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A self-assembling β-peptide hydrogel for neural tissue engineering

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We report a new class of β-peptide based hydrogel for neural tissue engineering. Our β-peptide forms a network of nanofibres in aqueous solution, resulting in a stable hydrogel at physiological conditions. The hydrogel shows excellent compatibility with neural cells and provides a suitable environment for cells to adhere and proliferate.

Hydrogels possess physical features of soft tissues and have been explored widely for their use in nerve regeneration and drug delivery[1]. Hydrogels are capable of providing a supportive 3-dimensional (3D) microenvironment for cells to adhere, infiltrate, migrate, and proliferate[2]. Such superior properties are credited to their hydrophilic nature which allows a significant amount of water to be absorbed and retained. This property, together with their high porosity, facilitates the rapid diffusion of nutrients and metabolites to and from cells[3]. Injectable hydrogels are of interest, since drugs and cells can be incorporated into the polymer solution by mixing prior to injection and can be implanted in the body with minimal surgical procedures[1e, 2f].

Hydrogels based on peptide self-assembly are extremely promising candidates to provide a suitable microenvironment for cells due to their ability to control the structural and functional properties of the end-product[4]. Self-assembly of peptides occurs either spontaneously or due to changes in the environmental conditions such as temperature, pH and ionic strength. This leads to the formation of nanofibres and subsequently a fibrillar network which absorbs water and forms hydrogels. Several modes of peptide self-assembly have been utilised, such as: ionic self-complimentary peptides, ß-hairpin peptides, multidomain peptides, Fmoc-protected peptides, acetylated aliphatic peptides and peptide amphiphiles[4a, 4b, 5], all based on α-amino acids. α-Peptide hydrogels have been used in neural tissue engineering where they have been investigated as injectable matrices to provide both a physical and biological support following injury to the brain[6]. However these materials suffer from degradation by proteolytic enzymes which limit their long-term application. For the potential repair of large brain lesions caused by traumatic brain injury, it may be preferable to use a slow-degrading peptide matrix, which could provide long-term physical support to the surrounding brain parenchyma[2b]. The superior metabolic stability of β-peptides makes them promising candidates to overcome the degradation observed in α-peptide-based hydrogels over time[7]. Single β-amino acid substitutions have been used to stabilise a wide range of α-peptides against proteolytic stability but still retaining their bioactivity[8]. This approach has also been applied to the stabilisation of α-peptide-based hybrid hydrogels[9]. Despite improvement in the proteolytic-stability of the hydrogel, the non-uniform fibre diameter and inappropriate stiffness limited their efficacy for optimum cell adhesion[9a, 9e]. Approaches reported so far for the formation of β-amino acid containing peptide-based hydrogels are based on external stimuli, including changes in pH and temperature, raising concerns over cell encapsulation in tissue engineering[9a, 9e]. Herein we report a β-peptide that does not require a pH or thermal trigger for gelation and have assessed its biocompatibility using neural cells. To the best of our knowledge, this is the first report of a peptide hydrogel comprising exclusively β-amino acids, which was used as a scaffold for tissue engineering.

We recently developed a completely new approach to peptide based self-assembly in which helical N-acetyl-β^3-peptides spontaneously form fibres ranging in size from nano- to macro-scale[10]. The peptide monomers self-assemble in a unique head-to-tail fashion which is driven by a 3-point hydrogen-bonding motif associated with the 14-helical structure of N-acetyl-β^3-peptides. These peptides self-assemble into helices irrespective

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of the monomer sequence, and can therefore be modified with specific functional groups without affecting their ability to form fibres. A number of groups have demonstrated that incorporation of a hydrophobic acyl tail within a peptide sequence to produce a peptide amphiphile directs their self-assembly into nano-cylinders with uniform diameter thereby leading to stable hydrogels[11]. In order to achieve a similar level of control of our β-peptide fibre morphology, we introduced an alkyl chain to the side chain of a β-peptide. The tri-β-peptide Ac-βAzβKβA-OH was synthesized (via solid-phase peptide synthesis) containing a β-homo-lysine (K) residue to enhance peptide solubility in aqueous buffer. The C_{14} alkyl chain, was then introduced on solid support by reducing the azide moiety using triphenylphosphine followed by acylation with myristic acid to form the final peptide structure (Fig. 1a,b & Fig. ESI-1).

