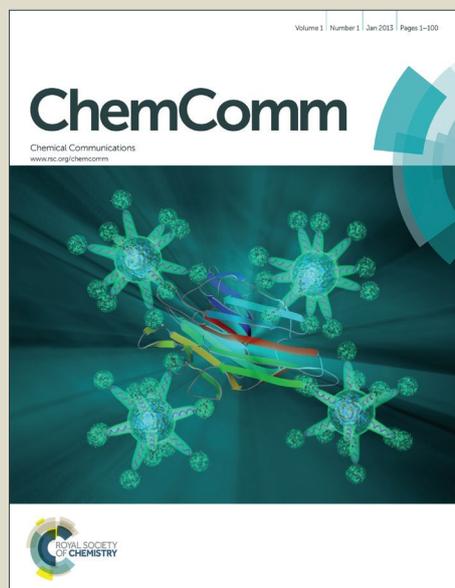


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COMMUNICATION

## Design of a hydrophobic tripeptide that self-assembles into amphiphilic superstructures forming a hydrogel biomaterial

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**We report the rational design of a heterochiral hydrophobic tripeptide self-assembling into amphiphilic D-superstructures that yield a self-supportive hydrogel at physiological pH. The material endures cell culture conditions and sustains fibroblast proliferation. Tripeptide superstructures are thoroughly analysed by several techniques.**

There is tremendous interest in the development of soft biomaterials through the hierarchical self-organisation of peptides, as hydrogels are an elected choice for a biomaterial to interface with, or even reconstitute, living tissue.<sup>1, 2</sup> Supramolecular hydrogels based on peptides are revolutionising medicine, as they are bringing innovation in areas spanning from new therapeutic paradigms used to direct cell fate,<sup>3, 4</sup> to improved immune response to vaccines<sup>5, 6</sup>, and to new tools to assist surgical procedures.<sup>7</sup> Peptides as short as three amino acids are attractive for biological use in light of their ability to encode powerful messages to cells, e.g. to induce adhesion, migration or differentiation.<sup>8</sup> Besides, such simple molecules are very convenient to prepare also by solid phase peptide synthesis, and at relatively low cost when compared to longer peptides.<sup>9</sup>

A popular approach to obtain supramolecular hydrogels consists of peptide derivatisation with aromatic, rigid units (e.g., Fmoc, Nap, Cbz, etc.) that exert  $\pi$ - $\pi$  stacking interactions.<sup>10, 11</sup> Phenylalanine plays a unique role in self-assembly: the amino acid forms fibrils,<sup>12, 13</sup> and the Phe-Phe dipeptide self-assembles on its own<sup>14, 15</sup> or when inserted within defined peptides.<sup>16, 17</sup> Yet, formation of macroscopic hydrogels is a different matter, and prediction of gelation of short peptides is still far from trivial.<sup>18, 19</sup> Even more difficult to foresee is the self-assembly behaviour of tripeptides devoid of

synthetic appendages, due to their flexible nature and ability to adopt many conformations. In 2015 an intense computational study screened all 8,000 combinations of the 20 natural amino acids as tripeptides and identified only four new sequences able to gel in water.<sup>20</sup> The elegant work correlated hydrophobicity with self-assembly behaviour, and highlighted how disparate supramolecular outcomes arise from subtle differences, such as the order of amino acids along the sequence. The authors ranked the top 20 triplets according to their aggregation propensity, based on two different indicators, for a total of 40 tripeptides. Interestingly, Phe-Leu-Phe was not amongst the identified 40 hits, which featured the two aromatic amino acids rather frequently. Besides, it was noted that hydrophobic tripeptides may be challenging to handle, due to their poor solubility in water. Therefore, introduction of a hydrophilic amino acid (e.g., Lys) next to two aromatic residues (e.g., Phe-Phe) offered a convenient strategy to identify the new four self-assembling motifs in water (i.e., Lys-Phe-Phe, Lys-Tyr-Phe, Lys-Tyr-Trp, and Lys-Tyr-Tyr).<sup>20</sup>

A subsequent work analysed the conformations adopted by Phe-X-Phe amphiphiles, where X is a hydrophilic residue, and confirmed the high stabilisation exerted by the terminal aromatic amino acids for the formation of anti-parallel beta sheets. A series of compounds was assessed for gelation. Amongst the unprotected tripeptides, Phe-Glu-Phe, Phe-Thr-Phe, and Phe-Cys-Phe could gel, although only in the presence of 10% hexafluoroisopropanol, which poses a serious limitation for biological use. In one case, Phe-Lys-Phe could gel without the organic solvent, yet all the reported supramolecular gels displayed an acidic pH within the range 3.5-5.<sup>21</sup> Clearly, these scientific efforts emphasise the inherent difficulties to achieve hydrogels for biological use from the self-organisation of simple tripeptides in water at neutral pH.

