

Aspects of the structural integrity of chondroitin sulphate after laser irradiation

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Abstract

The effects of laser irradiation on molecular mass and conformation of pure chondroitin sulphate dissolved in phosphate buffered saline (PBS) 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. © 2002 Elsevier Science Ltd. All rights reserved.

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1. Introduction

The extracellular matrix (ECM) 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 (Hardingham, 1981). 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 more novel applications of lasers in medicine (Spivak, Grande, Ben-Yishay, Menche & Pitman, 1992; Wang, Pankratov, Perrault & Shapshay, 1995; Wang, Perrault, Pankratov & Shapshay, 1996; Pullin et al., 1996; Collier et al., 1993; Helidonis et al., 1993; Sobol, 1995; Bagratashvili, Sobol, Sviridov, Omelchenko & Popov, 1997; Sviridov, Sobol, Jones & Lowe, 1998). It allows the potential treatment of

deformed cartilage without any dramatic alterations in the structure of the 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 by atomic force microscopy has shown additional pores of tens of nms in size arising as a result of laser irradiation (Sobol, Omel'chenko, Mertig & Pompe, 2000). A 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) (Sobol, Kitai, Jones, Sviridov, Milner & Wong, 1999). However, these components have as yet not been identified, and, as far as we are aware, their molecular masses and diffusion properties have not been studied. This article describes a study of 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 4-sulphate sodium salt from bovine trachea was obtained from Fluka BioChemika (Switzerland)

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

Sample	Solution temperature as a result of irradiation (°C)	M_w (Da)	$s_{T,b}$ (S)
Chondroitin sulphate, non-irradiated		$21,200 \pm 4800^a$	1.351 ± 0.001
Chondroitin sulphate, irradiated	70	$17,400 \pm 2400^b$	1.354 ± 0.001
Chondroitin sulphate, irradiated	90	$15,500 \pm 2500^c$	1.357 ± 0.001

^a Average of three injections.

^b Result from single injection.

^c Average of two injections.

(Product no. 27042) and dissolved at a concentration of 10 mg/ml in phosphate buffered saline (PBS, 0.01 M phosphate/0.15 M 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) ($\lambda = 2.1 \mu\text{m}$) at an energy density of 0.55 J/cm^2 and a pulse repetition rate of 5 Hz for 18–30 s to reach local temperatures of 70 or 90°C. The diameter of the laser beam was 14 mm which was larger than the thickness of the layer of the solution being irradiated (approx. 1 mm) and the temperature was measured with an accurately calibrated thermocouple.

In a second experiment, pieces of fresh porcine auricular cartilage (ten disk shaped samples of 7 mm in diameter and 0.5 mm in thickness) were immersed in PBS (pH 7.4) (5 ml) at the top of a syringe and held in that position during laser irradiation; the control samples (5 g in total) were held in a similar syringe for the same length of time.

Hundred micro litre 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 (Wyatt, 1993) and aliquots of the pure chondroitin sulphate samples were used for sedimentation velocity studies (Ralston, 1993).

2.1. SEC/MALS

The eluent (PBS as described above) was pumped at a flow rate of 0.8 ml/min through the column system consisting 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 5 mW He–Ne laser, and a Differential interferometric refractometer (Optilab 903, Wyatt Technology, Santa Barbara, USA). The injection volume was 100 μl 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 0.16 ml/g for the specific refractive index increment (dn/dc) (Kitchen & Cleland, 1978).

2.2. Sedimentation velocity in the analytical ultracentrifuge

Four hundred micro litre of sample and 400 μl of solvent were loaded into respective solution and solvent channels of 12 mm 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 50,000 rpm. Data acquisition was performed using the manufacturers software and sedimentation coefficients were determined using the DCDT routine developed by Stafford (1992) which also gives the sedimentation coefficient distributions.

2.3. Chemical analysis

It was important to determine if there was any material diffusing out of the cartilage matrix and if this was the case whether this material was (a) proteoglycan in nature and (b) sulphated. Two crude qualitative tests were therefore carried out on the solution from the laser irradiated cartilage: Toluene Blue was used to qualitatively detect the presence of proteoglycans and BaCl_2 was used to detect the presence of sulphate groups in the solution.

3. Results

It was the aim of this study to investigate the effect of laser irradiation on one of the components of the ECM of cartilage (chondroitin sulphate) and on cartilage itself with the techniques described above. SEC/MALS was used to describe the changes in molecular mass and sedimentation velocity was used to determine the sedimentation coefficient of non-irradiated and irradiated chondroitin sulphate.

