Graphical Abstract

B_G

$\uparrow C_s$

$\longrightarrow$
NF-High

I_G

$\uparrow C_s$

$\longrightarrow$
NF-Medium

N_G

Title

Neurofilament networks: salt-responsive hydrogels with sidearm-dependent phase behavior

Joanna Deek^{a,1,*}, Peter J. Chung^b, Cyrus R. Safinya^{b,*}

^a Department of Chemistry and Biochemistry, University of California, Santa Barbara, California 93106

¹ Current address: Lehrstuhl für Zellbiophysik E27, Technische Universität München 85748 Garching, Germany; email: joanna.deek@tum.de

^b Department of Physics, Department of Materials, and Department of Molecular, Cellular, & Developmental Biology, University of California, Santa Barbara, California 93106; email: cyrussafinya@ucsb.edu, pjchung@physics.ucsb.edu

* Corresponding author(s): joanna.deek@tum.de, cyrussafinya@ucsb.edu

Abstract

Background

Neurofilaments (NFs) – the neuron-specific intermediate filament proteins – are assembled into 10 nm wide filaments in a tightly controlled ratio of three different monomer types: NF-Low (NF-L), NF-Medium (NF-M), and NF-High (NF-H). Previous work on reconstituted bovine NF hydrogels has shown the dependence of network properties, including filament alignment and spacing, on the subunit composition.

Methods

We use polarized optical microscopy and SAXS to explore the full salt-dependent phase behavior of reconstituted bovine NF networks as a function of various binary and ternary subunit ratios.

Results

We observe three salt-induced liquid crystalline phases: the liquid-ordered B_G and N_G phases, and the disordered I_G phase. We note the emergent sidearm roles, particularly that of NF-H in driving the parallel to cross-filament transition, and the counter-role of NF-M in suppressing the I_G phase.

Conclusions

In copolymers of NF-LH, NF-H shifts the I_G to N_G transition to nearer physiological salt concentrations, as compared to NF-M in copolymers of NF-LM. For ternary mixtures, the role of NF-H is modulated by the ratio of NF-M, where beneath 10 wt% NF-M, NF-H drives the transition to the disordered phase, and above which NF-H increases interfilament spacing.

General Significance

Understanding the role of individual subunits in regulating the network structure will enable us to understand the mechanisms that drive the dysfunction of these networks, as observed in diseased conditions.

Keywords

Neurofilament
Network
Liquid crystal
Hydrogel
Sidearm-dependent
Salt-responsive

Abbreviations

IFs, intermediate filaments; NFs, neurofilaments; SAXS, small angle X-ray scattering; POM, polarized optical microscopy.

1. Introduction

Neurofilaments (NFs) are the cell-specific intermediate filaments (IFs) found in the neuronal cells of the spinal cord and the brain [1, 2], forming a space-filling liquid-crystalline aligned network in axons and dendrites [3]. NFs are implicated in the radial growth [4] and maintenance of the axonal caliber, which is critical for signal transduction from the neural head to the body [5, 6]. Moreover, a dynamic attribute to the network is of functional importance, specifically in enabling NF rearrangement to accommodate for the trafficking of material along microtubules interspersed within the NF framework [2, 7, 8]. The disruption of this expansive NF structure, particularly in association with the formation of cellular inclusions, is a hallmark of multiple diseases [9-11], most notably ALS [12, 13] and Charcot-Marie-Tooth disease [14, 15].

NFs are heteropolymers assembled from different monomer types: the neurofilament triplet proteins – NF-Low (NF-L), NF-Medium (NF-M), and NF-High (NF-H) – expressed most abundantly and ubiquitously, and α -internexin and peripherin more specifically expressed in the CNS and PNS, respectively [16-18]. The NF triplet proteins, the focus of this work and henceforth referred to as the NF subunits (or simply NFs), are obligate heteropolymers present in a mole ratio of 7:3:2 NF-L:NF-M:NF-H (NF-LMH) [19] (averaged over all parts of the axonal cell in bovine spinal cord). All three subunits, schematically depicted in Figure 1(A), share the characteristic tripartite structure of IF monomers, which includes unstructured N- and C-termini (the head and tail domains, respectively) flanking an alpha-helical rod domain (the body). The formation of coiled-coils via the alpha-helical rod domains drives the polymerization of the subunits. Assembly starts with a parallel dimer (of which NF-L is a necessary component), which then multimerizes into half-staggered tetramers. The mature filament, depicted in Figure 1(B), is formed of approximately 8 tetramers (i.e. ≈ 32 subunits in cross-section) and is ≈ 10 nm in diameter [20-22]. While the alpha-helical rod domains constitute the core of the filament, the tail domains of the subunits project radially outwards to form a space-filling corona at 22 nm intervals along the longitudinal axis of the filament [23]. The length, polyampholytic nature, and charge distribution of the subunit tails (also referred to as sidearms) determine the role that each of NF-L, NF-M, and NF-H confers to the resultant network properties. A detailed description of charge distribution along the sidearms, pre and post-translational phosphorylation, can be found in *Supplementary Figure S1*.

Empirical evidence has demonstrated a staggered temporal [24, 25] and spatial expression [3] pattern for each of the NF subunits (for a side-by-side comparison of axons and dendrites, see *Supplementary Figure S2*). Furthermore, mouse knockout studies show a variation in observed phenotypes, depending on the nature of the knocked-out subunit [26]. Thus, understanding the physiological structure-function relation between NF subunits (NF-L vs NF-M vs NF-H) to the system properties is critical and only achievable through reconstituted NF gels of controlled subunit compositions.

Fig. 1.: (A) Schematic representation of NF-Low, NF-Medium, and NF-High subunits, with unstructured N- and C-termini (the head and tail domain, respectively) flanking an alpha-helical rod domain (the body). Their main distinction lies in the length and charge of their tail domains (details on features in *Supplementary Figure S1*). The formation of coiled-coils via the alpha-helical rod domains drives the multimerization of the NF subunits into (B) the mature NF – 10nm in diameter. While the body domains form the core of the mature filament, the tail domains projecting radially outwards, enabling them to interact with those from neighbouring filaments (mainly through electrostatic associations), thus driving network formation. (C) The salt-dependence of NF hydrogel network properties, seen in the presence of three distinct phases: (left column) the bluish-opaque gel (B_G) phase, (center column) the isotropic gel (I_G) phase, and (right column) the nematic gel (N_G) phase. (i.) Bright-field microscopy images of 70:30 wt% NF-L:NF-M ($\approx 4:1$ mol ratio) hydrogels, with the B_G sample displaying the characteristic bluish tint (arising from light scattering). Scale bar is 1.5 mm. (ii.) POM images of the same samples in (i.), clearly revealing in both the B_G and N_G phases the average alignment of filaments responsible for rotating plane-polarized light. (iii.) Samples of 44:31:25 wt% NF-L:NF-M:NF-H ($\approx 7:3:2$ mol ratio) hydrogels in each of the three phases cut into cubes. Scale bar is 5 mm. The shape and water-retention properties of each of the hydrogels, coupled with POM images and scattering data, helped construct probable models for the relative strength of interfilament interactions and the average arrangement of constituent filaments, depicted in (iv.). Figure adapted from [27].

