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EFFECT OF X-RADIATION ON HYALURONIC ACID

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ABSTRACT

The effects of radiation on tissue have generally been investigated in the context of therapeutic irradiations, radiation protection and exposure to solar radiation. Typically attention has concentrated primarily on cells, less consideration being given to the extracellular matrix (ECM). ECM consists of collagen and elastin fibers immersed in a viscoelastic gel composed principally of hyaluronan (HA) and proteoglycans. The present study examines changes in the physical properties of the principle matrix micromolecules due to exposure to ionizing radiation. Freeze-dried rooster-comb HA (Sigma) were used to give doses of 10-100 Gy for HA, to cover the range of irradiation exposure during radiotherapy. The viscosity of HA (at 1.25% and 0.125% w/v) was measured by both cone and plate and capillary viscometry, the former providing measurement at uniform shear rate and the latter providing a more sensitive indication of changes. The Raman microspectrometry was employed to characterize these changes on ECM molecular conformation. In regard to the viscometry, both techniques (cone and plate and capillary viscometry) reveal a dose-dependent reduction in viscosity (from 340 ± 194 cP for controls to 1500 ± 88 cP at a shear rate of 2 s^{-1} and dose of 75 Gy), again suggesting depolymerisation.

ABSTRAK

Kesan sinaran ke atas tisu secara amnya telah dikaji dalam konteks sinaran terapiutik, perlindungan sinaran dan pendedahan kepada sinaran matahari. Lazimnya perhatian lebih tertumpu kepada sel-sel tetapi kurang perhatian terhadap matriks luar sel (ECM). ECM terdiri daripada kolagen dan gentian elastin yang terbenam di dalam gel viskoelastik yang terdiri daripada hyaluronan (HA) dan proteoglikan. Kajian ini mengkaji perubahan dalam ciri-ciri fizikal prinsip matriks mikromolekul yang disebabkan oleh pendedahan kepada sinaran mengion. Freeze-dried rooster-comb HA (Sigma) digunakan untuk memberi dos 10-100 Gy untuk HA, yang merangkumi julat pendedahan penyinaran semasa radioterapi. Kelikatan HA (pada kadar 1.25% dan 0.125% w / v) telah diukur oleh kedua-dua viskometer kon dan plat dan viskometer kapilari, pengukuran terdahulu menyediakan pada kadar ricih yang seragam dan memberikan petunjuk yang lebih peka terhadap perubahan. Raman mikrospektrometri telah digunakan untuk mencirikan perubahan bentuk molekul ECM. Dalam hal berkaitan dengan viskometer, kedua-dua teknik (viskometer kon dan plat dan viskometer kapilari) menunjukkan pengurangan dos bergantung kepada kelikatan (daripada 340 ± 194 cP bagi kawalan kepada 1500 ± 88 cP pada kadar ricih 2 s^{-1} dan dos 75 Gy), sekali lagi mencadangkan dipolimerasi.

Keywords: Hyaluronan; Viscometry; Raman Spectroscopy

INTRODUCTION

Hyaluronan is a high molecular-mass polysaccharide in ECM typically found in the connective tissues of vertebrates, which were formerly known as acid mucoplolysaccharide and are now designated glycosaminoglycan (GAG). GAG are unbranched single-chain polymers of disaccharide units containing N-acetylhexosamine and hexose. The second sugar is a hexuranic acid in all except keratin sulphate, which contains galactose instead. As biofluid, there is considerable need to measure the viscosity of it to understand the complex features of its rheology. Changes to hyaluronan rheological property can also be characterized by looking at changes in viscosity. As a part of this effort, it is important to understand the concept of newtonian and non-newtonian fluid in order to understand the rheology of HA.

Balazs (2007) demonstrated that the unique rheological properties of hyaluronan, which govern its biological performance, derive from its large molecular volume and from extensive interaction and entanglemment of the molecular coils. Under low shear rate, the network exhibits extremely high viscosity and at high oscillating frequencies has high elasticity (Coleman et al. 1999). low frequencies, corresponding to a knee joint almost at rest, intermolecular entanglements dynamically break and reform within time scale of measurements (Müller 1929). The rheological properties are, therefore, predominantly viscous. At high frequencies, the configurational readjustment of the chain cannot occur between the short periods of the oscillating strain. Under these conditions, the fluids rheological behaviour is predominantly elastic (Fung 1993).

RESULT AND DISCUSSION

Thus hyaluronan of a sufficiently high molecular weight can serve in the joint as an effective lubricant when movements are slow and as a shock absorber when movements are fast. Previous investigation has reported a decreases in viscosity value when hyaluronan (HA 4 mg/ml) is subjected to higher shear or oscillating frequency (Nishimura 1998).

