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Raman spectroscopic study on sodium hyaluronate: an effect of proton and γ irradiation

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Raman spectroscopy was applied to the analysis of structural changes in lyophilised sodium hyaluronate after proton and γ irradiation (0.5, 5, 50, 100, 200 and 600 Gy). Characteristic Raman bands of the polysaccharide were sensitive to irradiation. Significant damage was observed at doses of 50 Gy or higher. The spectral changes confirmed radiation-induced loss of native solution conformation, destruction of primary structure, fragmentation, cross-linking and elimination of functional groups. Differences in the effects of proton and γ radiation on sodium hyaluronate are discussed.

Supporting information may be found in the online version of this article.

Keywords: sodium hyaluronate; proton irradiation; γ irradiation; Raman spectroscopy

Introduction

Hyaluronic acid (hyaluronan) is a macromolecular component of connective, epithelial and neural tissues of animals. It is present in the extracellular matrix, synovial fluids and capsules of some bacteria. Hyaluronic acid is a linear polysaccharide (glycosaminoglycan) composed of an alternating sequence of 1,3-linked *N*-acetyl-β-D-glucosamine (GlcNAc) and 1,4-linked β-D-glucuronic acid (GlcA) (Fig. 1). In aqueous solutions, hyaluronic acid chains have an expanded random coil conformation.[1,2] The size of coils varies with pH and salt concentration, as would be expected for a flexible polyelectrolyte.[3] In aqueous solutions, the conformation of hyaluronic acid is stabilised by weak and transient hydrogen bonds, which are in rapid interchange with water molecules.[2,4,5] In particular, the acetamido group of GlcNAc is rotated to form a direct hydrogen bond with the adjacent carboxylate of GlcA unit[1], with participation of single water molecule bridging both groups. In the solid state, the conformation and packaging of hyaluronic acid macromolecules depends on the pH, temperature and extent of hydration. Thus, several helical conformations of this polysaccharide (two-, three- and fourfold) have been observed.[6,7]

As a principal constituent of the extracellular matrix, hyaluronic acid contributes significantly to cell proliferation and migration. By retaining a large amount of water, this polysaccharide is able to control tissue hydration. Due to its viscoelastic properties, hyaluronic acid protects tissues against overuse and shocks. Depletion of this polysaccharide in tissues is often associated with various pathological states.[8] Hyaluronic acid is well recognised as an important determinant of cancer cell behaviour. This polysaccharide is able to interact with tumour cells through specific surface receptor and hyaluronan-binding protein, inducing signalling events to tumour cell survival, growth and migration.[8–11] Increased synthesis of hyaluronic acid in cancer cells may lead to formation of a less dense matrix that enhances tumour cell motility and invasion.[12] All the effects mentioned above lead to tumour growth and increase the speed of metastasis. Therefore, like biopolymers such as DNA and proteins, hyaluronic acid is interesting as possible molecular target of cancer radiotherapy. Structural alteration and destruction of this polysaccharide component of the extracellular matrix could make a contribution to the effectiveness of ionising radiation therapy of cancer diseases.

Raman spectroscopy has been shown to be a powerful tool for structural characterisation of glycosaminoglycans, including hyaluronic acid.[13–18] Raman band assignments for hyaluronic acid and its monomeric components have been reported in the literature.[16,19–21] Changes in specific Raman marker bands are able to give detailed information about the structural and conformational alterations in hyaluronic acid. Irradiation experiments with model biomolecules and tissues monitored by infrared (IR) and Raman spectroscopy have been used in the characterisation of possible mechanisms of radiation damage.[22–26]

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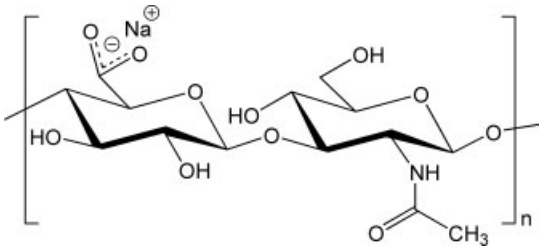

Figure 1. Structure of sodium hyaluronate.

