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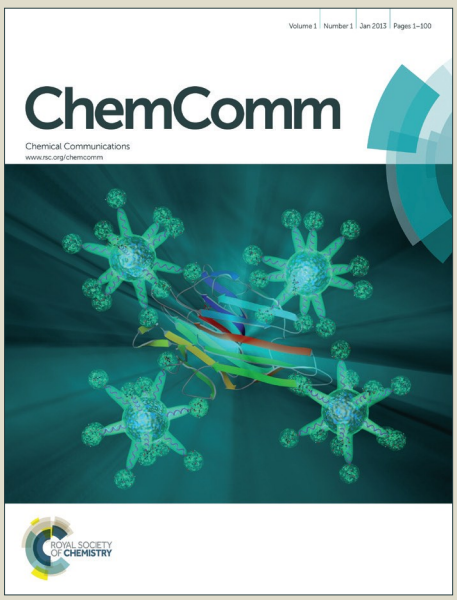

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COMMUNICATION

Peptide based hydrogels for cancer drug release: Modulation of stiffness, drug release and proteolytic stability of hydrogels by incorporating D-amino acid residue(s)

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Synthetic tripeptide based noncytotoxic hydrogelators have been discovered for releasing an anticancer drug at physiological pH and temparature. Interestingly, gel stiffness, drug release capacity and proteolytic stability of these hydrogels has been sucessfully modulated by incorporating D-amino acid residues, indicating their potential use for drug delivery in future.

Peptide based hydrogels[1] continue to attract attention among researchers due to their potential applications in various research fields.[2] Peptide molecules are not only good gelators due to the presence of various self-assembling units in their structures but also possess inherent biocompatibility which enhances their applicability.[3] The orientation of different functional groups in proper configuration is crucial to trigger an entropically disfavoured process like gelation.[1n] Amino acids, the basic building blocks of peptide molecules, are inherently chiral with at least one stereogenic centre dictating the spatial arrangement of various functional groups attached to them. So, all peptide based gelator molecules are examples of chiral gelator molecules,[4] whose chirality can be a tool for tuning the physical properties of the corresponding gels to make them smarter.[5] Furthermore, the simplicity and low cost of synthesis make these peptide-based materials a wonderful platform for studying chirality induced effects.

Doxorubicin is a potent anticancer drug routinely used in the advanced stage of breast cancer, gastric carcinoma and leukaemia. However, it has many side effects when it is administered in high dose.[7] Thus, selection of a delivery vehicle is very important to overcome this problem.[8] Hydrogels are important delivery vehicle for targeted delivery of Doxorubicin into cancer affected areas, where intravenous administration is not very promising (in the case of osteosarcoma patients).[9] Here, we study the slow and sustained release of Doxorubicin from a peptide-based thixotropic hydrogel Boc-(L)Phe-(L)Phe-(L)Phe-COOH (**P1**). However, the homochiral triphenylalanine system containing only α-L protein amino acid residues has a serious shortcoming due to its proteolytic instability,[10] this is because α-L amino acid residues can be easily recognized and cleaved by the proteolytic enzymes present inside the cell. So, in addition to the drug release ability, it is essential to optimise the proteolytic stability of the hydrogel by incorporating D-amino acid residues into the parent gelator molecule. The parent gelator molecule **P1** has three chiral centres in its structure, therefore alternation of chirality of Phe residue(s) might change the orientation of the corresponding aromatic ring and the direction of π-π stacking interaction, which can have a profound effect on the macroscopic properties of the corresponding gel. It is also of great interest to investigate how alternation of chiral residue(s) around the chiral centre(s) in the parent peptide affects the self-assembly, gelation, mechanical strength, proteolytic stability and drug release capacity of these gels. We have synthesized(detailed synthetic procedure and characterization data are given in the ESI) all possible stereoisomers of the parent compound and investigated their self assembly, drug release and mechanical properties as well as proteolytic stability in order to optimise their properties in future use.

Fig. 1 shows structures of peptides and images of the peptide-based gels in their respective vials. **P1** (LLL), **P2** (DLL), **P3** (LDL) and **P4** (LLD) are four diastereomers (chirality sequence in brackets) and **P5** (DDD), **P6** (LDD), **P7** (DLD) and **P8** (DDL) are their respective enantiomers. It can be noticed that **P1** (and its enantiomer **P5**) and **P2** (and its enantiomer **P6**) form gels instantaneously, whereas **P3** (and its enantiomer **P7**) takes 12 hours to form a gel and **P4** and its enantiomer **P8** are nongelator at physiological pH (7.46) and temperature. So

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Electronic Supplementary Information (ESI) available: [Details of synthesis and characterization of peptides, gelation study, FT-IR, CD, proteolytic and cytotoxicity studies, FE-SEM and injectability images, SAXS, XRPD and step-strain rheological plots, proposed model and detailed instrumentation]. See DOI: 10.1039/x0xx00000x

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chirality has some extensive effect on the gelation or non-gelation of the tripeptides.

