Efficiencies of fragmentation of glycosaminoglycan chloramides of the extracellular matrix by oxidising and reducing radicals: potential site-specific targets in inflammation?

Sambulelwe Sibanda, Almabrok Akeel, Stephen W Martin, Andrew W J Paterson, Ruth Edge[1], Saphwan Al-Assaf[2] and Barry J Parsons*

Faculty of Health and Social Sciences, Leeds Metropolitan University, Calverley Street, Leeds, LS1 3HE, UK

[1]Dalton Cumbrian Facility, The University of Manchester, Westlakes Science and Technology Park, CA24 3HA

[2]Phillips Hydrocolloids Research Centre, Glyndwr University, Wrexham, LL11 2AW , UK

*Author for correspondence

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Abstract

Hypochlorous acid and its conjugate base, hypochlorite ions, produced in inflammatory conditions, may produce chloramides of glycosaminoglycans, the latter being significant components of the extracellular matrix (ECM). This may occur through the binding of myeloperoxidase directly to the glycosaminoglycans. The N-Cl group in the chloramides is a potential selective target for both reducing and oxidising radicals, leading possibly to more efficient and damaging fragmentation of these biopolymers relative to the parent glycosaminoglycans. To investigate the effect of the N-Cl group, ionising radiation has been used to produce quantifiable concentrations of the reducing radicals, the hydrated electron and the superoxide radical and also of the oxidizing radicals, hydroxyl, carbonate and nitrogen dioxide, all of which have been reacted with hyaluronan and heparin and their chloramides in the current study . PAGE gels calibrated for molecular weight have allowed the consequent fragmentation efficiencies of these radicals to be calculated.

Hydrated electrons were shown to produce fragmentation efficiencies of 100% and 25 % for hyaluronan chloramide (HACl) and heparin chloramide (HepCl), respectively. The role of the sulphate group in heparin in the reduction of fragmentation can be rationalized using mechanisms proposed by Davies and co-workers (Rees et al. *J. Am. Chem. Soc.* **125**: 13719-13733; 2003) in which the initial formation of an amidyl radical leads rapidly to a C-2 radical on the glucosamine moiety. The latter is 100% efficient in causing glycodsidic bond breakage in HACl but only 25 % in HepCl, the role of the sulphate group being to favour the non-fragmentory routes for the C-2 radical. The weaker reducing agent, the superoxide radical, did not cause fragmentation of either HACl or HepCl although kinetic reactivity had been demonstrated in earlier studies.

Experiments using the oxidizing radicals, hydroxyl and carbonate, both potential *in vivo* species, show significant increases in fragmentation efficiencies for both HACl and HepCl, relative to the parent molecules. The carbonate radical has been shown to be involved in site-specific reactions at the N-Cl groups, reacting via abstraction of Cl, to produce the same amidyl radical produced by one-electron reductants such as the hydrated electron. As for the hydrated electrons, the data support fragmentation efficiencies of 100% and 29% for reaction of carbonate radicals at N-Cl for HACl and HepCl respectively. For the weaker oxidant, nitrogen dioxide, no fragmentation was observed, probably attributable to a low kinetic reactivity and low reduction potential. It seems likely therefore that the N-Cl group can direct damage to extracellular matrix glycosaminoglycan chloramides which may be produced under inflammatory conditions. The *in vivo* species , the carbonate radical is also much more likely to be site-specific in its reactions with such components of the ECM than the hydroxyl radical.

Graphical abstract
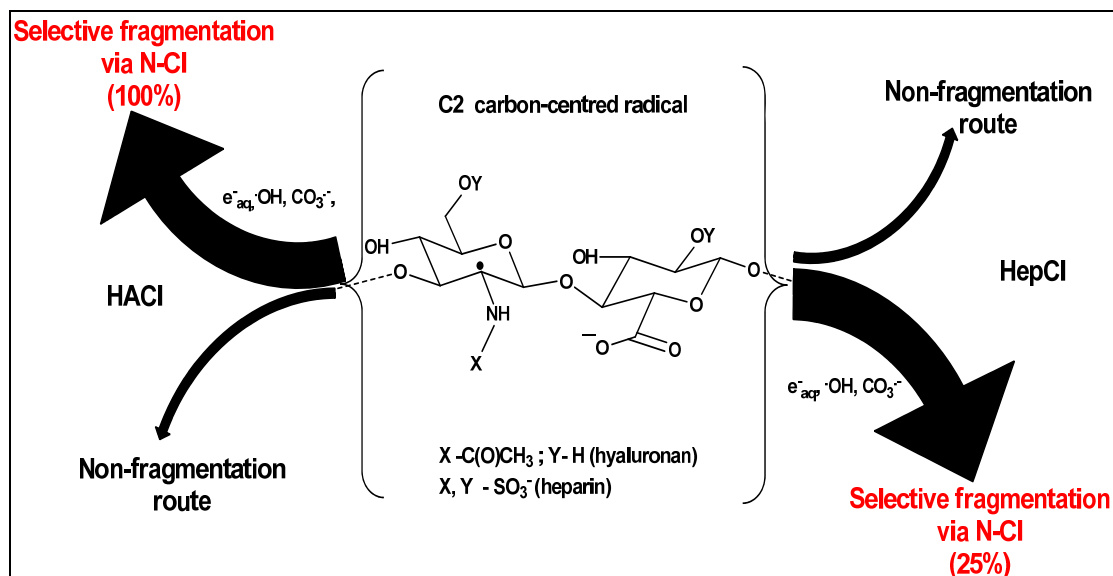
Introduction

The extracellular matrix (ECM) is made up of huge multi-molecular complexes with arrays of link proteins and aggrecan molecules along a central hyaluronan backbone. Hyaluronan (HA) is bound by a number of ECM and cell surface proteins [1,2]. With this central structural function, HA is a particularly important component of the ECM [3, 4], as demonstrated by the fact that a hyaluronan synthase-2 knockout is embryonically lethal in mice [5]. HA also provides a hydrated environment [6] for growing, moving and renewing cells and tissues [7], activates signalling events in cells and is involved in moderating many cellular processes, including proliferation, migration, adhesion and apoptosis [8-11]. HA appears to have a range of significant biological functions dependent upon its molecular mass. Large molecular mass fragments are involved in space-filling and immunosuppressive roles, whilst smaller HA fragments have been shown to be pro-inflammatory and angiogenic; oligosaccharides may be involved in cell signalling (reviewed in [12]).

Oxidative damage of the extracellular matrix components by either enzymatic or non-enzymatic pathways may have implications for the initiation and progression of a range of human diseases. These include arthritis, kidney disease, cardiovascular disease, lung disease, periodontal disease and chronic inflammation. Oxidative damage to hyaluronan by reactive oxidative species, and in particular by free radicals, has received much attention, largely through the ease of monitoring its fragmentation using viscometric techniques, which is reviewed in [13]. The potential mechanism of oxidative damage to the ECM and its role in human pathologies has also been discussed in a recent review [14].

Several reactive species may be formed at sites of inflammation, including superoxide (O_2^{-}) , hydrogen peroxide, hypochlorite (HOCl/ OCl$^-$) and peroxynitrite (ONOO$^-$ / ONOOH). The latter species may be formed *in vivo* by the diffusion-controlled reaction between superoxide and nitric oxide ($k= 6.7 \times 10^9$ M^{-1} s^{-1}) [15] where nitric oxide is generated by macrophage inducible nitric oxide synthase and endothelial nitric oxide synthase [16,17] Our previous studies on HA have measured HA fragmentation yields as a proportion of quantifiable fluxes of free radicals produced by ionising radiation. For this purpose, both viscosity changes and a combination of gel permeation chromatography with multi-angle laser light scattering were used to measure changes in molecular weight of the polydispersed hyaluronan. In this way, the efficiencies of fragmentation of HA by a range of free radicals and reactive oxidative species including hydroxyl radicals, carbonate radicals, dibromide and dichloride radical anions and peroxynitrite were determined [18,19].

The fragmentation of hyaluronan and other glycosaminoglycans has also been investigated intensively by Davies and co-workers using both electron paramagnetic resonance (EPR) spectroscopy and sensitive PAGE techniques. The use of the latter technique showed the novel and potentially biologically significant result that peroxynitrous acid, carbonate and hydroxyl radicals react largely in a site-specific process to produce an array of HA fragments, in a "ladder-type display" each separated from its neighbour by the molecular mass of the repeating disaccharide unit in HA, thus mimicking to a significant extent the action of the enzyme, hyaluronidase [20,21]. Similar site-selective fragmentation was also observed when glycosaminoglycan chloramides (formed through reaction with hypochlorite) were reduced by copper (I) ions and superoxide anion radicals [22,23].

The formation of chloramides and chloramines from the reaction of hypochlorite with amides and amines respectively was demonstrated in an early study [24] and is suggested to be a key process in inflammation, in which hypochlorite (from myeloperoxidase) may produce glycosaminoglycan chloramides. *In vitro* studies of the reactions of hypochlorite with glycosaminoglycans have indeed demonstrated that chloramides are produced in yields and rates of reaction which are dependent upon both pH and ratio of hypochlorite to glycosaminoglycan concentrations [25, 26] and have demonstrated that such derivatives may accelerate the fragmentation of glycosaminoglycans within the ECM [24]. Chloramides are weak oxidizing agents and are therefore potential biological targets for reducing radicals and other reducing agents through reaction at the N-Cl group. Indeed, it has been shown that both superoxide radicals and transition metal ions cause the fragmentation of HA through reaction with its chloramide derivative [22]. In the case of the heavily-sulphated heparan sulphate, both Cu(I) and Fe(II) were also found to produce fragmentation of its chloramide derivative. An estimate of the efficiency of the fragmentation process was made in a PAGE experiment using a standard octasaccharide for calibration of molecular weight in a completely decomposed chloramide: a yield of 50% was estimated [27].

