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Stabilization of hyaluronan-based materials by peptide conjugation and its use as a cell-seeded scaffold in tissue engineering

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ABSTRACT

New materials based on molecules naturally occurred in body are assumed to be fully biocompatible and biodegradable. In our study, we used hyaluronic acid (HA) modified with peptides, which meet all this criterion and could be advantageously used in tissue engineering. Peptides with RGD, IKVAV or SIKVAV adhesive motif were attached to HA-based fiber or non-woven textile through ester bond in the term of solid phase peptide synthesis. A linker between HA and peptide containing three glycine or two 6-aminohexanoyl units was applied to make peptides more available for cell surface receptors. Dermal fibroblasts adhered readily on this material, preferentially to RGD peptide with 6-aminohexanoyl linker. Contrary, the absence of adhesive peptide did not allow the cell attachment but maintained the material stability.

1. Introduction

Development of new biomaterials for tissue engineering is still a big task, especially when we focus on its biodegradability and biocompatibility, stability, multifunctionality, ease of preparation and low cost. Hyaluronic acid (HA), a glycosaminoglycan abundantly expressed on the cell surface, and its derivatives has been recently studied as a new materials for wound dressings (Longinotti, 2014), drug delivery systems (Šmejkalová et al., 2017), bone (Solchaga et al., 2005) or cartilage augmentation (Bauer et al., 2016) and as an artificial skin or mucosa (Galassi et al., 2000). To use HA scaffold with intended application, there is always a need to use some type of modification, since hyaluronan is unstable in aqueous solutions and is biologically intact. To enhance, modulate or control the therapeutic action, various chemical modifications of HA have been described. The syntheses were performed in various solvents, by different approaches and with different target sites of hyaluronan (Schanté, Zuber, Herlin, & Vandamme, 2011).

Novel properties of various polysaccharides can be reached by conjugation with biologically active peptides, which has been previously described. These modified polysaccharides include alginate (Marks, Abu-Rabeah, & Ben-Hamo Fadlon, 2012; Shachar, Tsur-Gang, Dvir, Leor, & Cohen, 2011), carrageenan (Popa, Gomes, & Reis, 2011), chitin (Neugebauer, Williams, Barbier, Brzezinski, & Willick, 2009), cellulose (Eichler, Beyermann, & Bienert, 1989; Fraczyk, Walczak, & Kaminski, 2018; Hilpert, Winkler, & Hancock, 2007) or cotton (Edwards et al., 2018; Eichler, Bienert, Stierandova, & Lebl, 1991; Orlandin, Formaggio, Toffoletti, & Peggion, 2014). Peptides prepared and presented on some of these scaffolds added significant and valuable properties to the mentioned biopolymers, e.g. antimicrobial (Orlandin et al., 2014) and antibacterial (Mateescu et al., 2015) activity or improvement of cell adhesion (Ning, Xu, Chen, & Schreyer, 2016). Unfortunately, most of these polysaccharides are non-biodegradable or non-biocompatible solid phase carriers which had to be removed after synthesis and peptides had to be detached for the further use. For these reasons, biocompatible and biodegradable HA modified by peptide can be theoretically utilize directly without peptide detachment.

Peptide motifs are often employed for the stimulation of cell adhesion on tissue surfaces (Huettner, Dargaville, & Forget, 2018; Lam, Truong, & Segura, 2014). Up to now (Bernstein-Levi, Ochbaum, & Bitton, 2016; Kim & Kumar, 2014; Ouasti, Kingham, Terenghi, & Tirelli, 2012), HA was modified by peptides in solution – HA was dissolved and the modification was performed. This HA-peptide was then used for preparation of scaffold, but this method did not allow the full accessibility of the peptide on the surface in comparison with usage of solid

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phase peptide synthesis (SPPS), where the peptide is present on the outer part of fibers, nanofibers, non-woven textile, or films prepared from HA (Betak et al., 2014; Burgert, Hrdina, Masek, & Velebny, 2012; Foglarova et al., 2016; Foglarová et al., 2016; Scudlova et al., 2014; Zapotocky et al., 2017). This type of modification then allows the peptides to be recognized by cellular membrane receptors for adhesion.

Different peptides supporting cellular adhesion have been already studied in literature. The mostly described RGD peptide sequence is known to regulate cellular activities by interacting with $\alpha_5\beta_1$, $\alpha_v\beta_5$ and $\alpha_v\beta_3$ integrins, which also contributes to the wound healing processes (Muzzarelli, 2009). Other peptides as (S)IKVAV, which is derived from laminin A protein chain, promote different biological activities as cellular adhesion, migration, differentiation, and growth (Tashiro et al., 1989). Adsorption of peptides lead to limited cell attachment (Bellis, 2011) however peptides anchored to the polymer via linkers such as hexa-leucine tail on polyethylene terephthalate or polytetrafluoroethylene (Olivieri & Tweden, 1999) or poly-L-lysine on poly(lactic acid) (Quirk, Chan, Davies, Tendler, & Shakesheff, 2001) mediate stronger cell adsorption and provide an easy coating procedure. For this reason, the spacing between the peptide sequence and the anchoring moiety is crucial for cell attachment (Beer, Springer, & Coller, 1992).

In this paper, we describe the preparation and analysis of hyaluronan in the form of fibers and non-woven textiles as the carrier for covalent bonding of peptides to primary hydroxyl group of *N*-acetyl-D-glucosamine unit in terms of SPPS. Short peptides enabling adhesion of cells were chosen for this study. A linker between HA and peptide containing three glycine unites or two 6-aminohexanoyl units was applied to make peptide motifs more available for cell surface receptors. This construct was then directly employed for the study of certain chemical and physical properties as well as for cellular adhesion tests.