Freeze-dried rooster comb hyaluronan (Sigma, molecular weight ~10^6 Da was made into a 2% (w/v) stock solution in 0.1molar sodium chloride at 7.2 pH. More dilute solutions down to 0.01% (w/v) were prepared by serial dilution with 0.1M sodium chloride. Purified water was used for the preparation of the solutions in all the experiments. The shear dependence of viscosity was investigated using a Brookfield DV III programmable cone and plate rheometer that imposes a uniform shear rate on the fluid. The cone used was the CP 40 with 0.8^o angle and a sample volume of 0.5ml. The samples were observed in epi illumination using lenses of X10 or X40 magnification using Raman Spectroscopy. Tissue samples were placed on an aluminised slide and kept moist under a quartz coverslip to minimise background effects. Hyaluronan samples were placed in an aluminised well-slide and covered with a quartz cover slip. Wave number calibration was carried out using the 520 cm^{-1} line of a silicon wafer. Hyaluronan used a wider range of doses using Linac, from 10-100 Gy, were delivered extending either side of the range of radiation exposures typically provided during a course of radical radiotherapy. The dose rate was adjusted to 4 Gy/minute. Hydrated tissue samples in deionized water and HA solutions were contained in 14 ml glass vials (RS,UK) and were irradiated with the support of two ‘solid-water' filled phantoms (Wellhofer-Scanditronix, Sweden).

EXPERIMENTAL METHOD

Most biofluid hyaluronan are viscoelastic and there are many ways the viscoelasticity of a fluid can be revealed, as follows:

Thixotropy and *shear-thinning* are among the features of vicoelastic fluid. The word thixotropy was introduced by H.Freundlich (Freundlich 1922) to describe sol-gel transformation of colloidal solutions. Many suspensions and emulsions (solid and liquid particles, respectively, dispersed in a liquid medium) and colloids show a fall in viscosity as the shear stress is increased. This recovery is called thixotropy. Rheologists also use the term thixotropy to describe all recoverable loses in consistency produced by shearing (Fung 1933).

If the consistency or viscosity falls when shearing increases, the material is said to show shear thinning (Fung 1933). The reverse effect, an increase in viscosity with increasing shear rate, is called shear thickening. Shear thinning is of often explained by breaking up of the structure by shearing

At higher shear rates the chains cannot adjust as rapidly as the oscillating strain and thus are deformed sinusoidally.

The viscoleasticity of hyaluronan solution in this investigation is measured by the capillary viscometer and a cone and plate viscometer. The capillary method is one of the simplest methods for measuring fluid viscosity and uses flow in a long cylindrical tube under the force gravity (Bowlt 1975). The laminar flow of liquids flows through a cylindrical capillary tube. The viscosity is determined by measuring the flow rate of the fluid flowing through the capillary tube and the pressure diferential between both ends of the capillary tube. This method also benefits from the consistency of viscosity reading even at lower concentration.

For constant shear and smaller amount of samples being is very suitable for the highly costly hyaluronan solutions and also demonstrating shear thinning behavior at higher shear rates, the cone and plate viscometer is used (Mills & Kokini 1984). The sample occupies the conical space. When the plate rotates, the linear velocity increase with the radial distance; but since the gap also increases linearly with the radius, the shear rate remains constant throughout if the cone remains stationary. The magnitude and phase relationship between the torque and shear rate will yield the complex modulus of a viscoelastic material.

The water viscosity is measured every time before a hyaluronan sample is tested in the cone and plate rheometer. This verification procedure is important in order to ensure that the cone (model CP 52) which is consistent with viscous fluid like hyaluronan are always consistent when the measurements take place. It is also important that before one starts the experiment the correct degree of gap between the cone and plate is obtained. Hence one uses water as a Newtonian fluid which the viscosity are always constant at 1cP [131] at any shear rates. This can be seen at figure 1 below.

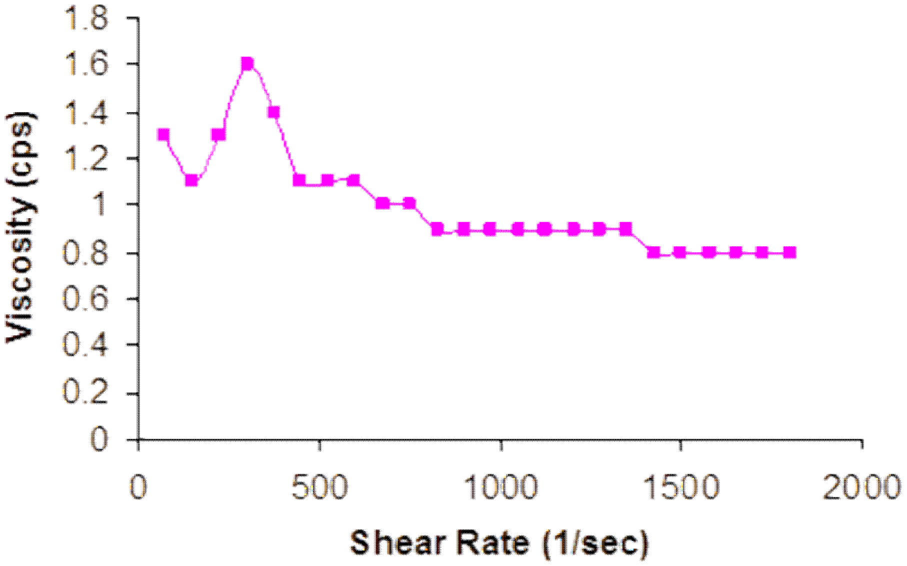


Figure 1: Results shows measurement of deionized water viscosity plotted against

shear rate using a cone and plate rheometer at room temeperature.