We reported the use of chirality as a new tool to favour (unprotected) tripeptide self-assembly into hydrogels at physiological conditions.<sup>22</sup> Introduction of a D-amino acid at the N-terminal of Phe-Phe bearing tripeptides resulted in the rapid formation of self-supporting hydrogels in phosphate buffer at pH 7.4, whilst their homochiral L-analogues did not

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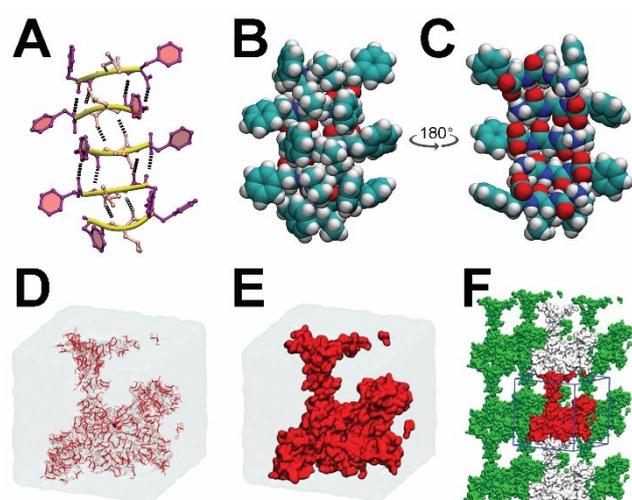
gel.<sup>22, 23</sup> This concept can be extended to similar tripeptide sequences in the D-L-L stereoconfiguration.<sup>23, 24</sup> Subsequent studies on complete stereoisomer series of hydrophobic tripeptides confirmed the pronounced self-organisation of the heterochiral compounds in the D-L-L stereoconfiguration, and a limited self-assembling propensity for L-D-L isomers.<sup>25, 26</sup> In the latter case, only Val-<sup>D</sup>Phe-Phe (or its enantiomer) formed a gel, albeit with a low level of supramolecular order, due to the presence of various peptide conformations. As a result, the soft material displayed modest rheological properties, and rapid dissolution in cell culture.<sup>26</sup>

Encouraged by these results, we reasoned that the order of supramolecular assemblies of L-D-L isomers could be improved by design assisted by computer simulations. First, we substituted valine with the more bulky leucine, recurrent in self-assembling motifs, to promote peptide interlocking through increased steric hindrance. This is confirmed by <sup>D</sup>Leu-Phe-Phe gelling within seconds<sup>23</sup> as opposed to minutes for <sup>D</sup>Val-Phe-Phe.<sup>22</sup> Second, we introduced leucine at the centre of the sequence to space the two Phe residues further apart. This is because presence of the aromatic amino acid at both termini should favour interaction between adjacent beta-sheets<sup>21</sup> through the formation of Phe-zippers.<sup>23, 27</sup> Stabilisation of beta-sheets<sup>28, 29</sup> and steric zippers<sup>30</sup> are both crucial parameters to improve the rheological properties of amyloid-based supramolecular materials. An added benefit for biological use is avoiding having two adjacent Phe residues that are a key amyloid motif in pathological states.<sup>14</sup>

