hydrogel precursor solution. Removal of the spheres with EDTA should leave larger pores in the crosslinked hydrogel.



3.7.1 Alginate nanospheres

Alginate is a polysaccharide derived from algae which polymerizes upon addition of calcium ions (Fig. 3.12 (A)). It can be depolymerized by adding EDTA or another chelator to remove the calcium. Alginate salts are available in a number of molecular weights and viscosities, making it a promising candidate for a porogen. If alginate nanospheres could be polymerized and added to the hydrogel precursor solution, they could later be removed with EDTA, leaving larger pores than would otherwise be present (Fig. 3.12 (B)).

A number of protocols exist for creating alginate microspheres, but fewer are appropriate for nanospheres, which is the scale that we would need in order to use them as a porogen. I made nanospheres following the method described in [4].

I prepared 10 mL of 0.6 mg/mL alginic acid sodium salt (Sigma, low-viscosity, product number A0628) in water. Even a small amount of sodium alginate added to water creates a viscous solution that takes a lot of time and stirring to dissolve. I used a sonicator with microtip to sonicate the sodium alginate solution while adding 2 mL of 0.67 mg/mL calcium chloride in water drop-by-drop. I then stirred the solution on a magnetic stir plate for 30 minutes before adding 2 mL of 0.3 mg/mL poly-L-lysine in water. The solution was then stirred 30 more minutes.

I tested several methods of preparing the nanospheres for addition to the hydrogel precursor solution. First, the nanospheres were left in the solution in which they were made. Second, the nanospheres were spun down out of that solution at 14400g for 10 minutes and immediately resuspended in deionized water. (The pellet becomes more difficult to resuspend if it is not immediately resuspended.) Finally, the nanospheres were lyophilized and resuspended in water. The resuspended solution remained cloudy even after several days, and so I didn't pursue lyophilizing the nanospheres.

The radius and diffusion coefficients of the both the non-spun and spun nanospheres were determined using a Titan DynaPro dynamic light scattering (DLS) system. The non-spun nanospheres had an average radius of 170 ± 10 nm, with an estimated diffusion constant of $1.1 \pm 0.1 \mu\text{m}^2/\text{s}$. The

nanospheres that had been spun and resuspended had two populations: almost entirely particles of radius 210 ± 10 nm and diffusion constant $1.05 \pm 0.06 \mu\text{m}^2/\text{s}$, and a small number of very large particles. The large particles are probably aggregates from centrifugation.

After measuring the size distribution of the two samples, I added EDTA in an effort to depolymerize the nanospheres. The maximum final EDTA concentration was 25 mM, and the samples were left to sit up to 30 minutes. Re-running the DLS data gave inconclusive results as to the presence or size of remaining particles. The fits did not change much, although the large aggregates appeared to have vanished from the spun sample. It appeared that at least some nanospheres remained.

Next, the nanospheres were added to hydrogel precursor solution, which was then crosslinked. Several nanosphere concentrations were tested before a condition was found in which the nanospheres appeared by eye to resuspend. An approximately 2 μL nanosphere pellet (spun down from 10 mL of solution) was resuspended in a final concentration of 0.11 mg/mL PEG-ene, 0.09 mg/mL 8K PEG dithiol linker, and 5 mM LAP, all in 50 mM MOPS buffer pH 7.4. The MOPS buffer was chosen because it has no divalent cations (and thus will not interfere with the alginate nanospheres). The isoelectric point of poly-L-lysine is at about pH 5, and the nanospheres should be kept above that pH so their coating remains intact.

However, even these gels were clearly inhomogeneous under 4x magnification. In an attempt to depolymerize the nanospheres, the resulting hydrogels were rinsed with PTB pH 5 (to remove any possible poly-L-lysine coating) and then soaked in 100 mM EDTA for two days. No change was observed under 4x magnification. For comparison, a macroscopic alginate droplet dissolved after 20 minutes in 100 mM EDTA and vortexing. New gels were made and soaked in a pH 5 buffer (to remove any poly-L-lysine coating) and then in a solution containing 100 mM EDTA and 200 mM sodium citrate. Gels remained cloudy and no change was observed.

Given the difficulty in resuspending and depolymerizing the alginate nanospheres, our focus shifted to developing a dextran/dextranase porogen system.

3.7.2 Dextran/dextranase porogen system

Following the alginate nanospheres attempt, we tried creating nanopores using high-molecular-weight (high-MW) dextran. Dextran is a branched, inert polymer that is commercially available at molecular weights up to 250 kD. We used two sizes as porogens, 70 kD ($R_H \approx 10$ nm) and 250 kD ($R_H \approx 15$ nm) [62].

As a proof of concept, I made and crosslinked precursor solutions containing 100 μ M 70 kD dextran-rhodamine or 250 kD dextran-fluorescein. The precursor contained 12% acrylamide and 3.3% bisacrylamide in 50 mM sodium acetate (NaOAc) buffer pH 5 as well as 2 mM LAP. The precursor solution was crosslinked as 1- μ L hydrogels in a 400- μ m tall PDMS gasket chamber for 20 s using the UV LED. After rinsing with NaOAc buffer, the chamber was filled with a freshly-made solution of 20 mg/mL dextranase (Sigma, D5884, dextranase from *Penicillium sp.*) in NaOAc buffer. Chamber was immediately placed in the Olympus widefield's environmental chamber, held at 37°C, and imaged overnight. The fluorescence intensity in the dextranase-treated gels decreased significantly more rapidly than that of gels which contained dextran but were not treated with dextranase (Fig. 3.13). In the case of 70 kD dextran, the fluorescence reached a steady value (equilibrated, same as that in the reservoir) in approximately 100 minutes, while the non-treated case wasn't equilibrated after an overnight incubation. For the 250 kD dextran, both the treated and non-treated gels reached an equilibrium in approximately the same amount of time, but the treated gel equilibrated much closer to the reservoir level than the non-treated gel. Both sets of gels indicate that dextranase is able to digest the dextran within the hydrogels, allowing a significant amount of dextran and digest products to leave the gel, as expected.

The next step in attempting this porogen system was recreating the effect in FSFG gels. Unfortunately, it became clear that the dextranase was digesting the FSFG as well as the dextran (Fig. 3.14). At this point, we stopped trying to use the dextran-dextranase porogen system for FSFG gels. However, we did investigate the effect of dextran-dextranase treatment on the pore size in acrylamide hydrogels without anchored proteins. Contrary to expectations, dextran-dextranase

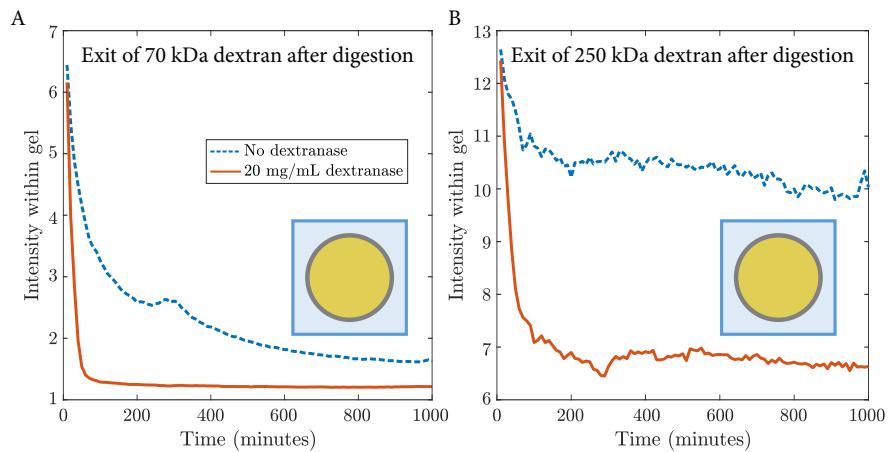


Figure 3.13: Exit of fluorescent dextran from hydrogels after digestion with dextranase. Each hydrogel originally contained 100 μM dextran and was treated with 20 mg/mL dextranase as described in the text. The total fluorescence intensity within the gel was monitored as a function of time as it approached equilibrium with the buffer in the reservoir. Intensity was normalized to initial gel fluorescence.

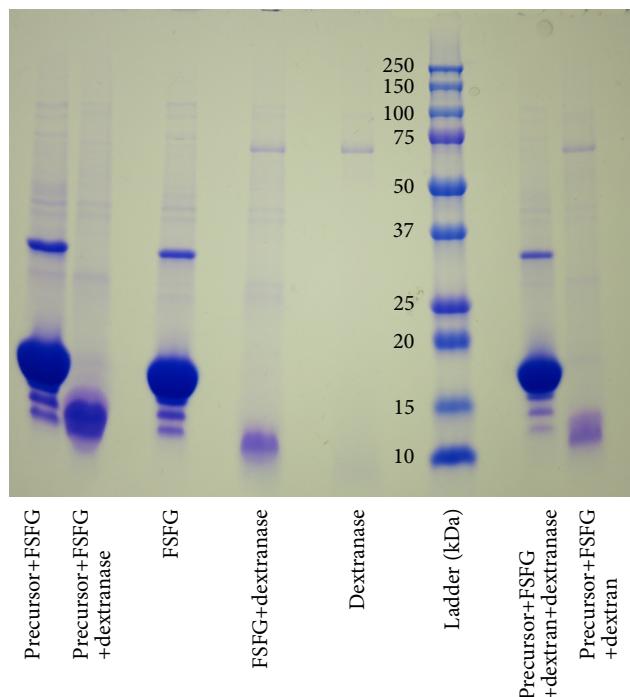


Figure 3.14: SDS-PAGE gel demonstrating dextranase digestion of FSFG. Three conditions are shown, with and without dextranase: precursor solution with FSFG, FSFG only, and precursor with FSFG and dextran. In each case, addition of dextranase destroys the FSFG band at 15 kDa and produces bands of smaller degradation products. Dextranase alone is shown for reference. AnyKd PAGE gel (BioRad), 100 V, 80 minutes.

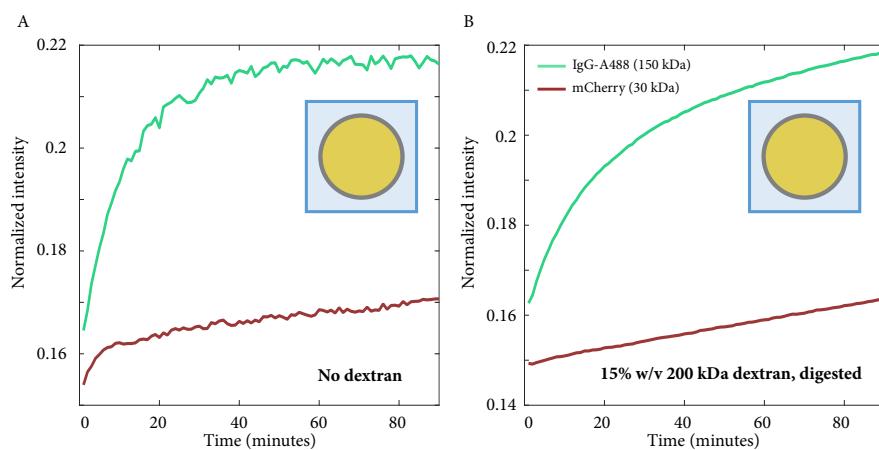


Figure 3.15: Comparison of protein accumulation within hydrogels made with or without dextran. Reservoirs contained 10 μM IgG-A488 and mCherry. (A) Accumulation in a 12% acrylamide hydrogel containing no Nups or dextran. (B) Accumulation in a 12% acrylamide hydrogel containing no Nups but made with 15% w/v 200 kDa dextran and digested for 2 hours at 37°C with 20 mg/mL dextranase.

treatment did not increase subsequent diffusion of fluorescent proteins into the treated hydrogels. For these experiments, I used 200 kD non-fluorescent dextran in the acrylamide precursor solution. After the gels (either 12% or 6% final acrylamide concentration) were crosslinked, I treated them with 20 mg/mL dextranase solution in sodium acetate buffer (freshly made solution) for 2 hours at 37°C in an incubator. Then I rinsed the gels with buffer and used a syringe pump to flow 10 mL of PTB through the chamber over the course of 10 hours. The next day, I challenged the gels with a solution containing 10 μM each immunoglobulinG tagged with Alexa Fluor 488 (IgG-Alexa488, 150 kD) and mCherry (28 kD). I monitored the accumulation of each protein in the gel over time (Fig. 3.15). In both the 12% and 6% acrylamide gels, dextran-dextranase treatment did not improve influx of IgG-Alexa488 over corresponding non-treated gels. In 6% acrylamide gels, the influx of mCherry also remained approximately the same for treated and non-treated gels. This indicates that dextran-dextranase treatment does not significantly increase the pore size in acrylamide hydrogels. This is probably because significant dextran remains in the gel even after dextranase treatment, as suggested by Fig. 3.13.

