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Unexplored capabilities of chemiluminescence and thermoanalytical methods in characterization of intact and degraded hyaluronans

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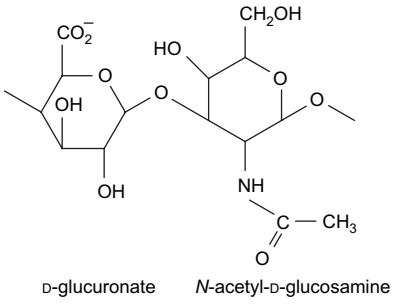
Abstract

Three intact and four degraded hyaluronans were investigated by using chemiluminometry, differential scanning calorimetry, and thermogravimetry. Degradation of hyaluronan was induced by a system containing H_2O_2 alone (882 mM); 55 mM H_2O_2 *plus* 1.25 μM $CuCl_2$; NaOCl alone (10 mM); and NaOCl *plus* $CuCl_2$ and ascorbic acid (10 mM, 0.1 μM, and 100 μM, respectively). The four different oxidative systems yielded biopolymer fragments represented by similar viscosity characteristics. The results obtained by using chemiluminescence and thermoanalytical methods indicate that hyaluronans of similar rheological properties could be distinguished from each other.

Keywords: Hyaluronan; Hyaluronan degradation; Chemiluminometry; Differential scanning calorimetry; Thermogravimetry; IR spectroscopy

1. Introduction

Hyaluronan (HA) is a linear glycosaminoglycan built of disaccharide repeating units comprising D-glucuronate linked through β-(1 → 3) glycosidic linkage to *N*-acetyl-D-glucosamine. These disaccharide structural units are linked via β-(1 → 4) glycosidic bonds.



Hyaluronan is an essential functional component of almost all tissues in the vertebrate organism. Various animal tissues – e.g. rooster combs, shark skin, bovine eyeballs – have been used as sources of isolation and production of high molar mass HAs. Since in biological materials hyaluronan is present in a complex system, linked and/or mixed with other biopolymers, several separation procedures have to be applied to isolate a pure compound [1,2]. The mean molar mass of the commercially available "extractive" HA preparations obtained from animal tissues is mostly in the range from several hundred thousands g/mol up to approximately 2500 kg/mol. To date, the demand for HA-based materials approved for applications in human medicine is being satisfied by high molar mass HAs prepared from rooster combs. For example, Healon® (Pharmacia & Upjohn, Inc., Peapack, NJ, U.S.A.) – used in viscosurgery at eye implant insertion – has a mean molar mass of about 2500 kg/mol.

Although animal tissues, primarily rooster combs, were involved at the early stages of production of the clinically utilizable materials approved by FDA, e.g. in eye surgery

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(Healon®), hyaluronans produced by microorganisms such as *Streptococcus zooepidemicus*, *Streptococcus equi*, etc. and isolated from the growth medium are currently offered by many companies at the market in the amount of several tons per year, as well. Some of these "fermentative" HA preparations meet the demand on molar mass in the range of several thousands of kg/mol [3,4]. The risk of mutation of the bacterial strains, possible "co-production" of various toxins, immunogens, etc., however, hinders the broader application of fermentative HAs in clinical practice. This is also the reason why hyaluronan originating from rooster combs is at present still preferred for human treatment in cases, when the material is to be introduced into the organism, e.g. in the eye, knee joint, etc. Microbial HAs have been approved for treatment of superficial wounds, as well as for the use in the cosmetic industry.

The mean molar mass of the commercially available HAs is distributed over a broad range, yet this parameter is often insufficiently specified for the marketed materials. Moreover, a frequently neglected fact is that both fermentative and extractive HAs may contain certain contaminating ingredients. A trace amount of proteins, e.g. in extractive HA samples, originates usually from the so-called "link proteins". The presence of "frozen"/crystalline water along with the traces of transition metal cations in the samples could pose potential risk of the reduction of the high molar mass of hyaluronans (even of those stored in the solid form) most probably due to their degradation by atmospheric O_2 [5] and the subsequent change of their properties. Hygroscopicity of dry hyaluronans is another complicating factor, when the solution with a precisely defined concentration of HA is required. Not only the ubiquitous bacteria or moulds, but also the accompanying contaminating substances (proteins, metal cations, etc.) must be critically assessed for their potential to degrade the polysaccharide chain of HA.

Under homeostatic conditions, HA exists as a high molar mass polymer that plays important role in maintaining structural integrity of the tissue. Under stress conditions, such as tissue injury, the biopolymer is readily degraded and a significant amount of lower molar mass fractions is formed. The biological properties of these fragments are different from those of the larger precursor molecules. While the high molar mass HA chains possess anti-angiogenic, anti-inflammatory, and immunosuppressive properties, intermediate-sized fragments act predominantly in an opposite way, i.e. they are highly angiogenic, pro-inflammatory, and immunostimulating.

Recently Camenisch and McDonald [6] pointed out the necessity to control the biological activity of commercial "intact" extractive and fermentative HAs of different molar masses, as well as that of the polymer fragments prepared by either physico-chemical methods or by partial digestion with hyaluronidases. They also proposed to validate the identity/differences of the samples by a set of certain bioanalytical procedures.

Validation of identity/differences of HA samples may be effected by various bioanalytical tools; several of established analytical methods being currently available for investigation of the polymers, including biopolymers. The approach based on the analysis of HA samples in the solid form by employing chemiluminometry, differential scanning calorimetry (DSC), and thermogravimetry (TG) was not explored yet when looking for the discrimination between the materials having similar molar mass characteristics. This is, however, an appropriate analytical procedure, in which a sample is heated from room temperature to the temperature approaching its thermal stability. An assumption has been put forward that any pre-degradation occurring prior to such testing should have specific impact on the resulting thermoanalytical characteristics of the material.

In the present study we have therefore prepared HA fragments by the degradation induced by H_2O_2 or NaOCl alone, as well as by their mixtures with $CuCl_2$ and ascorbic acid. This choice was determined by the fact that the above reagents may attack different structural units of hyaluronan. The degradation was carried out to attain the same final dynamic viscosity of HA solution, which was monitored by measuring the rotational viscosity. The potential of chemiluminometry, DSC, and TG to reveal the differences in the intact and degraded HAs has been evaluated for impacts of the methods to discriminate sample identities/differences.

