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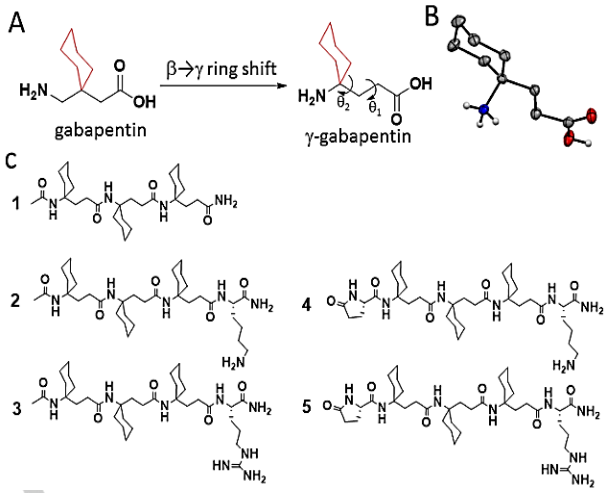
Remarkable modulation of self-assembly in short γ-peptides by neighboring ions and orthogonal H-bonding

Sandip V. Jadhav,*[a] Paolo Amabili,[a,c] Hans–Georg Stammler[b] and Norbert Sewald*[a]

Abstract: Gabapentin, an anti-epileptic drug, is known to form stable helical structures in short peptides. Distinctly, we report on the newly synthesized γ-analogue of gabapentin, i.e. γ-gabapentin (γ-Gpn), manifests β-sheet character at molecular and nanofibrous hydrogel at supramolecular level. We have undertaken the study to investigate the influence of proximally immobilized cationic amino acids (lysine and arginine) on self-assembly of backbone expanded tripeptide motif. Interestingly, arginine was found to be superior, both physically and mechanically, over lysine in driving hydrogelation. We have concluded that intrinsic and biochemically distinct properties of guanidinium ion of arginine (compared to ammonium ion of lysine) have contributed towards this effect. Furthermore, similar to pyroglutamyl (pGlu) modified amyloid β peptides, *N*-pGlu modification of our self-assembling tripeptide motif exerts dramatic influence on aggregation and exhibited enhanced β-sheet character, accelerated self-assembly kinetics, improved optical transparency and provided higher mechanical stiffness to peptide hydrogel.



Scheme 1. A) Chemical structures of gabapentin (Gpn) and γ-gabapentin (γ-Gpn). B) Crystal structure of γ-Gpn. C) Structures of synthesized peptides on solid phase using Fmoc chemistry.

Introduction

Smart and selective therapeutic approaches are of high current interest because of worldwide increased incidence of neurodegenerative diseases and cancer. Efficient use of supramolecular biomaterials show outstanding promise in this context.[1] In recent years, peptide based biomaterials experienced increased attention as new generation tools to tackle these issues.[2] Low production cost, biocompatibility, control over structural reversibility and their straightforward chemical synthesis render peptides very attractive candidates in nanomedicine and nanotechnology.[3] In recent years peptide based hydrogel/nanofiber materials have been developed for selective cytotoxicity,[4a] for 3D bioprinting,[4b] as vaccine adjuvant,[4c] for suturing ultrasmall vessels,[4d] as immunomodulators,[4e] etc. Moreover, hydrogels are capable to mimic the three-dimensional environment of the extracellular matrix (ECM) which is essential e.g. for cell culture in vitro.[5] Peptide-hydrogel based ECM surrogates are being extensively used and are also commercially available, including HydroMatrix (Sigma), PuraMatrix (Corning) and PGmatrix (PepGel LLC).

