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Gelator length precisely tunes supramolecular hydrogel stiffness and neuronal phenotype in 3D culture

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Abstract

The brain is one of the softest tissues in the body with storage moduli (G') that range from hundreds to thousands of pascals (Pa) depending upon the anatomic region. Furthermore, pathological processes such as injury, aging and disease can cause subtle changes in the mechanical properties throughout the central nervous system. However, these changes in mechanical properties lie within an extremely narrow range of moduli and there is great interest in understanding their effect on neuron biology. We report here the design of supramolecular hydrogels based on anionic peptide amphiphile nanofibers using oligo-L-lysines of different molecular lengths to precisely tune gel stiffness over the range of interest and found that G' increases by 10.5 Pa for each additional lysine monomer in the oligo-L-lysine chain. We found that small changes in storage modulus on the order of 70 Pa significantly affect survival, neurite growth and tyrosine hydroxylase-positive population in dopaminergic neurons derived from induced pluripotent stem cells. The work reported here offers a strategy to tune mechanical stiffness of hydrogels for use in 3D neuronal cell cultures and transplantation matrices for neural regeneration.

Graphical Abstract

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Author Contributions

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Keywords

Hydrogels; Supramolecular; Mechanical properties; Neurons; Peptide amphiphiles

Introduction

Mechanobiology is drawing increasing attention for its potential utility in the field of biomaterials design for regenerative medicine^{1–7}. As we learn more about the interactions between cells with their extracellular matrix (ECM), it becomes increasingly evident that the mechanical properties of the ECM are critical in directing cell biology^{8–10}. Previous work has provided many examples of how stiffness (G') of a scaffold also strongly influences cell biology. For example, induced pluripotent stem cells (iPSCs) grown in stiff environments are more likely to develop a bone or muscle-like phenotype while softer environments tend to be favor neuronal and adipose phenotypes^{11–16}.

Neurons are sensitive to the mechanical properties of their environment and show better growth, survival and neuronal differentiation when they are cultured in soft environments with mechanical properties akin to the brain compared to environments that mimic other tissues^{6, 17, 18, 19–23}. It is currently difficult to probe small variations in G' that occur within the central nervous system (CNS) in order to better understand the effects that stiffness has on neuronal phenotype in different regions of the brain. Moreover, currently available materials to culture neurons do not allow the variation of G' while keeping other biologically important variables such as matrix morphology, cross-linking density and biodegradation rate constant^{24–31}. For example, the stiffness of commonly used matrices, such as PuraMatrix, collagen, and Matrigel, are varied by changing the concentration of matrix components, thus making it difficult to deconvolute the effects of chemical signals and mechanics. Another common method to change polymeric matrix stiffness is to control the degree of cross-linking^{32–34}. However, crosslinking can affect both matrix degradation kinetics and cell, which may be confounding factors in proliferation and differentiation^{33–37}.

Our group has developed a class of peptide amphiphiles (PAs) that can self-assemble into high aspect ratio nanofibers that resemble fibrous ECM^{6, 30, 38–45}. When multivalent ions are

introduced to solution, these nanofibers form bulk gels that can function as a reproducible and biodegradable artificial ECM for cells that will support neurons in a biocompatible environment^{30, 38–40, 46–49}. Furthermore, PA scaffolds are injectable, which makes them suitable for minimally invasive approaches. Previous studies have also demonstrated that the mechanical properties of PA scaffolds can be tuned by using different strengths and concentrations of ions to variably screen electrostatic repulsion among highly charged PA nanofibers⁴⁰.

Parkinson's Disease (PD) is caused by the progressive death of dopaminergic (DA) neurons within the brain, causing an inability to synthesize enough dopamine to regulate movement. It is the second most common neurodegenerative disease, affecting over half a million individuals in the United States alone⁵⁰. Replacing DA neurons lost to PD with DA neurons derived from induced pluripotent stem cells (iPSCs) show promise as a palliative implant for PD patients and this therapeutic approach is currently in clinical trials^{51, 52}. Unfortunately, current protocols for differentiating DA neurons from iPSCs rely solely upon biochemical factors to promote differentiation and require long periods of time in culture (several months) to produce significant quantities of tyrosine hydroxylase-expressing (TH+) cells^{22, 53–57}. Poor survival of DA neurons (1–3%) after injection additionally limits translation by requiring large numbers of grafted cells with little guarantee that therapeutic quantities of DA neurons will survive^{51, 58–61}. Retention of injected neurons is also problematic because, in the absence of a localizing scaffold, cells tend to migrate out of the target site of injection. Finally, these culture methods frequently yield undesirable neurons of other types (1–5%)^{22, 53, 57}, which can cause debilitating neurological sequelae if injected into patients with PD^{62–64}. As discussed below, we propose that the type of supramolecular biomaterials investigated here could eventually be integrated in cell transplantation therapies for PD and other neurodegenerative diseases.