The interaction of sidearm domains from neighboring filaments, mainly through electrostatic associations, drives the hydrogel network formation. Previous small-angle X-ray scattering (SAXS) work on *in vitro* reconstituted NF networks demonstrate the direct role subunits play in tailoring the interfilament interactions, and thus the network properties [27-30]. At near-physiological ionic strengths (86 mM monovalent salt, pH 6.8), and in the

absence of external osmotic pressure, heteropolymers of neurofilament-low-medium (NF-LM) at various mole ratios of NF-M form compact gels in the liquid-ordered nematic gel (N_G) phase. The N_G phase is characterized by parallel filament alignment, with predominantly attractive interfilament interactions, and interfilament spacings, $d \approx 500$ Å are measured for 30 wt% NF-M hydrogels. Yet under the same ionic strength conditions, heteropolymers of neurofilament-low-high (NF-LH) transition, with increasing mole fractions of NF-H, into a looser liquid-disordered isotropic gel (I_G) phase. The I_G phase, on the other hand, is characterized by random and crossed-filament orientations where the interfilament interactions are predominantly repulsive, and $d \approx 650$ Å is measured for 30 wt% NF-H hydrogels [29].

Continued SAXS-osmotic pressure experiments reveal the subunit-dependent variability in response to external osmotic pressure. Gels of NF-LH and NF-LMH (assembled at physiological mole ratio 7:3:2 NF-L:NF-M:NF-H) undergo an abrupt non-reversible transition from a gel-expanded ($d \approx 600 - 700$ Å) to a gel-condensed ($d \approx 400$ Å) state for $P > P_c \approx 20$ kPa, indicating an onset of pressure-induced and sidearm-mediated attraction. On the other hand, NF-LM hydrogels transition to the gel-condensed ($d \approx 400$ Å) state from a gel-collapsed ($d \approx 500$ Å) state for $P < P_c$, which exhibits only transient interpenetration of the NF-M sidearm chains [28]. These findings provide direct evidence for an attraction-dominated interpenetrating conformation, permitted by the formation of ionic bridges between residues of opposite charge along the anti-parallel oriented chains. The ability of NF hydrogels to resist osmotic compression over several orders of magnitude, enabled by attraction-driven sidearm interpenetration, highlights their role in resisting both internal and external stresses.

A more recent study combining polarized optical microscopy (POM) and SAXS experiments has revealed the striking presence of a re-entrant liquid-ordered phase upon varying conditions of ionic strength for fixed NF subunit ratios (findings summarized in Figure 1(C)). With decreasing salt concentration (C_s) from physiological conditions, intended to mimic charge variation achieved *in vivo* via phosphorylation or dephosphorylation, hydrogels in the N_G phase melt into the I_G phase, and back into a liquid-ordered phase, the bluish-opaque gel (B_G) phase [27]. The regulatory role of the sidearms is most evident for the I_G phase. While homopolymer NF-Low hydrogels lack the I_G phase entirely, NF-H in 70:30 wt% NF-L:NF-H (70:30 wt% NF-LH) hydrogels stabilizes the I_G phase over a much wider salt range ($\Delta C_s \approx 70$ mM) than NF-M ($\Delta C_s \approx 10$ mM) in 60:40 wt% NF-L:NF-M (60:40 wt% NF-LM) hydrogels. In addition, both sidearms play a concerted role in the ternary 7:3:2 NF-LMH system: NF-H determines the C_s transition from B_G to I_G , and NF-M plays a larger role in stabilizing the N_G phase, and thus the I_G to N_G transition [27]. This distinction in network substructure is also reflected in phase-dependent mechanical and water retention properties. The N_G and I_G phases had elastic plateau modulus (G_0) values $\approx 100 - 200$ Pa, comparable to other physical hydrogels [31], whereas the B_G phase had significantly larger G_0 values $\approx 3000 - 4000$ Pa (similar to chemically cross-linked hydrogels), presumably due to a much larger number and/or strength of crosslinks per unit volume [27, 32]. In addition, the water retention propensity (reported as a

percent of the normalized gel weight after 24 hrs of drying) of the B_G phase ($\approx 60\%$) is remarkably higher than that of the N_G ($\approx 40\%$) or the I_G (less than 10%) phase.

The degree of occupancy/association of the charged sidearms in the network determines the ability of the substructure to rearrange to accommodate for water loss, consistent with the relative hydrogel stickiness to the glass surface and maintenance of macroscopic shape. Accordingly, hydrogels in the I_G phase, where the transient sidearm interactions involve minimal overlap and are predominantly repulsive, are the stickiest and lose the most water. As there is no filament alignment in the I_G phase, drying also proceeds in the absence of a preferred orientation, resulting in lower conformity with the initial macroscopic shape. On the other hand, in the B_G phase, where sidearm interactions – with longer stretches of oppositely-charged residue overlap – are less transient, the gels are less sticky and retain most of their water content. In both the N_G and the B_G phases, water loss occurs along the nematic-director (the preferential direction for filament rearrangement) resulting in extraordinary shape-retention, especially for the B_G phase. As water loss from the hydrogel system proceeds in a positional-dependent manner, beginning with the highest rate from the outermost areas (at the gel-air interface) and propagating with dampened rates towards the center, this results in the formation of a skin-layer, a layer of more dehydrated elements that prevents further water loss from the rest of the gel – self-encapsulation [33, 34]. The gradual skin layer formation explains the rapid water loss in the first few hours, which later slows down and eventually levels off [27].

In this study, we present the full development of the monovalent salt-dependent phase behavior of various binary (NF-LM and NF-LH) and ternary (NF-LMH) compositions, diagramming the contribution of NF-L, NF-M and NF-H, in a dose-dependent manner, to the overall NF hydrogel system. We focus on the I_G to N_G phase transition. We show that in the binary NF-LH samples, increasing the wt% of NF-H from 5 wt% to 20 wt% correlates to a broadening of the I_G phase from 50 mM to 65 mM salt respectively. On the other hand, the I_G phase for binary NF-LM samples consistently spans a salt range of ≈ 10 mM, despite a gradual increase from 5 wt% to 30 wt% of NF-M. The varied contribution of sidearms NF-M and NF-H in their corresponding binary systems, given that both confer an almost equivalent increase in total charge of the system, strongly suggests that an additional facet of sidearm-dependent network regulation is involved. Interestingly, for the ternary NF-LMH systems, we find that NF-H plays a varied role depending on its ratio to NF-M. For compositions < 10 wt% NF-M, NF-H behaves as a plasticizer, driving the system into the I_G phase; but for compositions > 10 wt% NF-M, NF-H behaves as a spacer, increasing the d -spacing (for hydrogels still in the N_G phase) from ≈ 700 Å (in its absence) to ≈ 900 Å (at 20 wt% NF-H). In contrast to the phase behavior of the reconstituted systems, we report that the native hydrogels completely lack the I_G phase, even at low salt concentrations, reinforcing the need to have isolated the subunits to enable us to explore their individual properties.