The advantage of peptide-based biomaterial lies in the fact that overall properties of such materials can be easily tuned in a bottom-up approach. There are continuous approaches under investigation to discover new self-assembling peptide materials in order to address unmet therapeutic needs. Dynamic combinatorial libraries[6a] and computational tools[6b-c] have been recently employed to discover new hydrogel forming peptide motifs. The design of these biomaterials is widely inspired and elicited from self-assembling natural proteins. Therefore, such materials mostly differ by their amino acid sequence and, hence, varying side chain functionality.[7] However, only little efforts have been made in the design of backbone engineered peptide hydrogels. Along this line, organogels from short homo-oligomeric β- and γ-peptides have been previously reported.[8] Xu and co-workers have obtained one of the first hydrogel nanofibers from N-capped β-peptides which showed higher biostability than α-peptides.[9] Thus, knowing their ability to adopt defined secondary structure[10a] and excellent stability towards enzymatic degradation,[10b] we were motivated to explore backbone-expanded γ-peptides as new generation hydrogel biomaterials. Here we report the design-based discovery of supramolecular hydrogels from short γ-gabapentin oligomers. γ-Gabapentin is a newly synthesized γ-amino acid building block. The distinct influence of adjacent arginine and lysine residues on hydrogel formation has also been studied. Furthermore, similar to amyloid beta (Aβ), the

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N-pyroglutamyl modified peptide motif shows accelerated hydrogel formation leading to stable and mechanically superior hydrogel.

Results and Discussion

Gabapentin (Gpn) is an analogue of the neurotransmitter γ-aminobutyric acid (GABA) and is currently being used as an anti-epileptic drug and for treatment of neuropathic pain.[11] Gabapentin has been extensively utilized as building block in the synthesis of helical peptidomimimetics.[12a-b] In the present study, we have chemically synthesized the γ-isomer of gabapentin (γ-Gpn) by formally shifting the spirocycle from β to γ position (Scheme 1A). The trifluoroacetate salt of γ-Gpn crystallizes in monoclinic $P2_1/c$ space group with the amino group positioned axially as shown in Scheme 1B. The additional torsional angles θ_1 and θ_2 (around C^{α}-C^{β} and C^{β}-C^{γ}) adopt *anti* (171°) and *gauche* (63°) conformation, respectively. As different polymorphs of gabapentin are expected due to accessible different conformational states,[12c] we aimed at understanding the structural propensity of γ-Gpn in peptides. We first designed and synthesized the short tripeptide **1** on solid support (Scheme 1C). Interestingly, **1** adopts an extended sheet type of conformation as confirmed by 2D NMR experiments (see the Supporting Information). Absence of NOE cross peaks for inter-residue amide NH-NH interactions suggests lack of intramolecular hydrogen bond and strong evidence of NH^{i}-$C^{\alpha}H^{(i-1)}$ and NH^{i}-$C^{\beta}H^{i}$ interactions observed in ROESY spectra suggested the completely unfolded structure of **1**. Amide I stretching frequencies centered at 1628 cm^{-1} in FT-IR (see Supporting Information) further supports its extended sheet structure. Notably, while Gpn strongly favors helical conformation,[12a-b] γ-Gpn reported here displays high propensity towards extended or unfolded structures. Extended sheet conformations from homo-oligomers of other γ-amino acid building blocks have been reported previously.[8c,13]

Interestingly, upon standing overnight in DMSO, peptide **1** was observed to self-assemble into self-supported gel structure at 4mg/mL concentration. The FE-SEM image of the lyophilized gel sample shows assembly of **1** into nanofibers (Supporting Information). Variable temperature NMR experiment (20-80°C in DMSO) revealed that all backbone amide protons are involved in intermolecular H-bonding (Figure. 1A, upfield shift with rising temperature).[8b] Interestingly, a gradual downfield shift with increasing temperature observed for all spirocyclic ring protons as well as backbone methylene units confirms the involvement of hydrophobic interactions in driving self-assembly[14] (Figure 1B). The terminal amide protons (-$CONH_2$) are being exchanged with increasing temperature as seen in Figure 1A. Intriguingly, these protons are being rapidly exchanged by the protons from residual water as seen from strong NOE interactions between them and the solvent signal (Figure 1C). Primary requirement to impart gel formation on a hydrophobic peptide motif in aqueous environment is a hydrophilic surface functionality to increase

