

Notes on the mechanism of low-temperature laser tissue welding

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1 - Introduction

In this report we propose an hypothesis on the mechanism of low-temperature laser welding of a model connective tissue (cornea), based on the reorganization of the proteoglycans of the extracellular matrix.

2 - Low-temperature laser tissue welding

Low-temperature laser tissue welding is a technique used to provide immediate sealing of wounds: the cut is stained with a water solution of Indocyanine Green followed by irradiation with a near-infrared laser operated at low-power densities, which induces a localized heating of the tissue [1]. Ultrastructure analysis of corneal samples closed with the low-temperature laser welding technique showed intact collagen fibrils interwoven across the cut, indicating that temperature rise inside the stroma due to laser irradiation did not exceed the denaturation temperature of fibrillar collagen (i.e. about 65° C) [2]. This feature is in agreement with previous studies dealing with the experimental and theoretical description of the temperature dynamics during laser corneal welding [3]. The results indicated a typical operating temperature of 55-65° C, which is actually below the threshold value at which strong modifications to the collagen matrix are expected. Therefore, we may deduce that the mechanism of low-temperature laser welding is related to some structural modifications of the nonfibrillar components of the extracellular matrix (ECM).

3 - Proteoglycans

In connective tissues, collagen fibrils (fibrillar component of the ECM) and interfibrillar proteoglycans (PGs), which are the main nonfibrillar component of the ECM, form a dense and well-organized mutually interconnecting network. The interfibrillar PGs usually have a globular protein part (head) to which one glycosaminoglycan (GAG) is attached (tail) [4]. The interfibrillar PGs constitute a well-defined, complex molecular chain system providing rigid bridges between the fibrils and thereby being responsible for the maintenance of the regular array of collagen fibrils [4]. PGs are equidistant and orthogonally attached at specific sites of the collagen fibrils by their protein cores. The interaction of the PGs with collagen fibrils is thought to be noncovalent and is characterized by an affinity constant of $\sim 10^8$ M⁻¹ [5]. By using a specific proteoglycan staining for electron microscopy, GAGs have been shown to form antiparallel doublets that make it possible to maintain the relative position among the adjacent collagen fibrils [1,6]. It was proposed that 2 GAG chains, 1 from each PG, form duplexes, covering the space between fibrils, anchored by protein cores attached to each fibril [6] (this is called “the Scott’s model”).

4 - Hyaluronan and its aggregating properties

In a previous study [7] we carried out a multifaceted analysis which enabled us to bring to light the ability of the model GAG hyaluronan in physiological solution to generate stable superstructures. Because of its greater availability, HA is the archetypal GAG from which the structural properties of the other GAGs can be deduced in first approximation. While spectroscopic measurements of the less concentrated HA samples (< 50 mg/ml) suggested the presence of a temporary polymer network (in which the overlapping of individual HA domains does not lead to stable interactions), by increasing the concentration, the crowded local environment was supposed to promote the association of HA chains one to another. Spectroscopic, rheological and calorimetric analyses of highly concentrated HA solutions (100 mg/ml) revealed the presence of stable polymer networks, which dissolved reversibly upon heating above 60° C. Thus, we hypothesized a switching from temporary to stable HA networks as a function of the concentration. These latter networks are heat-labile, which is in accordance with the formation of junction zones mediated by weak interactions.

In physiological compartments the presence of a high number of macromolecules makes the space highly crowded. In practice this leads to the enhancement of the “effective” concentration of the individual macromolecules [8]. The intermolecular associations of highly concentrated HA

solutions can be viewed as a strategy of the system to reduce its free energy by maximizing the available volume (and minimizing the excluded volume), as normally observed in living systems. These observations underline the importance of taking into account the “effective” macromolecular concentration when dealing with the structuring of HA and of other GAGs in physiological conditions.

5 - Involvement of proteoglycans in the mechanism of laser tissue welding

Similar concepts can be taken into consideration for the interfibrillar PGs of corneal stroma, which is considered a model of soft-fibrous connective tissue. This biological environment is highly crowded by macromolecular species, mainly collagen fibrils and PGs themselves, as nicely depicted by the following scheme recently proposed by Müller and co-workers [9] (Fig. 1).