Injectable biomaterial scaffolds offer a solution to the issues of localization and survival by providing mechanical support during transplantation, minimizing cell loss to anoikis, injection-associated shear stress and poor localization. Mechanical optimization of the scaffold might further increase survival as well as impede the development of undesired cell types. For this reason, we hypothesize that a mechanically optimized, injectable hydrogel as a matrix to transplanted cell could increase survival of DA neurons while simultaneously decreasing unwanted cell types within the graft. Such an approach has the potential to synergistically improve clinical outcomes in combination with currently existing growth factor delivery approaches. In this work we investigate the effect of adding positively charged oligo-L-lysines (K_n) with variable length to PA nanofibers in order to create an artificial ECM with a precision-tunable G' and investigate the relationship between G' and phenotype on human iPSC-derived DA neurons. Mechanical properties of the bulk gels have been characterized using oscillatory rheometry, and biological characterization was performed using confocal microscopy and 3D reconstruction with Imaris software.

Experimental

Materials & Methods

Peptide synthesis—PA-1, K₆, K₁₀ and K₁₅ were synthesized using a CEM Liberty microwave-assisted peptide synthesizer, and a Rink Amide MBHA resin to tether the nascent PA-1 during synthesis. Wang resin was used to tether the nascent oligo-lysines during synthesis. For the addition of each amino acid, 5 equivalents of Fmoc-protected amino acid in *N,N*-dimethylformamide (DMF) was added to 5 equivalents of *O*-benzotriazole-*N,N,N',N'*-tetramethyluronium-hexafluorophosphate in DMF and 10 equivalents of *N*-methyl-2-pyrrolidone in DMF. Fmoc removal was accomplished using a solution of 20% piperidine in DMF and 0.1 M hydroxybenzotriazole. The default settings for microwave power and duration were used. The palmitic acid tail was added to PA-1 using the same coupling conditions.

Resin cleavage was accomplished by adding 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane and 2.5% H₂O. Vacuum-assisted concentration and subsequent precipitation of the product into cold diethyl ether afforded a crude product. Mass was verified via ESI-MS in both positive and negative scan mode on an Agilent model 6510 Quadrupole Time-of-Flight LC/MS spectrometer using direct injection. Each injection was 1 μ L. PA-1 was further purified by HPLC at 25 °C using a Phenomenex Kinetix column (C18 stationary phase, 5 μ m, 100 Å, 30.0 $\times$ 150 mm) on a Shimadzu model prominence modular HPLC system. The flow rate was 25 mL/min. Acetonitrile and water containing 0.1% NH₄OH (v/v) were used as eluents. Purity was assessed with analytical HPLC performed at 40° C using a Phenomenex Jupiter 4 μ m Proteo 90 Å column (C12 stationary phase, 4 μ m, 90 Å pore size, 1 $\times$ 150 mm) on an Agilent model 1200 Infinity Series binary LC gradient system. The flow rate for analytical HPLC was 50 μ L/min using a water/acetonitrile gradient containing 0.1% NH₄OH. K_n products were mixed with 3 M HCl and lyophilized to remove excess TFA. The K_n products were then dialyzed against 150 mM NaCl in a GE Life Sciences MiniDialysis kit (1 kDa cut-off) overnight before being lyophilized for a final time. For K_n ($n = 1-4$) and PLL, the chloride salt was ordered from Sigma-Aldrich and used as received.

PA Preparation—Lyophilized PA-1 was mixed with 150 mM NaCl to achieve a concentration of 10 mg/mL. 1 M NaOH was added dropwise until the pH reached 7.5 and the mixture was optically clear. Samples were then aliquoted into 400 μ L vials and stored at -40° C. Prior to use samples were thawed at room temperature for 8–12 hours and then annealed at 90° C for 30 minutes, followed by a controlled temperature gradient of 0.5° C/min down to 25° C to create long fibers.

Mechanical Testing (in situ)—All testing was performed on a MCR302 rheometer with a flat 8 mm plate attachment. PA-1 samples were diluted to 0.5 wt% with 150 mM NaCl. 25 μ L of PA solution was pipetted onto the bottom surface of the rheometer and 7 μ L of K_n solution (150/ n mM) was pipetted onto the surface of the measuring plate. The measuring plate was then lowered until a gap height of 0.5 mm was reached. The gel was cured 25° C for 5 minutes under small oscillatory strain ($\omega = 10$ rad/s, strain = 0.01%). We first

determined the linear viscoelastic region using an oscillatory stress sweep at $\omega = 10$ rad/s and 25° C. It was determined that all gels behaved in a viscoelastic manner at or below shear strains of 0.1%. For all further experiments, a shear strain of 0.1% was used.

The gap height was then adjusted to maintain a normal force of 0 N and samples cured for an additional 10 minutes under the previously stated conditions. Data points were collected every 6 seconds and the final 5 data points were averaged to collect G' and G'' for each sample. After samples cured, they were subjected to a frequency sweep ($\omega = 1 - 150$ rad/s, 40 data points collected per decade, strain = 0.1%) and then an amplitude sweep ($\omega = 10$ rad/s, 0.1 – 150% with 40 data points per decade). The gels were then exposed to rupturing strains of 400% for 30 seconds before the measuring parameters were returned to their initial values ($\omega = 10$ rad/s, strain = 0.1%) and the gel was allowed to equilibrate for 5 minutes before a “broken” value was collected.