Molecular masses and sedimentation coefficients obtained for the pure chondroitin sulphate sample are shown in Table 1 and the elution profiles for the non-irradiated and irradiated (irradiation temperature 70°C) pure chondroitin sulphate from SEC/MALS are shown in Fig. 1(a) and (b). The molecular mass of the pure chondroitin sulphate decreased with laser irradiation and this decrease was greater at higher irradiation temperatures. The elution profile for the non-irradiated pure chondroitin sulphate shows two peaks on the light scattering trace — one peak

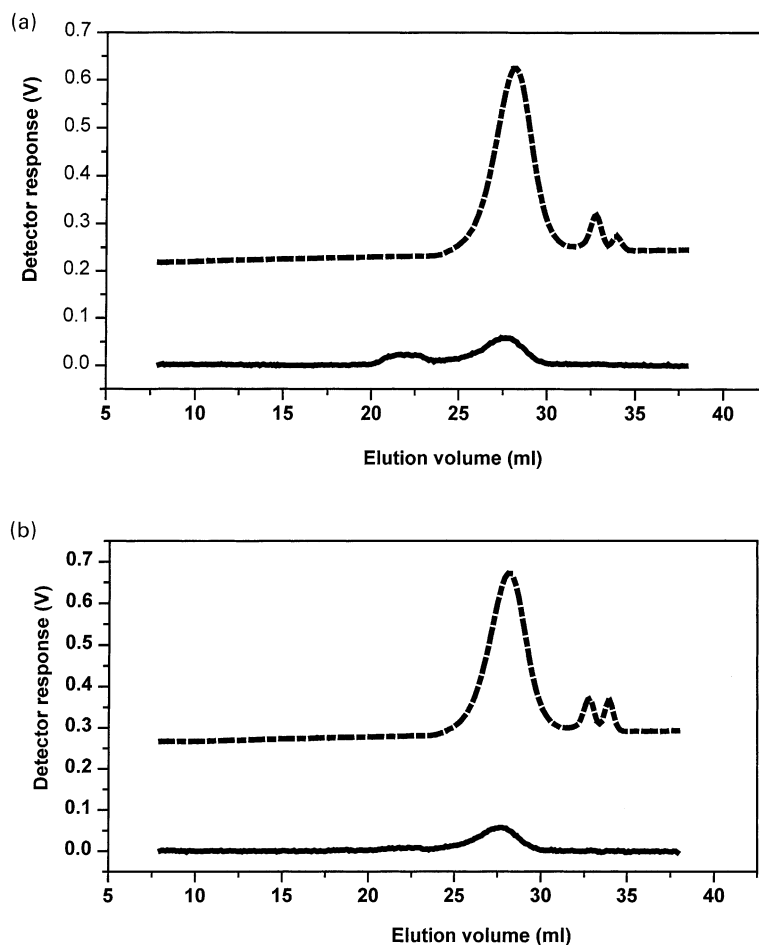


Fig. 1. Elution profiles from (a) non-irradiated and (b) irradiated (irradiation temperature 70°C) chondroitin 4-sulphate. — refers to profile from 90° light scattering detector, — refers to profile from concentration (refractive index) detector.

at an elution volume of approximately 22 ml without a corresponding peak on the RI trace and one at an elution volume of approximately 27.5 ml which has a corresponding concentration peak. Light scattering is very sensitive to small amounts of high molecular weight material (i.e. scattering intensity is proportional to $M \times \text{concentration}$) whereas the response on the RI trace depends on concentration alone. These traces therefore indicate that there is a small amount of aggregated material in the pure non-irradiated chondroitin-sulphate solution which disappears with irradiation (see Fig. 1(b)). The peak due to the aggregated material was not included in the molecular mass calculations, the reduction in molecular mass seen for the irradiated samples is therefore only due to changes in the non-aggregated material.

