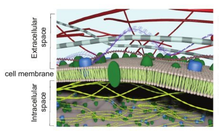
Deciphering Dynamics of Supramolecular Polymers with Multiphase and Multinuclear NMR Spectroscopy

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Supramolecular Plymers: Dynamic Assemblies of 'Dancing' Monomers



Supramolecular polymers (SPs) are formed by spontaneous bonding of monomers through noncovalent interactions, resulting in structures with high molar mass similar to covalent macromolecules. Such entities are omnipresent in Nature and play essential roles in biological systems. Most obvious examples are protein polymers in the dynamic cytoskeleton of cells and fibrils in extracellular matrices. Drawing inspiration from biology, supramolecular polymers hold great potential for developing new functional soft materials because noncovalent bonding allows for combining high degrees of internal order with tunable dynamics. This powerful combination opens up possibilities for novel functions that are yet unknown and also offers materials that can be easily depolymerized for recycling or biodegradation. …"Tuning the amplitude of supramolecular motion and selecting the necessary operative mechanisms will require a deeper understanding of dynamics so that monomer design and pathway-dependent polymerization strategies can be implemented..."

R. Z. Pavlovic, S. A. Egner, L. C. Palmer, S. I. Stupp, J. Polym. Sci **2023**, *61*(10), 870.

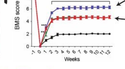
Addressing Challenges of High Societal Relevance with Supramolecular Polymers

Disruption of the nervous system

through acute injuries or diseaseses causes enormous suffering for patients, their families, and creates great socioeconomic burden. Creating biomaterials based on sophisticated synthetic supramolecular assemblies can offer advanced therapies capable of enhancing the healing processes of our bodies beyond their natural capabilities, which is a promise of regenerative medicine. The vision is that biomaterials can be administered in the body to act as scaffolds for tissue repair using endogenous cells improving the rate and extent of healing. Stupp laboratory developed biomaterials based on dynamic peptide amphiphile supramolecular polymers which show great promise in finding strategies to reverse paralysis caused by traumatic injuries or neurodegenerative disease.



Functional recovery from paralysis quantified by the Basso Mouse Scale (BMS) which measures hind limb walking ability in mice over a period of 12 weeks after spinal cord injury.



Reversing Paralysis



High mobility **More** recovery

Low mobility **Less** recovery

Alvarez et al. found that administering supramolecular polymers with peptide amphiphiles (PAs) bearing bioactive signals resulted in enhanced functional recovery in mice with severe spinal cord injuries (SCI), leading to a reversal of their paralysis. The recovery was more pronounced when the supramolecular polymers exhibited intense "supramolecular dancing," while slower movement of the signals in the polymers led to less significant recovery.

Alvarez et al. Science, 2021, 374, 848-856.

Growing Mature Neurons for Advanced Transplataion Therapies

Although previous researchers have differentiated stem cells to become neurons, those neurons were functionally immature — resembling neurons from embryonic or early postnatal stages. The limited maturation obtained with current stem cell culture techniques diminish their potential for neurodegeneration studies.

The IKVAV-**PA2** supramolecular polymers, featured with high degree of supramolecular motion, are showing high promise as an extracellular matrix mimmetics on which iPCSs can be cultured to provide functionally mature neurons. This can create many new opportunities for medical research and offer advanced transplantation therapies for neurodegenerative diseases and traumatic injuries.

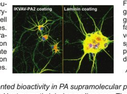
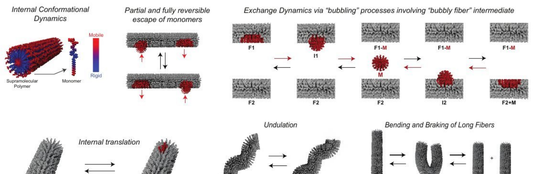


Figure on the left shows fluorescent micrographs of human neurons (stained with red, green and blue) growing on coatings with fast-moving molecules (left, PA2-IKVAV) or conventional laminin (right) for 60 days. Neurons spread homogenously and showed more complex branching on the highly mobile coating developed in The Stupp Laboratory.

Alvarez et al. Cell. Stem. Cell., 2023, 30, 219-238.