In this study, ionizing radiation was used to produce selected oxidizing and reducing free radicals whose concentrations can be both determined with both significant accuracy and precision, and can be controlled. In this way, free radicals can be reacted with the chloramide derivatives of hyaluronan and heparin, the latter being the most heavily-charged glycosaminoglycan. The oxidizing radicals were hydroxyl ($^{\cdot}OH$), carbonate ($CO_3^{\cdot-}$) and nitrogen dioxide ($NO_2^{\cdot}$) , all potential *in vivo* species produced via peroxynitrite and other reaction pathways [28,29]. The reducing radicals selected in

this study were the hydrated electron (e^-_{aq}) and superoxide (O_2^-). The hydrated

electron e^-_{aq} is strongly reducing and may be expected to be highly, perhaps

100%, selective in its attack. It acted as a model in this study for less strongly-

reducing agents such as glutathione disulphide anion radicals. Superoxide, a much

weaker reducing species, was also investigated in this study. The main aim was to

measure, for the first time, the efficiencies of fragmentation of hyaluronan and heparin

chloramides by all these free radicals and thereby to deduce, by comparison with

experiments carried out under the same conditions with the parent glycosaminoglycans,

whether the N-Cl group confers selectivity of attack to enhance fragmentation. Any

enhanced fragmentation efficiency seen with chloramide derivatives relative to parent

molecules is of clear relevance to inflammation within the extracellular matrix where

these derivatives are likely to be formed and may therefore present site-specific targets

for free radicals and other reactive species.

Materials and methods

(i) Materials

Sodium formate, hypochlorous acid, *tert*-butanol and sodium bicarbonate were all

analytical grade (Sigma Aldrich). Hyaluronan (80kDa) was a gift from Novozymes ;

heparin sodium salt (Alfa Aesar) was of research grade (the structures of hyaluronan

and heparin are shown in Scheme 1). De-ionised water used for buffer preparation was

prepared by a Select Purewater 300 system (resistivity 18 MΩ).
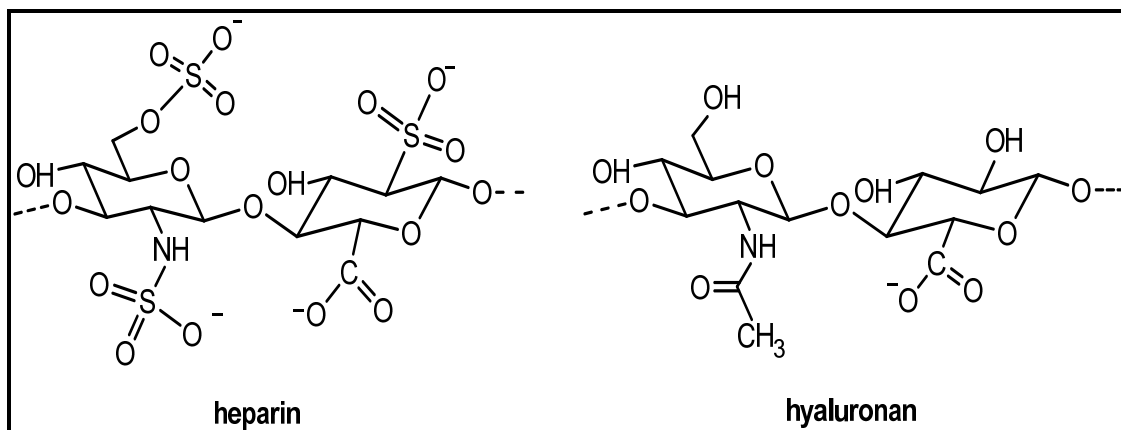
Scheme 1. Structures of hyaluronan and heparin.

(ii) Preparation of chloramide derivatives

The hyaluronan chloramide was prepared from the reaction of hyaluronan (4mg/ml) in phosphate buffer, with pH- adjusted HOCl (1mM) at 37°C for 300 minutes followed by 300 minutes incubation at room temperature to ensure all the HOCl reacted. After this period, the reaction was complete as determined by the UV absorption spectrum of the reaction solution. Heparin chloramide was prepared by reacting 9.7mM heparin with 9mM HOCl at pH 6.5 for 180 minutes followed by extensive dialysis. A typical hyaluronan chloramide preparation should be viewed as a substituted hyaluronan comprising 90% N-H groups and 10 % N-Cl (chloramide) groups. For HepCl, there are approximately 33% N-H groups and 66% N-Cl groups in the substituted polymer. All chloramide solutions in this study, referred to as either HACl or HepCl , contain N-H and N-Cl groups in these proportions. The probability of unsubstituted glycosaminoglycans (i.e. HA or Hep) is very low and can be ignored. The synthesised chloramides were stored at 4°C and used in gamma radiolysis experiments as soon as possible thereafter. The chloramide concentrations of both HACl and HepCl preparations prior to gamma radiolysis were determined using the TNB (5-thio-2-nitrobenzoic acid) assay. Dilutions
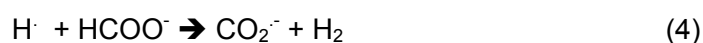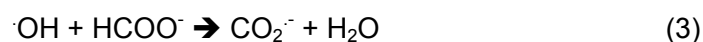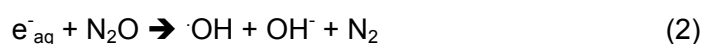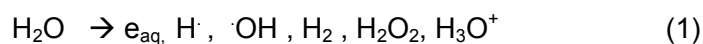
of the chloramide solutions were selected to match the 35µM-45µM concentrations of

TNB. After 30 minutes incubation, the absorbance of the TNB thiol oxidation product was

measured at 412nm ($\mathcal{E}$=13600 M^{-1} cm^{-1}, [24]).

(iii) Generation of selected radicals by water radiolysis

Reducing radicals

The hydrated electron , e^-_{aq}, ($E($ H$_2$O/ e^-_{aq}) = -2.9 V [30]) and the superoxide radical, O_2^-

, ($E(O_2/O_2^-)$ = -0.33 V [31] were produced by established radiation chemical techniques,

as follows:

Hydrated electrons, e^-_{aq}, were generated by the radiolysis of argon-saturated 0.1M *tert*-

butanol solutions.   Radiolysis of aqueous solutions produces the primary radicals , e_{aq} ,

hydrogen atoms (H$^.$) and hydroxyl radicals, ($^.$OH), as shown in reaction (1). In the

presence of *tert*-butanol, hydroxyl radicals are scavenged to produce non-reactive

species allowing the reactivity of the hydrated electron to be studied independently.

Superoxide radicals were produced by the radiolysis of oxygenated solutions of 0.1M

formate. Under these conditions both formate radicals and hydrated electrons react

rapidly with oxygen to produce superoxide radicals, as shown in reactions (1) to (6). The

yields of hydrated electrons and superoxide are 0.28 and 0.62 µmol J^{-1} respectively. All

solutions contained typically 0.1mM chloramide (HACl) or 0.4 mM chloramide (HepCl)

(based on average disaccharide molecular weights).

$$H_2O \;\rightarrow e_{aq,}\; H^.\;,\;\;^.OH\;,\;H_2\;,\;H_2O_2,\;H_3O^+ \qquad\qquad (1)$$

$$e^-_{aq} + N_2O \rightarrow\;^.OH + OH^- + N_2 \qquad\qquad (2)$$
$$^.OH + HCOO^- \rightarrow CO_2^- + H_2O \qquad\qquad (3)$$
$$H^.\; + HCOO^- \rightarrow CO_2^- + H_2 \qquad\qquad (4)$$

$$CO_2^{\cdot-} + O_2 \rightarrow O_2^{\cdot-} \tag{5}$$

$$e_{aq}^- + O_2 \rightarrow O_2^{\cdot-} \tag{6}$$

Oxidising radicals

The hydroxyl radical ($E(^{\cdot}OH/OH^-) = 1.9$ V [32]) , the carbonate radical ($E(CO_3^{\cdot-}/CO_3^{2-})$ =1.59 V [33]) and the nitrogen dioxide radical ($E(NO_2^{\cdot}/NO_2^-)$=1.04 V [34]) were produced as follows:

Hydroxyl radicals, $^{\cdot}OH$, were generated by the radiolysis of nitrous oxide-saturated solutions at pH 7.0, as in reactions (1) and (2). Carbonate radicals, $CO_3^{\cdot-}$, were produced by the radiolysis of nitrous oxide saturated solutions of 0.1 M sodium carbonate at pH 8.5. Under these conditions, hydrated electrons and hydroxyl radicals produce carbonate radicals, as shown by reactions (1), (2) and (7) [35,36] . The carbonate anion radical, $CO_3^{\cdot-}$, exists in equilibrium with the protonated form, $HCO_3^{\cdot}$ with a very low pK_a (< 0 [37]), and is therefore the predominant species at the pH values covered in this study . Nitrogen dioxide radicals, $NO_2^{\cdot}$, were produced by the radiolysis of nitrous oxide saturated solutions of 0.1 M sodium nitrite [38]. Under these conditions, nitrogen dioxide radicals are formed efficiently via reactions (1), (2) (and (8). The yields of $^{\cdot}OH$, $CO_3^{\cdot-}$ and NO_2 radicals are all 0.56 µmol J^{-1} . All solutions contained typically 0.1mM -1mM chloramide (HACl) or 0.4mM chloramide (HepCl) (based on average disaccharide molecular weights) of hyaluronan chloramide or heparin chloramide).
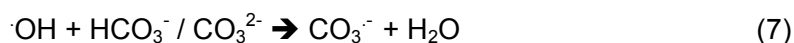
$$^{\cdot}OH + HCO_3^- \, / \, CO_3^{2-} \rightarrow CO_3^{\cdot-} + H_2O \tag{7}$$

$$^{\cdot}OH + NO_2^- \rightarrow NO_2 + H_2O \tag{8}$$
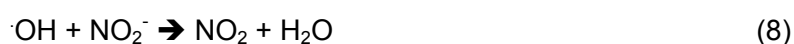
Irradiation doses of up to 100Gy were delivered to solutions of HA, Hep, HACl and HepCl under the conditions described above. Thus, concentrations of free radicals up to 60 µM could be achieved, the dose delivered being adjusted according to the amount of biopolymer fragmentation observed.