In previous works[27,28], we reported that proton and γ irradiation led to evident but dissimilar changes in the Raman markers of dsDNA and serum proteins. Observed differences reflect specific lesions of macromolecules caused by protons and γ-rays dependent on the mechanism of the radiation effect. In this work, we use Raman spectroscopy to estimate specific dose-dependent alterations in sodium hyaluronate after proton and γ irradiation (0.5–600 Gy) in diluted aqueous solutions.

Experimental

Chemicals

Sodium hyaluronate was obtained from Sigma, USA. Its aqueous solution (~2% w/w) was prepared in deionised water and used for proton and γ irradiation experiments.

Irradiation Procedure

Proton radiation

Negative ions H^- were accelerated to an energy of 25 MeV by the isochronous cyclotron U-120M at the Nuclear Physics Institute ASCR, Czech Republic. Electrons were then stripped in a carbon foil and the negative ions were converted into protons with 100% efficiency. This arrangement enables high currents of extracted ions to be reached. Moreover, the extraction of negative ions from the acceleration chamber is simpler and more efficient in comparison with the extraction of positive ions by a deflection system.[29] The proton flux was shown to be uniform (±2%) over a circular beam area of 4-cm diameter with the actual measured points at the centre and at the distance of ±2 cm from the centre on the horizontal level. The proton dose was controlled by a UNIDOS electrometer with calibrated ionisation chamber NE 2581 located at a distance of 2 cm from the centre of the proton beam and corrected for the actual atmospheric pressure and temperature at the measurement site. The measured doses 49.5, 100.8, 201.0 and 599.9 Gy delivered in exposure times lasting between 17 and 180 or 240 s did not differ from the planned ones (50, 100, 200 and 600 Gy) by more than 1%.

γ radiation

Solutions of sodium hyaluronate were irradiated using a Chisotat S01 ^{60}Co-source (maximum activity 13 TBq). The ^{60}Co-source emits photons with energies of 1.17 and 1.33 MeV. The delivered doses of γ-rays were controlled by a calibrated monitor and comprised 0.5, 5, 50, 100, 200 and 600 Gy in exposure times lasting between 0.79 and 946 min.

The proton and γ irradiation procedures for smaller doses of 0, 0.5, 5 and 50 Gy were repeated two to three times.

Raman Spectroscopic Measurement

After radiation exposure, the solutions were shock frozen in liquid N_2, lyophilised and stored at −20 °C. Lyophilised samples and the standard, which underwent all these procedures without irradiation, were used for the spectroscopic analyses. Raman spectra were recorded by a Dilor-Jobin Yvon-Spex Raman spectrometer equipped with an Olympus BX 40 system microscope with 100× objectives. A He/Ne ion laser system with an excitation line at 632.8 nm and an excitation power of 16 mW was used for the measurements. Spectra are the accumulated averages of 10–15 exposures of 180 s each at room temperature. Average spectra from several experiments were smoothed by a 5 cm^{-1} filter, corrected by a polynomial baseline using LabSpec software and then used for analysis. Second derivatives of spectra were applied to detect the positions of overlapping bands. Some spectral regions of the average spectra were also analysed by the normalised least-squares curve-fitting procedure (PeakFit module of Origin 6.0 software) using multiple Voigt (Gaussian–Lorentzian mix) curves. The band positions obtained from the second derivative algorithm were used as the initial guesses for curve fitting of the smoothed and baseline corrected spectra. Best curve fitting was determined by the lowest possible χ^2 values.

Results

The FT Raman spectra of sodium hyaluronate in the 600–1800 cm^{-1} region before and after proton and γ irradiation are shown in Fig. 2, and assignments of Raman bands are summarised in Table 1.[1,19,30–35] The vibrational spectra in the presented range can be divided into several subregions: (1) stretching vibrations of carbonyl compounds (1800–1500 cm^{-1}), (2) deformation modes of CH_2 and C–OH (1500–1200 cm^{-1}), (3) CO and CC stretching vibrations (1200–950 cm^{-1}) and (4) complex skeletal out-plane vibrations (950–600 cm^{-1}).[30] Decreasing and broadening effects in the carbonyl stretching region (1800–1500 cm^{-1}) were more pronounced for γ-irradiation, whereas proton irradiation led to more evident band decreases in the next two regions (1500–1200 and 1200–970 cm^{-1}).