2. Experimental

2.1. Biopolymers

The three samples of intact high molar mass hyaluronans used throughout the study were provided by Dr. E. Orviský, formerly affiliated with the National Institute of Rheumatic Diseases, Piešťany, Slovak Republic (sample F1750762, $M_w = 1378$ kg/mol; $M_w/M_n = 1.61$), by Dr. K. Thacker from Lifecore Biomedical Inc., Chaska, MN, U.S.A. (sample LIFECORE P9710-2, $M_w = 1215$ kg/mol; $M_w/M_n = 1.79$), and by the company CPN Ltd., Ústí nad Orlicí, Czech Republic (sample CPN, $M_w = 659.4$ kg/mol; $M_w/M_n = 1.88$) [7].

2.2. Chemicals

NaCl and $CuCl_2 \cdot 2H_2O$, analytical purity grade, were from Slavus Ltd., Bratislava, Slovakia, ascorbic acid was from Merck KGaA, Darmstadt, Germany. Ethanol ($\approx$96%, v/v) and aqueous solution of H_2O_2 ($\approx$30%) were purchased from Chemapol, Prague, Czech Republic. Aqueous solution of NaOCl ($\approx$1 M; containing 6–14% active chlorine) was the product of Riedel de Haen AG, Seelze, Germany. The actual concentrations of both H_2O_2 and NaOCl aqueous solutions were determined by photometric methods [8,9]. Water used was of redistilled deionised quality.

2.3. Preparation of the solutions for HA degradation

The solution of hydrogen peroxide (8.82 M) was prepared by dissolving NaCl in the commercial H_2O_2 solution to a salt concentration of 0.15 M. Similarly, the solution of cupric chloride (16.0 μM) and that of ascorbic acid (16.0 mM) were prepared in 0.15 M water solution of NaCl. The solution of

sodium hypochlorite (392 mM) was prepared by diluting the commercial NaOCl solution in redistilled de-ionized water (dissociation of NaOCl at neutral pH due to the value of $pK_a = 7.53$ results in nearly equimolar concentration of undissociated acid (HOCl) and the ions of ClO^-).

2.4. Degradation studies by the method of rotational viscometry

Four different reaction systems were employed in the studies of degradation of the high molar mass HA sample – LIFECORE P9710-2: (a) containing 882 mM H_2O_2, (b) containing 55 mM H_2O_2 *plus* 1.25 μM $CuCl_2$, (c) containing 10 mM NaOCl, and (d) containing 10 mM NaOCl *plus* 0.1 μM $CuCl_2$ and 100 μM ascorbic acid.

The biopolymer – 20.0 mg – was dissolved in 0.15 M aqueous NaCl. The dissolution procedure was carried out overnight in the dark at room temperature in two steps. First, 4.0 ml solvent was added. Next, 3.2 ml (system a), 3.325 ml (system b), 3.796 ml (system c), or 3.696 ml (system d) of the solvent was added after 6 h. After 24 h, 800 μl of the H_2O_2 solution was added to the formed gel-like solution under moderate stirring during 30 s (system a). For investigating the system involving H_2O_2 *plus* $CuCl_2$, 625 μl of the $CuCl_2$ solution was added into the gel-like HA solution 9 min before application of 50 μl of the H_2O_2 solution. As for the system involving 10 mM NaOCl, 204 μl of the NaOCl solution was added into the gel-like HA solution and stirred moderately for 30 s (system c). For the system involving NaOCl *plus* $CuCl_2$ and ascorbic acid, 50 μl of the $CuCl_2$ solution was added into the gel-like HA solution 9 min before application of 50 μl of ascorbic acid solution, and 204 μl of NaOCl solution (system d) was added subsequently.

The resulting solution (8.0 ml) was immediately transferred into the Teflon® cup reservoir of the rotational viscometer. The record of the viscometer output parameters started 2 min after the experiment began. The changes of dynamic viscosity (η) and torque were monitored at 25 ± 0.1 °C by using a digital rotational viscometer Brookfield DV-II+ PRO (Brookfield Engineering Labs., Inc., Middleboro, MA, U.S.A.) equipped with a cup-spindle pair built of Teflon® at our laboratory [10]. At the spindle rotational speed of 180 rpm, the shear rate equaled 237.6 s^{-1}. The LIFECORE P9710-2 sample degradation was monitored until the nominal η value 5.8 mPa s was reached. Within the monitored interval of η values, the torque ranged between 72 and 36%.

To assay the reaction outcome, the reservoir content (at the nominal η value 5.8 mPa s) was poured into 20 ml ethanol, which led to precipitation of the polymer. On the following day, the polymer precipitate was washed out with 20 ml ethanol, centrifuged, and dried in a desiccator. The pellet prepared from the dried polymer obtained from the degradation system involving hydrogen peroxide was very firm. On the other hand, the pellet prepared from the dried polymer obtained from the degradation system involving sodium hypochlorite was brittle, and the odour of chlorine was felt even after the precipitation of the degraded sample.

2.5. Chemiluminometry

Chemiluminescence measurements were performed with a photon-counting instrument Lumipol 3 manufactured at the Polymer Institute of the Slovak Academy of Sciences. The sample was placed on an aluminium pan in the sample compartment. The gas flow (pure oxygen or nitrogen) through the sample cell was 3.0 l/h. The temperature in the sample compartment of the apparatus was raised from 40 up to 220 °C, with a linear gradient 2.5 °C/min. The signal of the photocathode was recorded at 10 s data collection interval. The amount of samples used for each measurement ranged from 0.98 to 1.62 mg.

2.6. Differential scanning calorimetry

Calorimetric measurements were performed using a Mettler–Toledo DSC 821[e] differential scanning calorimeter. Indium was used for calibration of temperature and heat of fusion. Glass transition temperature (T_g) and thermal stability of the samples were evaluated from the second heating of samples from room temperature up to 550 °C (10 °C/min) in a nitrogen atmosphere (50 ml/min). The first heating (from room temperature up to 170 °C) was used for water removal. Thermooxidative decomposition was investigated in a temperature range from room temperature up to 550 °C (10 °C/min) in an oxygen flow (50 ml/min). The amount of samples applied ranged between 0.80 and 2.77 mg. At least three parallel runs were performed for each sample.

