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Reaction of superoxide radicals with glycosaminoglycan chloramides: A kinetic study

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Abstract

Hypochlorous acid and its acid-base counterpart, hypochlorite ions, produced in inflammatory conditions, may produce chloramides of glycosaminoglycans, perhaps through the binding of myeloperoxidase directly to the glycosaminoglycans. The N-Cl group in the chloramides is a potential target for reducing species such as Cu(I) and superoxide radicals. Laser flash photolysis has been used here to obtain, for the first time, the rate constants for the direct reaction of superoxide radicals with the chloramides of hyaluronan and heparin. The rate constants were in the range $2.2 - 2.7 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$. The rate constant for the reaction with the amino acid, taurine, was found to be much lower at $3.5 - 4.0 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$. This demonstration that superoxide anion radicals react directly with hyaluronan and heparin chloramides may support the mechanism first proposed by Rees et al, *Biochem. J.* **381**: 175-184; 2004 for an efficient fragmentation of these glycosaminoglycans in the extracellular matrix under inflammatory conditions.

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. These HA-binding proteins are termed hyaladherins and share a homologous HA binding domain with the link protein, which is referred to as the Link homology domain [1]. Examples include aggrecan, versican, and tumor necrosis factor alpha stimulated gene 6 (TSG-6) [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 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 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].

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 have been 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 peroxyxynitrite have been determined [15,16].

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 peroxyxynitrous 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 the neighbour by the molecular mass of the repeating disaccharide unit in HA, thus mimicking to a significant extent the action of the enzyme, hyaluronidase [17,18].

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 [19,20].

The formation of chloramides and chloramines from the reaction of hypochlorite with amides and amines respectively was demonstrated in an early study [21] and is suggested to be a key process in inflammation, in which hypochlorite (from myeloperoxidase) may produce glycosaminoglycan chloramides. Such derivatives may accelerate the fragmentation of glycosaminoglycans within the ECM [22]. Chloramides are weak oxidising agents and are therefore potential biological targets for reducing radicals and other reducing agents. Indeed, it has been shown that superoxide radicals cause the fragmentation of HA via reaction with its chloramide derivative [19].

Strong reducing agents such as the hydrated electron, e^-_{aq} , may be expected to react rapidly with chloramines and chloramides. Pulse radiolysis studies of the simplest chloramine, NH_2Cl , showed that the hydrated electron reacts with it at diffusion-controlled rates ($k = 2.2 \times 10^{10} M^{-1} s^{-1}$) [23,24]. In more recent pulse radiolysis studies of chloramines and chloramides of amino acid derivatives [25] and of chloro- and bromo-derivatives of model compounds such as N-bromoglutarimide (NBG) and N-bromosuccinimide (NBS) [26], hydrated electrons were also shown to react at near diffusion-controlled rates. In the latter study, superoxide radicals were also reacted with NBG and NBS and found to follow complex chain reaction pathways. In the case of N-chlorosuccinimide, a direct kinetic measurement could be made, yielding a rate constant of $8 \times 10^5 M^{-1} s^{-1}$. An indirect method based on EPR data, has also allowed an estimate of the rate constant for the reaction of superoxide radicals with taurine monochloramine to be made ($k = 5-6 \times 10^2 M^{-1} s^{-1}$) [27].

It seems clear, therefore, that there is no direct, unambiguous kinetic measurement of the rate constants for the reaction of superoxide radicals with the centrally-important chloramides of key glycosaminoglycans of the ECM. Consequently, in the present work we have used the laser flash photolysis technique to measure rate constants for the chloramide derivatives of both hyaluronan and heparin, the latter being a model for sulphated glycosaminoglycans of the ECM.

Materials and methods

a) Materials

The following materials were purchased as indicated : hyaluronan (Novozymes), heparin (Alfa Aesar) , hypochlorite solution, taurine (Sigma Aldrich), superoxide dismutase from bovine erythrocytes , > 3kU/mg protein (Sigma Aldrich). All other reagents were of analytical grade.

b) Preparation of chloramides and chloramines

The chloramide derivative of hyaluronan (HACl) was produced by reaction of 4 mg/ml (10 mM in disaccharide units) hyaluronan (HA) in chelex treated 0.1 M phosphate buffer pH 8.5 with 9 mM hypochlorite for 300 minutes at 37°C. The reaction was monitored at 292 nm to ensure that there was less than 0.3 mM unreacted HOCl which was then quenched by the addition of 2 mM taurine (conditions leading only to taurine monochloramine formation as confirmed by its characteristic absorption maximum at 251 nm) , followed by extensive dialysis in 9.5 mM borate buffer.

The [HACl] was also confirmed by TNB assay. In this assay, A solution of 5-thio-2-nitrobenzoic acid (TNB) was prepared by hydrolysis of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in 50 mM NaOH through gentle stirring in a dark container until the yellow colour development reached a

maximum, which took about 5 minutes. This TNB solution was diluted 40 times in 0.1M phosphate buffer (pH 7.4) prior to any assays and added to (10-25 μ l) of the chloramides to be assayed. These samples were then incubated in the dark at room temperature for 15 minutes after which absorbances were read spectrophotometrically at 412 nm and concentrations of chloramides measured using an extinction coefficient for TNB (ϵ_{412} 14,100 M⁻¹ cm⁻¹). In this reaction 1 mol of chloramides oxidizes 2 mol of TNB to 1 mol of DTNB [21].

