Three-dimensional cell culture of chondrocytes on modified di-phenylalanine scaffolds

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Abstract
The design of self-assembled peptide-based structures for three-dimensional cell culture and tissue repair has been a key objective in biomaterials science for decades. In search of the simplest possible peptide system that can self-assemble, we discovered that combinations of di-peptides that are modified with aromatic stacking ligands could form nanometre-sized fibres when exposed to physiological conditions. For example, we demonstrated that a number of Fmoc (fluoren-9-ylmethylxycarbonyl) modified di- and tri-peptides form highly ordered hydrogels via hydrogen-bonding and $\pi-\pi$ interactions from the fluorenyl rings. These highly hydrated gels allowed for cell proliferation of chondrocytes in three dimensions [Jayawarna, Ali, Jowitt, Miller, Saiani, Gough and Ulijn (2006) Adv. Mater. 18, 611–614]. We demonstrated that fibrous architecture and physical properties of the resulting materials were dictated by the nature of the amino acid building blocks. Here, we report the self-assembly process of three di-phenylalanine analogues, Fmoc-Phe-Phe-OH, Nap (naphthalene)-Phe-Phe-OH and Cbz (benzyloxycarbonyl)-Phe-Phe-OH, to compare and contrast the self-assembly properties and cell culture conditions attributable to their protecting group difference. Fibre morphology analysis of the three structures using cryo-SEM (scanning electron microscopy) and TEM (transmission electron microscopy) suggested fibrous structures with dramatically varying fibril dimensions, depending on the aromatic ligand used. CD and FTIR (Fourier-transform IR) data confirmed $\beta$-sheet arrangements in all three samples in the gel state. The ability of these three new hydrogels to support cell proliferation of chondrocytes was confirmed for all three materials.

Spontaneous formation of macroscopic hydrogels from small molecule building blocks via self-assembly is a very powerful tool for the preparation of novel materials with well-defined properties at the molecular level. Peptides are particularly interesting as building blocks for these materials and self-assembled nanowires, fibres, sheets and tubes have all been described. These peptide-based biomaterials are gaining interest as a result of their programmability, biodegradability, and bioresorbability. A number of strategies exist to design molecular biomaterials based on self-assembled peptides and their derivatives [1]. These include materials based on a variety of structural motifs including coiled-coils, $\beta$-sheets, $\beta$-hairpins and peptide amphiphiles usually containing at least ten amino acids per peptide chain [2]. Significant progress in applying these systems in three-dimensional cell culture and tissue engineering has been made by the groups of Zhang et al. [3] and Stupp and co-workers [4]. It has been known for some time that using aromatic components allows the use of much smaller peptides by taking advantage of $\pi$-stacking interactions [5]. We are interested in using self-assembled gels from short peptide derivates as highly tuneable systems for three-dimensional cell culture. Systems based on short peptides have a number of advantages including opportunities for molecular design based on their more predictable behaviour, predictable degradability and lower cost.

Thus we study the molecular-level engineering of simple di-peptides to make supramolecular hydrogel structures. Our previous research demonstrated that combinations of di-peptides that are modified with aromatic groups could form nanometre-sized fibres when exposed to physiological conditions. For example, we demonstrated that when modified at the N-terminus with an Fmoc (fluoren-9-ylmethylxycarbonyl) group, a number of simple di-peptides form highly ordered hydrogels that allow cell proliferation of chondrocytes in three dimensions [6]. The $\pi-\pi$ interactions from the fluorenyl rings when stabilized by the hydrogen bond interactions drive the self-assembly process to form fibrous networks. We further demonstrated that fibrous architecture and physical properties of the assembled structures were dictated by the nature of the amino acid building blocks used in the self-assembly process. Combinations of Fmoc-di-peptides were identified which formed fibrous hydrogels that were (i) stable under cell culture conditions (ii) of similar dimensions to the fibrous components of the extracellular matrix and (iii) capable of supporting cell culture of chondrocytes in three dimensions.

Here, we report the self-assembly process of three di-phenylalanine analogues, Fmoc-Phe-Phe-OH, Nap

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Key words: cell proliferation, chondrocyte, fluoren-9-ylmethylxycarbonyl (Fmoc), hydrogel, self-assembly, three-dimensional cell culture

Abbreviations used: Cbz, benzyloxycarbonyl; Fmoc, fluoren-9-ylmethylxycarbonyl; FTIR, Fourier-transform IR; Nap, naphthalene; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

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Figure 1 | Electron microscopy analysis of fibre structures

Cryo-SEM images of the three structures (1a, 1b, 1c) and TEM images of Fmoc-Phe-Phe-OH and Nap-Phe-Phe-OH (2a, 2b) suggested fibrous structures. Fibre diameters that were observed lie well within the range found for extracellular matrix components which is 5-300 nm.

In Figure 2, a) Fmoc-Phe-Phe-OH, b) Nap-Phe-Phe-OH, and c) Cbz (benzyloxycarbonyl)-Phe-Phe-OH, to compare and contrast the self-assembly properties and cell culture conditions attributable to their protecting group difference.

Fibre morphology analysis of the three structures using cryo-SEM (scanning electron microscopy) and TEM (transmission electron microscopy) (Figure 1) suggested fibrous structures with dramatically varying fibril dimensions, depending on the aromatic ligand used. The CD and FTIR (Fourier-transform IR) (Figure 2) data also confirmed β-sheet arrangements in all three samples in the gel state and indicate the molecular models attached to each structure.

The ability of the three hydrogels to support cell proliferation of chondrocytes was also confirmed for all three materials. LDH (lactate dehydrogenase) assays performed to assess the number of metabolically active cells to study cell proliferation gave results that showed the existence of metabolically active cells in all three structures up to 10 days (Figure 3).
Figure 2 | Spectroscopic characterization of di-phenylalanine analogues
(A) CD spectrum of Fmoc-Phe-Phe-OH gave rise to peaks at 310 nm, indicative of Fmoc orientation, and the peak at 218 nm indicates a β-sheet structure. (B) FTIR analysis of both Fmoc-Phe-Phe-OH (a) and Cbz-Phe-Phe-OH (b) shows peaks in the 1700–1600 cm⁻¹ wavelength region. These are indicative of antiparallel β-sheets.

Figure 3 | Cell-proliferation assay
All three structures confirmed the existence of metabolically active cells up to 10 days. Fmoc, Fmoc-Phe-Phe-OH; Nap, Nap-Phe-Phe-OH; CBZ, Cbz-Phe-Phe-OH.

Figure 4 | Confocal microscopy of di-phenylalanine analogues
(A, B) Live/dead staining of Fmoc-Phe-Phe-OH and Nap-Phe-Phe-OH respectively. (C) Results of the Collagen II antibody staining of cell-seeded Fmoc-Phe-Phe-OH scaffold.

The results presented above therefore confirmed that when protected by different aromatic stacking ligands, di-phenylalanine can self-assemble into nanofibrous hydrogels via interlocked β-sheets/π-stacks. The variation of stacking group has resulted in the formation of nanofibres of varying curvature, branching and diameter, having similar dimensions to fibrous components of the extracellular matrix. The results also confirmed that all three modified di-phenylalanine hydrogels support chondrocyte cell culture in both two and three dimensions.

References

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