Supporting Information

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

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Peptide Synthesis and Purification

The tripeptide (Ac-β-homo-azidoalanine-β-homo-lysine-β-homo-alanine-OH) was synthesized on a 0.1 mmol scale using standard Fmoc chemistry on Wang resin. The Fmoc-protected β-homo-alanine (3.1 eq. to resin loading) along with HBTU (O-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate), DIPEA (N,N-diisopropylethylamine) and DMAP (4-dimethylaminopyridine) (3, 4.5 and 0.1 eq. to resin respectively) were dissolved in DMF (N,N-dimethylformamide) and the reaction proceeded overnight. The resin was then washed with DMF to remove unreacted amino acids. 20% Piperidine/DMF (2×20 min) was added to remove the Fmoc protecting group. To couple β-homo-lysine (Boc)-OH, it was added to resin along with HBTU and DIPEA in DMF for 60 min. The resin was then washed and the same steps were repeated to add β-homo-azidoalanine to the sequence. Upon successful removal of Fmoc-protecting group of β-homo-azidoalanine, acetic anhydride and DIPEA in DMF (2×20min) were added to acetylate the N-terminus. To reduce the azide group to an amino group, triphenylphosphine (TPP) (3 eq. to resin) dissolved in tetrahydrofuran (THF) and H_2O (4:1) was added to the resin and incubated overnight. The resin was then washed with THF/H_2O (4:1), dichloromethane (DCM) and DMF. To add the C14 alkyl chain to the reduced azidoalanine side chain, myristic acid, HBTU and DIPEA (3, 3 and 4.5 eq. to resin respectively), were dissolved in DMF and added to the resin and the reaction was continued overnight. To cleave the peptide, resin was treated with 5%v/v water in trifluoroacetic acid (TFA) for 3h and the cleaved peptide in TFA was collected. The TFA was evaporated under a stream of N_2 gas and the peptide was lyophilized. The peptide was purified using reverse-phase HPLC (Agilent HP1200). The collected fractions were then analysed by analytical HPLC (Agilent HP1100) to assess peptide purity and confirmed by mass spectrometry (Agilent 1100 MSD SL, negative ion mode) (Fig ESI-1)[1]. The obtained mass for the peptide was 596.3, which is in agreement with the calculated mass of 597.4.

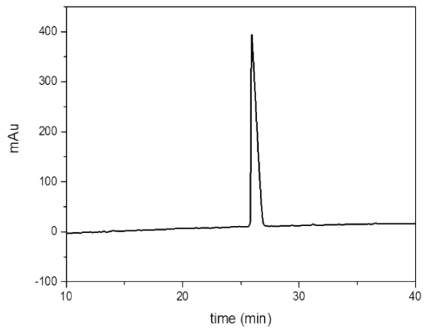

Fig. ESI-1. HPLC chromatogram. HPLC retention time was 25.91 min with a solvent gradient of 0-70% acetonitrile over 40 min.

Hydrogel Formation

100µL PBS (pH 7.4) was added to 1.0mg of peptide to give a concentration of 10mg ml^{-1}. The solution was then vortexed and a stable hydrogel was formed within a few minutes. The hydrogel formation was assessed by an inversion test.

Atomic Force Microscopy (AFM) Analysis

2 µL of peptide solution in water (0.25mg mL^{-1}) was placed on a clean mica surface. The sample was incubated under a petri dish for 30 minutes and then dried with gentle stream of N_2 gas. The sample was then imaged with a Nanoscope IV AFM with a Multimode head (Veeco, Santa Barbara, CA, USA) using a vertical engage 'E' scanner. Images were obtained in H_2O *via* tapping mode with NSC-15 'B' silicon cantilevers (Micromasch, Tallinn, Estonia) with a nominal force constant of 40 N/m. Topographic, phase and amplitude images at a resolution of 512 x 512 were simultaneously obtained using scan frequency of 1 Hz with typical scan sizes of 5 µm x 5 µm and 2 µm x 2 µm. Images were processed with a sequence of plane fitting and offset flattening using Gwyddion 2.29 (www.gwyddion.net) software.

Transmission Electron Microscopy (TEM) Analysis

A 10 µl drop of solution containing peptide was placed onto parafilm. A formvar-coated grid was placed face down on the solution and left for five minutes. The sample was gently blot dried and left to dry further for 30 min. The samples were imaged using a Hitachi H6500 Transmission Electron Microscope.