In conclusion, dextranase is capable of digesting a significant fraction of high-MW dextran within an acrylamide hydrogel. However, a significant amount of the dextran still remains in the gel even after an overnight rinse, meaning that dextran-dextranase treatment does not result in noticeably larger pores overall. In addition, the dextranase we used digested the FSFG peptide

as well as dextran. Dextran-dextranase treatment is not suitable as a porogen system for our hydrogels.

3.8 Polymerization using confocal microscope

Given the problems that arose using photomasks and a UV LED to make hydrogels, we tested a crosslinking method using a 405 nm laser on the Nikon A1R spinning disc confocal microscope in the JSCBB microscopy facility. Once a region of interest has been defined with the Nikon software, the photobleaching setting can be used to selectively expose regions of the field of view to UV light. The precursor solution therefore crosslinks in the region of interest only. This method is similar to that used in [53], but the masking is done using the A1R software instead of a physical mask in the back focal plane.

We were able to “draw” hydrogels of arbitrary shapes using the 10x objective on the confocal along with the 405 nm laser at 100% power. Figure 3.16 shows hollow rings with an outer diameter of about 600 μm and an inner diameter of 500 μm , as well as lines with a width of about 50 μm . In order to crosslink the precursor solution, two raster-scans of the laser across the region of interest were needed, with the longest-allowable dwell time per pixel. (In the microscope settings, the shortest dwell time is set as ‘1’ and the longest as ‘1/32’.) The precursor solution contained 0.5 mM LAP, 110 mg/mL 20kD 8-armed PEG-norbornene, 11 mg/mL 1kD PEG-dithiol, and 1 mM TCEP in PTB; it was used to fill 70- μm -thick sticky-tape or NOA flow chambers.

Crosslinking with the confocal has several advantages over LED crosslinking. Significantly smaller features are possible using the confocal. 50- μm features are consistent and reproducible, and features down to approximately 25 μm are possible, in contrast with the effective 100- μm lower limit using the LED and photomasks. Additionally, arbitrary shapes, including shapes with inner cavities, are possible using the confocal. Multiple small hydrogels can be created in the same chamber, including hydrogels of varying composition, created by removing the excess precursor solution and refilling the chamber with a different solution. Finally, the degree of crosslinking can potentially be varied by changing the photobleaching settings.

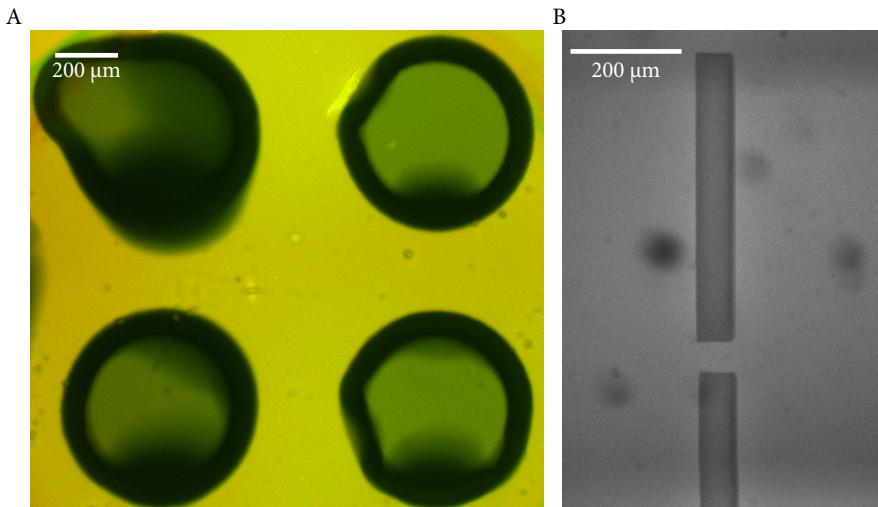


Figure 3.16: Image of laser-written hydrogels. (A) Ring hydrogels containing no Nups. Reservoir contains $20 \mu\text{M}$ NTF2-A488 and mCherry. (B) Line hydrogels containing FSFG-A647. Reservoir contains precursor solution with FSFG-A647. Photobleaching within gel by 405 nm laser is evident.

The most appealing geometry made possible with confocal crosslinking is the hydrogel ring, as shown in Figs. 3.16 and 3.18. Unlike all other hydrogel-chamber geometries, the inner reservoir is small enough to equilibrate in only a few hours (Fig. 3.17). Ideally, the hydrogel rings could have been used to test selective flux through the NPC mimics, a possibility not offered by other hydrogel-chamber geometries, which are optimized for testing influx into the hydrogels only. Long thin lines can also be written with the confocal by repeatedly moving the field of view and re-crosslinking, overlapping the new segment with the previous. This geometry could be useful in creating counter-propagating flow chambers with hydrogel windows.

Confocal crosslinking does not damage FSFG, as demonstrated in Fig. 3.18. Hydrogels were made using the precursor solution described above with the addition of 10 mg/mL FSFG-cys. After soaking in PTB buffer overnight, a mixture of $25 \mu\text{m}$ each NTF2-Alexa488 and mCherry was added to the outer reservoir. After two hours of equilibration, the FSFG hydrogels showed a partition coefficient greater than one for NTF2-Alexa488 but smaller than one for mCherry. This indicates that FSFG is anchored into the gel and that NTF2-A488 is able to bind it.

Finally, Norland Optical Adhesive (NOA) can also be crosslinked using this method. Complicated flow chambers can be created, although it is labor-intensive and difficult to remove all of the excess NOA afterwards.

Despite the advantages of confocal crosslinking, several obstacles combined to ultimately

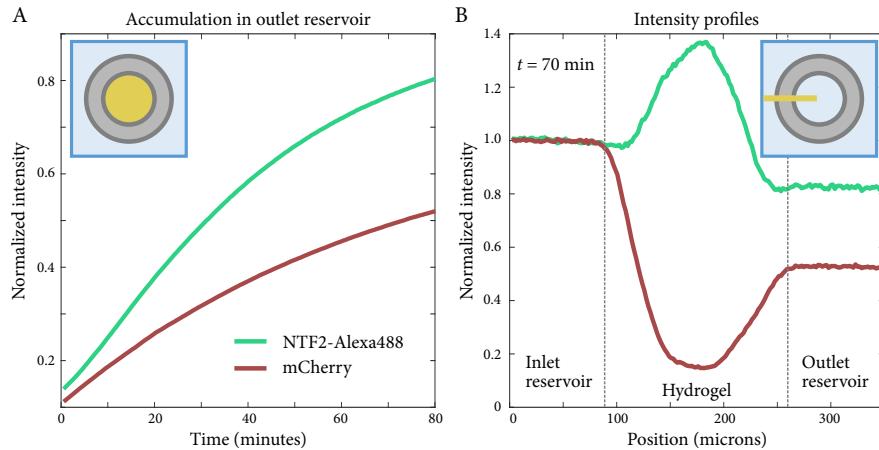
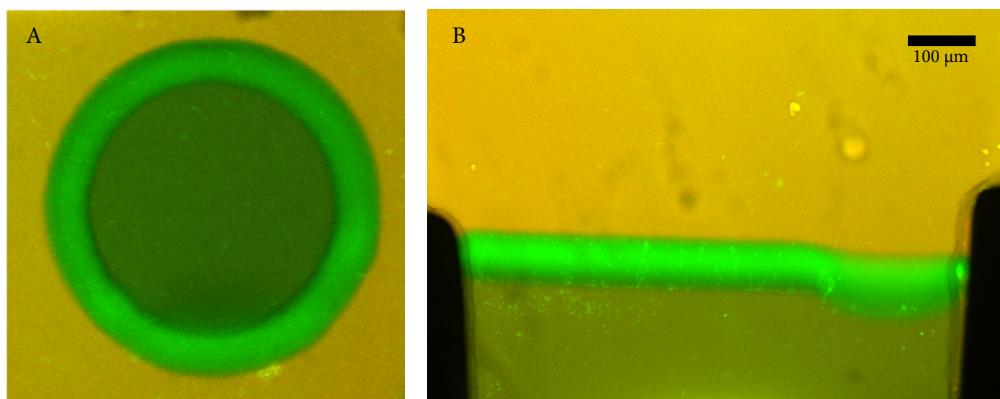


Figure 3.17: Sample (A) accumulation and (B) intensity profile plots for a 50 μm thick confocal-crosslinked ring nominally containing 10 mg/mL FSFG. Intensity normalized to inlet reservoir. Inlet contains 25 μM NTF2-Alexa488 and mCherry in PTB.

Figure 3.18: Images of laser-written hydrogels nominally containing 10 mg/mL FSFG. Reservoirs contain 20 μM NTF2-A488 and mCherry. (A) Hydrogel ring, likely with lightly-crosslinked inner reservoir. (B) Hydrogel bar separating large inlet and small outlet.



make this method unusable for our purposes. The most serious problem was that of stray crosslinking. Areas outside of the defined region of interest were often unpredictably crosslinked, as can be seen in Fig. 3.16. Stray crosslinking outside of the rings is limited by rinsing the gels within 5 minutes of crosslinking, removing any excess precursor solution [53]. However, rinsing the chamber does not remove precursor solution from the ring's inner reservoir. Stray crosslinking in this reservoir is often more difficult to detect and more damaging to the experimental results. Figure 3.18 illustrates the problem: the ring has been equilibrated with NTF2-A488, which has preferentially entered the inner reservoir over mCherry. The concentration of NTF2-A488 is, in fact, higher in the inner than the outer reservoir, indicating that there is some low concentration of FSFG available in the inner reservoir for binding to NTF2. As the hydrogel was soaked in buffer overnight after crosslinking, any mobile FSFG remaining from the precursor solution has been removed, meaning that the remaining FSFG is most likely anchored into a lightly-crosslinked hydrogel that fills the inner reservoir. The presence of this gel alters the results of an equilibration experiment by artificially increasing the final NTF2 concentration in the inner reservoir.

With help from Danielle Konetski and Christopher Bowman, we attempted to address the stray crosslinking by adding a photoinhibitor to the precursor solution. The radical inhibitor 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO) can be used in aqueous solution to limit crosslinking [51]. We tested the effect of the photoinhibitor using a precursor solution as described above with the addition of 0.5 mM TEMPO. While the edges of the resulting hydrogels became marginally sharper, stray crosslinking was still evident, especially in the inner reservoir.

Another significant problem was swelling and buckling of the hydrogels. Despite the hope that thinner hydrogels would swell and equilibrate more easily, buckling of the rings and lines was pervasive and difficult to predict (Fig. 3.16). Ring hydrogels in particular often developed minor leaks due to buckling. Despite a great many attempts to improve the swelling problem (see various other sections), the rings ultimately could not be used for selective transport experiments.

