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Amphiphilic Peptide-based Supramolecular, Non-Cytotoxic Stimuli-responsive Hydrogels with Antibacterial Activity

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ABSTRACT

A series of peptides with a long fatty acyl chain covalently attached to the C-terminal part and a free amine ($-NH_2$) group at the N-terminus have been designed, so that these molecules can be assembled in aqueous medium by using various non-covalent interactions. Five different peptide amphiphiles with a general chemical formula $[H_2N-(CH_2)_nCONH-Phe-CONHC_{12}(n=1-5, C_{12}= \text{dodecylamine})]$ have been synthesized, characterized and examined for self-assembly and hydrogelation. All of these molecules [**P1** ($n=1$), **P2** ($n=2$), **P3** ($n=3$), **P4** ($n=4$), **P5** ($n=5$)] form thermo-responsive hydrogels in water (pH 6.6) with nano-

fibrillarnetwork structure. Interestingly, the hydrogels obtained from compound **P4** and **P5** exhibit potential anti-microbial activity against Gram positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*) and Gram negative bacteria (*Escherichia coli*). Dose dependent cell-viability studies using MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) by taking human lung carcinoma (A549) cells vividly demonstrates the non-cytotoxic nature of these gelator molecules *in vitro*. Haemolytic studies show non-significant or little haemolysis of human erythrocyte cells at minimum inhibitory concentration (MIC) of these tested bacteria. Interestingly, it has been found that these antibacterial non-cytotoxic hydrogels exhibit proteolytic resistance towards the enzymes proteinase K and chymotrypsin. Moreover, the gel strength and gel recovery time have been successfully modulated by varying the alkyl chain length of the N-terminally located amino acid residues. Similarly, the thermal stability of these hydrogels has been nicely tuned by altering the alkyl chain length of the N-terminally located amino acid residues. In the era of antibiotic resistant strain of bacteria, the discovery of this new class of peptide-based antibacterial, proteolytically stable, injectable and non-cytotoxic soft-materials hold future promise for the development of new antibiotics.

INTRODUCTION

The aggregation and network formation of low molecular weight gelators(LMWGs)¹⁻⁵has become one of the most expanding areas in current research. Supramolecular hydrogels⁶⁻¹¹are constructed through the self-assembly of individual molecules by using various non-covalent interactions including hydrogen bonding, π - π interactions, van der Waals interactions, hydrophobic interactions and others to form a micro-/ nano- network structure with a lot of cavities inside, and under suitable conditions water molecules are immobilized within the network structure to form hydrogels. Peptide-based hydrogels¹²⁻¹⁷attract particular attention

not only due to their capacity to form hydrogels via various non-covalent interactions through molecular self-assembly, but also owing to their special characteristics including bio-functionality. They have wide-spread applications in sustained release of drugs and important biomolecules,¹⁸⁻²² cell culture,^{23,24} tissue engineering,²⁵⁻²⁹ pollutant removal from wastewater,^{30,31} oil spill recovery,^{32,33} wound healing,³⁴ as well as being effective antimicrobial agents³⁵. Most of these supramolecular gels are endowed with stimuli responsiveness that allows them to be used as a carrier of drugs³⁶ that can be released in response to an external stimulus like pH, heat, light, mechanical stress or others. Though there are many examples of thermo-,³⁷ photo-³⁷ and chemo-responsiveness³⁸ of peptide based gels, the making of a mechano-responsive peptide-based hydrogel still remains a challenging task.³⁹ The hydrogel which exhibits 'thixotropy',^{40,41} i. e., shear-thinning property is of particularly importance for industrial and bio-medical applications,⁴² as by applying mechanical stress or strain the system undergoes gel-to-sol transition, but immediately recovers back into gel state on removal of the stress. Rheological properties are particularly important for designing injectable scaffolds because the stiffness and recovery of the gel network scaffold regulates the injected system to remain localized in a well-defined approach. One of the major challenges in this field is to modulate the rheological properties along with thixotropic properties of the hydrogels.⁴³⁻⁴⁷ This is because different injectable therapeutic approaches require hydrogels with different rigidity to minimize unwanted leakage and flow of the injected fluid.⁴²

The increasing prevalence of bacterial strains which are completely resistant to conventional antibiotics present today possesses a great threat in modern society.³⁵ There is thus a crying need for new types of antibiotics to combat different types of bacteria. Several silver nanoparticle embedded antibacterial hydrogels are reported in the literature so far.⁴⁸⁻⁵⁰ However, these types of gels have several side effects like pigmentation of skin or eyes, oxidative DNA

damage or inflammation.⁵¹ Other conventional methods like delivery of antimicrobials loaded into or covalently attached to soft biomaterials often suffer from their limitations of either burst release or reduced activity of these antimicrobials.⁵² The biocompatible nature of supramolecular peptide-based gels make them a convenient starting platform to selectively develop potent antimicrobial agents.^{34,53,54} Several antibacterial agents based on polycationic peptide containing lysine or arginine residues are also well-known.^{55,56} Cationic and hydrophobic residue-containing amphiphilic peptides can be used as the molecular building blocks for antimicrobial functional supramolecular hydrogels, as they can mimic the structure of natural antimicrobial peptides.⁵² In this context, short peptide-based amphiphiles have become increasingly attractive in the development of peptide therapeutics. This is because these peptide amphiphiles can be easily synthesized with fewer steps and relatively low cost associated with their synthesis. The study of free N-terminal short peptide based amphiphiles that exhibit interesting functional properties is relatively rare.⁵⁷ Keeping this in mind and the rising demand of peptide-based antibacterial agents, we have designed a new class of simple and synthetic peptide-based amphiphile without lysine or arginine residues. A series of peptides with a common formula $[H_2N-(CH_2)_nCONH-Phe-CONHC_{12}(n=1-5, C_{12}=dodecylamine)]$ have been synthesized, well-characterized and studied for hydrogelation and for potential antibacterial activity. Figure 1 illustrates the chemical structures of these gelator molecules (**P1**, **P2**, **P3**, **P4** and **P5**). An attempt has been made to investigate systematically whether the increase in the alkyl chain length of these peptide-based gelator molecules can alter their self-assembly and gelation behaviour and also to examine the dependency of thermal and mechanical properties of these gels on the increase of the number of $-CH_2$ units in this series of hydrogelators. The structural change associated with an increment in alkyl chain length of the N-terminally located amino acid residue can affect the supramolecular organization of these gelator peptides and their affinity towards bacterial

membranes.⁵²The cationic charge density over the nano-fibrils and the improved rigidity of the hydrogels through the simple variation of structural domain can provide a mechanical support to the gel fibers to direct their adherence towards bacteria and it may boost up the antimicrobial activity.⁵²All of these hydrogelators were studied for antibacterial activity and interestingly, among all gelators in the series only the gel obtained from the longer alkyl chain containing gelators (**P4** and **P5** in the Figure 1) than the other gelator analogues exhibit potential antibacterial activity against Gram positive (*Staphylococcus aureus* and *Bacillus subtilis*) as well as Gram negative (*Escherichia coli*) bacteria. Both of these hydrogels also show negligible lytic effect against human erythrocytes. Furthermore, these hydrogels show significant stability towards proteolytic enzymes present inside the cells, thus enhancing the hydrogel's applicability for practical purposes in future. These gels also exhibit an excellent bio-compatibility with no significant cytotoxicity towards human lung carcinoma (A549) cells indicating the probable use of this new type of peptide-based amphiphiles as next generation antibiotics.

EXPERIMENTAL SECTION

L-Phenyl alanine (L-Phe), L-Glycine (L-Gly), β -Alanine (β -Ala), γ -amino butyric acid (γ -Aba), δ -amino valeric acid (δ -Ava), 6-amino caproic acid (Acp) and 7-amino heptanoic acid (Ahp) were purchased from Aldrich. Dodecylamine (C_{12}), 1-hydroxybenzotriazole (HOBt), N, N'-dicyclohexylcarbodiimide (DCC) and all solvents were purchased from SRL, India. Details of the synthetic procedures of gelator peptide, instrumentation details and spectroscopic analysis are given in the Supporting Information.