Typical stock solutions prepared in this way were stored at 4 °C and contained 3.4 mM HAcI + 1.6 mM HA.

The chloramide derivative of heparin (HepCl) was synthesised from the reaction of 6.4 mg/ml heparin (10 mM in disaccharide units) hydrated in chelex treated 0.1M phosphate buffer pH 6.5 with 9 mM hypochlorite for 180 minutes at 37°C. The unreacted hypochlorite (less than 0.1 mM) in the reaction was quenched by addition of 1mM taurine (conditions again ensuring the formation of the monochloramine only). The taurine monochloramine that was formed was removed by extensive dialysis 10 mM borate buffer (pH 9.5). HepCl was then assayed using TNB to confirm its concentration. Typical stock solutions prepared in this way were stored at 4°C and contained 6.8 mM HepCl + 2.2 mM Hep.

For solution preparation for the laser flash photolysis experiments, all dilutions were carried out in chelex-treated borate buffer (pH 9.5) and contained 5 μ M EDTA. The sodium formate and sodium persulphate stock solutions were also prepared in chelex -treated borate buffer (pH 9.5). In all the experiments, the reagents were added just prior to any saturation with oxygen or air, with the exception of the cytochrome c competition experiments where the cytochrome c was added immediately before laser excitation to prevent possible cytochrome c oxidation by persulphate.

c) Laser flash photolysis

For laser photoexcitation experiments, 1 ml samples were excited using the 4th harmonic (266 nm) of a Q-switched Nd-YAG laser (Brilliant B, Quantel) in a 1 ml quartz cuvette of 1 cm pathlength. The energy output of each laser pulse was approximately 40 mJ and pulses were 6-8 ns in duration. Data was collected using an Applied Photophysics LKS-60 flash photolysis instrument with detection system at right angles to the incident laser beam. The probe light (150 W xenon lamp) was passed through a monochromator before and after passage through the sample. Absorbance changes were measured using a photomultiplier tube and kinetic transients were typically collected over 200 ms. For measurements over faster timescales (typically < 1 ms), the output of the xenon arc lamp was pulsed using a xenon arc pulser (Applied Photophysics) and transients were measured using an Infiniium oscilloscope model no. 54830B (Agilent Technologies).

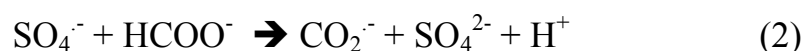
Results

Formation of superoxide radical using laser flash photolysis:

Laser flash photolysis was used to produce the superoxide radical in yields of up to 4.1×10^{-5} M. This was achieved by excitation, at 266 nm, of solutions containing 30 mM persulphate ($\text{S}_2\text{O}_8^{2-}$) and 10-50 mM formate (HCOO^-) in the presence of either air or oxygen and 50 μM EDTA. Upon excitation at 266 nm, persulphate (extinction coefficient of approximately $20 \text{ M}^{-1} \text{ cm}^{-1}$) produces sulphate anion radicals ($\text{SO}_4^{\cdot-}$) with a quantum efficiency close to 2 [28-30] (reaction 1). $\text{SO}_4^{\cdot-}$ has a peak of maximum absorption at 455 nm but several different values have been measured for its extinction coefficient [28- 37]. The reasons for the wide range of reported values have been discussed in detail [30, 33] and based on these findings, a value of $1600 \text{ M}^{-1} \text{ cm}^{-1}$ at 455 nm has been used in the current work.



In the presence of formate, $\text{SO}_4^{\cdot-}$ oxidises formate to $\text{CO}_2^{\cdot-}$, with a rate constant for reaction (2) of 1.1 to $1.7 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ [38, 39].



At 10 mM formate, the decay of the $\text{SO}_4^{\cdot-}$, monitored at 450 nm , was complete within $2\text{-}3 \mu\text{s}$, consistent with the above second-order rate constants. Addition of hyaluronan, heparin, hyaluronan chloramide and heparin chloramide at concentrations of up to 4 mM did not increase the rate of decay of $\text{SO}_4^{\cdot-}$, indicating an upper limit of $4 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ for reactions with these substrates. Hence, it is unlikely that reactions of the sulphate radical with these glycosaminoglycans and their chloramide derivatives will compete significantly with the formation of $\text{CO}_2^{\cdot-}$ at the formate concentrations employed. Using an extinction coefficient of $1600 \text{ M}^{-1} \text{ cm}^{-1}$ at 450 nm for the sulphate radical, typical initial yields at the end of the laser pulse were in the range $3.5 - 4.1 \times 10^{-5} \text{ M}$. In the presence of oxygen, $\text{CO}_2^{\cdot-}$ reacts rapidly ($k = 2.4 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$) [40] to produce the superoxide radical (reaction 3).



In oxygenated solutions (approximately 1 mM oxygen), superoxide radicals would thus be produced within about $2 \mu\text{s}$.

