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Enhanced cell adhesion on silk fibroin via lectin surface modification

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ABSTRACT

Various tissue engineering (TE) approaches are based on silk fibroin (SF) as scaffold material because of its superior mechanical and biological properties compared to other materials. The translation of one-step TE approaches to clinical application has generally failed so far due to the requirement of a prolonged cell seeding step before implantation. Here, we propose that the plant lectin WGA (wheat germ agglutinin), covalently bound to SF, will mediate cell adhesion in a time frame acceptable to be part of a one-step surgical intervention. After the establishment of a modification protocol utilizing carbodiimide chemistry, we examined the attachment of cells, with a special focus on adipose-derived stromal cells (ASC), on WGA-SF compared to pure native SF. After a limited time frame of 20 min the attachment of ASCs to WGA-SF showed an increase of about 17-fold, as compared to pure native SF. The lectin-mediated cell adhesion further showed an enhanced resistance to trypsin (as a protease model) and to applied fluid shear stress (mechanical stability). Moreover, we could demonstrate that the adhesion of ASCs on the WGA-SF does not negatively influence proliferation or differentiation potential into the osteogenic lineage. To test for in vitro immune response, the proliferation of peripheral blood mononuclear cells in contact with the WGA-SF was determined, showing no alterations compared to plain SF. All these findings suggest that the WGA modification of SF offers important benefits for translation of SF scaffolds into clinical applications.

1. Introduction

Classical tissue engineering (TE) approaches such as matrix-assisted autologous chondrocyte transplantation are based on a two-stage process: in the first step, cells from a harvested tissue are isolated and cultured until an adequate cell number is attained; and in the second step, the cell-seeded biomaterial is implanted [1,2]. As a consequence, these approaches involve two surgical interventions: tissue harvest and implantation. To avoid disadvantages such as high economical costs and low patient comfort (two operations with anesthesia), an alternative would be a so-called one-step surgical procedure for tissue regeneration [3]. Recently, the feasibility of this one-step procedure has been proven [4,5]. In a one-step procedure cells are harvested and added to a biomaterial in the same operation in which they are implanted. Essentially required biomaterial properties for such a one-step procedure include prompt and robust cell adhesion to ensure an adequate initial cell seeding efficiency.

In general, the promotion of cell adhesion onto surfaces of biomaterials is of great interest for TE applications. Many attempts have been made to tailor material surfaces with bioactive molecules such as cell-binding peptide sequences including arginine–glycine–aspartic acid (RGD) or biomolecule-derived substances such as gelatin or fibronectin to improve cell adhesion [6,7]. The search for efficient strategies to improve cell–material adhesion is not restricted to the TE field but comprises a wide range of biopharmaceutical applications, e.g. the surface modification of polymeric drug delivery systems to promote the interaction with the intended treatment site [8]. In this context, the capability of carbohydrate-specific lectins to mediate site-directed targeting has been extensively investigated [9–11].

Lectins are generally defined as proteins or glycoproteins of non-immunological origin that bind carbohydrates [12], including

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glycosylated membrane components (glycoproteins and glycolipids) exposed at the surface of mammalian cells. In previous studies [13,14], the dose-dependent improvement of cell adhesion on plastic culture dishes pre-treated with diluted solutions of various lectins was shown. Besides coating of material surfaces solely by physical interactions, lectins have also been chemically coupled to various materials, without interfering with the carbohydrate-binding capability [11,15,16].

Here, we describe the functionalization of silk fibroin (SF) with wheat germ agglutinin (WGA), a plant lectin isolated from *Triticum vulgare*. This non-toxic, dietary glycoprotein shows binding affinity primarily for N-acetyl-D-glucosamine and sialic acid residues. Both sugar structures are ubiquitous in the glycocalyx of mammalian cells and therefore might provide versatile targets for adhesion improvements.

In the last decade, SF has attracted attention as a biomaterial in various TE applications due to its mechanical properties, processability in various forms and its good biocompatibility [17–19]. Dependent on the preparation process, two types of SF are distinguished in TE applications: regenerated SF and native SF cocoon fibres. In both cases, the inherent inflammatory-response-eliciting sericin must be extracted. This outer gum-like cover of raw silk fibres is typically removed by a so-called degumming process, most often via boiling in alkaline solutions. SF is a protein of more than 5000 amino acids mainly composed of repetitive motifs of glycine and alanine. Nevertheless, SF does contain a sufficient fraction of reactive amino acids such as aspartic and glutamic acid, tyrosine, serine and threonine, all accessible for chemical modifications [20]. As the modification of SF with other biomolecules via carbodiimide chemistry has shown excellent results in previous studies, this form was chosen in this experiment. For example, carbodiimide binding chemistry has been used to couple SF with bone morphogenetic protein 2 (BMP-2) to induce bone formation [21,22], and to RGD peptide to promote cell attachment [23,24]. Moreover, the carbodiimide-mediated coupling of WGA was shown to result in stable immobilization of the dimeric protein with at least half of the binding domains being freely accessible for cell-associated carbohydrates [25].