The C_{14} acylated tripeptide resulted in the formation of fibres with highly consistent diameter as confirmed by atomic force microscopy (AFM) and transmission electron microscopy (TEM) shown in Fig. 1c,d. It can be seen that the peptide self-assembled into a flexible fibrous mesh even at a low concentration of 0.25mg ml^{-1} in water. An interwoven network of numerous nanofibres with bundles entraps water, resulting in the formation of a stable β-peptide hydrogel. The β-peptide started to form a hydrogel at a concentration of 7.5 mg mL^{-1} in PBS buffer (pH 7.4) and maintained a stable structure under physiological conditions over weeks at an acceptably low concentration of 10 mg mL^{-1}, comparable to previous studies[12]. The inversion test shown in Fig. 1e provides strong visual evidence of the formation of highly stable hydrogel in buffer, demonstrating that the hydrogel retained its 3D structure and was able to support its own weight.

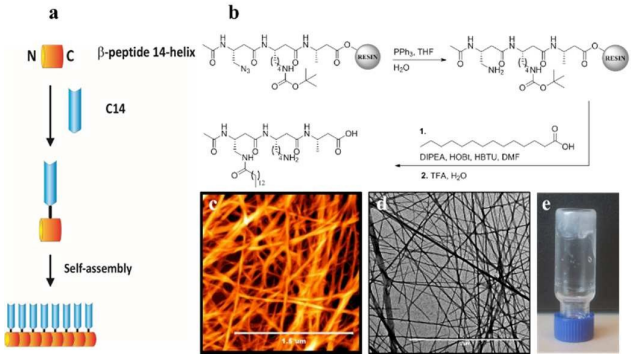

Fig. 1. (a) Schematic of supramolecular self-assembly of N-acetylated β^3-peptide. Helices of β^3-tripeptides when N-acetylated provide six axially oriented hydrogen bonds, which leads to nanofibre formation. (b) Solid phase peptide synthesis of the C_{14} acylated, N-acetyl-β^3-tripeptide. (c) AFM characterization of nanofibrous network of self-assembled peptide (0.25mg ml^{-1}) after 24 hr incubation time (200-400 kHz probe resonance, 10-50 nm probe amplitudes); (d) TEM characterization of nanofibrous network of self-assembled peptide (0.25mg ml^{-1}) (e) Inversion test showing the formation of a highly stable hydrogel that retains its 3D structure and supports its own weight.

To determine the suitability of our β-peptide hydrogel in nerve regeneration applications, the mechanical properties of the hydrogel was investigated by rheological testing. The elastic response component, *G′* (storage modulus), of 5 to 11 times higher than the viscous response component, *G″* (loss modulus), demonstrated a phase transition into a viscoelastic material and further confirmed hydrogel-like behaviour of our material. It was not possible to record the initial phase transition from sol-to-gel state in the rheology study due to the rapid formation of hydrogel. The hydrogel reached its plateau value of 1.2 kPa after 1 hour (Fig. ESI-3a). The frequency sweep curve showed broad linear viscoelastic properties with the storage modulus dominating across the whole range of frequencies (0.1-100 rad s^{-1}), indicating a wide processing regime for the hydrogel (Fig. ESI-3b). Furthermore, as far as we can ascertain, there is no report in the literature that describes such superior hydrogel stiffness for β-peptide hydrogels[9a, 9e].

Cycles of step-strain oscillatory rheology were performed to investigate the injectability of the hydrogel. Fig. 2 represents step-strain measurements of the hydrogel. In order to disturb the hydrogel 3D network and to ensure the gel-to-sol conversion, the hydrogel was exposed to high strain (100%) for 1 minute. The hydrogel recovery behaviour upon relaxation under low strain value (1%) was then monitored. The hydrogel exhibited shear-thinning behaviour at high strains, developing fully injectable properties[13]. Upon reduction of strain, the hydrogel showed exceptionally rapid and complete recovery within seconds which would allow facile surgical handling during implantation and maintain appropriate shape after implantation[3b]. The fast recovery of the hydrogel stiffness was confirmed by 3 cycles, demonstrating the reversible and robust nature of hydrogel structures. The rapid recovery of the hydrogel is attributed to hydrogen bonding and non-covalent interactions which are responsible for the formation of the 3D network. Interestingly, high strain breaks the fibrous network by perturbing the non-covalent interactions, without destroying the fibres, resulting in the breakdown of the gel into a sol state. After reducing the strain, the fibres start to re-organize the 3D network of the hydrogel in a relatively short time.