RESULTS AND DISCUSSION

Gelation Study:

A series of structurally related peptide-based molecules with free N-termini with a common formula $[H_2N-(CH_2)_nCONH-Phe-CONHC_{12}]$ ($n= 1-5$, $C_{12}= \text{dodecylamine}$) have been synthesized and studied for their respective self-assembly and gelation in aqueous medium (ultrapure water, pH = 6.6). All these peptide amphiphiles have been found to form hydrogels. All these gelator molecules share a common motif – a phenylalanine (Phe-) residue, an amino acid residue at the N-terminal site (without any protection) and a C-terminus blocked with a long alkyl chain (Figure 1). They contain a polar amine group at the N-terminus, intervening hydrogen bonding motif CO-NH and hydrophobic Phe- residue at the side chain as well as C-terminally located long chain alkyl amine. The purpose of incorporating Phe-residue is to promote self-assembly and gelation using π - π interaction and hydrophobic interaction.¹⁹ These new type of peptide-based amphiphiles self-assemble in aqueous medium and under suitable conditions they form gels. Figure 1a represents the common structure of the five gelator molecules in the series which contain N-terminally located amino acid residues, varying from glycine to β -alanine to 4-aminobutyric acid, to 5-aminovaleric acid to 6-aminocaproic acid residues accordingly. These peptide-based molecules are termed **P1**, **P2**, **P3**, **P4** and **P5** respectively. All the five gelators were found to form self-supporting hydrogels in ultrapure water (pH= 6.6) and the corresponding vials pictures of these peptide-based hydrogels (3.65mM) are shown in Figure 1b. The peptide gelator with higher 'n' value ($n= 6$, named as **P6**) did not form any gel under similar conditions, but a translucent viscous aggregate was appeared (Figure S19). For preparation of the hydrogels, 0.05mmol of each of the gelators were dissolved in 1400 μL of ultrapure water in separate glass vials by heating on a hot plate, homogeneous solutions were

obtained, then they were slowly cooled to room temperature (25 °C) and the respective hydrogels were formed within a few minutes (Figure 1). All the hydrogels are thermo-reversible in nature (Figure S20) and can be stored at room temperature for several months without degradation. Gels obtained from gelator molecules **P1**, **P2**, **P3**, and **P4** are transparent in nature, while **P5** forms a translucent hydrogel under similar conditions. Interestingly, it has been observed that except for **P1**, the hydrogels (**P2** to **P5**) are thixotropic. These hydrogels can be broken by simple shaking of the gel vials and the gel phase reappears within several minutes. This shear-thinning feature of these hydrogels enable them as injectable hydrogels and makes them attractive for systematic use in peptide therapeutics. Figure S21 clearly indicates the injectable nature of the hydrogel (3.65 mM) obtained from the gelator **P4**. The detailed mechano-responsiveness of the gels obtained from **P2** to **P5** has been investigated thoroughly in the succeeding section of this paper (Mechanical Stability and Thixotropic Property of Gels).

A survey of the gelation behaviours of the gelators has been summarized in Table 1. The minimum gelation concentration (MGC) of the gelator **P5**, is the smallest of any of the investigated hydrogels in this series. **P2** molecule bears the highest MGC value in this series. The MGC values decrease with increasing alkyl chain length of the gelator molecules starting from **P2** to **P5**. It is clear from the table that except hydrogel **P1**, all other hydrogels in this series shows the following trend for the minimum gelation concentration (MGC) value: **P2**>**P3**>**P4**>**P5**. Thus, the MGC value decreases with an increase in the alkyl chain length of the gelator molecules from **P2** to **P5**. However, the MGC value of the gel obtained from **P1** shows an anomaly and for this case the MGC value is 0.83 % (w/v), which is higher than **P5**, but lower than **P2** to **P4** gels. It was also observed that the peptide gelator with longest alkyl chain (**P5**) in the series underwent gelation very slowly, and it required around 40 min to form a stable gel (Table 1). While a decrease in the alkyl chain length in other peptide

variants causes a faster gelation rate for the other gels (from **P2** to **P4**) in the series under similar conditions (except for **P1**). The exact reason for the anomalous behaviour of **P1** gel is yet to be explored.

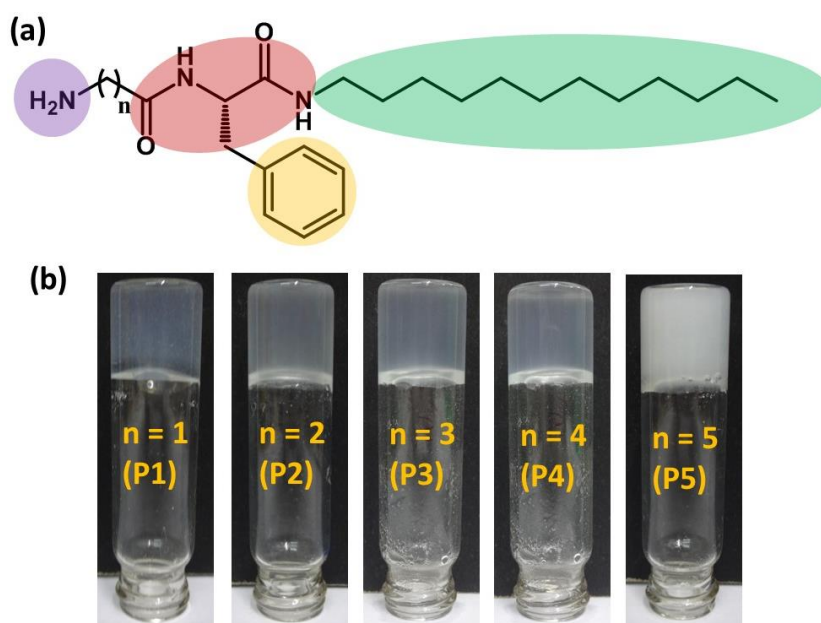


Figure 1. (a) The general molecular structure of the gelator peptides. (b) Pictures of vials containing hydrogels of **P1**, **P2**, **P3**, **P4** and **P5** at 3.65 mM concentration.

Thermal Stability of Gels:

To evaluate the thermal stability, gel-to-sol transition temperatures (T_{gel}) of the hydrogels in the series were measured (Table 1). It is evident that the gel melting temperature (T_{gel}) is the lowest for the hydrogel obtained from **P1** and the highest for **P5** hydrogel, keeping the concentration fixed in all cases. The thermal stability of the hydrogel is thus dependent on the molecular structure of the gelator molecule and is related to the alkyl chain length of the N-terminally located amino acid residues. The T_{gel} value shows the following trend: **P5** > **P4** > **P3** > **P2** > **P1** (Figure S22a). It can be envisaged that the **P5** molecules may pack better

among themselves in the gel state than other gelator molecules in the series and this can be due to the presence of more van der Waals interaction sites than other gelator molecules (**P1** to **P4**). This can be because **P5** has the most -CH₂ units in this series. The hydrogel **P1** with the N-terminally located Gly-residue has the least van der Waals interaction sites which may cause it to have the lowest T_{gel} value.