One interesting feature of confocal crosslinking is the degree of control it affords over the illumination method. In the A1R's photobleaching mode, the 405 nm laser is raster-scanned over

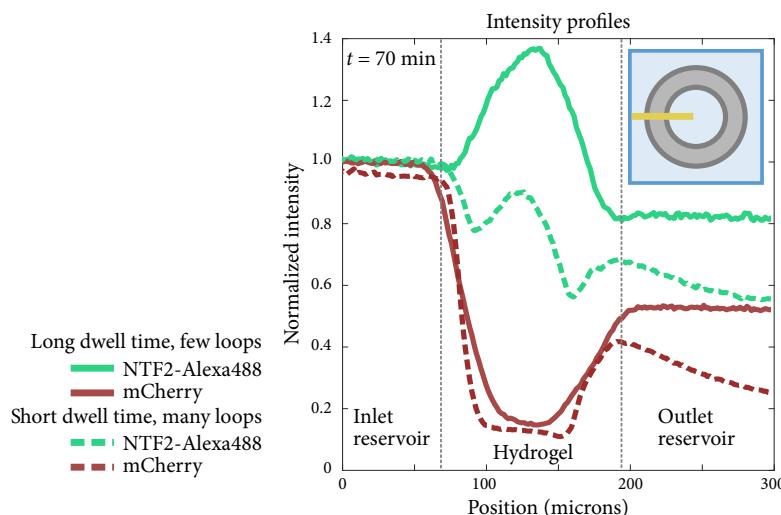


Figure 3.19: Effect of dwell time and loop number on edge-dip effect in equilibrated, confocal-crosslinked ring hydrogels. Nominal FSFG concentration is 10 mg/mL. Reservoirs contain 20 μM NTF2-A488 and mCherry in PTB. Intensity profiles are shown for a hydrogel polymerized with $t_{\text{dwell}} = 1/32$ and two loops (solid lines) and a hydrogel polymerized with $t_{\text{dwell}} = 1$ and 64 loops.

each pixel in the field of view. The shutter is toggled in order to illuminate only pixels within the predefined region of interest. The controls permit the laser intensity, dwell time at each pixel, and number of raster scan loops to be varied. The laser intensity was always kept at 100%, but changing the dwell time and loop number had a dramatic effect on the final properties of the hydrogel.

Generally speaking, a longer dwell time at each pixel and a low number of loops gave the best results. Figure 3.19 shows post-equilibration profiles for two hydrogel rings made with different illumination settings. Each gel received the same total illumination time, but in one case the dwell time was reduced 32-fold and the loop number increased by the same factor. In both cases, the hydrogels were soaked in PTB buffer overnight and then challenged with 25 μM NTF2-Alexa488 and mCherry. After 70 minutes, sufficient NTF2 had accumulated in the gel to make the differences between gels obvious. The low-dwell-time hydrogel accumulated less NTF2 and displayed noticeable dips in NTF2 accumulation at both its inner and outer edges. The edge-dip is reminiscent of those noted in the gels crosslinked by photomasking and the UV LED. While the cause of the dip is unclear, it may be the result of diffusion of fresh precursor solution into the edge region over the course of the crosslinking process. The fresh precursor then crosslinks, leading to a dense hydrogel edge that excludes NTF2 and mCherry. This hypothesis is consistent with the observation that a longer dwell time and fewer raster loops largely eliminated the edge dip. With fewer raster loops, there is less opportunity for diffusion of uncrosslinked precursor solution into the gel edge. It

should also be noted that the edge dip does not form when an entire droplet of precursor solution is crosslinked with the UV LED (i.e. no mask is used), further supporting the diffusion explanation. In any case, long dwell times and low loop numbers are clearly preferable with confocal crosslinking.

In conclusion, confocal crosslinking has a number of advantages over LED crosslinking, and a corresponding number of obstacles. Despite the attraction of testing selective transport using small hydrogel rings, we ultimately chose to use a much simpler hydrogel geometry and crosslinking method.

3.9 Dye-labeling and free dye

NTF2 must be labeled with a fluorescent dye before it can be used in diffusion experiments. Alexa Fluor 488 or fluorescein have both been used. When photobleaching is needed, as in the case of fluorescence recovery after photobleaching, fluorescein should be used, due to the extreme photostability of Alexa Fluor 488. There are several choices of labeling chemistry, including both NHS and SDP esters, which label the multiple exposed lysines of NTF2, and maleimide, which labels the cysteine of an engineered NTF2-cys. Both labeling chemistries result in bonds that eventually hydrolyze, cleaving the dye from the protein.

Free dye is a significant problem for diffusion experiments, since free dye (about 1kD) diffuses much faster than dye bound to a protein and is experimentally identical. Non-negligible levels of free dye would give the impression that NTF2 is equilibrating significantly faster than it actually is. With help from Annette Erbse, Eric Verbeke and I tested several protocols to optimize the dye-labeling process and minimize the presence of free dye. We found that the maleimide-labeling chemistry is more stable, but in practice not as efficient as the ester-labeling protocol. Ultimately we chose to use esters for labeling. Detailed protocols are described in Appendix A. Extensive washing during the protein re-purification step is crucial, as is immediate aliquoting and freezing of the labeled protein. Labeled protein should be used within a few days of thawing.

As soon as possible after a reaction, a BCA is performed to quantify the protein concentration in the labeled sample. Within 24 hours, the labeled protein is aliquoted and frozen. A small

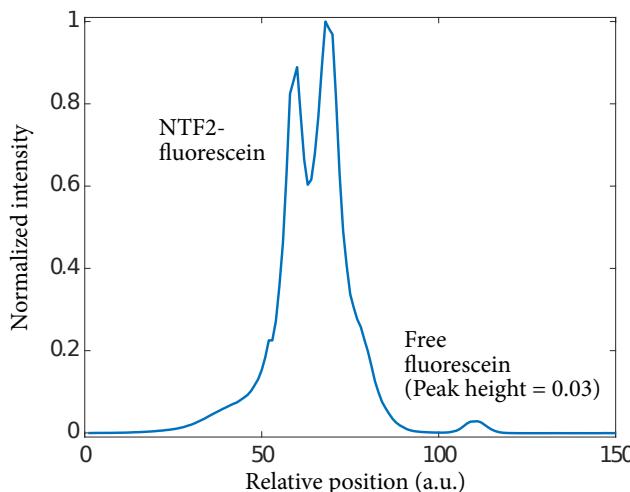


Figure 3.20: Line scan of fluorescence Typhoon image of native PAGE gel containing freshly-labeled NTF2-fluorescein sample. The height of the free dye peak is 3% that of the labeled protein peak.

sample is thawed and run on a native PAGE gel along with a sample of free dye. The gel is then imaged using a Typhoon fluorescence imager and stained with Coomassie. The intensity of each fluorescence band in the labeled protein sample is estimated with a line scan. Figure 3.20 shows a representative batch of labeled protein. Typically, the free dye band is 1-3% the amplitude of the labeled-protein band. Finally, I measure the absorbance of the labeled protein at 494 (check this wavelength) nm to calculate the concentration of Alexa488. I compare this measurement with the protein measurement from the BCA to calculate a labeling efficiency. Typical labeling efficiencies are 50-70%, lower than the reported values of 90% or more.

Mathematical analysis also suggests that free dye is minimal when the optimized protocol is used. A large dataset of accumulation curves was fit to either single or double exponentials and the resulting parameters and goodness of fit analyzed. While the accumulation curve should in fact be related to the error function (Sec. 4.2.1), exponentials were used to simplify the analysis.

If there is no free dye, as in the case of the mCherry accumulation, the accumulated intensity $I(t)$ can be approximated as

$$I(t) = A_1 \exp(-t/\tau_1) + C \quad (3.1)$$

with some amplitude A_1 , equilibration lifetime τ_1 , and constant offset C . In this case, a non-zero value of C is likely due to background fluorescence. If, on the other hand, a sample contains a population of small, free dye molecules as well as large labeled protein, both populations will

equilibrate at different rates, leading to an accumulated intensity of

$$I(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2) + C \quad (3.2)$$

where each population has an equilibration lifetime as well as an amplitude related to its abundance in the sample.

I fit a collection of 43 accumulation experiments to both Eqns. 3.1 and 3.2. I compared the resulting parameters as well as the adjusted R-square value of each fit. Adding more parameters to the fit will always improve the fit, but the adjusted R-square is intended to account for the effect of adding more parameters to a model. A higher adjusted R-square therefore means a better fit.

The adjusted R-square values did not significantly improve when the mCherry data was fitted to a double instead of a single exponential. The adjusted R-square values changed by an average of -0.04 ± 0.03 . In addition, the lifetimes and amplitudes of each component of the two-component fit were apparently uncorrelated. Both results support the fact that there is no free dye in the red channel, since the only fluorescence is coming from mCherry.

On the other hand, the NTF2 fits did result in a significantly higher (0.019 ± 0.006) adjusted R-square value on average with the double-exponential fit. Strikingly, the two components sorted themselves into two categories: a low-amplitude, short-lifetime component and a high-amplitude, long-lifetime one. The most straightforward interpretation is that the low-amplitude component is the free dye, which should be present in low amounts and equilibrate much more rapidly than labeled protein, thanks to its small size.

The median amplitude of the free-dye signal was 1.2% that of the labeled-protein signal, using a sample of 43 experiments, a result comparable to that seen in Fig. 3.20. The median free-dye equilibration lifetime was 50 minutes, as compared to approximately 2000 minutes for the labeled-protein lifetime. These results confirm that the new, more stringent dye-labeling protocol is successful in removing almost all free dye from the labeled-protein sample, and that the hydrolysis of the dye is negligible on the time scale of the experiment and enforced shelf life of the labeled protein.

Chapter 4

Bound diffusion measurements

Following up on the principles of the bound-diffusion theory, we wanted to see whether we could measure bound diffusion in a biomaterial inspired by the nuclear pore complex. In order to do that, we made Nup-filled hydrogels and measured the diffusion of transport factors and inert proteins within them. We measured the diffusion in a few different ways: monitoring the concentration profile and total accumulation as the proteins diffused into the cell, and using fluorescence recovery after photobleaching (FRAP).

4.1 Experimental setup

4.1.1 Hydrogels

Applying the lessons learned in the previous chapter, the nuclear pore mimics were 6% acrylamide hydrogels with FSFG-bis anchored in. The precursor solution had a final concentration of 6% premixed acrylamide/bisacrylamide (29:1) in PTB pH 7.0. The crosslinker was either 1 mM LAP or 0.1% ammonium persulfate and 0.5% TEMED (check these concentrations). Lyophilized FSFG-bis was resuspended in PTB, allowed to sit at room temperature for at least 20 minutes, and added to the precursor solution. I used nominal FSFG concentrations between 5 and 10 mg/mL, depending on whether it was FSFG concat 1 or FSFG concat 2. If a photoinitiator was used, it was added in the darkroom, and the solution was protected from light as much as possible afterwards.

After the precursor solution was thoroughly mixed, it was degassed in a vacuum desiccator for 10 minutes and immediately pipetted into a disassembled 400- μ m-thick PDMS gasket chamber.

Drops between 0.5 and 2 μL were carefully pipetted onto the plastic slide and the chamber assembled around the drops. Typically, each chamber measured a few centimeters on a side and contained a control gel with no Nups as well as one or more Nup-filled gels. The chamber was then illuminated with UV light at some intensity that I will look up as uniformly as possible for 30 seconds using a ThorLabs etc. LED. Condensation around the gels indicated that they had crosslinked. The chamber was immediately rinsed with at least 100 μL of PTB, filled with fresh PTB, and sealed with PDMS and clingwrap. The gels were left to soak at 4°C for at least 12 hours so that any remaining precursor solution and protein could leave the gel.

4.1.2 Influx experiments

After soaking in buffer, the buffer solution was removed by pipette or wicking with a Kimwipe (not by aspirating) and a fluorescent reservoir solution added. The reservoir solution contained 20 μM each NTF2-fluorescein (NTF2-F) and mCherry in PTB. The chamber was resealed after adding the solution. If a profile and accumulation experiment was being run, the experiment was run immediately after adding the reservoir solution. Experiments were run on an Olympus widefield using a 4x objective with FITC and TRITC filter cubes (should I put exact specifications here). Typical experiments were run for 120 minutes at a rate of one frame per minute. Exposure times were usually 30-100 ms with 3-8 dB gain in both channels. Minimal photobleaching took place over the course of these experiments. Data was analyzed as described below. After the end of an influx experiment, the chamber could be stored at 4°C, protected from light, for a further 12-24 hours in order to equilibrate. A FRAP experiment could then be performed.

4.1.3 Fluorescence recovery after photobleaching

Fluorescence recovery after photobleaching (FRAP) relies on the redistribution of fluorophores after patterned photobleaching in order to determine their diffusion constant. After a small portion of the hydrogel is bleached, the bleached spot gradually exchanges with the non-bleached fluorophores outside, and the average fluorescence intensity within the bleached spot recovers. The

recovery lifetime can be used to determine the fluorophore's diffusion constant, and the final recovered intensity as compared to the intensity outside the bleach spot can be used to determine the mobile fraction of fluorophore.

