Acta Biomaterialia 38 (2016) 11–22

Contents lists available at ScienceDirect

Acta Biomaterialia

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

Full length article

Characterisation of minimalist co-assembled fluorenylmethyloxycarbonyl self-assembling peptide systems for presentation of multiple bioactive peptides

Conor C. Horgan [a,1], Alexandra L. Rodriguez [a,1], Rui Li [b], Kiara F. Bruggeman [a], Nicole Stupka [c], Jared K. Raynes [d], Li Day [d,e], John W. White [f], Richard J. Williams [g,2,*], David R. Nisbet [a,2,*]

[a] Research School of Engineering, The Australian National University, Canberra 2601, Australia
[b] Centre for Chemistry and Biotechnology, Deakin University, Waurn Ponds 3217, Australia
[c] School of Medicine, Deakin University, Waurn Ponds 3217, Australia
[d] CSIRO Food and Nutritional Sciences, Werribee 3030, Australia
[e] AgResearch Ltd, Grasslands Research Centre, Palmerston North 4442, New Zealand
[f] Research School of Chemistry, The Australian National University, Canberra 2601, Australia
[g] School of Aerospace, Mechanical and Manufacturing Engineering, RMIT University, Melbourne 3001, Australia

ARTICLE INFO

Article history:
Received 29 November 2015
Received in revised form 17 April 2016
Accepted 26 April 2016
Available online 27 April 2016

Keywords:
Self-assembling peptides
Hydrogels
Co-assembly
Fmoc
Tissue engineering

ABSTRACT

The nanofibrillar structures that underpin self-assembling peptide (SAP) hydrogels offer great potential for the development of finely tuned cellular microenvironments suitable for tissue engineering. However, biofunctionalisation without disruption of the assembly remains a key issue. SAPS present the peptide sequence within their structure, and studies to date have typically focused on including a single biological motif, resulting in chemically and biologically homogenous scaffolds. This limits the utility of these systems, as they cannot effectively mimic the complexity of the multicomponent extracellular matrix (ECM). In this work, we demonstrate the first successful co-assembly of two biologically active SAPs to form a coassembled scaffold of distinct two-component nanofibrils, and demonstrate that this approach is more bioactive than either of the individual systems alone. Here, we use two bioinspired SAPs from two key ECM proteins: Fmoc-FRGDF containing the RGD sequence from fibronectin and Fmoc-DIKVAV containing the IKVAV sequence from laminin. Our results demonstrate that these SAPs are able to co-assemble to form stable hybrid nanofibres containing dual epitopes. Comparison of the co-assembled SAP system to the individual SAP hydrogels and to a mixed system (composed of the two hydrogels mixed together post-assembly) demonstrates its superior stable, transparent, shear-thinning hydrogels at biological pH, ideal characteristics for tissue engineering applications. Importantly, we show that only the coassembled hydrogel is able to induce *in vitro* multinucleate myotube formation with C2C12 cells. This work illustrates the importance of tissue engineering scaffold functionalisation and the need to develop increasingly advanced multicomponent systems for effective ECM mimicry.

Statement of Significance

Successful control of stem cell fate in tissue engineering applications requires the use of sophisticated scaffolds that deliver biological signals to guide growth and differentiation. The complexity of such processes necessitates the presentation of multiple signals in order to effectively mimic the native extracellular matrix (ECM). Here, we establish the use of two biofunctional, minimalist self-assembling peptides

* Corresponding authors.
E-mail addresses: richard.williams@rmit.edu.au (R.J. Williams), david.nisbet@anu.edu.au (D.R. Nisbet).
[1] Equal first author.
[2] Equal contribution.

http://dx.doi.org/10.1016/j.actbio.2016.04.038

(SAPs) to construct the first co-assembled SAP scaffold. Our work characterises this construct, demonstrating that the physical, chemical, and biological properties of the peptides are maintained during the co-assembly process. Importantly, the coassembled system demonstrates superior biological performance relative to the individual SAPs, highlighting the importance of complex ECM mimicry. This work has important implications for future tissue engineering studies.

1. Introduction

The successful application of biomaterials to tissue engineering requires the fabrication of carefully controlled physical and chemical properties presented on biologically relevant scales to promote cellular regeneration. The most promising candidates for tissue engineering should provide a three-dimensional (3D) microenvironment with support for cells as well as biochemical cues to promote and regulate cell behaviour such as cell adhesion, migration, proliferation and differentiation [1–3]. These biomaterials must also exhibit little or no immune response, and be biocompatible and non-cytotoxic after degradation [1,4]. These requirements present significant challenges in the design of tissue engineering materials, and provide the design inspiration for materials that can be applied to cell regeneration and repair.

