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We demonstrate an in-situ ultrasonic approach to influence self-assembly across the supramolecular to micron length scales, showing enhancement of supramolecular interactions, chirality and orientation, which depends on the peptide sequence and solvent environment. This is the first successful demonstration of using oscillating pressure waves to generate anisotropic organo- and hydro- gels consisting of oriented tripeptides structures.

Peptides are versatile building blocks for the formation of structured materials for a range of applications in biomedicine and bionanotechnology. However, while they may be highly organized at the supramolecular (nanoscale) level, most self-assembling materials are disordered at the macro-scale. The ability to control their orientation across the length scales opens up ways to better control mechanical, optical and electronic properties.

A number of approaches have been used to align both inorganic and organic materials. These include Langmuir-Blodgett techniques, lithography, chemical vapor deposition, magnetic and electric fields. Several successful examples have been reported to generate anisotropic gels but few methods allow for in-situ alignment in solution or the gel-phase. Stupp’s group demonstrated a thermal pathway to process self-assembling peptide amphiphiles into highly aligned macrostructures. Yan and co-workers used heating to trigger covalent crosslinking of cyclo-dipeptides leading to highly ordered peptide crystals. Xu and co-workers reported an enzymatic self-assembly approach which, by nature of the aromatic side chains of peptides used, resulted in alignment of fibers through aromatic-aromatic interactions for generating anisotropic hydrogels. Ultrasound, i.e. high frequency (>20kHz) pressure waves, is commonly used in supramolecular chemistry to overcome energy barriers and disrupt intermolecular interactions, which has been used routinely to control self-assembly and gelation processes, including most recently the demonstration of dynamic and transient reconfiguration of peptide nanostructures. We reasoned that the directionality of sound waves - longitudinal pressure waves which lead to oscillating compression of the solvent volume in the same direction as the wave itself- may provide opportunities to introduce anisotropy in gel-phase, especially if the ultrasound is applied directionally. Thus, in this work we demonstrate the use of ultrasound to trigger the self-assembly and alignment of tripeptide nanostructures resulting in thermodynamically stable anisotropic organo- and hydrogels (Scheme 1).

Scheme 1 (a) Chemical structures of tri-peptide derivatives of DFFI and DFFD. (b) Cartoon of self-assembly of DFFD and DFFI altering from disordered short fibers to highly oriented fibrous clusters after ultrasound exposure in methanol (DFFD) and buffer solution (pH 8, DFFI).

The D-L-L stereochemistry in tripeptides was previously shown to give rise to self-supporting hydrogels by Hartley’s group without the need for modification with aromatic or aliphatic groups normally required for short peptide gelation. Here, we use tripeptides
We further investigated these observations using diffusion-ordered spectroscopy (DOSY). These experiments reveal that the diffusion becomes slower upon application of ultrasound, suggesting supramolecular reorganization towards formation of larger structures (Figure S1).

To gain insights into the chiral organization of the peptide structures, the presence of chirally organized phenyl moieties and orientation of self-assembled structures within the tripeptide before and after ultrasound, circular (CD) and linear dichroism (LD) spectroscopy were used. As Figure 2a shows, a CD and LD silent spectrum can be obtained for 3FFD before ultrasound. Remarkably, sound waves induced supramolecular chirality by significantly enhancing the peaks at 240 and 270 nm in the organogel, which may be attributed to the supramolecular organization of chiral centers within π-π stacking of phenylalanines. In 3FFD, LD spectra report that a minimum at 205 nm and a maximum at 255 nm are observed which indicates both perpendicular and parallel orientation of phenyl moieties to the axis exist in self-assembled structures (Figure 2b). Infrared spectroscopy in the amide I region of the spectrum (1600-1700 cm\(^{-1}\)) was used to determine the hydrogen bonding networks within the fibrous structures. Spectra of the samples with and without ultrasound treatment are displayed in Figure 2c. The suspended precipitate of 3FFD in methanol before and after ultrasound irradiation. The storage modulus (G') exceeds the loss modulus (G'') by factor of 12 demonstrating the tripeptides formed viscoelastic gels. Overall, these results revealed that the size transition of peptide self-assembled structures from nano- to micron scale was acoustically induced, where supramolecular chirality and orientation of peptide microtubes and hydrogen bonding interactions were dramatically enhanced (Scheme 1).

In water, gelation was not observed for 3FFD, most likely due to the hydrophilicity of charged aspartic acid. The peptide sequence was therefore adapted to increase the hydrophobicity. By changing aspartic acid (3FFD) to more hydrophobic isoleucine (3FFI), the ultrasonic alignment can be used to fabricate anisotropic hydrogels, indicating that the anisotropic arrangement of supramolecular interactions.