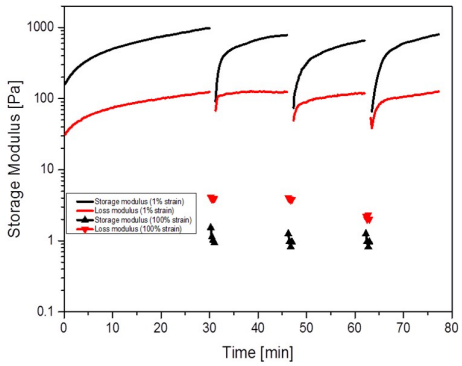

Fig. 2. Rheological behaviour of the β-peptide hydrogel (10mg ml^{-1}) showed fast and complete recovery after network disruption by high strain (100%) for 1 minute. High strain = 100%; low strain = 1%; Frequency = 1 Hz; Temperature = 35°C.

In neural tissue engineering the implanted hydrogel should provide a favourable 3D microenvironment for neural cells and should therefore be non-toxic and allow cells to adhere and proliferate[2a, 2c]. To investigate the cytotoxicity of our hydrogel, a thin layer of sterilized hydrogel was seeded with SN4741 cells, a clonal substantia nigra dopaminergic neuronal progenitor cell line[14]. To investigate the interaction of cells with the hydrogel, cells were stained with calcein AM/ethidium, imaged using fluorescence microscopy after one day and were compared with cells cultured on uncoated tissue culture plate (TCPS) as the positive control. After one day, some live cells were seen on top of the hydrogel equilibrated with PBS; although they had a rounded morphology compared to the elongated cells in the TCPS (Fig. ESI-6a), suggesting little or no interaction with the material. It was hypothesized that the low level of viable cells on top of the hydrogel is due to the absence of a bioactive motif in the peptide sequence and not the cytotoxicity of the β-peptide hydrogel.

Upon implantation in the body, the hydrogel would be immersed in the body fluid and proteins, which can induce cell attachment to the material. To mimic *in vivo* conditions, the hydrogel was equilibrated with culture medium, with and without serum for one day. We hypothesized that the proteins in the serum would deposit on top of the hydrogel and promote cell attachment. After one day of cell culture, cells were able to attach, spread and elongate on top of the hydrogel equilibrated with serum (Fig. 3a). By contrast, the attached cells on top of the hydrogel equilibrated without serum (Fig. ESI-6b) exhibited less spreading morphology than that of TCPS (Fig. 3b) and hydrogel equilibrated with serum. These results confirm that proteins derived from serum make the hydrogel a promising environment for cells to adhere and proliferate. A very high viability of cells on top of the hydrogel equilibrated with serum similar to TCPS also demonstrates the non-toxicity of the hydrogel to cells (Fig. ESI-7).

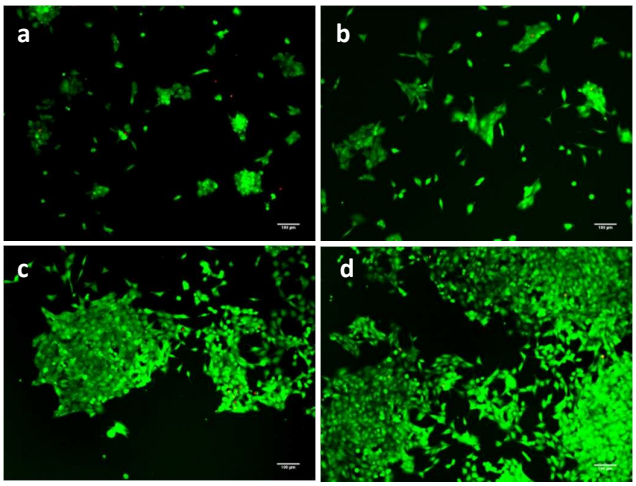

Fig. 3. Growth of SN4741 cells and live/dead cells assay on peptide hydrogels equilibrated with media containing serum for one day at two time points (1 day and 3 days cell culture) – Green cells represent live cells, stained with calcein AM. Red cells represent dead cells, stained with ethidium homodimer. a) Peptide hydrogel equilibrated with culture media containing serum for 1 day – 1 day culture, b) TCPS – 1 day culture, c) Peptide hydrogel equilibrated with culture media containing serum for 1 day – 3 days culture, d) TCPS – 3 days culture. Scale bar 100µm. Higher magnification images ESI-5.