Morphological Study:

Field emission scanning microscopy (FE-SEM) was performed in order to examine the nanoscale morphologies of the xerogels prepared from the corresponding hydrogels of the peptide gelators (**P1** to **P5**). FE-SEM images (Figure 2) of these xerogels showed the formation of intertwined nano-fibrillar assemblies for all the hydrogel matrices. The images revealed that the nanofibers obtained from the xerogels were approximately 200 nm to 250 nm in width and several micrometres in length. These fibres were entangled with each other on large length scales to form a three dimensional nano-fibrillar gel network structure and entrapping water molecules to form a hydrogel. The xerogels obtained from hydrogels **P3**, **P4** and **P5** gave a striking morphology that was completely different from hydrogels obtained from **P1** and **P2**. Careful inspection of FE-SEM images (Figure 2) revealed the formation of an ordered array of nano-spheres fusing into nano-fibres for the gelators with longer alkyl chain containing amino acid residues (**P3**, **P4**, **P5**) in the series.

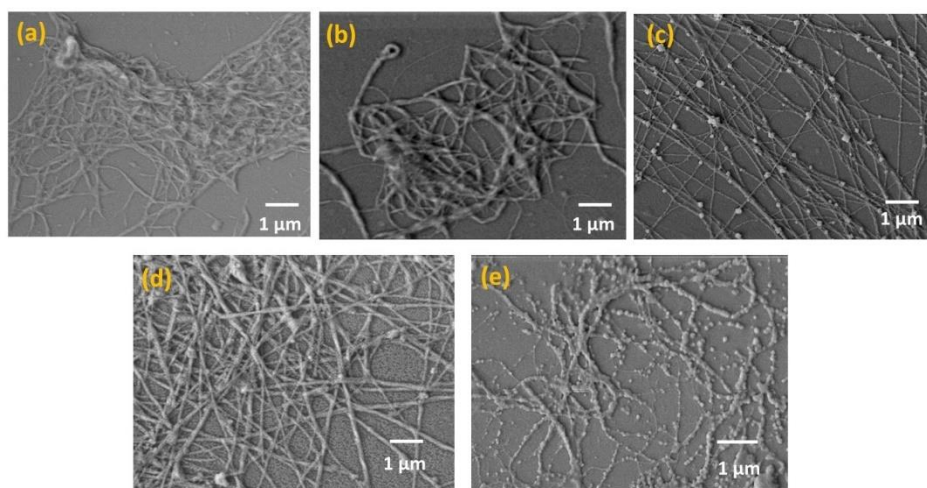


Figure 2.FE-SEM images of the xerogels obtained from(a) **P1**, (b) **P2**, (c) **P3**, (d) **P4** and(e) **P5**hydrogels.

Mechanical Stability and Thixotropic Property of Gels:

Rheological studies were performed to examine the visco-elastic behaviour, gel stiffness and mechano-responsiveness of all the hydrogels. All rheological studies have been done for gels **P1** to **P5** in similar concentrations (3.65mM) and similar conditions. It has been found that that the modulus gradually increases with an increase in the alkyl chain length of the N-terminally located amino acid residues except for the hydrogel **P1**(Figure S23). The hydrogel **P1** showed exceptional behaviour regarding gel formation and mechanical rigidity. At an angular frequency 1 rad/sec, a G' value of 1.43×10^2 Pa was obtained for freshly prepared **P1** hydrogel, and the gel was continued to stiffen ($G' = 1.26 \times 10^4$ Pa) upon aging upto 7days (Figure S24). Furthermore, hydrogel **P1** does not show any mechano-responsive behaviour. The mechanical strength of other hydrogels in this series show the following trend: **P5**>**P4**>**P3**>**P2**(Figure S22b). The storage modulus for the freshly prepared **P5** gel (2.7×10^2 Pa) indicates its higher mechanical strength compared to hydrogels obtained from **P2**, **P3** and **P4**. It is interesting to note that except **P1**, other hydrogels show thixotropic property, i. e., each of them are broken under the influence of mechanical shaking (stress/strain) and the gel phase reappears in each case upon the withdrawal of the external strain within several minutes. The mechanical strength (storage and loss modulus) and the gel recovery time after the complete cessation of the destructive strain are listed in Table 1. One noteworthy feature of this series of thixotropic gel is that the gel recovery time after the first cessation of large amplitude strain follows the order: **P5**>**P4**>**P3**>**P2**(Figure 3). This data clearly indicates that

P5 gel has the highest mechanical strength and the gel recovery time can be successfully modulated by increasing the alkyl chain length of the N-terminal residue. Both the mechanical strength and gel recovery time can be controlled by the alkyl chain length of the N-terminal residue. It is evident from Table 1 that both the gel recovery time and the gel strength increases with in the number of $-\text{CH}_2$ units of the N-terminally located amino acid residues of the respective gelator molecules. An increase in alkyl chain length of N-terminal amino acid probably enhances more van der Waals interactions among gelator peptides in their respective gel states. This can serve as a factor for the increased gel stiffness with an increase in alkyl chain length in this series of gelators.

	MGC (%) (w/v))	Gel formation time (min)	T_{gel} (3.65 mM) (° C)	G' (Pa)	G'' (Pa)	Recovery time after cessation of large amplitude strain (sec)
P1	0.83	15	48	143.7	16.8	-
P2	1.12	8	52	82.4	11.9	420
P3	0.99	10	58	99.3	13.7	480
P4	0.9	15	64	216.0	39.4	520
P5	0.77	40	68	272.2	46.8	680

Table 1. Comparison among gelation kinetics, thermal (gel melting temperature) and rheological properties of the freshly prepared hydrogels **P1**, **P2**, **P3**, **P4** and **P5**. The concentration of hydrogels was 3.65 mM for all these cases. G' and G'' values were measured at a fixed angular frequency 1 rad/sec (at 25 °C).

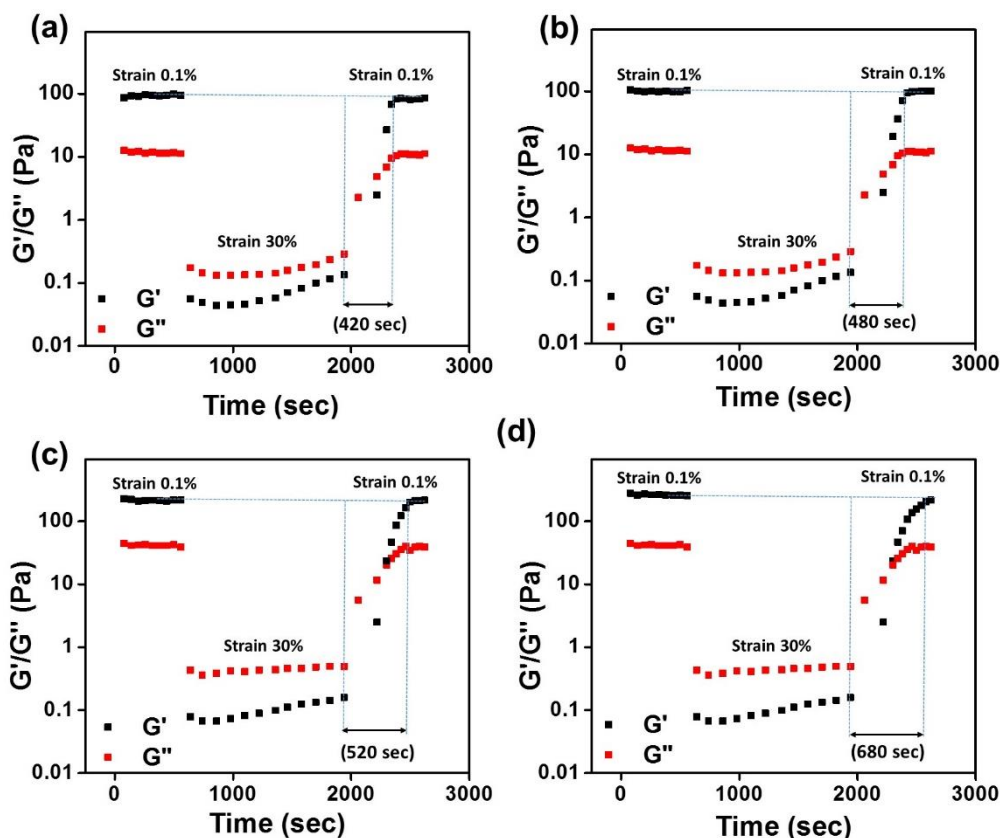


Figure 3: Time dependent step-strain rheological analysis of the thixotropic hydrogels (a) **P2**, (b) **P3**, (c) **P4** and (d) **P5** at 3.65 mM concentration at a fixed angular frequency of 1 rad/sec (at 25 °C). G' remains higher than G'' before the application of shear and after the complete removal of shear for each cases.

