Hydrodynamic study of the behavior of chondroitin sulphate under nondestructive laser irradiation of cartilage

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ABSTRACT

The effects of laser irradiation on molecular mass and conformation of pure chondroitin sulphate dissolved in phosphate buffered saline were investigated using size exclusion chromatography/multi-angle light scattering (SEC/MALS) and sedimentation velocity in the analytical ultracentrifuge. In addition, cartilage pieces immersed in buffer were irradiated with a laser in order to study whether cartilage components may diffuse away from the matrix and into the surrounding aqueous medium as a result of laser treatment. Size exclusion chromatography/multi-angle light scattering and sedimentation velocity measurements showed that (a) laser irradiation decreases the molecular mass of chondroitin sulphate and (b) laser irradiation of cartilage induces diffusion of macromolecules into the medium. The results obtained allow us to understand the mechanism of stress relaxation and structural alterations in cartilage under non-destructive laser radiation.

Keywords: Laser, cartilage, molecular mass determination, sedimentation coefficient.

1. INTRODUCTION

The extracellular matrix in cartilage accounts for more than 90% of the volume of the tissue. It is composed of a dense network of fine collagen fibres which are embedded in a highly concentrated solution of aggregates in which proteoglycan molecules are attached to hyaluronic acid chains. The proteoglycans bind by a specific site at one end of the protein backbone which has a high affinity for a decasaccharide unit of hyaluronate[1]. In addition, link-protein molecules form an integral part of the aggregate structure and are supposed to increase the stability of the aggregate structure by bridging the proteoglycan molecule and the hyaluronate.

Stress relaxation and reshaping of cartilaginous tissue under non-destructive laser radiation is one of the novel applications of lasers in medicine[2-10]. It allows the potential treatment of deformed cartilage without any dramatic alterations in the structure of the extracellular matrix (ECM). A number of studies have been performed *in vitro* as well as *in vivo*, but the exact mechanism of laser induced stress relaxation in cartilage is not yet fully understood. Examination of the fine structure of ECM irradiated with atomic force microscopy has shown additional pores of tens of nms in size arising as a result of laser irradiation[11]. A phenomenological theoretical model of laser-induced alteration of cartilage structure has been presented which considers both the heating of the tissue and the diffusion of some components of ECM which are freed when tissue temperature was raised to some critical point (approx. 70° C)[12]. However, these components have as yet not been identified, and their molecular masses and diffusion properties have not been studied as far as we are aware. This paper describes a study on the effect of laser irradiation on pure chondroitin sulphate in solution and the effect of laser irradiation on the diffusivity of macromolecules through the cartilage matrix.

2.MATERIALS AND METHODS

Chondroitin sulphate was obtained from Sigma (No. 27042) and dissolved at a concentration of 10mg/ml in phosphate

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buffered saline (PBS, 0.01M phosphate/ 0.15M chloride, adjusted to pH 7.4). One aliquot of the solution (of 1.9 or 4 ml in volume) was irradiated using a Holmium:YAG laser (Verso Pulse 3000 Coherent) at an energy density of 0.55J/cm^2 at a pulse repetition rate of 5 Hz for 18 to 30 s to reach local temperatures of 70° C or 90° C. The temperature was measured with an accurately calibrated thermocouple.
In a second experiment, pieces of fresh porcine auricular cartilage (ten samples of a disk shape of 7 mm in diameter and 0.5 mm in thickness) were placed on the surface of 5ml of PBS (pH 7.4) in a syringe and held in that position during laser irradiation, the control samples (5g in total) was held in a similar syringe for the same length of time.
100µl of the pure chondroitin sulphate samples (treated and untreated) and the PBS (pH 7.4) from the irradiated and non-irradiated cartilage samples were injected into a SEC/MALS system in order to determine their absolute molecular masses[13] and aliquots of the pure chondroitin sulphate samples were used for sedimentation velocity studies[14].

2.1 SEC/MALS

The eluent (PBS as described above) was pumped at a flow rate of 0.8ml/min through the column system consiting of TSK G6000PW, TSK G5000PW, TSK G4000PW analytical columns protected by a guard column. The eluting fractions were monitored by a Dawn DSP multi-angle light scattering photometer (Wyatt Technology, Santa Barbara, USA) fitted with a 5mW He-Ne laser, and a Differential interferometric refractometer (Optilab 903, Wyatt Technology, Santa Barbara, USA). The injection volume was 100µl and the system was run at ambient temperature. Weight average molecular masses were obtained using the Zimm plot method in the dedicated ASTRA™ software using a value of 0.16ml/g for the specific refractive index increment (dn/dc)[15].