Nanostructured 3D matrix scaffolds are some of the most promising and important biomaterials that have been applied to tissue engineering [4,5]. Developing design rules for these materials allows them to be purpose-built to provide a suitable cellular microenvironment that mimics the native ECM [1,2,4,6]. A variety of scaffolds have been investigated, including electrospun nanofibres [7–9], biologically derived and synthetic hydrogel scaffolds [10–16], and self-assembling peptide (SAP) hydrogel scaffolds [4,17,18].

SAP hydrogel scaffolds are a novel class of biomimetic materials consisting of structure forming low molecular weight peptide sequences [19,20]. SAP hydrogel scaffolds have proven to be particularly promising due to their inherent biocompatibility, tuneable mechanical and chemical properties, ability to fill irregularly shaped voids, and their porous, nanofibrous structure, making them ideal candidates for applications in regenerative medicine [4,21,22].

Recently, the potential of minimalist SAP systems for biomedical applications has gained much interest, due to their short length and ability to present biochemical cues at high densities through careful control of the amino acid sequence, allowing functionality to be included without sacrificing structure [17,21,23]. Understanding the mechanisms underpinning the structural formation of one group of these systems, fluorenylmethyloxycarbonyl (Fmoc) SAPs, has enabled the introduction of such biochemical cues via spontaneous assembly [23,24]. Here, under appropriate conditions, the Fmoc groups interact through π-π stacking, forming the backbone of the Fmoc-SAP structure, and the pendant peptides align through hydrogen bonding to form a network of stabilising β-sheets (Fig. 1) [25–27]. These assemblies subsequently stack to form nanotubes with the peptide sequence exposed at high density within beta strands [23,26,28]. Individual nanotubes then align longitudinally, resulting in the spontaneous formation of nanofibre bundles decorated with the peptide sequence of interest constituting the hydrogel scaffold [21]. The minimalist nature of Fmoc-SAP systems is advantageous as they are relatively inexpensive and quick to produce [21,24]. In addition, the Fmoc moiety itself has been shown to possess anti-inflammatory properties, a valuable property for the development of adjuvant scaffolds in cellular applications [29]. While a recent *in vitro* study has indicated the cytotoxicity of degradation products from high concentrations of Fmoc-F and Fmoc-FF due to the poor stability of these systems [30], and the possible formation of aggregates and nanocrystalline structures [31,32]; these studies, and recent work by our group have highlighted the need for optimisation and functionalization, thereby making these systems suitable for attachment based cell culture *in vitro* [33], and neural stem cell transplantation and viral vector delivery *in vivo* [34].

Another promising advantage of Fmoc-based SAP systems over other, longer SAP systems is their heightened potential for biofunctionalisation. Previously, biofunctionalisation of non-Fmoc peptides (such as RADA16) and peptide amphiphiles (PA), achieved through capping with short bioactive sequences (epitopes), has shown some promise in eliciting an improved cellular response *in vitro* and *in vivo* [17,35–37]. The capping of PAs with IKVAV in particular has seen selective promotion of cell differentiation, neurite extension, and neural differentiation, as well as reduced development of astrocytes (an inflammatory cell within the central nervous system (CNS)), indicating the formation of an appropriate cellular microenvironment for tissue engineering, in this case within the CNS [17,38–40]. While these results are promising, the relative size of the bioactive epitope to the PAs results in an inherently low-density presentation of the epitope [21,40,41]. What is more, work on biofunctionalised PAs has shown that density, rather than dimensionality, of the biofunctional epitope presentation plays a more important role in cell differentiation and is likely to play a wider role in the biofunctionality of the introduced material [35,42].

Our previous research has focused on the high-density presentation of RGD and IKVAV epitopes (Fig. 2a & b) [21,43,44]. The laminin-derived IKVAV and the fibronectin-derived RGD sequences are common components of the mammalian ECM [21,42,45]. These Fmoc peptides have been rationally designed to form SAP hydrogels at physiological pH [21], allowing for control of various cellular processes including survival, adhesion, differentiation, migration and proliferation [4]. Incorporation of these sequences has enabled us to develop an Fmoc-SAP scaffold that more closely mimics the ECM, both physically and chemically. However, while biofunctionalisation of SAP scaffolds represents a significant advancement in the development of cell scaffold materials, cellular control is somewhat limited in this instance by the presentation of a single biochemical cue [42].

The combination of multiple signals in a single, homogenous scaffold offers the potential for a synergistic effect on cellular response through the improved spatial and chemical arrangement of multiple bioactive signals [46]. A range of co-assembled systems, mixed, and self-sorting have been reported [22,47–50], however, bioactive co-assembly has only previously been investigated in PA systems, with limited success [46], and in an Fmoc-SAP system where a non-gelating Fmoc-RGD molecule was used to decorate fibrils of Fmoc-FF [51]. In each case, the mechanism by which the SAPs molecularly pack into the fibres is not well understood and the design of such co-assembled systems continues to prove challenging [52].

Here, we present a first step towards the development of truly co-assembled minimalist bioactive Fmoc-SAP scaffolds for the high-density presentation of multiple biochemical cues, in this