Measurement of deionized water viscosity is adopted as a standard procedure before measuring viscosity of hyaluronan. This is to ensure that the rheometer and the cone used are providing correct and consistent results. Water is a Newtonian fluid and the actual viscosity of water is 1cP at 20°C [131].

Measurement of Hyaluronan Viscosity Using a Cone and Plate Rheometer and Capillary Viscometer

Hyaluronan Viscoelasticity

A typical result of the measurement of viscosity of hyaluronan is shown in the Fig 2 using cone-plate viscometer. The viscosity has been measured in units of cP and is plotted against the shear rate for 1.25% w/v HA in deionized water. It is seen that viscosity decreases at higher shear rates (from $100s^{-1}$- $480s^{-1}$). Thus hyaluronan solutions are predominatly viscous at low shear rate and predominantly elastic at higher shear rates. This may suggest that at lower shear rates configurational adjustments of the polysaccharide chain, through Brownian motion, are rapid enough to allow the chains to maintain the random configurations under the imposed strain and to slip by each other, resulting in viscous flow. At higher shear rates the chains cannot adjust as rapidly as the oscillating strain and thus are deformed sinusoidally.

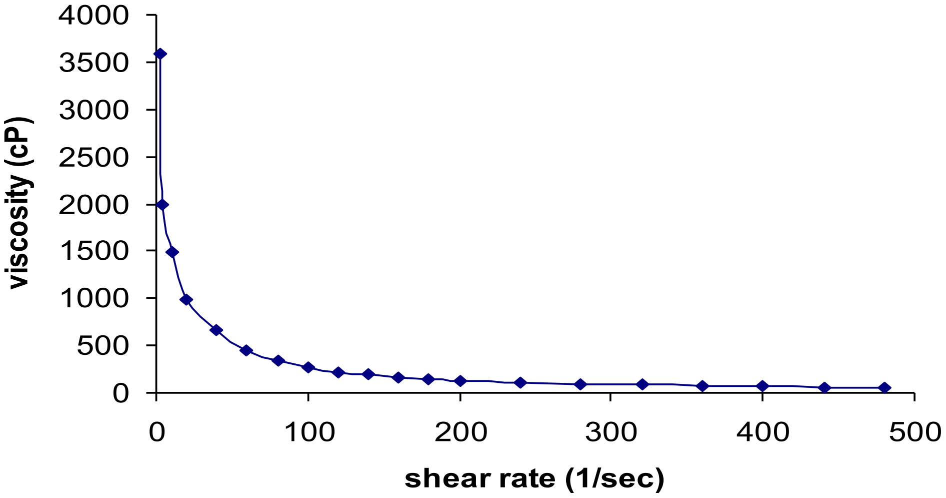


Figure 2: The above figure indicates a plot of viscosity as function of shear rate for 1.25% w/v of hyaluronan in deionized water. The non linear relation between viscosity and shear rate describes the non-Newtonian behaviour of this amorphous viscoelastic gel. At lower shear rates shear thinning is observed and the thixotropic property of hyaluronan is revealed.

Evaluation of intrinsic viscosity, η_{int}, provides measurement of the ratio of effective hydrodynamic volume of a polymer molecule to its molecular weight. This ratio increases with polymer chain length (Laurent 1962). The value is obtained by extrapolation of the specific reduced viscosity back to zero concentration as previously described in the methods chapter.

The weight-average molecular weight, M_w of a polymer such as hyaluronan is related to [(η_{int}) by the Mark-Houwink equation:

$$\eta_{int} = kM_w^a$$

where k and a are the Mark-Houwink constants (0.012 and 0.86, respectively) determined in 0.15M saline [P. J. Coleman, 2000 #26].

The intrinsic viscosity of the hyaluronan evaluated in this study has also being dissolved in normal saline containing 0.15M of NaOH. This enables us to use a similar constant in estimating the Mw. Viscosity is measured using a miniature U-tube viscometer (M5) with nominal overall length 250mm (DC Scientific Glass, USA); sample of approximate volume 3ml was used. The measurement is conducted in constant temperature in a water bath thermostatted at 25°C.

At higher shear rates the chains cannot adjust as rapidly as the oscillating strain and thus are deformed sinusoidally.