2. Materials and Methods

2.1. Crude NF purification

NFs were purified from bovine spinal cord, as presented elsewhere [27-29, 35]. The fresh spinal tissue (kept on ice) was homogenized in equal parts with buffer A (100 mM MES, 1 mM MgCl_2 , 1 mM EGTA, 0.1% NaN_3 , adjusted to pH 6.8 with NaOH), and then centrifuged to remove the cellular debris, with the clarified NFs remaining in the supernatant. The NF-enriched solution was brought to a final concentration of 50% glycerol, and centrifuged again; the product of the glycerol spin was further clarified on a non-continuous sucrose gradient (0.8 M and 1.5 M sucrose in buffer A). The final product is a native hydrogel of NFs and co-sedimenting proteins, referred to as the crude product.

2.2. Subunit isolation

In order to separate the subunits, the integrity of the filament (kept intact for the crude purification process) is disrupted: the crude protein gel is homogenized in buffer B (8 M urea, 100 mM sodium phosphate, 0.1% β -mercaptoethanol, pH 6.5). A first round of ion exchange chromatography, using a DEAE-cellulose resin (Sigma Aldrich, St Louis, MO, USA), produces two main fractions: an NF-H-enriched fraction that is eluted with buffer B, and a second fraction enriched in both NF-M and NF-L that is eluted with buffer B with 55 mM NaCl, pH 7.0. Each of these fractions is then applied separately to a second ion-exchange column – hydroxylapatite resin (Bio-Gel HT gel, BioRad, Hercules, CA, USA) – resulting in pure fractions for each of the subunits. Using Centricon tubes (Millipore, Billerica, MA, USA), the subunit fractions were concentrated, and their purity tested with 7.5% SDS-PAGE (for exemplary gel, see *Supplementary Figure S3*). The final sample concentrations were determined using the Bradford Assay.

2.3. Hydrogel assembly and salt buffer preparation

Hydrogel pre-cursor mixes are assembled from the individual subunits – NF-L, NF-M, and NF-H – still in buffer B, to the desired ratios, reported for subunit NF-M or NF-H as a wt% of the total protein mass (a.k.a. the grafting density) and/or their mole ratio to NF-L. To enable filament assembly, the NF mixes are dialyzed against buffer A for 24 h to remove the urea. The post-assembly monovalent salt concentration (≈ 85 mM NaOH for buffer A) is reduced by further dialyzing the assembled filament mixes against 40 mM MES buffer A (which requires only ≈ 30 mM NaOH to reach pH 6.8) for 12 h. The starting salt concentration for the assembled filament mixes is adjusted to fit the desired conditions before the hydrogels are formed: from ≈ 30 mM 1:1 salt concentration, milliQ water and a stock of KCl solution were used to lower and increase the salt content, respectively. In like manner, buffer C (buffer A lacking sodium azide), which is used to equilibrate and layer hydrogel samples, was adjusted to the desired 1:1 salt concentrations. The concentration of MgCl_2 is fixed at 1 mM for all the samples. The assembled filament mixes, now in the desired buffer conditions, are adjusted to a final concentration of $4 \text{ mg} \cdot \text{mL}^{-1}$. Single filament AFM studies of the reconstituted filaments reveal that they share the characteristic native beaded structure [36], but have a shorter contour length of ≈ 200 nm compared to the native

system (reported as a few microns) [28, 37]. We have demonstrated that the shorter contour length does not lead to significantly different properties with reference to the native system [27-29]. All chemicals were purchased from Sigma Aldrich and used without further purification.

2.4. Capillary sample preparation

The concentration-adjusted assembled filament mixes are then aliquoted to ensure a final protein content of 150 μg of NFs per capillary sample. The 150 μg samples are spun down using a Beckman Airfuge (rotor A-10/30) at 100,000 g at room temperature for 1 h. The excess buffer is removed, and exchanged with an excess of fresh buffer C to allow the hydrogels to swell (equilibrate). The equilibrated hydrogels are then transferred to 1.5 mm quartz capillaries (Hilgenberg, GmbH, Germany), layered with a pre-set volume of fresh buffer C and sedimented at low speeds. For samples used to monitor hydrogel phase reversibility, the capillaries remain unsealed, to enable a further buffer exchange. For all remaining samples, the capillaries are flame-sealed.

2.5. Polarized Optical Microscopy

A Nikon 282130 microscope fitted with a 5X objective was used to observe the equilibrated capillary samples, which in turn are imaged with a Nikon Coolpix P5000 camera fitted with a MDC lens.

2.6. Small Angle X-ray Scattering

SAXS experiments were conducted on the equilibrated hydrogel capillary samples (in the same quartz capillaries) at the Stanford Synchrotron Radiation Lightsource (SSRL BL4-2), using X-rays with wavelength 1.3776 $\text{\AA}$ and a sample-to-detector distance of ≈ 3.5 m. The scattering data was acquired on a MarCCD 165 (Fiber Optic-Taper coupled CCD) detector. Test samples were run to determine the optimal exposure time, with the experimental samples run for half the exposure time where radiation damage was first observed. The data was azimuthally-averaged using 'Nika' for Igor Pro [38] to yield the scattering profile, with correlation peaks positions, $q_{NF} = 2\pi/d$. An arbitrary background (set as an exponential decay of intensity versus q -value, i.e. a line in log-log plots) was subtracted from the individual scattering profiles, and the outcome fit either to Gaussians or Lorentzian functions to determine the peak positions, and as a result the interfilament d-spacing. Both methods yielded comparable peak values, to within $\pm 2\%$. Variations in proteins prepared from different batch purifications, presumably due to the variation in population distribution of phosphorylation states (in terms of degree of phosphorylation), are a main source of error in d-spacing determination. For this reason, multiple samples from different batches were prepared at the same nominal salt concentration in the different hydrogel phases – bluish-opaque gel (B_G), isotropic gel (I_G), and nematic gel (N_G) phases – and their difference in d-spacing was found to be in the range of $\pm 5\%$ or less. As the hydrogel phase behavior is probed for small salt intervals, all samples of each composition are prepared from the same protein batch, ensuring that the observed trends are due to changes in C_S , with $n = 1$ for each data point.

3. Results and discussion

3.1. Optical characterization of the salt-induced phase behavior of equilibrated NF hydrogels

In the hydrogel state, NFs form lyotropic liquid crystals, where their phase behavior is a function of the water-to-protein content (see *Supplementary Figure S4*). Therefore, in order to discern the salt-dependent phase behavior of the NFs, i.e. the effect of the apparent charge of the filaments on the overall network properties, the hydrogels – assembled at various subunit compositions – were pre-equilibrated with an excess of buffer (at specific 1:1 salt concentrations) prior to their characterization. All gels had ≈ 1 wt% protein content.