Sodium hyaluronate has acetamide and carboxylate groups originating from GlcNAc and GlcA units, respectively. The region of carbonyl stretching vibrations (1800–1500 cm^{-1}) of hyaluronate contains several highly overlapped bands. The main maximum was observed at 1651 cm^{-1} (amide I); shoulders at 1630 and 1600 cm^{-1} were assigned to antisymmetric stretching of COO^- and peaks and shoulders at 1535–1580 cm^{-1} to amide II vibration. Proton and γ irradiation led to subsequent dose-dependent decrease in intensity in this region. The curve-fitting analysis at 1800–1500 cm^{-1} was used for better band assignment and for following the changes in the components caused by irradiation. Results of the curve fitting are illustrated in Fig. S1 and Table S1 (Supporting Information). Assignment of the components obtained was made based on the work of Haxaire *et al.*[31,32] devoted to IR spectroscopic investigation of hyaluronan hydration. We assume that, as already reported for IR bands, the position of Raman bands of amide I (1674–1647 cm^{-1}), amide II (1596–1532 cm^{-1}) and antisymmetric stretching COO^- (1638–1601 cm^{-1}) vibrations will depend on the participation of these groups in the formation of intra- and intermolecular hydrogen bonds.

Relative areas of all the Voigt components were calculated, and expected trends of dose-dependent dynamics in their changes

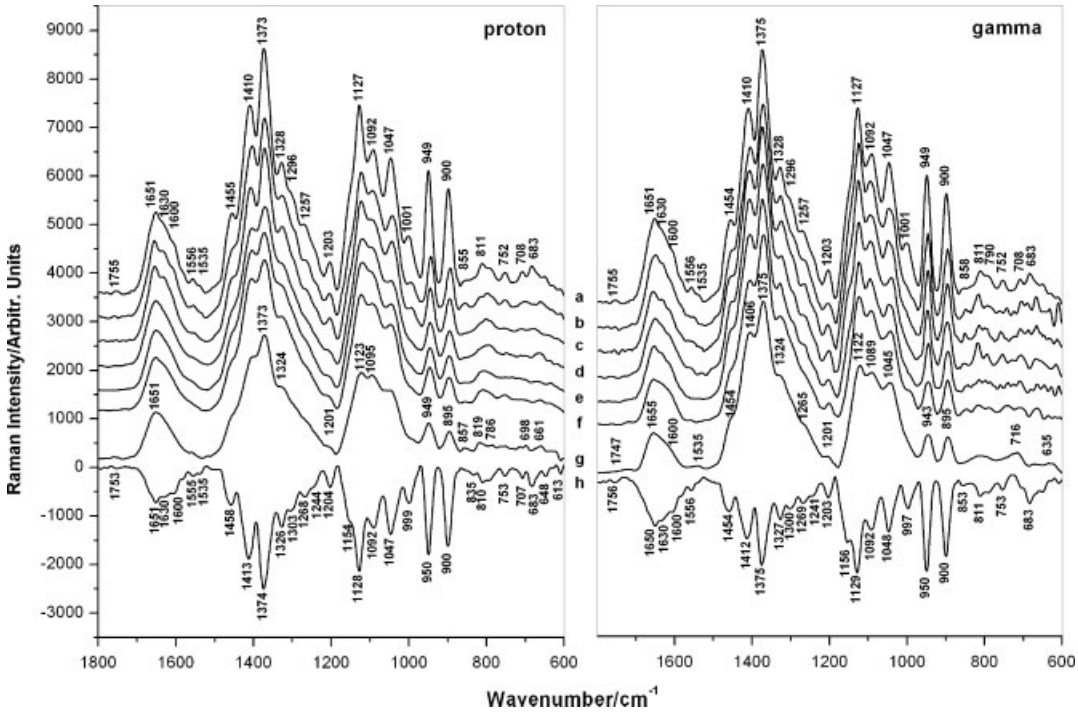

Figure 2. FT Raman spectra (1800–600 cm^{-1}) of sodium hyaluronate before and after proton (left panel) and γ (right panel) irradiation at different doses: (a) 0 Gy, (b) 0.5 Gy, (c) 5 Gy, (d) 50 Gy, (e) 100 Gy, (f) 200 Gy, (g) 600 Gy and (h) difference spectrum: (g) minus (a).

