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The collaggrecan: Synthesis and visualization of an artificial proteoglycan

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ABSTRACT

An artificial aggrecan-like proteoglycan has been designed and synthesized in vitro. At variance with natural proteoglycans, whose glycosaminoglycan chains are always *O*-linked via a tetrasaccharide bridge to the serine residues of a specific protein core, the present structure consists of chondroitin-6-sulfate chains directly bound to the lysine and hydroxylysine residues of a collagen molecule backbone. The resulting macromolecule has been characterized by histochemistry, atomic force microscopy and FTIR. The number of variables involved (e.g., length and type of the collagen backbone, glycosaminoglycan species, sulfation type and pattern, molecular weight, number and length of side chains, etc.) makes possible to conceive an almost endless variety of artificial proteoglycans, each precisely tailored to a specific functional role. In addition to their use as biomaterials, glycated collagens interact with cells in complex ways and a previous study has already shown the ability of a glycated collagen to redirect fibroblastoma cells from proliferation to differentiation. The research is still underway.

1. Introduction

Of the many different macromolecules that make up the extracellular matrix, collagen and proteoglycans are really ubiquitous and they are present, in different forms and amounts, in each and every connective tissue. Proteoglycans have a long evolutionary history and can be found in all members of the Bilateria [1,2], so they have been around for more than half a billion years.

From a chemical standpoint a proteoglycan is simply defined as a protein core covalently linked to one or more heavyweight polysaccharide chains. This broad definition encompasses extremely different molecules, either intra-, peri- or extracellular, and a comprehensive classification is only slowly emerging [3,4].

In particular the collagen-bound small proteoglycans of the extracellular matrix have been the subject of active research because of their multiple role as modulators and organizers of the matrix architecture [5–8], while relatively less attention has been paid to the large proteoglycans such as aggrecan and hyaluronic acid (this latter not really a proteoglycan, but similar in physiological role and in distribution), which mostly belong to cartilages and to highly hydrated extracellular spaces such as the joints of the locomotory apparatus. These are, however, structures of some interest because of the high morbidity of arthritic phenomena in an aging population [9] and the difficulty, if not impossibility, to restore ailing joints to the original functionality.

While natural hyaluronic acid can be easily obtained in industrial amounts and is already widely used in the clinical practice as well as in the cosmetic industry, aggrecan is rare and expensive; in addition, neither is free from concerns about toxicity, antigenicity, pirogenicity, and prion or virus contamination, not unlike other biomaterials of biological origin [10,11]. The availability of engineered, custom-designed molecules with different glycosaminoglycan side chains and different functional behavior seems promising. It must be noted that because of the many parameters involved (e.g., glycosaminoglycan species, sulfation type, sulfation pattern, molecular weight, number of side chains, concentration, etc.) it is possible to conceive an almost endless variety of aggrecan-like molecules, and to obtain types precisely tailored to each specific functional demand.

In the present research a large neoproteoglycan made of chondroitinsulfate chains covalently linked to a collagen backbone was synthesized in vitro as a first attempt in that direction. The resulting macromolecule was tentatively named *collaggrecan*. In addition, a

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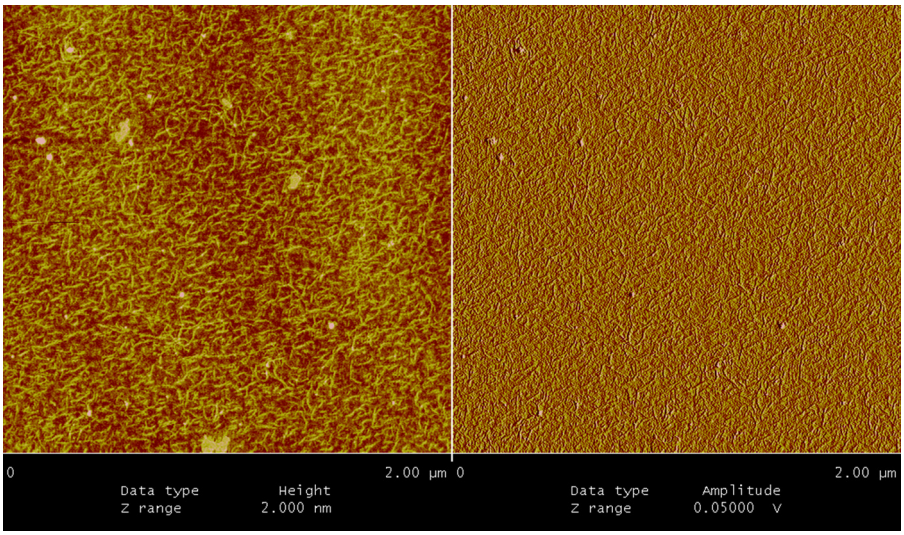

Fig. 1. Atomic force micrograph of collaggrecan molecules adsorbed to mica. The molecules form an uniform monolayer on the substrate.