The sedimentation coefficient is a measure of the velocity of a macromolecule as it moves through a solution in a centrifugal field — its value is therefore dependent on both molecular mass and shape of the molecule. Chondroitin sulphate is a linear sulphated glycosaminoglycan with molecular mass of approx. 2×10^4 Da (Luscombe & Phelps, 1967; Mathews, 1971; Wasteson, 1971; Hopwood & Robinson, 1975). The fact that it is negatively charged

suggests that it will adopt an elongated conformation in solution as the presence of like charges on the molecule prevent it from forming a compact conformation. Comparison of the measured sedimentation coefficient for chondroitin sulphate (1.35S) with that of a globular molecule such as lysozyme (molecular mass 14,400 Da, sedimentation coefficient 1.91S) confirms that the molecule adopts a more extended conformation. Table 1 shows that there is virtually no change in the sedimentation coefficient for the irradiated and non-irradiated chondroitin-sulphate samples — such behaviour is fairly 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 macromolecular material present in the diffusate from the non-irradiated cartilage (Fig. 2(a)), two small peaks are visible on the light scattering trace from the irradiated cartilage (Fig. 2(b)). Peak 1 of Fig. 2(b) is indicative of large molecular weight material and the second peak to which a concentration peak can be assigned (see Fig. 2(b) peak 2) elutes at an elution volume slightly higher than that of the pure chondroitin sulphate

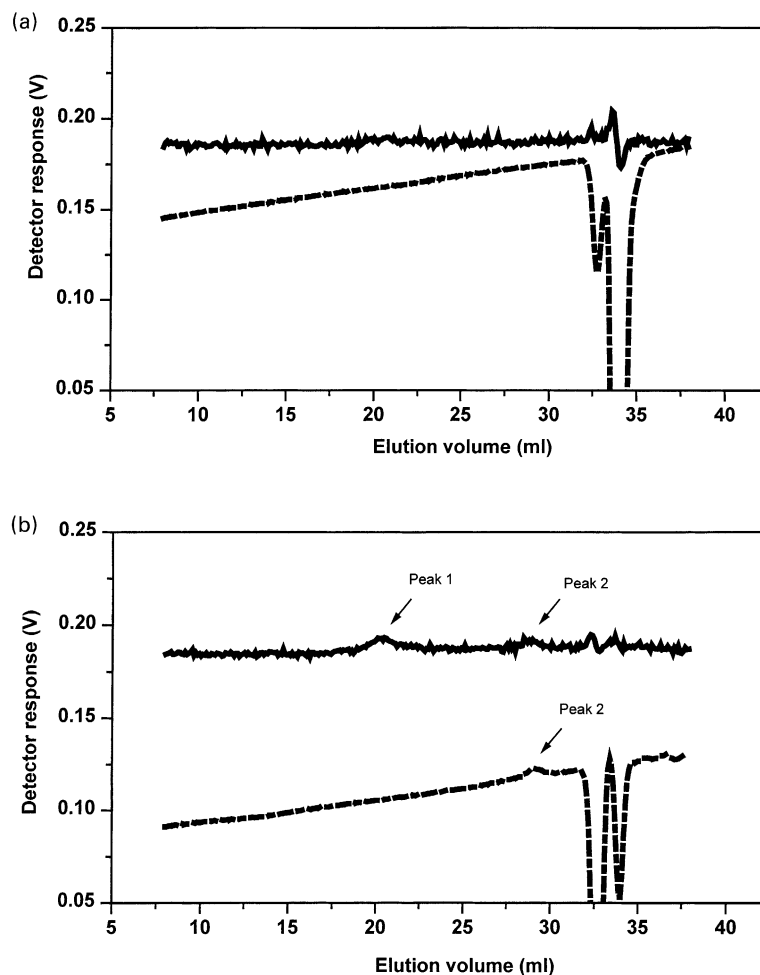


Fig. 2. Elution profiles from the diffusate from the (a) non-irradiated and (b) irradiated cartilage samples — refers to profile from 90° light scattering detector, — refers to profile from concentration (refractive index) detector.

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 macromolecular components have 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 negative results for the non-irradiated cartilage indicating (a) that there are proteoglycan components migrating out of the ECM and (b) that they are likely to be sulphated.

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 (Hardingham, 1981), 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 ECM 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 for the health of the cartilage tissue that these flow properties are maintained at the correct level. Previous experiments have shown (Sobol et al., 2000) 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 supports this hypothesis by indicating that proteoglycans diffuse away from the cartilage matrix after laser irradiation. Moreover, it appears that these proteoglycans contain sulphate groups which suggests that it could be either keratan sulphate or chondroitin sulphate which diffuses out of the matrix. The chemical analyses performed on the diffusate were very crude; it is therefore impossible to make any definite deductions regarding the nature of the macromolecules detected and further work is required to identify these materials. However, if these findings could be proven and the macromolecules identified they would support the theory of laser-induced alterations in cartilage structure (Sobol et al., 1999b) and give some insight into the molecular mechanism of the diffusion limitation of structural alterations.

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