Mechanical testing (ex situ)—25 μ L of 1 wt% PA-1 were placed on one half of a Bio-Rad 0.5 mm glass polyacrylamide mold. 7 μ L of K_n solution (150/n mM, where n = the repeat number of K_n) were pipetted onto the corresponding surface of the opposite glass plate and the solutions cured for 30 minutes before manipulating. A razor blade was used to remove gels from the glass and transfer them into PBS where they were equilibrated overnight prior to treatment. Gels which were deformed during the handling process were discarded. To stiffen gels, 400 μ L of PLL (4 mg/mL) was added to each gel and allowed to sit for >4 hours. The excess PLL was then aspirated and PBS was used to rinse the gel. To soften gels, 400 μ L of 0.05 wt% trypsin was added to each gel and allowed to sit for 30 minutes at 37° C. The trypsin was removed and gels were immediately treated with 10% BSA in PBS for 10 minutes to neutralize the remaining trypsin. Each gel was then washed with PBS and stored until measurement.

Gels were carefully transferred to the MCR rheometer with a razor blade. Gels that were deformed during the transfer process were discarded. The gap size was adjusted to maintain $F_n = 0$ N (approximately 0.5 mm) and the storage/loss moduli were measured under the same conditions previously described.

Dynamic Light Scattering—PA-1 was diluted to 0.05 wt% in PBS and mixed with an equivalent volume of pH-balanced K_n (150 mM NaCl) and allowed to equilibrate for 2 hours. 100 μ L of sample was placed in a Starna ZEN2112 cuvette and measured in a Malvern Zetasizer Nano ZSP at 25° C (the measurement angle was 173°). Three sets of measurements were taken of each sample, with the number of runs per measurement automatically determined by the instrument. The general purpose analysis model (normal resolution) on the Malvern software was used to calculate parameters.

Zeta potential—Samples of fibers were diluted to 0.05 wt% in PBS and mixed with an equivalent volume of K_n (150 mM NaCl, pH = 7.4) and allowed to equilibrate for 2 hours. 700 μ L of sample was placed in a DTS1070 disposable cuvette and bubbles were removed through gentle agitation. Three sets of measurements were taken of each sample, with the number of runs per measurement automatically determined by the instrument. Each measurement was collected at 25° C and fitted with the Smoluchowski model.

Line 1 – 3D Cell culture—iCell DopaNeurons (Line 1 - CDI) were received frozen and thawed according to manufacturer's instructions. The cells were re-suspended in serum-free iCell Neural Base Medium 1 supplemented with Cell® Neural Base Medium 1 and iCell® Neural Supplement B per the manufacturer's instructions media at a concentration of 20 million cells/mL and then mixed with an equal volume of PA-1 (1 wt%). Ten μ L of the resulting material was pipetted into each well of and Ibidi μ -Slide Angiogenesis plate. 4 μ L of gelling solution (40/n mM K_n) was pipetted into each well and the gel was allowed to cure for 10 minutes prior to the addition of 50 μ L media (iCell media). Media was exchanged every 2–3 days after plating until cells were harvested at 1 or 14 days for live/dead assays, immunocytochemistry or Western blot.

Line 2 – 3D cell culture—The iPSC line no. 2 from a healthy control subject was already generated and published elsewhere^{55, 65}. iPSCs were cultured in mTeSR media (Stem Cell Technologies) and passaged every 5 to 7 days on Matrigel (Corning) coated plates.

Differentiation of iPSCs into midbrain dopaminergic neurons was done according to published protocols⁶⁶ and as previously described^{55, 67}. Briefly, cells were passaged mechanically at day 12 into 2mm² blocks and plated into Poly-D-Lysine/laminin coated dishes. At day 25, these neural blocks were passaged by accutase into single cells and plated onto either poly-D-lysine/laminin coated plates or suspended in 3D cell culture. The cells were re-suspended in Neurobasal Media (Thermo Fisher Scientific #21103–049) with Neurocult SM1 supplement (Stemcell Technologies #5711) at a concentration of 20 million cells/mL and then mixed with an equal volume of PA-1 (1 wt%). Ten μ L of the resulting material was pipetted into each well and Ibidi μ -Slide Angiogenesis plate. 4 μ L of gelling solution (40/n mM K_n) was pipetted into each well and the gel was allowed to cure for 10 minutes prior to the addition of 50 μ L media. Media was exchanged every 2–3 days after plating until cells were harvested at day 26 or day 40 for live/dead assays, immunocytochemistry or Western blot.