Proteolytic Stability

The stability of the newly synthesised β-peptide was compared to that of angiotensin II in a 24 hour digestion study in trypsin. Enzymatic degradation was performed by incubating 0.2 mg peptide with 0.01 mg enzyme in 200µL PBS at 37°C for 24 hours. Several aliquotes (20µL) were taken at 0, 1, 3 and 24 hours and degradation was monitored using LC/MSD. After 3 hours of digestion, only 25% of angiotensin II was remaining. (Fig. ESI-2) In contrast, 100% of the β-peptide was present following 24 hours incubation.

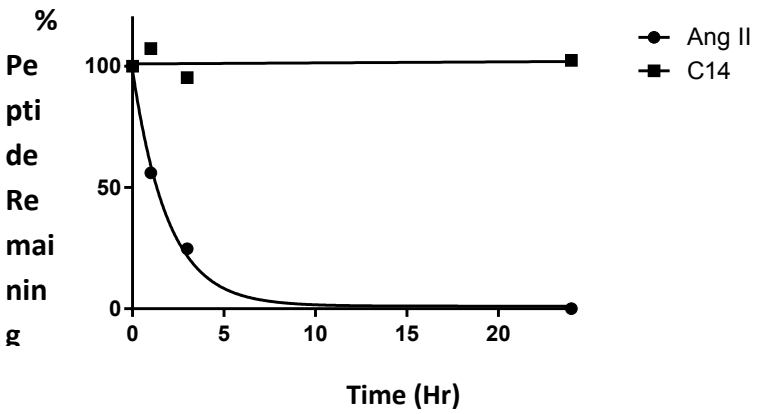

Fig. ESI-2. Stability assay data showing the digestion over time of the novel β-peptide as well as the α-peptide angiotensin II over 24 hours treated with trypsin.

Rheometry

Rheological studies were conducted using a Anton Paar rheometer (Physica MCR 501) with an 8 mm parallel plate. The temperature was maintained at 35 °C for all experiments. Peptide samples were dissolved in PBS to give a concentration of 10 mg ml^{-1}. Experiments were immediately performed with a plate gap of 0.1 mm. The gelation of the peptide over time was examined for 3 hours and the storage (elastic) and loss (viscous) moduli were reported as a function of time. When the hydrogel reached an equilibrium state, its response to frequency variation was examined. All experiments were repeated three times and the average values were reported. The injectability of the hydrogel was examined by its ability to recover after network disruption under high strain. The network of the stable hydrogel was disrupted under high strain (100%) for 1 min and the recovery of hydrogel properties was checked when the strain returned to its standard value (1%). The experiment was repeated three times, each time after 15 minutes[2].

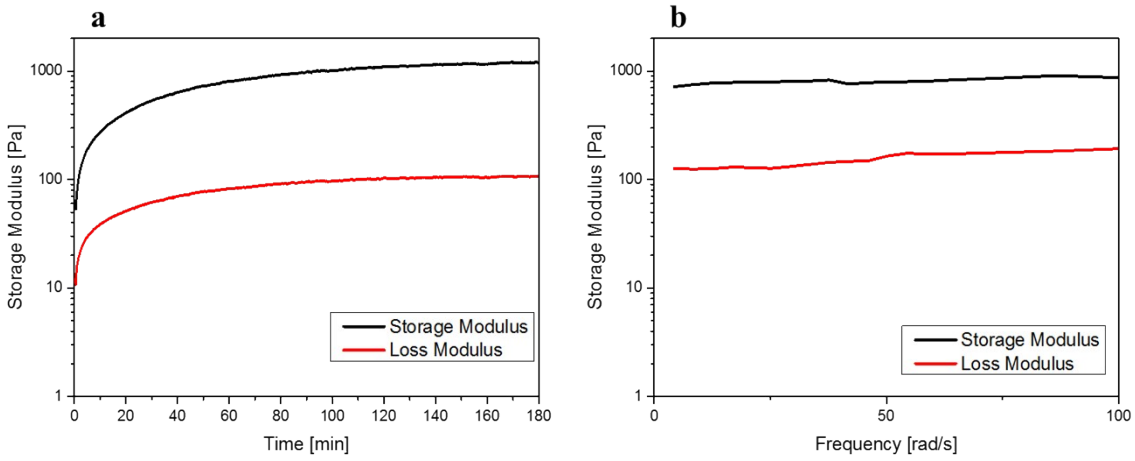

Fig. ESI-3 a) The dominance of elastic modulus over viscous modulus proved that the material formed a solid-like hydrogel. The hydrogel reached its plateau value of 1.2kPa after one hour; b) The hydrogel showed linear viscoelastic behaviour over the entire range of frequencies (1-100rad s-1); strain = 1%; Temperature = 35°C.