(iv) Irradiation of chloramide solutions

Gamma radiolysis studies were carried out using a model 812 Cobalt-60 source (Foss Therapy Services, Inc), capable of supplying an absorbed dose of up to 450 Gy per minute. The dose rate used in these experiments was 14-28 Gy per minute. All experiments were carried out at room temperature. Solutions for irradiation experiments involving the hydrated electron, hydroxyl radicals, carbonate radicals, nitrogen dioxide and superoxide radicals were saturated, as appropriate, with research grade nitrous oxide, argon or oxygen.

(v) Analysis of changes in the molecular weight distributions

The samples were analysed to determine the extent of fragmentation of the glycosaminoglycan using modified poly-acrylamide gel electrophoresis (PAGE), (Min and Cowman, 1986). 10 % and 20% vertical slab polyacrylamide gels (0.1x16x20 cm) were run using a Bio-Rad Protean II xi multi cell system. 45µl of the chloramide sample was added to 5µl of loading buffer (10x TBE (Tris-Borate- EDTA) buffer, 0.1M tris/0.25M borate/0.001M EDTA) containing 2M sucrose. This 50µl sample volume was loaded onto gels covered with 1X TBE running buffer. 25µl of bromophenol blue dye (0.02% in 2M sucrose) was applied to a well with no sample and was used as a tracking dye. For hyaluronan,the gels were run initially at 125V (20mA) for 20 minutes and then at 250V

(40mA) for approximately 100 minutes. For heparin, the gels were run for 4.5 hours at 200 V and for 9 hours at 100V. At this point, the bromophenol blue tracking dye was within 1.5 cm of the gel bottom, and the gels were immediately transferred to shallow non-stick trays. The polymer samples were fixed in the gel matrix by soaking the gel in 0.5% alcian blue dye dissolved in deionised water for 30 minutes in the dark. The gels were then de-stained using several changes of deionised water. The fixed gel was scanned using a high resolution (6400 dpi x 9600 dpi) Epson Perfection V500 Photo flat bed scanner interfaced to a PC. The captured images were visualised and digitised using Quantiscan ® software v3 (BioSoft).

The gpc -MALLS system consisted of a degasser ERC-3215α (ERC, Japan), a constametric ® 3200 MS pump (Thermo separation Products, FL), an injection valve with 1000µl loop (Reodyne 7725i) fitted inside a temperature regulated oven (Gilson, Model 831, UK) and a DAWN-DSP multi-angle light scattering photometer (Wyatt Technology, Santa Barbara, USA) equipped with He-Ne laser (λ = 633nm). Simultaneous concentration detection was performed using a calibrated differential refractometer (RI 2000, Schambek, Germany). A refractive index increment dn/dc value of 0.150 was used in the calculations. The mobile phase was 0.1M NaCl containing 0.005% NaN_3 filtered through 0.2µm pore size cellulose nitrate membrane. The samples injected were subjected to prior filtration through a nylon filter of 0.45µm pore size. A set of two columns SB-803HQ & SB-806HQ (8mmx300mm, Shodex OHpak, Japan, exclusion limits $1x10^5$ and $2x10^7$ g/mol) was used for the separation. The flow rate for the eluent was 0.45 ml/min. The Berry fitting method with linear fit was used for data processing in ASTRA software (Version 4.90.08). All measurements were performed at room temperature.

To calibrate the Rf values in the PAGE scans for molecular mass , solutions of hyaluronan and heparin were irradiated in order to fragment these biomolecules .

Irradiation doses of upto 100 Gy were delivered and the molecular weight distribution determined using the gpc/MALLS technique. These solutions were also subjected to PAGE . By matching the molecular weight distribution from the gpc/MALLS experiments to the PAGE scans, correlations of molecular mass v Rf were obtained. This allowed the use of PAGE as a more efficient method of molecular mass distribution measurement for the other irradiation experiments in which various oxidising and reducing radicals were reacted with both hyaluronan and heparin.

The amount of biopolymer fragmentation, arising from reaction of the selected radicals ,was measured from the changes in the PAGE gels . Each gel, after densitometric scanning, could thus be transformed into a plot of stain intensity v molecular mass (M_i). By assuming that the stain intensity at a particular Rf (or M_i) value is directly proportional to the weight of biomolecules of that M_i (i.e. is proportional to N_iM_i where N_i is the number of molecules of mass M_i), the number average molecular weight, M_n can be calculated from the expression, $\sum N_iM_i / \sum N_i$.

Results and Discussion

(i) Reducing radicals

Figure 1a shows a typical PAGE gel obtained when hydrated electrons (up to 16 µM at a 50Gy dose) are reacted with 0.1mM hyaluronan chloramide (HACl). The gel shows a clear transition towards lower molecular weights of the biopolymers in the solution with increasing radiation dose. The preparation of HACl (see Materials and Methods) produces a substituted hyaluronan in which 1 out of 10 N-H groups are replaced by an N-Cl group. Since hydrated electrons and other reducing agents react very slowly, if at all, with the parent molecule, HA [39], (also confirmed in this study, but data not shown,

in a control experiment in which the parent biomolecule, HA, was reacted with hydrated electrons which showed no change in the PAGE scans relative to the unirradiated HA), only the N-Cl group reacts, with anticipated rate constants close to those expected for a diffusion-controlled reaction [40]. Figure 1b shows the respective densitometric scan and stain intensity v Rf plots for reaction of the hydrated electron with HACl . In the latter plots, only M_i values greater than about 9000 Da could be measured. From each of the molecular weight distributions in Figure 1c, the M_n value was calculated and hence the number of chain breaks per biomolecule could be determined from the expression, $(M_n^0 - M_n^p) / M_n^p$ where M_n^0 and M_n^p are the number average molecular weights at zero dose and at a specified radiation dose respectively. The concentration of chain breaks ([CB]) is therefore equal to CB per biomolecule x $[HA]_0$ where the latter is calculated from the M_n value of the unirradiated hyaluronan. A plot of the [CB] v $[e_{aq}^-]$, s shown in Figure 2 in which there is a linear relationship between the two variables. The slope of plot therefore represents the efficiency of fragmentation by the hydrated electron. Unlike many irradiation experiments where the yields of product are often only linear in the lower dose ranges, attributable to the target free radical starting to react with the product and not the substrate, here the products, smaller fragments of the biopolymers, are similar , if not otherwise identical, to the parent target molecule and so a linear relationship is more likely. The slope in Figure 2 for HACl indicates that e_{aq}^- reacts with an efficiency of 108+/- 11 %, that is, every reaction leads to fragmentation of hyaluronan chloramide. In similar experiments, in which the hydrated electron was reacted with solutions containing 0.4 mM heparin chloramide (HepCl), the fragmentation efficiency was found to be much lower at 25 +/- 3 % as shown in Figure 2.