Immunocytochemistry—For immunocytochemistry, gels were rinsed 2x with PBS prior to fixation with 4% paraformaldehyde (30 minutes). Gels were rinsed 3x with PBS and then permeabilized with blocking buffer (BB – 2% normal goat serum, 0.2% Triton-X) for 30 minutes. Primary antibodies (tyrosine hydroxylase, β 3-tubulin, human nuclear antibody, MAP2, DAT and Nurr1) were incubated at 4° C overnight prior to washing with PBS and incubation with the appropriate secondary antibodies for 3 hours. Samples were washed to remove background stain and fixed with Ibidi mounting media prior to imaging on a Nikon A1R Ga-Asp confocal microscope. Image quantification was performed using ImageJ. 3D stacks were compressed into a single image using maximal z-projection. Manual background subtraction was performed after measuring the background fluorescence of each gel where no cell was identified. Cells were then manually counted.

Western Blot—Media was aspirated from each well 15 days after encapsulation. 50 μ L of N-PER supplemented with phosphatase and protease inhibitor was mixed with each sample. The gels were manually triturated with a 100 μ L pipette to ensure even mixing and dissolution of the gel and centrifuged at 10,000 rpm for 10 min at 4° C. The supernatant was collected and a sample diluted by a factor of 10.5 uL was run in a preliminary gel to assess

GAPDH levels. Samples were normalized based on this data and gels were run again. Quantification was performed using ImageJ and statistical analysis was performed using GraphPad PRISM.

Conventional Transmission electron microscopy (TEM)—TEM was performed on 0.05% (w/v) PA nanofiber suspensions, diluted from 1% (w/v) solutions with 150 mM NaCl and 33/n mM K_n . A 10 μ L aliquot of the nanofiber suspension was pipetted on to a 300-mesh copper grid with a lacey carbon support and allowed to deposit for 3 minutes. Excess sample was removed by rinsing 3x with filtered water. Samples were then stained twice for 30 sec with 2 wt% uranyl acetate in H_2O filtered through a 45 μ m PTFE filter. Excess stain was removed by rinsing 3x with filtered water. Samples were then allowed to dry before imaging on FEI Spirit G2 TEM.

Cryogenic TEM—Cryo-TEM (JEOL 1230 TEM with LaB6 filament, 100 kV) was performed on 0.05% (w/v) PA nanofiber suspensions, diluted from 1% (w/v) solutions with 150 mM NaCl and 33/n mM K_n . Samples were prepared for cryo-TEM using a Vitrobot Mark IV vitrification robot at 95–100% humidity at 21 °C. An aliquot of 4–5 μ L of the nanofiber suspension was deposited on a 300-mesh copper grid with a lacey carbon support. The sample was then plunged from a humid environment directly into liquid ethane and was subsequently transferred and stored under liquid nitrogen. Samples were then transferred to the Gatan 626 cryogenic TEM holder using the Gatan transfer stage.

Results & Discussion

PA self-assembly & gelation

PA-1 is a previously characterized peptide amphiphile with a hydrophobic palmitoyl tail, six β -sheet forming amino acids, and three glutamic acid residues (Fig 1A)⁶⁸. It spontaneously self-assembles under physiologic conditions (150 mM NaCl at pH 7.4) to form long, soluble nanofibers. When multivalent cations such as Ca^{2+} or Gd^{3+} are added to this solution, a gel is formed in which the storage modulus (G') depends on cation valency and concentration⁴⁰. However, differences in the hydration spheres and electronic structures of different metal ions mean that there is a significant deviation from the linear relationship between G' and charge⁴⁰. Inspired by the relationship between electrostatic charge and G' , we chose to use variably sized oligo-L-lysines K_n ($n = 4$ –15, Fig 1A) and a poly-L-lysine (PLL, $n = 120$ –140) as cationic gelators because the electronic structures and hydration spheres of K_n and K_{n+1} are similar enough to allow us to probe the relationship between electrostatics and G' in isolation. Using an organic polymer, we are also able to test cations with a wider range of charges than metal ions alone. These molecules are also non-toxic and easily synthesized with a range of oligomer lengths^{69, 70}. Importantly, K_n composed of L-lysine residues (as opposed to D-lysine residues) may also be degraded by cells, allowing for matrix reorganization *in vitro* independent of cellular degradation of the PA nanofibers. Numerous studies have shown that matrix remodeling is necessary for cell growth and survival^{1, 2, 36, 37}. When solutions of K_n were added to a solution of 0.5 wt% PA-1, we observed the formation of self-supporting hydrogels with fibrillar morphology observed by SEM (Fig 1C). Rheological measurements revealed that G' of these gels increased linearly

with respect to n at a rate of 10.5 Pa per lysine residue ($r^2 = 0.98$, Fig 1D). For the various K_n samples tested, G' ranged from 18.6 Pa for K_3 to 1276 Pa for PLL ($p < 0.001$), which spans the range of G' found in human CNS tissues (30–3,000 Pa)^{71–75}. Recent work has demonstrated that G' is not the sole determining factor in cell mechanobiology. Shear strain response, flow behavior and stress relaxation are also critically important variables in determining how extracellular mechanics affect cell behavior^{33–35, 76, 77}. Importantly, while n modulated G' , it did not change any of the other mechanical properties we measured. For PA-1/ K_n gels, the rate of shear thickening was independent of n over the range of frequencies tested (5–100 Hz, Fig 1E, $p = 0.42$). Similarly, strain behavior is independent of n within the range of strains tested (0.1 – 5%, Fig 1F, $p = 0.52$). Stress relaxation is also independent of n (Fig 1G, $T_{1/2}$: $K_4 = 110s$, $K_{10} = 89s$, $K_{15} = 124s$, $p = 0.76$). It is worth noting that these relaxation rates fall within the fast relaxation regime shown to be conducive to matrix reorganization by Chaudhuri et al³³, suggesting that this system is ideally suited for 3D cell culture. Therefore, when we vary n , we find that G' is the only parameter that changes in these hydrogels. This has therefore allowed us to isolate this variable in the response of cells cultured in these artificial scaffolds.