Structural Study:

Fourier transform infrared spectroscopic (FT-IR) studies have been carried out to obtain an insight into non-covalent interactions among the self-assembling gelator molecules during the gel formation. Figure S25 shows the FTIR spectra of different xerogels obtained from gelator peptides **P1**, **P2**, **P3**, **P4** and **P5**. It is observed that two peaks corresponding to both non-hydrogen bonded and hydrogen bonded NH-stretching frequencies have been observed at 3457 cm^{-1} and 3297 cm^{-1} for all xerogels obtained from these hydrogels. However, the intensity of the peak at 3457 cm^{-1} is more prominent for **P4** and **P5** gels than that of other

hydrogels and the peak intensity at 3297 cm^{-1} is most intense for **P1** and **P2** than the other hydrogels. The characteristic hydrogen bonded C=O stretching frequencies at 1640 cm^{-1} is observed in all cases and NH- bending (corresponding to hydrogen bonded) at 1544 cm^{-1} has also been seen for all five peptide based gels. The FTIR data revealed the presence of a hydrogen bonded sheet-like structure in the gel state during the self-assembly.¹⁸

To gain insight about the molecular arrangement within the supramolecular network structure, SAXS on gels and wide-angle powder XRD (WPXRD) of the xerogels obtained from the hydrogels were carried out. From the small-angle X-ray scattering (SAXS) (Figure 4a) measurements for the wet gel, a peak appeared corresponding to a d value around 24 Å . This value is higher than the calculated molecular length (21.5 Å), but smaller than double the calculated molecular length. Therefore, it can be assumed that the molecules stack among themselves in an inter-digitated pattern during their self-assembly and a probable model is illustrated in Figure 4c. The d value (around 26 Å) obtained from the SAXS (Figure S26a) of hydrogel **P1** is also in good agreement with a probable stacking pattern similar to that of **P2**. For the hydrogels **P3**, **P4** and **P5**, the corresponding d values derived from SAXS were around 19 Å , 24 Å and 16 Å (Figure S26b,c,d) respectively. In the WPXRD of the xerogels obtained from the hydrogel **P2** (Figure 4b), the peak corresponding to the d spacing of 3.82 Å indicates the existence of π - π stacking among the gelator molecules in their self-assembled state. The appearance of the peak corresponding to $d = 4.45\text{ Å}$ suggests the presence of a β -sheet-like structure. The WPXRD data derived from the xerogels obtained for the hydrogels **P1**, **P3**, **P4** and **P5** (Figure S27) are also well supported with the π - π stacking arrangement and a sheet-like structure formation in their respective self-assembled gel state.

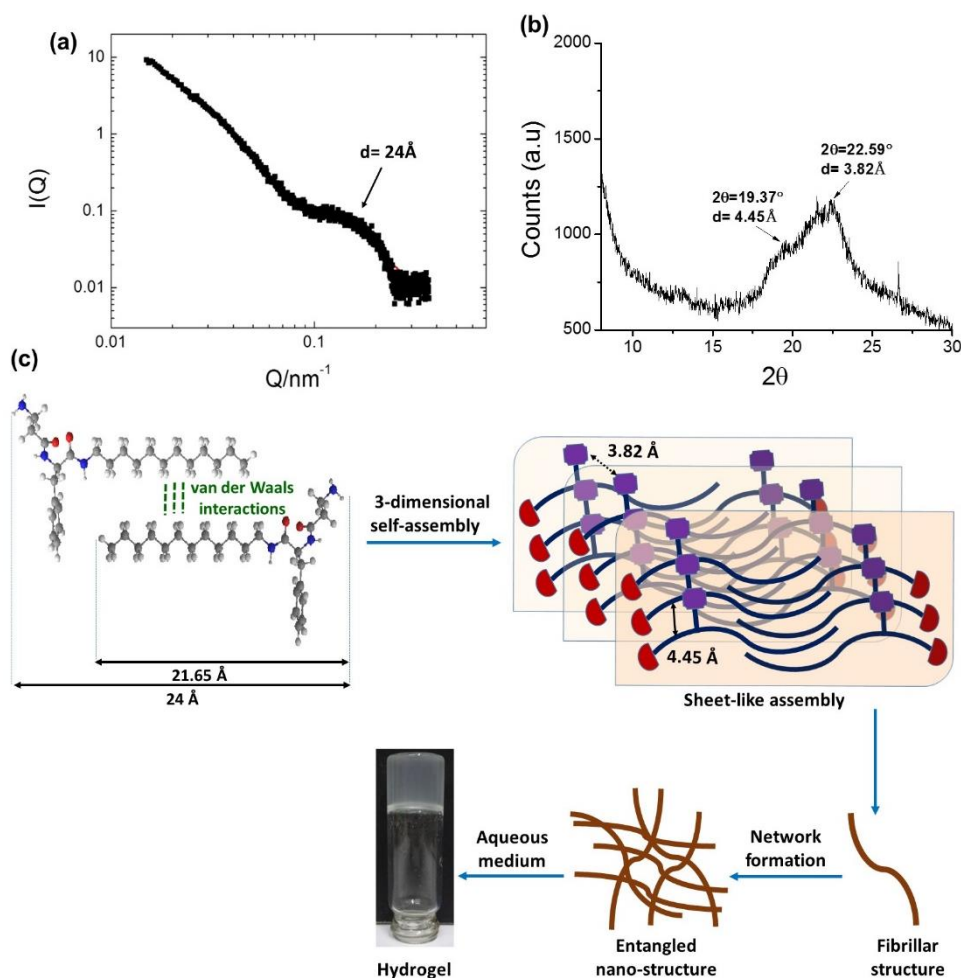


Figure 4. (a) SAXS intensity profile obtained from **P2** hydrogel shows a peak corresponding to $d = 24 \text{ \AA}$, which matches well with the calculated molecular length for packing of gelator molecules in an interdigitated form. (b) WAXRD of the xerogel obtained from the hydrogel **P2**. (c) A tentative model of the packing arrangement obtained from the stacking of the peptide molecules of **P2** based on SAXS, WAXRD data.

Antibacterial Study:

It is interesting to notice that some of these gelator peptides exhibit potential antibacterial properties. Another important point is that these peptides belong to a new class of peptide antibiotics bearing a unique positive charge at the N-terminus without any lysine or

arginine residues. Many of the peptide antibiotics bear either one or more lysine or arginine residue or both of these residues to exhibit antibacterial properties.^{55,56} There is a class of antimicrobial cyclic peptide with alternating D- and L- residues with no cationic residues.⁵⁸ Antimicrobial activity of the hydrogels (purified compound) were investigated against pathogenic test bacteria of Gram positive (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram negative (*Escherichia coli* and *Pseudomonas aeruginosa*) using the agar disc-diffusion method. The hydrogels **P4** and **P5** were found active against both Gram positive (*B. subtilis* and *S. aureus*) and Gram negative bacteria (*E. coli*), while no antibacterial activity was observed for the other hydrogels (**P1**, **P2** and **P3**) even at concentration of 500 µg/mL. Figure 5 shows that the samples **P4** and **P5** are quite active against *S. aureus* and *E. coli* but less active against *B. subtilis*. The inhibition zone was formed in the screening test indicating the anti-bacterial activity of both the hydrogels (**P4** and **P5**) against Gram-negative bacteria *E. coli* and Gram-positive bacteria *S. aureus* (Table 2). The hydrogel **P4** also produced a relatively smaller inhibition zone against Gram-positive bacteria *B. subtilis*. The micro-dilution technique was used to determine minimum inhibitory concentration (MIC) for the hydrogel. The MIC varies with the test organisms and it was found that the hydrogels **P4** and **P5** were quite effective against Gram-negative bacteria *E. coli* and Gram-positive bacteria *S. aureus*. The MIC value is around 50-100 µg/mL for both the hydrogels against *S. aureus*. The MIC value against *E. coli* was found around 100-200 µg/mL for both **P4** and **P5** gels. MIC value ranged from 100-200 µg/mL of **P4** gel for *B. subtilis*. All the hydrogels were ineffective against Gram-negative bacteria *Pseudomonas aeruginosa* even upto a concentration of 500 µg/mL.