Kinetic measurements of the decay of superoxide radical absorbance

The superoxide radical exists in equilibrium with its protonated form, HO_2^{\cdot} ($\text{pK}_a = 4.8$) [41]:



At pHs above the pK_a therefore, the principal species is the superoxide radical anion. The major route for its decay in the absence of substrates is via reaction with its protonated form, $HO_2\cdot$. As the pH is increased, it has been shown that the observed second-order rate constant decreases logarithmically [41]. At the pH of 9.5, chosen for the experiments in the current study, a second-order rate constant in the range 10^3 to 10^4 $M^{-1} s^{-1}$ may be anticipated [41]. The absorption spectrum due to superoxide only can be observed at pHs above 7, with an absorption maximum at 240 nm and an extinction coefficient of $2430 M^{-1} cm^{-1}$. In the current study, superoxide formation and decay was monitored at 270 nm, where an extinction coefficient of $1500 M^{-1} cm^{-1}$ was assumed [41].

Figure 1 shows the formation and decay of the superoxide radical at 270 nm in the absence and presence of 3 mM hyaluronan, heparin and also the amino acid taurine. In the absence of substrates, the initial concentration of superoxide is $4.1 \times 10^{-5} M$ and is therefore consistent with all $CO_2^{\cdot-}$ radicals reacting with oxygen. Its decay was simulated kinetically using the IBM Chemical Kinetics Simulator v1.01 programme by assuming a simple dismutation reaction for the overall reaction given in (5):



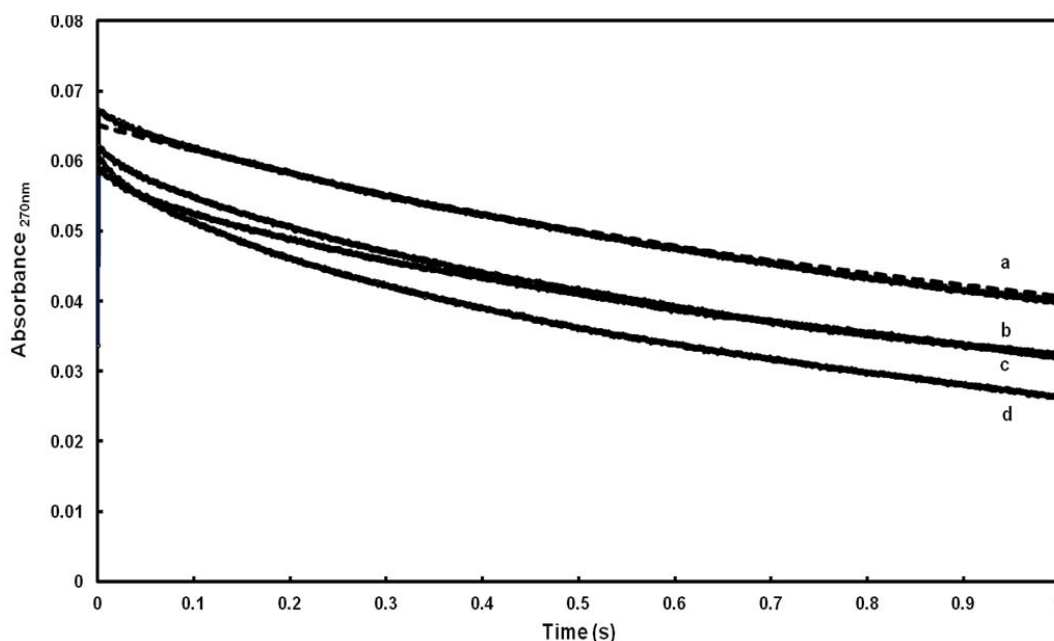


Figure 1

Decay of the superoxide radical absorbance at 270 nm in the absence (a) and presence of 3 mM hyaluronan (b), 3 mM heparin (c) and 3 mM taurine (d). All solutions contained 30 mM sodium persulphate, 50 mM sodium formate, 5 μ M EDTA in 10 mM borate buffer at pH 9.5, and were oxygen saturated.

A good fit to the experimental data was obtained in this way using $k(5) = 5.5 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$, consistent with the values anticipated at pH 9.5 [41]. This demonstrates that there can be little or no reaction of superoxide radicals with trace amounts of transition metal ions such as copper (I) and copper(II), whose simple aquo complexes react at diffusion controlled rates with superoxide. The use of EDTA to complex any such trace metal ions produces EDTA complexes which are relatively unreactive.

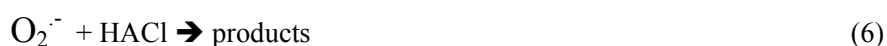
Reactions of superoxide with HA, Hep and Tau

In the presence of 3 mM HA and Hep, there is a small decrease in the initial concentration of superoxide, which is attributable to minor variations in laser pulse energy and also some small (<10%) reaction of $\text{SO}_4^{\cdot-}$ with HA or Hep. For these glycosaminoglycans, there is clearly no detectable reaction with superoxide radicals as trial simulations indicate that $k(\text{O}_2^{\cdot-} + \text{HA or Hep}) < 5 \text{ M}^{-1} \text{ s}^{-1}$ (*i.e.* is unreactive). In the presence of 3 mM taurine, there is a small increase in the

rate of decay of the superoxide radical absorbance at 270 nm. Simulation of this decay indicates that $k(\text{O}_2^- + \text{taurine}) = 10 \pm 5 \text{ M}^{-1} \text{ s}^{-1}$, which can be compared with the existing known value of $< 10^3 \text{ M}^{-1} \text{ s}^{-1}$ measured at pH 7.4 [42].