We also considered whether G' was dependent on the concentration of K_n so we measured the G' PA-1 mixed with a series of concentrations of various K_n molecules (K_4 , K_{10} and K_{15}). We found that, for all K_n molecules tested, there was a concentration of K_n above which G' no longer increased. Additionally, the concentration at which maximal G' was achieved depended upon chain length (Fig 1H). For K_4 , the highest G' was achieved using 0.9 mM K_4 or 3.6 mM total lysine units ($K_n * n$) while K_{10} reached a maximum G' using 0.22 mM K_{10} , (2.2 mM of total lysine units). In K_{15} , the plateau concentration was found to be 0.07 mM or 1 mM total lysine units. For all measurements performed here, we used a total lysine unit concentration of 33 mM to make sure we were at this plateau concentration.

The brain is one of the softest tissues in the body with a G' of different regions measuring in 100s to 1000s of Pa depending on the measurement parameters^{73, 78}. Within the brain, there is significant structural and regional heterogeneity in G' , even for different parts of the same structure. For example, subcortical gray matter stiffness varies by several hundred Pa depending on the specific structure measured^{72, 73, 79}. Similarly, tissue stiffness is highly dependent on age⁸⁰. Because PD is a disease of aging, it is important to probe how these relatively small changes in mechanical properties affect DA neurons.

To test the effect of chemical modification of K_n molecules on gelation, we conjugated the fibronectin-mimetic peptide RGDS to K_6 and K_{10} (Fig 2A). We observed that while K_6 GGRGDS produced a weaker gel with PA-1 than un-modified K_6 , when K_{10} GGRGDS was mixed with PA-1, it produced a gel with an equivalent G' to K_{10} (Fig 2B, $p < 0.001$). We hypothesized the difference in G' between PA-1 gels made with K_6 GGRGDS and K_6 could be explained by the difference in hydrophobicity between K_6 GGRGDS and K_6 , because the three hydrophobic G residues in K_6 GGRGDS would repel the highly charged surface of the PA-1 nanofiber, making it less effective at screening charges than K_6 . For each peptide, we therefore calculated the grand average of hydropathy (GRAVY)⁸¹, which yields more negative values as hydrophilicity increases. The GRAVY of K_6 GGRGDS (−2.79) is significantly less than that of K_6 (3.9, Fig S1), which supports our hypothesis that

hydrophobicity decreases G' . Meanwhile, the proportional difference in hydrophobicity between $K_{10}GGRGDS$ (GRAVY = -3.09) and K_{10} (-3.9) is much smaller, leading to a smaller difference in the observed G' .

Because the interaction between PA-1 and K_n is non-covalent, we hypothesized that the binding interaction between positively charged K_n and negatively charged PA-1 would not irreversibly reorganize the PA-1 nanofibers. Specifically, we considered that the gelling interaction between nanofiber and K_n depends solely on the length of the K_n bound and the system does not retain a “memory” of previous gelators used. To test this, we inserted a photocleavable 2-nitrophenylalanine group into a K_{12} chain to produce a peptide ($K_6F_{2N}K_6$) that degrades into two K_6 sequences upon exposure to UV light (Fig 2C and S2). Mixing $K_6F_{2N}K_6$ and 1 wt% PA-1 produced strong gels with $G' = 4500$ Pa. Following UV cleavage of the 2-nitrophenylalanine residue under acidic conditions (0.5 M HCl) (Fig S2), G' decreased to 1900 Pa ($p < 0.001$). This modulus is comparable to a gel under made the same conditions using K_6 (Fig 2I). We also modified G' enzymatically. Trypsin is a protease that selectively cleaves peptides and proteins at lysine sites, which we used to selectively to degrade K_n molecules. To reversibly tune gel stiffness, we added PLL (4 mg/mL, PBS) to a 1 wt% PA-1 gel made with K_6 (Fig 2J). The storage modulus increased substantially ($G' = 7600$ Pa, $p < 0.001$) after overnight exposure to PLL. Treating the resulting stiff gel with trypsin returned the gel to a G' comparable with that of the original gel ($G' = 1700$ Pa, $p < 0.001$). This process was repeatable over at least three cycles without loss of gel integrity (Fig 2J). In both the UV- and enzymatically-cleaved systems, we showed that stiff gels made by long K_n molecules could be softened by decreasing n . In both cases, the softened gels were comparable to PA-1/ K_6 gels that were never exposed to longer K_n molecules. Furthermore, adding long K_n back to the system restored G' to its original value before enzymatic degradation. All these observations lead us to suggest that the interaction between PA-1 and K_n is completely reversible.