2.7. Thermogravimetric analysis

Thermogravimetry was performed using a Mettler–Toledo TGA/SDTA 851[e] instrument in a nitrogen or oxygen flow (30 ml/min) using a heating rate of 10 °C/min in a temperature range from room temperature up to 550 °C. Indium and aluminium were used for temperature calibration. The amount of samples applied ranged between 0.28 and 3.45 mg. Two parallel runs were performed for each sample.

2.8. FTIR spectroscopy

The FTIR spectra were acquired by using a NICOLET Magna 750 spectrometer equipped with DGTS detector and OMNIC 7.1 software (Thermo Electron Corp., Madison, WI, U.S.A.). ATR (Attenuated Total Reflectance) accessory with ZnSe crystal was used for scanning the spectra. Number of scans was 128, at a resolution of 4 cm^{-1}.

3. Results and discussion

3.1. Rotational viscometry

Fig. 1 shows the loss of HA solution viscosity during the sample treatment on applying the four different oxidative systems (a), (b), (c), and (d). As can be seen, in all cases the viscosity of the solution decreases rather quickly.

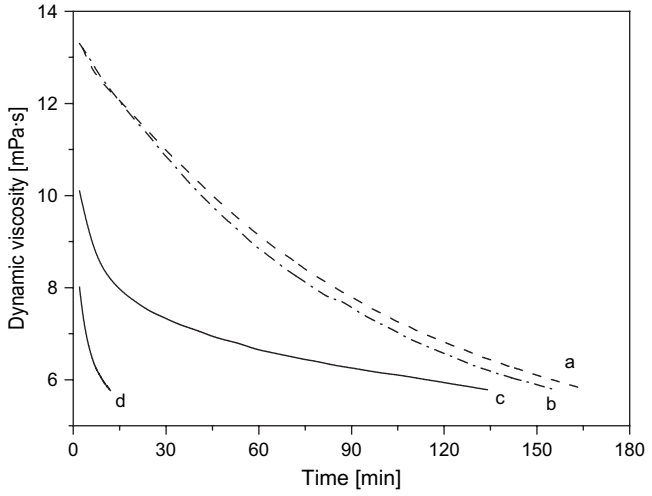


Fig. 1. The dynamic viscosity versus time profile of a 0.25% (w/v) solution of LIFECORE P9710-2 sample incubated with (a) 882 mM H_2O_2; (b) 55 mM H_2O_2 *plus* 1.25 μM $CuCl_2$; (c) 10 mM NaOCl; (d) 10 mM NaOCl *plus* 0.1 μM $CuCl_2$ and 100 μM ascorbic acid.

Solution of the Lifecore P9710-2 sample (2.5 mg/ml) upon application of the aqueous solution of hydrogen peroxide (882 mM ≡ 3%) has shown a rapid decline of dynamic viscosity value of 5.8 mPa s, which lasted about 165 min (curve a in Fig. 1). As previously reported [11], reduction of H_2O_2 concentration from 882 to 55 mM led to a slower decrease of the molar mass; however, addition of a micromolar amount of transition metal (namely 1.25 μM Cu^{2+}) contributed to the viscosity decrease so significantly that its degradative effect could be compared to that of 882 mM H_2O_2 (cf. Fig. 1, curves a and b). As evidenced by our results, the two different experimental conditions (a) and (b) resulted in practically identical kinetics of η decrease.

Sodium hypochlorite (10 mM) added to the solution of Lifecore P9710-2 sample also resulted in a rapid decrease of the dynamic viscosity. The magnitude $\eta = 5.8$ mPa s was reached after 134 min (cf. curve c in Fig. 1). However, as can be seen, introduction of Cu^{2+} (namely 0.1 μM $CuCl_2$) together with ascorbic acid (100 μM) resulted in an even more accelerated degradation of the high molar mass hyaluronan, and the similar decrease of η value to 5.8 mPa s is being attained in less than quarter of an hour (12 min; cf. Fig. 1, curve d).

The reduction in the viscosity of the above mentioned samples is closely related to the reduction of molar mass of HA, which is induced by the respective reagents. Below the suggestions of the possible mechanisms of this phenomenon are provided.

3.2. Overview of the potential reactions leading to the changes of the molar mass of HA samples

An idea may be adopted, which is quite common in polymer chemistry, that scission of the hyaluronan macromolecule occurs as a β-scission of oxyl radicals at lower temperatures. Oxyl radicals, which appear on HA molecule are usually the reaction intermediates from the decomposition of polymer hydroperoxides. Polymer hydroperoxides of hyaluronan could be formed during the sample storage in the presence of air or during the HA treatment with free-radical initiators at the pre-degradation sequence of reactions. This simple assumption opens new possibilities for investigation of HA characteristics.

Hydrogen peroxide − one of the reagents used − does not react with hyaluronan directly. However, traces of transition metals present in HA samples may catalyze the decomposition of H_2O_2 with the generation of hydroxyl radicals ($HO^{\bullet}$). In the LIFECORE P9710-2 sample, among other transition metals (iron = 13 ppm, chromium < 3 ppm, cobalt < 3 ppm, nickel < 3 ppm, cadmium < 1 ppm, arsenic < 1 ppm, mercury < 1 ppm), a 4 ppm concentration of copper ions has been claimed [“Certificate of Analysis” (Lifecore Biomedical Inc., Chaska, MN, U.S.A.)]. The sequence of reactions of transition metal ions with hydrogen peroxide involves the well known Haber−Weiss scheme, in which the transition metal ions in lower and higher oxidation states enter the redox reaction with hydrogen peroxide leading to the generation of very reactive hydroxyl radicals. The formation of hydroxyl radicals is particularly important in the system containing hydrogen peroxide and a given concentration of transition metal ions, especially copper ions, which significantly accelerate degradation of HA [11].

Hydroxyl radicals attack hyaluronan macromolecule with formation of alkyl radicals $A^{\bullet}$ having the free-radical sites in the positions 1 and 4 of the D-glucuronate unit. In the presence of oxygen, these alkyl radicals rapidly react with it to give peroxyl radicals, which enter the reaction with HA to yield hydroperoxides

$$HO^{\bullet} + HA \rightarrow H_2O + A^{\bullet}$$

$$A^{\bullet} + O_2 \rightarrow AOO^{\bullet}$$

$$AOO^{\bullet} + HA \rightarrow AOOH + A^{\bullet}$$

The decomposition of AOOH, either spontaneous or induced by transition metal ions, yields alkoxyl radicals, which are presumed intermediates of the main chain splitting

$$AOOH \rightarrow AO^{\bullet} + {}^{\bullet}OH$$

$$AOOH + Cu^{+} \rightarrow AO^{\bullet} + {}^{-}OH + Cu^{2+}$$

The attack of hydroxyl radicals on D-glucuronate or *N*-acetyl-D-glucosamine moieties of HA can also lead to the “opening” of rings without breaking the polymer chain [12,13]. However, subsequent transformation of the generated *C*-centred radicals to peroxyl radicals leads to hydroperoxides as well, and subsequently results in the biopolymer fragments having a reduced dynamic viscosity.