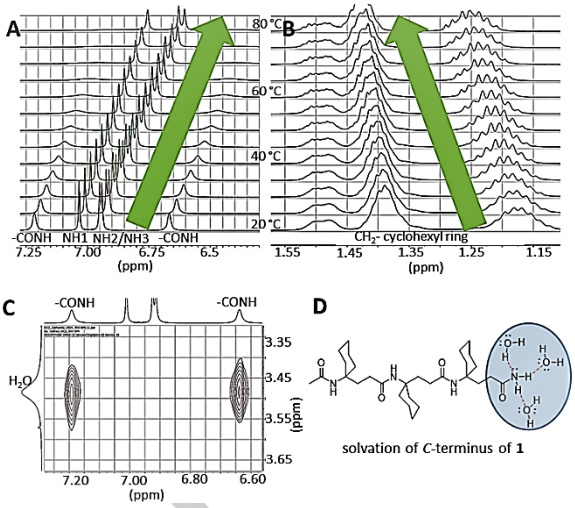


Figure 1. Variable temperature (20-80°C) ^{1}H-NMR spectra (DMSO-d_6) suggest self-assembly of **1** driven by A) intermolecular backbone H-bonding and B) side chain hydrophobic interactions. C) Partial ROESY spectra depicting strong NOE between terminal amide protons of **1** and residual solvent water. D) Schematic representation of the solvent-exposed *C*-terminus of **1.**

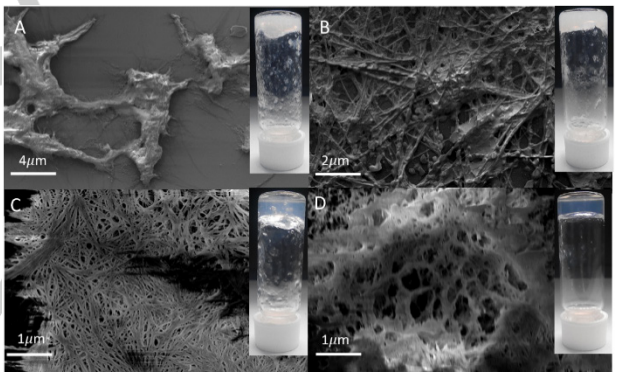


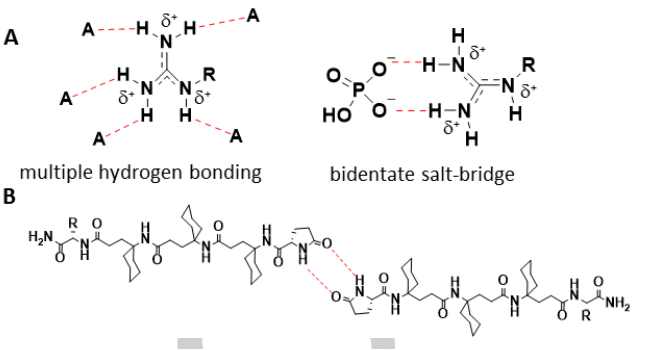
Figure 2. FE-SEM morphologies of A) **2**, B) **3**, C) **4** and D) **5** (inset: inverted vial test showing self-supported hydrogel formation).

water solubility. The hydrophilic *C*-terminus of the otherwise highly hydrophobic peptide **1** (Figure 1D) motivated us to introduce a charged polar amino acid head group at the solvent exposed end. Hence, we synthesized peptides **2** and **3** with lysine and arginine residues at the *C*-terminus, respectively (Scheme 1C). As anticipated, both peptides were completely dissolved in water, but failed to self-assemble into a hydrogel even at higher concentration. FE-SEM images in water suggest that **2** and **3** do not undergo nano-fibrillar self- assembly and form spherical aggregates (see the supporting Information). This can be explained by the fact that repulsion between positively charged lysine in **2** and arginine in **3** impedes their association and thereby halts hydrogel formation.