Our data demonstrate a monotonic relationship between G' and the length of a peptide gelator (n) in self-assembled hydrogels. In several previous studies across multiple self-assembling peptide systems, the strength of assemblies was found to increase with length of the peptide and a maximum strength achieved between 8–12 residues^{82–85}. Beyond that length, β -sheet-forming peptide assemblies tend to become more unstable, which has been explained by the increasing entropic cost of maintaining the necessary linear conformation for β -sheet formation compared to the enthalpy gain⁸⁵. Our system is unique because gelation depends on overcoming electrostatic repulsion among nanofibers by electrostatic screening after β -sheet formation has already occurred. We hypothesize that there is a minimal entropic penalty for K_n to shield charge at the PA-1 nanofiber surface because a linear conformation is not necessary for electrostatic interactions. Once electrostatic repulsion is overcome, nanofiber aggregation is entropically favored due to the exclusion of bound water molecules, thus driving gelation. In this regard, we demonstrated previously that PA nanofibers contain significant amount of bound water on their surfaces⁸⁶. We also previously demonstrated that the Irving-Williams series, which is derived from the exchange of water ligands for other ligands (in this case PA-1) within a metal complex, accurately predicts which divalent metallic ions will form PA gels with the highest G' ⁴⁰. Therefore, the enthalpy of favorable electrostatics between the supramolecular polymer of PA-1 and K_n ,

combined with the entropy gain of excluding water molecules from the PA-1 nanofiber surface should consistently outweigh entropy loss for $n > 8$.

Longer K_n bundle nanofibers more efficiently

To investigate the relationship between K_n -mediated charge screening and gelation, we turned to dynamic light scattering (DLS) to study nanofiber aggregation as a function of K_n concentration. Adding K_n to PA-1 (0.43 mM in 150 mM NaCl) increased the zeta potential (ζ) of the nanofibers (Fig 3A). The ζ of nanofibers increased most rapidly with the longest n , which correlated with the predicted charge of the peptide at pH 7.4 (Fig S1). DLS demonstrated that this change in ζ was correlated with a change in hydrodynamic radius, indicating nanofiber aggregation. Concentrations as low as 0.0025 mM K_{10} (0.025 mM individual lysine units), drove aggregation in dilute PA-1 solutions (0.43 mM) (Fig 3B). Adjusting for the number of residues per K_n , the overall number of lysine monomer units needed to induce gelation in a solution of PA-1 increased as n decreased. This is consistent with observations that more positively charged cations tend to be more efficient at gelling PA nanofibers and create stiffer gels⁴⁰. It is also consistent with observations that polyelectrolytes (including PLL) with increased charge induce bundling of actin (another negatively charged nanofiber) filaments more efficiently than polyelectrolytes with fewer positive charges^{87–89}.

By both conventional and cryo-TEM, PA-1 formed well-dispersed nanofibers in the absence of a cationic gelator (Fig 4A, E). Upon addition of K_n , the nanofibers aggregated into bundles with radii on the order of 100 nm regardless of n (Fig 4B–D). Cryo-TEM also shows nanofiber aggregation, indicating that this phenomenon is not a drying artefact (Fig 4E–H). Small angle x-ray scattering (SAXS) also demonstrates nanofiber bundling upon the addition of K_n (Fig 4). When only PA-1 is present, the slope in the low q region is approximately 1 indicating the formation of 1D nanostructures, which is consistent with the individual nanofibers observed on TEM. Upon the addition of K_n , the slope in this region decreases to -2 , indicating the formation of nanostructures with higher dimensionality (Fig 4I, Fig S3).

G' controls neuronal phenotype

As described above, K_n co-gelators allowed us to precisely and accurately tune G' of PA-1 gels over the range of moduli found in healthy adult CNS without changing the loss factor, stress relaxation, or rate of shear thickening. Additionally, given the nature of our experimental system, changing G' does not require modifying the peptide content of samples, or even the number of positively charged K residues within the system as G' is dependent upon the length of the K_n gelator rather than concentration. By comparing PA-1/ K_4 gels with PA-1/ K_{10} gels, we are able to study how cells survive, mature and express functionally relevant proteins when cultured in soft mechanical environments with differences in G' that are representative of the differences in G' between different regions of the brain.