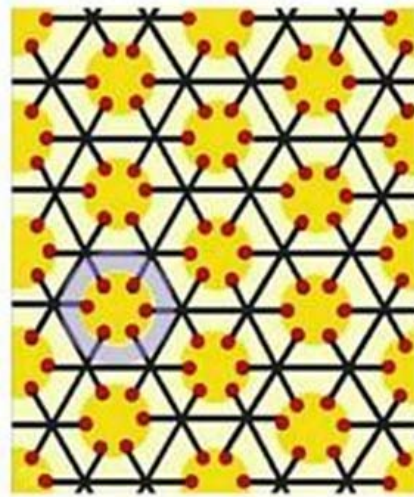


Fig. 1 - Schematic visualization of stromal organization as proposed by Müller [9]. Hexagonal arranged collagen fibrils are interconnected at regular distances with their next nearest neighbours by groups of six PGs, attached orthogonally to the circumference of the fibrils. In this way a regular meshwork of ring-like (intermediate between hexagonal and circular) 11 nm thick structures (bluish halo) enwrapping the collagen fibrils is formed.

In this scheme, at equidistant sites along their circumferences, six core proteins of PGs are attached to the hexagonal arranged collagen fibrils. The GAG chains of PGs join next nearest neighbour collagen fibrils and form a ring-like structure around each collagen fibril of approximately 11 nm in thickness (i.e. a gel-like shell surrounding individual collagen fibrils). The paper from Müller et al. [9] also furnishes quantitative parameters of PGs and collagen fibrils in the stromal cornea, achieved by electron microscopy analysis. These are reported in Tab. 1 and used for the following calculations:

Tab. 1 - Quantitative parameters of proteoglycans and collagen fibrils in the human cornea ([9]).

Diameter of ring-like structures	45.0 ± 4.2 nm	(~45 nm)
Thickness of PGs	10.5 ± 1.7 nm	(~11 nm)
Length of PGs	53.9 ± 5.4 nm	(~54 nm)
Diameter of collagen fibrils	22.7 ± 1.8 nm	(~23 nm)
Distance between adjacent collagen fibrils		(~43 nm)

Now, we can tentatively try to calculate the “effective” concentration of the PG component within the corneal stroma taking into account of the space occupied (and thus subtracted) by the main extracellular stromal macromolecules (fibrillar collagen and PGs themselves).

Corneal stroma is composed by 78% of water, 15% of collagen, 1% of PGs, and 5% of other components (% in weight/weight). Considering the density of corneal stroma equal to 1 g/ml (the exact value is 1.062 g/ml [10]), we can estimate a concentration of stromal PGs of about 10 mg/ml (that we call “apparent concentration”).

Let’s consider a volume inside the stroma of 54 nm (in length) \times 43 nm (in width) \times 100 nm (in height) = (232103) nm³, that we call the “apparent volume”, where the length is equal to that of each individual PG (along the X-axis), the width is the interfibrillar distance (along the Y-axis) and the height is arbitrarily chosen (along the Z-axis) (see the scheme of Fig. 2).

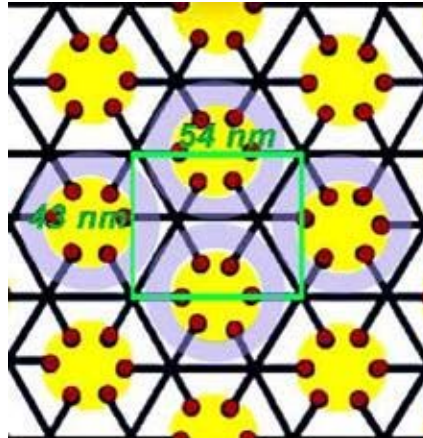


Fig. 2 - Calculation of “effective” stromal PG concentration. We consider a (54 \times 43 \times 100) nm³ volume (the green square defines the (54 \times 43) nm² area laying on the XY plane) of corneal stroma. Excluded areas are represented by ring-like structures (bluish halos) surrounding each fibril

Inside the considered area of (54 \times 43) nm² on the XY plane, two half ring-like structures (two semicircles of 795.2 nm² area) plus two other fractions of ring-like structures (two circular segments of 236.5 nm² area) are present. These correspond to a total occupied volume of [(795.2 \times 236.5) \times 2] \times 100 = (2106) nm³, which represents the total “excluded volume”. Thus, the “effective volume” is (2103) nm³, which represents about 10% of the “apparent volume”. We can thus estimate that the “effective PGs concentration” in corneal stroma is 10 times higher than the “apparent concentration”, that is \sim 100 mg/ml. This is in accordance with the concentration value at which the model GAG hyaluronan has been proved to aggregate in physiological solution. We may note that this is a very rough estimation which, among others, does not take into account the electrostatic repulsion between neighbour PGs and the interaction of each core PG protein with the collagen fibril or other extracellular components, which both can alter the aggregation process.

We can thus infer that the Scott’s model [1,6], which provides the presence of PG doublets connecting two adjacent fibrils (i.e. aggregates formed by the interaction of two GAG chains), is reasonable for the corneal stroma in physiological conditions. It is worth noting that this is true if we extend the results found for the archetypal GAG (hyaluronan) studied in crowded in vitro environments to the corneal GAGs in vivo. Thus, we ought to point out that this conclusion can be intrinsically subjected to some fault.