Peptide Stability under UV Sterilisation

Prior to the cell viability assay, peptide powder was sterilized under a UV lamp for 30min as a standard sterilization step for biological samples. To ensure that UV irradiation did not degrade the peptide, the purity and molecular weight of the irradiated peptide powder was examined using LC-MS. Only one peak corresponding to the molecular mass of 596.3 was observed following UV irradiation (Fig. ESI-4), which was the molecular mass of the peptide, thus indicating that UV irradiation did not break down the peptide structure.

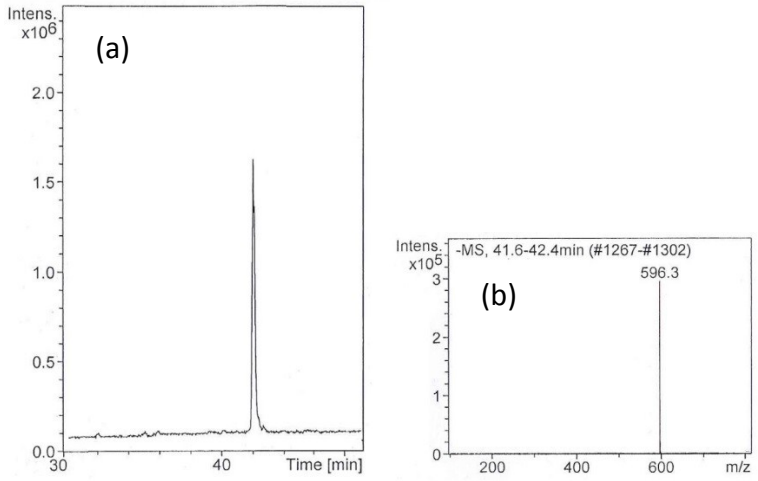

Fig. ESI-4 a) HPLC chromatogram of β-peptide following UV sterilisation (HPLC retention time was 42.0 min with a solvent gradient of 10-60% acetonitrile over 60 min). b) Total ion chromatogram of β-peptide following UV sterilisation (Agilent 1100 MSD SL, negative ion mode).

Moreover, the rheology of the formed hydrogel remained unchanged after UV sterilization showing a storage moduli of 1.2kPa, in agreement with our reported rheology for our peptide hydrogel.

Cell Viability Analysis

SN4741, a substantia nigra dopaminergic neuronal progenitor cell line, was used to examine the viability of cells on the hydrogel[3]. The sterilised peptide was dissolved in PBS and added to each well of a 96-well plate to completely cover the bottom of the well and the plate was incubated overnight. To extract residual TFA, the formed hydrogels were equilibrated with PBS for one hour and the PBS was then extracted from the top of the hydrogel. Hydrogels were equilibrated with culture media with and without serum for one day and the media was then transferred to another well. Three wells were prepared for each condition. The cells were trypsinized to give a cell suspension and approximately 1,000 cells were added on top of each hydrogel. Control experiments were performed by adding the same population of cells into three empty wells. To determine the possibility of cytotoxic components leaching out, the hydrogels were equilibrated in PBS or media, and the same population of cells cultured in the wells containing the equilibrated solutions. To determine cell viability, cells were stained with calcein AM (live cells) and ethidium homodimer (dead cells). Live and dead cells of each well were counted and the average ratio of live cells for each condition were compared to each other and TCPS as previously described[4, 5].

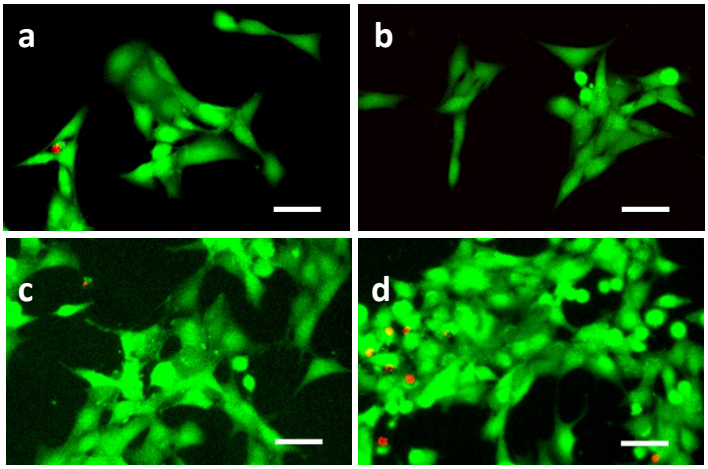

Fig. ESI-5. Higher magnification images for growth of SN4741 cells and live/dead cells assay on peptide hydrogels equilibrated with media containing serum for one day at two time points (1 day and 3 days cell culture) – Green cells represent live cells, stained with calcein AM. Red cells represent dead 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 50µm.