The influence of specific ions on short peptide based supramolecular hydrogels have been previously studied.[15a] To understand their self-assembling propensity in the presence of guest ions also referred to as the *Hofmeister effect*, we investigated **2**

and **3** dissolved in phosphate buffer at pH 7.4. Notably, both peptides form self-supported and opaque hydrogels upon standing overnight. FE-SEM images of lyophilized hydrogel samples clearly show that both peptides form fibrillar structures (Figure 2A-B). Besides the elongated fibers, spherical and filamentous aggregates were also observed. This non-uniform and irregular aggregation might have caused the opaque nature of the hydrogel (Figure 2A-B, inset). Peptide **3** manifested distinct and elongated fiber formation compared to **2**. The minimum gelation concentration (MGC) for **2** and **3** was determined as 15 mg mL^{-1} (23.2 mM) and 3mg mL^{-1} (4.45 mM), respectively. This striking difference in MGC and the distinct FE-SEM morphologies prompted us to investigate the exceptional ability of arginine over lysine for driving the self-assembly process in short peptides. This can be presumably explained by comparing properties of arginine over lysine.[15b-d] The arginine sidechain is more basic compared to the lysine sidechain - the guanidinium group has a higher pK_a value (≥12) and therefore contributes towards stronger electrostatic interactions in water. The distinct planar geometry of the guanidinium ion in arginine is capable to form a greater number of H-bonds and stable bidentate salt bridges with guest ions like hydrogen phosphate (HPO_4^{2-}; Scheme 2A). The ability of arginine to form electrostatically defying like-charge Arg-Arg pairing also promotes their intermolecular association in water.[15c] Taken together, Arg has advantages over Lys to favorably stabilize supramolecular hydrophobic interactions in **1**. Our observation is in agreement with the postulate that hydrophobic interactions are modulated by proximally located ions and salt bridges in aqueous environment.[16]

The hydrogels obtained from **2** and **3** are metastable; on sheer stress, these hydrogels rapidly exude water to leave behind a semi-solid gel (Figure 3A). Hence, we decided to introduce a supramolecular crosslink via additional orthogonal H-bonding to increase the elasticity of these hydrogel materials. Instead of *N*-acetyl capping in **2** and **3**, we chose to incorporate pyroglutamyl residues as pendant element. It has been shown that *N*-pyroglutamyl modified amyloid beta (Aβ) peptides trigger early seeding to enhance fibril aggregation leading to increased toxicity in Alzheimer's disease.[17] The effect of such modifications on small aggregating peptide motifs has not yet been tested. To address this issue, we synthesized peptides **4** and **5** (Scheme 1C). Introduction of an *N*-terminal pyroglutamyl residue on solid support was efficiently achieved by PyBOP-mediated coupling of pyroglutamic acid to the free *N*-terminus of resin-bound peptides **2** and **3**. Other uronium based coupling reagents failed to yield quantitative couplings. To our surprise, peptides **4** and **5** formed optically transparent and mechanically stable hydrogels at physiological pH. The effect of the *N*-pyroglutamyl modification was also transmitted on their nano-scale morphologies. FE-SEM images show comparatively uniform fibrous and highly entangled morphology for both **4** and **5** (Figure 2C-D). In contrast to **2** and **3**, exceptional structural homogeneity on the nanoscale and absence of irregular aggregates helped **4** and **5** to form optically transparent hydrogels (Figure 2C-D, inset). Interestingly, **5**



Scheme 2. A) H-bonding and salt bridge offered by guanidinium ion of arginin side chain (A=acceptor, R= arginine). B) Plausible self-complementary H-bonding originated from pyroglutamyl.