It is apparent from the antibacterial studies that these types of peptide-based amphiphile gels are more effective against Gram-positive bacteria (*S. aureus*, *B. subtilis*) than Gram-negative

bacteria (*E. coli*), as the gelator peptide is effective against Gram negative bacteria only in higher concentration.

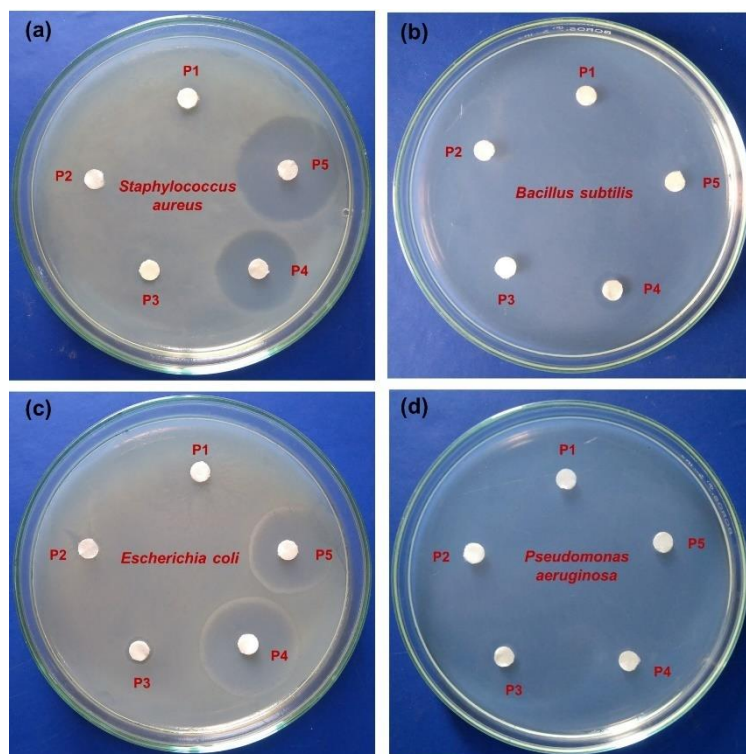


Figure 5. Effect of hydrogel on Gram-negative and Gram-positive bacteria by agar-diffusion assay method: various bacteria (a)*Staphylococcus aureus*, (b) *Bacillus subtilis*, (c) *Escherichia coli* and (d)*Pseudomonas aeruginosa* were spread on an Agar plate. The concentration of the hydrogels was 500 µg/mL for all case.

	P4		P5	
	Zone of inhibition diameter	MIC	Zone of inhibition diameter	MIC
<i>Staphylococcus aureus</i>	21 mm	50-100µg/ml	26 mm	50-100µg/ml

<i>Bacillus subtilis</i>	17 mm	100-200µg/ml	-	100-200µg/ml
<i>Escherichia coli</i>	24 mm	100-200µg/ml	21 mm	100-200µg/ml
<i>Pseudomonas aeruginosa</i>	-	-	-	-

Table 2. List of bacterial inhibition zone (diameter) (by disc-diffusion method using hydrogel at 0.5% (w/v) concentration) and minimum inhibitory concentration by **P4** and **P5** hydrogels on Gram negative and Gram positive bacteria. The concentration of the hydrogels was 500 µg/mL for all case.

Proteolytic Stability:

The peptide gelators **P4** and **P5** contain both a non-proteinogenic residue, at the N-terminus of the molecules and a proteinogenic alpha amino acid (Phe) residue. Due to the absence of peptide linkage between two alpha proteinogenic amino acid residues,⁵³ these peptide gelators are expected to show resistance towards proteolysis with proteolytic enzymes, proteinase K and chymotrypsin. To study the proteolytic stability, the peptide gelators were incubated with proteinase K and chymotrypsin in HEPES buffer (pH 7.46) at physiological temperature (37 °C). The enzymatic degradation of each peptide has been monitored at different time intervals by using mass spectrometry. The result is summarized and presented in Figure S28. No change in the mass spectral analysis of these gelator molecules was observed during various time intervals upto 36 hr. It can be concluded that these gelator peptides are proteolytically stable and have high biostability. Hence, the stability of these gelator peptides make them applicable for use in real situations in near future.

Haemolytic Assay:

It is important to find out whether these antibacterial gelator peptides exhibit any haemolytic activity or not at different concentrations of the hydrogels (Figure 6). Interestingly, these gelator peptides **P4** and **P5** do not exhibit any significant haemolysis at the minimum inhibitory concentration (MIC) for *S. aureus* and they show a small amount of haemolysis at the twice of the MIC for *S. Aureus*(Figure 6c, d). However, both of the gelator peptides show a small amount of haemolysis (13% and 11.7%) at MIC for *E. coli* and *B. subtilis*. The dose-dependent haemolysis of **P4** and **P5** is summarized in Table 3. It can be said that both of these gelator peptides are very effective for the bacteria *S. aureus* and even at four times the MIC of *S. aureus* only about 40-44% haemolysis occurs (Figure 6a,b). The minimum haemolytic concentration (MHC) for **P4** and **P5** hydrogels were found as 40-50 µg/ml and 30-40 µg/ml respectively (Figure S29).

These results from the antibacterial and haemolysis study are comparable to the previously reported cationic antibacterial peptides.^{55,56} However, the molecular structure of these cationic peptide amphiphiles are different from previously reported antibacterial agents. This is because these peptides neither contain lysine nor arginine residue, but, they have N-terminally located Gly- and its higher homologues (β -Alanine, 4-aminobutyric acid, 5-aminovaleric acid and 6-aminocaproic acid) with a free N-terminal position and the C-terminus is blocked with a fatty acyl amine with an intervening peptide residue (Phe-). This represents a new antibacterial motif.

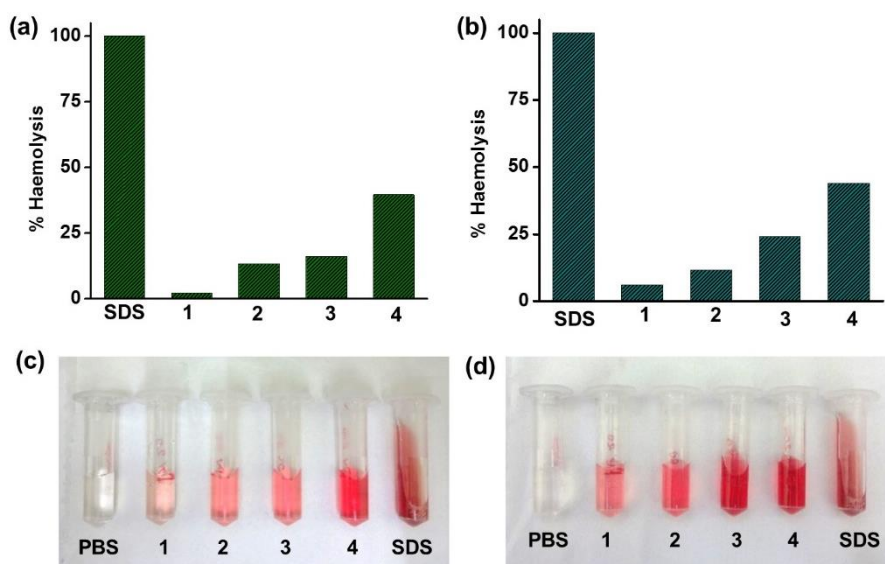


Figure 6. Dose–response plot for the haemolysis of human red blood cells by hydrogels of (a) **P4** and (b) **P5**. Dose-response images for the haemolysis of human red blood cells by hydrogel (c) **P4** and (d) **P5**. ‘1’ denotes 50 µg/ml, ‘2’ denotes 100 µg/ml, ‘3’ denotes 200 µg/ml, ‘4’ denotes 500 µg/ml concentration of the hydrogels.