The degradative action of hypochlorous acid on HA has a somewhat different chemistry. Recently Rees et al. [14] showed that the reaction of HOCl with the *N*-acetyl group of *N*-acetyl-D-glucosamine moieties of HA led to the formation

of long-lived *N*-chloroamides. The reaction then proceeds with the production of the *N*-centred amidyl radicals via both metal ion-dependent and ion-independent processes. These *N*-centred radicals undergo rapid isomerisation to give carbon-centred radicals at *C*-2 of the *N*-acetyl-D-glucosamine units (via a 1,2-hydrogen atom transfer) and at *C*-4 of the neighbouring D-glucuronate moieties (via a 1,5-hydrogen atom transfer). In the presence of oxygen, peroxyl radicals and hydroperoxides may be formed as well. This may represent the initial events for the reduction of the molecular mass of the HA.

In our opinion, however, another alternative exists, namely the formation of an intermediate product – hyaluronan hypochlorite. This might be formed as the product of the reaction of hydroxyl groups on hyaluronan moieties (AOH) with HOCl according to the following reactions

$$AOH + HOCl \rightarrow AO{-}Cl + H_2O$$

$$AOH + Cl_2 \rightarrow AO{-}Cl + HCl$$

where Cl_2 is the ''active'' chlorine present in NaOCl solution or chlorine formed due to the reaction between dissociated HOCl and chloride anions (NaCl solution used).

Such intermediate hypochlorites decompose readily to yield alkoxyl radicals and chlorine atoms (cf. system c)

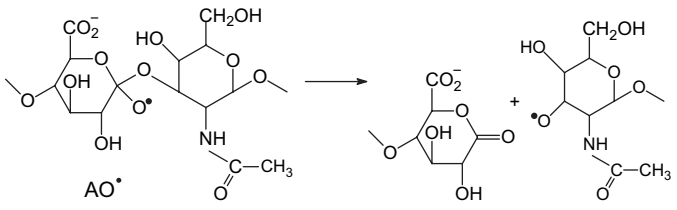
$$AO{-}Cl \rightarrow AO^{\bullet} + Cl^{\bullet}$$

On applying system d, due to a high reducing capability of ascorbic acid, the copper cations are present in their lower oxidation states. The cuprous ions thus decompose the intermediate hypochlorites by producing alkoxyl radicals much faster

$$AO{-}Cl + Cu^{+} + H^{+} \rightarrow AO^{\bullet} + HCl + Cu^{2+}$$

The fragmentation of the generated alkoxyl radicals ($AO^{\bullet}$) and the reduction of the molar mass of HA should be similar to that depicted by Scheme 1.

The FTIR analysis (ATR technique) of the HA samples, treated by four different oxidative systems, did not, however, reveal any changes in the spectral characteristics of the biopolymers. As can be anticipated from Scheme 1, the HA fragments formed would be most probably the macromolecules with a halved degree of polymerization, of which one fragment should have a terminal carbonyl group and the other one a terminal hydroxyl which have been formed from the terminal alkoxyl radical. However, FTIR analysis of such a ''bicomponent'' mixture was not capable of revealing differences that would arise from a single scission of the high molar mass sample. For Lifecore P9710-2 having



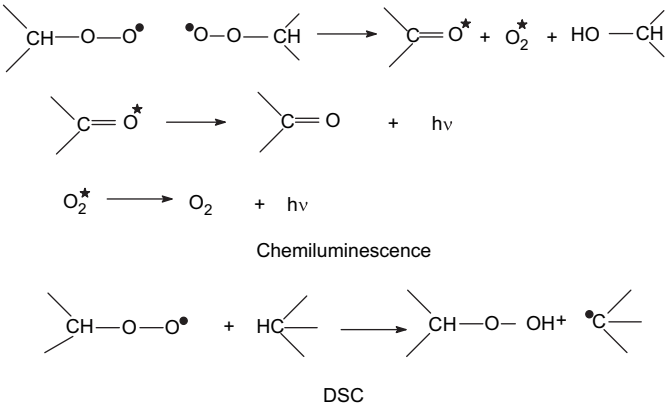
Scheme 1.

$M_w = 1215$ kg/mol and $M_w/M_n = 1.79$, the number of the repeating disaccharide units can be calculated as ca. 1700, taking into account $M_n \approx 680$ kg/mol and the molar mass of the disaccharide ≈ 400 g/mol. This means that the chemical modification – introduction of a new carbonyl group – represents a change smaller than 600 ppm.

3.3. Treatment of intact and degraded hyaluronans in the inert and oxidizing atmospheres at steadily increasing temperatures

3.3.1. Comparison of the intact samples

Chemiluminescence and DSC records of three different intact samples of hyaluronans in oxygen and nitrogen are presented in Figs. 2 and 3. At the highest temperature used (220 °C), the chemiluminescence intensity is much more pronounced in oxygen than in nitrogen, the same being valid for the rates of the heat release. This may be an indication that both chemiluminescence and heat release (DSC) are governed predominantly by the reactions of peroxyl radicals or eventually by the decomposition of the hydroperoxides formed. In the case of chemiluminescence, the most probable candidate reaction providing light emission is the disproportionation of peroxyl radicals [15–24], whereas in the case of DSC it is propagation reaction of peroxyl radicals



Asterisks denote the excited states of ketones (triplet) and oxygen (singlet), which are transferred to the ground state with the emission of light.

The chemiluminescence method, which reflects the disproportionation of peroxyl radicals, monitors the progress of the oxidation in a material under examination. The rate of disproportionation (termination) in a free-radical process is directly proportional to the rate of initiation. The signal intensity I depends on the rate of the oxidation process w according to the following relationship

$$I = \Phi w$$

The proportionality coefficient Φ is not constant when switching from one material to another; it depends on the geometry of the experiment, on the presence of quenchers of excited states, as well as on several other factors, which become known only after a detailed study of the respective system.