2.2 Sedimentation velocity in the analytical ultracentrifuge

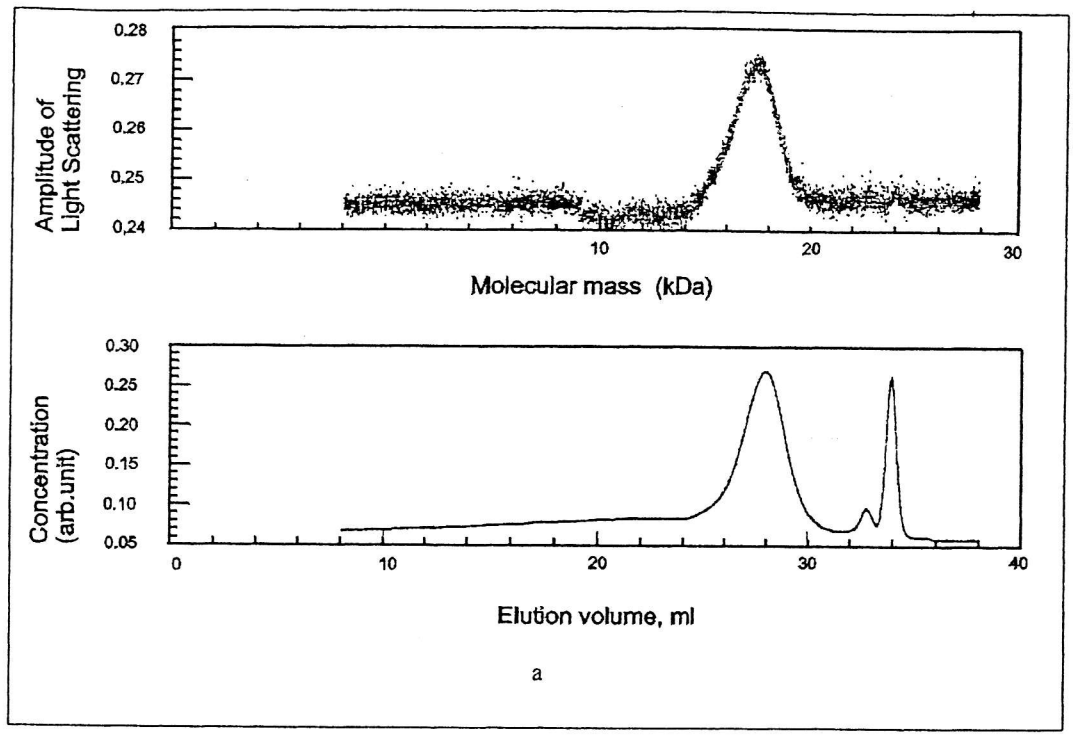
400µl of sample and 400µl of solvent were loaded into respective solution and solvent channels of 12mm pathlength double sector ultracentrifuge cells. Samples were run in a Beckman Optima XL-I analytical ultracentrifuge (Beckman, Palo Alto, USA) fitted with a Rayleigh interference optical system at a temperature of 20° C and a speed of 50000rpm. Data acquisition was performed using the manufacturers software and sedimentation coefficients were determined using the DCDT routine developed by Stafford[15] which also gives the sedimentation coefficient distributions.

2.3 Chemical analysis

Toluidine Blue has been used to detect Proteoglycans of macromolecules diffused out of the cartilage matrix into the in phosphate buffered saline. $BaCl_2$ has been used to manifest sulphas groups in the solution.

3.RESULTS

Molecular masses and sedimentation coefficients obtained for the pure chondroitin sulphate sample are shown in table 1 and the elution profiles obtained from the pure chondroitin sulphate (irradiated and non-irradiated) and the aqueous medium ('diffusate') surrounding the irradiated and non-irradiated cartilage samples are shown in figures 1 and 2.
The molecular mass of the pure chondroitin sulphate decreased with laser irradiation and this decrease was greater at higher irradiation temperatures. The sedimentation coefficient however, remained fairly constant: this is typical for an elongated molecule, where loss of mass is compensated by loss in asymmetry (and hence frictional resistance).
The diffusates from the irradiated and non-irradiated cartilage samples showed significant differences. Whilst there is no indication of any material present in the diffusate from the non-irradiated cartilage, two peaks are visible on the light scattering trace from the irradiated cartilage. Peak 1 of figure 2(b) is indicative of large molecular weight material and the second peak to which a concentration peak can be assigned (see figure 2(b) peak 2) elutes at an elution volume slightly higher than that of the pure chondroitin sulphate leading to the conclusion, that the majority of molecules which have diffused out of the cartilage matrix are slightly smaller in size than would be expected for chondroitin sulphate. Unfortunately, these 'diffusate' peaks are not sufficiently large to give sensible molecular mass values, but they qualitatively indicate that a macromolecular component has diffused from the cartilage into the buffer. Macromolecular concentrations of the diffusate were also too low for sedimentation velocity experiments.
Chemical analysis for the determination of sulphate groups and proteoglycan gave positive results for the PBS from the irradiated cartilage but were found to give negative results for the non-irradiated cartilage.