To investigate how G' impacts neuronal differentiation, we encapsulated human iPSC-derived DA neurons at 40 days post-differentiation in PA-1 and gelled the resulting solution

with the chloride salts of K_4 (10mM), K_{10} (4 mM), or Ca^{2+} (20 mM) to create 3D gels. The total concentration of lysine monomers was kept constant in K_n -based gels in order to control for potential differences in cell viability and growth due to potential lysine toxicity^{24, 69, 90}. We also plated DA neurons on poly-D-lysine (PDL)/laminin coated coverslips as a positive control to ensure batch-to-batch consistency as well as on coverslips covered with only PLL. Because the 3D gels are prepared with oligo-L-lysines and do not incorporate laminin, it was established that PLL-coated coverslips would be the most analogous 2D control because they present a similar cationic surface for adhesion. At 1 and 14 days *in vitro* (DIV), we performed live/dead staining to compare the viability of neurons cultured in PA-1 gels made with K_n compared to PA-1 gelled with Ca^{2+} (Fig S4). At 1 DIV, both K_n samples demonstrated increased survival compared to Ca^{2+} (30% for Ca^{2+} , 43% for K_{10} and 50% for K_4 , $p < 0.0001$) and K_4 demonstrated increased viability compared to K_{10} . At 14 DIV, this difference in viability between K_4 and K_{10} was even more pronounced (K_{10} = 25%, K_4 = 38%, $p < 0.0001$), suggesting that the softer PA-1/ K_4 gel better supports neuron viability in both short- and long-term culture.

In addition to viability staining, samples were fixed and stained for the expression of DA and neuronal markers TH and TUBB3 (Fig 5, movies 1 & 2 in SI). As with viability, PA-1/ K_4 gels proved better for cells as the percentage of TH+ neurons increased (Fig 5G–I, M - 75%) compared to stiff PA-1/ K_{10} gels (Fig 5J–M - 49%). In two dimensions, 40% of neurons expressed TH in the absence of laminin, and this number increased to 95% when laminin was included in the surface coating. Interestingly, this increase in the percentage of TH+ neurons was similar to the magnitude of the increase observed when we used softer PA-1/ K_4 gels compared to PA-1/ K_{10} gels despite the absence of laminin in these samples. Essentially, a difference in G' of 70 Pa is therefore sufficient to trigger change in the population of TH+ cells comparable to the addition of laminin. To confirm the observed trends, we repeated these experiments with another iPSC-derived DA line (line 2) starting at 25 days post-differentiation. Neurons derived from line 2 also demonstrated increased survival and TH expression in PA-1 gels made with shorter K_n (Fig 6, S5) confirming the results obtained with line 1 (compare Fig. 5).

In addition to increasing the number of neurons expressing TH, neurons grown in 3D using PA-1/ K_4 gels displayed enhanced neurite outgrowth compared to PA-1/ K_{10} . The average length of the longest neurite in PA-1/ K_4 gels was $95.0 \pm 8.1 \mu m$ compared to an average of $64.2 \pm 5.1 \mu m$ in PA-1/ K_{10} gels (Fig 5H and N, $p < 0.01$). Total neurite length also increased in PA-1/ K_4 gels with an average arbor of $368 \pm 29 \mu m$ compared to $284 \pm 23 \mu m$ in PA-1/ K_{10} (Fig 5N and O, $p < 0.05$). Moreover, neuronal morphology of neurons grown in PA-1/ K_4 gels was different from those grown in PA-1/ K_{10} gels, with a greater ratio between longest neurite and total neurite area (Fig 5I, $p < 0.05$). We further quantified these differences with a volumetric Sholl analysis (log-log), and the resulting linear fits confirm that DA neurons grown in PA-1/ K_4 and PA-1/ K_{10} gels are morphologically distinct (Fig. S6, $p < 0.001$).

Previous work by Keung and colleagues showed that changes in G' between 100, 700 and 75000 Pa changed the expression of the neuronal marker TUBB3, however changes in other markers for neuronal subtype were not observed (including TH), which led the authors to

conclude that neuronal subtype was insensitive to matrix stiffness¹⁸. Other studies have also investigated the ability of soft extracellular environments to support neural stem cells or induce neural differentiation in stem cells, but they exclusively examine maturity and maintenance of stemness, not achievement of a particular neuronal phenotype^{17, 23, 91, 92}.

Conclusions

We have developed a strategy to control G' in bulk PA gels using peptide gelators of variable length. Oligo-L-lysine (K_n) gelators with increasing length screen electrostatic charge more efficiently at the surface of negatively charged PA nanofibers, resulting in more mechanically robust gels. This method of stiffening hydrogels as biomaterials is remarkably precise since adding a single lysine unit to the oligomeric gelator increases G' by ~ 10 Pa, which is a level of precision unmatched by other 3D cell culture methods that typically rely on peptide content or cross-linking density to control G' . The effect of gelator length is so dominant that within a pre-formed gel cleaving long K_n chains softens the gel *in situ* and a G' can be obtained which is characteristic of the shortened K_n . Likewise, addition of longer K_n chains increases G' until it matches that of gels made with the longest chain. Furthermore, we found that it is possible to incorporate bioactive peptide sequences onto the K_n gelators without affecting the general trend of increasing G' with increasing n . This work also revealed the great sensitivity of midbrain dopaminergic neurons in terms of survival and phenotype to changes in their mechanical environment. This precise tunability of 3D gel stiffness without changing other key variables could offer an important tool to optimize function, phenotype and outgrowth in dopaminergic neuron transplants using hydrogels for the treatment of Parkinson's disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PD Parkinson's Disease

DA	dopaminergic
CNS	central nervous system
iPSCs	induced pluripotent stem cells
TH	tyrosine hydroxylase
PA	peptide amphiphile
SEM	scanning electron microscopy
K_n	oligo-L-lysine with n lysine repeats
PLL	poly-L-lysine
GRAVY	grand average of hydropathy
TEM	transmission electron microscopy
PDL	poly-D-lysine
DIV	days <i>in vitro</i>

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Figure 1.