Reactions of superoxide with HAcI, HepCl and TauCl

Figure 2 shows the decay of the superoxide radical absorbance at 270 nm in the presence of 1mM and 2 mM HAcI (with respective HA concentrations of 0.32 mM and 0.64 mM). There is little or no effect of HAcI at these concentrations on the initial amount of superoxide observed, confirming that there is no significant competition of the chloramide for either SO_4^- or CO_2^- , perhaps attributable to the electronic repulsion of the ionized carboxyl groups close to the N-Cl functions in the biopolymers. However, it is clear from the 1 mM and 2 mM HAcI experiments that the rate of decay of superoxide increases dramatically in the presence of the chloramide. Simulation of the decay of superoxide, assuming only dismutation of superoxide (reaction 5) and a reaction with HAcI, produced good fits to the data by using values of $k(\text{O}_2^- + \text{O}_2^-)$ equal or close to $9.0 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$. The second-order rate constants for the reaction of superoxide with HAcI were thus calculated to be $2.2 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ at 1 mM HAcI and $2.4 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ at 2 mM HAcI. There was no need to include the reactions of superoxide with the parent glycosaminoglycans in the simulation due to their very low rate constants. Hence, for the first time, a direct kinetic measurement has established a value of $2.4 \pm 0.3 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ for the reaction:



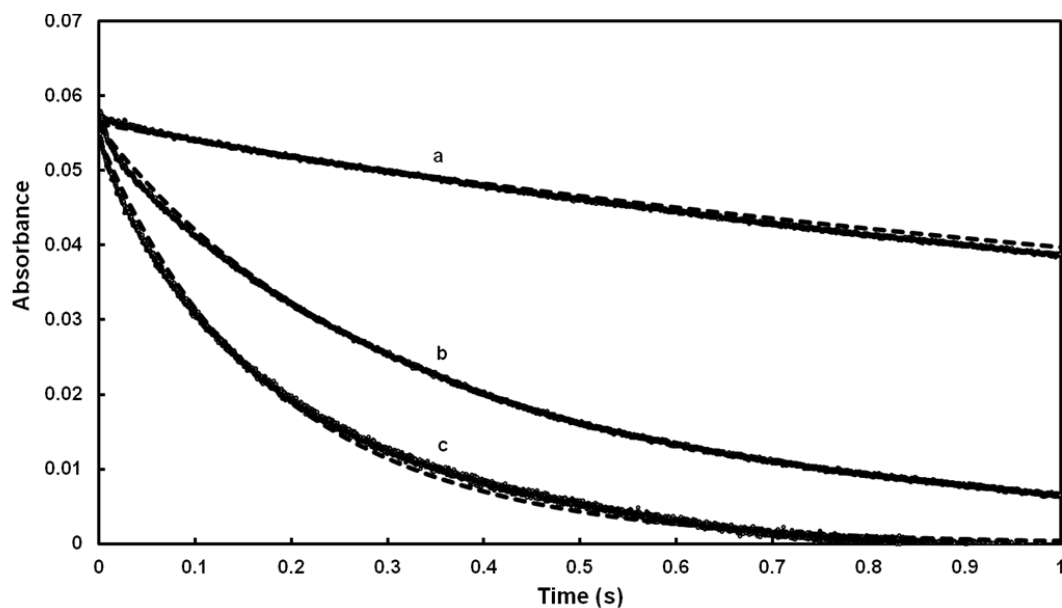


Figure 2

Decay of the superoxide radical anion absorbance at 270 nm in the absence or presence of HAcI. a) 0 mM HAcI fitted to $k(\text{O}_2^{\cdot-} + \text{O}_2^{\cdot-}) = 5.5 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ (---); b) 1 mM HAcI fitted to $k(\text{O}_2^{\cdot-} + \text{HAcI}) = 2.2 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ and $k(\text{O}_2^{\cdot-} + \text{O}_2^{\cdot-}) = 8.0 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ (---); c) 2 mM HAcI fitted to $k(\text{O}_2^{\cdot-} + \text{HAcI}) = 2.4 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ and $k(\text{O}_2^{\cdot-} + \text{O}_2^{\cdot-}) = 9.0 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ (---). All solutions contained 30 mM sodium persulphate, 50 mM sodium formate, 5 μM EDTA in 10 mM borate buffer at pH 9.5, and were oxygen saturated.