FRAP is often performed with a confocal microscope, but we were able to manage it with the Olympus widefield. In order to photobleach the gel, a 40x objective was used and the field of view exposed to near-UV (the DAPI cube - find precise specifications) at maximum power for 5 seconds. The objective was rapidly changed to 4x and a time series begun. Typical time series consisted of 15-30 frames taken as rapidly as possible (5-10 seconds per frame) and 30-60 frames taken at a slower rate (1-2 minutes per frame). Total experiment time ranged from 1 to 4 hours. Typical exposure times were 10 ms for NTF2-F and 40 ms for mCherry with a gain of 3 dB for both channels.

We had problems equilibrating the gels. Sometimes the gels were crosslinked inhomogeneously, and sometimes they didn't fully equilibrate even after 24 hours. Smaller gels ($0.5 \mu\text{L}$) helped solve the equilibration problem, but then the exchange of fluorescent proteins with the buffer was a major problem, because a significant fraction of the gel area was being bleached. We accounted for these problems during the data analysis, described below.

4.2 Influx analysis

In principle, the diffusion constants of NTF2-F and mCherry within the NPC mimics can be determined using the concentration profiles or total fluorescence accumulation within the hydrogel over time. We used several mathematical models to fit the profiles and accumulation in an attempt to extract diffusion coefficients. The noise inherent in the data made this difficult, and ultimately we used FRAP instead to find the diffusion constants.

Fickian diffusion was always assumed, although in practice the presence of binding and of the hydrogel network lead to slightly non-Fickian behavior. It was also assumed that the length of a binding event was negligible on the timescale of diffusion, so that the observed diffusion constant of the NTF2-F could be treated as Fickian diffusion and written as a weighted average of the diffusion

constants while free and bound. This is a good approximation because we think the dissociation constant of NTF2 and FSFG is $K_D \approx 1$ mM, with a diffusion-limited on-rate and correspondingly high off-rate.

4.2.1 Profile analysis

Using these assumptions, the method of [63] was followed in order to fit the profile and accumulation data. This method assumes that an arbitrary shape is initially empty but held in an infinite reservoir kept at a constant concentration. The shape fills according to the diffusion equation. Although there are slightly different numerical factors for different shapes, they show that a circle is a good approximation for many shapes. Since our gels are usually nearly circular, the circular solution is a good one. Using this method, the protein concentration $c(r, t)$ as a function of radial distance r from the gel center and time t is given by

$$\frac{c(r, t)}{c_0} = 1 - 2 \sum_{n=0}^{\infty} \frac{J_0(\alpha_n r)}{\alpha_n a J_1(\alpha_n a)} \exp(-\alpha_n D t) \quad (4.1)$$

where c_0 is the equilibrium concentration within the shape, D is the diffusion constant, a is the shape radius, and $\alpha_n a$ is the n th zero of the Bessel function of the first kind J_0 .

Mortensen et al define a characteristic timescale for equilibration $\tau = (\mathcal{A}/\mathcal{P})^2(\pi/4D)$ where \mathcal{A} and \mathcal{P} are the shape's area and perimeter, respectively. For a circle $\mathcal{A}/\mathcal{P} = a/2$, but this ratio can be numerically calculated for our hydrogels using their precise shapes. For times $t \ll \tau$, the concentration profile can be approximated as

$$c(r, t) = c_0 \operatorname{erfc} \left(\frac{r}{\sqrt{4Dt}} \right) \quad (4.2)$$

In order to fit to these equations, I averaged the intensity of a thin slice cutting through the reservoir and the gel to the center of the gel. I normalized the data by dividing by the average reservoir intensity (the entire reservoir in the field of view, not just the thin slice). I normalized on a time-point basis to eliminate drift in the illumination or reservoir concentration. After normalizing, I tried to fit the first time-points to Eqn. 4.2, both at a fixed time for many positions and vice

versa. The fits worked fairly well for the NTF2-F plots, as those had a well-defined gel edge. It was more difficult for the mCherry fits.

I also tried to use the full equation, Eqn. 4.1. I fit to the first 100 terms using Matlab, and that worked slightly better, but it was still difficult for the mCherry fits.

4.2.2 Accumulation analysis

Next, I tried to fit to the total accumulation. From Mortensen et al, the averaged intensity inside the gel $N(t)$ is

$$\frac{N(t)}{N_0} = 1 - \sum_{n=0}^{\infty} \frac{4}{(\alpha_n a)^2} \exp\left(-\frac{(\alpha_n a)^2 \pi t}{16\tau}\right) \quad (4.3)$$

I fit the first 100 terms of this in Matlab, but it didn't work terribly well either. This one worked better for mCherry than for NTF2-F, likely because binding was throwing off the NTF2 results. Overall, the most important data points were near the edge of the gel and the beginning of the experiment, and the edge effects and uncertainty in experiment start time meant that both of those types of points had lower signal-to-noise than the rest of the experiment.

4.2.3 Gel dimension estimations

In order to fit the influx and FRAP data, I needed to estimate several parameters of the gel geometry: the radius of the gel, its perimeter, and its area. Although the gels were not perfectly circular, most were close, and so I took the radius to be half of the longest dimension in the video's field of view. Typically, the entire gel did not fit into the field of view. To estimate the radius, I used the mask of the entire gel and counted the number of non-zero elements by row. I took the maximum number of nonzero elements, divided by two, and multiplied by the pixel spacing: 1.58 μm per pixel for the 4x objective on the Olympus widefield.

To estimate the area and perimeter, I embedded the image in a larger frame, and created a ROI that covered the entire gel, estimating where necessary. I summed the non-zero elements of the ROI and scaled using the pixel spacing. For the perimeter, I called the Matlab function that returns only the outline of an ROI, and summed and scaled that.

4.2.4 Partition coefficient and bound probability calculations

In order to calculate the bound diffusion coefficient, I needed to estimate the amount of time that a given transport factor spends bound to Nups. When the system is in chemical equilibrium, the concentration of free transport factor (T), free Nup (N), and transport factor - Nup complex (C) is related to the dissociation constant K_D by $K_D = NT/C \approx N_T T/C$ in the linear approximation $N \approx N_T$ when N_T is the total Nup concentration, both free and bound, and is a constant. The fraction of transport factors that are bound is then given by

$$p_B = \frac{C}{C+T} = \frac{C}{C + \frac{CK_D}{N_T}} = \frac{1}{1 + \frac{K_D}{N_T}} \approx 1 - \frac{K_D}{N_T} \quad (4.4)$$

where the final step applies if $K_D \ll N_T$, which might be the case but now I'm confused again.

It's difficult to measure the total Nup concentration anchored to the hydrogels, so we'd like to remove that term in favor of an experimental quantity that's easier to measure. The partition coefficient is much easier to measure. The partition coefficient is the ratio of a fluorescent protein's concentration just inside and just outside an equilibrated hydrogel. To measure it, just take the ratio of the average intensity within the gel to that in the reservoir. The concentration c_0 of the inert protein and the transport factor is equal in the reservoir. If γ_T is the partition coefficient of the transport factor and γ_I that of the inert protein, then the transport factor concentrations can be expressed as

$$T = \gamma_I c_0 \quad (4.5)$$

$$C = T_T - T = \gamma_T c_0 - \gamma_I c_0 \quad (4.6)$$

The total transport factor concentration within the gel is $T_T = T + C$ and is a constant. Therefore, within the gel, the chemical equilibrium condition can be expressed as

$$\frac{K_D}{N_T} = \frac{T}{C} = \frac{\gamma_I c_0}{\gamma_T c_0 - \gamma_I c_0} = \frac{\gamma_I}{\gamma_T - \gamma_I} \quad (4.7)$$

Combining Eqns. 4.4 and 4.7, the bound probability can be expressed only in terms of the partition coefficients as

$$p_B = \frac{1}{1 + \frac{K_D}{N_T}} = \frac{1}{1 + \frac{\gamma_I}{\gamma_T - \gamma_I}} = 1 - \frac{\gamma_I}{\gamma_T} \quad (4.8)$$

4.2.5 Bound-diffusion calculation

Once the observed diffusion constants for NTF2 and mCherry have been calculated, along with the bound probability (using the partition coefficients), the bound diffusion is given straightforwardly by the weighted average

$$D_{\text{obs,TF}} = p_B D_B + (1 - p_B) D_F \quad (4.9)$$

Taking the free diffusion coefficient of the transport factor to be approximately equal to the observed diffusion of the inert protein ($D_F = D_{\text{obs,I}}$), the bound diffusion coefficient of the transport factor is

$$D_B = \frac{D_{\text{obs,TF}} - (1 - p_B) D_{\text{obs,I}}}{p_B} \quad (4.10)$$

4.3 FRAP analysis

4.4 Discussion

Chapter 5

FG Nup aggregation under crowded conditions

Disordered proteins are often prone to aggregation, often causing disease, and the aggregation behavior of FG Nups is important to understand. It is not clear what the aggregation state of Nups is within the pore or how their aggregation might play into nuclear transport [?]. *In vitro*, many Nups spontaneously aggregate into amyoids over the course of a few hours, but there is evidence that this aggregation does not happen in the cellular environment [?, 17]. We investigated aggregation behavoir of an aggregation-prone Nup fragment in a number of different crowders. Inert crowders such as PEG and PVP help mimic the extremely crowded cellular environment, but they may interact different with the protein than nonspecifically-binding crowders such as cell lysate or BSA. We wanted to understand how different crowders affected the aggregation properties of Nups.

We used a 124-amino-acid fragment of the Nup Nsp1. The FG-repeat segment of Nsp1 contains an aggregation-resistant portion (used as the basis for the FSFG peptide) as well as one that aggregates in buffer over the course of a few hours. This aggregation-prone portion is the basis of the FG124 peptide used in the following aggregation experiments.

We used aggregation timecourses with thioflavin T as a readout, as well as fluorimetry, NMR, and x-ray scattering. Our conclusion is something about the phenylalanines and how they behave differently in when crowded with PEG than with PVP (which also has an aromatic ring).

5.1 Results

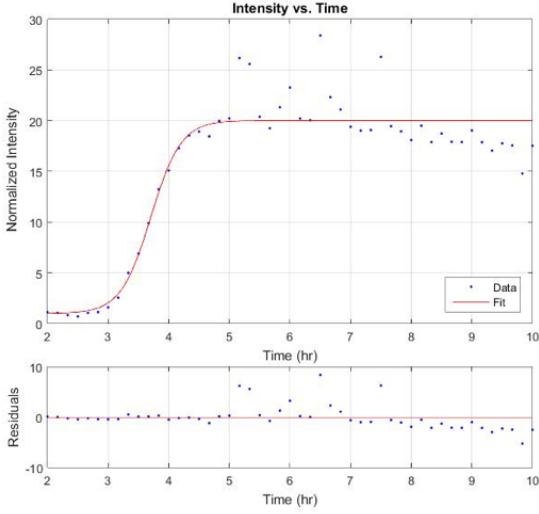
5.1.1 Thioflavin timecourses

We analyzed aggregation dynamics using thioflavin T, a fluorophore whose excitation and emission maxima shift and which grows much brighter in the presence of thioflavin. Samples containing FG124, crowding agent, and thioflavin T were incubated while shaking overnight in a 96-well plate and their fluorescence intensity recorded every ten minutes. The resulting traces show a typical aggregation pattern, with a lag phase, burst phase, and saturation phase.

Thioflavin T (ThT) is a dye that grows much brighter when bound to amyloids. Upon binding, its absorption maximum shifts from 385 nm to 450 nm and its emission maximum from 445 nm to 482 nm [64]. Although ThT is a more reliable indicator of amyloids than other fluorescence methods, notably Congo red stain, it suffers from reproducibility problems. There is no consensus on the mechanism of ThT binding to amyloids. Some proposed mechanisms rely on the presence of ThT micelles, which form above a critical concentration of about 4 μ M, while others advocate for avoiding micelles [65, 66]. There is some evidence that amyloid fibrils can adsorb to the plastic surface of a multiwell plate, decreasing ThT fluorescence intensity as the fibrils mature [67]. Often in our timecourse experiments, the ThT fluorescence did reach a maximum and then decrease. The fluorescence intensity also depends on the sample pH, an effect which we noticed in our timecourses. Despite these challenges, thioflavin T is the most consistent dye for detecting the process of amyloid formation.