are illustrated in Fig. 3. Two components **A1** (1674–1665 cm^{-1}) and **A2** (1657–1647 cm^{-1}) were assigned to amide I vibration of amide groups, which accept, respectively, zero to one and one to two hydrogen bonds. These components have near equal areas in the case of non-radiated hyaluronate. After radiation, the area of both components decreased significantly; **A1** decayed more evidently for protons and **A2** for γ-rays. Therefore, non-destructed amide C═O groups accept more hydrogen bonds after proton irradiation and less after γ-irradiation. The next three components at 1638–1628 cm^{-1} (**B1**), 1616–1601 cm^{-1} (**B2**) and 1586–1581 cm^{-1} (**B3**) were assigned to carboxylates accepting, respectively, zero, one or two hydrogen bonds. The former component may have some contribution from the bending vibration of residual water in freeze-dried samples of sodium hyaluronate. The ratio of component areas was 4.4 : 2.3 : 1 for non-irradiated hyaluronate. Proton irradiation caused a moderate dose-dependent decrease in the **B1** and **B2** areas (with an exception of a slight increase in the **B2** area after 0.5 Gy) and an increase in the **B3** area. Significant decay of the **B1** area was observed for γ-irradiated hyaluronate, whereas **B2** and **B3** areas increased at several doses. Comparing the effects of proton and γ irradiation on these components and based on the decrease in the total area of **B1–B3**, we assume that destruction of the carboxylate group is more pronounced for γ-irradiation. In both cases, the rearrangement of residual carboxylates resulting in the formation of more hydrogen bonds may take place. The position of **B3** was shifted to 1596–1592 cm^{-1} after the highest doses of γ-radiation (200 and 600 Gy) – this may be explained by contributions by the new groups (COO^-, NH_2 or aromatics) appearing after destruction of the polysaccharide. The last two components at 1563–1550 cm^{-1} (**C1**) and at 1538–1532 cm^{-1} (**C2**) were assigned to amide II vibrations. Both the components are very weak because the amide II vibration is forbidden in Raman; larger **C1** characterises amide groups donating NH for hydrogen bonding and smaller **C2** arises from free amide NH groups. In most cases, irradiation led to significant decrease in or even disappearance of these components which could be a result of direct destruction of amide groups and package disordering by multiple molecular lesions. The decline was more expressive after proton irradiation then after treatment with γ-rays. Probably, protons cause ionisation of NH in amides, whereas γ-irradiation destructs the environment of these groups to a greater extent. In contrast, noticeable (slight) increase in **C1** area was observed after 100 Gy of γ (proton) irradiation that could be explained by the formation of new hydrogen bonds involving non-destructed amide NH groups as proton donors.

The next spectral region (1500–1200 cm^{-1}) contains several peaks assigned mainly to deformational modes of CH and C–OH. A symmetric stretching vibration of COO^- was found at 1410 cm^{-1}. This vibration is sensitive to hydrogen bonding[31–33], and the shift of this peak to 1406–1403 cm^{-1} caused by irradiation could be explained by the formation of more hydrogen bonds with participation of carboxylate. Peaks at 1455 and 1373 cm^{-1} were assigned to antisymmetric and symmetric bending vibrations of CH_3 groups; the former band has a contribution from CH_2 scissoring vibration. Subsequent decrease in these bands after irradiation points to the destruction of aliphatic groups (CH_3 and CH_2OH) of GlcNAc in hyaluronate. A peak at 1328 cm^{-1} was assigned mainly to an amide III vibration. It was shifted to 1325–1323 cm^{-1} after 5 Gy of irradiation; the shift was less pronounced for higher doses. A marked decrease in and broadening of this band was observed for 600 Gy for both protons and γ-rays.