Estimation of Intrinsic viscosity and molecular weight of hyaluronan

The graph shown in figure 3 below is of measured for reduced specific viscosities (as described previously in methods) of hyaluronan solutions in 0.15M NaCl, as a function of concentration 0.125%-0.008%. As expected, these non-Newtonian solutions show an increase in viscosity with concentration. These data enable estimation of η_{int}, from which an estimate of molecular size of this long chained polymer can be estimated.

Linear regression and extrapolation gave value of reduced viscosity at zero shear rates. The latter is plotted on a logarithmic scale as a function of C in Fig. 1.3. The intercept, intrinsic viscosity at zero shear rate is 11.746 lg^{-1} or 11746 ml g^{-1} in methods used by McDonald and Levick 1995 [133]. Molecular weight of hyaluronan sample is estimated from [η_{int}]=11746 ml g-', predicts a sample mean Mw of 9.246×10^6, which is roughly in line with the normal Mw hyaluronan as reported in previous literature (Coleman et al. 1999; Dahl et al. 1952).

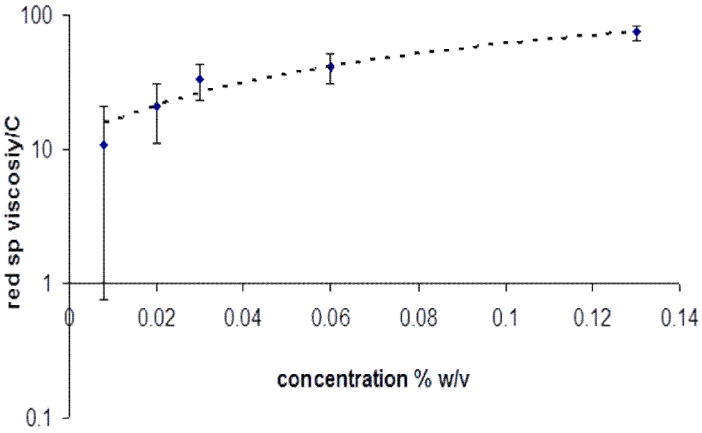


Figure 3: The above figures indicate the plot of reduced specific viscosity (η_{sp}/C), versus concentrations used for various concentrations hyaluronan in normal saline containing 0.15M NaCl. The value of reduced viscosity is derived from the value of relative viscosity v using a BSUM M5 miniature capillary viscometer thermostatted at 25°C. The graph is extrapolated at zero concentrations to estimate the value of intrinsic viscosity, yielding 11.746 lg^{-1}.

Effects of x-radiation on hyaluronan rheology

The predominant effect of irradiation of polysaccaharides (i.e hyaluronan), either in solution or dry state, is degradation. In aqueous solution this is infered from decrease in viscosity and the formation of reducing sugars (Bailey 1968).

Results of irradiation in the presence of 0.1M NaCl indicate the observed fall in viscosity of hyaluronan to be largely a post irradiation effect. The molecular weight, determined by light scattering falls, indicated the fall to result from main chain-scissions. The fall in viscosity was attributed to reaction with hydroxyl radicals, which causes main chain-scissions (Go Matsumura & Ward 1966).

Hyaluronan irradiated with gamma radiation from a Co-60 source showed a progressive degradation leading to a decrease in viscosity average molecular weight. This decrease was observed to occur with an increasing ·OH radical concentration produced by irradiation (Al-Assaf et al. 2000). Ziva Lurie et al. in 2003 also reported gamma irradiation of hyaluronan solution using a Cs-137 source to induce loss of hyaluroan viscosity. Such degradation leads to subsequent decrease in the effectiveness of HA as a joint lubricating agent.

At higher shear rates the chains cannot adjust as rapidly as the oscillating strain and thus are deformed sinusoidally.

Viscosity changes in hyaluronan in this investigation were measured using cone and plate and capillary viscometers. In figure 4, cone and plate viscometry showed the non-linear dependence on shear rate and concentration that is expected of a long-chain polymer. The asymptotic value and that measured in the capillary viscometer were both reduced after irradiation. The magnitude of the effect depended on the level of exposure. At a shear rate of 2 s^{-1} the viscosity of 1.25% w/v HA in deionized water fell from 3400 ±194 cP for controls to 1500 ± 88cP at a dose of 75 Gy. When the HA was further diluted in deionized water at 0.125% w/v and viscosity measured using a capillary viscometer decrease of HA viscosity was observed for irradiated HA, the magnitude of the effect depending on the level of doses given, as shown in figure 5.

