Biomaterials 32 (2011) 6099–6110

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Functional fibrils derived from the peptide TTR1-cycloRGDfK that target cell adhesion and spreading

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ARTICLE INFO

Article history:
Received 18 March 2011
Accepted 5 May 2011
Available online 1 June 2011

Keywords:
Self assembly
Bioactivity
RGD peptide
Nanotopography
Adhesion
Cell spreading

ABSTRACT

Peptide self-assembly offers a route for the production of fibrous nanomaterials with advanced bioactive properties that promote specific cell interactions. In this study the peptide TTR1-cycloRGDfK was designed to form amyloid-like fibrils that display the functional cyclic RGDfK pentapeptide ligand to target mammalian cell surface $\alpha_V\beta_3$ integrin receptors. The $TTR_{105-115}$ (or TTR1) sequence was used as the self-assembling domain. Once assembled, TTR1-cycloRGDfK fibrils display a characteristic cross-β core structure by X-ray fibre diffraction that was preserved following dehydration. Thin films of fibrils were characterised by infrared synchrotron mapping, scanning electron microscopy and atomic force microscopy. Cell adhesion and spreading were promoted on thin films of TTR1-cycloRGDfK fibrils via specific interactions with the cyclic RGDfK ligand. Low levels of non-specific interactions were also observed between cells and non-functionalised fibrils. TTR1-cycloRGDfK fibrils are an advance on bioactive fibrils previously designed to interact with a range of RGD binding integrins and our findings show that the assembly of amyloid-like fibrils based on the TTR1 sequence is robust and can be directed to form materials with specific properties.

1. Introduction

Amyloid fibrils have been described as a robust class of self-assembling protein fibres defined by a common cross-β core structure [1]. This structure imparts a number of properties that are advantageous for materials science including compressive strength [2] and resistance to conditions that typically destabilise globular proteins, such as high pressure or proteolytic digestion [3–5]. Historically these fibrillar assemblies have been known for their association with diseases involving protein conformation but many recent studies suggest the mature fibril is inert and that a diverse range of polypeptide sequences can adopt a cross-β fibrillar structure [6–10]. Amyloid-like fibrils with positive functions have been identified in nature [11–16], further supporting the idea that fibrils can be engineered for utility [17] and that these fibres represent a valuable addition to the repertoire for material development. Studies on model peptide and protein systems also indicate that the fibril cross-β core structure is stable after dehydration [18], although there is potential to further screen sequences for optimal stability under conditions such as repeated dehydration on a surface [19].

The self-assembly pathway that leads to amyloid-like fibrils provides the dual benefit of a robust scaffold with the possibility of functionalisation. A stretch of amino acids with high β-sheet propensity is used to drive fibril assembly into a cross-β core. This sequence may be combined with a sequence of low β-sheet propensity allowing functionalisation. Functional fibrils have been formed based on this principle using the TTR1 (also known as $TTR_{105-115}$; YTIAALLSPYS in single letter amino acid code) sequence and the functional sequence RGD (R is arginine, G is glycine, D is aspartic acid) [20] that promotes cell adhesion [21]. Functionalisation can be directed to the amino or carboxyl terminus or certain amino acid side chains. The degree of functionality can also be tuned by incorporating a greater or lesser concentration of the functionalised peptide with the base assembling peptide [10]. The molecular basis for fibril functionalisation is not limited to amino acid based molecules [22,23] and this diversity recommends these fibrils for a range of applications in materials science.

The RGD sequence has been used to functionalise a range of materials including those with a fibrous morphology, increasing bioactivity by targeting the integrin receptors that mediate cell

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doi:10.1016/j.biomaterials.2011.05.021

attachment [20,24–26]. The linear sequence RGD and similar peptides such as GRGDS, interacts with a broad range of integrin receptors with varied affinity [26,27]. Cyclic variants of the RGD sequence have also been designed to target specific integrin pairs. For example, the cyclic pentapeptide RGDfK (f is D-phenylalanine, K is lysine), designed by Kessler and co-workers, has a high selectivity and affinity to the $\alpha_V\beta_3$ cell surface integrin than the linear RGD sequence [28,29]. This cyclic RGDfK ligand has been successfully used to target cell attachment, spreading and the delivery of therapeutics [30–33]. The $\alpha_V\beta_3$ integrin receptor has also been the focus of studies seeking new therapeutics due to the involvement of this receptor in many cell-adhesion processes, such as bone remodelling [28]. The availability of cyclic peptide ligands suggests they could be used to functionalise fibrils allowing the fine tuning of cellular interactions.