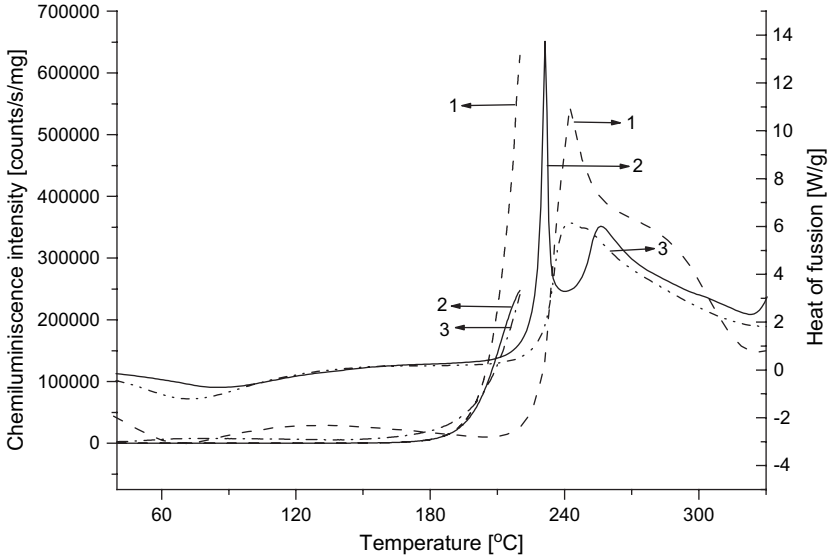


Fig. 2. The chemiluminescence and DSC non-isothermal runs for intact hyaluronans in oxygen. Samples: F1750762 (1), LIFECORE P9710-2 (2), and CPN (3).

The rate of oxidation, however, is a real variable that depends on the mechanism of the oxidation (degradation) process.

By the nonlinear regression analysis of chemiluminescence – temperature course assuming that the rate of degradation is composed of several processes having different rates of initiation, the rate constants of the oxidative degradation may be calculated. This procedure was described in detail elsewhere [25]. The average values of rate constants for 40, 100, and 200 °C are given in Table 1. From the values for the intact samples, one can immediately compare the residual quality of the respective samples. For example, at both 100 and 200 °C the best rating is obtained for F1750762 and the worst one for CPN sample.

The areas below the curves for chemiluminescence measurements in the temperature range 40–220 °C and the areas of exotherms for DSC measurements within the range 40–330 °C (Table 2) are much more pronounced in oxygen than in nitrogen. DSC curves at temperatures higher than 330 °C revealed further exothermic peaks – which, however, belonged to the carbonization process of the originally cross-linked residues – the case is not discussed here.

The most intense chemiluminescence signal at 220 °C was observed for the sample F1750762 in both the nitrogen and oxygen atmospheres (cf. Figs. 2 and 3). Taking into account an assumption that the total area below the chemiluminescence curves corresponds to the amount of oxidizable sites, one may conclude that Lifecore P9710-2 sample has the lowest, while the sample F1750762 has the highest concentration of oxidizable sites (cf. Table 2). Sample CPN, whose molar mass is lower than those of both the Lifecore P9710-2 and

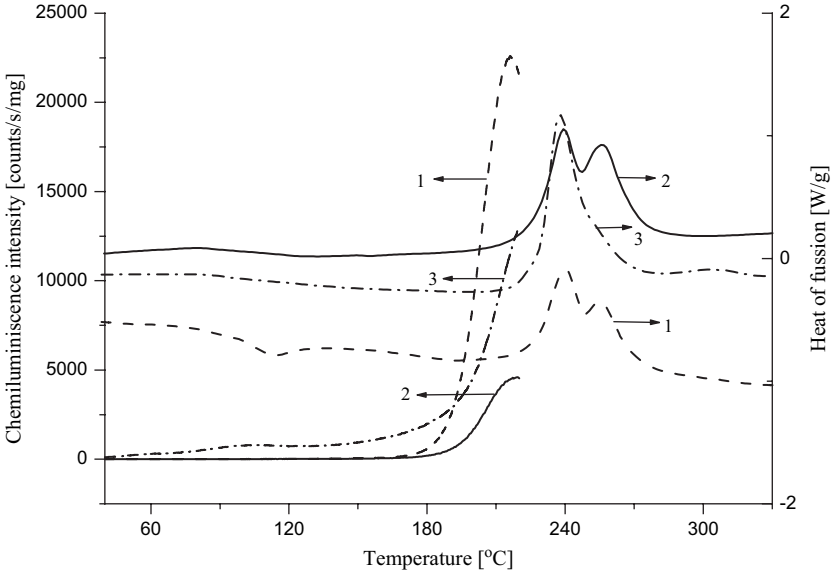


Fig. 3. The chemiluminescence and DSC non-isothermal runs for intact hyaluronans in nitrogen. Samples: F1750762 (1), LIFECORE P9710-2 (2), and CPN (3).

Table 1
The rate constants (in s^{-1}) of oxidation in oxygen for intact and degraded hyaluronans determined from non-isothermal chemiluminescence measurements

Sample	Temperature (°C)		
	40	100	200
F1750762	1.6×10^{-9}	2.8×10^{-8}	6.1×10^{-5}
CPN	7.3×10^{-6}	8.1×10^{-5}	1.3×10^{-3}
Lifecore P9710-2	1.1×10^{-9}	8.3×10^{-8}	2.8×10^{-4}
HP882[a]	3.9×10^{-10}	1.7×10^{-6}	3.9×10^{-4}
HP55-CU1.25[a]	5.3×10^{-8}	4.7×10^{-6}	9.5×10^{-4}
HOCL10[a]	1.7×10^{-8}	4.0×10^{-7}	2.0×10^{-3}
HOCL10-CU0.1-AA100[a]	3.7×10^{-8}	1.7×10^{-6}	1.1×10^{-3}

[a] Sample codes HP882, HP55-CU1.25, HOCL10, and HOCL10-CU0.1-AA100 relate to HA fragmented by using systems a, b, c, and d, respectively.