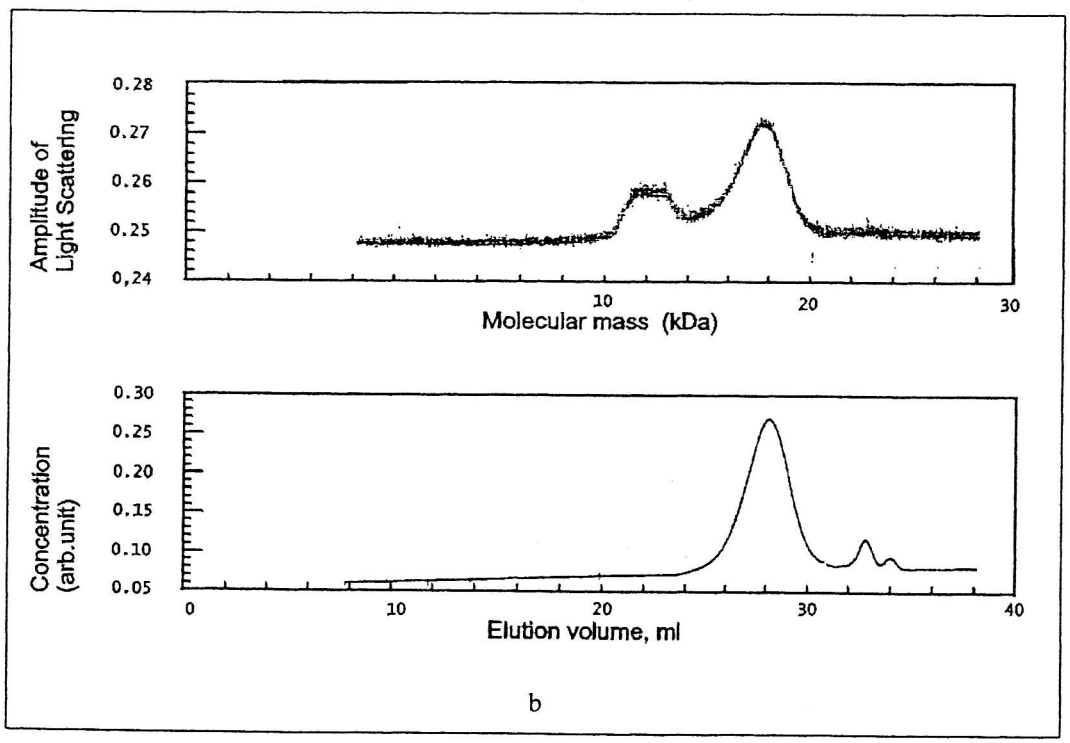
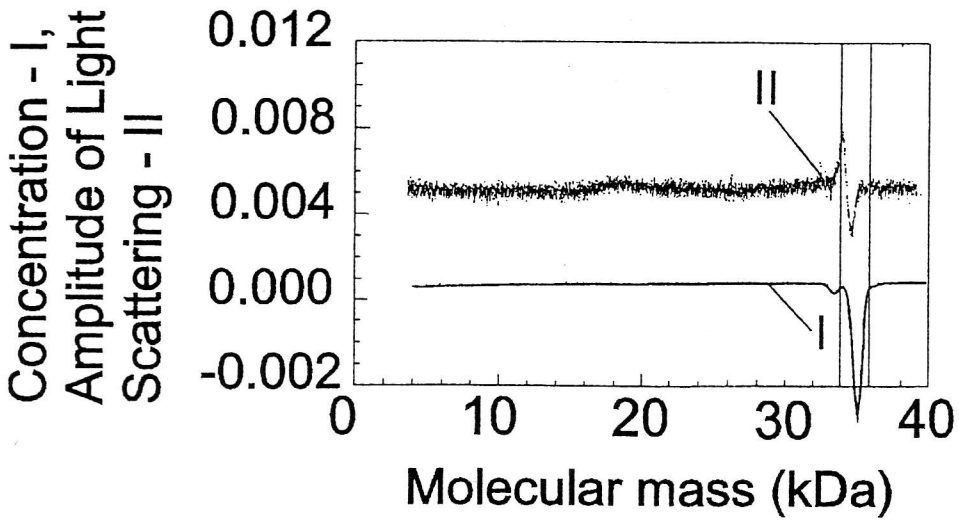
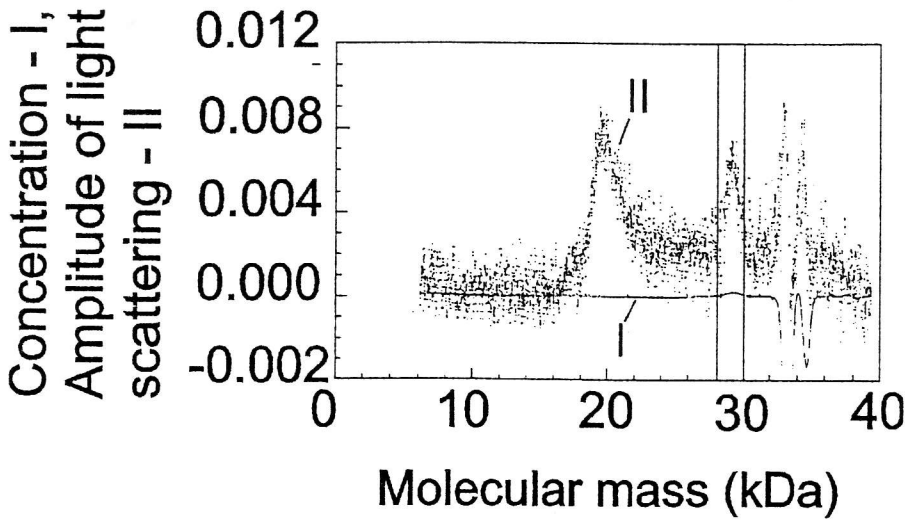