Raman bands in the 1200–1000 cm^{-1} region originate mainly from CO and CC stretching vibrations. Peaks at 1092 and 900 cm^{-1} were assigned to antisymmetric and symmetric COC vibrations of β-glycoside linkages[36], the latter one has contributions from C1H and COH bending vibration.[37,38] The corresponding Raman

Table 1. Changes in the Raman markers of sodium hyaluronate after proton and γ irradiation

Wavenumber (cm^{-1})	Dose-dependent changes		Assignment[1,19,30–35]
	proton	γ	
1669sh	↓	↓	Amide I
1651	→1655, 1651	1648←, 1655	Amide I
	1638sh	1645sh	$\delta(H_2O)$
1630sh	↓	↓	$\nu_{as}(COO^-)$
1602sh	↓	↓	$\nu_{as}(COO^-)$
1588sh	↓	↓	$\nu_{as}(COO^-)$
1556	↓	↓	Amide II
1536sh	↓	↓	Amide II
1455	↓, →1461sh	↓, →1461sh	$\delta(CH_2)$, $\delta_{as}(CH_3)$
1410	→1402, ↓	→1406, ↓	$\nu_s(COO^-)$
1373	↓	↓	$\delta_s(CH_3)$
1328	↓, →1319	↓, →1324	Amide III, δ(CH), δ(COH)
1297sh	↓, →1288	↓, →1294	δ(CH), δ(COH), δ(CCH)
1271	↓, →1257sh	↓, →1262sh	$\omega(CH_2)$, δ(COH), δ(CCH)
1238sh	1242	1237	δ(COH), δ(CCH), δ(HCO)
1203	↓	↓	$\tau(CH_2)$, δ(CCH)
1156sh	↓	↓	ν(CO), ν(CC)
1127	↓, →1123	↓, →1122	ν(CO), ν(CC), δ(COH)
1092	1095←, ↓	1096←, ↓1089	ν_{as}(COC) – glycosidic bonds, δ(COH)
1047	→1042, ↓1047	1050←, ↓1045	ν(CO), ν(CC), δ(COH)
1001	→990, ↓	↓	$\rho(CH_3)$ – GlcNAc
950	→943, ↓949	→943, ↓	Amide V – ν(CNC)
900	→893, ↓895	↓, →895	ν_s(COC), $\delta(C_1H)$ – β-glycosidic bonds
853	857←, ↓	→843, ↓	$\rho(CH_2)$
811	820←, ↓	817←, ↓	$\delta(COO^-)$, $\gamma(COO^-)$, δ(CCO)
787sh	↓	801←, ↓	δ(OCO)
752	↓	↓	$\delta(COO^-)$
708	↓, 713, 698	→706, ↓, 716	γ(CCO), γ(CCH)
683	↓	689, 667, ↓	Ring breathing
650	659←, ↓	→646, ↓	γ(CCO), γ(CCH)
631	↓	↑↓	γ(CCO), γ(CCH)
610	↓	↑↓	γ(C=O), γ(CO), γ(CCO)

↓, ↑: decrease or increase in intensity; ←, →: shift to higher or lower wavenumber.

bands were found in the spectra of cellulose (β-1,4-glucan) and its oligosaccharides but were absent in the spectrum of β-D-glucose.[36] The assignment of the intense band at 949 cm^{-1} is not unequivocal. It has been assigned to the skeletal COC linkage and CC vibrations[1,19] as well as to the rocking vibration of CH_2.[39] This band, however, is absent in the Raman spectra of cellulose and laminarin (β-1,4- and β-1,3-glucans), so it cannot be an attribute of β-glycosidic bonds. However, the Raman spectra of *N*-acetyl-D-glucosamine[19,39] and chitin[40] have a very intense band at 973–953 and 950–954 cm^{-1}, respectively. Deacetylation of chitin leads to a shift to lower wavenumbers (942–948 cm^{-1}), a significant decrease in or even the disappearance of this band in the Raman spectra of chitosans depending on the degree of acetylation remaining. According to these observations, we suggest that the band of hyaluronate at 949 cm^{-1} could arise from vibration of the acetamide groups (amide V) in the GlcNAc units.