F1750762 samples, contains very reactive degradable sites, which is evidenced by a well measurable chemiluminescence at the temperatures as low as 90 °C (Figs. 2 and 3). The chemiluminescence intensity thus reflects the history of the sample indirectly indicating the previous extent of oxidation. It is of interest that chemiluminescence signals evidently precede those recorded by DSC (Figs. 2 and 3). The latter method obviously monitors not only the oxidation but also other exothermic processes like decomposition of hydroperoxides and crosslinking. The rather large amount, 19–43%, of charred residue (cf. Table 3), which remained after treatment of the samples at high temperatures indicates that degradation of the sample creates reaction by-products, which may crosslink at the elevated temperatures.

DSC runs for CPN sample in oxygen and nitrogen also revealed only one main exothermic peak situated around 240 °C, whereas both F1750762 and Lifecore P9710-2 samples show an additional exothermic shoulder at around 260 °C (cf. Figs. 2 and 3). The latter may indicate a two-step mechanism in formation of the final charred residue, which again may have some relation with the sample history.

Thermal characteristics obtained from the DSC and thermogravimetric analyses in both nitrogen and oxygen are collected in Table 3. As evident, for intact samples only slight differences between the temperatures corresponding to the first decomposition peaks (T_{exo1}) were found. However, a marked decrease (by 35 °C) of glass transition temperature values (T_g) in the samples order CPN > Lifecore P9710-2 > F1750762 is evident. As can be seen from Table 2 and chemiluminescence measurements in nitrogen, the higher extent of peroxidation obviously increases T_g of HA probably because of enhanced stiffness of the polymer chains due to introduction of more polar groups. Thus, T_g of different HA samples may also serve as the first criterion for their discrimination.

Experiments in nitrogen show even much higher discrimination between different samples (Fig. 3). According to the chemiluminescence measurements, the sample CPN contains a noticeable amount of instable peroxides, which start to decompose already at 90 °C. This may be deduced from the increased level of chemiluminescence intensity at temperatures below 90 °C for sample CPN (line 3 in Fig. 3).

3.3.2. Comparison of the degraded samples

Figs. 4 and 5 show chemiluminescence and DSC non-isothermal records in oxygen and nitrogen for the sample Lifecore P9710-2 degraded in the presence of hydrogen peroxide. When compared with the intact Lifecore P9710-2 sample, considerably lower intensities of chemiluminescence at 220 °C were found. Integral chemiluminescence (S_{CL}) for

Table 2
The surface (S) under chemiluminescence (S_{CL}) (40–220 °C) and DSC (S_{DSC}) (40–330 °C) curves for intact and degraded hyaluronans

Sample	Atmosphere	S_{CL} (counts/g)	S_{CLO}/S_{CLN}[b]	S_{DSC} (J/g)	S_{DSCO}/S_{DSCN}[c]
F1750762	O_2	1.38×10^{11}	13.4	3399	23.1
	N_2	1.03×10^{10}		147	
CPN	O_2	1.08×10^{11}	13.4	1479	7.9
	N_2	8.05×10^{9}		188	
Lifecore P9710-2	O_2	8.37×10^{10}	40.4	1560	8.2
	N_2	2.07×10^{9}		191	
HP882[a]	O_2	4.23×10^{10}	13.2	1268	8.8
	N_2	3.19×10^{9}		143	
HP55-CU1.25[a]	O_2	1.44×10^{10}	17.0	860	4.2
	N_2	8.49×10^{8}		203	
HOCL10[a]	O_2	3.90×10^{10}	34.5	n.a.	n.a.
	N_2	1.13×10^{9}		n.a.	
HOCL10-CU0.1-AA100[a]	O_2	3.64×10^{10}	25.1	n.a.	n.a.
	N_2	1.45×10^{9}		n.a.	

n.a. – The HA samples initially containing hypochlorite(s) were not analyzed by DSC method because of the corrosive effect of released chlorine and/or HCl vapors on the instrument.

[a] Sample codes HP882, HP55-CU1.25, HOCL10, and HOCL10-CU0.1-AA100 relate to HA fragmented by using systems a, b, c, and d, respectively.

[b] S_{CLO} and S_{CLN} are the surfaces under chemiluminescence curves observed in the atmospheres of oxygen and nitrogen, respectively.

[c] S_{DSCO} and S_{DSCN} are the surfaces under DSC curves observed in the atmospheres of oxygen and nitrogen, respectively.

Table 3
DSC and thermogravimetry characteristics of intact and fragmented hyaluronans

Method	HA material code						
Parameter	F1750762	LIFECORE P9710-2	CPN	HP882[a]	HP55-CU1.25[a]	HOCL10[a]	HOCL10-CU0.1-AA100[a]
Rotational viscometry							
η (mPa s)	12.26	12.08	5.80	5.8	5.8	5.8	5.8
Differential scanning calorimetry (in N_2)							
T_g (°C)	90	106	125	141	89	n.a.	n.a.
T_{exo1} (°C)	240	240	238	211	238	n.a.	n.a.
ΔH (J/g)	323	366	390	340	451	n.a.	n.a.
Differential thermogravimetric analysis (in N_2)							
T_{onset} (°C)	229	228	232	196	228	n.a.	n.a.
Residue (%)	31	29	19	36	45	n.a.	n.a.
Differential scanning calorimetry (in O_2)							
T_{exo1} (°C)	241	232	241	229	232	n.a.	n.a.
ΔH (J/g)	6770	3171	2516	2477	2131	n.a.	n.a.
Differential thermogravimetric analysis (in O_2)							
T_{onset} (°C)	228	225	230	196	227	n.a.	n.a.
T_{DTG1} (°C)	240	240	237	209	235	n.a.	n.a.
Residue (%)	40	37	43	65	46	n.a.	n.a.

n.a. – The HA samples initially containing hypochlorite(s) were not analyzed by DSC method because of the corrosive effect of released chlorine and/or HCl vapors on the instrument. Temperatures: T_g = glass transition; T_{exo1} = first exothermic peak; T_{onset} = onset; T_{DTG1} = first DTG peak. ΔH = exothermic heat for the whole temperature interval under investigation. Residue = charred residue after the temperature run.