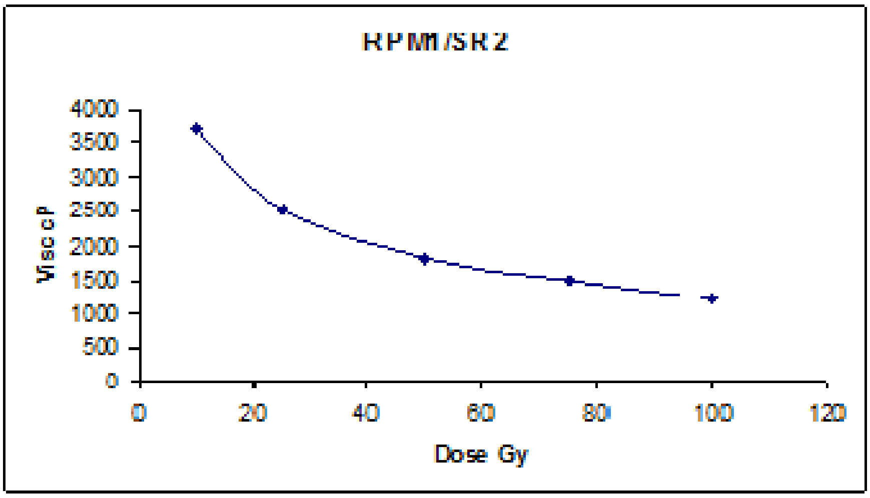


Figure 4: shows plot of viscosity as a function of shear rate on 0.125% w/v hyaluronan in deionised water using a cone and plate viscometer.

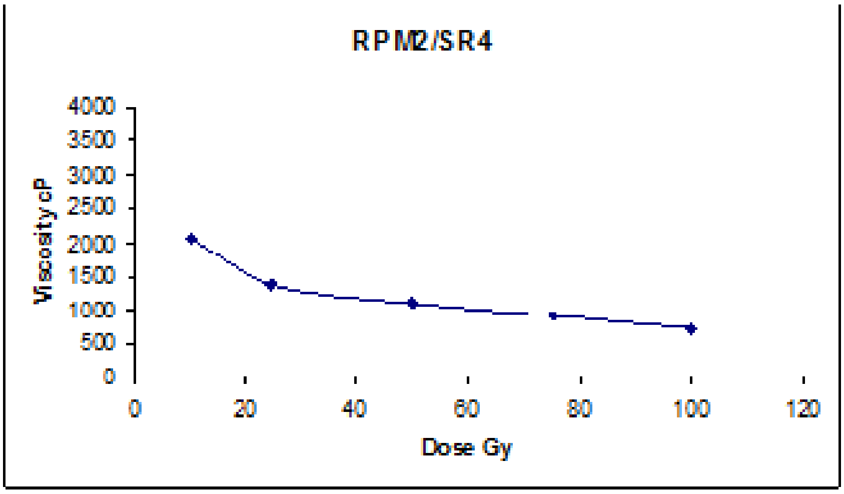
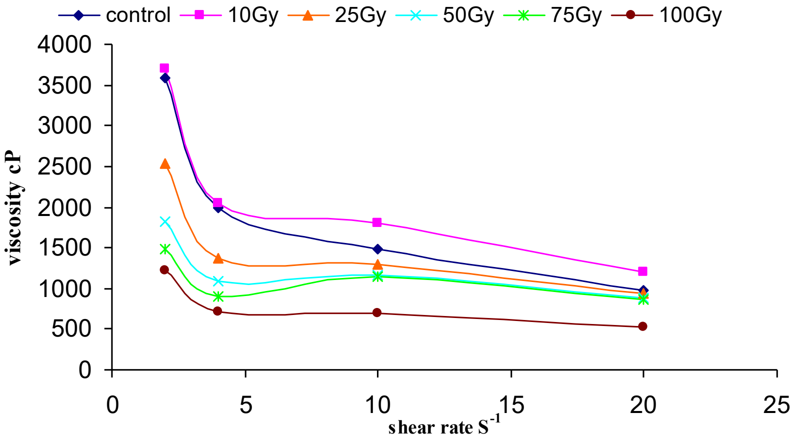
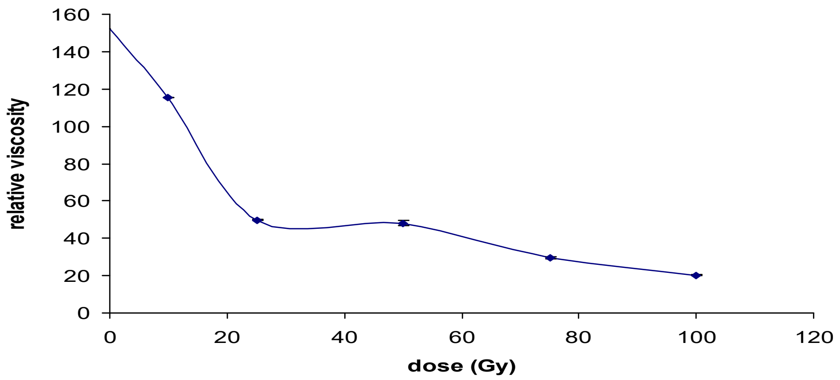


Figure 5: shows plot of viscosity as a function of x-ray dose on 0.125% w/v hyaluronan in deionised water using a miniature u-tube capillary viscometer.