2. Materials and methods

2.1. Materials

Sodium hyaluronate and hyaluronan lyase from *Streptococccus pneumoniae* (SpHyl, 7500 IU. mL^{-1}) were obtained from Contipro a.s., (Czech Republic). Fmoc-6-Ahx-OH, Fmoc-L-Ala-OH, Fmoc-L-Arg(Pbf)-OH, Fmoc-L-Asp(*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-L-Ile-OH, Fmoc-L-Lys (Boc)-OH, Fmoc-L-Lys(Fmoc)-OH, Fmoc-L-Nle-OH, Fmoc-L-Ser(*t*Bu)-OH, Fmoc-L-Val-OH, trifluoroacetic acid (TFA), OxymaPure®, *N*,*N*´-diisopropylcarbodiimide (DIC), *N*,*N*-dimethylformamide (DMF) and Wang resin were purchased from Iris Biotech (Germany), while anisol and methyl fenyl sulfide (thioanisol) were obtained from Merck (Czech Republic) and dichloromethane (DCM), diethyl ether (DEE), acetic anhydride, pyridine, methanol were obtained from LachNer (Czech Republic). Commercial-grade solvents were used without further purification. Material for cell cultivation as Dulbecco's Modified Eagle Medium (DMEM) low glucose (1 $g.L^{-1}$), D-glucose, L-glutamine, trypsin-EDTA, penicillin-streptomycin solution and fetal bovine serum are commercially available products from Merck (Germany).

2.2. Fibers formation

The fibers were prepared by wet spinning according to our patented procedure (Betak et al., 2014; Burgert et al., 2012; Scudlova et al., 2014). The fiber-forming hyaluronan (Mw 600 kDa) was dissolved in demineralized water in concentration of 5% (*m/m*). The polymer solution was transferred into piston syringes and deaerated in a centrifugal field (1200 rpm, 20 min). The deaerated polymer solution was extruded (200 $L.min^{-1}$) using a Nexus 6000 automatic linear extruder (Chemyx) into the coagulation bath consisted of a mixture of propan-2-ol and lactic acid (volume ratio 80/20). The resulting fibers were washed with absolute ethanol and stabilized in acetone. Finally, the fibers were dried at room temperature. The obtained dry fibers had the linear mass density of 10 tex (10 $mg.m^{-1}$).

2.3. Non-woven textile fabrication

The non-woven textiles were prepared according to our patented procedure (Burgert et al., 2013). The fiber-forming hyaluronan (Mw 0.83 MDa) was dissolved in demineralized water in concentration of 1% (*m/m*) to obtain a homogenous, well flowing, viscous solution suitable for spinning. This solution was spun by the wet method into the spinning bath comprising propane-2-ol. The forming fiber was collected to the maturation bath consisting of propane-2-ol where the coagulation of the fiber was completed. Then fibers were poured into the Microtron-MB-800-B knife mixer and the fibers were shortened. The shortened fibers were filtrated off, the obtained filtration cake was compressed to the form of a non-woven textile and the product was dried at the approx. temperature 70 °C for 15 min.

2.4. Methods

2.4.1. Attachment of amino acid to HA carrier

The attachment of first amino acid (Fmoc-L-Nle-OH) to HA carrier was performed in a syringe reactor in DMF using DIC and OxymaPure® in 3 M excess (meq) of Fmoc amino acids (reaction conditions are given in Table 1). Couplings passed for 2 h. For elimination of the Fmoc group, 20% piperidine in DMF (5 and 20 min) was used. After each coupling, residual amino groups on the peptide were capped by acetic anhydride. The Fmoc-release test served as a measure of coupling yield.

2.4.2. Synthesis of lysine dendrimers on HA fiber

The first amino acid (Fmoc-L-Lys(Fmoc)-OH) was attached to HA fiber in DMF as solvent, using DIC and OxymaPure® in 3 M excess (meq). Coupling time was 2 h under laboratory temperature. The both Fmoc groups were removed by 20% piperidine in DMF (5 and 20 min) and subsequent residues of Fmoc-L-Lys(Fmoc)-OH were then attached to the growing peptide-HA conjugate to obtain branched dendrimeric peptides. After each coupling, residual amino groups on the growing peptide chains were capped by acetic anhydride. More detailed reaction conditions are given in Table 1. All reactions were carried out in a syringe reactor. The Fmoc-release test was used to measure coupling yield.

Table 1
Protocol for attachment of amino acid or protected peptide on HA carrier.

Step	Process	Volume	×	No	Time
1.	Washing				
	DMF	5 mL	×	3	5 min
2.	Coupling				
	3 meq of amino acid/peptide	5 mL			overnight
	3 meq of OxymaPure®				
	3 meq of DIC				
	0.3 meq of DMAP[a]				
3.	Washing				
	DMF	5 mL	×	5	10 min
	DCM	5 mL	×	3	5 min
	IPA	5 mL	×	3	5 min
	DMF	5 mL	×	5	10 min
4.	Deprotection				
	20% piperidine in DMF	5 mL	×	2	5 + 20 min
5.	Washing				
	DMF	5 mL	×	5	10 min
	DCM	5 mL	×	3	5 min
	IPA	5 mL	×	3	5 min
	DMF	5 mL	×	5	10 min
6.	Repetition of steps 2 to 5 for next amino acid				

[a] for 1st coupling only.