The final concentration of crowding agent was 19% serine w/v, 13% PEG, and 13% PVP. Lysate concentration varied between time courses and was in the 1-10 mg/mL range. Two timecourses were run with varying PEG and PVP concentrations: 25%, 20%, 13%, and 5%. Samples with no crowding agent ('buffer' samples) were run as a reference, and samples in 7M guanidine hydrochloride (GuHCl) with no crowding agent were run as negative controls in each timecourse. No aggregation was observed in the GuHCl samples. In every case, blanks were run alongside the sample conditions. In the blanks, the FG124 was omitted.

Figure 5.1: Sample sigmoid fit. Look up conditions. (Pulled this from Sophie's thesis but I did the fit)



First the data were normalized to the blanks. In nearly all cases, the blank intensity remained steady over time, as expected. In those cases, the mean blank intensity was subtracted from the corresponding data. (In cases where the blank intensity changed over time, it was subtracted pointwise from the data.)

Then the normalized curves were fit to a sigmoid given by

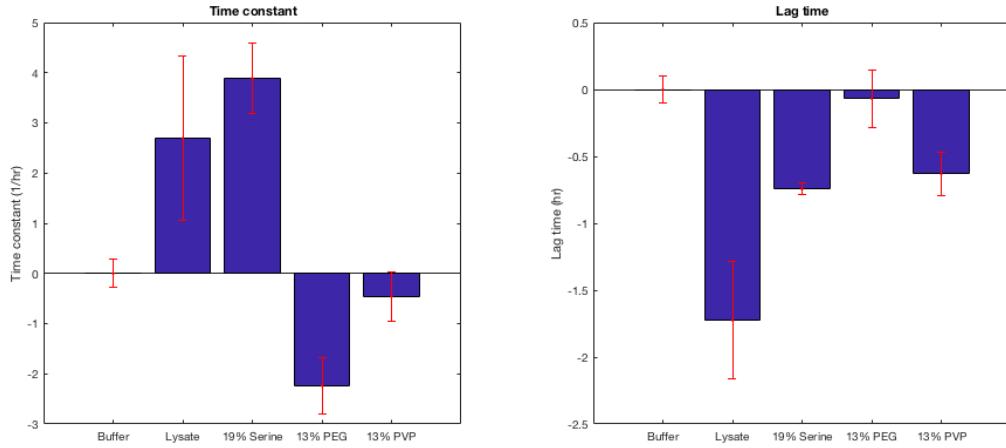
$$I(t) = C + \frac{A}{1 + \exp(-k(t - T_{1/2}))} \quad (5.1)$$

where $I(t)$ is the normalized fluorescence intensity as a function of time. The useful physical parameters for our purposes are the time constant k , which gives a measurement of the steepness of the slope at the beginning of the burst phase, and the lag time T_l . The lag time is calculated as

$$T_l = T_{1/2} - \frac{2}{k} \quad (5.2)$$

and represents the duration of the lag phase [].

Ideally, we expect $C = 1$, as aggregation should not have begun at the start of the experiment, so there should be no increase in sample fluorescence over that of the blank. Experimentally, C usually ranged between 1 and 2, up to about 10 for some sample conditions. This might have been

Figure 5.2: test

because aggregation had already begun, but this seems unlikely because the lag phase continued for some time. I don't know why C wasn't close to unity for all conditions and replicates.

The saturation phase asymptotes to an intensity given by $I_{\text{sat}} = C + A$. We found significant variation in I_{sat} for the same condition between timecourses, and the relative magnitudes of different conditions also varied between timecourses. I think this is a limitation of the thioflavin tests. Therefore, we do not consider I_{sat} in our analysis, leaving k and T_l as parameters of interest.

Next we normalized once again to account for differences between timecourses. It was impossible to hold the concentration exactly fixed between timecourses, and there were probably some other environmental variables we couldn't control perfectly, so it was important to normalize again. Within each timecourse, we averaged the fit parameters for all buffer replicates and subtracted that average from each other replicate.

We then combined replicates from all timecourses by condition and took the final average and standard error of the mean for both fit parameters. The results are shown in Fig. 5.2. I ran a one-factor ANOVA to reject the null hypothesis that all means were the same, and then two-sample t-tests on each pair of conditions to find which differences were statistically significant. For the time constant, all pairs of conditions were significantly different from each other with $p < 0.05$.

except buffer-PVP. For the lag time, all pairs of conditions were significantly different from each other with $p < 0.05$ **except** buffer-PEG and serine-PVP.

I followed the same process for the timecourses involving the concentration series for PEG and PVP, but there were fewer significant differences between concentrations, as seen in Fig. 5.3. The ANOVA for the PEG time constants gave $p = 0.0024$ with the t-test showing differences between: buffer-25% PEG, buffer-13% PEG, and 25% PEG-20% PEG. The ANOVA for the PEG lag times was likewise significant with $p = 0.0102$. The significant pairs were: buffer-25% PEG, buffer-20% PEG, buffer-5% PEG, and 25% PEG-13% PEG.

For PVP, there were no significant differences in the time constant. For the lag time, after the ANOVA and t-tests, there were differences between: buffer and everything except 5% PVP, 25% PVP and everything but 20% PVP, and 20%PVP-13% PVP.

For both conditions, it looks roughly like the lag time increases with the crowder concentration, but given the large error bars, I wouldn't read too much into it. The time constants don't follow any particular trend and are mostly indistinguishable anyway.

We tested the effects of pH on FG124 aggregation by running several experiments in buffer at varying pH. We ran six replicates of each condition, for pH values between 5 and 8. No significant differences were found in the lifetimes or lag times of any pH condition. Results are summarized in Table 5.1. We concluded that aggregation is not affected by pH in the range 5-8.

Table 5.1: FG124 aggregation lifetime and lag time with varying pH. Each condition run with 6 replicates in PTB buffer. One-way ANOVAs show no statistically significant differences between conditions. Standard errors are shown.

pH	Lifetime τ (hr)	Lag time T_{lag} (hr)
5	0.34 ± 0.09	6.8 ± 0.1
6	0.35 ± 0.12	6.2 ± 0.2
7	0.38 ± 0.14	6.8 ± 0.4
8	0.50 ± 0.14	6.6 ± 0.2

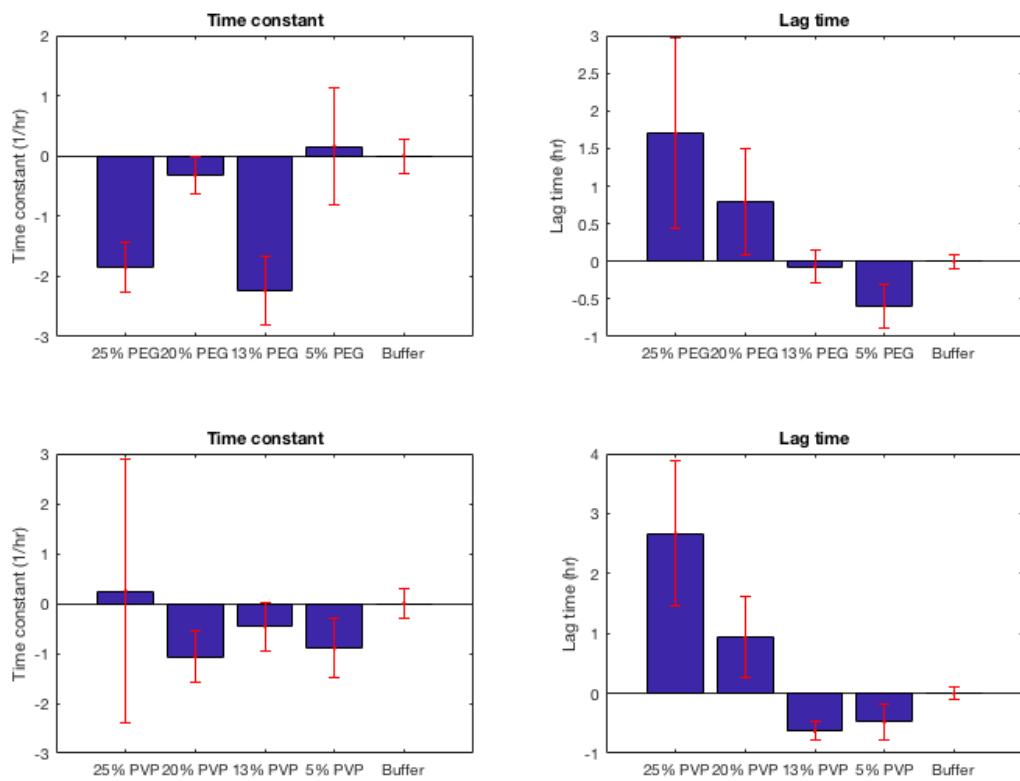
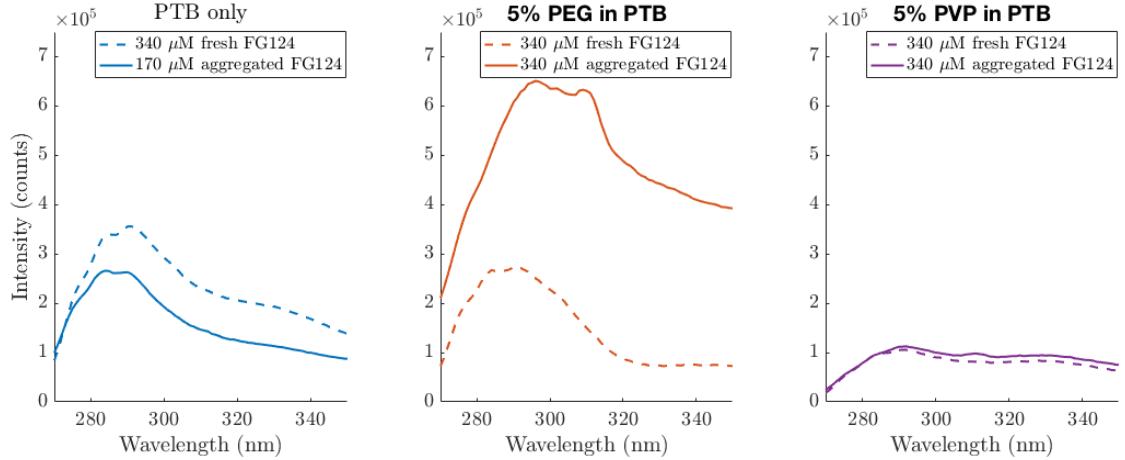
Figure 5.3: test

Figure 5.4: Emission scan of fresh and aggregated FG124 in crowded conditions. Data normalized by subtracting blank sample.



5.1.2 Fluorimetry

We hypothesized that the crowder-dependent difference in aggregation might arise from the aromatic ring in PVP, which could interact with the phenylalanine in FG124 in a ring-stacking interaction. To test this idea, I collected emission spectra of fresh and aggregated FG124 in PEG and PVP crowders near the phenylalanine peak wavelength (see Fig. 5.4). Only 5% PEG and PVP in PTB were tested because PEG has a peak near the FG124 peak which dominates at higher PEG concentrations. Data were normalized by averaging over two runs and subtracting a blank run (containing crowder and buffer but no protein). The PEG sample showed the largest difference upon aggregation, both in peak height and location. Figure 5.5 shows the same data, normalized to a maximum amplitude of one and offset, in order to emphasize the changes in peak shape. In both crowder conditions, but not in the buffer condition, a small peak appears in the aggregated FG124 near 310 nm.

Additionally, I compared the phenylalanine peaks in FSFG and fresh and aggregated FG124 to those of pure phenylalanine [68], as seen in Fig. 5.6. Data are blanked and normalized to a maximum intensity of one. The peak slightly shifts toward longer wavelengths as the data progress from phenylalanine to FSFG to fresh and aggregated FG124. I also measured PEG and PVP

Figure 5.5: Emission scan of phenylalanine, FSFG, and FG124. Excited at 240 nm. Phe data is not mine, need to check reference. Data normalized to a maximum amplitude of 1.