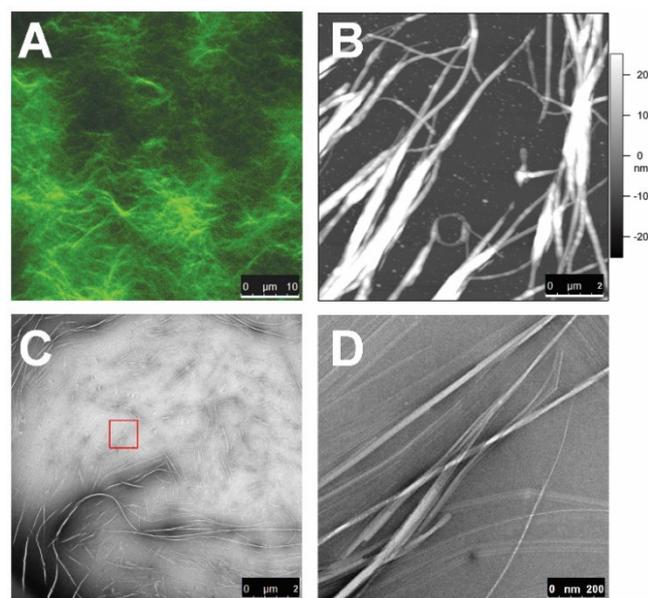
It should be noted that despite Phe-X-Phe amphiphiles' tendency towards lateral association into assemblies, no hydrogel has been reported thus far for sequences where X is either a hydrophobic amino acid, or a D-amino acid, or both. In our rationale, X is not required to be hydrophilic to achieve amphiphilic superstructures, as long as it is a D-amino acid sandwiched between two L-residues. As a result, the supramolecular beta-sheets display the amide bonds exposed

on the opposite surface relative to the hydrophobic side chains, effectively creating an amphiphilic superstructure with a hydrophilic and a hydrophobic surface, as confirmed by modelling (Fig. 1A-C). This is effectively the first amphiphilic superstructure reported to arise from a heterochiral tripeptide composed of hydrophobic amino acids. Remarkably, the average inter-strand distance of 5.0 Å is in very good agreement with the observed XRD signal at 4.9 Å that is a typical value for similar beta-strands (see ESI).<sup>23, 25, 26</sup>

Molecular dynamics simulations in explicit water confirmed a propensity towards aggregation leading to fibrils (Fig. 1D-F), whilst maintaining the antiparallel beta-sheet organisation in amphiphilic superstructures (see Fig. S4, page S8 of ESI for details). Phe-<sup>D</sup>Leu-Phe is the first unprotected tripeptide in the L-D-L stereoconfiguration to yield self-supportive hydrogels at physiological conditions. Following a pH-trigger method,<sup>22</sup> the compound is dissolved at alkaline pH as anion, thanks to the repulsion between the carboxylates. As the pH is lowered to 7.4, the zwitterion immediately gels reaching a storage modulus  $G'$  of 7 kPa and a loss modulus  $G''$  of 0.9 kPa within an hour (see ESI for rheometric analyses). The minimal gelling concentration is 5 mM or 0.2 wt %. The gel has also a remarkable stability to temperature as assessed by DSC (see ESI), with the transition to a solution starting at  $70 \pm 2$  °C, as confirmed by visual observation in separate tests. Extended hydrogen bonding between peptide termini likely plays a role, as suggested by FT-IR data (see ESI) for the amide II region, with a signal centred at  $1550\text{ cm}^{-1}$ .<sup>22</sup> In the amide I region, maxima at  $1645\text{ cm}^{-1}$  and  $1685\text{ cm}^{-1}$  indicate the formation of antiparallel beta-sheets, as expected for a Phe-X-Phe tripeptide.<sup>21</sup> Secondary conformation was confirmed by fluorescent amyloid-stain microscopy imaging (Fig. 2A), and circular dichroism (see ESI). The latter revealed a D-type supramolecular chirality due to the D-amino acid in position



**Fig. 1.** Antiparallel  $\beta$ -sheets of Phe-<sup>D</sup>Leu-Phe (A) display a hydrophobic (B) and a hydrophilic (C) surface. Molecular dynamics simulations (D-F) of hundreds of tripeptides in explicit water (grey box) reveal propensity for the peptide (backbone in red superposed to stick model, D) to form aggregates (surface rendered in red, white or green in E,F) that self-assemble to form fibrils (F). Repetition of aggregates is shown in white or green along different directions.