Bright-field and POM observations (representative images depicted in Figure 2) reveal the presence of the three salt-induced, reversible phases: (in order of decreasing monovalent salt concentration) the liquid-ordered N_G phase, the disordered I_G phase, and the re-entrant anisotropic B_G phase. Phase reversibility was demonstrated (as presented previously) by exchanging the buffer on the pre-equilibrated hydrogels, and simultaneously imaging them with POM. The near-parallel orientation of the constituent filaments in the N_G and B_G phases rotates plane-polarized light, where the gels appear bright even through the perpendicular polarizer orientation. Domains with slightly different nematic order parameters are seen as local regions of variable brightness within the hydrogels. The bluish opacity characteristic of the B_G phase, and apparent under bright-field microscopy, arises from the scattering of visible light by structures on the order of hundreds of nanometers in size, assumed to be distinct regions of low and high protein content (Figure 1(C,iv.), left schematic) [27].

The nature and strength of the interfilament interactions drive filament alignment: repulsive interactions favor crossed-filament orientations (I_G) to maximize separation of like charges, whereas attractive interactions favor more parallel filament orientations (N_G) [27-29]. But in the absence of sufficient buffer salt for charge screening, opposite charges within the sidearm chains of neighboring filaments condense, creating distinct protein-rich and water-rich regions (B_G). Thus the network phase behavior reflects the charge state of the filaments, and how it changes as a function of subunit composition.

3.2. SAXS experiments on the equilibrated NF hydrogels

Small-angle-X-ray-scattering (SAXS) experiments were performed on the pre-equilibrated hydrogels of the various subunit compositions, at different monovalent salt conditions. Representative SAXS data, plotted as azimuthally-averaged intensity versus q -value, is depicted in Figure 3. Two-dimensional phase plots combining the phase information (obtained from POM imaging) with the measured interfilament spacings, $d (= 2\pi/q)$ of the

Fig. 2.: Representative optical microscopy images, and SAXS data for various NF hydrogel samples. (A,B) Optical microscopy images (x 5 magnification) of equilibrated hydrogels in each of the three monovalent-salt-induced phases – B_G , I_G , and N_G – in 1.5mm quartz capillaries. Hydrogels of (A) copolymer NF-LH 70:30wt% ($\approx 4.5:1$ mol ratio) and (B) homopolymer NF-L under (*i.*) bright-field and (*ii.*) polarized optical microscopy. (B,*ii.*) The BF image of the NF-L B_G hydrogel is taken against a black background to more clearly display the bluish tint characteristic of the B_G phase. Scale bar in (A,*ii.*) is 1.5mm.

Fig. 3.: Representative SAXS data for copolymer NF-LH 80:20wt% ($\approx 7.5:1$ mol ratio) in various salt conditions. The dotted red profile is an example of background subtracted from SAXS data, and the dotted black lines through the correlation peaks are guides to the eye.

corresponding hydrogels are detailed in Figure 4. The salt lines highlight the sidearm-dependent phase transition boundaries in addition to the interfilament separation for a given C_S (specific sample details tabulated in *Supplementary Table One to Seven*). The phase behavior work (and in particular, determining the phase boundary C_S) relied entirely on the POM method, where the distinct phases show different textures. Furthermore because the textures in the two oriented birefringent (N_G , B_G) phases are completely different (where e.g. the B_G phase shows much smaller domains), simple visual inspection was sufficient to immediately determine the phase. The SAXS profiles only gave us the interfilament spacing and often could not distinguish between different phases.

Figure 4(A,i.) schematics represent the average filament alignment in the three phases, each with distinct background shade colors: yellow (and closed triangles) for the B_G phase, grey (and open circles) for the I_G phase, and green (and closed squares) for the N_G phase. As a general trend, the d -spacing increases with the increasing C_S from B_G to I_G , and then decreases closer to the I_G to N_G transition, leveling off in the N_G phase beyond ≈ 200 mM salt. In copolymers NF-LH, the average interfilament spacing in the N_G phase increases with increasing NF-H wt% (Figure 4(A)), a feature not observed for copolymers of NF-LM (Figure 4(B)). Interestingly, the difference in spacing to copolymer NF-LM becomes notable for NF-LH copolymers with > 10 wt% NF-H (i.e. where ≈ 2 of the 32 subunits are NF-H). As previously reported [27], NF-H and NF-M, in copolymers NF-LH and NF-LM respectively, control both the interfilament spacing, as well as the phase transition boundaries, Figure 5. The salt range over which the I_G phase persists for NF-LH is much larger than for NF-LM, with an increase in the C_S of the I_G to N_G transition for NF-LH in a NF-H dose-dependent manner, an effect not observed for NF-M in NF-LM. These observations delineate the distinct role that each of the sidearms play, where the I_G phase is absent from homopolymer NF-L systems, and the I_G to N_G boundary is shifted towards more physiological salt concentrations by NF-H. The B_G to I_G transition, on the other hand, is independent of the wt% of NF-H or NF-M.

3.3. Salt-dependent phase behavior of reconstituted and isolated ternary NF-LMH equilibrated hydrogels

In order to explore the concerted contribution of both NF-M and NF-H to the network structure, copolymers of all three subunits at multiple ratios were reconstituted and equilibrated in various monovalent C_S buffers. At 5 mM, 45 mM, 150 mM and 240 mM monovalent salt (represented in *Supplementary Figure S5*), the reconstituted NF-LMH hydrogels are in the B_G , I_G , N_G , and N_G phases respectively – identical to the observed phases for each of the NF-LM and NF-LH copolymer systems. Yet, at 65 mM salt – where copolymers of NF-LM are well in the N_G phase, and copolymers of NF-LH are far from the I_G to N_G boundary – the reconstituted ternary copolymers manifest NF-H-like behavior. NF-LMH hydrogels at 65 mM salt persist in the I_G phase until the composition of NF-M exceeds 10 wt%, which is marked by an abrupt

Fig. 4.: Monovalent salt phase diagrams of NF hydrogels. (Column A, *i.*) Schematics of average filament orientation in each of the three salt-dependent phases: parallel filaments in the liquid-ordered blue gel (B_G) and nematic gel (N_G) phases (yellow and green backgrounds, triangle and square symbols, respectively), and randomly-oriented filaments in the disordered Isotropic gel (I_G) phase (grey background, open-circle symbols). Phase diagrams for copolymers of (Column A) NF-LH and (Column B) NF-LM as a function of monovalent salt concentration. (Column A) Hydrogels of copolymer NF-LH at various weight percentages of subunit NF-L:NF-H (*ii.*) 95:5 wt% ($\approx 36:1$ mol ratio), (*iii.*) 90:10 wt% ($\approx 17:1$ mol ratio), and (*iv.*) 80:20 wt% ($\approx 7.5:1$ mol ratio). (Column B) Hydrogels of copolymer NF-LM at various weight percentages of subunit NF-L:NF-M (*i.*) 95:5 wt% ($\approx 31.5:1$ mol ratio), (*ii.*) 90:10 wt% ($\approx 15:1$ mol ratio), (*iii.*) 80:20 wt% ($\approx 6.5:1$ mol ratio), and (*iv.*) 70:30 wt% ($\approx 4:1$ mol ratio). The y-axis plots the measured d-spacing for the samples, $n = 1$. The salt concentrations of the I_G phase boundaries are cited (in mM) and indicated with blue notches drawn to the x-axes. All hydrogel samples included 1 mM $MgCl_2$ in addition to the (1:1) salt concentration reported along the x-axis. For detailed sample information, please refer to *Supplementary Tables 1 – 7*.