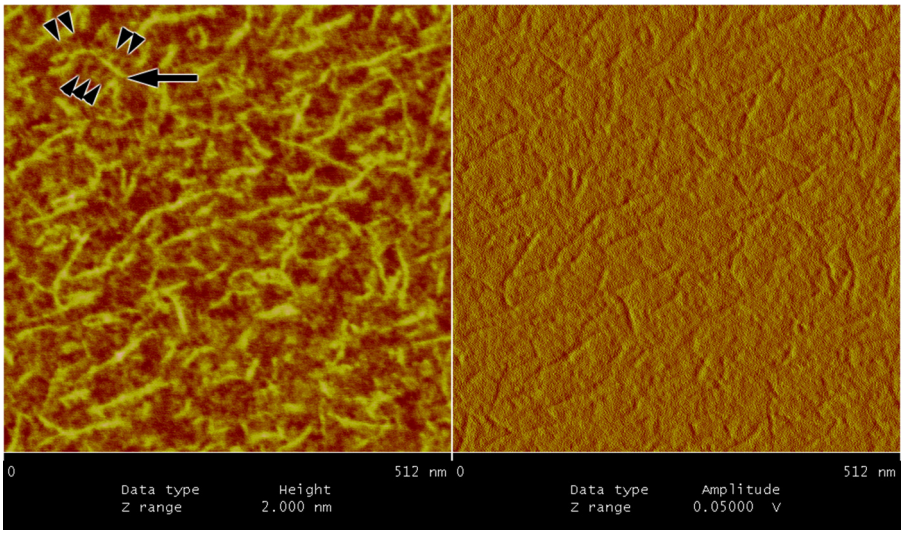

Fig. 2. A higher magnification of the same specimen reveals the single molecules. A collagen molecule (indicated by the arrow) is covalently bound to several, barely visible chondroitinsulfate side chains (arrowheads) approximately orthogonal to the collagen axis.

pre-assembled collagen film was functionalized the same way in order to obtain an insoluble collaggrecan membrane.

2. Materials and methods

2.1. Synthesis of soluble collaggrecan

100 mg of soluble type I bovine collagen (Kensey Nash, from bovine) was dissolved in 10 mL of 0.16 mM chondroitin-6-sulfate from shark cartilage (CAS number 12678-07-8) aqueous solution followed by the sequential addition of 0.08 mM $NaBH_3CN$ in citrate buffer (pH 6.00) and reacted for 24 h at room temperature. The reaction mixture was purified with Vivaspin 20 centrifugal concentrators (MWCO 100,000 Da), 4 washings for 15 min at 3000 rpm (i.e., until no unreacted chondroitin-6-sulfate was detectable by FT-IR), and lyophilized.

2.2. Synthesis of insoluble collaggrecan membrane

Type I collagen films from bovine Achilles tendon (Sigma–Aldrich, catalog no. C9879) were produced by a solvent-casting method as previously described [12]. Briefly, the collagen was dissolved in acetic acid 0.5 M for 4 h at 40 °C. The suspension was homogenized with a mixer for 2 min at maximum speed. 40 mL of collagen solution was poured into an $8.5 \times 12.5\,cm^2$ culture multiwell lid and the solvent evaporated in the fume hood for 3 days. The collagen matrices were produced as thin transparent films ($1\,mg/cm^2$).

A piece of collagen matrix (1 mg) was immersed in 2 mL of 1.5 mM chondroitin-6-sulfate solution followed by the addition of 0.75 mM $NaBH_3CN$ in citrate buffer (pH 6.00) and reacted overnight at room temperature. The collagen matrix was then washed with 2 mL of HCl 0.1 M for 10 min, 2 mL of NaOH 0.1 M for 10 min, 2 mL of milliQ water three times for 20 min, and finally with 2 mL of ethanol for 10 min.

2.3. Visualization

A freshly cleaved mica was treated for 30 min with 50 µL of a 1 N solution of $MgCl_2$ and thoroughly washed with milliQ water. 50 µL of a collaggrecan solution in milliQ water (approximately 1 µg/mL) were then deposited onto the pretreated mica and allowed to