Two intense and well-resolved bands at 949 and 900 cm^{-1} mentioned above were chosen as a probe for amide and glycoside bonds, respectively. Proton and γ irradiations led to significant dose-dependent decrease, shift to lower wavenumbers and some broadening of these bands. The maximal decrease in two intense bands at 949 and 900 cm^{-1} was comparable after 600 Gy irradiations for both protons and γ-rays. Proton irradiation of 0.5 Gy led to the maximal shift (6–7 cm^{-1}) of both bands; for γ-irradiation of the same dose, the shift was less pronounced (2–5 cm^{-1}). The former band was shifted to 945 cm^{-1} after γ-irradiation and the latter band to 895 cm^{-1} for both types of radiation (600 Gy). Decay of the integral intensities of these bands could be owing to a cleavage of amide and glycosidic bonds, respectively; band broadening and shifts indicate changes in native conformation and package of macromolecules owing to destruction of molecular environment. Dose-dependent changes in the relative area and width of Raman bands at 949–945 and 900–895 cm^{-1} are illustrated in Fig. 4. The decline in the relative area of both peaks is more pronounced in the case of proton irradiation, especially for the minimal dose of 0.5 Gy. However, the effects were similar for both types of irradiation at the maximal dose of 600 Gy. The effects of proton and γ radiation on broadening of both bands were dissimilar. After 0.5 Gy of proton radiation, the widths were unchanged, but higher doses caused subsequent broadening. In the case of γ-radiation, a threshold dose of band broadening was 200 Gy for 950 cm^{-1} and 50 Gy for 900 cm^{-1}; at