These very ecent results from our laboratory clearly demonstrate unprecedented bioactivity in PA supramolecular polymers to address the enormous scientific challenge of finding strategies to combat neurodegenerative disorders caused by traumatic injuries or disease. Therefore, we suggest that future opportunities exist for novel functions in supramolecular polymers by tuning dynamic behavior through the chemical design of monomers, and also by gaining an understanding of the precise spatiotemporal mechanisms linked to supramolecular motion in these emerging polymers.

Motion in PA Assemblies

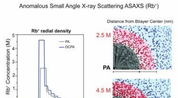


R. Z. Pavlovic, S. A. Egner, L. C. Palmer, S. I. Stupp, J. Polym. Sci. **2023**, *61*(10), 870.

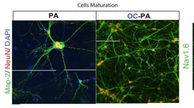
Boosting Development and Excitabilityof Neurons with Ion Cloud Nanostructures

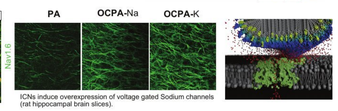
The development of the human brain and spinal cord is governed by the maturation of neurons as they establish the circuitry necessary for motor skills, learning, and memory. Neuronal circuitry is critically dependent on the controlled transport of ions in and out of cells. Abnormal ion availability and transport underlie devastating neurological pathologies and injuries, which could be mitigated by elevated ion concentrations, but excessive extracellular sodium or potassium can cause neuronal death. We report here the discovery of a strategy to stabilize a dense ion cloud around a nanoscale supramolecular assembly that can harbor a concentration of these ions that is ~50-fold higher than the lethal dose in solution but unexpectedly enhances neuronal development and accelerates action potential propagation. This was accomplished by embedding a small organic cation in a negatively charged peptide amphiphile nanostructure that leads to full ionization of monomers without disassembling the supramolecular polymer. We cultured mouse and human neurons on the fully charged filaments covered by the ion clouds and observed rapid neurite branching and functional maturation based on upregulation of p-CREB, a master transcription factor of cyclic-AMP responsive gene expression programs. Furthermore, rat hippocampal brain slices revealed that ion clouds can increase and sustain the frequency of action potential propagation. Our study has provided a vision for the potential use of synthetic systems to boost neurotransmission.

Anomalous Small Angle X-ray Scattering ASAXS (Rb^+)



Cells Maturation





ICNs induce overexpression of voltage gated Sodium channels (rat hippocampal brain slices).

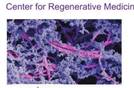
At this stage, we asked if multiphase and multinuclear NMR approach will be able to tell us more regarding what happens with supramolecular motion in PAs coassembled with organic cations (OC_6)? Questions we asked are: a) what happens to local molecular fluctuations of PA monomers in OCPA assemblies, b) how mobile is the OC_6 why the OCPAs precipitate larger number of positively charged monovalent ions relative to PAs, c) are there any differences in dynamics of ion binninding in PA vs. OCPA filaments?

Acknowledgments



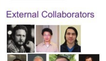
Peptide Synthesis Core at NU

Center for Regenerative Medicine at NU



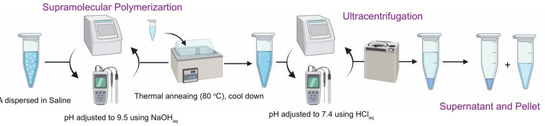
NSF CHE-2102662

External Collaborators



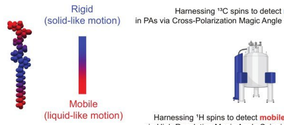
Northwestern SQI SIMPSON QUERREY INSTITUTE

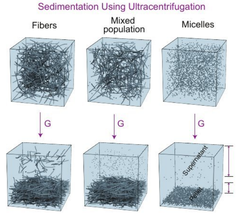
The objective of this work *is to to provide an in depth understanding on structural dynamics of supramolecular polymers obtained from PAs that are of high relevance for regenerative medicine.* ***The experimental approach*** *involves supramolecular polymerization at physiological conditions, ultracentrifugation and analyses of the obtained supernatants and pellets using multiphase and multinuclear magnetic resonance (NMR) spectroscopy in combination with small angle X-ray scattering (SAXS), transmission electron (TEM) and scanning electron (SEM) microscopies.*



