Biomaterials 33 (2012) 6650–6659

Contents lists available at SciVerse ScienceDirect

Biomaterials

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

Cell adhesion and proliferation on RGD-modified recombinant spider silk proteins

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ARTICLE INFO

Article history:
Received 22 February 2012
Accepted 30 May 2012
Available online 22 June 2012

Keywords:
Silk
Genetic engineering
Chemical coupling
Cell adhesion
Cell proliferation
RGD peptide

ABSTRACT

Due to the biocompatibility and biodegradability as well as the mechanical properties of the fibers, spider silk has become an attractive material for researchers regarding biomedical applications. In this study, the engineered recombinant spider silk protein eADF4(C16) was modified with the integrin recognition sequence RGD by a genetic (fusing the amino acid sequence GRGDSPG) as well as a chemical approach (using the cyclic peptide c(RGDfK)). Both modified silk proteins were processed into films, and thereafter characterized concerning secondary structure, water contact angle and surface roughness. No influence of the RGD-modifications on any of these film properties could be detected. However, attachment and proliferation of BALB/3T3 mouse fibroblasts were significantly improved on films made of the RGD-modified silk proteins. Interestingly, the genetically created hybrid protein (with a linear RGD sequence) showed similar or slightly better cell adhesion properties as the silk protein chemically modified with the cyclic RGD peptide.

1. Introduction

Recently, our knowledge on wound healing has been greatly increased, mediating the development of new materials for wound coverage and tissue engineering. In tissue engineering materials have been used of natural origin including collagen, chitosan, silk, hyaluronan and fibrin as well as synthetic biodegradable polymers such as polyurethanes, polyesters (polyglycolide (PGA), polylactic acid (PLA)), polyethylene oxide (PEO) or polyvinyl alcohol [1].

For biomaterial applications the interaction of a material's surface with cells is of critical importance. It determines cell attachment, as well as the spreading behavior, proliferation and differentiation. The interaction strongly depends on the physico-chemical properties of the surface, such as its hydrophilicity, roughness, or presence of functional groups, its microstructure and mechanical properties [2]. However, the affinity of cells for certain surfaces differs depending on the cell type. Fibroblasts, for example, prefer surfaces with an intermediate wettability [3], whereas osteoblasts favor highly hydrophilic surfaces [4]. Several tools have been investigated to improve the interaction between cells and biomaterials. One way of promoting cell attachment is to change the morphology of the material, for example by introducing pores (as in foams or non-woven mats) or by employing patterned surfaces [5,6]. Another strategy is to chemically modify the surface, e.g. by plasma treatment, functionalization with chemical functional moieties like amine, carboxyl, hydroxyl or carbonyl groups, or by grafting of polymers onto the surface, for instance poly-acrylic acid or chitosan [7,8]. Furthermore, immobilization of biomolecules such as growth factors or cell adhesive peptides (e.g. containing the sequence RGD) has been shown to increase cell affinity [9–11]. RGD peptides originate from sequences of fibronectin and vitronectin, proteins of the extracellular matrix acting as integrin ligands. RGD peptides have been coupled to diverse biomaterials like hyaluronan and PEG-based hydrogels, titanium implants, polyurethane and PDMS surfaces [12–16]. Also various recombinant silk proteins have been genetically modified with RGD domains [17–20]. A wide variety of different RGD peptides has been employed, ranging from the minimal recognition sequence RGD to GRGDS, GRGDY or longer variants like RGDSPASSKP or Ac-CGGNGEPRGDYRAY-NH_2. Cyclic RGD peptides were identified to show even higher affinity and receptor selectivity for integrins than linear ones [21–24], and they can be designed to optimally address the integrin subtype of interest [25].