[a] Sample codes HP882, HP55-CU1.25, HOCL10, and HOCL10-CU0.1-AA100 relate to HA fragmented by using systems a, b, c, and d, respectively.

sample HP882 in nitrogen (3.19×10^9 counts/g) exceeded the value for intact Lifecore P9710-2 sample (2.07×10^9 counts/g; cf. Table 2), which resulted from a considerably higher level of peroxides – giving a quite distinct decomposition peak at 130–140 °C (line 2 in Fig. 5). The large extent of sample peroxidation by a (highly) concentrated hydrogen peroxide is of interest. Such oxidative treatment with H_2O_2 evidently leads to the formation of a less stable material documented by a considerably smaller area below the DSC curve in O_2 (cf. Table 2; 860 or 1268 J/g versus 1560 J/g). The observation that only small amount of the intact HA remained for the test procedure is generally valid for all three methods applied – chemiluminescence, DSC, and thermogravimetry. The products of degradation due to the treatment with hydrogen peroxide (and with NaOCl) apparently tend to undergo crosslinking reactions, occurring at elevated temperatures of the test, which explains the presence of a higher amount of the charred residue in the final stages of the thermal treatments, namely 36 or 45% versus 29% in nitrogen and 65 or 46% versus 37% in oxygen (cf. Table 3).

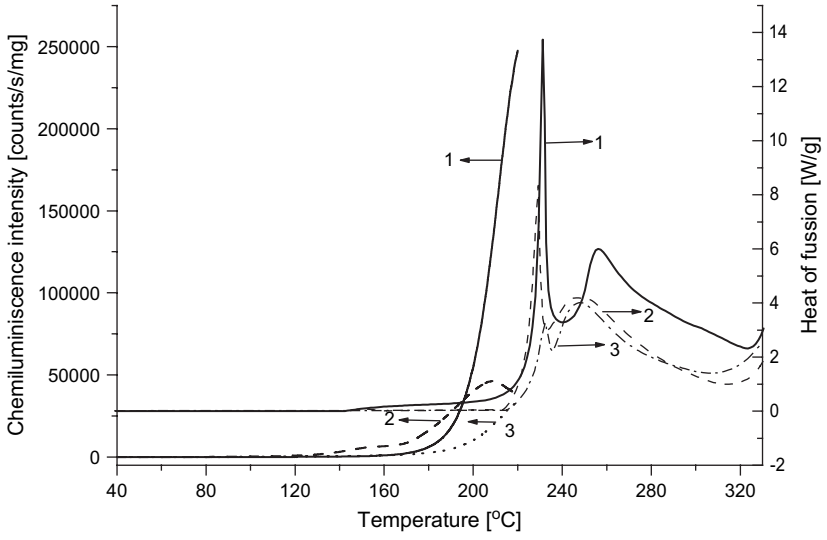


Fig. 4. The chemiluminescence and DSC non-isothermal runs in oxygen for the sample Lifecore P9710-2 degraded in the presence of hydrogen peroxide. Samples: LIFECORE P9710-2 (1), HP882 (2), and HP55-CU1.25 (3). Sample codes HP882 and HP55-CU1.25 relate to HA fragmented by using systems a and b, respectively.

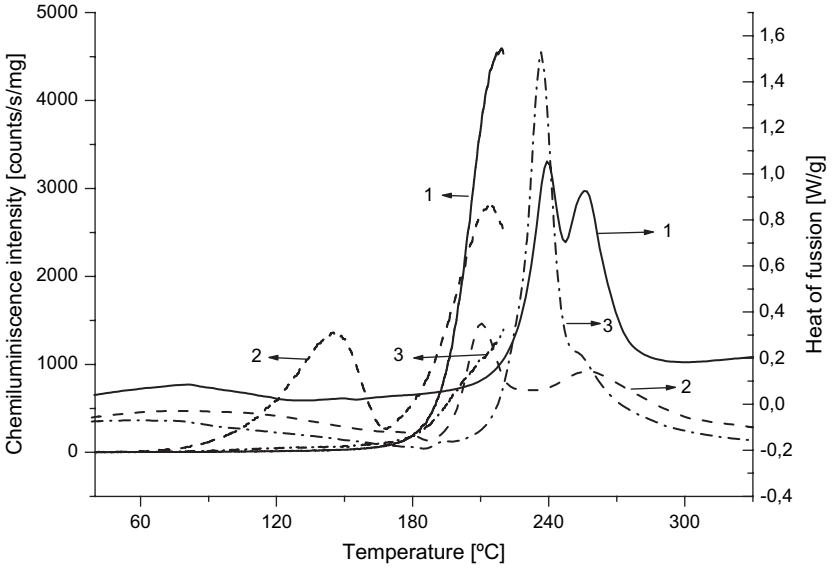


Fig. 5. The chemiluminescence and DSC non-isothermal runs in nitrogen for the sample Lifecore P9710-2 degraded in the presence of hydrogen peroxide. Samples: LIFECORE P9710-2 (1), HP882 (2), and HP55-CU1.25 (3). Sample codes HP882 and HP55-CU1.25 relate to HA fragmented by using systems a and b, respectively.

The presence of a minute amount of Cu^{2+} ions in the degradative system (system b) resulted in a change in the DSC curve of the recovered sample HP55-CU1.25, where one main decomposition peak with a small shoulder was observed (Fig. 5). The onset temperatures of the Lifecore P9710-2 sample and that of the sample treated with H_2O_2 *plus* $CuCl_2$ are identical; however, the sample modified with H_2O_2 alone showed a significant decrease of T_{onset} (Fig. 6, Table 3), which was due to the effect of the larger amount of peroxides formed in the samples treated with 882 mM hydrogen peroxide (cf. line 2 in Fig. 5). Due to this fact, the sample treated with 882 mM H_2O_2 had the lowest thermal stability.

The average rate constant at 40 °C of oxidation of the degraded samples (Table 1) is the highest for the sample oxidized by the system comprising 55 mM H_2O_2 *plus* 1.25 μM $CuCl_2$ ($5.3 \times 10^{-8}\ s^{-1}$), followed by the sample prepared by a treatment with NaOCl *plus* $CuCl_2$ and ascorbic acid ($3.7 \times 10^{-8}\ s^{-1}$), and the sample, at preparation of which NaOCl alone was applied ($1.7 \times 10^{-8}\ s^{-1}$). The lowest rate constant at 40 °C ($3.9 \times 10^{-10}\ s^{-1}$) was established for the sample prepared by applying a rather high H_2O_2 concentration. This order is in a rather good agreement with the degradation schemes proposed above. The peroxides on the HP882 sample at 40 °C are still quite stable; however, the concentration of reactive sites after the treatment of the sample Lifecore P9710-2 is significantly reduced so that the rate constant is even lower than for the intact hyaluronan ($1.1 \times 10^{-9}\ s^{-1}$). On the other hand, at higher temperatures, such as e.g. at 200 °C, the lowest stability was found for the sample Lifecore P9710-2 pre-degraded with NaOCl alone (the rate constant is $2.0 \times 10^{-3}\ s^{-1}$), followed by the sample prepared by the tri-component mixture of NaOCl *plus* $CuCl_2$ and ascorbic acid ($1.1 \times 10^{-3}\ s^{-1}$). The sequence is ended by the two samples, at preparation of which hydrogen peroxide was applied – namely $9.5 \times 10^{-4}\ s^{-1}$ for the HP55-CU1.25 sample and for hyaluronan treated by (highly) concentrated hydrogen peroxide $3.9 \times 10^{-4}\ s^{-1}$.