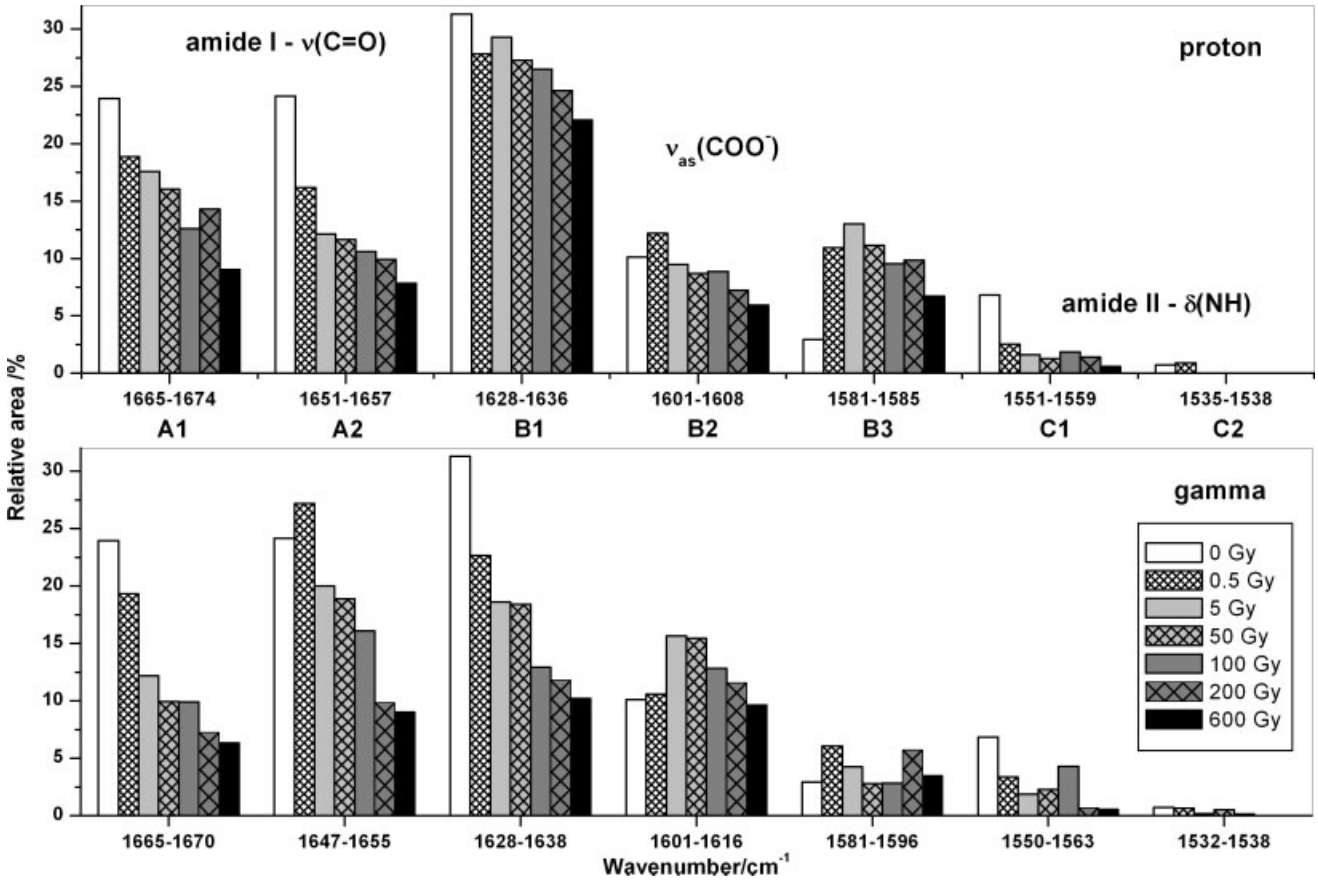

Figure 3. Relative areas (%) of the Voigt components for sodium hyaluronan before and after proton (top panel) and γ (bottom panel) irradiation at different doses (0, 0.5, 5, 50, 100, 200 and 600 Gy).

higher doses, the effect was the same as for the threshold dose (1.3 and 1.1, respectively). Maximal broadening of the band at 950 cm^{-1} (1.4 for protons, 1.3 for γ) was somewhat higher than that of the band at 900 cm^{-1} (1.2 for protons, 1.1 for γ).

The region of complex skeletal vibrations (853–600 cm^{-1}) underwent significant changes after proton and γ irradiation. This is an evidence of marked structural and conformational alterations in hyaluronate. Band assignment is very difficult in this region, and the literature data are poor. Thus, we cannot assign specific changes in Raman bands, only the overall effect on the region. Proton radiation led to a subsequent decrease in bands with broadening and wavenumber shifts in some cases. In contrast, γ rays caused an intensity increase (0.5- to 100-Gy doses) and the appearance of new bands.

Discussion

Ionising radiation produces a wide spectrum of lesions in hyaluronate: cleavage or modification of functional groups, chain fragmentation by cleavage of glycosidic bonds, appearing of new functions as a result of pyranoid ring rupture etc. It is well known that radiobiological effects depend on the nature of the radiation. In this context, a comparison of the effects of protons and γ-rays on sodium hyaluronate may help in understanding the damage mechanisms. In dilute aqueous solutions, the indirect effects of ionising radiation, i.e. those mediated by the solvent molecules, make the main contribution to the whole impact on dissolved biomolecules. Thus, the efficacy of protons and γ-rays in destructing specific molecular targets will depend on the relationship between primary and secondary products of water radiolysis, which attack selectivity-specific molecular targets.[41,42]

Irradiation effects of ultraviolet (UV) and γ-rays on polysaccharides have been investigated much more than those of proton radiation.[43–46] UV radiation reduces the viscosity of hyaluronic acid solutions and leads to its degradation.[43,45] Induction of oxygen radicals and singlet oxygen by UV radiation causes cleavage of glycosidic bonds and decarboxylation of hyaluronate. Oxygen radicals induce the cleavage of polysaccharide chains, whereas singlet oxygen causes change in the tertiary and quaternary structures and loss of their elastic properties.[46] The H· and ·OH radicals formed by water radiolysis are able to accelerate the molecular chain scission of hyaluronate. Reaction between the above-mentioned free radicals and polysaccharide molecules leads to rapid degradation of HA in aqueous solution.[45] The damaging effect of radiolytic radicals on the depolymerisation of hyaluronic acid is in the order of $HO\cdot > e^-_{aq} > O_2^-$. Model experiments confirmed that degradation of this polysaccharide by reactive radical species proceeds primarily at glucuronic acid and/or at *N*-acetylglucosamine residues depending on the reaction conditions and radical specificity.[47–49] Acid- and base-catalysed as well as pH-independent mechanisms of HO-induced degradation of hyaluronic acid and similar compounds have been suggested.[48] The latter process probably leads to breakage of both the types of glycosidic bonds in hyaluronate. Secondary processes, i.e. formation of macroradicals and their reactions, lead to