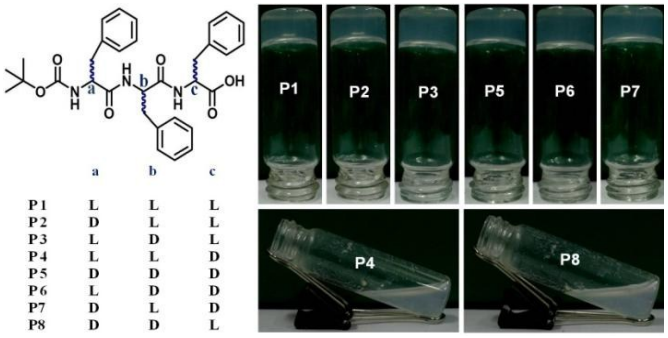

Fig. 1 Chemical structures of tripeptides and their gelation nature in pH 7.46 phosphate buffer.

Gel morphology was examined using field emission scanning electron microscopy (FE-SEM). FE-SEM images of all xerogels and dried aggregated solutions of all tripeptides show similar fibrillar morphology (Fig. S26). However it is difficult to predict gelation (or non-gelation) ability just from a fibrillar morphology observed from microscopic experiments, rather efficient 3D networks formed by these fibres leads to gelation which needs entanglement of fibres to a long range.

Comparison of FT-IR spectra of xerogels and dried solutions provides information on the difference in H-bonding interactions for different tripeptides. All the enantiomeric pair show a similar pattern (as shown in Fig. S27 of ESI), so properties of only four diastereomeric peptides **P1**, **P2**, **P3** and **P4** are discussed here. The peak around 1649 cm^{-1} corresponds to the hydrogen bonded stretching frequency of the amide carbonyl groups which are present in all four cases,[9a] while the peak for hydrogen bonded urethane C=O which appears at 1690 cm^{-1} is prominent only for **P1** and **P2** xerogels. However, it is weak for **P3** and almost non-existent for dried **P4** solution. This indicates the absence of hydrogen bonding for the urethane carbonyl group of **P4** and only weak H-bonding interaction for **P3**. The amide stretching peaks corresponding to 3343 and 3422 cm^{-1} are assigned to hydrogen bonded and non-hydrogen bonded amide N-H respectively.[11] Interestingly, in this study **P1** and **P2** show both types of peaks, suggesting the presence of both hydrogen bonded and free N-H, while **P3** and **P4** do not show any significant peak at 3343 cm^{-1} indicating the presence of free N-H groups only. **P4** is unable to form H-bonding at both urethane and amide N-H sites resulting in its inability to form a gel.

Small angle X-ray scattering (SAXS) is a useful technique to obtain information on molecular packing. For **P1** gel and its enantiomer **P5** a broad peak was observed at d= 28 Å, (Fig. S28) which is double the calculated length of a single molecule (calculated from Chem-Draw 3D Ultra software).

Wide-angle X-ray powder diffraction (XRPD) studies were done for all samples in xerogel form. A characteristic peak at 4.7 Å (2θ= 18 Å) can be assigned to a *β*-sheet like arrangement in the molecular packing of gelator molecules which is revealed by the diffraction pattern of all the xerogels. The peak at d= 3.7 Å (2θ = 23.5) corresponding to the π-π packing interaction[9a] is only prominent for the **P1** xerogel, while the intensity of the peak is much weak for **P2**, **P3** and **P4** in their dried form. Fig. S30 summarizes the XRPD pattern of gelator molecules **P1**, **P2**, **P3** and the nongelator molecule **P4** and their corresponding enantiomers. So, we can notice a poor π-π interaction in the molecular packing of peptides containing residues of mixed chirality. It confirms that molecular packing is significantly perturbed for the heterochiral sytems where molecules pack less efficiently than that for the homochiral systems. On the basis of FT-IR, SAXS and XRPD data a schematic model can be proposed for **P1** and **P2** (Fig. S31).