Radiation dose (Gy)
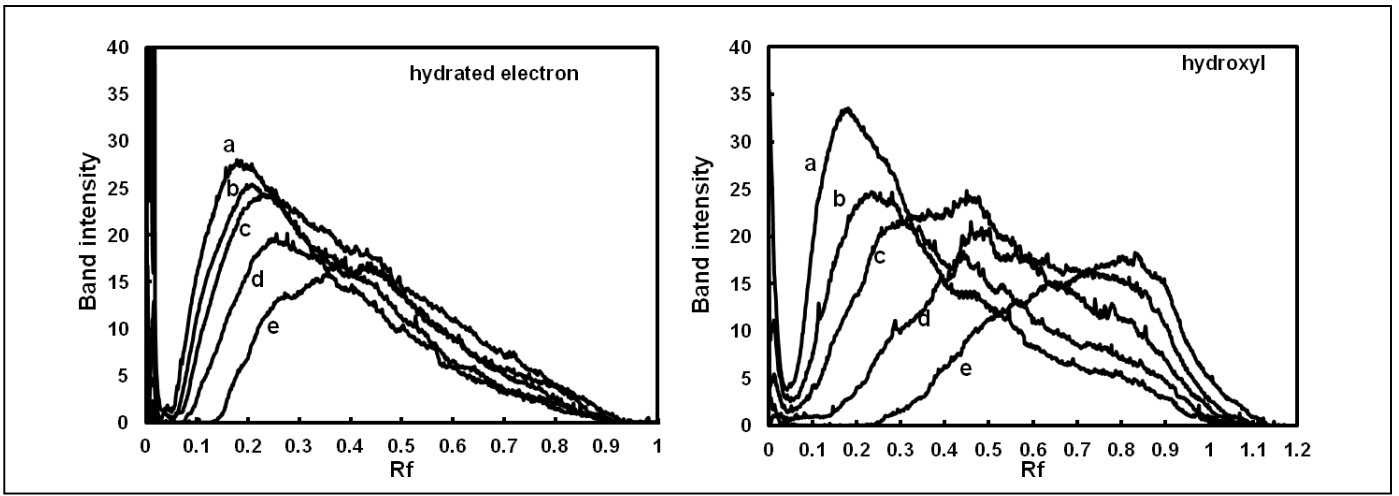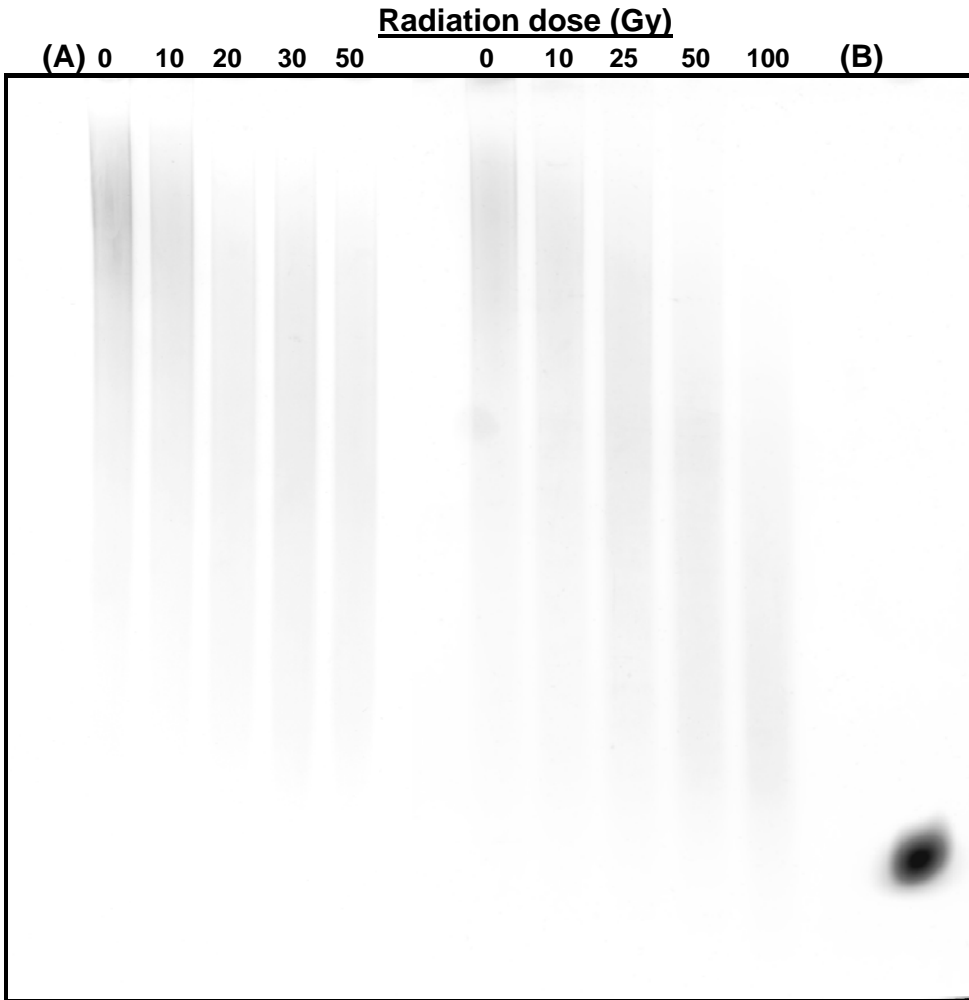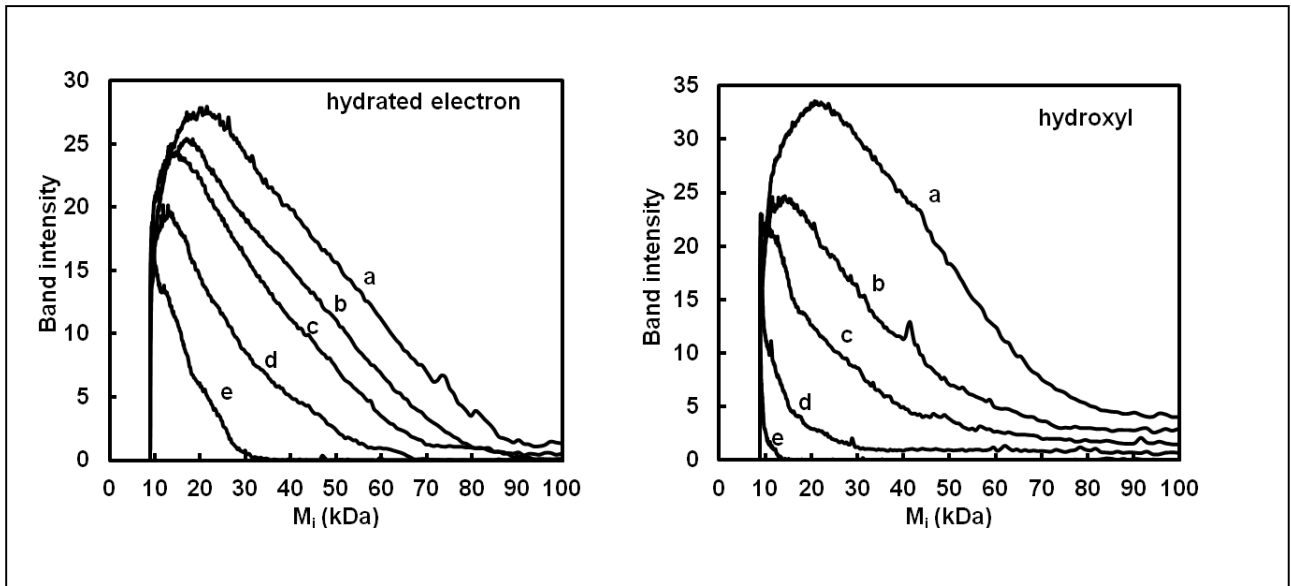
Fig. 1. (a) Alcian blue-stained PAGE gel showing depolymerization of HACl by hydrated electrons (side A) and hydroxyl electrons (side B) at pH 7.5 (also shown is the migration of the tracking dye bromophenol blue). (b) The densitometric scans of the gels in (A) showing dose-dependent depolymerization of HACl by the hydrated electron at 0, 10, 20, 30, and 50 Gy and also by the hydroxyl radical at irradiation doses of 0, 10, 25, 50, and 100 Gy. (All solutions were prepared in pH 7.5, 0.01 M phosphate buffer). (c) Stain intensity vs Mi for the hydrated electron- and hydroxyl radical-induced fragmentation of HACl.
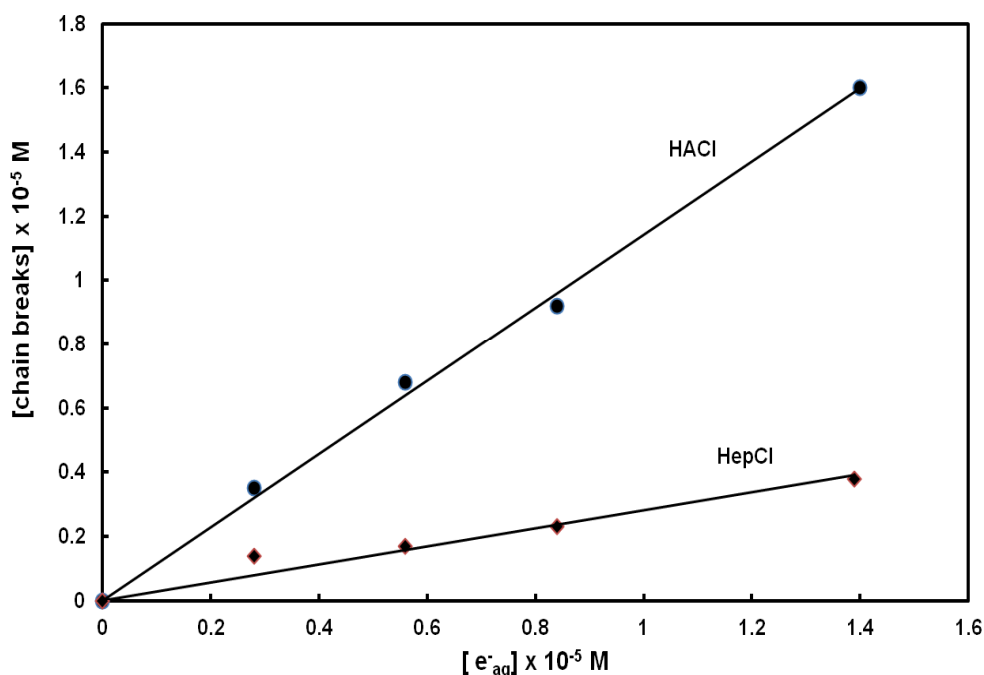
Fig. 2. The effect of [e could be calculated. Studies of alkylamino radicals in aq] on the fragmentation yields of HACl and HepCl

In previous pulse radiolysis and laser flash photolysis studies, it has been shown that e^-_{aq} reacts rapidly with HACl and HepCl with second-order rate constants of $2.2 \times 10^9 \, M^{-1} \, s^{-1}$ and $7.2 \times 10^8 \, M^{-1} \, s^{-1}$ respectively [34]. In that study, it was assumed that the hydrated electron reacted solely at the N-Cl moiety to eliminate chloride, as shown in reaction (9). This was supported by the lack of any demonstrable reaction of these species with the parent molecule, HA, and also by the absorption spectra of transient species attributable to carbon-centred free radicals formed on the glucosamine moiety [41]. The latter observation provided support for the 1,2 hydrogen shift mechanism for HACl proposed by Davies and co-workers as shown in Scheme 2 in which a chloride ion is eliminated by one-electron reductants to form a nitrogen-centred radical in the first instance followed by rapid re-arrangement to produce the C-2 radical on the glucosamine moiety

and/or C-4 radicals on the uronic acid moiety [22]. Both EPR data and ion-exchange

chromatography have been used to detect chloride in the one-electron reduction of

glycosaminoglycan chloramides as well as in pulse radiolysis studies of the reaction of

the hydrated electron with chloramines and amides which confirm that chloride ion is

produced in yields of 100% [27].