From the same extension (of the HA behaviour to that of the other GAGs), we can conclude that, by heating aggregated corneal PGs beyond the temperature threshold of \sim 60 $^{\circ}$ C, a reversible disaggregation of the GAG chains should occur.

Another expected effect of the temperature rise is the denaturation of the protein core of the PGs, which could lead to suspect a possible detachment of the PGs from the fibrils during heating. Thermal denaturation of the PG core was previously investigated by P.G. Scott [11]. He proved that thermal denaturation of interfibrillar PGs, in particular of decorin, actually occurs at a low temperature (i.e. at around 45 $^{\circ}$ C), but this does not affect the linkage between the protein core of the PGs and the collagen fibril. The only addition of a chemical denaturant was associated with the breakage of the PG-collagen interaction.

Hence, we can tentatively hypothesize a model of the corneal welding process taking place at the typical low powers of diode laser irradiation. The GAGs bridges connecting collagen fibrils in native stroma are probably broken at the characteristic temperatures of diode laser welding (in the

55-65 °C range), as suggested by our results. The individual GAG strands, freed upon heating, could successively create new bonds with other free strands during the cooling phase. In practice, a number of disaggregated PGs of one side of the wound could interact with other PGs of the other side generating new bonds, which are probably of noncovalent nature. The weld is held by groups of interwoven fibrils joining the sides of the cut as shown by microscopy data. The interwoven fibrils are supposed to be connected by several newly-formed GAG bridges. A scheme of the proposed mechanism of laser welding is reported in Fig. 3.

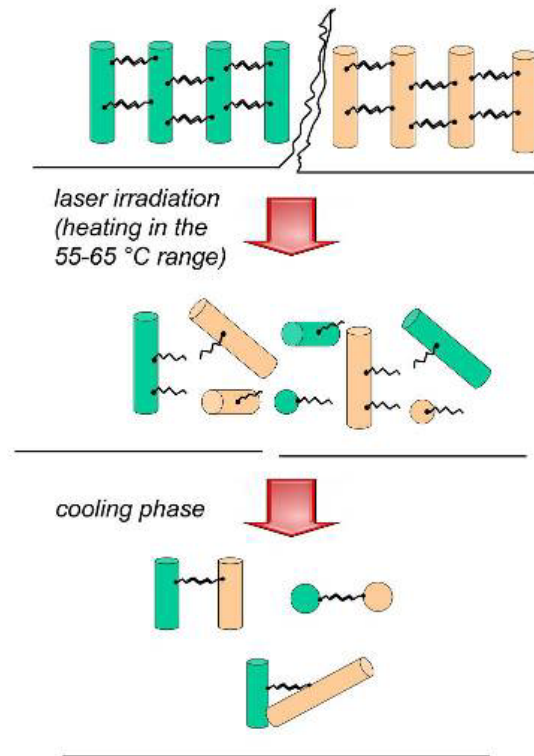


Fig. 3 - Proposed scheme of low-temperature laser welding of corneal stroma. During the laser irradiation a local heating in the 55-65 °C range is induced. This leads to the disruption of GAGs aggregates (doublets) of the two side of the wound to be welded and thus to a fibrillar rearrangement. During the cooling phase, GAG strands freed upon heating may interact with each other generating new bonds between the fibrils at the two sides of the cut and thus originating the weld.

6 - Perspectives

Further investigations on the mechanism of low-temperature laser welding are needed to prove our hypothesis. These could follow a twofold approach. From one side, it is necessary to carry out a more detailed analysis in order to prove the feasibility of self-assembly, and mainly of duplexing, of the interfibrillar PGs. This could be achieved mainly by means of calorimetric and spectroscopic methods, as we employed for the hyaluronan molecule. However, this investigation may bring some concerns. The scarce availability of commercial PGs necessarily requires their extraction directly from the tissue. Extraction protocols of interfibrillar PGs were previously proposed, even if they are troublesome in obtaining a highly-purified product on the microgram scale which is needed to prove the aggregating tendency of PGs. A more feasible strategy is to extract only the GAG part of PGs, which is obtained following simpler protocols. Furthermore, more convincing data are expected in this case due to the lack of the protein part (PG core) that could strongly affect the results.

On the other side, a non-traditional electron histochemistry approach could be tried, based on staining protocols specifically used for the PGs detection. This, in principle, could lead to a description of the final based on these non-traditional electron histochemistry methods could fail in the case of our welded samples because of the wide usage of highly-reactive reagents employed during the fixation and the resin embedding process. These could strongly affect the local tissue architecture, thus compromising the detection of the exact structuring of the PGs at the weld site.

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