Fig. 5.: The salt concentrations of phase transitions, as a function of the sidearm grafting density and the corresponding linear charge densities. The salt concentrations of the phase boundaries between B_G and I_G ($C_{B_G-I_G}$, filled symbols) and between I_G and N_G ($C_{I_G-N_G}$, open symbols) are plotted for the various grafting densities of NF-M (square symbols) in copolymer NF-LM, and likewise of NF-H (circular symbols) in copolymer NF-LH. The linear charge density, λ_Q , corresponding to the sidearm fractions and computed for 32 subunit tails, is plotted on the top axis. The green asterisk on the top axis marks the λ_Q for homopolymer NF-L: $-59.6 \text{ e}^-/\text{nm}$. The phase transitions between the phases for hydrogels of copolymer NF-LM – 95:5 wt% ($\approx 31.5:1$ mol ratio), 90:10 wt% ($\approx 15:1$ mol ratio), 80:20 wt% ($\approx 6.5:1$ mol ratio), 70:30 wt% ($\approx 4:1$ mol ratio), and 60:40 wt% ($\approx 2.5:1$ mol ratio) – are independent of the weight percent of NF-M. Conversely, hydrogels of copolymer NF-LH – 95:5 wt% ($\approx 36:1$ mol ratio), 90:10 wt% ($\approx 17:1$ mol ratio), 80:20 wt% ($\approx 7.5:1$ mol ratio), and 70:30 wt% ($\approx 4.5:1$ mol ratio) – transition from the B_G to the I_G phase at roughly the same concentration, but exhibit a strong dependence of the I_G to N_G transition on the weight ratio of NF-H.

transition into the N_G phase, Figure 6(A). Interestingly, at 90 mM monovalent salt, a number of the ternary compositions with < 10 wt% NF-M are in the N_G phase, *Supplementary Figure S5(B)*. This displays the effect of constituent charge on network properties, and how added salt reduces (by charge screening) the repulsive interactions that stabilize the I_G phase.

For the purpose of correlating wt% of NF-M and NF-H to the resultant interfilament spacing, reconstituted ternary copolymers are subdivided based on the wt% of NF-L, where the sum of the large sidearms is $100 - \text{NF-L wt\%}$, and the ratio of NF-M:NF-H is varied within this sum, Figure 6. Colored dotted lines in Figure 6(A) are drawn through the composition lines of fixed NF-L wt%, and the corresponding d -spacing results presented in Figure 6(B). The d -spacing results for the reconstituted ternary copolymers mirror the phase transitions with increasing NF-M wt%, where the spacings decrease from $\approx 1000 \text{ \AA}$ in the I_G phase to $\approx 750 \text{ \AA}$ in the N_G phase, Figure 6(B). Per wt% of NF-L, the NF-H dose-dependent increase of $\approx 200 \text{ \AA}$ in d -spacing in the N_G phase, before then transitioning into the I_G phase, nicely demonstrates NF-H's “spacer-role”.

Fig. 6.: Salt-dependent phase behavior of ternary NF hydrogels with variable ratios of NF-L:NF-M:NF-H. (A) Phase diagram of ternary NF hydrogels (pre-swelled and equilibrated) at 65mM monovalent salt. The phase behavior indicates that any ternary mix containing at least 10 wt% NF-M forms a nematic gel. NF-H dominates the features of the network (transitioning the gel into an isotropic state) when NF-M is present in ratios less than 10 wt%. For information on how to find the ratio of all three subunits at each data point, as well as further phase diagrams at various monovalent salt concentrations, please refer to *Supplementary Figure S5*. (B) The contribution of the larger sidearms is not only exhibited in the phase of the hydrogels, but also in their interfilament spacing. Plots of d-spacing (Å) as a function of variable NF-M and NF-H ratios, at a fixed wt% of NF-L, demonstrate that the gradual increase in wt% of NF-H (specifically in the regime of NF-M $\geq$ 10 wt%, delineated with a blue vertical dotted line as a guide to the eye) results in an increase in d-spacing from $\approx$ 700 Å to $\approx$ 900 Å. For each data point, $n = 1$.

The nature and dose-dependent sidearm contributions to the NF network structure could only be elucidated in reconstituted networks of variable sidearm compositions. To emphasize the applicability and need for the systematic approach of NF subunit isolation and later reconstitution into networks, we draw the parallel between the salt-dependent behavior of reconstituted ternary NF-LMH networks at the physiological subunit ratio of 7:3:2 to that of isolated NFs (the crude system), Figure 7. The crude system displays salt-dependent phase behavior mirroring that of the 7:3:2 NF-LMH system, clearly reinforcing the picture of charge (and hence composition) dependent filament interactions that drive phase order. At near-physiological C_S (≈ 150 mM 1:1 salt), the crude, like the 7:3:2 NF-LMH system, is in the liquid-ordered N_G phase, Figure 7(A). With decreasing salt conditions, the crude system transitions into the B_G phase – marked by an increase in the opacity of the hydrogel – and bypasses the disordered I_G phase that is otherwise manifested by the reconstituted 7:3:2 NF-LMH system at intermediate C_S , Figure 7(B). Notably, the opacity of the B_G phase for the crude system is significantly reduced in comparison to the 7:3:2 NF-LMH system for hydrogels of comparable protein wt%.

The crude NF system is composed of non-disrupted filaments, which are expected to be much longer than the self-assembled reconstituted 7:3:2 NF-LMH filaments. Filament rearrangement governs the transition from one phase to another, whereby more entanglements restrict the filaments from sliding past one another and assuming different conformations. There is a much larger energetic cost to rearrange larger and more intertwined filaments. At C_S where interfilament interactions are mainly transient, the enhanced degree of filament entanglement plays a larger role in determining crude hydrogel properties, by dampening the characteristic filament rearrangements that drive the phase shifts. This picture is substantiated by the larger (and essentially constant) interfilament spacings of ≈ 800 Å for crude compared to ≈ 650 Å for reconstituted NF-LMH (at high monovalent C_S), as well as larger domains observed with POM for crude hydrogels. The filament intertwining would also hold interacting groups in proximity increasing the frequency of the transient interfilament interactions, thus strengthening the associations. Furthermore, the co-isolating proteins (as seen in Figure 7(C), inset) are expected to screen the repulsive interactions otherwise responsible for driving the hydrogels into the I_G phase, thus masking the NF-dependent salt responsiveness.