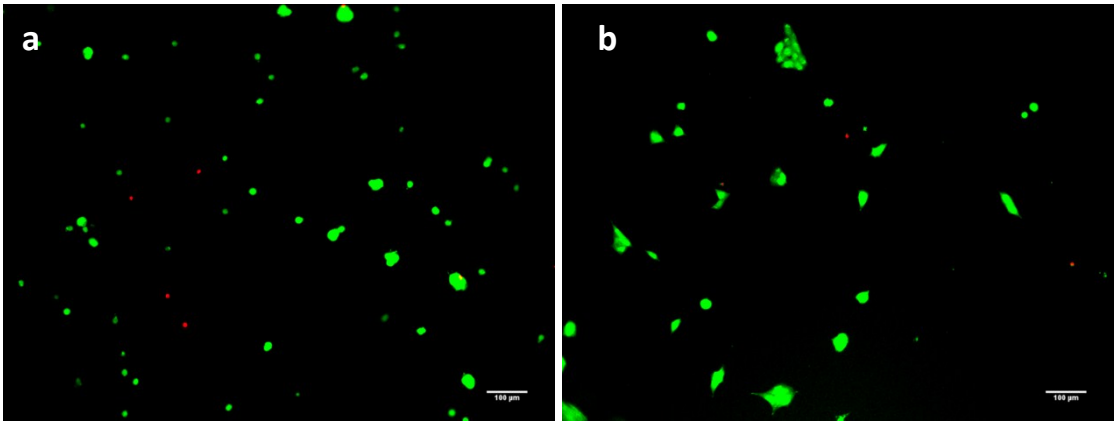

Fig. ESI-6. Growth of SN4741 cells and live/dead cells assays on peptide hydrogels using different equilibration conditions after 1 day cell culture – Green cells represent live cells, stained with calcein AM. Red cells represent dead cells, stained with ethidium homodimer. a) Peptide hydrogel, b) Peptide hydrogel equilibrated with culture media without serum for one day. Scale bar 100µm.

Statistical Analysis

The cell viability on top of the peptide hydrogels using different equilibration conditions was quantified by counting the number of live cells in each condition. Live cells per cm^2 were expressed as mean ± standard deviation in Fig. ESI-7. Equal variances in different groups were confirmed by Levene's Median Test. Then the groups were compared using one-way ANOVA with Turkey's post hoc testing (GraphPad Prism Version 6.01). $P<0.05$ was used to determine statistical significance.