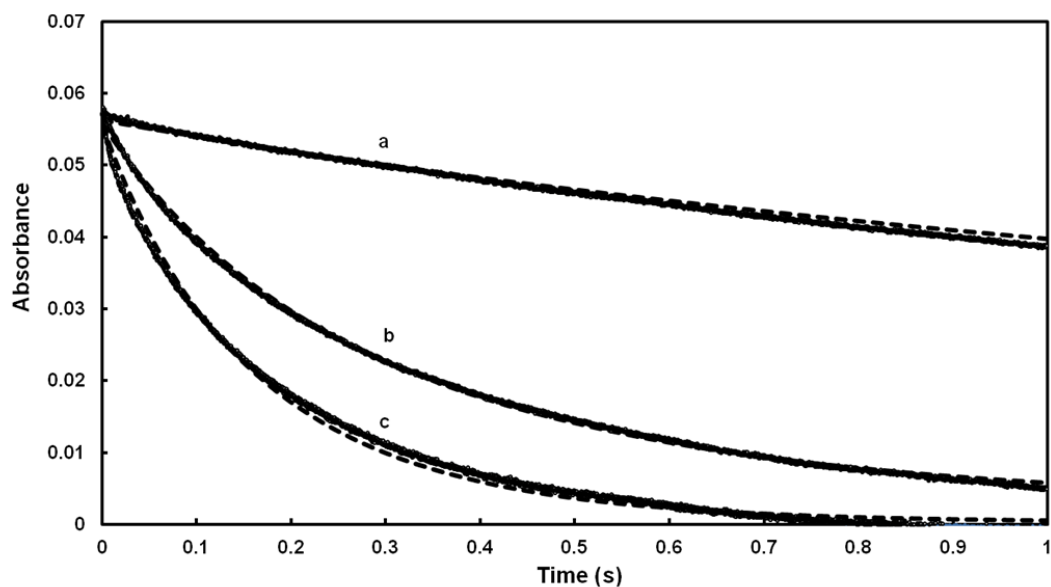


Figure 3

Decay of the superoxide radical anion absorbance at 270 nm in the absence or presence of HepCl. a) 0 mM HepCl fitted to $k(\text{O}_2^{\cdot-} + \text{O}_2^{\cdot-}) = 5.5 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ (---); b) 1 mM HepCl fitted to $k(\text{O}_2^{\cdot-} + \text{HepCl}) = 2.5 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ and $k(\text{O}_2^{\cdot-} + \text{O}_2^{\cdot-}) = 9.0 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ (---); c) 2 mM HepCl fitted to $k(\text{O}_2^{\cdot-} + \text{HepCl}) = 2.6 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ and $k(\text{O}_2^{\cdot-} + \text{O}_2^{\cdot-}) = 9.0 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ (---). All solutions contained 30 mM sodium persulphate, 50 mM sodium formate, 5 μM EDTA in 10 mM borate buffer at pH 9.5, and were oxygen saturated.

Similar experiments were carried out using 1 mM and 2 mM HepCl solutions (also containing 0.25 mM and 0.5 mM Hep respectively). The kinetic data are shown in figure 3 and demonstrate a definite reaction of superoxide with HepCl. Kinetic simulations produce good fits to the data, indicating $k(\text{O}_2^{\cdot-} + \text{HepCl}) = 2.5 \pm 0.3 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$.

The effect of the monochloramine of the amino acid, taurine, on the decay of the superoxide radical was also studied using 1 mM and 2 mM TauCl. Figure 4 shows the kinetic data at 270 nm. Unlike the experiments with HAcI and HepCl, the initial concentration of superoxide is significantly affected by the presence of the chloramine, decreasing relative to the control experiments, by approximately 50% and 66% in the 1 mM and 2 mM TauCl experiments respectively.