In the current study we focused on working with adipose derived stromal cells (ASCs) as this cell type can be obtained by minimally invasive surgical procedures in acceptable cell numbers allowing one-step procedures. Besides ASCs we also worked with primary anterior cruciate ligament fibroblasts (ACLFs) as we want to use these primary cells in future ligament TE studies of our group. In upcoming examinations we envision to use the herein described WGA-modified SF as raw material for ligament scaffolds.

We hypothesized that the coupling of WGA to SF would result in a robust and significantly higher cell adhesion compared to non-treated SF, facilitating the potential use of SF in one-step surgical procedures for tissue regeneration.

2. Materials and methods

If not indicated otherwise, all reagents were purchased from Sigma (Vienna, Austria) and were of analytical grade. Fluorescein-labelled and pristine WGA was obtained from Vector Laboratories (Burlingham, CA, USA). HEPES (2-(4-(2-hydroxyethyl)-1 piperazinyl)-ethane-sulfonic acid) and urea were bought from Merck (Darmstadt, Germany).

2.1. Silk fibres and films

White raw *Bombyx mori* silkworm fibres of 20/22 den, 250 T m^{-1}, were purchased from Testex AG (Zürich, Switzerland). Prior to experiments the fibres were extracted from silk sericin, as described previously, by cooking in Na_2CO_3 solutions [26]. Bundles of silk fibres have been used to study the modification of silk scaffolds with WGA.

To prepare silk films the degummed fibres were dissolved by boiling them in a ternary system consisting of calcium chloride, ethanol and ddH_2O in a molar ratio of 1:2:8 for 6 h. The solution was then filtered (0.22 μm, Rotilabo®, Roth (Karlsruhe, Germany) and dialysed against ddH_2O using a Slide-a-Lyzer® dialysis cassette form Pierce Biotechnology (Rockford, USA) with a molecular weight cutoff of 3.500 Da. The aqueous SF solution was lyophilized and the regenerated SF dissolved in hexafluoro-2-propanol to give a SF solution of 25 mg ml^{-1}. This solution was used to prepare silk films in 96-well microtitre plates according to Sofia et al. [21]. In detail, 24 μl of the 25 mg ml^{-1} silk solution was used to coat one well of the 96-well plate, resulting in 600 μg dry fibroin per well. Prior to surface modification, SF films were fixed with $MeOH/ddH_2O$ (9+1) to induce the irreversible rearrangement of β-sheet structures, and air-dried under atmospheric pressure.

2.2. Modification of SF films/fibres with glycine, bovine serum albumin and WGA via carbodiimide chemistry

Both films and fibres were hydrated in 20 mM HEPES/NaOH (pH 7.0) overnight (o/n) and thoroughly washed in the same buffer. Carboxyl groups were activated by immersing the films/fibres with a solution of 5.0 mg ml^{-1} 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 7.0 mg ml^{-1} *N*-hydroxysuccinimide (NHS) in 20 mM HEPES/NaOH (pH 7.0), and incubated for 2 h. After activation of the films/fibres, the supernatant was discarded and the films/fibres were washed with 20 mM HEPES/NaOH (pH 7.0). SF films/fibres were then incubated o/n with a solution of either glycine (0.1 mg ml^{-1}), BSA (0.015 mM) or WGA (0.015 mM) in HEPES/NaOH (pH 7.4) at 4 °C. In order to demonstrate the independency of enhanced cell adhesion due to the treatment of the SF films with the coupling reagents the glycine-modified SF was used as control group in the adhesion and proliferation experiments. We further included BSA-modified SF as control groups in the experiments in which possible cell behaviour changes (differentiation potential, in vitro immune response test) due to the modification process were investigated.

After rinsing to remove excessive ligands, BSA- and WGA-modified films/fibres were immersed in a 0.1 mg ml^{-1} solution of glycine in HEPES/NaOH pH 7.4 for 30 min to saturate unreacted coupling sites. Finally, all films/fibres were thoroughly rinsed with 6 M urea and 20 mM HEPES/NaOH (pH 7.4), prior to storage in 20 mM HEPES/NaOH (pH 7.4) at 4 °C for up to 2 weeks. Control samples reacting with the same amount of fluorescein-labelled lectin (fWGA) instead of WGA were included in each batch as a readily traceable ligand to check for homogeneity and reproducibility of the coupling procedure. Images of silk fibres were taken using a Zeiss Epifluorescence Axio Observer.Z1 deconvolution microscopy system (Carl Zeiss, Oberkochen, Germany) equipped with LD Plan-Neofluar objectives and the LED illumination system "Colibri®". fWGA was monitored at excitation/emission 485/525 nm, and overlays with differential interference contrast images were acquired to facilitate spatial orientation. Samples incubated via the same protocol but without prior activation with EDC/NHS served as a control to assess unspecific adhesion of fWGA to the silk protein. For all samples, imaging conditions were kept constant in order to allow for direct comparison.

2.3. Cells

2.3.1. NIH/3T3

The NIH/3T3 cell line was purchased from ECACC (European Collection of Cell Cultures, UK) and served as a control. NIH/3T3