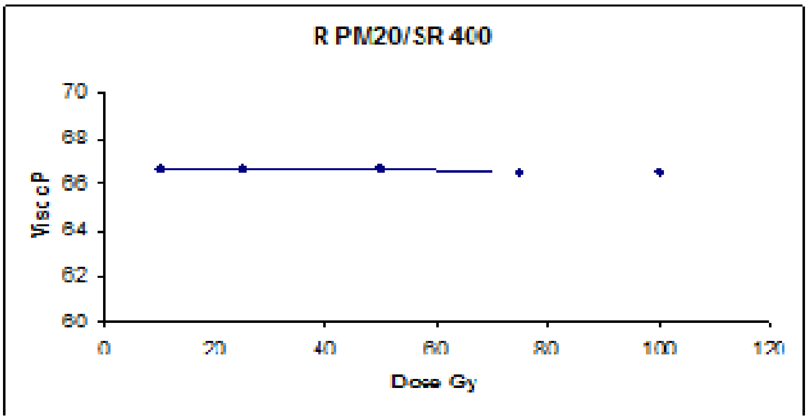
The graph below (figure 6) characterizes the viscosity of hyaluronan after exposure to x-ray using a cone-plate viscometer. These further elucidate details of the reductions of hyaluronan viscosity after irradiation. A further important feature that is observed that changes of viscosity of hyaluronan occur at lower shear rates as in higher shear rates the macromolecules tend to aligned and deformed because of the shearing forces.



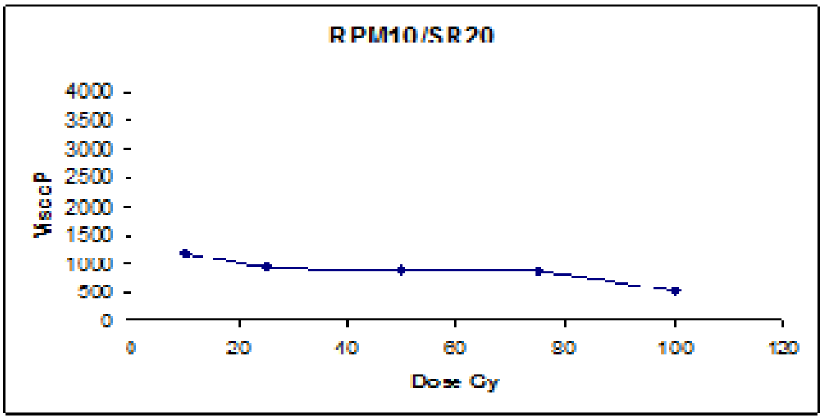
A



B



C



D

Figure 1.6: The viscosity of 1.25% hyaluronan w/v as a function of X-radiation doses for 1.25% w/v hyaluronan at lower shear rates (A at $2s^{-1}$,B-$4s^{-1}$,C-$20s^{-1}$, and D at $400s^{-1}$) when hyaluronan is exposed to 10,25,50 and 100Gy of x-radiation,. Shear rates are proportionate with the RPM value which is the rheometer motor rotational speed per minute. These graphs demonstrate that a significant reduction in hyaluronan viscosity is only to be observed at the lower shear rates while at higher shear rates the relationship is complex.

Raman spectroscopy has been used in characterising HA before and after exposure to ionizing radiation. Spectra were obtained for 1.25% w/v HA solution in deionized water, the sample being produced on a quartz slide covered by a quartz cover slip. The reported spectra are the means of 10 acquisitions at a laser power of 100 % on the sample, obtained at an objective lens magnification of 40X. Preliminary experiments showed that these conditions did not produce photodamage to the samples. A silicon standard was used to perform a frequency calibration.

Background subtraction, using a cubic spline interpolation through intensity minima, and other spectral manipulations were performed using the proprietary software WIRE 2.0 (Renishaw, Wooton under Edge, U K).

Figure 1.7 shows Raman spectra for control and 100 Gy irradiated hyaluronan. Analysis of the Raman spectra for the control HA revealed there to be a dominant water peak. When these peaks were fitted to a 100% Gaussian using peak parameters obtained from the available literature (Leikin et al. 1997), as described by Ellis (personal communication, 2007), no significant differences were observed for control and irradiated HA. After peak fitting water peaks were deconvulved and revealed at a frequency of 3209 cm^{-1} and a more minor peak at 3403 cm^{-1}, produced primarily by OH-NH stretch vibrations. In irradiated tissues, the two observed bands at 3208 cm^{-1} and 3402 cm^{-1} result from a different mode of OH vibrations. All parameters of the OH spectra, calculated with Gaussian deconvolution, and the width of the N-H peaks have approximately quantify the observed spectral changes is not an artefact but rather a manifestation of changes in the structure of water in HA. The C=C stretch mode was prominent at 1639 cm^{-1} in the control tissue and at 1636 cm^{-1} in the irradiated samples.