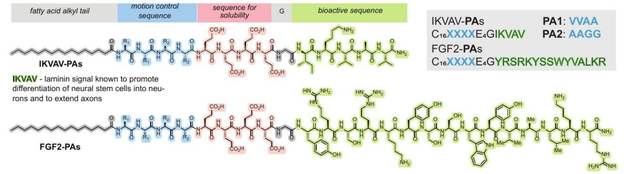
Supramolecular polymerization *at physiologically relevant conditions.* ***Ultracentrifugation*** *helps packing NMR samples which will be analyzed via magic angle spinning.* ***TEM and SEM*** *provide morphological insight which is easily understood while* ***SAXS*** *gives information about supramolecular polymer nanostructure in its antive environment.*

Multiphase and Multinuclear NMR *allows for harnessing ^{1}H and ^{13}C spins available in chemically unperturbed PAs to give insights into relevant chemical processes, supramolecular polymer dynamics, and translational mobility of PAs in their native environment. Additionally, we have utilized it to interogte monovalent ion binding to supramolecular filaments.*





Supramolecular Polymers Decorated with Signaling Peptide Sequences



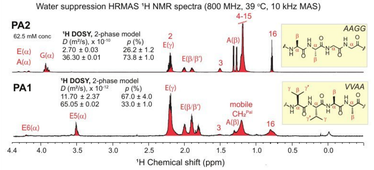
IKVAV-**PAs** **PA1**: VVAA
C_{16}XXXE$_4$GIKVAV **PA2**: AAGG
FGF2-**PAs**
C_{16}XXXE$_4$GYRSRKYSSWYVALKR

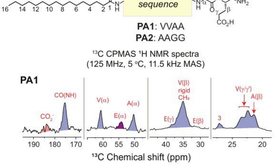
FGF2 *- the fibroblast growth factor 2 mimetic peptide activates the receptor FGFR1 to promote cell proliferation and survival. This angiogenic signal helps with growth of new blood vessels following SCI and also with proliferation of oligodendrocytes which produce myelin ensuring optimal transmission of electrical signals by neurons.*

Supramolecular Assembly Structure and Dynamics Tunded by Mutatations in "Motion Control" Domain

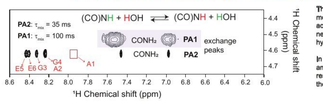
Our journey of exploring the supramolecular motion in PA assemblies began with supramolecular polymers of **PA1** and **PA2**. Both **PA1** and **PA2** were designed without signaling peptides, terminated with two glutamic acids for enhanced solubility, and containing VVAA and AAGG tetrapeptides in the "motion control" domain. This selection was inspired by Alvarez et al.'s 2021 report, which demonstrated that bioactive PAs containing these tetrapeptides hold great promise for advanced supramolecular nanotherapeutics. We were extremely surprised to find that the two PAs yield supramolecular assemblies with distinct morphologes, exhibiting significantly different supramolecular dynamics.

Water suppression HRMAS ^{1}H NMR spectra (800 MHz, 39 °C, 10 kHz MAS)





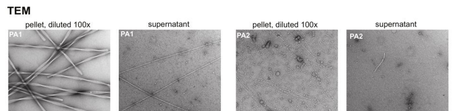
Getting insight into supramolecular polymer "lubrication" by internal water molecules via HRMAS ^{1}H-^{1}H 2D EXSY NMR.



The NH/H$_2$O ^{1}H magnetization transfer measurements offer unique insights into the accessibility of water molecules to amide groups along the peptide backbone. Within this context, the ease of water molecule access to these amide groups becomes apparent. In the case of PA2, water molecules can readily access nearly all residues, with the slowest exchange observed at the A1 location. This access to water disrupts the hydrogen bonding network, resulting in enhanced mobility of PA2 molecules.

In contrast, the PA1 EXSY spectrum displays water proton exchange exclusively with the terminal carboxy-amide ($CONH_2$) group, occurring on a millisecond timescale. This observation strongly indicates that other residues in PA1 experience substantial restriction due to hydrogen bonding interactions. Consequently, the exchange of NH/H$_2$O takes place on timescales exceeding one second for these residues in PA1.

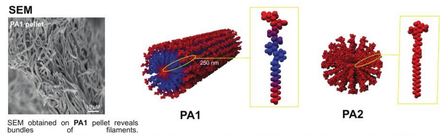
TEM



PA1 pellet shows predominantly filaments (i.e., wider twisted nanoribbons), while spherical micelles and thiner filaments are observed in the supernatant.