	50µg/ml	100µg/ml	200µg/ml	500µg/ml
P4	1.9	13.4	16.2	39.6
P5	2.2	11.7	24.0	44.0

Table 3. Haemolysis assays (percentages) of **P4** and **P5** hydrogels at different concentrations on human red blood cells.

Cell Viability Assay:

Cell proliferation assay was carried out using human lung carcinoma, A549 by using yellow coloured 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction into purple formazon (**Figure 7**). First, A549 cells were seeded at a density of 10,000 cells per well in a 96-well plate for twenty-four hours prior to treatment with hydrogelator. After that, DMEM medium containing different concentrations (600 µM, 300 µM, 150 µM, 75 µM, 37.5 µM, 18.75 µM, 9.375 µM, 4.68 µM, 2.34 µM, 1.17µM and 0.58 µM) of hydrogelator were treated with the cells and kept for twenty-four hours (Figure 7a, c). Next, MTT solution was added and the solutions were stored for four hours at 37 °C in an incubator. Finally, cell viability was checked by absorbance study at 550 nm wave length.

Percentage cell viability = $\frac{[A550 \text{ (treated cells)} - \text{background}]}{[A550(\text{untreated cells}) - \text{background}]} \times 100$.

Cell morphologies of A549 cells were checked after the hydrogelator treatment. Cells were seeded on a confocal disk at a density of 5,000 per disk for twenty-four hours prior to hydrogelator treatment. Then DMEM medium, containing two different concentrations (600 μM and 18.75 μM) of the compound was added and kept for twenty four hours at 37 °C incubator (Figure 7c,d and S30b,c). One confocal disk remained untreated for the control study (Figure S30a). Next, cellular morphologies were observed using an inverted microscope (Olympus IX83 fluorescence microscope, at 40X objective) in DIC mode.

To check cytotoxicity of both the compounds, cell viability assay has been performed upto 600 μM . It has been observed that no cytotoxicity has been observed in both the gel compounds. Moreover, we have examined the morphology of cells at two different gelator concentrations (600 μM and 18.75 μM) with respect to untreated. It has been observed that A549 cellular morphology remains healthy and unaffected after treatment of two compounds for 24 h.

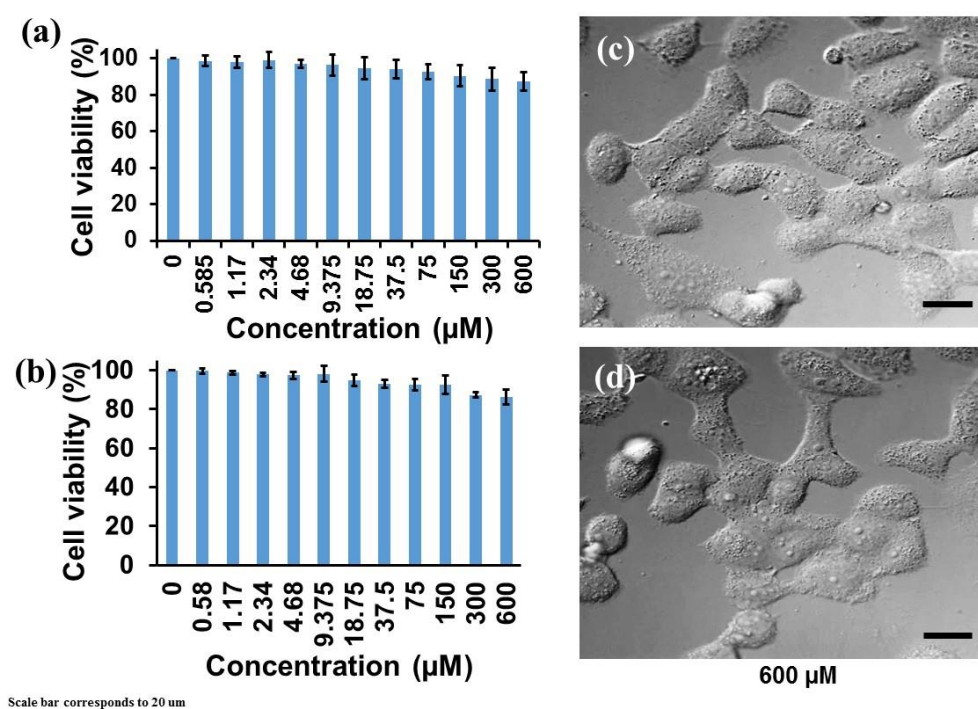


Figure 7. Cell viability of human lung carcinoma, A549 cells after 24 hours treatment with different concentrations of gel as calculated from the MTT assay. Scale bar corresponds to 20 μm .

CONCLUSION

In this study, a series of amphiphilic peptides have been discovered to form thermo-responsive hydrogels with a new structural motif containing a free amino group at the N-terminally located glycine/ ω -amino acid residues and a C-terminus blocked with a covalently linked dodecylamine residue. Most of these peptide-based hydrogels exhibit a mechano-responsive (thixotropic) property and this thixotropy as well as thermal stability of these hydrogels have been successfully tuned by increasing the alkyl chain length of the N-terminally located amino acid residues. Two of these five peptide-based hydrogels exhibit remarkable antibacterial activity against both Gram positive (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram negative (*Escherichia coli*) bacteria with a non-significant/minimum haemolytic activity at their respective minimum inhibitory concentration of different bacteria. An MTT cell viability assay exhibits no cytotoxicity of these antibacterial peptide gelators. Moreover, these gels are proteolytically stable and injectable in nature. This suggests that the discovery of this new type of peptide amphiphile based hydrogels hold a future promise for the development of new soft materials as antibiotics.

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publication website at DOI: bm-2017-01006x.R1

Instrumentation, Synthetic procedures, NMR, HRMS, Thermo-reversibility study, Injectibility study, Rheological study, Spectroscopic study, FTIR, SAXS, WPXRD and Cell viability study.

REFERENCES

- (1) Raeburn, J.; Adams, D. J.Multicomponent low molecular weight gelators.*Chem. Commun.***2015**, 51, 5170-5180.
- (2) Cornwell, D. J.; Smith, D. K.Expanding the scope of gels-combining polymers with low-molecular-weight gelators to yield modified self-assembling smart materials with high-tech applications.*Mater. Horiz.***2015**, 2, 279–293.
- (3)Pettignano, A.; Grijalvo, S.;Häring, M.;Eritja, R.; Tanchoux, N.;Quignard, F.;Díaz, D. D. Boronic acid-modified alginate enables directformation of injectable, self-healingand multistimuli-responsive hydrogels.*Chem. Commun.***2017**, 53, 3350-3353.
- (4) Vemula, P. K.; John, G.Crops: A Green Approach toward Self-Assembled Soft Materials.*Acc. Chem. Res.* **2008**, 41, 769-782.