Circular dichroism (CD) measurements were performed to examine the chiroptical behaviour of all compounds in aggregated state (Fig. S32). **P1**, **P2** and their enantiomers show two peaks at 203 nm and 223 nm, whereas **P3** and **P4** show only one weak peak at 220 nm, indicating that **P1** and **P2** may have a slightly more ordered structure in comparison with **P3** and **P4**.[5a] Interestingly, it has been found that the intensity of the peak at around 220 nm decreases gradually from **P1** to **P4**. This indicates that the π-π interaction prevails in the order **P1** > **P2** > **P3** > **P4**. Moreover, the chirality of the terminal amino acid residue (whether it is "D" or "L") dictates the directions of the CD signals at 220 nm(positive or negative). For **P1**, **P2**, **P3** and **P8**, the terminal amino acid residue has (L)-configuration and the peak is oriented in positive direction, whereas for **P4**, **P5**, **P6** and **P7**, the terminal amino acid residue has (D)-configuration and the peak is oriented in the negative direction.[5a] So, this observation indicates how chirality of the terminal residue (whether it is "D" or "L") governs the supramolecular assembly of all these peptides.

To check how the different self-assembly patterns are reflected in the macroscopic properties of these gelator peptides, rheological measurements for all hydrogels were carried out to measure the gel strength keeping the gelator concentration the same [1 % (w/v)]. From a frequency sweep experiment (at constant strain of 0.1%) (Fig. 2 and Fig. S33 in ESI), it is

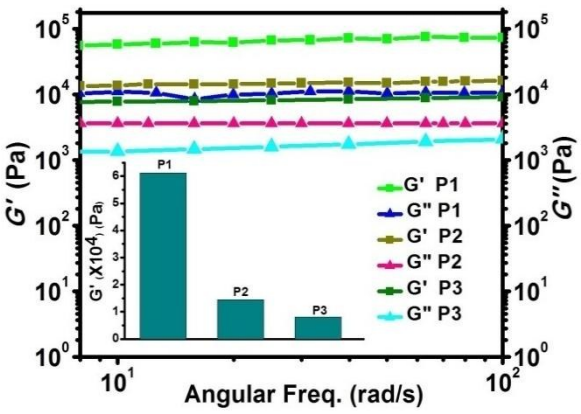

Fig. 2 Frequency sweeps of dynamic shear modulus for hydrogels **P1**, **P2** and **P3** (gels made at 1 % (w/v), G' and G'' values for the enantiomers are given in the ESI Fig. S27). Inset: Comparison of mechanical strength with change in chirality of residues at constant frequency 12.595 rad/sec.

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observed that all hydrogels formed by **P1**, **P2**, **P3** show storage modulus (G') > loss modulus (G'') and also G' and G'' values are weakly dependent on angular frequency over the range studied indicating the formation of a stable gel. Again the enantomeric counterpart of each tripeptide shows similar rheological data, and therefore we are focusing only on diastereomers **P1**, **P2**, **P3** and **P4**. It is notable that mechanical strength as indicated by G' decreases gradually in the series **P1**> **P2**> **P3** (**P4** does not gel) (Fig. 2). It is evident from the study that gel strength (G') gradually decreases as the D-Phe residue is moved from the N to the C terminus. This agrees well with previous observations which shows the decreasing trend of H-bonding and π-π stacking interaction in the order **P1** ~ **P2** > **P3** > **P4.** This remarkable observation shows that it is possible to modulate the mechanical strength of a particular gelator by changing molecular chirality and placing the D-residue in the proper position (at or towards the C terminus). All the hydrogels are thixotropic in nature, and have been checked by repetitive shaking and resting cycles. Thixotropy of the gels have been confirmed by using time dependent step-strain rheology experiments, keeping the gelator concentration fixed in all cases. At first the strain on the gel was kept constant at 0.1% then after 200 seconds it was rapidly increased to 10%, at this point the gel was broken which is indicated by crossover of G'' values over G'. Again after 200 seconds strain was decreased to 0.1% where reformations of the gels was observed. The relative strength recovery of gels of **P1**, **P2**, **P3**, **P5**, **P6** and **P7** is near 100% and recovery times lies in the range 410-440 seconds range (Fig. S34).

The differences in molecular level chirality and packing lead to changes in macroscopic properties, as exemplified by mechanical and dynamic properties of the gels. Hydrogels obtained from **P1** to **P3**, **P5** and **P6** show thixotropic behavior (Fig. S34) and this has been exploited for injectable studies. (Fig. S35).

P1, **P2**, **P5** and **P6** hydrogels can encapsulate an anticancer drug, Doxorubicin and their release properties have been studied. For each gel, the same amount of drug was loaded into the same volume of gel with same molar concentration of gelator. Then the same volume of supernatant buffer solution was added at the top of the drug loaded hydrogel to examine the drug release. It is observed that though the release profile of **P1** and **P2** (also of **P5** and **P6**) are similar, the total amounts of released drug are different for gels obtained from different diastereomers, whereas enantiomeric pair show the same amount of release. Anticancer drug-loaded hydrogels **P2** and **P6** show the maximum release of 76% after 79 hours, then reaching a plateau. However, both the gels **P1** and **P5** show a maximum release of 65% within a total span of 52 hours and after that each of these gels started rupturing gradually. Interestingly, stiffer gels release less drug sustainably (because they break down within 52 hours and after that there is a random release) than the weaker gels(Fig. 3).