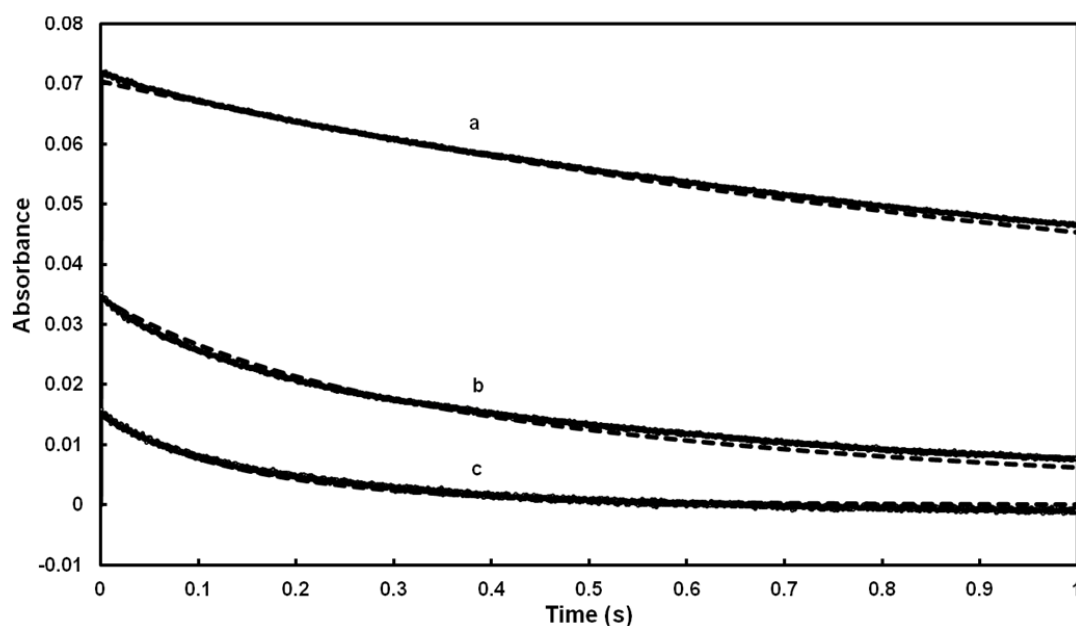


Figure 4

Decay of the superoxide radical anion absorbance at 270 nm in the absence or presence of TauCl. a) 0 mM TauCl fitted to $k(\text{O}_2^{\cdot-} + \text{O}_2^{\cdot-}) = 7.0 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ (---); b) 1 mM TauCl fitted to $k(\text{O}_2^{\cdot-} + \text{TauCl}) = 3.5 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$, $k(\text{O}_2^{\cdot-} + \text{O}_2^{\cdot-}) = 4.0 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ and $k(\text{O}_2^{\cdot-} + \text{RO}_2) = 1.0 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ (---); c) 2 mM TauCl fitted to $k(\text{O}_2^{\cdot-} + \text{TauCl}) = 4.0 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$, $k(\text{O}_2^{\cdot-} + \text{O}_2^{\cdot-}) = 5.0 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ and $k(\text{O}_2^{\cdot-} + \text{R}) = 2.1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ (---). All solutions contained 30 mM sodium persulphate, 50 mM sodium formate, 5 μM EDTA in 10 mM borate buffer at pH 9.5, and were oxygen saturated.

The most likely explanation is that CO_2^- , a strong reducing agent (the one-electron reduction potential of CO_2 being -1.9 V [43]), reacts faster with TauCl than with the glycosaminoglycans chloramides. By simple competition of this reaction with the reaction between CO_2^- and oxygen (reaction (3) above), and using $k(3) = 2.4 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ [40], it can be estimated that $k(\text{CO}_2^- + \text{TauCl}) = 2.4 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$.

Figure 4 also shows that an increase in the concentration of TauCl does increase the rate of decay of superoxide. Kinetic simulations of the kinetic data for the 1 mM and 2 mM TauCl experiments using only the superoxide dismutation (reaction (5)) and the reaction between superoxide and TauCl did not produce consistent $k(\text{O}_2^- + \text{TauCl})$ values. In view of the significant competition of CO_2^- for both oxygen and TauCl, it seemed plausible that the peroxy radicals (designated here as RO_2^-) formed in the $\text{CO}_2^- / \text{TauCl}$ reaction could also react with superoxide radicals. Inclusion of this latter reaction with rate constants in the range of $1\text{-}2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ produced good fits and consistent values for $k(\text{O}_2^- + \text{TauCl}) = 3.5\text{-}4.0 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$.

Kinetic measurements of the reduction of ferricytochrome c by the superoxide radical

An additional approach to determine the rate of reaction of superoxide with the glycosaminoglycan chloramides and taurine monochloramine involved monitoring the rate of reduction of ferricytochrome c (cyt c) at 550 nm in the absence and presence of the latter substrates. Laser flash photolysis of oxygenated solutions containing 30 mM persulphate, 50 mM formate, 50 μM cyt c, 50 μM EDTA at pH 9.5 produced a growth in absorbance at 550 nm over 1 s . Under these conditions, CO_2^- reacts much more rapidly with oxygen ($k = 2.4 \times 10^6 \text{ s}^{-1}$) than with ferricytochrome c ($k = 3.5 \times 10^4 \text{ s}^{-1}$ [50]) and the growth was therefore assigned to the reduction of ferricytochrome c by superoxide (see figure 5a). To provide additional support for the latter assignment, the experiment was repeated in the presence of 0.5 μM superoxide

dismutase. The resultant kinetic trace (5d and also the inset) shows that the amount of ferrocytochrome c formed is now only 3% of that seen in figure 5a indicating that the enzyme has removed superoxide radicals effectively. Using a difference extinction coefficient of $2.11 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ for the ferri- and ferro- forms of cytochrome c [44], the yield of ferrocytochrome c reduction is $1.5 \times 10^{-5} \text{ M}$. This is lower than the superoxide concentrations observed at similar laser energies in the absence of cytochrome c. This can be attributed to a significant proportion of the laser energy being absorbed by the cytochrome c, which has a strong absorption at the laser excitation wavelength of 266 nm. However, it should be noted that the laser flash photolysis of cytochrome c solutions alone did not produce any long-lived transient species which could interfere with the superoxide absorbance. The rate of reduction of ferricytochrome c, shown in figure 5 was $2.0 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, which is consistent with the value of $1.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ determined at pH 9.5 in a key study of the effect of pH on the reaction of cytochrome c with superoxide [45].