In this study the peptide TTR1-cycloRGDfK was designed to produce bioactive fibrils that display the cyclic RGDfK ligand to target $\alpha_V\beta_3$ integrins, by covalently coupling the amyloid forming TTR1 peptide and the cyclic RGDfK peptide. These fibrils are an advance on previously described bioactive fibrils that were RGD functionalised with the linear GRGDS peptide [20]. Fibrils assembled from the TTR1-cycloRGDfK peptide are expected to show advanced bioactivity due to the display of the cyclic RGDfK ligand on the fibril surface.

2. Materials and methods

All chemicals were purchased from Sigma–Aldrich Inc. (Australia) unless specified.

2.1. Peptide synthesis

The peptide TTR1-cycloRGDfK was produced in house at the Bio21 Molecular Science and Biotechnology Institute (Australia). Amino acid sequences YTIAALLSPYSC-NH_2 and cyclised RGDfK were independently synthesised by standard Solid-Phase Peptide Synthesis chemistry using a Liberty microwave peptide synthesiser (CEM Corporation, North Carolina, USA).

The sequence YTIAALLSPYSC-NH_2 was assembled with standard 9-fluorenylmethyloxycarbonyl (Fmoc)-protected amino acid derivatives purchased from GL Biochem Ltd (Shanghai, China). It was then cleaved with trifluroacetic acid (TFA) and purified by High Performance Liquid Chromatography (HPLC) on a C18 reversed-phase column in a 0.1% TFA buffer system with a CH_3CN gradient of 1% per minute. Peptide fractions were pooled, lyophilised and shown to be >95% pure by analytical HPLC and mass spectrometry.

Synthesis of the cyclic RGDfK peptide with the linker molecule 3-maleimidopropionic acid attached to the side chain of the lysine required a complex synthetic strategy. The peptide was synthesised on the Liberty microwave peptide synthesiser on very-acid labile chlorotrityl resin with (1-(4,4-dimethyl-2,6-dioxocyclohex-L-ylidene)-3-methylbutyl) (ivDDE) protection on the lysine side chain. The peptide resin at the completion of synthesis possessed a free N-terminus and this was reprotected with the very-acid labile Trityl group to allow the selective removal of the ivDDE group from the lysine side chain and the attachment 3-maleimidepropanoic acid at this position. The pentapeptide was then cleaved from the resin with 2% TFA/dichloromethane leaving the arginine and aspartic acid side chain protecting groups intact. The protected linear pentapeptide was cyclised in solution with *O*-(7-Azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) purchased from GL Biochem Ltd. (Shanghai, China) and then deprotected with a solution of 95% TFA/2.5% triisopropylsilane/2.5% water. The cyclised peptide was purified using the same conditions used for YTIAALLSPYSC-NH_2. The cyclised RGDfK peptide without the linker molecule was also synthesised and purified as described for control experiments.

Equimolar amounts of purified linear YTIAALLSPYSC-NH_2 and cyclic [RGDfK(Mal)] (Mal is maleimidopropionate) were dissolved in ammonium acetate buffer (pH 6.5) and the reaction followed by mass spectrometry. Within 15 min the peptides had quantitatively coupled together to form a stable thioether linkage. The reaction solution was immediately purified preparatively to yield the desired product >95% purity.

TTR1-RGD, TTR1-RAD and TTR1 peptides were synthesised and purified to >95% by CS Bio Co. (California, USA) according to the sequences described in Gras et al. [20].

2.2. Fibril formation

Fibrils were assembled by resuspending each TTR1-based peptide separately at 10 mg/ml with 10% (v/v) CH_3CN in high purity Milli-Q water of resistivity 18 MΩcm (dH_2O), incubating this solution at 37 °C for 24 h and maturing the solution at room temperature for 28 days [20]. The conversion from free TTR1-cycloRGDfK peptide to fibrils was assessed at 28 days maturity by centrifuging samples at 313,000 g and 4 °C for 50 min in a Beckman XL-1 ultracentrifuge (Beckman Coulter, Inc., USA) to separate fibrils from free peptide. The amino acid concentration in the supernatant was determined by amino acid analysis using a ninhydrin-based detection technique.

Thioflavin T (ThT) fluorescence was measured for TTR1-cycloRGDfK fibrils or freshly dissolved peptide. Samples were prepared at a concentration of 2 mg/mL in 10 mM potassium phosphate and 150 mM NaCl, pH 7.0 containing 47 μM ThT. This preparation was incubated for 10 min before 200 μL of each sample was added to a black 96-well plate (Nalge Nunc International, NY, U.S.A.). The fluorescence intensity was measured using a FLUOstar OPTIMA Microplate Reader (BMG Labtech, NC, U.S.A.) with an excitation and emission wavelength of 480 nm and 440 nm respectively.