$$e^-_{aq} + HACl / HepCl \rightarrow HA^. / Hep^. + Cl^- \qquad (9)$$

Although reaction (9) must produce N-centred radicals in the first instance, these must

be short-lived as only carbon-centred radicals could be detected [22,41].
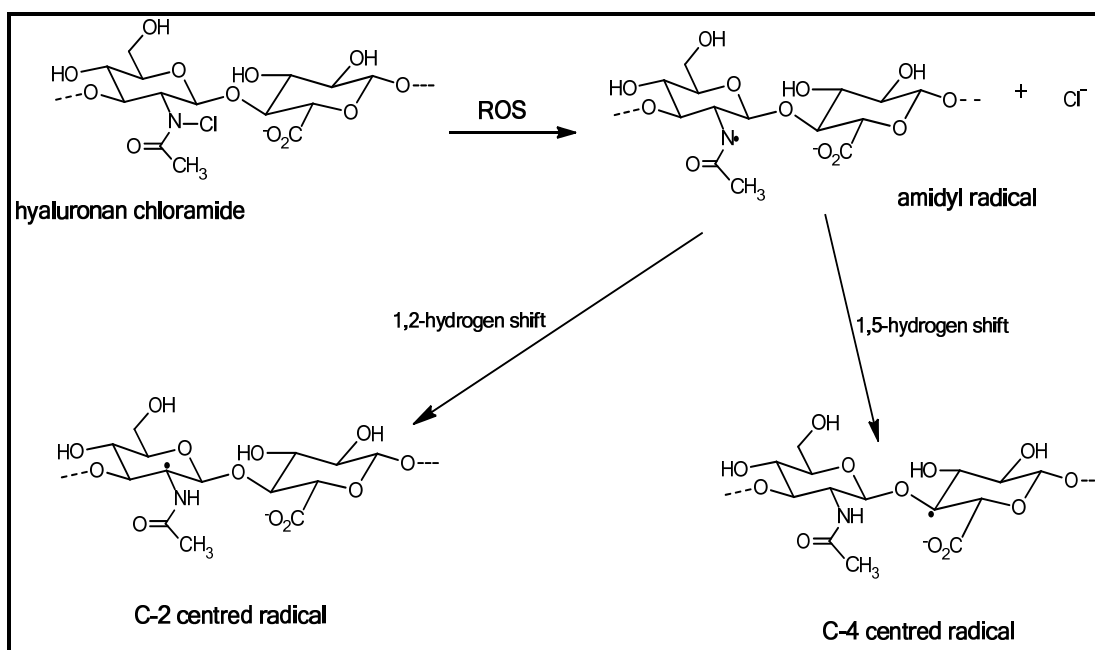
Scheme 2. Conversion of amidyl radical to the C-2 and C-4 radicals [27].

In the current study, the high rate constants for the reaction of the hydrated electron with HACl and HepCl ensure that there can be no competing reactions with sub-micromolar concentrations of oxygen (as an impurity) which may be present in the argon used to de-gas these solutions. The difference in fragmentation efficiencies of 108 +/- 11 % and 25 +/- 3 % for HACl and HepCl respectively is therefore significant and presumably is attributable to the effect of the presence of sulphate groups and the associated higher negative charge in HepCl relative to HACl. These two biopolymers have large differences in charge density with heparin containing on average 2-3 sulphate groups per disaccharide unit whereas hyaluronan has one negative charge per disaccharide unit. From EPR studies of glycosaminoglycans, including sulphated derivatives such as heparin sulphate, two modes of transformation of the N-centred radical, the amidyl radical, have been proposed . One involves 1,2-hydrogen shift to produce a C-2 radical on the glucosamine moiety, the other is a 1,5-hydrogen shift to produce a C-4 radical on the glucuronic moiety. The EPR evidence appears to favour the production of C-4 radicals [22,27]. However, evidence from time-resolved studies appears to support the formation of C-2 radicals since the transient spectra observed are consistent with those expected for carbon-centred radicals associated with an adjacent nitrogen atom such as $RCONRCH_2^{\cdot}$ (where R can be H- or CH_3-) [41]. It seems probable therefore that the 100% fragmentation yield observed for HACl occurs via the C-2 radical on the glucosamine moiety with the subsequent β-scission being 100% effective. In the case of HepCl , two possibilities can be proposed; (i) that the latter β-scission process is not 100% efficient and is in fact close to a value of 25% and that 75% of the C-2 radicals are involved in non-fragmentary routes; (ii) that the 1,5-hydrogen shift is favoured over the 1,2-hydrogen shift to produce C-4 radicals on the uronic acid moiety with, for instance, 75% of the amidyl radicals following this route. In the latter scenario, however, the efficiency of fragmentation would be close to zero via the 1,5-hydrogen shift route. On

balance, the transient spectra observed on the reaction of hydrated electrons with both

HACl and HepCl support the formation of C-2 radicals [41], and thus the large difference

in fragmentation efficiencies measured here are likely to be attributable to the effect of

negatively charged sulphate groups on the β-scission process of the C-2 radicals in

which a non-fragmentary pathway competes effectively. Scheme 3 shows these possible

mechanisms.
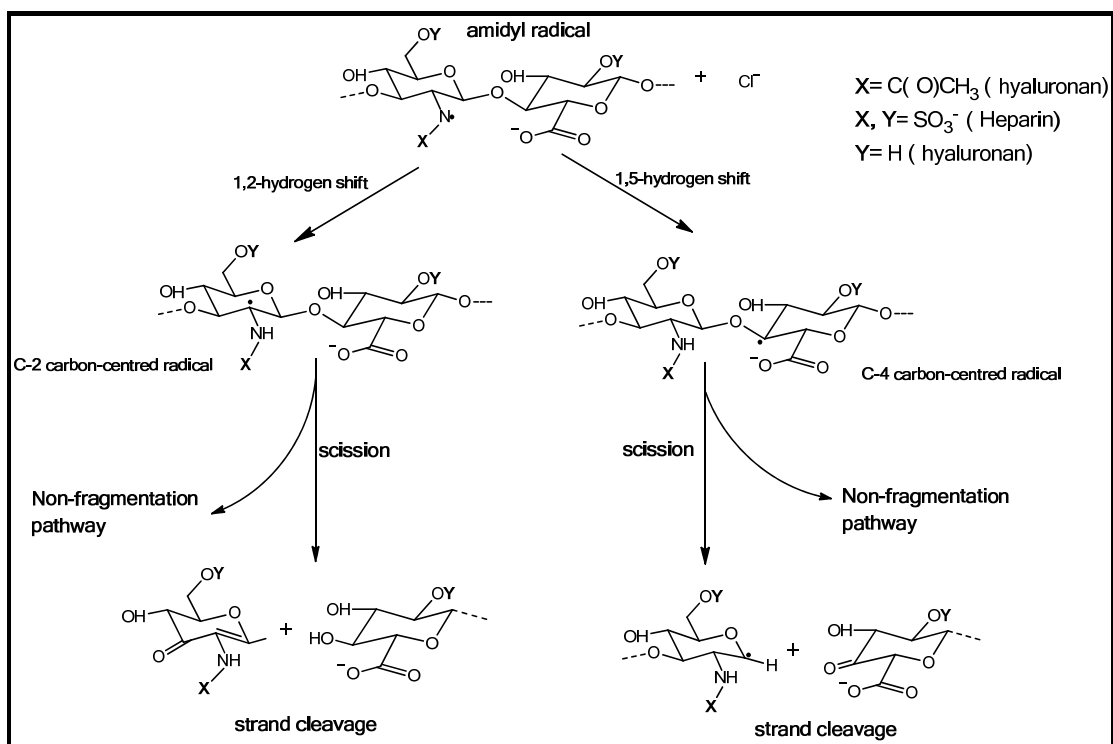
Scheme 3. Possible reactions pathways for C-2 and C-4 radicals after formation of

hyaluronan and heparin amidyl radicals [27].

The weak reducing agent, the superoxide radical was produced by the irradiation of oxygen-saturated 0.1mM and 0.8mM chloramide (HACl) and 0.4mM chloramide (HepCl) solutions containing 0.1M formate at pH 9.5, as in reactions (1) and (3) – (6). Under these conditions, all primary radiolysis radicals produce superoxide. At chloramide (HACl) concentrations of 0.1mM and 0.8mM and chloramide (HepCl) concentrations of 0.4mM, <1% of e^-_{aq} react directly with the chloramide . PAGE scans from these experiments (Figure 3 shows typical scans for 0.1mM HACl) show no change in molecular weight up to a dose of 100 Gy (equivalent to 62 µM superoxide) . At the higher 0.8mM HACl concentration, a barely-detectable movement of HACl polymers was observed in the PAGE gels (not shown) with a limit of fragmentation efficiency based on the superoxide yield of less than or equal to 2%. This small amount of fragmentation is probably attributable therefore to the minor competing reaction of e^-_{aq} with HACl. Direct measurements of the rate of reactions of superoxide with the chloramides of both hyaluronan and heparin have been made by generating superoxide through the laser flash photolysis of air or oxygen-saturated persulphate solutions containing formate and either HACl or HepCl at pH 8.5 in borate buffer. The rate constants for the reaction of superoxide with HACl and HepCl were found to be similar, in the range 2.2 -2.7 x 10^3 M^{-1} s^{-1} [42]. Despite this demonstration of a reaction, it would appear from the current study that it does not lead to fragmentation of HACl. In contrast, in a study by Rees et al [23], using SOTS-1 as a thermal source of superoxide, fragmentation of the chloramides of hyaluronan and chondroitin sulphate was observed. These workers concluded, through the inhibitory effect of EDTA, that the superoxide reaction was mediated, at least in part, by Cu^{2+}_{aq} , presumably through the redox cycling of Cu(II) and Cu(I) leading to reduction of the N-Cl group by Cu(I). An identical conclusion was also made for the chloramines and chloramides of heparan sulphate [27]. This indirect mechanism for reaction of superoxide with glycosaminoglycan chloramides may be in competition with the direct