Fig. 7.: The salt behavior of crude NF hydrogels, with comparison to reconstituted gels. (A,B) Optical microscopy images (x 5 magnification) of equilibrated hydrogels at 5 mM, 40 mM, and 150 mM monovalent salt concentrations in their corresponding monovalent-salt-induced phases – B_G , I_G , and N_G – in 1.5 mm quartz capillaries. Hydrogels of (A) the crude mixture and (B) reconstituted copolymer NF-LMH with a 7:3:2 wt% ratio under (i.) bright-field and (ii.) polarized optical microscopy. Large differences between the crude and reconstituted systems are apparent at the macroscopic level, namely that the crude system is not observed in the isotropic state, and that the opacity of the blue gel phase is significantly more pronounced in the reconstituted system. Scale bar is 1.5 mm. (C) Monovalent salt phase diagram of equilibrated crude NF hydrogels, exhibiting only the liquid-ordered states: the blue gel (B_G) phase at low salt, and the nematic gel (N_G) at higher salt concentrations (for detailed sample information, please refer to *Supplementary Table 8*). The system does not melt into the liquid-disordered isotropic gel (I_G) at intermediate salt concentrations, as observed in the reconstituted system. The differently colored section in the phase diagram, between $\approx 40 - 65$ mM salt, is the salt range in which sample phase varies with protein batch, where depending on the preparation, the B_G to N_G phase transition shifts within this window. (Inset) Coomassie-stained SDS-PAGE gel, both lanes loaded with NFs from the crude preparation. Multiple co-sedimenting proteins (including tubulin) are observed. All hydrogel samples included 1 mM $MgCl_2$ in addition to the (1:1) salt concentration reported, with $n = 1$ for every data point.

4. Conclusions

The temporal and spatial variation in NF composition in neurons (*in vivo*) demonstrates a pre-set contribution from each sidearm to the overall network features. The approach of deconstructing the NF network system has shed further light on the nature of the subunit-regulated interfilament interactions, as well as their interdependent properties in the physiological network. In this study, we explore the full phase behavior of reconstituted NF hydrogels by varying the subunit composition in addition to the 1:1 C_S of the equilibrium buffer. Our findings clearly demonstrate the fine-tuning of filament interaction strength and alignment by subunit composition, in a ratio-dependent manner.

The ability of the NF polymer system to transition to and from the plastic state is key for network adaptability and remodeling. The means with which NF sidearms fulfill their physiological role as modular structural elements, as well as exhibit responsiveness to salt, is dependent on their propensity to assume different chain, and thus overlap, conformations. This is made possible by the fact that the sidearms are intrinsically unstructured: the dimension and structure of intrinsically disordered proteins is a function of the charge distribution along the amino acid chain [39]. The absence of structure enables the chains to adopt conformations resulting from different intrachain-interactions, which can further be tuned by phosphorylation. In addition, strong polyelectrolytes show chain expansion at physiological salt conditions (≈ 100 mM), which would explain why NF spacings are maintained at large core to core distances [40]. The source for qualitative divergence between the sidearms, in addition to the length, is charge distribution along the chain, which dictates the tendency and modes of intrachain-association.

As a function of decreasing C_S , we observe the occurrence of three liquid-crystalline phases: the liquid-ordered N_G phase, the liquid-disordered I_G phase, and a re-entrant liquid-ordered B_G phase. The presence of subunit NF-M or NF-H is necessary for the existence of the I_G phase, as well as its salt range, and each plays a distinct role in the onset of the phase transitions – B_G to I_G , and I_G to N_G . Yet, the crude system is not observed in the I_G phase, even within the I_G salt range for the ternary 7:3:2 NF-LMH system, a result that we believe is due to the lesser parameter control in the system formulation. The non-disruptive nature of the crude isolation results in the incorporation of filaments of all subunit compositions, as well as a distribution of phosphorylation states (both of which would contribute to the *in vitro* measurements proportionally to their fraction). Our work also highlights the relevance and need for studying the reconstituted hydrogels to assess the individual subunit contributions to the system.

For copolymers NF-LM, presence of NF-M expands the salt range for the B_G phase from $C_S \approx 10$ mM (for homopolymer NF-L) to ≈ 40 mM, and does so independently of the wt% of NF-M. The NF-LM systems transition from I_G into N_G consistently at $C_S \approx 45$ mM, demonstrating the effect NF-M has on suppressing the I_G phase. On the other hand, NF-H plays the prominent role in enhancing interfilament repulsions, manifested as a significantly reduced $C_{B_G-I_G} \approx 15$ mM as well as an expansive I_G salt range ($C_S \approx 50 - 65$ mM),

closely correlated with its increasing wt% in NF-LH copolymers. The average filament alignment observed for each of the NF-LM and NF-LH copolymer systems (around physiological salt conditions) nicely corresponds to *in vivo* observations. In insect Sf9 cells co-transfected with binary subunit systems, NF-M plays a crucial role in parallel filament assembly [41], and together with NF-L forms more aligned cellular networks than those of NF-L and NF-H [42, 43]. Furthermore, Lee *et al.* predict by molecular modeling of the effects of ionic strength on NF architecture that the sidearms are highly extended at non-physiological low salt (1 mM and 10 mM, compared to 100 mM), where NF-M is much more extended than NF-H. This change in sidearm extension at C_S characteristic of the B_G phase could explain why the onset of the I_G phase is significantly more delayed for NF-M than for NF-H [44].

Despite having relatively similar charge features (Figure 5), NF-M and NF-H confer differing properties to the hydrogel network. While NF-M drives the interfilament attraction and close-packing, NF-H contributes the repulsive and expansive component. These characteristics are clearly reflected in the average d -spacing trend for the copolymer systems in the N_G phase. Homopolymer NF-L systems have d -spacings of ≈ 350 Å, and the addition of NF-M only increases the interfilament separation by ≈ 100 Å (for all wt% compositions of NF-M), contrary to the addition of NF-H, which increases the separation from ≈ 100 Å to ≈ 400 Å with increasing wt% compositions of NF-H. In this regime, the short-range interfilament overlap conformations for like subunits vary in computed association energetics, with the most favorable being “tip-to-center” for NF-L, “beyond-full-overlap” for NF-M, and “tip-to-tip” for NF-H [28, 45], corresponding to the d -spacing results.

Reconstituted ternary NF-LMH systems reveal an interesting interplay in subunit contributions to overall networks properties. For < 10 wt% NF-M, NF-H behaves as a plasticizer, where the hydrogels are in the I_G phase. But for >10 wt%, NF-M stabilizes the N_G phase, and NF-H serves as an interfilament spacer, even at higher wt% composition relative to NF-M. The “spacer-effect” of NF-H suggests that the prominent functional sidearm feature is chain conformation. This means that NF-H creates the larger area of excluded volume compared to NF-M, effectively extending the distance range before which sidearms from neighboring filaments can overlap. Changes in chain excluded volume have been observed empirically with regards to the degree of phosphorylation of NF-H [46, 47]. Furthermore, simulations on NF tail domains suggest that phosphorylated NF-H extends from the filament core, and assumes a cross-bridge-conductive conformation [48, 49]. This, combined with the observation that the last 191 amino acids of the C-terminal domain of NF-H play a key role in cross-bridge formation [42], could explain how NF-H sets the interfilament spacing in the ternary systems. Interestingly, and in support of this salt-dependent sidearm behavior, Lee *et al.* show that for ionic strengths > 50 mM, NF-H is now more extended than NF-M. This is in nice agreement with the larger measured spacings for the copolymer NF-LH systems and the observed “spacer-effect” in the ternary NF-LMH systems [44].