Finally it should be pointed that the rate constants at 200 °C for any degraded HA samples ranging between $3.9 \times 10^{-4}\ s^{-1}$ and $2.0 \times 10^{-3}\ s^{-1}$ are higher than the value estimated for the intact Lifecore P9710-2 sample – $2.8 \times 10^{-4}\ s^{-1}$. Thus, the thermal stability at 200 °C of these degraded samples is rather low as compared to that of the parent sample Lifecore P9710-2.

4. Conclusions

The samples of hyaluronans prepared from different sources may be well discriminated by chemiluminescence and thermoanalytical methods. The most sensitive of the methods appears to be chemiluminescence, which in nitrogen atmosphere detects the presence of various relative levels of initially present peroxides. These may be the decisive reactive groups determining the final properties of hyaluronans including the resulting molar mass. The artificial degradation of hyaluronan with hydrogen peroxide, hydrogen peroxide and Cu ions, NaOCl, and other agents promotes the emergence of differences between the individual samples. While molar mass of hyaluronans in living organisms is somewhere around 2000–3000 kg/mol, the manufacturers supply the samples of molar mass at 1500 kg/mol and less. Isolation procedures and efforts to prepare hyaluronans with lower molar mass may contaminate the supplied material with metal ions and subsequently with peroxides. From this viewpoint, discrimination between the different samples as demonstrated by the above described methods of thermal analysis and particularly by

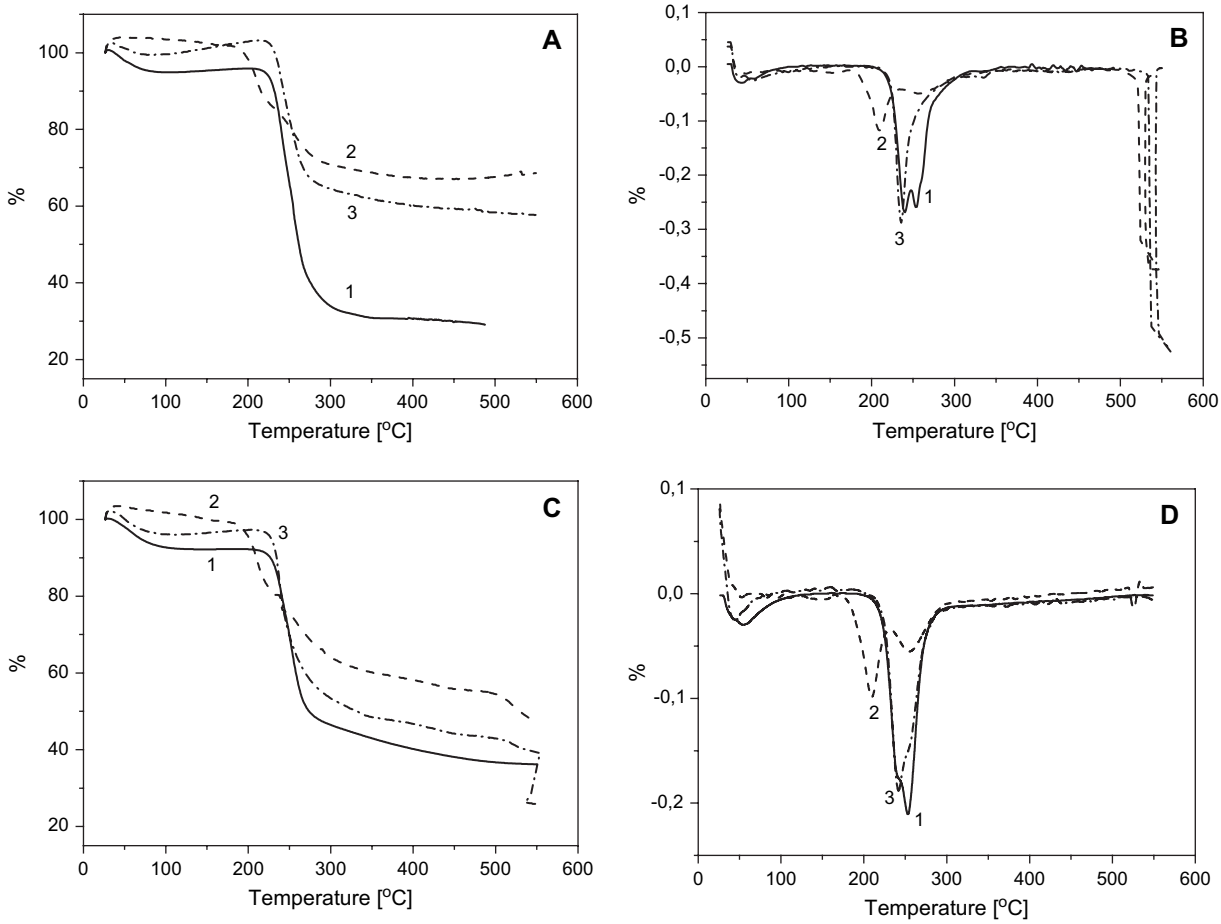


Fig. 6. Non-isothermal thermogravimetry runs for intact and degraded Lifecore P9710-2 in oxygen (panel A) and derived data (panel B), and in nitrogen (panel C) and derived data (panel D). (The initial decline of non-isothermal TG line is due to the loss of water evaporated from the sample at the beginning stages of temperature increase.) Samples: LIFECORE P9710-2 (1), HP882 (2), and HP55-CU1.25 (3). Sample codes HP882 and HP55-CU1.25 relate to HA fragmented by using systems a and b, respectively.

chemiluminescence, appears to be of high importance when hyaluronans are destined for a direct application in human body.

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