A significant difference observed between control and irradiated tissue was the loss of a broad shoulder between 1300 cm^{-1} and 1400 cm^{-1}, arising from C-H deformation. The symmetric COO$^-$ cation vibrational mode from hyaluronan can be seen as a shoulder at 1411 cm^{-1} in the spectrum of the control sample. In irradiated HA, a wavelength shift can be seen for this shoulder of COO$^-$, to 1421 cm^{-1}. At a Raman shift of 1378 cm^{-1}, a weak shoulder is observed, being attributable to CH_2 deformation. For irradiated hyaluronan, the peak is shifted to 1380cm^{-1}. A strong peak at a frequency of 1219 cm^{-1} in control HA was reduced in intensity in the 100 Gy irradiated sample and shifted to 1222 cm^{-1} (Ellis 2007).

In the spectra of control HA, a peak corresponding to the vibrational mode of C-O-C is seen at a frequency of 947 cm^{-1}. In irradiated HA this suffers a slight shift, to 954 cm^{-1}. For control HA, the C-O-C stretching, O-H deformation and C=O deformation modes combine to form a peak at 894 cm^{-1}, shifted to 847 cm^{-1} in the sample irradiated to a dose of 100 Gy. Another strong sharp peak is seen at 791 cm^{-1} in control HA, changing to 801 cm^{-1} in irradiated HA, the height of the peak also decreasing in the irradiated samples.This peak is attributed to the C-H bonds (Ellis 2007).

The C-C-C def (deformed) vibrational mode can be seen at 543cm^{-1} for control HA. In irradiated HA there is a prominent peak at 599 cm^{-1}, resulting either from a peak shift of the CCC def peak or from a skeletal vibration mode of HA. Additional peaks in the irradiated samples that can be ascribed to radiation crosslinking on hyaluronan were suspected to be found within peak 600-900 cm^{-1}.

Radiation has damaged the long polymer chains of hyaluronan, presumably through the action of free radicals (Lurie et al. 2003). This would impair its physiological function as a lubricant, and possibly in forming the complexes with aggrecan that are important to ECM biomechanics and physiology.

In our study, hyaluronan has been irradiated in the presence of water. Thus it is important to note that radiation has indirectly reacted with hyaluronan macromolecules through formation of free radicals originated from radiation interaction with water. Radicals produced by ionizing radiation are randomly distributed rather than site specifically, reacting randomly in tissue (Lurie et al. 2003). Many studies (Bates et al. 1984; Schiller et al. 2003; Yamazaki K et al. 2003) have demonstrated the role played by active-oxygen-derived species (ROS) in depolymerization of hyaluronan. ROS initiate or participate in numerous physiological and pathophysiological processes, such as inflammation, ischemia-reperfusion injury and aging as well as in rheumatoid arthritis in which degraded hyaluronan is found in affected articular joints.

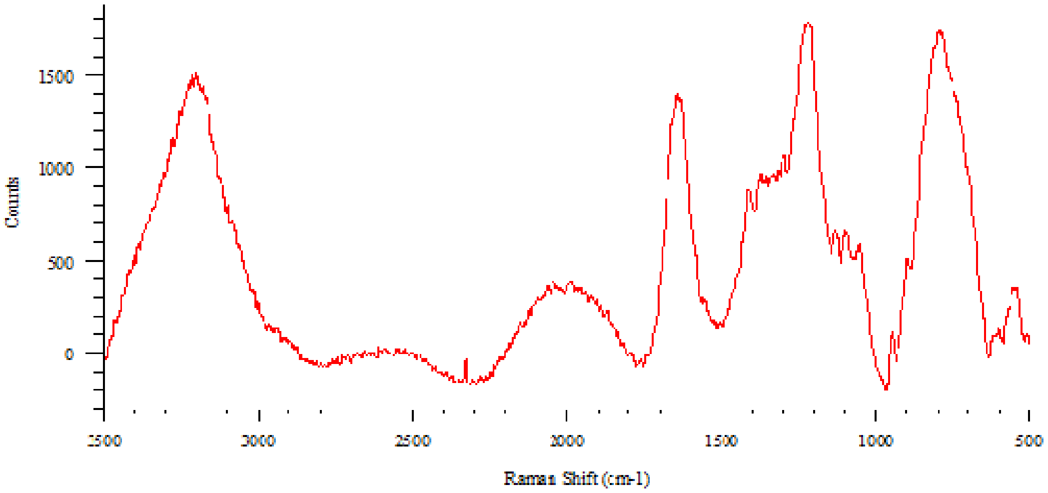
Lamberts et al. as cited by Go et al. has pointed out that the indirect action of irradiation under physiological conditions is generally to be due to the hydroxyl radicals. The sensitivity of hyaluronan to ionizing radiation, as determined by physical techniques has generally been attributed to glycosidic bond cleavage. Although chain scissions reduce the molecular weight, and hence the viscosity, contraction of molecular volume would also account for the observed effects apart from the bond rupture.