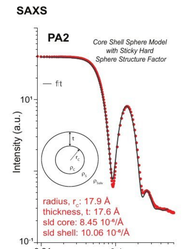
A large number of micelles are observable in TEM of **PA2**, along with thin filaments and protofilaments.

SEM



SEM obtained on **PA1** pellet reveals bundles of filaments.

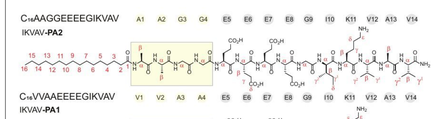
SAXS



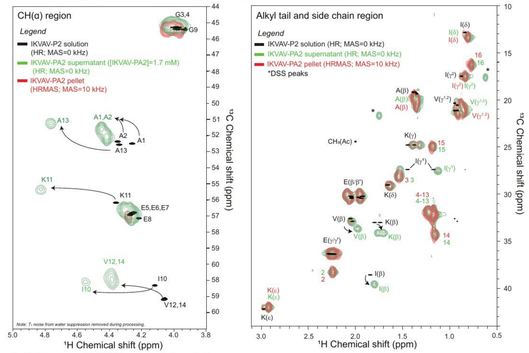
A spherical micelle form factor dominates the-SAXS profile of the **PA2** "pellet" collected at the bottom of the ultracentrifuged tube.

The "motion control" domain's peptide sequence significantly affects the morphology and dynamics of PA-based supramolecular polymers synthesized under physiological conditions. PA1 with the VVAA tetrapeptide forms filaments with robust β-sheet, while PA2 featuring the AAGG peptide adopts spherical nanostructures. These findings underscore the potential of biodegradable PAs for designing custom-shaped nanostructures with desired behaviors, crucial for developing advanced future therapies based on synthetic supramolecular polymers.

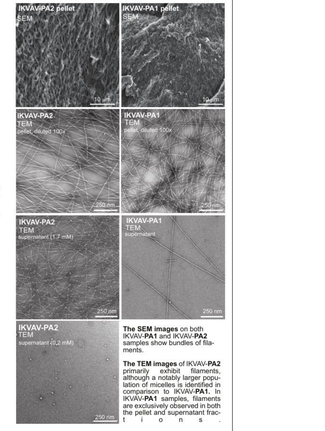
Bioactive Supramolecular Polymers with Laminin Signal IKVAV



Multiphase ^{1}H-^{13}C HSQC NMR Spectroscopy Reveals Structuring of IKVAV Signals

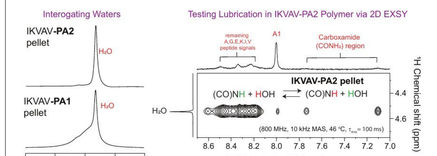


Morphological Differences in KVAV-PA1 and IKVAV-PA2

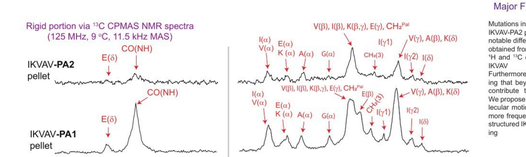


The SEM images on both IKVAV-**PA1** and IKVAV-**PA2** samples show bundles of filaments.

The TEM images of IKVAV-**PA2** primarily exhibit filaments, although a relatively larger population of micelles is identified in comparison to IKVAV-**PA1**. In IKVAV-**PA1** samples, filaments are exclusively observed in both the pellet and supernatant fractions.



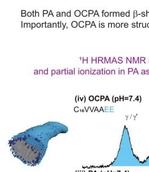
The alkyl tail and amino acid sidechain resonances are much sharper in IKVAV-PA2 relative to IKVAV-PA1. This reavealed that local molecular fluctuations (e.g. establishing and breaking noncovalent side chain interactions) are occuring much more frequently in the IKVAV-PA2 relative to IKVAV-PA1. Amide resonances are detectable in IKVAV-PA2, which is in sharp contrast to IKVAV-PA1 material. Disruption of hydrogen bonding network is not fast compared to MAS 10 kHz time scale in IKVAV-PA1. PAs undergo ~100 times faster translational diffusion in IKVAV-PA2 relative to IKVAV-PA1. Importantly, IKVAV-PA1 fits well only into a one-phase model, while IKVAV-PA2 gave satisfactory fit with two phase model.