- (5) Das, A. K.; Bose, P. P.; Drew, M. G. B.; Banerjee, A. The role of protecting groups in the formation of organogels through a nano-fibrillar network formed by self-assembling terminally protected tripeptides. *Tetrahedron* **2007**, *63*, 7432-7442.
- (6) Draper, E. R.; Wallace, M.; Schweins, R.; Poole, R. J.; Adams, D. J. Nonlinear Effects in Multicomponent Supramolecular Hydrogels. *Langmuir* **2017**, *33*, 2387-2395.
- (7) Nebot, V. J.; Escuder, B.; Miravet, J. F.; Smets, J.; Fernandez-Prieto, S. Interplay of Molecular Hydrogelators and SDS Affords Responsive Soft Matter Systems with Tunable Properties. *Langmuir* **2013**, *29*, 9544-9550.
- (8) Weiss, R. G. The Past, Present, and Future of Molecular Gels. What Is the Status of the Field, and Where Is It Going? *J. Am. Chem. Soc.* **2014**, *136*, 7519-7530.
- (9) Li, J.; Du, X.; Hashim, S.; Shy, A.; Xu, B. Aromatic–Aromatic Interactions Enable α -Helix to β -Sheet Transition of Peptides to Form Supramolecular Hydrogels. *J. Am. Chem. Soc.* **2017**, *139*, 71-74.
- (10) Du, X.; Zhou, J.; Shi, J.; Xu, B. Supramolecular Hydrogelators and Hydrogels: From Soft Matter to Molecular Biomaterials. *Chem. Rev.* **2015**, *115*, 13165-13307.
- (11) Gorai, T.; Maitra, U. Luminescence resonance energy transfer in a multiple component, self-assembled supramolecular hydrogel. *Angew. Chem. Int. Ed.* **2017**, DOI: 10.1002/ange.201704738.
- (12) Decandio, C. C.; Silva, E. R.; Hamley, I. W.; Castelletto, V.; Liberato, M. S.; Oliveira, Jr., V. X.; Oliveira, C. L. P.; Alves, W. A. Self-Assembly of a Designed Alternating Arginine/Phenylalanine Oligopeptide. *Langmuir* **2015**, *31*, 4513–4523.

- (13) Fichman, G.; Adler-Abramovich, L.; Manohar, S.; Mironi-Harpaz, I.; Guterman, T.; Seliktar, D.; Messersmith, P. B.; Gazit, E. Seamless Metallic Coating and Surface Adhesion of Self-Assembled Bioinspired Nanostructures Based on Di-(3,4-dihydroxy-L-phenylalanine) Peptide Motif. *ACS Nano* **2014**, 7, 7220-7228.
- (14) Singh, N.; Kumar, M.; Miravet, J. F.; Ulijn, R. V.; Escuder, B. Peptide-Based Molecular Hydrogels as Supramolecular Protein Mimics. *Chem. Eur. J.* **2017**, 23, 981-993.
- (15) Frederix, P. W. J. M.; Scott, G. G.; Abul-Haija, Y. M.; Kalafatovic, D.; Pappas, C. G.; Javid, N.; Hunt, N. T.; Ulijn, R. V.; Tuttle, T. Exploring the sequence space for (tri-)peptide self-assembly to design and discover new hydrogels. *Nat. Chem.* **2015**, 7, 30-37.
- (16) Dooling, L. J.; Tirrell, D. A. Peptide and Protein Hydrogels. Polymeric and Self Assembled Hydrogels: From Fundamental Understanding to Applications. RSC Publishing, Cambridge, **2013**, 93-124.
- (17) Jonker, A. M.; Löwik, D. W. P. M.; van Hest, J. C. M. Peptide- and Protein-Based Hydrogels. *Chem. Mater.* **2012**, 24, 759-773.
- (18) Li, I. -C.; Moore, A. N.; Hartgerink, J. D. "Missing Tooth" Multidomain Peptide Nanofibers for Delivery of Small Molecule Drugs. *Biomacromolecules* **2016**, 17, 2087-2095.
- (19) Basu, K.; Baral, A.; Basak, S.; Dehsorkhi, A.; Nanda, J.; Bhunia, D.; Ghosh, S.; Castelletto, V.; Hamley, I. W.; Banerjee, A. Peptide based hydrogels for cancer drug release: modulation of stiffness, drug release and proteolytic stability of hydrogels by incorporating D-amino acid residue(s). *Chem. Commun.* **2016**, 52, 5045-5048.
- (20) Geisler, I. M.; Schneider, J. P. Evolution-Based Design of an Injectable Hydrogel. *Adv. Funct. Mater.* **2012**, 22, 529-537.

- (21) Naskar, J.; Palui, G.; Banerjee, A. Tetrapeptide-Based Hydrogels: for Encapsulation and Slow Release of an Anticancer Drug at Physiological pH. *J. Phys. Chem. B* **2009**, *113*, 11787-11792.
- (22) Kaufmann, L.; Kennedy, S. R.; Jones, C. D.; Steed, J. W. Cavity-containing supramolecular gels as a crystallization tool for hydrophobic pharmaceuticals. *Chem. Commun.* **2016**, *52*, 10113-10116.
- (23) Yan, C.; Mackay, M. E.; Czymmek, K.; Nagarkar, R. P.; Schneider, J. P.; Pochan, D. J. Injectable Solid Peptide Hydrogel as a Cell Carrier: Effects of Shear Flow on Hydrogels and Cell Payload. *Langmuir* **2012**, *28*, 6076-6087.
- (24) Liyanage, W.; Vats, K.; Rajbhandary, A. Benoit, D. S. W.; Nilsson, B. L. Multicomponent dipeptide hydrogels as extracellular matrix-mimetic scaffolds for cell culture applications. *Chem. Commun.* **2015**, *51*, 11260-11263.
- (25) Moore, A. N.; Hartgerink, J. D. Self-Assembling Multidomain Peptide Nanofibers for Delivery of Bioactive Molecules and Tissue Regeneration. *Acc. Chem. Res.* **2017**, *50*, 714-722.
- (26) Lee, K. Y.; Mooney, D. J. Hydrogels for Tissue Engineering. *Chem. Rev.* **2001**, *101*, 1869-1879.
- (27) Zhou, M.; Smith, A. M.; Das, A. K.; Hodson, N. W.; Collins, R. F.; Ulijn, R. V.; Gough, J. E. Self-assembled peptide-based hydrogels as scaffolds for anchorage-dependent cells. *Biomaterials* **2009**, *30*, 2523-2530.
- (28) Marchesan, S.; Vargiu, A. V.; Styan, K. E. The Phe-Phe Motif for Peptide Self-Assembly in Nanomedicine. *Molecules* **2015**, *20*, 19775-19788.