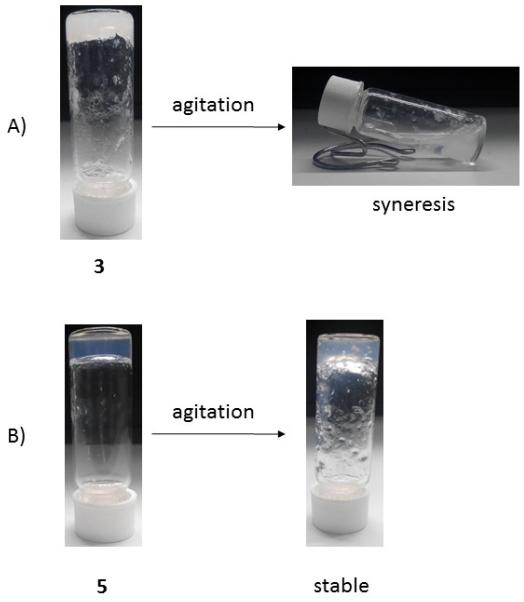


Figure 3. A) Metastable hydrogel **3** exudes water on agitation and B) stal hydrogel **5** retains self-supported nature after vigorous agitation (no syneresis).

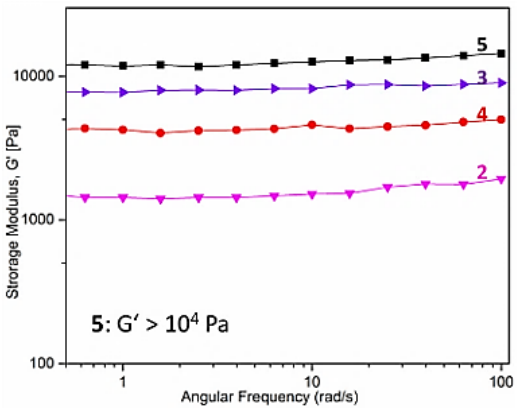


Figure 4. Frequency sweep rheology experiment performed on hydrogels **2**-**5** at 25mM concentration after 2 days of aging.

shows a highly porous structure capable of retaining high water content (>99 wt%). MGC for **4** and **5** was found to be 8 mg mL^{-1} (11.18 mM) and 2 mg mL^{-1} (2.69 mM). *N*-Pyroglutamyl modification was also observed to acutely accelerate the gelation rate. Peptide **4** and **5** form self-supported hydrogels in PBS within one hour (confirmed by inverted vial experiment). Moreover, these hydrogels were found to be physically stable even after vigorous agitation (Figure 3B). The MGC for the peptides was observed in the order **2** > **4** > **3** > **5**. The gelation rate was observed in the order **5** > **4** > **3** > **2** with peptide **5** form fastest gel within 10 minutes. Such a dramatic influence of *N*-acetyl to *N*-pyroglutamyl modification on stability, optical transparency and aggregation kinetics on otherwise metastable and opaque hydrogels encouraged us to hypothesize on the self-assembly mechanism of **4** and **5**. We presume that additional supramolecular crosslinks originated from self-complementary hydrogen bonding offered by the pyroglutamyl residue arrange the self-assembling peptide in ordered manner (Scheme 2B and Scheme S1 in the Supporting Information) and thereby impart material stability. Such dynamic and non- covalent crosslink could prove useful to coax reversible properties into peptide materials.

Moreover, to know the hydrogel formation at different pH values, we tested the self-assembly of these peptides in PBS at different pH. The peptides **2**-**5** manifested hydrogel structures over the pH range of 6-14. However, peptide **2** and **4** formed soft semi-solid hydrogel at pH 6. The viscous solutions were obtained for the pH values < 6.

Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) was used to further study the final conformation of peptide aggregates. Inspection of the amide I (1700-1600 cm^{-1}) and amide II (1600-1500 cm^{-1}) bands supports the extended β-structure of peptides (see the Supporting Information). Freeze-dried hydrogel samples of **2**-**5** show amide I stretching frequencies centered at 1637-1634 cm^{-1} and amide II frequencies centered at 1555-1550 cm^{-1}, which are typical for β-type extended conformations.[18] Shoulders with reduced intensities in amide I region appeared in the range of 1676-1685 cm^{-1} can be attributed to the antiparallel nature of β-sheet. Interestingly, peptide **2** shows an additional non-β component at 1665 cm^{-1}. This peak might be assigned to a turn in the peptide backbone.