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**Figure 1** TEM images of 3FFD in methanol (a) before and (b) after ultrasound exposure. Inset photographs are 3FFD forming precipitation or gel without or with ultrasound in methanol. (c, d) SEM and (e) Two-photon microscopy images of the ultrasonicated 3FFD peptide gels. The scale bars are (a) 100 nm, (b) 100 nm, (c) 10 µm, (d) 5 µm and (e) 5 µm. The concentration is 25 mM.

**Figure 2** (a) CD, (b) LD and (c) FT-IR spectra of 3FFD in methanol before and after ultrasound irradiation. (d) Rheology of the elastic modulus (G') and viscous modulus (G'') of 3FFD organogel after ultrasound irradiation. The concentration is 25 mM.

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We used nuclear magnetic resonance (NMR) Spectroscopy to obtain insights into the supramolecular transitions induced by ultrasound. As Figure S1 shows, after exposure of 3FFD to ultrasound, a downfield shift was observed suggesting change in the chemical environment with enhanced supramolecular interactions.
interactions is highly related to the peptide sequences and solvent environment. 12.5 mg of \(^5\)FFI was dissolved in 1 ml phosphate buffer solution (pH 8) (30 mM). The solubility was enhanced by heating up to 80 °C. After cooling down to room temperature, a precipitate consisting of entangled short nanofibers was observed (Figure 3a). The solubility of \(^5\)FFI in buffer solution determined by UV-Vis spectroscopy is 15.5 mM. When in-situ ultrasound treatment was introduced to the \(^5\)FFI buffer solution for 30 seconds during the cooling process, a hydrogel was obtained. TEM images show that the self-assembled peptide structure changes to bundles of highly oriented microfibers (Figure 3b). SEM images indicate that the macroscopic alignment of peptide fibers up to 1 µm of thickness and 100 µm of length are achieved (Figure 3c). Two-photon microscopy image showed the formation of micro-structure of \(^5\)FFI in the gel-phase (Figure 3d).

Fig. 3 TEM images of \(^5\)FFI in phosphate buffer (pH 8) (a) before and (b) after ultrasound irradiation. Inset photographs are \(^5\)FFI forming solution or gel without or with ultrasound in buffer. (c) SEM image and (d) Two-photon microscopy images of the ultrasonicated \(^5\)FFI peptide gels. The scale bars are (a) 100 nm, (b) 1 µm, (c) 10 µm and (d) 2 µm. The concentration is 30 mM.

To acquire more insights at the supramolecular level in the aqueous medium, the samples were characterized by spectroscopic methods. Significant enhancement of CD and LD signal at 215 nm revealed the presence of chiral centers within \(\beta\)-sheet type arrangement and perpendicular orientation of phenyl moieties to the axis in self-assembled structures after ultrasonic exposure (Figure 4a, b). IR absorption spectra were taken by using deuterated phosphate buffer at pD 8 and show a broad band from 1635 to 1650 cm\(^{-1}\) (Figure 4c), indicating a less ordered H-bonding pattern between the peptide backbones.\(^7\) Figure 4d shows the linear viscoelastic responses of the \(^5\)FFI hydrogels. The storage modulus (\(G'\)) exceeds the loss modulus (\(G''\)) by factor of 22 demonstrating that the tripeptides formed viscoelastic gels. The results indicate that using one-directional ultrasound, which provides differential influences of self-assembly of peptides in both organic and aqueous media by dramatically increasing the hydrophobic stacking and hydrophilic hydrogen bonding interactions, gives rise to the enhancement of chirality, orientation and gelation process.

Following the same experimental procedure as mentioned above, we used a standard sonication bath (The frequency is around 40 KHz with varied acoustic pressure and amplitude,) as a control experiment. Figure S2 shows that the sonication bath can also trigger the gelation of \(^2\)FFD in methanol and \(^5\)FFI in buffer solution. However, disordered fibrous structures are observed by SEM (Figure S2), which proves that oscillating pressure waves give rise to the highly oriented peptide self-assembly.

Conclusions

In conclusion, we demonstrate the use of ultrasonic waves as an in-situ approach to induce the self-assembly and alignment of tripeptides forming highly ordered micro- fibrous structures in both organic and aqueous media. Two different peptides were studied, \(^2\)FFD and \(^5\)FFI with the former forming an anisotropic organogel in methanol, while the latter forms an anisotropic hydrogel, showing that the anisotropic arrangement of supramolecular interactions is highly related to the peptide sequences and solvent environment. These results indicate that the influence of ultrasound transcends the molecular to nano- and micron length scales. The ultrasonic approach may give rise to facilitate amelioration of organization and mechanical properties supramolecular nanostructures.

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Notes and references