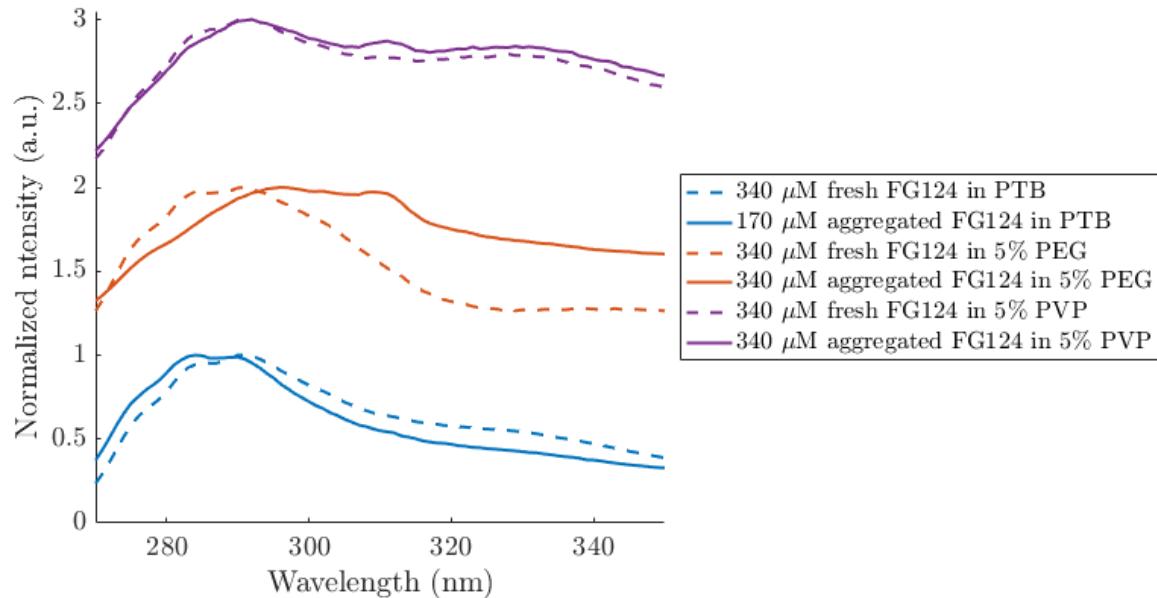


Figure 5.6: Emission scan of phenylalanine, FSFG, and FG124. Excited at 240 nm. Phe data is not mine, need to check reference. Data normalized to a maximum amplitude of 1.

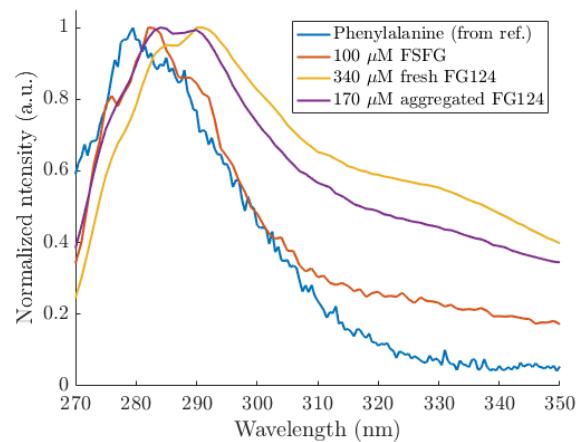
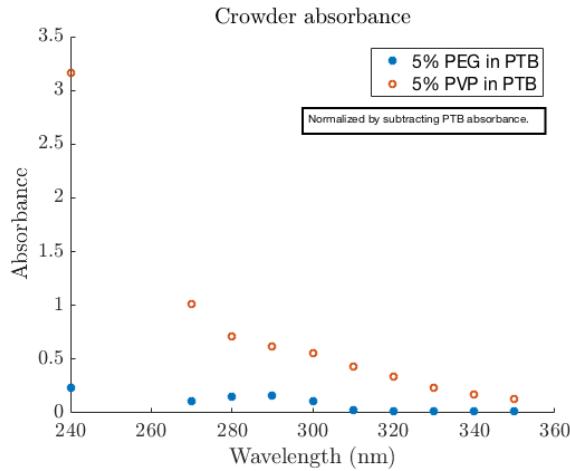


Figure 5.7: Absorbance of 5% PEG and PVP solutions in PTB. Normalized by subtracting PTB absorbance.



absorbance near 240 nm (see Fig. 5.7). PVP has a very high absorbance in that range, which might explain why the recorded counts were lowest for the PVP conditions.

5.2 Discussion

It's possible that the differences in aggregation time between PEG and PVP come from changes in viscosity. The two samples do have widely different viscosity, as measured by Steve Whitten. A 13% PEG solution in PTB has a dynamic viscosity of 15.87 mPa s, while that of a 13% PVP solution in PTB is 7.34 mPa s. The literature doesn't entirely agree on what happens to aggregation as a function of viscosity, but what we see could be due to that change. (Check in lab book where I have lit search notes.)

5.3 Materials and Methods

5.3.1 Buffers

Potassium transport buffer (PTB) (150 mM KCl, 20 mM HEPES, 2 mM MgCl₂) was used for all timecourse and NMR samples.

5.3.2 FG124 preparation

His-tagged FG124 was expressed in *E. coli* in the plasmid pRSF. Cultures were grown in LB and induced at 37 degrees C for 2-4 hr with 1 mM IPTG at OD 0.6-0.8. Periplasmic matrix was removed prior to lysis. Cells were then lysed via sonication and FG124 purified using TALON cobalt resin. All purification buffers were PTB with 7M GuHCl and PIC. The elution buffer also contained 250 mM imidazole.

5.3.3 Timecourse preparation

Stocks of PEG and PVP in PTB were prepared at 20 or 40% w/v; serine stocks were prepared at 30% w/v. PEG and serine were at pH 7; PVP was pH 7 or pH 5. A pH series with no crowding agent showed no significant differences based on pH. Lyophilized lysate was prepared by homogenizing BL21 DE3 Gold cells and spinning them down. The supernatant was lyophilized in a decomposing ammonium bicarbonate buffer and resuspended in PTB to the desired concentration when needed. A 10 mM stock solution of ThT in PTB was prepared and filtered no more than a week before the timecourse, stored at room temperature and protected from light. Immediately prior to starting the timecourse, FG124 was desalted into PTB to remove the imidazole and GuHCl. Samples were promptly prepared containing the appropriate percentage of crowder, a final concentration of 1-2 mg/mL FG124, and 200 uM thioflavin T. All samples in the same timecourse had the same concentration of FG124, including the buffer sample, which contained no crowding agent. Blanks were prepared with crowding agent and thioflavin T, but no FG124. Samples were pipetted into black, flat-bottomed, clear-bottomed 96-well plates with 150 uL per replicate. Each sample yielded four to six replicates. Only one blank replicate was used per condition. One negative control and corresponding blank were prepared per timecourse containing 7M GuHCl and no crowding agent but using the same protein sample as all other conditions. Each well contained a 3mm-diameter glass or teflon bead. The plate was sealed with a PCR seal and taken to a Safire II plate reader. The fluorescence was measured from the bottom at 10-minute intervals with an

excitation wavelength of 450 nm, emission wavelength of 482 nm, and 5 nm bandwidths. The plate shooook orbitally at high speed between measurements and was held at a temperature of 30 degrees C. The time between desalting and beginning the plate reader measurements was typically about an hour; the time of desalting was taken as $t = 0$ for the purposes of calculating lag time. In parallel with the sample preparation, the concentration of the desalted FG124 was measured with a BCA assay.

5.3.4 NMR sample preparation

5.3.5 NMR experiments

5.3.6 Fluorimetry

FG124 was purified as described above and stored in PTB with 7M GuHCl. Immediately before use, 130 uL of 520 uM FG124 was desalted with a Zeba spin desalting column to remove the GuHCl. The resulting stock was used in the crowder samples, which had a final concentration of 340 uM Fg124. I used the shared-instrumentation fluorimeter. I tested PTB, 5% PEG (MW, source, purity?) in PTB, and 5% PVP (same questions?) in PTB as blanks. I measured 340 uM FG124 in PTB and the two crowders as well. Between runs, I cleaned the cuvette (micro quartz cuvette from Kaar lab (sample volume?)) with ethanol 3x, then 5x with DI water, and gently blotted the outside with ethanol. Fluorimeter settings: 4 nm slits, 1000 V PMT, excitation wavelength of 240 nm. Step size = 1nm, average over 2 runs. After taking this data with fresh FG124, I let the samples sit at room temp overnight to aggregate (no shaking) and most were cloudy in the morning. I took similar data with the aggregated samples. I needed to rinse with 7M GuHCl, let soak in 7M GuHCl for 5 minutes, and then perform the same cleaning procedure as the previous day in order to remove the aggregates from the cuvette.

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Appendix A

Protein expression and purification

A.1 Purification of his-tagged protein with metal affinity column

Most proteins used in this work contain a C-terminal 6x histidine tag. These proteins can be purified using a cobalt or nickel affinity column. Proteins purified with this protocol include all Nup variants (FSFG, FG124, etc.), NTF2, mCherry, and GFP. This protocol is designed for the purification of one 0.5-L cell pellet. If running more than one column at once, adjust the total buffer volumes accordingly.

This protocol is used for all Nup variants. For FSFG variants, use the urea concentrations noted in the buffer table. For FG124 purifications, use 7M guanidine hydrochloride in all buffers (including elution) instead of urea. Always make urea and guanidine hydrochloride solutions the same day they will be used. **Do not use urea, guanidine hydrochloride, or other denaturants when purifying ordered proteins.**

To prevent aggregation, BME must be used for any protein that contains two cysteines. **Do not use BME if not necessary; any BME solutions must be disposed of in the hazardous waste container in the fume hood.**

Add the protease inhibitor cocktail (PIC) stock in DMSO immediately before using the buffer. PIC has a lifetime of about 30 minutes in aqueous solution. Store all buffers on ice before use. Incubations should be done at 4°, but the column can be used at room temperature.

Figure A.1: Buffer guide for cobalt or nickel affinity column purifications.

Buffer	Use (and volume)	Composition
Lysis Buffer	for cell lysis (15 mL)	Buffer of choice 1x (15** mL) 1:1000 PIC (15uL) (8M Urea) (7.2 g) (3mM BME) (3 uL)
Wash I	for wash I (20 mL)	Buffer of choice 1x (20** mL) 1:1000 PIC (20uL) (3M Urea) (4 g) (3mM BME) (4uL)
Wash II *the imidazole is optional; can be useful for removing junk bound to column	for wash II (20 mL)	Buffer of choice 1x (20** mL) 1:1000 PIC (20 uL) (10mM Imidazole) (.013 g) (3mM BME) (4uL)
Elutions	20 mL total	Buffer of choice 1x (20** mL) 1:1000 PIC (20uL) 250mM Imidazole (.32 g) (3mM BME) (4uL)
Handy Molecular Weights	Imidazole: 68.08 g/mol Urea: 60.06 g/mol BME: 14.3 M GuHCl: 95.53 g/mol	**Bring up to volume with buffer. Will not need full 20- <u>25mL</u> if using urea

- (1) Remove the periplasmic matrix, if not already done (Sec. B.2).
- (2) Lyse the cells. Add 15 mL of lysis buffer to thawed 0.5-L pellet. Resuspend pellet by pipetting up and down or vortexing and then lyse by sonicating. Keep solution on ice. lookup: sonicator brand. Sonicate for at least two minutes total, in 30s-on, 60s-off pulses. Power delivered to sample should be at least 20 W. Centrifuge resulting lysate for about 15 mins on top speed of either floor centrifuge to pellet cellular debris.
- (3) Prepare the metal-affinity column. Gently resuspend the resin into a slurry by slowly turning the bottle. The beads will be crushed if shaken vigorously. Into a disposable plastic column, pipette enough slurry to contain 3.5-4 mL of beads once the storage buffer has drained out (typically 6-8 mL of slurry, if beads and buffer are stored in a one-to-one mixture).
Equilibrate the column by running 5-10x the bed volume (25-50 mL) of buffer through the column. Do not let the column run dry at any point in the purification.
- (4) Add supernatant to column and nutate for one hour at 4°C.
- (5) Drain the column. Add wash I buffer and nutate at 4°C for 10 minutes.
- (6) Drain the column. Add Wash II buffer, nutate at 4°C for 10 minutes, and drain.
- (7) Elute and collect the protein. Three elution methods can be used:
 - (a) *Fractional elution:* Prepare a row of eppendorfs. Add 5-10 mL elution buffer to open column and catch the draining liquid in fractions with 0.5-1.0 mL (8-16 drops) per eppendorf. Do not let the column dry. After all elutions are completed, use the Bradford test to pool the fractions with similar protein concentrations. **The Bradford test is unreliable for Nup variants and batch elution should be used.** Fractional elution gives the highest protein concentration and should be used where possible.