Our spacing and alignment findings for the various copolymer and ternary systems reproduce *in vivo* observations for composition-dependent network variations. Dendrites (*Supplementary Figure S2(A.ii)*) are depleted in NF-H, where dendritic NF networks are more tightly packed and highly aligned, in accord with *d*-spacings of ≈ 450 Å for NF-LM copolymers for all compositions and over a wide salt range. On the other hand, the axonal NF network (*Supplementary Figure S2(A.i)*) is looser, seen in *d*-spacings of ≈ 650 Å for 7:3:2 NF-LMH ternary mixes (and *d*-spacings of ≈ 800 Å for the crude system). Nevertheless, trafficking of micron-sized vesicles along the axon (*Supplementary Figure S2(B)*) necessitates a rearrangement of the NF network, which appears more disordered in the area surrounding the vesicle. The loss of internal order makes it more energetically favorable to rearrange the network. Unlike the other cytoskeletal systems, changes to filament alignment for NFs can occur independently of associated proteins, as we have shown here, with NF-H playing a critical role in enabling the order to disorder transition. We find that NF-H pushes the N_G to I_G transition to the physiological salt regime, and that NF-M could serve to enhance the attractive component, helping to drive the system back together after rearrangement. The observations of NF hydrogels in the B_G phase, albeit at non-physiological salt conditions, represent the interaction modality characteristic of highly stretched filaments. These sidearm conformations, physiologically resulting from increasing their charge density, could explain the formation of hyperphosphorylation-associated inclusions in the cell. Extrapolating to observed aberrant physiological conditions associated with subunit over or under-expression or hyperphosphorylation, the effect of such parameter shifts may be the favoring of interpenetration states of the NFs with reduced dynamic properties, adversely affecting their ability to fulfill their structural role.

Acknowledgements

We gratefully acknowledge support by the US Department of Energy (DOE), Office of Basic Energy Sciences (BES), Division of Materials Sciences and Engineering under award number DE-FG02-06ER46314 (stimuli responsive biological assemblies) and the US National Science Foundation under award DMR-1401784 (protein phase behavior). JD is also grateful to the Alexander von Humboldt foundation for their postdoctoral fellowship support. We acknowledge use of UC-Santa Barbara NSF-DMR-MRSEC facilities (NSF-DMR-1121053, a member of the NSF-funded Materials Research Facilities Network www.mrfn.org). The X-ray diffraction work was carried out at the Stanford Synchrotron Radiation Lightsource, a Directorate of SLAC National Accelerator Laboratory and an Office of Science User Facility operated for the US DOE Office of Science by Stanford University.

References

- [1] L.M. Godsel, R.P. Hobbs, K.J. Green, Intermediate filament assembly: dynamics to disease, *Trends in cell biology*, 18 (2008) 28-37.
- [2] P.A. Coulombe, P. Wong, Cytoplasmic intermediate filaments revealed as dynamic and multipurpose scaffolds, *Nature cell biology*, 6 (2004) 699-706.
- [3] N. Hirokawa, M.A. Glicksman, M.B. Willard, Organization of mammalian neurofilament polypeptides within the neuronal cytoskeleton, *The Journal of Cell Biology*, 98 (1984) 1523-1536.
- [4] M.L. Garcia, C.S. Lobsiger, S.B. Shah, T.J. Deerinck, J. Crum, D. Young, C.M. Ward, T.O. Crawford, T. Gotow, Y. Uchiyama, M.H. Ellisman, N.A. Calcutt, D.W. Cleveland, NF-M is an essential target for the myelin-directed "outside-in" signaling cascade that mediates radial axonal growth, *J Cell Biol*, 163 (2003) 1011-1020.
- [5] P.N. Hoffman, D.W. Cleveland, J.W. Griffin, P.W. Landes, N.J. Cowan, D.L. Price, Neurofilament gene expression: a major determinant of axonal caliber, *Proceedings of the National Academy of Sciences of the United States of America*, 84 (1987) 3472-3476.
- [6] A. Yuan, M.V. Rao, Veeranna, R.A. Nixon, Neurofilaments at a glance, *Journal of Cell Science*, 125 (2012) 3257-3263.
- [7] N. Hirokawa, *The Neuronal Cytoskeleton*, Wiley-Liss, 1991.
- [8] N. Hirokawa, Organelle transport along microtubules — the role of KIFs, *Trends in cell biology*, 6 (1996) 135-141.
- [9] E. Fuchs, The cytoskeleton and disease: genetic disorders of intermediate filaments, *Annual review of genetics*, 30 (1996) 197-231.
- [10] M.B. Omary, P.A. Coulombe, W.H.I. McLean, Intermediate Filament Proteins and Their Associated Diseases, *New England Journal of Medicine*, 351 (2004) 2087-2100.
- [11] R. Perrot, J. Eyer, Neuronal intermediate filaments and neurodegenerative disorders, *Brain Research Bulletin*, 80 (2009) 282-295.
- [12] J.-P. Julien, J. Kriz, Transgenic mouse models of amyotrophic lateral sclerosis, *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, 1762 (2006) 1013-1024.
- [13] S. Xiao, J. McLean, J. Robertson, Neuronal intermediate filaments and ALS: A new look at an old question, *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, 1762 (2006) 1001-1012.
- [14] J. Brownlee, S. Ackerley, A.J. Grierson, N.J.O. Jacobsen, K. Shea, B.H. Anderton, P.N. Leigh, C.E. Shaw, C.C.J. Miller, Charcot–Marie–Tooth disease neurofilament mutations disrupt neurofilament assembly and axonal transport, *Human Molecular Genetics*, 11 (2002) 2837-2844.
- [15] G.M. Fabrizi, T. Cavallaro, C. Angiari, L. Bertolasi, I. Cabrini, M. Ferrarini, N. Rizzuto, Giant axon and neurofilament accumulation in Charcot–Marie–Tooth disease type 2E, *Neurology*, 62 (2004) 1429-1431.
- [16] Y. Yan, K. Jensen, A. Brown, The polypeptide composition of moving and stationary neurofilaments in cultured sympathetic neurons, *Cell Motility and the Cytoskeleton*, 64 (2007) 299-309.
- [17] A. Yuan, M.V. Rao, T. Sasaki, Y. Chen, A. Kumar, Veeranna, R.K.H. Liem, J. Eyer, A.C. Peterson, J.-P. Julien, R.A. Nixon, α -Internexin Is Structurally and Functionally Associated with the Neurofilament Triplet