The culture was continued for 3 days to investigate the persistence of cell proliferation for an extended time period on top of the hydrogel. As can be seen in Fig. 3c, cells developed an extensive network, quite similar to the cells on TCPS (Fig. 3d) and also maintained their dopaminergic phenotype (Fig. ESI-8). However, the population of cells on top of the hydrogel were lower than the TCPS. This is due to the fact that cells penetrated into the hydrogel over time and it was difficult to capture a focused image. Interestingly, microscopic observation showed excellent integrity of the hydrogel over three days, with no visible fractures in the whole layer of hydrogel. This confirms the high stability of the β-peptide hydrogel in comparison to α-peptide hydrogels, where the latter starts to show fractures within similar time periods, possibly due to proteolytic degradation of the α-peptide building blocks[15]. Moreover, the resistance of our β-peptide to proteolysis (Fig. ESI-2) and UV (Fig. ESI-4) degradation further highlights the stability of this β-peptide material. The non-toxicity, high cell viability and excellent structural integrity of our β-peptide hydrogel could therefore lead to outperforming traditional α-peptide hydrogels for nerve regeneration applications.

Conclusions

We have introduced a new class of peptide-based hydrogels solely composed of β-amino acid building blocks via a facile single step protocol. Our β-peptide hydrogels rapidly self-assemble in aqueous environment, can be chemically modified at the monomer level and are mechanically stable and biocompatible. These properties combine to generate a hydrogel which is non-toxic, with a similar stiffness to brain tissue, and provides a suitable environment for cells to adhere and proliferate. The enhanced self-healing property of the hydrogel will allow minimal post-operative damage upon implantation in the body, resulting in rapid recovery.

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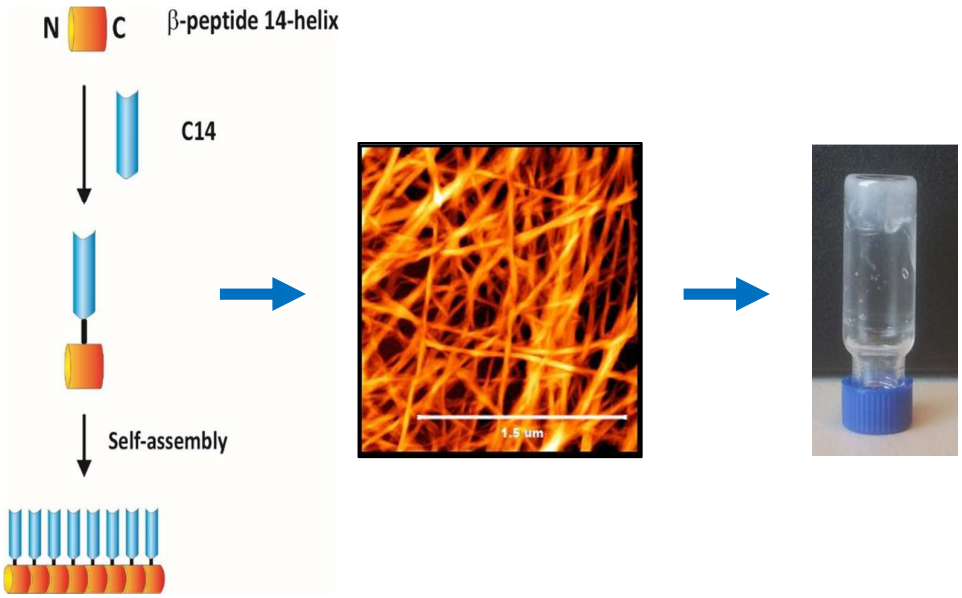
Graphical Abstract



We have synthesised the first helical β^3-peptide amphiphile and shown its ability to form an injectable, stable and biocompatible hydrogel.