Far UV CD signatures of peptides **2**-**5** in trifluoroethanol (TFE) were determined (see the Supporting Information). All peptides showed spectra characteristic for β-conformations with negative and positive Cotton effects centered around 216 nm and 193-195 nm, respectively. However, **3** did not exhibit minimum at 216 nm at given concentration. The cross-over for other peptides occurred at 208 nm. Peptide **5** shows the most intense CD signature compared to all other candidates suggesting that, similar to the Aβ, the β-structure is pronounced due to the *N*-pyroglutamyl modification.[17] CD spectra of peptides with increasing percentage of water in TFE show gradual transition to random structures. It underlines the role of TFE in stabilization of β-conformation in solution. Noteworthy, TFE is known to stabilize helical conformations and turns of β-hairpins in polypeptides and proteins.[19] However, stabilization of β-sheet structure in TFE is poorly documented. Nonetheless, the stabilization of β-sheet structures of a short homo-oligomeric peptides in TFE was previously reported.[20]

Oscillatory rheology is one of the best methods to measure the visco-elastic behaviour of soft materials like peptide hydrogels.[21] We quantitatively determined the stiffness of the hydrogels of peptide **2**-**5** in frequency sweep rheological studies. Identical molar concentrations were used to facilitate direct comparison of their visco-elastic properties (Figure 4). Peptide **5** forms the stiffest and most homogeneous gel with the highest elastic modulus (G'>10^4 Pa). This can be attributed to the dual contribution of the guanidinium moiety and the pyroglutamyl mediated supra- molecular crosslink that effectively hold solvent water compared to other peptides (also seen in FESEM morphology). Peptide **2** showed the lowest G' value among the tested peptides. The metastable gels from **2** and **3** show syneresis and release water to yield fibrous aggregate after the rheological test. The more stable gels from **4** and **5** retain their solid-gel like structure. Higher G' values for **3** and **5** over **2** and **4** prove the strong ability of arginine to drive stable supramolecular assembly.

Conclusions

In conclusion, our data constitute an interesting example how the positional displacement of the spirocycle from β to γ position of gabapentin exerts profound impact on the structure of a short oligomer both on molecular and supramolecular level. Differential impact of Arg versus Lys on peptide self-assembly gives notion of ion and salt-bridge dependent modulation of hydrophobic interactions on the nanometer scale in proteins.[16] Furthermore, self-complementary H-bonding driven by *N*-pyroglutamyl capping is observed to abolish irregular aggregation yielding optically transparent hydrogels on one hand and increasing its mechanical stiffness on other. Such a material is an ideal ECM surrogate as it facilitates the smooth movement of ex vivo cultured cells and allow free exchange of gases and nutrients required for their normal growth. The hydrogel obtained from peptide **5** shows the morphology with high water content (>99 wt%), high optical transparency, acceptable mechanical stiffness, and known proteolytic stability, which qualifies it for tissue engineering applications.[5] Moreover, *N*-Pyroglutamyl capping have previously shown early seeding and enhanced cytotoxicity of amyloid β fibrils in Alzheimer's.[17] The short self-aggregating peptide system described here could be useful motif to modulate, both therapeutically and diagnostically, the aggregation properties of amyloid β. Moreover, the results presented here should guide the future bottom-up design of short peptide based biostable material for their context dependent use in biomedicine and tissue engineering.