- Proteins in the Mature CNS, *The Journal of Neuroscience*, 26 (2006) 10006-10019.
- [18] A. Yuan, T. Sasaki, A. Kumar, C.M. Peterhoff, M.V. Rao, R.K. Liem, J.-P. Julien, R.A. Nixon, Peripherin Is a Subunit of Peripheral Nerve Neurofilaments: Implications for Differential Vulnerability of CNS and Peripheral Nervous System Axons, *The Journal of Neuroscience*, 32 (2012) 8501-8508.
- [19] G. Shaw, *Neurofilaments*, Springer, Berlin, 1998.
- [20] J.A. Cohlberg, H. Hajarian, T. Tran, P. Alipourjeddi, A. Noveen, Neurofilament Protein Heterotetramers as Assembly Intermediates, *Journal of Biological Chemistry*, 270 (1995) 9334-9339.
- [21] E. Fuchs, K. Weber, Intermediate filaments: structure, dynamics, function, and disease, *Annual review of biochemistry*, 63 (1994) 345-382.
- [22] M.K. Lee, D.W. Cleveland, Neuronal intermediate filaments, *Annual review of neuroscience*, 19 (1996) 187-217.
- [23] P.A. Janmey, J.-F. Leterrier, H. Herrmann, Assembly and structure of neurofilaments, *Current Opinion in Colloid & Interface Science*, 8 (2003) 40-47.
- [24] R.A. Nixon, T.B. Shea, Dynamics of neuronal intermediate filaments: A developmental perspective, *Cell Motility and the Cytoskeleton*, 22 (1992) 81-91.
- [25] R.C. Lariviere, J.P. Julien, Functions of intermediate filaments in neuronal development and disease, *Journal of neurobiology*, 58 (2004) 131-148.
- [26] J.-P. Julien, Neurofilament functions in health and disease, *Current Opinion in Neurobiology*, 9 (1999) 554-560.
- [27] J. Deek, P.J. Chung, J. Kayser, A.R. Bausch, C.R. Safinya, Neurofilament sidearms modulate parallel and crossed-filament orientations inducing nematic to isotropic and re-entrant birefringent hydrogels, *Nature communications*, 4 (2013) 2224.
- [28] R. Beck, J. Deek, J.B. Jones, C.R. Safinya, Gel-expanded to gel-condensed transition in neurofilament networks revealed by direct force measurements, *Nat Mater*, 9 (2010) 40-46.
- [29] J.B. Jones, C.R. Safinya, Interplay between liquid crystalline and isotropic gels in self-assembled neurofilament networks, *Biophysical journal*, 95 (2008) 823-835.
- [30] C.R. Safinya, J. Deek, R. Beck, J.B. Jones, Y. Li, Assembly of Biological Nanostructures: Isotropic and Liquid Crystalline Phases of Neurofilament Hydrogels, *Annual Review of Condensed Matter Physics*, 6 (2015) 113-136.
- [31] H.E. Warriner, S.H. Idziak, N.L. Slack, P. Davidson, C.R. Safinya, Lamellar biogels: fluid-membrane-based hydrogels containing polymer lipids, *Science (New York, N.Y.)*, 271 (1996) 969-973.
- [32] E.A. Kuru, N. Orakdogan, O. Okay, Preparation of homogeneous polyacrylamide hydrogels by free-radical crosslinking copolymerization, *European Polymer Journal*, 43 (2007) 2913-2921.
- [33] M.K. Ebara, Y.; Narain, R.; Idota, N.; Kim, Y. -J.; Hoffman, J.M.; Uto, K.; Aoyagi, T., *Smart Biomaterials*, in: *Smart Hydrogels*, NIMS Monographs, Springer Japan, 2014.
- [34] J. Thomas, K. Gomes, A. Lowman, M. Marcolongo, The effect of dehydration history on PVA/PVP hydrogels for nucleus pulposus replacement,

- Journal of biomedical materials research. Part B, Applied biomaterials, 69 (2004) 135-140.
- [35] R.K.H. Liem, [36] Purification of neurofilaments and their constituent polypeptides, in: *Methods in Enzymology*, vol. Volume 134, Academic Press, 1986, pp. 380-388.
- [36] S. Hisanaga, N. Hirokawa, The effects of dephosphorylation on the structure of the projections of neurofilament, *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 9 (1989) 959-966.
- [37] R. Beck, J. Deek, M.C. Choi, T. Ikawa, O. Watanabe, E. Frey, P. Pincus, C.R. Safinya, Unconventional salt trend from soft to stiff in single neurofilament biopolymers, *Langmuir : the ACS journal of surfaces and colloids*, 26 (2010) 18595-18599.
- [38] J. Ilavsky, Nika: software for two-dimensional data reduction, *Journal of Applied Crystallography*, 45 (2012) 324-328.
- [39] N. Srinivasan, M. Bhagawati, B. Ananthanarayanan, S. Kumar, Stimuli-sensitive intrinsically disordered protein brushes, *Nature communications*, 5 (2014).
- [40] R.K. Das, R.V. Pappu, Conformations of intrinsically disordered proteins are influenced by linear sequence distributions of oppositely charged residues, *Proceedings of the National Academy of Sciences*, 110 (2013) 13392-13397.
- [41] T. Nakagawa, J. Chen, Z. Zhang, Y. Kanai, N. Hirokawa, Two distinct functions of the carboxyl-terminal tail domain of NF-M upon neurofilament assembly: cross-bridge formation and longitudinal elongation of filaments, *J Cell Biol*, 129 (1995) 411-429.
- [42] J. Chen, T. Nakata, Z. Zhang, N. Hirokawa, The C-terminal tail domain of neurofilament protein-H (NF-H) forms the crossbridges and regulates neurofilament bundle formation, *J Cell Sci*, 113 Pt 21 (2000) 3861-3869.
- [43] T. Gotow, T. Tanaka, Y. Nakamura, M. Takeda, Dephosphorylation of the largest neurofilament subunit protein influences the structure of crossbridges in reassembled neurofilaments, *J Cell Sci*, 107 (Pt 7) (1994) 1949-1957.
- [44] J. Lee, S. Kim, R. Chang, L. Jayanthi, Y. Gebremichael, Effects of molecular model, ionic strength, divalent ions, and hydrophobic interaction on human neurofilament conformation, *The Journal of Chemical Physics*, 138 (2013) 015103.
- [45] M.J. Stevens, J.H. Hoh, Interactions between Planar Grafted Neurofilament Side-Arms, *The Journal of Physical Chemistry B*, 115 (2011) 7541-7549.
- [46] H. Aranda-Espinoza, P. Carl, J.F. Leterrier, P. Janmey, D.E. Discher, Domain unfolding in neurofilament sidearms: effects of phosphorylation and ATP, *FEBS letters*, 531 (2002) 397-401.
- [47] S. Kumar, J.H. Hoh, Modulation of repulsive forces between neurofilaments by sidearm phosphorylation, *Biochemical and biophysical research communications*, 324 (2004) 489-496.
- [48] E.B. Zhulina, F.A. Leermakers, A self-consistent field analysis of the neurofilament brush with amino-acid resolution, *Biophysical journal*, 93 (2007) 1421-1430.
- [49] E.B. Zhulina, F.A.M. Leermakers, The Polymer Brush Model of Neurofilament Projections: Effect of Protein Composition, *Biophysical journal*, 98 (2010) 462-469.