- (29) Lee, S. S.; Hsu, E. L.; Mendoza, M.; Ghodasra, J.; Nickoli, M. S.; Ashtekar, A.; Polavarapu, M.; Babu, J.; Riaz, R. M.; Nicolas, J. D.; Nelson, D.; Hashmi, S. Z.; Kaltz, S. R.; Earhart, J. S.; Merk, B. R.; McKee, J. S.; Bairstow, S. F.; Shah, R. N.; Hsu, W. K.; Stupp, S. I. Gel Scaffolds of BMP-2-Binding Peptide Amphiphile Nanofibers for Spinal Arthrodesis. *Adv. Healthcare Mater.* **2015**, *4*, 131–141.
- (30) Basak, S.; Nandi, N.; Paul, S.; Hamley, I. W.; Banerjee, A. A tripeptide-based self-shrinking hydrogel for waste-water treatment: removal of toxic organic dyes and lead (Pb^{2+}) ions. *Chem. Comm.* **2017**, *53*, 5910-5913.
- (31) Okesola, B. O.; Smith, D. K. Applying low-molecular weight supramolecular gelators in an environmental setting – self-assembled gels as smart materials for pollutant removal. *Chem. Soc. Rev.* **2016**, *45*, 4226-4251.
- (32) Bhattacharya, S.; Krishnan-Ghosh, Y. First report of phase selective gelation of oil from oil/water mixtures. Possible implications toward containing oil spills. *Chem. Commun.* **2001**, 185-186.
- (33) Basak, S.; Nanda, J.; Banerjee, A. A new aromatic amino acid based organogel for oil spill recovery. *J. Mater. Chem.* **2012**, *22*, 11658-11664.
- (34) Yang, Z.; Liang, G.; Ma, M.; Abbah, A. S.; Lu, W. W.; Xu, B. D-Glucosamine-based supramolecular hydrogels to improve wound healing. *Chem. Commun.* **2007**, 843-845.
- (35) Veiga, A. S.; Schneider, J. P. Antimicrobial Hydrogels for the Treatment of Infection. *Peptide Science* **2013**, *100*, 637-644.
- (36) Baral, A.; Roy, S.; Dehsorkhi, A.; Hamley, I. W.; Mohapatra, S.; Ghosh, S.; Banerjee, A. Assembly of an Injectable Noncytotoxic Peptide-Based Hydrogelator for Sustained Release of Drugs. *Langmuir* **2014**, *30*, 929-936.

- (37) Xie, F.; Qin, L.; Liu, M. A dual thermal and photo-switchable shrinking–swelling supramolecular peptide dendrongel. *Chem. Commun.* **2016**, 52, 930-933.
- (38) Segarra-Maset, M. D.; Nebot, V. J.; Miravet, J. F.; Escuder, B. Control of molecular gelation by chemical stimuli. *Chem. Soc. Rev.* **2013**, 42, 7086-7098.
- (39) Basak, S.; Nanda, J.; Banerjee, A.; Multi-stimuli responsive self-healing metallohydrogels: tuning of the gel recovery property. *Chem. Commun.* **2014**, 50, 2356-2359.
- (40) Terech, P.; Yan, M.; Maréchal, M.; Royal, G.; Galvez, J.; Velu S. K. P. Characterization of strain recovery and “self-healing” in a self-assembled metallo-gel. *Phys. Chem. Chem. Phys.* **2013**, 15, 7338-7344.
- (41) Liu, K.; Steed, J. W. Triggered formation of thixotropic hydrogels by balancing competitive supramolecular synthons. *Soft Matter* **2013**, 9, 11699–11705.
- (42) Guvendiren, M.; Lu, H. D.; Burdick, J. A. Shear-thinning hydrogels for biomedical applications. *Soft Matter* **2012**, 8, 260–272.
- (43) Mallia, V. A.; Weiss, R. G. Structural bases for mechano-responsive properties in molecular gels of (R)-12-hydroxy-N-(ω -hydroxyalkyl)octadecanamides. Rates of formation and responses to destructive strain. *Soft Matter* **2015**, 11, 5010-5022.
- (44) Mallia, V. A.; Weiss, R. G. Correlations between thixotropic and structural properties of molecular gels with crystalline networks. *Soft Matter* **2016**, 12, 3665-3676.
- (45) Castelletto, V.; Kaur, A.; Kowalczyk, R. M.; Hamley, I. W.; Reza, M.; Ruokolainen, J. Supramolecular Hydrogel Formation in a Series of Self-Assembling Lipopeptides with Varying Lipid Chain Length. *Biomacromolecules* **2017**, DOI: 10.1021/acs.biomac.7b00057.

- (46) Bowerman, C. J.; Liyanage, W.; Federation, A. J.; Nilsson, B. L. Tuning β -Sheet Peptide Self-Assembly and Hydrogelation Behavior by Modification of Sequence Hydrophobicity and Aromaticity. *Biomacromolecules* **2011**, *12*, 2735-2745.
- (47) Castelletto, V.; Kaur, A.; Hamley, I. W.; Barnes, R. H.; Karatzas, K.-A.; Hermida-Merino, D.; Swioklo, S.; Connon, C. J.; Stasiak, J.; Reza, M.; Ruokolainen, J. *RSC Adv.* **2017**, *7*, 8366-8375.
- (48) Liu, Y.; Ma, W.; Liu, W.; Li, C.; Liu, Y.; Jiang, X.; Tang, Z. Silver(I)-glutathione biocoordination polymer hydrogel: effective antibacterial activity and improved cytocompatibility. *J. Mater. Chem.* **2011**, *21*, 19214-19218.
- (49) Hu, Y.; Xu, W.; Li, G.; Xu, L.; Song, A.; Hao, J. Self-Assembled Peptide Nanofibers Encapsulated with Superfine Silver Nanoparticles via Ag^+ Coordination. *Langmuir* **2015**, *31*, 8599–8605.
- (50) Fullenkamp, D. E.; Rivera, J. G.; Gong, Y.; Lau, K. H. A.; He, L.; Varshney, R.; Messersmith, P. B. Mussel-inspired silver-releasing antibacterial hydrogels. *Biomaterials* **2012**, *33*, 3783–3791.
- (51) Yeo, E. D.; Yoon, S. A.; Oh, S. R.; Choi, Y. S.; Lee, Y. K. Degree of the Hazards of Silver-Containing Dressings on MRSA-Infected Wounds in Sprague–Dawley and Streptozotocin-Induced Diabetic Rats. *Wounds* **2015**, *27*, 95-102.
- (52) Jiang, L.; Xu, D.; Sellati, T. J.; Dong, H. Self-assembly of cationic multidomain peptide hydrogels: supramolecular nanostructure and rheological properties dictate antimicrobial activity. *Nanoscale* **2015**, *7*, 19160-19169.

- (53) Baral, A.; Roy, S.; Ghosh, S.; Hermida-Merino, D.; Hamley, I. W.; Banerjee, A. A. Peptide-Based Mechano-sensitive, Proteolytically Stable Hydrogel with Remarkable Antibacterial Properties. *Langmuir* **2016**, *32*, 1836-1845.
- (54) Pazos, E.; Sleep, E.; Pérez, C. M. R.; Lee, S. S.; Tantanitti, F.; Stupp, S. I. Nucleation and Growth of Ordered Arrays of Silver Nanoparticles on Peptide Nanofibers: Hybrid Nanostructures with Antimicrobial Properties. *J. Am. Chem. Soc.* **2016**, *138*, 5507-5510.
- (55) Ji, E.; Parthasarathy, A.; Corbitt, T. S.; Schanze, Kirk S.; Whitten, D. G. Antibacterial Activity of Conjugated Polyelectrolytes with Variable Chain Lengths. *Langmuir* **2011**, *27*, 10763-10769.
- (56) Uppu, D. S. S. M.; Samaddar, S.; Hoque, J.; Konai, M. M.; Krishnamoorthy, P.; Shome, B. R.; Haldar, J. Side Chain Degradable Cationic–Amphiphilic Polymers with Tunable Hydrophobicity Show in Vivo Activity. *Biomacromolecules* **2016**, *17*, 3094-3102.
- (57) Nandi, N.; Baral, A.; Basu, K.; Roy, S.; Banerjee, A. A dipeptide-based superhydrogel: Removal of toxic dyes and heavy metal ions from waste water. *Peptide science*, **2017**, *108*, 108:e22915 (1-9).
- (58) Fernandez-Lopez, S.; Kim, H. –S.; Choi, E. C.; Delgado, M.; Granja, J. R.; Khasanov, A.; Kraehenbuehl, K.; Long, G.; Weinberger, D. A.; Wilcoxen, K. M.; Ghadiri, M. R. Antibacterial agents based on the cyclic D,L- α -peptide architecture. *Nature*, **2001**, *412*, 452-455.

GRAPHICAL ABSTRACT