reaction demonstrated in the time-resolved study [35]. At the relatively low concentrations of chloramides, 200–300 µM used in the earlier studies [23,27], as little as 1 nM of Cu^{2+}_{aq}, involved in effective redox cycling at pH 7.4, would be the main channel of reaction, since superoxide reacts at diffusion-controlled rates with Cu (I/II) aquo complexes. At the pH of 9.5 (where the lifetime of superoxide is much longer than at pH 7.4) and at the higher mM chloramide and chloramine concentrations used in the time-resolved study, the direct reaction would be dominant. In the current study, superoxide is generated by steady-state radiolysis where the steady-state concentrations of superoxide would be extremely low (sub-nanomolar) and so decay of superoxide via dismutation through reaction with its acidic form, $HO_2^.$ would be unlikely to be significant. More likely, is a reaction with sub-nanomolar concentrations of redox metal ions, particularly Cu^{2+}_{aq}. However, this should lead to fragmentation as found by Rees et al [23]. It is thus difficult at present to reconcile the kinetic data [42], the SOTS-1 studies [23,27] and the fragmentation data presented here. It may be that superoxide reduces the chloramide directly, as confirmed for other reducing radicals in the previous kinetic study [41] to produce the amidyl radical which may react rapidly with oxygen to produce a nitroxide species as found in previous studies of sterically-hindered amidyl and aminyl radicals [43,44]. Ultimately a stable glycosaminoglycan nitroxide may be formed, as shown tentatively in Scheme 4. An estimate of the rate of conversion of the amidyl radical to a C-centred radical can be made from the kinetic study of the reaction of formate radicals with the chloramides in which the C-centred radical spectra are produced in a pseudo 1st order process which is [chloramide] dependent [41]. This suggests a rate of at least 5×10^6 s^{-1} for the amidyl to C-centred radical conversion. Thus, to ensure a good competition to form a glycosaminoglycan NO_2 species, as in Scheme 4, in the presence of 1 mM oxygen, a second order rate constant of 5×10^9 M^{-1} s^{-1}could be calculated. Studies of alkylamino radicals in non-aqueous solvents have

shown that these radicals react rapidly with oxygen, in one case a rate constant of 2 x 10^8 M^{-1} s^{-1} was measured [44]. It is therefore possible that fast reaction of the glycosaminoglycan amidyl radical with oxygen may also occur in the superoxide experiments. An alternative explanation is that the rapid transformation is too fast to allow the amidyl radical to react with oxygen and the C-2 radical produced would rapidly be converted into a peroxy radical though reaction with oxygen. If the latter occurred, this would indicate that no fragmentation can take place from C-2 peroxy radicals.
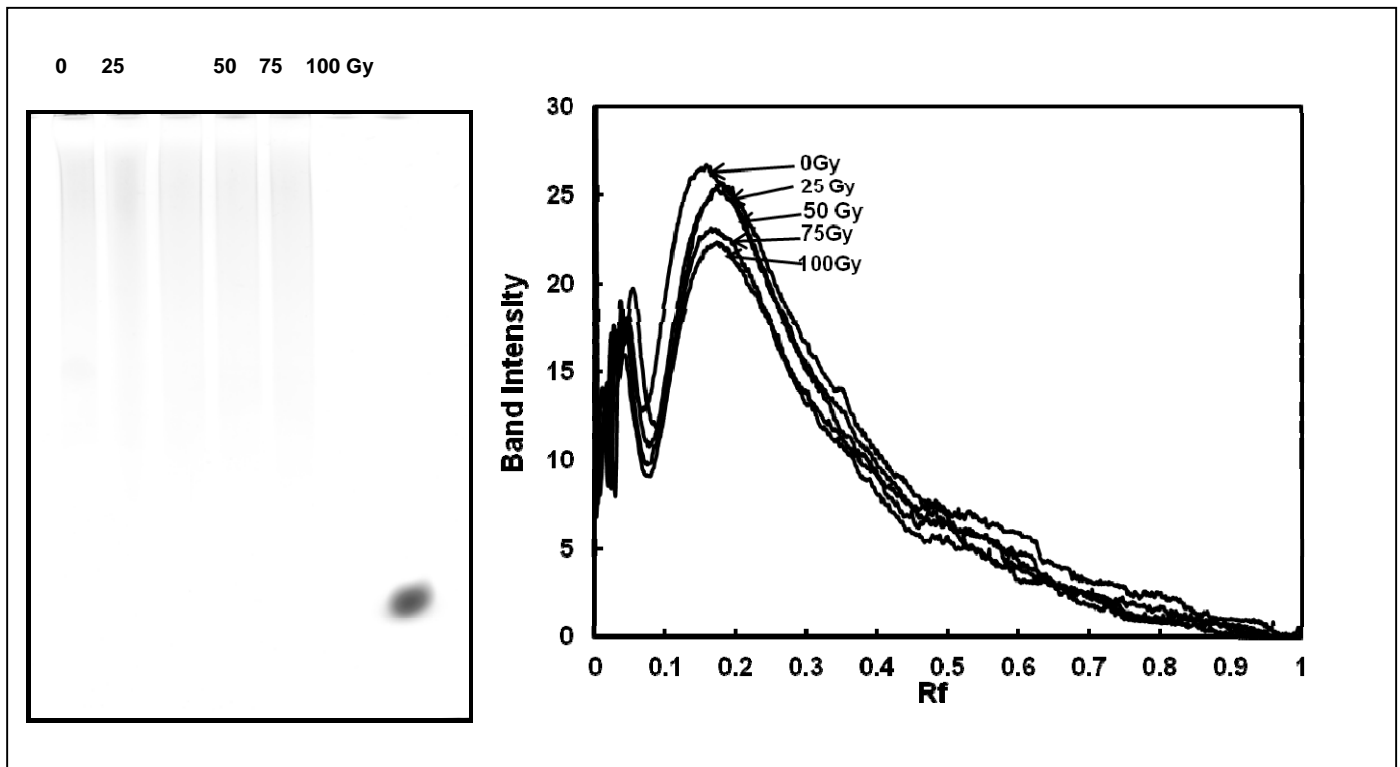
Fig. 3. Densitometric scans of the Alcian blue-stained PAGE gels showing lack of depolymerization of HACl by the superoxide radical anion (0.8 mM HACl, pH 9.5, 0.01 M borate buffer).
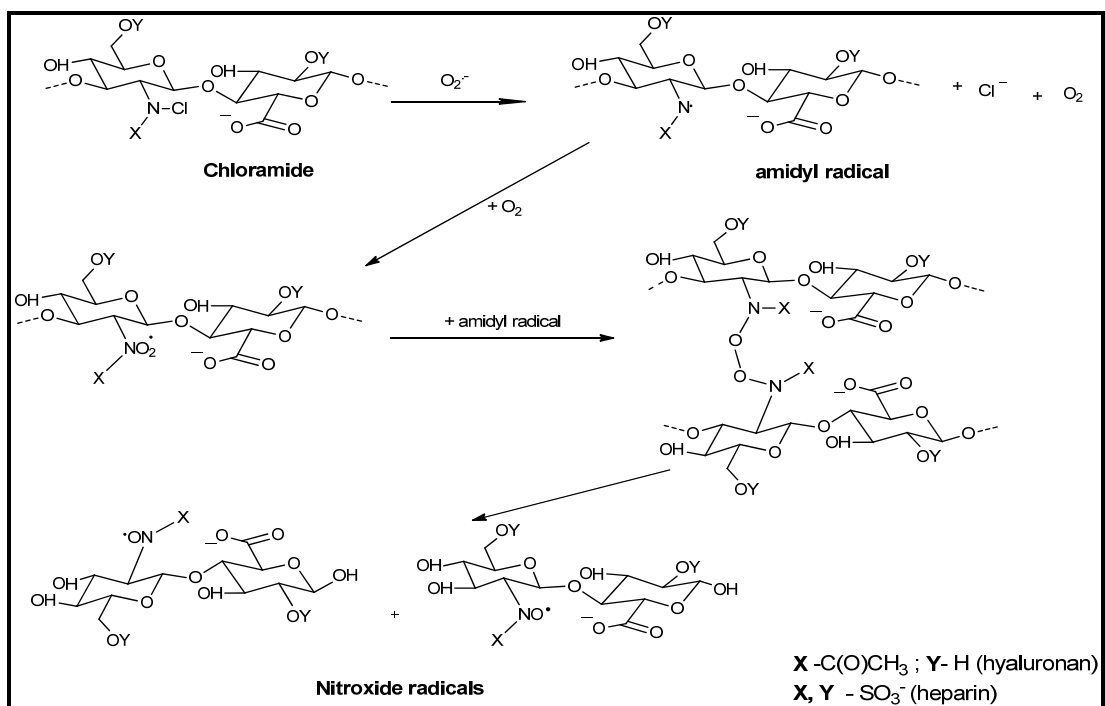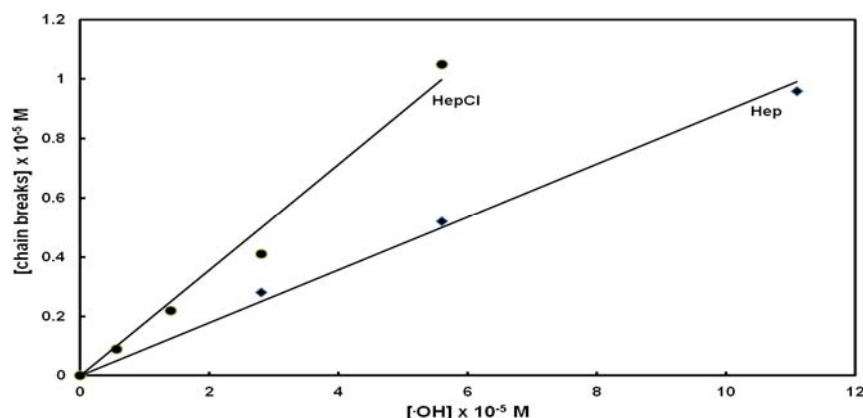
Scheme 4. Possible reaction pathway to produce glycosaminoglycan nitroxides after reduction of the chloramide derivatives by superoxide radicals.
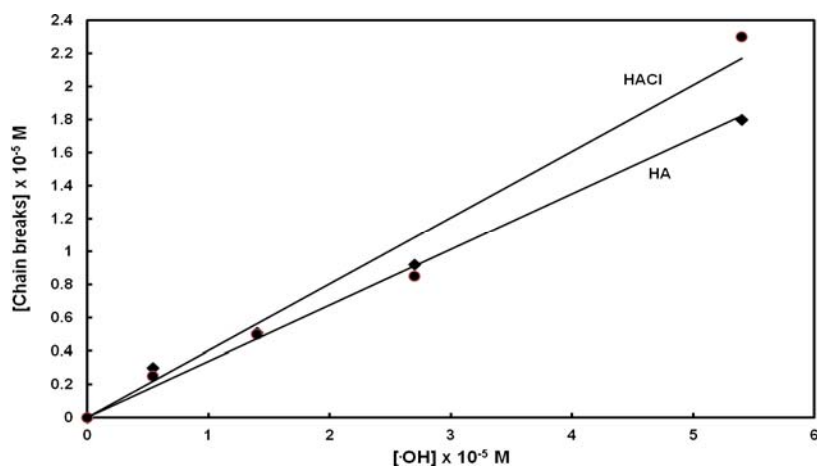
Fig. 4. Effect of hydroxyl radical concentration on the fragmentation yields of HA and HACl.