The HSQC spectrum of IKVAV-**PA2** supernatant is featured with distinct chemical shift corresponding to ^{13}C(α) and ^{1}H(α) from the IKVAV signal when compared to the corresponding IKVAV-**PA2** (N-acetylated peptide of the same sequence, found to be intrinsically disordered). Importantly, most ^{13}C(α)-^{1}H(α) correlations from the IKVAV-**PA2** are missing in the HRMAS HSQC spectrum recorded on the corresponding pellet, which can be prescribed to enhanced rigidity due to directional noncovalent interactions which underlie the formation of IKVAV-**PA2** supramolecular filaments.

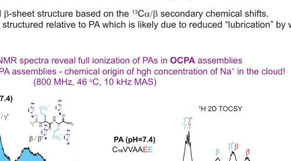
The HSQC spectrum of IKVAV-**PA2** supernatant is featured with distinct chemical shift corresponding to ^{13}C and ^{1}H signals from the sidechains of IKVAV when compared to the corresponding IKVAV-**PA2**. Importantly, some of these signals are missing in the HRMAS HSQC spectrum recorded on the corresponding pellet, which reveals that local molecular fluctuations in those regions of PA molecules are slow relative to MAS frequency.

Interogating Waters



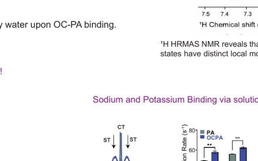
Chemical environment and dynamics of protons from water molecules in the two materials is dramatically different. Water dynamics are surprisingly heterogenous in IKVAV-**PA1**.

Testing Lubrication in IKVAV-PA2 Polymer via 2D EXSY



NH/H$_2$O ^{1}H magnetization transfer measurements demonstrate that water molecules can access amide groups along the IKVAV-**PA2** peptide backbone (millisecond timescale). This type of proton exchange, which should happen upon the disruption of PA-PA hydrogen bond interactions, informs us that water molecules residing within the IKVAV-**PA2** supramolecular nanostructures are responsible for measured highly dynamic nature of IKVAV-**PA2** polymers.

Rigid portion via ^{13}C CPMAS NMR spectra
(125 MHz, 9 °C, 11.5 kHz MAS)





^{13}C CPMAS NMR spectra reveal complementary situation relative to ^{1}H HRMAS NMR spectra. The ^{13}C NMR spectrum of IKVAV-**PA1** is featured with much signals relative to IKVAV-**PA2**, which can be attributed to significantly lower frequency at which local molecular fluctuations are happening in IKVAV-**PA1** relative to IKVAV-**PA2**. This suggest much stronger supramolecular cohesion in IKVAV-**PA1** relative to IKVAV-**PA2**.

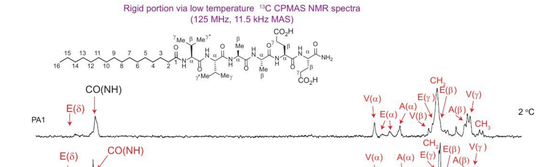
Major Findings

Mutations in motion control domain significantly affect supramolecular dynamics. IKVAV-PA2 polymers are much more dynamic relative to IKVAV-PA1. There are notable differences in morphological distribution of supramolecular nanostructures obtained from the two PAs as well.

^{1}H and ^{13}C chemical shift of α- and sidechain CH groups is a clear evidence that IKVAV signal is highly structured in IKVAV-PAs. Furthermore, E/K nuclear Overhauser effect (NOE) correlations are evident, indicating that beyond hydrogen bonding, electrostatic interactions between side chains contribute to the supramolecular arrangement of peptide amphiphiles (PAs). We propose that high bioactivity of IKVAV-**PA2** featured with high degree of supramolecular motion comes from the fact that its monomers can undergo partial escape more frequently than in the IKVAV-**PA2** filaments. This sort of motin exposes highly structured IKVAV signals to the transmembrane receptors, therefore effectively triggering biological processes of interest.