Fig.1(a),(b). Molecular mass distribution and light scattering in the chondroitin sulphate solution (a) - non-irradiated; (b) - irradiated.



a



b

Fig.2 (a),(b) Molecular mass distribution and light scattering in the aqueous medium surrounding cartilage sample. (a) - non-irradiated; (b) - irradiated.

Table 1: Results from SEC/MALS and sedimentation velocity experiments for pure chondroitin sulphate

Sample	Solution temperature as a result of irradiation	M_w, Da	$S \times 10^{13}$, s^{-1}
Chondroitin sulphate, non-irradiated		21200±4800	1.351±0.001
Chondroitin sulphate, irradiated	90° C	15500±2500	1.357±0.001
Chondroitin sulphate, irradiated	70° C	17400±2400	1.354±0.001

4.DISCUSSION

The question central to this study was how laser treatment would effect components of cartilage - either the pure components or those within the cartilage itself, although we restricted ourselves initially to the investigation of chondroitin sulphate. The molecular mass obtained by SEC/MALS for the non-irradiated pure chondroitin sulphate agreed very well with literature values[1], whereas a decrease in molecular mass was detected after laser treatment, i.e., there is some disruption of the polymer chain. The SEC/MALS traces also revealed some aggregated material in the control sample which was no longer present after irradiation, a clear indication of disruption of the aggregates.

The more interesting finding was that of the diffusivity of material from cartilage into the buffer following laser treatment. Besides providing the structural framework for the cartilage, the extracellular matrix which contains the proteoglycans also forms a fluid compartment in which channels allow the flow of nutrients, ions, hormones etc. It is, therefore, very important the health of the cartilage tissue that these flow properties are maintained at the correct level. Previous experiments have shown[11] that laser treatment of cartilage induces channel formation and it was suggested that proteoglycan units detaching themselves from the hyaluronic acid backbone would be responsible for this channel formation. Our study has supported this hypothesis by clearly indicating that proteoglycans diffuse away from the cartilage matrix after laser irradiation. This finding consists with the theory of laser-induced alterations in cartilage structure[12] and allows to understand the molecular mechanism of the diffusion limitation of structural alterations. Moreover, it appears that these proteoglycans contain sulphate groups which suggests that it could be either keratan sulphate or chondroitin sulphate which diffuse out of the matrix. Comparison of chromatograms from the pure chondroitin sulphate and the 'diffusate' indicates a slightly higher elution volume for the latter, suggestive of lower molecular masses (even when compared to the irradiated chondroitin sulphate) However, according to Newman[17] the length of the chondroitin sulphate attachment region may vary depending on the age o the cartilage. Keratan sulphate would also fulfill the criteria of a sulphate bearing proteoglycan, however the molecular mas of this material has been quoted as approx.5500 Da which would suggest that the elution volume of this material would b higher than that measured, especially, when some degradation of the material must be expected due to laser irradiation Obviously, full chemical analysis is required to determine the true nature of the diffusing component.

5.CONCLUSIONS

We have shown that (a) laser irradiation decreases the molecular mass of chondroitin sulphate and (b) laser irradiaton o cartilage induces diffusion of macromolecules into the medium. The results obtained allow us to understand the mechanisr of stress relaxation and structural alterations in cartilage under non-destructive laser radiation.

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