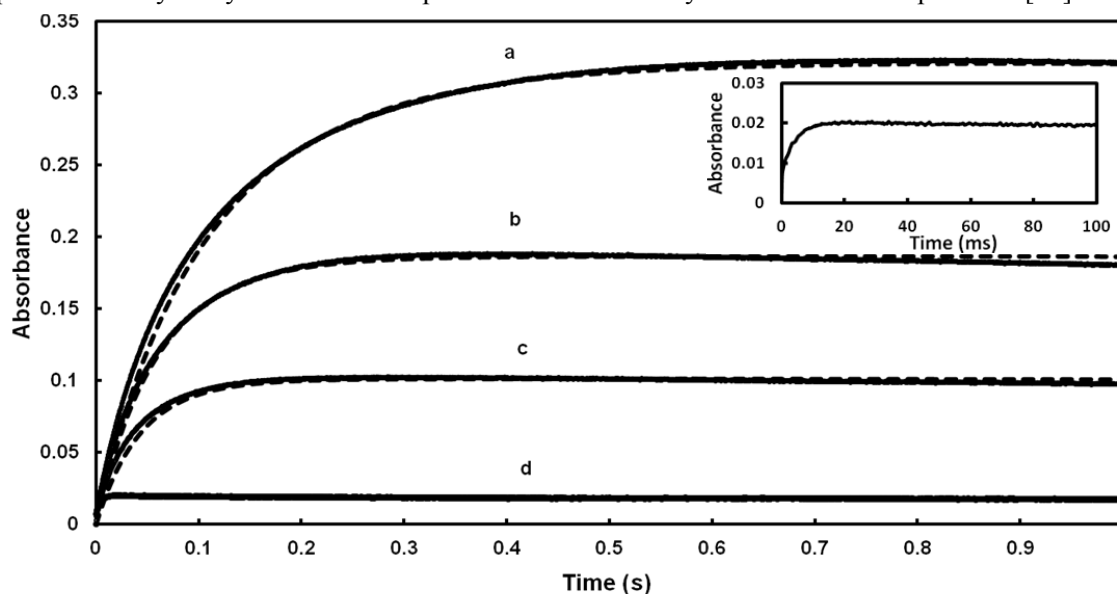


Figure 5

Reduction of ferricytochrome c by the superoxide radical observed at 550 nm in the absence or presence of HAcI. a) 0 mM HAcI fitted to $k(\text{O}_2^- + \text{O}_2^-) = 1.0 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ and $k(\text{O}_2^- + \text{cyt c}) = 2.0 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ (---); b) 2 mM HAcI fitted to $k(\text{O}_2^- + \text{O}_2^-) = 1.0 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, $k(\text{O}_2^- + \text{cyt c}) = 2.0 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $k(\text{O}_2^- + \text{HAcI}) = 2.4 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ (---); c) 3 mM HAcI fitted to $k(\text{O}_2^- + \text{O}_2^-) = 1.0 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, $k(\text{O}_2^- + \text{cyt c}) = 3.0 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $k(\text{O}_2^- + \text{HAcI}) = 2.7 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ (---). All solutions contained 30 mM sodium persulphate, 10 mM sodium formate, 50 μM ferricytochrome c, 5 μM EDTA in 10 mM borate buffer at pH 9.5, and were oxygen saturated; d) as for a) with addition of 0.5 μM superoxide dismutase (the inset shows an enlarged portion of this kinetic trace)

Reduction of ferricytochrome c in the presence of HAcI, HepCl and TauCl.

Figure 5 also shows the effect of 2 mM and 3 mM HAcI on both the rate and the yield of reduction of ferricytochrome c. As [HAcI] is increased, the rate increases and the yield decreases. Simulations, using only the dismutation of superoxide (reaction 5) and the reaction of superoxide with cyt c, yielded $k(\text{O}_2^- + \text{HAcI})$ values of 2.4×10^3 and $2.7 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ respectively. These values are very similar to the rate constants obtained using the decay of superoxide absorbance at 270 nm. Similar experiments were also carried out with solutions containing 2 mM HepCl and 4 mM HepCl and these are shown in figure 6. Kinetic simulation yielded values of $k(\text{O}_2^- + \text{HepCl})$ values of 2.2×10^3 and $2.7 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ respectively, again similar to those obtained in the 270 nm experiments.

Figure 7 shows the rates of reduction of ferricytochrome c in the presence of 1 mM, 2 mM and 4 mM TauCl. The effect is more dramatic than that observed in the HAcI and HepCl experiments, which is attributable to the reduction in superoxide yield that arises from the competition of CO_2^- for oxygen and TauCl. In the absence of TauCl, the ferrocyanochrome c yield of $1.1 \times 10^{-5} \text{ M}$ has also been used as the initial superoxide radical yield. Consequently, the initial yields of superoxide radical in the TauCl for the 1mM and 2 mM TauCl experiments can be calculated as $5.5 \times 10^{-6} \text{ M}$, $3.7 \times 10^{-6} \text{ M}$. However, unlike the more direct measurements of superoxide decay measured at 270 nm in the absence of cytochrome c (see figure 4), simulation of the kinetic data in figure 7 using the same reaction scheme, did not produce reasonable fits. The extra complexity, due to the presence of both peroxy radicals of taurine (from the reaction of CO_2^- with TauCl) and ferricytochrome c, meant that accurate fitting of the data was not possible. Nevertheless, the reduction in ferrocyanochrome c yields as [TauCl] was increased is consistent with a reaction of superoxide radicals with TauCl.