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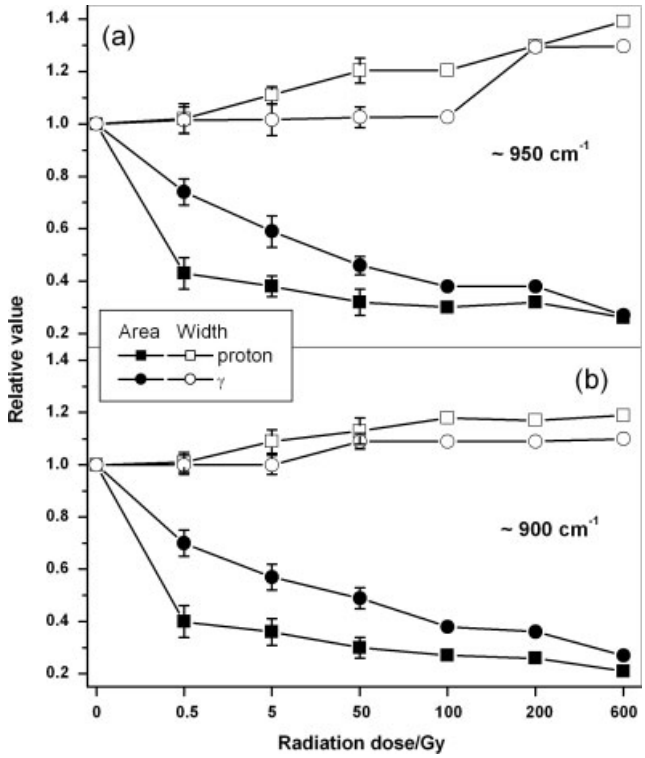

Figure 4. Relative values of area and width of the Raman bands at ~950 cm^{-1} (a) and ~900 cm^{-1} (b) as function of radiation dose (0–600 Gy) for proton- and γ -irradiated sodium hyaluronate.

further subsequent degradation of the polysaccharide. Two such macroradicals have been derived from HO attack at C5 on the glucuronic acid and at C6 on *N*-acetylglucosamine units.[50]

Kim *et al*.[44] reported that γ-irradiation decreases the molecular weight, viscosity and pH of hyaluronic acid in aqueous solution and changes it to more intense yellowish in colour. UV and FTIR spectra confirmed the formation of C=C bonds and new COOH groups in the polysaccharide solution after γ-irradiation (1–50 kGy). The structural changes in γ-irradiated macromolecules provide some evidence of the formation of a pyrancarboxylic acid ring. The radiation doses used in this study (0.5–600 Gy) were much less than those used by Kim *et al*.[44], so structural alterations detected by Fourier transform Raman were not so pronounced. Nevertheless, the analysis of the Raman spectra showed significant dose-dependent changes with a certain similarity for sodium hyaluronate irradiated by protons and γ-rays.

Various Raman marker bands of hyaluronate functional groups, i.e. carboxylates, *N*-acetyls and CH_2OH, were sensitive to irradiation. A shift in wavenumber position and/or broadening of these bands was explained as a result of alteration in conformation and packaging of the polysaccharide macromolecules; intensity decrease is evidence of the destruction of functional groups. Lower doses of radiation led to conformational changes in amide and carboxylate groups, whereas at higher doses, decarboxylation and cleavage of CN bonds occurred. In any case, destruction of native structural elements and the appearance of new functional groups led to rearrangement of the macromolecular package. Peak-fitting analysis of the carbonyl stretching region (1800–1500 cm^{-1}) led us to the conclusion that the whole hydrogen bond system of hyaluronate became stronger after irradiation. This effect could be connected with covalent cross-linking of polysaccharide chains induced by irradiation. Covalent linkages between macromolecules can draw together donor and acceptor groups supporting the formation of hydrogen bonds.

Despite the common dose-dependent trends in Raman spectra of irradiated sodium hyaluronate, specific changes in the characteristic bands of functional groups and glycosidic bonds indicate some differences in the effects of proton and γ radiation. Based on the analysis of dose-dependent spectral changes, we can conclude that decarboxylation (decrease at 1603–1600 cm^{-1}) and cross-linking (intensity increase and new bands at the far region) were more pronounced in the case of γ-rays, whereas proton radiation was more effective in cleavages of amide and glycosidic bonds (changes in the bands at 949 and 900 cm^{-1}). Therefore, we conclude that specific structural parts of sodium hyaluronate differ in their sensitivity to protons and γ-rays.

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Supporting information

Supporting information may be found in the online version of this article.

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