There has been considerable interest in the mechanism of degradation of hyaluronan by oxygen derived reactive species (ODRS). It has been suggested that the depolymerization of the molecule may account for the loss of synovial fluid viscosity which is a classic feature of infallammatory arthritis (Bates et al. 1984) Recent investigation has also concluded for hyaluronan that as the ·OH radical concentarations increase, there is an observed progressive degradation leading to a decrease in viscosity average molecular weights (Al-Assaf 2000).

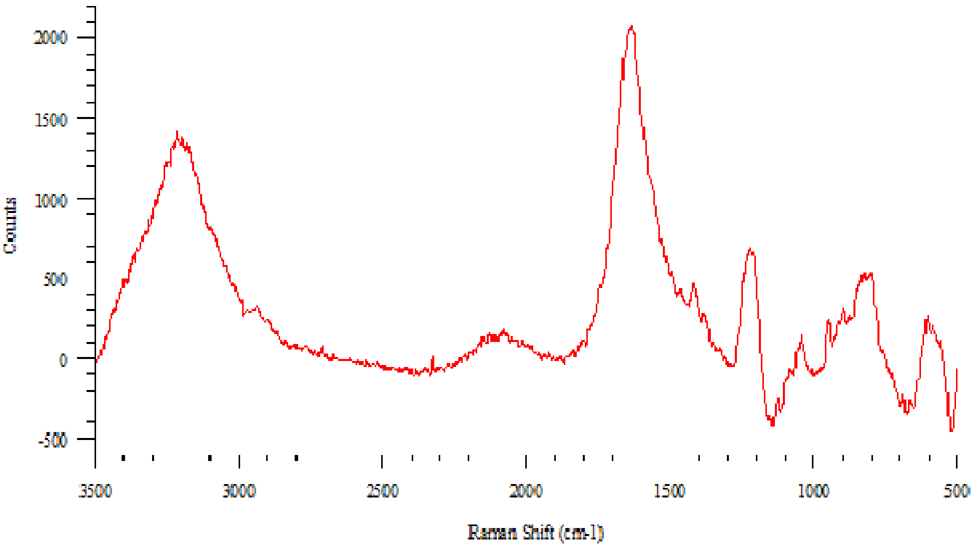
Protective agents against free radicals that induce depolymerization of hyaluronan are important. Mannitol and benzoate has been found to arrest the synovial fluid degradation by inhibiting deterioration of singlet oxygen radicals (Puig-Pallareda & Planas 1978). The scavenging effect of 2,6-diisopropylphenol on hydroxyl radicals produced by xanthine oxidase has also been found useful (Kvam et al. 1993). Study of hyaluronan when degraded by peroxynitrite has shown that thiourea is extremely effective as a protective agent, dimethyl sulfoxide is moderately effective and sodium benzoate and mannitol are only slightly effective (Li et al. 1997). Efforts are continuing in finding prospective drugs that display high capacity for inhibiting the free radical induced depolymerization reaction of the high-molecular-weight hyaluronan, especially in understanding the hyaluronan deficiency in joints and cartilage that concerns mostly inflammatory arthritis (Lal 1985; Mendoza et al. 2007; Carlin et al. 1985; Lisa A Plugge & Peter A Petillo 1999).

Raman Spectroscopic Study of Hyaluronan

The C-C-C def (deformed) vibrational mode can be seen at 543cm^{-1} for control HA. In irradiated HA there is a prominent peak at 599 cm^{-1}, resulting either from a peak shift of the CCC def peak or from a skeletal vibration mode of HA. Additional peaks in the irradiated samples that can be ascribed to radiation crosslinking on hyaluronan were suspected to be found within peak 600-900 cm^{-1}.



Control HA



100 Gy HA

Figure 1.7 Raman spectra of 0.25% w/v (in deionized-water) solution samples of hyaluronan, over the spectral range 500 – 3500 cm^{-1} for: (A) control HA and (B) HA irradiated to a dose of 100 Gy using high energy x-rays (B). Each spectrum is an average of at least 10 acquisitions (see text).

CONCLUSION

Exposure of ECM components to x-radiation has resulted in significant biomechanical and rheological modification. In the case of collagen, even at the low dose end of the range of x-ray doses under study (at 10Gy) significant increase in the initial modulus and decrease in the linear modulus has been observed. Similar effects were observed at 20 -50 Gy. However, we were not able to observe any pattern of changes which might easily be related to a dose dependency. For elastin, exposure to x-rays at doses ranging from 10 – 50 Gy results in a decrease in the Young's modulus value that again appears to be independent of the dose given. Conversely, in present studies involving exposure of hyaluronan to x-rays within the dose range 10 - 100 Gy, .the observed decrease in viscosity of hyaluronan is found to be dose dependent. Even at lower doses of ~ 10Gy, a reduction in viscosity is seen, although the largest decrease in viscosity is observed at 100 Gy. In future such studies, it is intended that the work concentrate on study of changes of viscosity at doses of ~ 1- 2 Gy, commensurate with the sorts of daily fractionated doses delivered in external beam teletherapy, in treatment of cancer patients.

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