2.3. Biophysical characterisation of TTR1-cycloRGDfK fibrils

2.3.1. Transmission electron microscopy

Micrographs were acquired using a FEI Company Tecnai TF30 transmission electron microscope (FEI Company, Eindhoven, The Netherlands) operated at 200 kV and fitted with a Gatan US1000 2k × 2K CCD camera (Pleasenton, Ca, USA). Transmission electron microscopy (TEM) grids were made hydrophilic by glow-discharge under a reduced atmosphere for 10 s. Fibrils were diluted (1:50) with dH_2O and 3 μL was applied onto a carbon-coated, Formvar film layered 300 mesh copper grid (ProSciTech, Australia) and allowed to adsorb for 1 min. Grids were then rinsed twice with dH_2O, negatively stained with aqueous uranyl acetate (2% w/v) for 20 s and air dried. The TEM scale bar was calibrated with a metal standard. Fibril dimensions were measured using ImageJ software (NIH, Bethesda, MD, USA) and the pixel size was calibrated using the imprinted scale bar.

2.3.2. Fourier transform infrared spectroscopy

TTR1-cycloRGDfK fibrils were examined on the Infrared (IR) beamline at the Australian Synchrotron using transmission Fourier transform infrared (FTIR) spectroscopy. Spectra were collected with a Brucker Vertex V80 vacuum FTIR spectrometer and Hyperion 2000 IR microscope (Bruker Optics GmbH., Ettlingen, Germany) using the Bruker OPUS version 6.5 software. The microscope and sample were purged with dry air to minimise water vapour contributions in the spectra. All data was collected at ambient conditions with a spectral resolution of 4 cm^{-1} and 128 scans co-added.

FTIR data was collected for fibril samples matured at 40 mg/mL with 10% (v/v) anhydrous CH_3CN in D_2O (Cambridge Isotopes Inc., USA) and a 2 μL aliquot was placed between CaF_2 windows separated by a ~6 μm spacer and scanned. Background scans were obtained under identical conditions without fibrils then subtracted from the sample data. The maximum absorbance of each spectrum was between 0.1 and 1.0 absorbance units. Data was normalised to the maximum absorbance in the 1700–1600 cm^{-1} range (amide I).

2.3.3. X-ray fibre diffraction

Wide angle X-ray scattering (WAXS) and small angle X-ray scattering (SAXS) patterns were collected for a TTR1-cycloRGDfK fibril stalk and a hydrated fibril pellet on the Macromolecular Crystallography [34] and the SAXS/WAXS beamlines respectively at the Australian Synchrotron. WAXS patterns were acquired with a sample-to-detector distance of 300 mm, a wavelength of 0.95363 Å and sample exposure time of 15 s. SAXS patterns were acquired with a sample-to-detector distance of 3338.7 mm, a wavelength of 1.0332 Å and sample exposure time of 5 s.

Fibril stalks were prepared by air drying 10 μL of TTR1-cycloRGDfK fibrils between two wax-filled capillary ends, as described previously [35]. A hydrated fibril pellet was prepared using a centrifugal concentrator (Millipore Amicon Ultra 10 kDa cut-off) then placing 10 μL of concentrated fibrils into a thin-walled quartz capillary with an inner diameter of 0.3 mm (Hampton Research Co, USA). Fibrils were aligned by centrifuging the capillary at 500 g for 5 min and capillary WAXS patterns were acquired immediately. Background WAXS patterns were collected by filling an equivalent capillary with aqueous acetonitrile (10% v/v).

WAXS images were converted to tiff files using the program fit-2d (Hammersley/ESRF) and radially integrated to generate one dimensional scattering patterns using a Matlab coded integration tool [18] in ImageJ. The integration tool was calibrated with a WAXS pattern of high density polyethylene collected using identical conditions. Fibril WAXS patterns were radially integrated in both the equatorial and axial direction using a 30° sector in the azimuthal direction on either side of the reflection. Averaged one dimensional scattering patterns were scaled at 0.15 $Å^{-1}$ as described previously [18]. For the hydrated sample the background scattering profile was subtracted from the fibril profile. All radial intensity profiles were normalised to the maximum intensity value and plotted against reciprocal space (1/d $Å^{-1}$) and peaks were used to determine the position of reflections. The error in the peak position for each sample was calculated by the difference in position between two equatorial and two axial profiles. The error in the calibrant was determined by the same process and was multiplied by the error in the peak position as an estimate of the overall error. SAXS diffraction patterns were converted to a one dimensional