- (b) *Batch elution:* Add a bed volume of elution buffer to column and let drain to remove waste buffer from column. Watch resin color change carefully so as not to lose protein. Add 3-5 mL of elution buffer and collect flow-through.
- (c) *Nutated elution:* Add 5-10 mL of elution buffer to sealed column and nutate for 10-30 minutes. Collect flow-through.

Early elutions should be fractional or batch elutions.

9) Clean and store the column. Run 5 bed volumes of MES (lookup: precise components) through the column, then 5 bed volumes DI water. Run about 20 mL MES buffer through the column, then 20 mL DI water. Store 1:1 in 20% ethanol.

A.2 Periplasmic matrix removal (PPMR)

The periplasmic matrix (PPM) contains proteases and debris that binds to metal affinity columns. Removing the PPM before protein purification significantly increase the yield of his-tagged disordered proteins.

Note: Keep both solutions on ice. Resuspension of pellets should be done by gently pipetting up and down and swirling the tubes. Do not vortex to resuspend. Rough treatment of the cells may lyse them.

- (1) Spin down cell culture at 4000g (Sorvall centrifuge, GSA rotor) for 10 minutes at 4°C.
- (2) Resuspend pellets in at least 50 mL cold SHE buffer (20% Sucrose, 50mM HEPES, 1mM EDTA pH 7.9) per liter of culture. Keep tubes on ice.
- (3) Spin down for 10 minutes at 5000 rpm at 4°C.
- (4) Resuspend pellets in at least 50 mL cold 5mM MgSO₄ per liter of culture.
- (5) Incubate tubes on ice for 10 minutes.
- (6) Spin down for 10 minutes at 5000 rpm at 4°C.

- (7) Proceed to purification or flash-freeze tubes in liquid nitrogen and store in ultra-low freezer.

A.3 Lyophilization

Lyophilization refers to freeze-drying proteins. It's a good way to store a known mass of protein before resuspension in hydrogel precursor solution. All lyophilization in this work was done using a Labconco freeze dry system. Lyophilization concentrates any salts in the sample buffer. Therefore, whenever possible, a decomposing buffer should be used. A 25 mM ammonium bicarbonate buffer was used for all lyophilized Nup variants. This buffer decomposes into carbon dioxide, ammonia, and water when lyophilized or above 36°C.

- (1) Dialyze sample into 25 mM ammonium bicarbonate if possible.
- (2) Perform a BCA to quantify the protein concentration in the sample. Prepare eppendorf aliquots that contain the desired mass of protein (usually 100 or 200 µg).
- (3) Cover the aliquots with parafilm and use a needle to punch a hole in the covering.
- (4) Flash-freeze the aliquots.
- (5) Follow lyophilizer instructions. Keep samples frozen and load as quickly as possible to avoid thawing. Ensure that the vacuum is below 50×10^{-3} mBar.
- (6) Leave aliquots on lyophilizer at least 12 hours.
- (7) Remove aliquots from lyophilizer. Remove parafilm and close eppendorfs. Store with desiccant in ultra-low freezer.

Appendix B

Calculation of PEG hydrogel pore size

The following equation was used to estimate pore size in a 10 wt % PEG hydrogel with 20-kDa 8-armed PEG-norbornene and 1-kDa PEG-dithiol crosslinker [69].

$$\frac{1}{M_c} = \frac{2}{M_n} - \frac{\left(\frac{\bar{v}}{v_1}\right) (\ln(1 - v_{2,s} + v_{2,s} + \chi v_{2,s}^2))}{v_{2,r} \left(\left(\frac{v_{2,s}}{v_{2,r}}\right)^{1/3} - \frac{1}{2} \left(\frac{v_{2,s}}{v_{2,r}}\right) \right)} \quad (\text{B.1})$$

with:

The average molecular weight between crosslinks as M_c .

The average polymer molecular weight before crosslinking $M_n = 20$ kDa.

The specific volume of polymer $\bar{v} = 0.8 \text{ cm}^3/\text{g}$ for 1.5K PEG.

The molar volume of the swelling agent $v_1 = 18 \text{ g/mol}$ for water.

The Flory PEG-water interaction parameter $\chi = 0.4$ [70].

The polymer volume fraction before swelling $v_{2,r} = 0.10$ for a 10 wt % hydrogel.

The polymer volume fraction after swelling $v_{2,s} = (\rho_p(\frac{Q_m}{\rho_s} + \frac{1}{\rho_p}))^{-1}$ [71] with:

The solvent density $\rho_s = 1 \text{ g/cm}^3$ for water.

The polymer density $\rho_p = 1.2 \text{ g/cm}^3$ for 20-kDa PEG (Santa Cruz Biotech).

The mass ratio of solvent to polymer $Q_m = 9$.

This calculation gives me $M_c \approx 210$ Da as the average molecular weight between crosslinks.

The mesh size ξ can be estimated using [69]

$$\xi = v_{2,s}^{-1/3} \ell \left(\frac{2M_c}{M_r} \right)^{1/2} c_n^{1/2} \quad (\text{B.2})$$

with:

The carbon-carbon bond length $\ell = 1.54 \text{ \AA}$.

The molecular weight of the repeating polymer unit $M_r = 67 \text{ Da}$ for PEG.

The “characteristic ratio” for PEG $c_n \approx 4$.

The result is an estimated mesh size on the order of 1 nm.

Appendix C

Bis-labeling Nup fragments for incorporation into acrylamide hydrogels

Before FSFG-cys or any other cys-labeled Nup variant can be tethered to an acrylamide hydrogel, it needs to be labeled with bisacrylamide or PEG-DA. This protocol describes the labeling procedure for either chemical group. The cysteines must be fully reduced for the labeling to occur. No BME, TCEP, or other reducing agent can be present in the reaction mixture. FSFG and other disordered peptides form disulfide bonds within minutes of being removed from reducing agents, so begin the reaction as quickly as possible after reducing. This protocol was developed with the help of Benjamin Fairbanks. Following labeling, the extent of labeling can be quantified using an Ellman's reagent assay.

C.1 Bis-labeling reaction protocol

- (1) Begin with a stock of at least 1 mg/mL FSFG with a terminal cysteine in PBS pH 7.8. Typical reactions use 1 mL of approximately 2 mg/mL FSFG.
- (2) Equilibrate an equal amount of immobilized TCEP resin slurry in a disposable 5-mL column which can be spun in a conical tube. Refer to Thermo-Pierce product reference sheet for resin volume and nutation time if necessary. Equilibrate with 20-30 bed volumes of PBS pH 7.8 using gravity, then spin at 161g for 10 s to remove remaining buffer.
- (3) Immediately add the FSFG stock and nutate at 4°C for 1 hour.

- (4) Prepare a conical tube with 40 μL of triethanolamine (TEA, in fume hood) and 50 μL 2% bisacrylamide solution. This will lead to a 10-fold molar excess of bisacrylamide over protein and 300 mM TEA in the final reaction mixture. Place column in conical tube and spin down 1000 rpm for 1 minute.
- (5) Immediately vortex thoroughly. Nutate at room temperature for 30 minutes.
- (6) Dialyze into 25 mM ammonium bicarbonate buffer to remove excess bisacrylamide and prepare for lyophilizing.
- (7) Perform a BCA to quantify protein concentration. Prepare 100 or 200 μg aliquots, freeze, and lyophilize. Store lyophilized protein with a desiccant in ultra-low freezer.

C.2 Ellman's reagent assay protocol

This protocol was taken from Thermo-Fisher's protocol and modified for a microwell plate. Due to the rapid disulfide bond formation of FSFG, the reactants must be mixed very rapidly once reducing agent is removed.

- (1) Equilibrate 80 μL TCEP resin slurry in each of two disposable 1-mL spin columns which can be spun in an eppendorf centrifuge. Refer to Thermo-Pierce product reference sheet for resin volume and nutation time if necessary. Equilibrate with 20-30 bed volumes of PBS pH 7.8 using gravity, then spin at 2300g for 10 s to remove remaining buffer.
- (2) Add 80 μL of bis-labeled FSFG in 25 mM ammonium bicarbonate to one column and a known concentration of unlabeled FSFG cys (as a control) in 25 mM ammonium bicarbonate to the other. Nutate for two hours.
- (3) While incubation proceeds, prepare the reaction buffer (0.1 M sodium phosphate buffer pH 8.0 with 1 mM EDTA) and the Ellman's reagent solution (ERS, 4 mg/mL Ellman's reagent in reaction buffer).

- (4) Prepare a 96-well plate with wells containing 1.8 μM ERS and 29.5 μM reaction buffer. Prepare wells for the FSFG-bis and FSFG cys samples but do not add the protein until everything has been prepared. Prepare a well containing ERS, reaction buffer, and 68 μL of 25 mM ammonium bicarbonate buffer. Finally, prepare a well containing ERS, reaction buffer, and 68 μL of unlabeled FSFG cys of a known concentration that has not been reduced.
- (5) Spin down both columns of TCEP solution 2300g for 30 s in an eppendorf centrifuge and collect the flow-through. Very rapidly, add 68 μL of flow-through to the appropriate wells and mix by pipetting up and down.
- (6) Incubate plate at room temperature for 15 minutes.
- (7) Measure the absorbance at 412 nm using a plate reader.
- (8) Calculate labeling efficiency. Use the buffer well's absorbance to blank the absorbance of the reduced FSFG cys sample and the labeled FSFG sample. Divide the blanked absorbance of the labeled sample by that of the reduced FSFG cys sample and subtract the resulting ratio from 1. The unreduced FSFG sample is not directly used but is a good check on the results.

Appendix D

Hydrogel precursor solutions

This appendix contains details of hydrogel precursor solutions. Lyophilized protein should be resuspended in the buffer component of the precursor solution and incubated at room temperature for at least 20 minutes before adding the other components. No-Nup control gels simply omit the protein. Precursor solutions must be degassed for 10 minutes and promptly polymerized. All solutions containing photoinitiator must be protected from light at all times. Mix under red light only. Prepare precursor solutions immediately before use.

D.1 PEG hydrogel precursor recipes

All PEG hydrogels were 10 wt % PEG with and 0.5 thiol-ene ratio. For more accurate pipetting, stock solutions were designed to be combined in equal volumes.

Table D.1: PEG hydrogel precursor stocks

Component	Concentration	Buffer
20-kDa PEG-norbornene	438 $\mu\text{g}/\mu\text{L}$	Water
1-kDa PEG-dithiol crosslinker	45 $\mu\text{g}/\mu\text{L}$	Water
8-kDa PEG-dithiol crosslinker	360 $\mu\text{g}/\mu\text{L}$	Water
Irgacure 2959	2 mM	Water
LAP	2 mM	Water
TCEP	4 mM	4x PTB

D.2 Acrylamide hydrogel precursor recipes

The monomer and crosslinker were bought premixed from BioRad (acrylamide/bisacrylamide 30% 29:1) but potentially could be prepared in different ratios and mixed separately. Any additional components, such as dextran or photoinhibitor, should be made into a stock with PTB and used instead of the buffer component in the recipe. Acrylamide hydrogels were mostly crosslinked with LAP photoinitiator, but sometimes with the APS/TEMED chemical crosslinking system. Results of several APS/TEMED concentrations in a 6% acrylamide precursor solution are shown in Table D.5.