Fig. 5. Effect of hydroxyl radical concentration on the fragmentation yields of Hep and HepCl.

(ii) Oxidizing radicals

Figures 1a, 1b and 1c show the PAGE gels, densitometric scans and molecular weight distributions for the hydroxyl radical induced fragmentation of HA and HACl. Figure 4 shows the fragmentation yields , expressed as plots of chaín break concentration, [CB] v [˙OH], after reaction of 1 mM HA and 0.1 mM HACl with hydroxyl radicals, showing respective fragmentation efficiencies of 32% and 42%. In the kinetic study of the reactivity of hydroxyl radicals, it was shown that there was little difference in the measured rate constants of hydroxyl radical with HA and the N-Cl groups in HACl , values in the range $(2.2 – 4.0) \times 10^8$ M^{-1} s^{-1} being calculated [42]. The hydroxyl radical thus reacts rapidly by H-abstraction with the many possible (up to 11) reactive C-H bonds in hyaluronan, each having a potential route to fragmentation. In the case of the HACl preparation, considered here as hyaluronan in which 1 out 10 N-H groups is substituted by N-Cl, it seems likely that attack at N-Cl is a more efficient route to fragmentation. Abstraction of Cl, for example, would lead to the formation of the same nitrogen-centred radicals that are formed by reaction of the hydrated electron and which lead to 100% efficient fragmentation as already demonstrated in this study (as depicted

in Scheme 2). However, the kinetic data do not allow a calculation of the degree of selectivity of attack and it is not possible to calculate the efficiency of fragmentation following abstraction of Cl. It is clear however, that the overall increase in efficiency from 32% to 42% when only 1 in 10 of N-H is substituted by N-Cl, does support a high fragmentation efficiency, perhaps approaching 100%.

For heparin and the 0.4 mM chloramide (HepCl) preparation, hydroxyl radicals produce fragmentation efficiencies of 8% and 19 % respectively (Figure 5). Again, as for HA and its chloramide preparation, HACl, there is no kinetic evidence for selectivity of attack at N-Cl [34]. In the 0.4mM chloramide (HepCl) preparation, the degree of N-Cl substitution is much greater than for HACl with 7 out of 10 N-H groups replaced by N-Cl. Although the kinetic data do not allow a calculation of the fragmentation efficiency following abstraction of Cl, it is nevertheless clear that, in the more highly substituted heparin biopolymer, the efficiency will be much lower than the 100% value proposed above for HACl.

In studies of the fragmentation of the HACl and HepCl preparations by carbonate radicals, more marked increases in fragmentation of the chloramides relative to the parent molecules were found. The densitometric scans of the PAGE gels are shown in Figures 6a and 6b for the reactions of carbonate radicals with HA and HACl, with efficiencies of 11% and 32% for HA and HACl respectively, as calculated from the slope of the [chain breaks] v [CO_3^-] plots in Figure 7. It has been shown from the earlier kinetic study that CO_3^- reacts with the N-Cl moiety (1.2×10^5 M^{-1} s^{-1}), i.e. about 2.9 x faster than with the HA molecule (3.5×10^4 M^{-1} s^{-1}) and it was thus concluded that carbonate radicals are likely to react in a site-specific mode at the N-Cl moiety [41]. Application of the latter rate constants to the concentrations of HA and the N-Cl groups (in HACl) indicates that about 25% of the carbonate radicals would react at N-Cl with the

remainder reacting with other groups such as –CH(OH)- and N-H. Assuming that the

fragmentation efficiencies for reaction at N-Cl and in HA are 100% and 11%

respectively, a combined fragmentation efficiency of 32% can be anticipated for the

HACl preparation as found in Figure 7. Thus abstraction of Cl by carbonate radicals to

form N-centred radicals is the most probable mode of reaction and one that leads to

100% fragmentation efficiency, analogous to the reaction of hydrated electrons which

produce the same N-centred radicals via elimination of chloride ion.
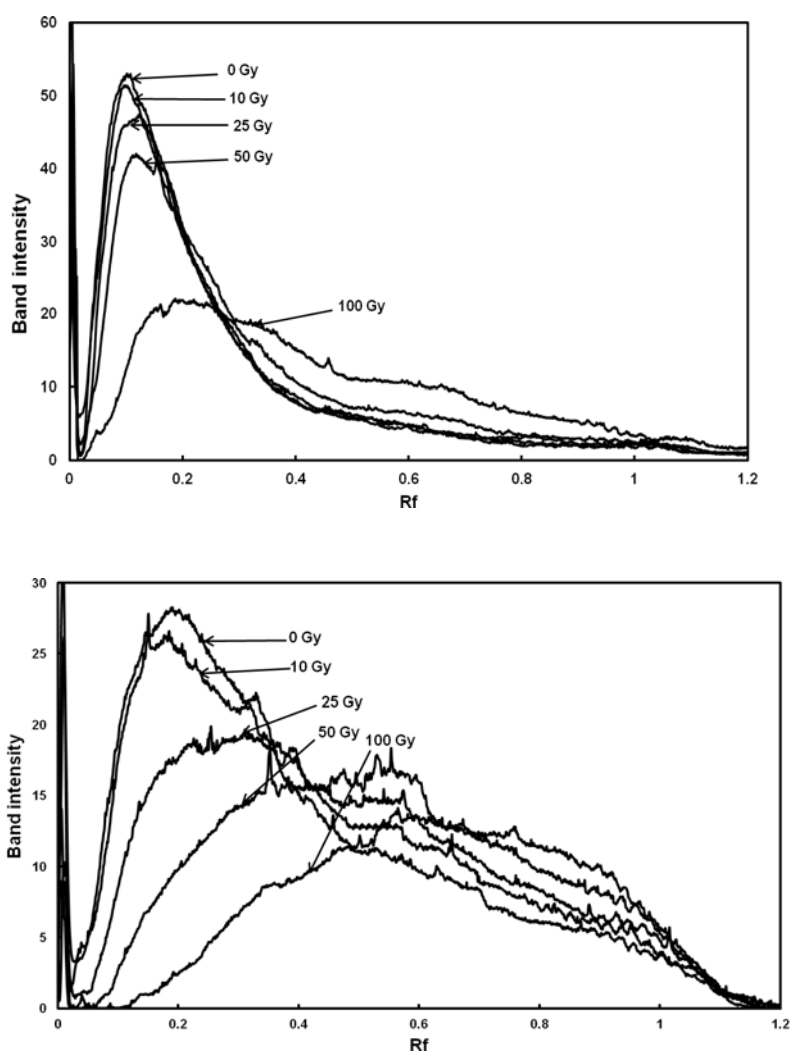
Fig. 6. Densitometric scans of the Alcian blue-stained PAGE gels for reaction of carbonate radicals with: (a) HA and (b) HACl (0.1 mM preparations, pH 7.0, doses 0.100 Gy).
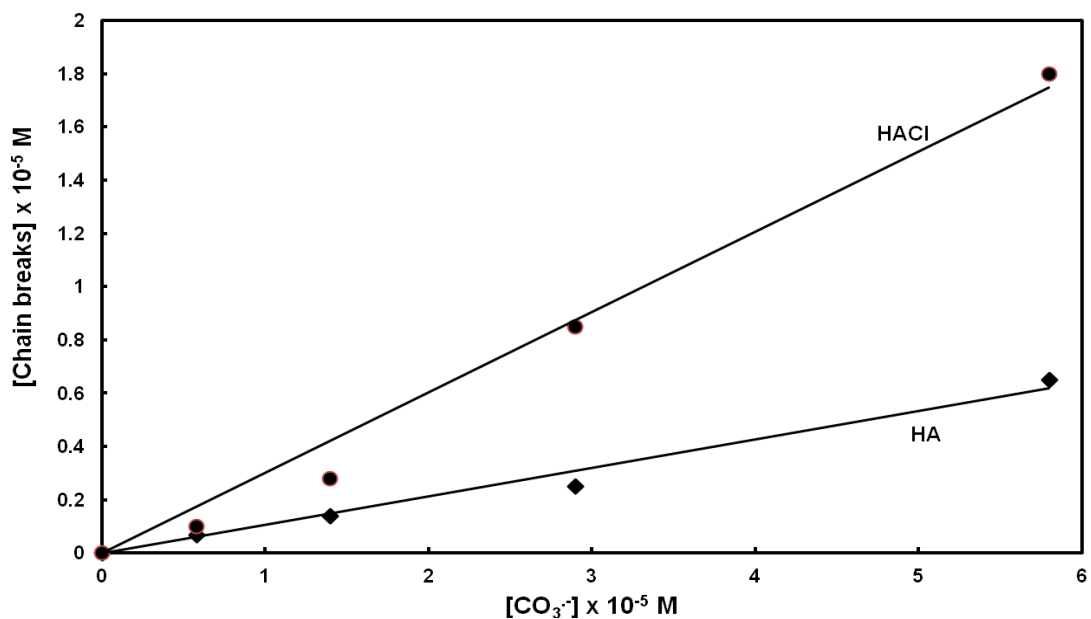
Fig. 7. Effect of carbonate radical concentration on the concentration of chain breaks after γ-irradiation of HA and HACl solutions (pH 8.5, 0.01 M phosphate buffer).
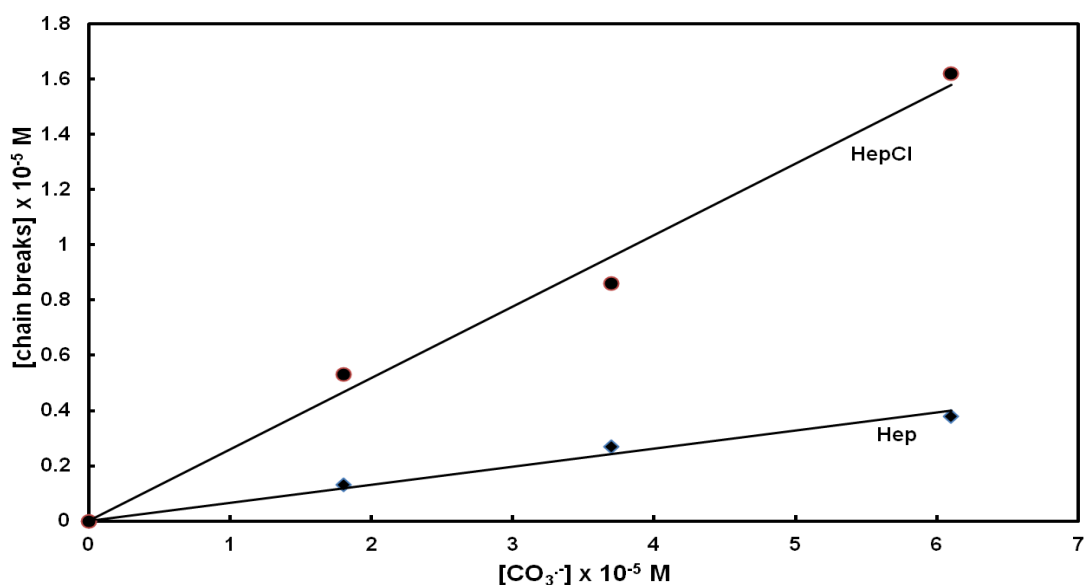
Fig. 8. Effect of carbonate radical concentration on the concentration of chain breaks after γ-irradiation of Hep and HepCl solutions (pH 8.5, 0.01 M phosphate buffer).