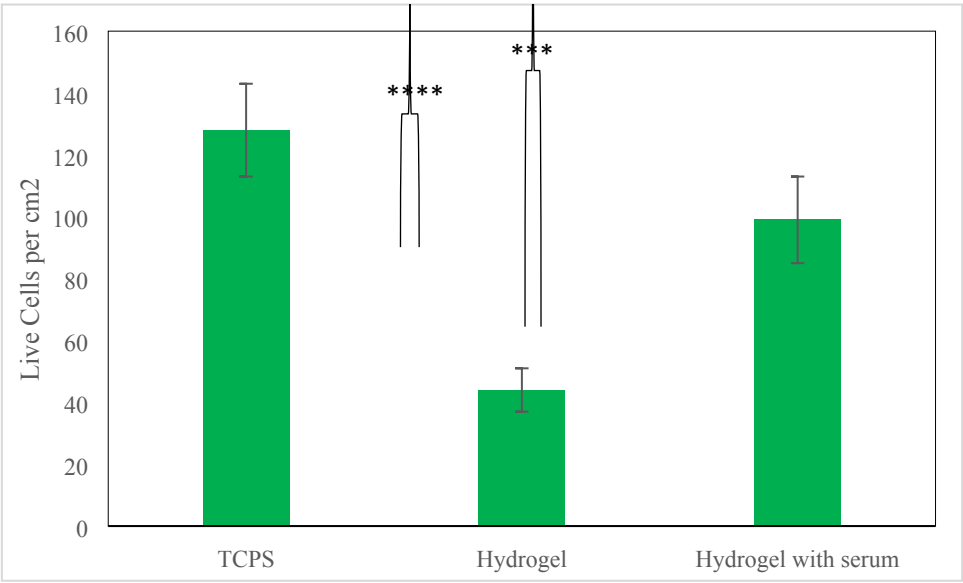

Fig. ESI-7. The number of live cells after 1 day culture on peptide hydrogel, peptide hydrogel equilibrated with culture media containing serum for 1 day and TCPS. (*** means P ≤ 0.001 and **** means P ≤ 0.0001)

Tyrosine hydroxylase (TH) Staining

To investigate whether the dopaminergic neuronal cells maintain their phenotype while seeding on top of the peptide hydrogel, a set of cell culture experiments was performed on top of the hydrogel equilibrated with media containing serum for 1 day, according to the method described on page 5 of the Supp. Info. Immunostaining with TH, as one of the most widely-used dopaminergic neuron markers[3, 6], was performed on cells after 1 day and 3 days and the staining on the hydrogel was compared with cells on TCPS as a control.

Cells were fixed with 4% paraformaldehyde for 10 min at room temperature and then rinsed with PBS for 3×5min. Cells were permeabilised in 0.3% Triton-X100 for 5 min and washed in PBS for 3×5min. The nonspecific antibody binding was blocked with 10% NGS (Normal goat Serum) including 1% BSA, 0.2% Tween20 in PBS for 1h at room temperature followed by PBS wash. Cells were then stained with mouse TH antibody (1:200) in 1% BSA in PBS at 4°C overnight. The samples were rinsed thoroughly with 0.2% Tween 20 in PBS on the following day and incubated with anti-mouse Alexa Flour 568 (1:1000) in 0.05% Tween 20 in PBS at 37°C for 1h. After thorough washing with 0.2% Tween 20 in PBS, the cells were counterstained with DAPI for 5 min and after thorough washing the cells were imaged using a fluorescent microscope. TH was expressed on the cytoplasm of cells (Fig. ESI-8). To ensure specific antibody binding, control samples were stained only with secondary antibody. There was no fluorescence response, showing that the staining procedure was specific. Fig. ESI-8 showed that the cells had similar phenotype on top of hydrogel and TCPS, confirming that live cells on top of our hydrogel kept their dopaminergic phenotype.

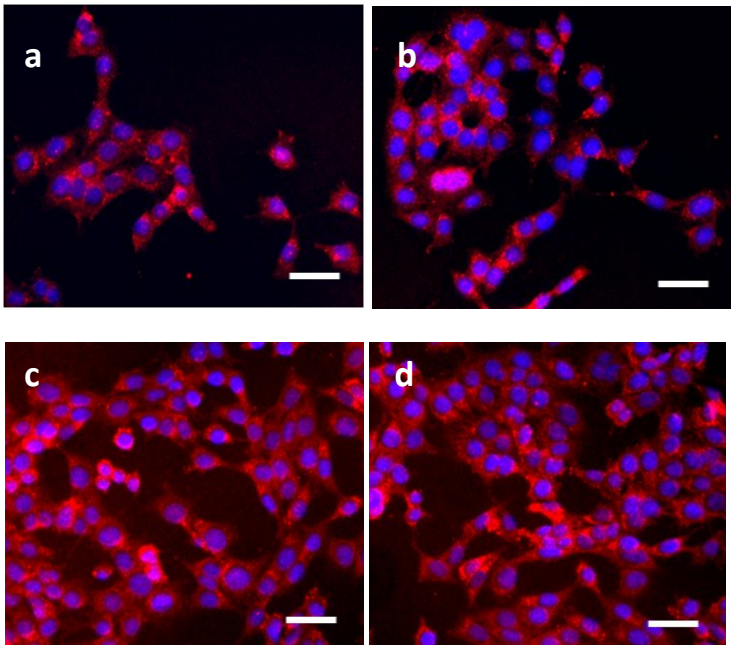

Fig. ESI-8. Representative images for the expression of TH by immunofluorescence (red) and DAPI counterstaining (blue) of SN4741 cells on peptide hydrogels equilibrated with media containing serum for one day at two time points (1 day and 3 days cell culture). a) Peptide hydrogel equilibrated with culture 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 50µm.

References

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