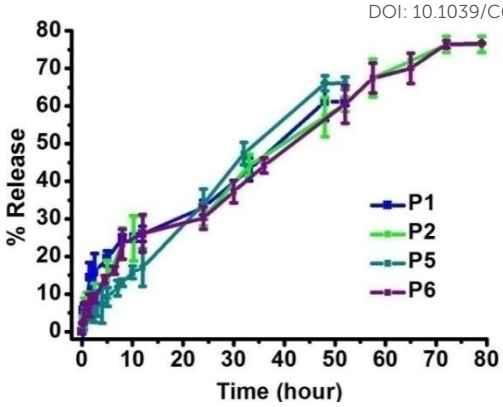

Fig. 3 Release of Doxorubicin from hydrogels of (a) **P1**, **P2** and (b) **P5**, **P6** (in all cases gel was loaded with 46 μg of drug and error bars are calculated after three experiments in each case).

The proteolytic stability of the four peptides, which can encapsulate and release Doxorubicin (**P1**, **P2**, **P5** and **P6**) were examined in the presence of a proteolytic enzyme Proteinase K. Degradation of each peptide was monitored periodically using mass spectrometry. The results (detailed procedure given in ESI, see Fig. S36, and Fig. S37-S40) show that **P1** and **P2** are proteolytically very much unstable and undergo degradation within 24 hours, but **P5** and **P6** remained stable. This stability makes **P5** and **P6** applicable for use in real situations. But out of them, **P6** has the greater delivery efficacy. The results of the above studies lead to the conclusion that among all the peptides discussed here **P6** has the optimised properties we are looking for and this makes it the most efficient Doxorubicin delivery vehicle. Here we have successfully designed hydrogels for drug delivery and also optimized their properties. To check their practical applicability, cytotoxicity has been studied. The minimum gelation concentrations (MGCs) of the reported hydrogels are around ~900 μM. Cytotoxicity has been studied up to 900 μM and no apparent cytotoxicity has been found. Fig. 4 shows the cell viability assay and cell morphology when treated with the proteolytically stable and most efficient drug releasing gelator **P6**. Results from cytotoxicity studies with other gelators is shown in Fig. S41. Our study vividly demonstrates that the gel obtained from gelator **P6** does not show significant toxicity towards cancerous cells, while a Doxorubicin loaded gel of P6 kills breast cancer cells more efficiently than that of the free drug (i.e. Doxorubicin) alone.

In summary, we have created soft biomaterials for cancer drug release at physiological pH and temperature having no cytotoxicity. Remarkably, the modulation of stiffness and proteolytic stability of these hydrogels has been achieved by replacing one or more L-Phe residue by D-Phe residue(s) and also by changing the position of the D-residue in the tripeptide. The incorporation and location of D-residues determine the mechanical stability of a gel as well as its drug release capacity. Moreover, it is the number and position of D-amino acid residues that determines the proteolytic stability of

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the gel. After trying all possible combinations of D and L amino acids, the most efficient gelator molecule has been identified as **P6**. So, it can be concluded that by incorporating D- instead of L-residues and by placing them in the proper position, proteolytic stability and mechanical strength of these soft biomaterials can be optimized for designing future drug delivery vehicles.

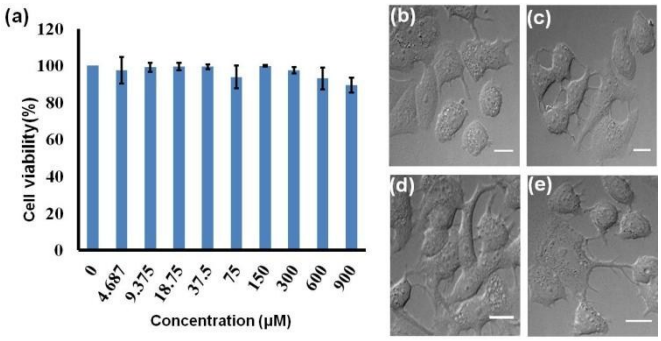

Fig. 4 (a) Cell survival study (by MTT assay) of MCF-7 cells after treatment with most efficient hydrogelator, **P6**. Morphologies of MCF-7 cells (at 40 X objective) after 24 h. (b) without treatment with the compound as control. Morphologies of the cells after treatment with (c) 600 μM (d) 37.5 μM and (e) 4.68 μM. Scale bar corresponds to 20 μm.

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