For the Hep and HepCl preparations, the fragmentation efficiencies were calculated as 6% and 23% respectively, as determined from Figure 8. From the earlier kinetic study, the rate constants for the reactions of carbonate radicals with Hep and with the N-Cl group in HepCl were 5×10^4 M^{-1} s^{-1} and 8×10^4 M^{-1} s^{-1} respectively [41]. Assuming a substituted heparin in which 7 out 10 N-H groups are replaced by N-Cl, it can be calculated that about 75% of carbonate radicals react at the N-Cl group. It can thus be estimated from the respective fragmentation efficiencies of 6% and 23%, that reaction at N-Cl leads to 29% fragmentation. This value is close to the value of 25% found for the reaction of hydrated electron with N-Cl and supports the proposal made above for HACl that abstraction of Cl by carbonate radicals also leads to the N-centred amidyl radical and that whether produced by electron reduction or by abstraction by oxidizing agents , the fragmentation efficiency should be the same , as found here for HepCl preparations. Unlike N-Cl in HACl however, the efficiency is not 100% but much lower at 25-29%.

When NO_2 radicals were reacted with HA and HACl and also with Hep and HepCl , no movement at all was seen in the PAGE gels (data not shown) indicating that no fragmentation by NO_2 occurs in any of the biomolecules. There are no kinetic data on the reactions of NO_2 with HA, HACl, Hep or HepCl and so it is not possible to say here that, like the superoxide experiments described above, there is kinetic reactivity but no resultant fragmentation. NO_2 is a weak oxidizing agent and reacts, for example, with good reducing agents such as ascorbate and the Trolox C anion with rate constants of 3.5×10^7 M^{-1} s^{-1} and 5.0×10^8 M^{-1} s^{-1} respectively [45,46]. Rate constants for reaction with glycosaminoglycans can therefore be expected to be much lower, particularly if reacting via H-abstraction. Chloramides are also weak oxidizing agents and hence unlikely to react via electron transfer to NO_2. It may be therefore that the reaction is too slow to produce biopolymer products.

Conclusions

The combination of fragmentation efficiency data for both reducing and oxidizing radicals has highlighted the significant role that N-Cl groups play in glycosaminoglycan chloramides in controlling site-specific attack. In particular, the fragmentation produced by the strong reducing agent, the hydrated electron is seen to determine the fragmentation efficiency arising from reactions of the N-centred amidyl radical. Thus in HACl, it is 100% efficient whereas in HepCl it is 25% efficient. It is clear therefore that the heavily-charged, sulphate-containing, heparin derivative has a large effect on the fate of either the amidyl radical (1,2- vs 1,-5-hydrogen shifts) or on the fates of the C-2 or C-4 radicals arising these hydrogen shifts respectively. From the spectral data of the earlier kinetic study [41], it seems that the 1,2-hydrogen shift is favoured for both HACl and HepCl and that therefore, for HepCl ,the fragmentation route of C-2 radicals is much less favoured than non- fragmentation routes (see Scheme 3).

From the data on oxidizing radicals, particularly that from reaction of carbonate radicals, it also seems likely that abstraction of Cl from N-Cl occurs to produce the same N-centred amidyl radical as produced by the hydrated electron, with the same fragmentation efficiencies consequent via this moiety of 100% and 29% for N-Cl groups in HACl and HepCl respectively.

The weaker reducing agent, the superoxide radical and the weaker oxidizing agent, nitrogen dioxide produced no detectable fragmentation. The previous observations of kinetic reactivity of superoxide with glycosaminoglycan chloramides [23,27,42] are difficult to reconcile with the absence of fragmentation. Acting as a one-electron reductant, superoxide would be expected to produce the amidyl radical with consequent fragmentation. Similarly, acting as an oxidant, it should also produce fragmentation. A possible explanation is that the amidyl radical reacts with oxygen to produce ultimately a

glycosaminoglycan nitroxide which may be stable to fragmentation. Alternatively, the C-2 peroxy radical may be formed instead which does not lead to fragmentation. In the case of nitrogen dioxide, it may be that the reaction is too slow and other reactions, presumably self-reactions occur instead.

The above discussion indicates that the carbonate radical is likely to be a potent and site-specific *in vivo* free radical in the damage of glycosaminoglycan components of the extracellular matrix, particularly the chloramide derivatives that are likely to be formed in inflammation. Reaction of these reactive species with glycosaminoglycans, which may be heavily-substituted by N-Cl groups under inflammatory conditions, is perhaps the key damaging process to these biopolymers which have structural and other important properties. These include roles in the activation of signaling events in cells and in the modulation of cellular processes, including proliferation, migration, adhesion and apoptosis. In the case of hyaluronan, the fragmented products of such damaging processes have also been shown to be pro-inflammatory and angiogenic.

The evidence presented here also shows that sulphated glycosaminoglycans of the extracellular matrix such as heparan sulphate are more resistant to fragmentation by reactive oxidative and reductive species. The mechanisms proposed by Davies and co-workers [23,27] help to rationalize this, as summarized in Scheme 3.

Although any strong reducing agents produced *in vivo* will be likely to compete kinetically with both oxygen and the chloramides minimizing the potential for fragmentation, local concentrations of chloramides may be sufficiently high to compete with in vivo oxygen levels. Any reaction with glycosaminoglycan chloramides, particularly HACl, will produce efficient fragmentation. Relatively high concentrations of HOCl are likely to be formed *in vivo* under both physiological and pathological conditions. It has been estimated, for

example, that activation of 5×10^6 neutrophil cells ml^{-1} generates 300–400 μM HOCl over 1–2 hours [47,48], with 2.5–5 mM HOCl produced at sites of inflammation [49]. HOCl is produced in inflammation by the highly basic protein myeloperoxidase which is known to bind, via electrostatic interactions, to negatively charged materials such as the polyanionic glycosaminoglycans [50]. In perlecan, for example, it binds to the heparin sulphate side-chains of this proteoglycan [51]. Thus, such a mechanism for the localised production of HOCl in the extracellular matrix may favour a reaction at glycosaminoglycans to produce glycosaminoglycan chloramides and not to produce protein-derived chloramines and chloramides at neighbouring linked proteins. Hence, high local concentrations of chloramides may prevail, making reactions of strong reducing agents more likely. Although, weaker reducing agents such as superoxide radicals will not be affected by reaction with oxygen, they do not appear to produce fragmentation directly: however, other weak one-electron reductants such as Cu(I) may do so.

In conclusion, this study has presented quantitative data which supports a significant role of glycosaminoglycan chloramides in directing site-specific attack by free radicals and other reactive species and one which can lead to extensive fragmentation of these important *in vivo* components of the extracellular matrix. The study has also highlighted the dramatic effect that sulphate groups have in minimizing fragmentation of these components.

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