A. Chemical structure of PA-1 and K_n molecules. B. SEMs of PA-1 (0.5 wt%, 150 mM NaCl, pH = 7.4) gelled with K_4 (left), K_{10} (middle), and K_{15} (right). C. Storage modulus (G'_0) of PA-1 nanofibers with K_n ($n > 3$) under reference conditions (the linear fit is shown). D. $\tan \delta$ of PA-1 nanofibers gelled with K_n . E. Frequency sweep of PA-1 gelled with K_4 , K_{10} and K_{15} ($\omega = 2-100$ rad/s) normalized to G'_0 ($\omega = 10$ rad/s). F. Strain sweep of PA-1 gelled with K_4 , K_{10} and K_{15} ($\gamma = 0.1-5\%$) normalized to G'_0 ($\gamma = 0.5\%$). G. Stress relaxation of PA-1 gelled with K_4 , K_{10} or K_{15} after 5% compression ($\omega = 10$ rad/s, $\gamma = 0.1\%$). H. G'/G'_0 of PA-1 gelled with different concentrations of K_4 , K_{10} and K_{15} (the x axis represents total lysine units in solution). Error bars are mean $\pm$ standard error of the mean. One-way ANOVA with Tukey post-hoc test was applied to the graph shown in G.

Figure 2.

A. Chemical structure of K_nGGRGDS. B. G' of PA-1 (0.5 wt%, 150 mM NaCl, pH = 7.4) gelled with K_nGGRGDS or equivalent K_n. C. Chemical structure of photo-cleavable hexa-L-lysine-2-nitrophenylalanine-hexa-L-lysine (K₆F₂NK₆). D. G' of PA-1 gels (1 wt%, 0.5M HCl, pH = 2) before and after exposure to UV light compared to PA-1/K₆ gel under equivalent conditions. E. G' of PA-1 treated sequentially (from left to right): K₆ (25 mM), PLL (5 mg/mL – 12h), trypsin (0.05% – 30 min), PLL (5 mg/mL – 12h), trypsin (0.05%, 30 min) and PLL (5 mg/mL – 12h). Error bars are mean $\pm$ SEM. * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001, one-way ANOVA with Tukey post-hoc test (B, D, E).

Figure 3.

A. ζ of 0.43 mM PA-1 upon serial addition of K_n . B. Dynamic light scattering of 0.43 mM PA upon serial addition of K_n ; the isoelectric point where the number of lysine subunits in solution is equivalent to the number of glutamic acid residues on the PA-1 is marked by the dashed line.

Figure 4.

A. Conventional TEM of annealed (heated to 30 minutes at 90°C and cooled at 1°C/min to room temperature) PA-1 (0.05 wt%, 150 mM NaCl, pH = 7.5) shows long, single nanofibers when treated with an OsO₄ stain; upon addition of K₄ (B), K₁₀ (C), or K₁₅ (D) to the solution leads to aggregation of PA-1 nanofibers. This same trend is observed with cryogenic TEM (E–H). I. Small angle x-ray scattering of PA-1 (1 wt%, 150 mM NaCl) nanofiber gels (scale bar = 25 nm for conventional TEM and 100 nm for cryo-TEM).

Figure 5.

A - C. Images of iPSC-derived DA neurons (150,000 cells/cm²) after 14 days of culture on PDL/laminin-coated coverslips. D-F. DA neurons after 14 days on PDL-coated coverslips without laminin. G-I. DA neurons cultured in 3D PA-1/K₄ gels (14 DIV, 40,000 cells/μL, 0.5 wt% PA-1). J-L. DA neurons cultured for 14 days in 3D PA-1/K₁₀ gels. M. Percentage of neurons (TUBB3+ cells) expressing TH after 14 days of culture. N. Average length of longest neurite for each neuron. O. Sum of length of all neurite extensions per neuron. Scale

bar = 100 μm . Error bars, mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, Student's t test (N-O) or one-way ANOVA with Tukey post-hoc test (M).

Figure 6.

A. iPSC-derived DA neurons (plated at day 25 post-differentiation, 10,000 cells/ μ L, 0.5 wt% PA-1) after 15 days of culture on PDL/laminin, in PA-1/K₄ gels, or PA-1/K₁₀ gels. B. Integrated fluorescence of TH (green). C. Representative western blot of TH/GAPDH ratio. D. Relative expression of TH as quantified by Western blot. Scale bar = 100 μ m. Error bars, mean $\pm$ SEM. * $P < 0.05$, Student's t test (B-D).