Spider silk reflects a promising material for biomedical applications, due to its biocompatibility, biodegradability and mechanical properties of the silk fibers [26]. Spider webs were already used

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0142-9612/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2012.05.069

by ancient Greeks to cover wounds and stop bleeding [27]. Although showing good properties, industrial use of spider silk is hampered by the cannibalistic behavior of spiders and therefore a limited availability. We, amongst others, established recombinant production of spider silk proteins in *Escherichia. coli* [28–31]. One of the recombinantly produced proteins is eADF4(C16) based on one of three major ampullate spidroins (MaSp) of the dragline silk of the European garden spider (*Araneus diadematus*). This recombinant silk protein consists of 16 repeats of a consensus (C-)module, mimicking the repetitive core domain of native ADF4 of *A. diadematus* (Fig. 1).

eADF4(C16) can be transformed into various morphologies such as films [32,33], particles [34,35], capsules [36], hydrogels [37] or non-woven mats [5]. Apart from organic solvents, eADF4(C16) can be processed from aqueous solutions under ambient conditions [38], which contributes to its suitability as a biomaterial.

Recently, we observed low adhesion and a lack of proliferation of BALB/3T3 mouse fibroblasts on eADF4(C16) films [5], which is not uncommon for silk protein matrices [39,40], reflecting a drawback for certain applications in tissue engineering. Therefore, the aim of this study was to improve cell adhesion and proliferation by creating a chemically or genetically modified variant of the protein containing the RGD sequence. While genetic approaches only allow the introduction of linear RGD domains, chemical functionalization enables coupling of cyclic RGD peptides. Here, we directly compared both approaches and their impact on cell binding and proliferation.

2. Materials and methods

2.1. Production of eADF4 (C16)

The recombinant spider silk protein eADF4(C16) is based on the consensus sequence of one of three spidroins of the dragline silk of the European garden spider (*A. diadematus*). The consensus motif (C-module) of ADF4 (GSSAAAAAAAASGPGGYGPENQGPSGPGGYGPGGP) is repeated 16 times in the recombinant protein (Fig. 1B). For detection, an N-terminal T7-tag is fused to the molecule. Production in *E. coli* and purification was performed as described by Huemmerich et al. [28]. Briefly, for the purification of eADF4(C16), cells were incubated in 50 mM Tris/HCl 100 mM NaCl buffer, pH 7.5 containing 0.2 mg/ml lysozyme at 4 °C for 30 min and lysed by ultrasonication. After centrifugation of cell fragments, soluble *E. coli* proteins were precipitated by heat denaturation at 80 °C for 20 min and removed by centrifugation. Silk proteins remained soluble and were salted out with 20% ammonium sulfate at room temperature (RT).

2.2. Genetic modification of eADF4 (C16)

DNA cassettes encoding RGD and a spacer sequence were created by annealing two synthetic oligo-nucleotides. For the RGD-tag GATCCATGGGCGGTCGTGGTG ACTCTCCGGGTTAATGAA and AGCTTTCATTAACCCGGAGAGTCACCACGACCGCCCATG and for the spacer sequence GATCCATGGGCGGTGGCTCTGGTTAATGAA and AGCTTT CATTAACCAGAGCCACCGCCCATG were used. The resulting amino acid sequence for the specific tag spRGD was GGSGGRGDSPG (Fig. 1B). The insertion of the DNA sequences into the cloning vector and the ligation with the gene encoding eADF4(C16) were accomplished by a seamless cloning strategy as described previously [28].

The gene encoding the RGE control silk protein was created by PCR-based site-directed mutagenesis using the plasmid *pET29-C16spRGD* as a template. The triplet GAC encoding Asp was changed to GAG encoding Glu by employing the forward primer GCTCTGGCGGTCGTGGTGAGTCTCCGGGTTAATG and the reverse primer GCTTTCATTAACCCGGAGACTCACCACGACC (the mutated base pair is underlined). 80 ng of vector DNA, 100 nM of each of the two primers, 200 μM of dNTPs, 1.25 U *Pfu*-DNA-Polymerase (Promega, Madison, Wisconsin, USA), 5 μl of 10× reaction buffer (200 mM Tris/HCl (pH 8.8), 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 1% Triton® X-100, 1 mg/ml BSA) were mixed in a total volume of 50 μl. PCR amplification parameters: incubation at 95 °C for 95 s, followed by 25 cycles at 95 °C for 45 s, 64 °C for 30 s, and 68 °C for 7.5 min. The PCR product was purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin, USA), and the template DNA was digested by *DpnI* at 37 °C for 60 min.

The DNA sequences of the genetically engineered *C16spRGD* and of *C16spRGE* were confirmed by sequencing. Protein production and purification procedures were identical to that of eADF4(C16).