Table D.2: Sample precursor soution recipe (10 wt % PEG, 10 mg/mL nominal Nup concentration)

Stock	Amount
20-kDa PEG-norbornene	2.5 μ L
1-kDa PEG-dithiol crosslinker	2.5 μ L
Irgacure 2959	2.5 μ L
TCEP in 4x PTB	2.5 μ L
Lyophilized FSFG cys	100 μ g

Table D.3: Acrylamide hydrogel precursor stocks

Component	Concentration	Buffer
Acrylamide monomer	30%	Premixed
Bisacrylamide crosslinker	1%	Premixed
LAP	20 mM	Water
APS	10% w/v	PTB
TEMED	1% w/v	PTB
PTB buffer	1x	PTB

Table D.4: Sample precursor soution recipe (6% final acrylamide concentration)

Stock	Amount
Premixed acrylamide/bis	2 μ L
LAP	1 μ L
PTB	7 μ L
Lyophilized FSFG cys	100 μ g

Table D.5: APS/TEMED chemical crosslinking tests

APS concentration	TEMED concentration	Degas time (min)	Results
1%	0.5%	0	Gelled in under 10 s.
0.1%	0.5%	0	Gelled in 5 minutes.
0.1%	0.1%	5	Gelled in 5 minutes (while degassing).
0.1%	0.1%	0	Gelled in 10 minutes.
0.1%	0.05%	0	Did not gel.
0.1%	0.05%	5	Gelled in 10 minutes.

Appendix E

Protein and peptide sequences

Table E.1: FG Nup fragments

Description	Notes	Sequence
FSFG his	('FSFG concat-1')	MGTSATSKPAFSFGAKSDENKAGATSKPA FSFGAKPEEKDDNSSKPAFSFGAKSN EDKQDGTAKPASFSGAKPAEKNNNET SKPAFSFGAKSDEKKDGDASKPAFSF GAKPDENKASATSKPASHHHHHH
cys FSFG his		MGCTSATSKPAFSFGAKSDENKAGATSKPA FSFGAKPEEKDDNSSKPAFSFGAKSNED KQDGTAKPASFSGAKPAEKNNNETSKPA FSFGAKSDEKKDGDASKPAFSFGAKPDEN KASATSKPASHHHHHH
cys FSFG cys his	Aggregates unless kept in reducing agent.	MGCTSATSKPAFSFGAKSDENKAGATSKPA FSFGAKPEEKDDNSSKPAFSFGAKSNED KQDGTAKPASFSGAKPAEKNNNETSKPA FSFGAKSDEKKDGDASKPAFSFGAKPDEN KACATSKPASHHHHHH
ybbR FSFG cys his	Most often used for hydrogel experiments; ybbR tag is intended for site-specific labeling but not used in the hydrogel experiments.	MGDSLEFIASKLATSATSKPAFSFGAKSDEN KAGATSKPAFSFGAKPEEKDDNSSKPA FSFGAKSNEDKQDGTAKPASFSGAK PAEKNNNETSKPAFSFGAKSDEKKDGDAS KPAFSFGAKPDENKACATSKPASHHHHHH
ybbR FSFG concat- 2 cys his		MGDSLEFIASKLATSATSKPAFSFGAKSDEN KAGATSKPAFSFGAKPEEKDDNSSKPA FSFGAKSNEDKQDGTAKPASF FGAKPAEKNNNETSKPAFSFGA KSDEKKDGDASKPAFSFGAKP DENKASATSKPASATSKPAFS FGAKSDENKAGATSKPAFSF GAKPEEKDDNSSKPAFSFGAKSNED KQDGTAKPASFSGAKPAEKNNNET SKPAFSFGAKSDEKKDGDASKPAFSF GAKPDENKACATSKPASHHHHHH

Table E.2: FG Nup fragments, continued.

Description	Notes	Sequence
ybbR FSFG concat- 3 cys his	Did not express well in my hands.	MGDSLEFIASKLATSATSKPAFSFGAKSDEN KAGATSKPAFSFGAKPKEKK DDNSSKPAFSFGAKSNEDKQDGTAK PAFSFGAKPAEKNNNETSKPAFSF GAKSDEKKDGDASKPAFSFGAKPD ENKASATSKPASATSKPAFSFGAKS DENKAGATSKPAFSFGAKPKEKKDDN SSKPAFSFGAKSNEDKQDGTAKPAFSF GAKPAEKNNNETSKPAFSFGAKSDEKK DGGASKPAFSFGAKPDENKASATSKPA SATSKPAFSFGAKSDENKAGATSKPAFS FGAKPKEKKDDNSSKPAFSFGAKSNEDKQ DGTAKPAFSFGAKPAEKNNNETSKPAF SFGAKSDEKKDGDASKPAFSFGAKPDEN KACATSKPASHHHHH
ybbR SSSG cys his	Nonbinding control.	MGDSLEFIASKLATSATSKPASSSGAKSDEN KAGATSKPASSSGAKPKEKKDDNSSKPA SSSGAKSNEDKQDGTAKPASSSGAK PAEKNNNETSKPASSSGAKSDEKKDGDAS KPASSSGAKPDENKACATSKPASLEHHH HHH
FG124 his	Aggregates unless kept in denaturant	MAPNNTNNANSSITPAFGSNNTGNTAFGN SNPTSNVFGSNNSTTNTFGSNSAGT SLFGSSSAQQTKSNGTAGGNT FGSSSLFNNNSTNSNTTKPAFGLNFGGGN NTTPSSTGNANTSNNLFGATASHHHHHH
cys FG124 his	Aggregates unless kept in denaturant.	MGCTSAPNNTNNANSSITPAFGSNNTGN TAFGNSNPTSNVFGSNNSTTNT FGSNSAGTSLFGSSSAQQTKSNGTAGGNT FGSSSLFNNNSTNSNTTKPAFGLNFGGGN NTTPSSTGNANTSNNLFGATASHMHHH HHH

Table E.3: Other sequences

Description	Notes	Sequence
NTF2 his	Wild-type yeast NTF2.	MALDFNTLAQNFTQFYNNQFDTDRSQLGN LYRNESMLTFETSQLQGAKDIVEKLVS LPFQKVQHRITTLDAQPASPNGDVLVMIT GDLLIDEEQNPQRFSQVFHLIPDGN SYYVFNDIFRLNYSAAHHHHH
Pho4	Nuclear localization sequence.	KVDKLGGSGSANKVTKNKSNSSPYLNKRR GKPGPDSLE
Spo12	Nuclear localization sequence.	KVDKLGGSGKSTSNLKSSHTTSNLVKKT FKRDLLKQDPKRKLQLQQRFASPTDR LVSPCSLKLE

Appendix F

Dye-labeling protocols

The following protocols were used to label proteins with various fluorophores. Dyes should always be stored in the ultra-low freezer in anhydrous DMSO. Labeled proteins should be aliquoted within 24 hours of labeling and stored in the ultra-low freezer until just before use. These protocols were adapted from Thermo-Fisher amine and cysteine labeling protocols with help from Eric Verbeke and Annette Erbse.

If better efficiency is needed, the next improvement would be to carry out the protocols in a glove bag under nitrogen.

F.1 Labeling NTF2 with fluorescein-NHS

- (1) Resuspend lyophilized fluorescein-NHS at 100 mg/mL in anhydrous DMSO in the dark-room. Discard the remaining DMSO aliquot. Make 100- μ L aliquots of dye solution. Store with desiccant in ultra-low freezer, protected from light.
- (2) Mix NTF2 in PTB pH 7.0 and 100 mg/mL fluorescein-NHS in DMSO with around 15-fold molar excess dye. Several other buffers can be used as well (see Thermo protocol). A typical labeling reaction used 0.5 mL of 16 mg/mL NTF2 and 18.6 μ L dye stock mixed in an Eppendorf with an Eppendorf stir bar.
- (3) Incubate mixture, stirring, protected from light, at room temperature for one hour.

- (4) Equilibrate TALON cobalt resin with PTB in a column that can be spun in a centrifuge. TALON resin has a stated capacity of 5-15 mL protein per mL resin. Using significantly more than needed can lead to nonspecific binding of free dye. A typical reaction required 1.6 mL of the resin slurry. Equilibrate with at least 10 bed volumes PTB.
- (5) Cap column and add reaction mixture. Nutate at 4°C for one hour, protected from light.
- (6) Allow column to drain and wash with approximately 100 bed volumes of PTB, protected from light as best as possible. Occasionally cap column and resuspend resin to remove free dye from column cap and sides. Test flow-through using UV light to ensure that no dye is visible by the end of the wash. Resin should still be bright yellow.
- (7) Spin column at 160g for one minute to remove remaining wash buffer. Immediately cap and elute with 1 mL of 300 mM imidazole in PTB. Nutate 4°C for half an hour and collect elution. Resin should return to pink.
- (8) Dialyze elution against PTB to remove imidazole. No more than 24 hours after labeling, aliquot and freeze labeled NTF2.
- (9) Run a sample of labeled NTF2 on a native PAGE gel along with a sample of fluorescein-NHS. Use the Typhoon to compare the concentration of free dye to labeled protein in the protein sample.

F.2 Labeling NTF2 with Alexa Fluor 488 - SDP or FSFG with Alexa Fluor 647 - SDP

- (1) Resuspend lyophilized Alexa Fluor 488 at 10 mg/mL in anhydrous DMSO in the darkroom. Discard the remaining DMSO aliquot. Make 10- μ L aliquots of dye solution. Store with desiccant in ultra-low freezer, protected from light.
- (2) Mix NTF2 in 0.1 M sodium bicarbonate buffer and 10 mg/mL fluorescein-NHS in DMSO. A typical labeling reaction used 200 μ L of 16 mg/mL NTF2 and 20 μ L dye stock mixed in

an Eppendorf with an Eppendorf stir bar.

- (3) Incubate mixture, stirring, protected from light, at room temperature for one hour.
- (4) Equilibrate TALON cobalt resin with PTB in a column that can be spun in a centrifuge. TALON resin has a stated capacity of 5-15 mL protein per mL resin. Using significantly more than needed can lead to nonspecific binding of free dye. Equilibrate with at least 10 bed volumes PTB.
- (5) Cap column and add reaction mixture. Nuteat at 4°C for one hour, protected from light.
- (6) Allow column to drain and wash with approximately 100 bed volumes of PTB, protected from light as best as possible. Occasionally cap column and resuspend resin to remove free dye from column cap and sides. Test flow-through using UV light to ensure that no dye is visible by the end of the wash. Resin should still be bright yellow.
- (7) Spin column 2300g for 20 s to remove remaining wash buffer. Immediately cap and elute with 300 μ L of 500 mM imidazole in PTB. Nuteat 4°C for half an hour and collect elution. Resin should return to pink.
- (8) Dialyze elution against PTB to remove imidazole. No more than 24 hours after labeling, aliquot and freeze labeled NTF2.
- (9) Run a sample of labeled NTF2 on a native PAGE gel along with a sample of fluorescein-NHS. Use the Typhoon to compare the concentration of free dye to labeled protein in the protein sample.

F.3 Labeling NTF2-cys with Alexa Fluor 488 - maleimide

- (1) Resuspend lyophilized Alexa Fluor 488 at 10 mg/mL in anhydrous DMSO in the darkroom. Discard the remaining DMSO aliquot. Make 10- μ L aliquots of dye solution. Store with desiccant in ultra-low freezer, protected from light.

- (2) Mix protein in PTB pH 7.0 and 50 μ M TCEP (with no other reducing agent present) and 10 mg/mL dye in DMSO. Typical amounts were 50 μ L of 100 μ M NTF2-cys into 50 μ L dye stock.
- (3) Incubate mixture, stirring, protected from light, at room temperature for two hours.
- (4) Equilibrate TALON cobalt resin with PTB in a column that can be spun in a centrifuge. TALON resin has a stated capacity of 5-15 mL protein per mL resin. Using significantly more than needed can lead to nonspecific binding of free dye. Equilibrate with at least 10 bed volumes PTB.
- (5) Cap column and add reaction mixture. Nutate at 4°C for one hour, protected from light.
- (6) Allow column to drain and wash with approximately 100 bed volumes of PTB, protected from light as best as possible. Occasionally cap column and resuspend resin to remove free dye from column cap and sides. Test flow-through using UV light to ensure that no dye is visible by the end of the wash. Resin should still be bright yellow.
- (7) Spin column 2300g for 20 s to remove remaining wash buffer. Immediately cap and elute with 300 μ L of 500 mM imidazole in PTB. Nutate 4°C for half an hour and collect elution. Resin should return to pink.
- (8) Dialyze elution against PTB to remove imidazole. No more than 24 hours after labeling, aliquot and freeze labeled NTF2.
- (9) Run a sample of labeled NTF2 on a native PAGE gel along with a sample of fluorescein-NHS. Use the Typhoon to compare the concentration of free dye to labeled protein in the protein sample.