Experimental Section

Synthesis of peptides 1-5

Peptides **1-5** were synthesized by manual solid-phase peptide synthesis on a Rink Amide resin by using Fmoc chemistry (0.25 mmol scale). Coupling reactions were performed by using the HBTU/HOBt activation protocol. Fmoc deprotections were done with 20 % piperidine in DMF. The *N*-terminus of the peptide (peptides **1**-**3**) was capped with an acetyl group. The pyroglutamyl capping (peptides **4** and **5**) was achieved by PyBOP/HOBt activation protocol. Peptides were cleaved from the resin by using 95 % trifluoroacetic acid, 2.5 % water and 2.5 % triisopropyl silane cleavage mixture. Precipitation of the crude products in cold diethyl ether and subsequent drying under reduced pressure yielded crude powders. Reverse-phase HPLC purification (detector: 220 nm) on a C18 column (90 % methanol in 40 min) at a flow rate of 1.5 mL min^{-1} was carried out to obtain the pure peptides. The exact mass of peptides were determined using MALDI TOF/TOF.

Crystal structure of γ-Gpn

Crystals of trifluroacetate salt of γ-Gpn were grown by slow evaporation from a solvent composition of methanol/water (3:1). A single crystal, rectangular in shape (0.192 × 0.18 × 0.05 mm^3), was mounted on a loop. The X-ray data were collected at 99.98(10) K on Agilent SuperNova, Dual, Cu at zero, Atlas diffractometer. Space group $P2_1/c$; a =10.8879(5), b = 9.5689(3), c = 13.6666(6) Å; α = γ = 90° and β = 110.500 (5)°, Volume, V = 1333.69(10) $Å^3$, monoclinic $C_9H_{17}NO_2 \cdot C_2HF_3O_2$; Z = 4; ρ-calcd. =1.421 $mgmm^{-3}$; absorption coefficient, μ = 1.165 mm^{-1}; F (000)=600. The final R value (all data) was 0.0357 (wR_2=0.0878) with zero restraints; The largest deference peak and hole were 0.33 and -0.26 e $Å^{-3}$, respectively.

Circular Dichroism (CD)

CD spectra were collected with a JASCO-810 CD spectrophotometer fitted with a Peltier temperature controller, using a rectangular quartz cuvette with an optical path length of 1 mm. The scanning speed of 50 nm/min was used to aquire 5 accumulations at 20 °C. The samples were prepared by dissolving 0.5 mg of peptide in 1 mL solvent. Trifluoroethanol (TFE), water and 50% TFE-water mixtures were chosen to obtain the spectra. The spectra recorded in phosphate buffer produced higher voltage (HT) values.

FE-ESEM

The field emission environmental scanning electron microscope (Philips XL 30) was used to analyse nanoscale morphologies of peptide hydrogels. The freeze dried hydrogel samples were placed on clean silicon surface fixed on metal stubs. Samples were sputter-coated with Pd and imaged.

Rheology

Measurements were performed on MCR-101 (Physica, Anton-paar) rheometer. Hydrogel samples were prepared and aged for 48 h before measurement. The visco-elastic properties were recorded using 25 mm parallel plate geometry and as a function of frequency sweeps between 0.1 and 100 Hz at constant strain (0.1%). All peptide hydrogels were prepared at 25 mM concentration for rheological studies.

Preparation of hydrogel

The pre-weighed HPLC purified peptides were dissolved in PBS (10 mM) by vortexing for 1 min and gently warmed, if needed. The vial containing peptide solution was kept standing and undisturbed. The hydrogel formation was confirmed by inverted vial test.

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Keywords: hydrogel • peptides • biomaterial • foldamer • supramolecular chemistry

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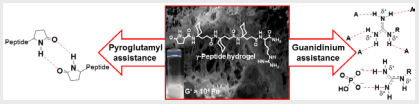

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Remarkable modulation of self-assembly in short γ-peptides by neighboring ions and orthogonal H-bonding

Bio(stable)material: The bottom-up design and systematic modulation of material properties of hydrogel obtained from short γ-peptide foldamer is discussed. On accounts of its intrinsically distinct biochemical properties, arginine demonstrates favourable influence on hydrogel material over lysine. Similar to amyloid β, the impact of *N*-pyroglutamyl modification on aggregation of short γ-peptides motif is investigated. Such modification imparts dramatic stiffness into hydrogel.