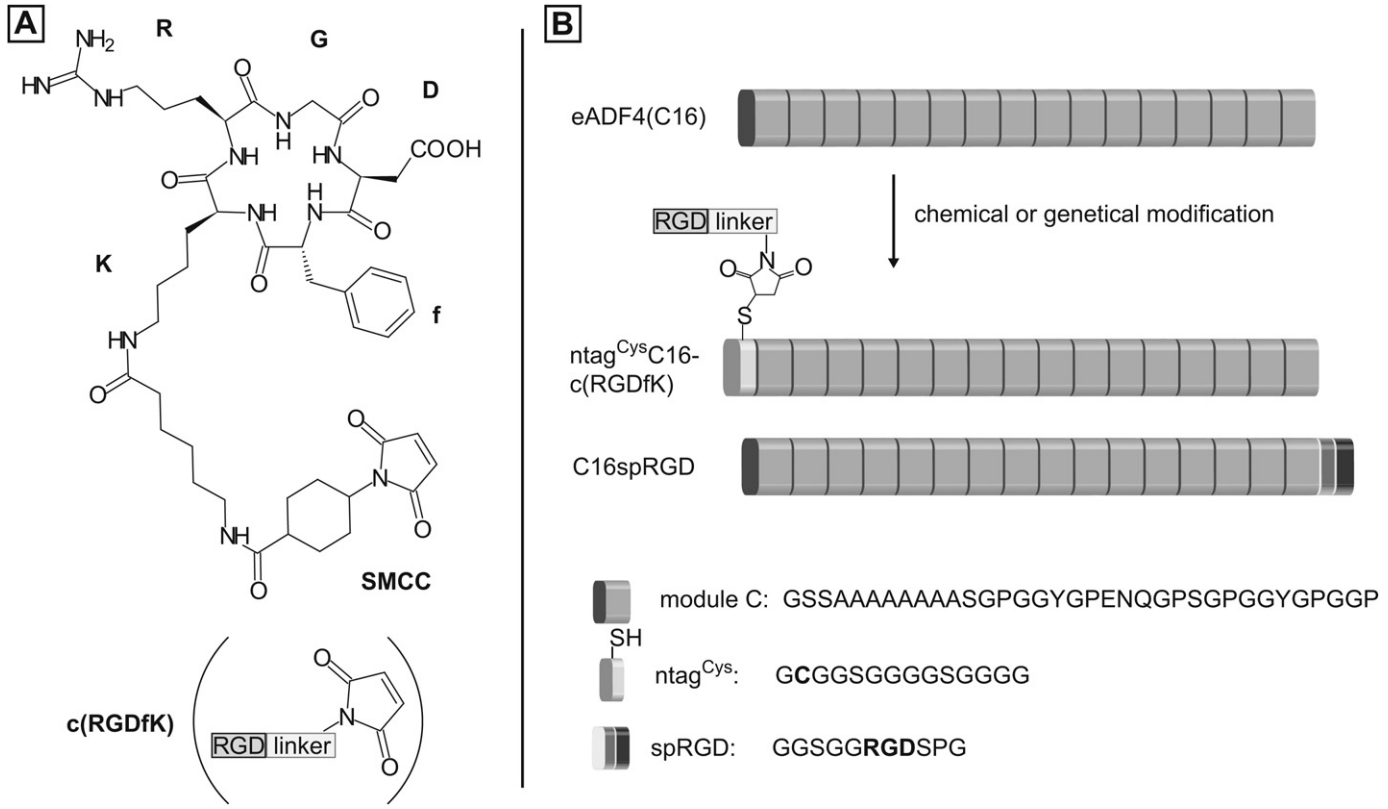

Fig. 1. A: Chemical structure of the synthetic cyclic RGD peptide c(RGDfK) employed for chemical modification of ntag^{Cys}C16. B: eADF4(C16) and the RGD-containing variants ntag^{Cys}C16-c(RGDfK) (chemically modified) and C16spRGD (genetically modified). For ntag^{Cys}C16-c(RGDfK), c(RGDfK) was covalently coupled to the thiol group of a cysteine residue of ntag^{Cys}C16 [33]. C16spRGD was modified by genetic engineering hybridizing a spacer and an RGD domain with eADF4(C16).