Multiphase and Multinuclear NMR in Fishing for the Origin of Bioactivity of Ion Cloud Nanostructures

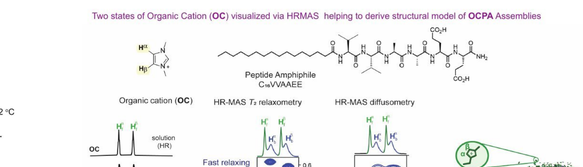
Rigid portion via low temperature ^{13}C CPMAS NMR spectra
(125 MHz, 11.5 kHz MAS)



Both PA and OCPA formed β-sheet structure based on the ^{13}Cα/β secondary chemical shifts.

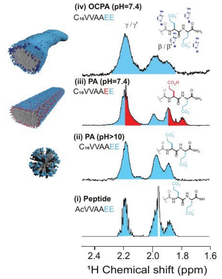
Importantly, OCPA is more structured relative to PA which is likely due to reduced "lubrication" by water upon OC-PA binding.

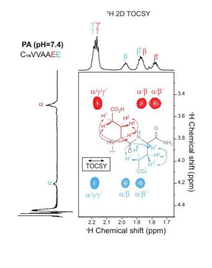
Two states of Organic Cation (OC) visualized via HRMAS helping to derive structural model of OCPA Assemblies



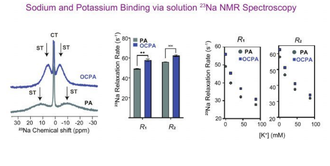
^{1}H HRMAS NMR reveals that **OC** resides in two distinct chemical envirnoments. Additionally, T_2 relaxometry together with ^{1}H diffusion measurments show that two states have distinct local molecular fluctuations and translational diffusion properties . Therefore, they are assigned as surface (s) and core (c)-bound OC states.

^{1}H HRMAS NMR spectra reveal full ionization of PAs in OCPA assemblies and partial ionization in PA assemblies - chemical origin of high concentration of Na$^+$ in the cloud!
(800 MHz, 46 °C, 10 kHz MAS)

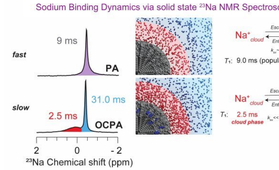




Sodium and Potassium Binding via solution ^{23}Na NMR Spectroscopy



Sodium Binding Dynamics via solid state ^{23}Na NMR Spectroscopy



Solution ^{23}Na NMR spectra of PA and ICN samples revealed the expected isochronous central transitions (CTs) as well as satellite transitions (STs) caused by residual ^{23}Na quadrupolar coupling, observed when interactions occur between Na$^+$ ions and oppositely charged structures such as proteins and polymers. We found that STs in **ICN** samples were sharper and less separated from the CT relative to **PA** samples, which we attribute to the presence of an enhanced electrostatic potential experienced by Na$^+$ ions within the cloud of ICN assemblies. These sharper ST peaks in ICNs indicate a higher degree of interaction and ordering of Na+ ions near the assembly surface, where ASAXS revealed a higher concentration of monovalent ions. Furthermore, the narrower ST linewidth for ICN samples indicate the presence of less dynamic Na$^+$ ions. Next, the longitudinal (R_1) and transverse (R_2) 23Na relaxation rates were measured for **PA** and **ICN** samples prepared at the same total Na+ concentration and constant Na$^+$/**PA** ratio. Consistent with our suggestion above, ICN assemblies showed higher 23Na relaxation rates relative to PA nanostructures (Fig. 2f), which indicates more binding of Na$^+$ cations within the cloud. We were also interested in investigating if other biologically relevant monovalent ions like potassium could form an **ICN**. Since we cannot measure binding of potassium ions directly by NMR, we utilized 2^{3}Na NMR relaxometry in a Na$^+$/K$^+$ competitive binding assay (Fig. 2g, Fig. S7) by incremental addition of KCl to each sample. These relaxometry measurements revealed the gradual liberation of Na$^+$ ions into solution as more KCl was added and K+ ions became condensed in the cloud, as indicated by a reduction in 23Na R1 and R2. We conclude that **OC** interactions with **PA** assemblies promote enhanced levels of charge density without disrupting their cohesive supramolecular structure. Additionally, 23Na snNMR spectra recorded on the pellets show that sodium exchange dynamics differs greatly in the two materials. This in turn enables a unique state of monovalent ion condensation that must be connected to the biological activity discovered here in the maturation of neurons.