**Fig. 2.** Fluorescence (A), Atomic Force (B), and Transmission Electron (C-D) microscopy images of tripeptide superstructures forming the hydrogel. D is a 10x magnification of the red square in C. Thicker fibers derive from the convergence of multiple fibrils (D).

two.<sup>25</sup> The CD signal at 227 nm can be ascribed to  $\pi$ - $\pi$  interactions,<sup>21-23</sup> and the maximum in the near-UV region (240-280 nm) was observed for self-assembling tripeptides bearing Phe with tertiary structure.<sup>22</sup> Microscopy data revealed numerous fibrils converging in bundles to form thicker fibers (Fig. 2), suggesting lateral interaction between sheets.

The hydrogel was tested for fibroblast cell culture over 3 days (Fig. 3). Remarkably, no significant difference was observed in terms of cell viability between control and gel of unnatural D-type supramolecular chirality. Cells penetrated within the hydrogel and instances of spreading were present (Fig. 3F, 3J, 3H, 3L). Overall, cell spreading occurred to a notably minor extent in the soft gel relative to tissue-culture plastics (TCPS,  $G' \sim 10^6$  Pa), in accordance to previous works where soft, non-adhesive substrates led to less spread, or even round, fibroblasts, albeit with high viability.<sup>31, 32</sup> It is worth noting that culture on soft gels of Young modulus  $\sim 7$  kPa, as opposed to stiffer gels, can serve the scope to de-activate fibroblasts from a reactive type that, if persistent, can lead to fibrosis.<sup>33</sup>

In our cell culture experiments, the hydrogel mass did not deteriorate (Fig. 3M), in contrast with the only other L-D-L peptide gel reported,<sup>26</sup> which rapidly dissolved. No cytotoxicity was apparent for the peptide in solution, with no effect on fibroblast adhesion to TCPS (see ESI), although it should be noted that this test was possible only at lower peptide amounts (*i.e.*, up to 1 mg/ml) relative to the critical gelling concentration (*i.e.*, 2 mg/ml). Indeed, we cannot exclude that peptide higher concentrations may lead to cytotoxicity, and future investigations call for further analysis in this sense.

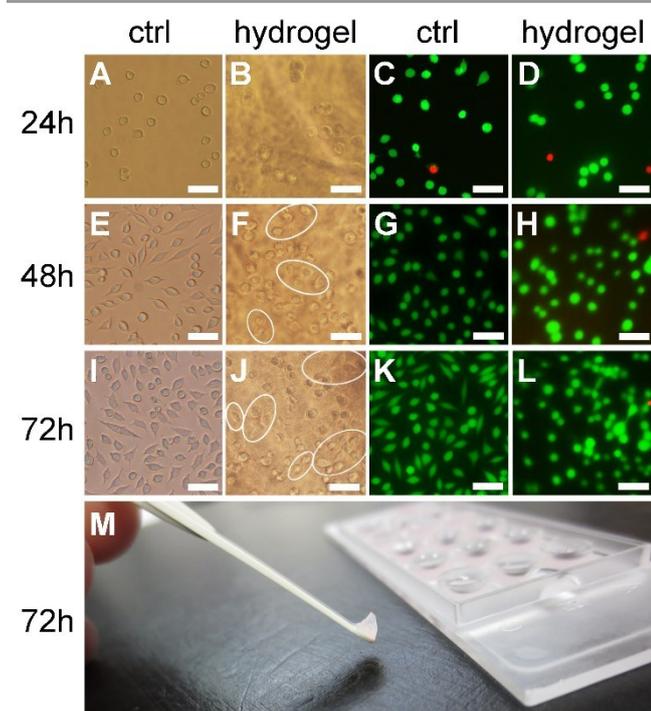
In conclusion, the emerging principles of chirality dictating self-assembly behaviour of unprotected tripeptides can be applied

for the rational design of supramolecular hydrogels that persist in cell culture and support fibroblast cell growth. Rational design allows the generation of supramolecular amphiphiles from hydrophobic amino acids, as long as D- and L-amino acids are appropriately positioned. We believe this approach can be extended to other sequences towards a platform of simple and convenient building blocks for supramolecular materials. It will be interesting to correlate peptide sequence with supramolecular and rheological behaviour. Current studies in our laboratories are proceeding in this direction, and are also motivated by the advantages that may follow the introduction of D-amino acids in biomaterial building blocks.<sup>34</sup>

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**Fig. 3.** Fibroblast cell culture on the hydrogel. Bright-field (left) and fluorescence (right) imaging confirmed cell proliferation and instances of spreading (circles).

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