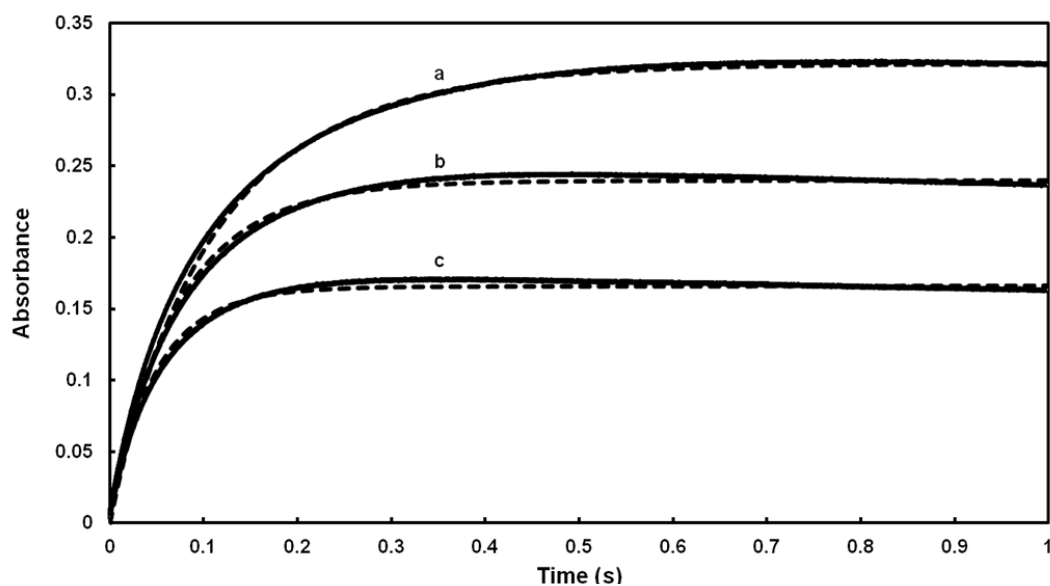


Figure 6

Reduction of ferricytochrome c by the superoxide radical observed at 550 nm in the absence or presence of HepCl. a) 0 mM HepCl fitted to $k(\text{O}_2^{\cdot-} + \text{O}_2^{\cdot-}) = 1.0 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, $k(\text{O}_2^{\cdot-} + \text{cyt c}) = 2.0 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ (---); b) 2 mM HepCl fitted to $k(\text{O}_2^{\cdot-} + \text{O}_2^{\cdot-}) = 1.0 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, $k(\text{O}_2^{\cdot-} + \text{cyt c}) = 2.2 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ and $k(\text{O}_2^{\cdot-} + \text{HepCl}) = 2.2 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ (---); c) 4 mM HepCl fitted to $k(\text{O}_2^{\cdot-} + \text{O}_2^{\cdot-}) = 1.0 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, $k(\text{O}_2^{\cdot-} + \text{cyt c}) = 2.5 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ and $k(\text{O}_2^{\cdot-} + \text{HepCl}) = 2.7 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ (---). All solutions contained 30 mM sodium persulphate, 10 mM sodium formate, 50 μM ferricytochrome c, 5 μM EDTA in 10 mM borate buffer at pH 9.5, and were oxygen saturated.

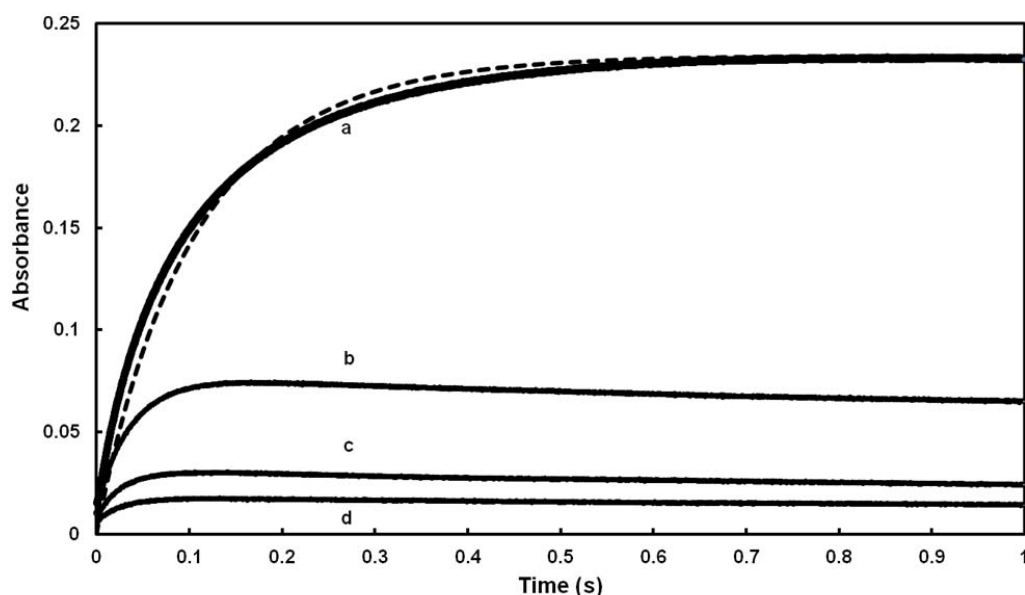


Figure 7

Reduction of ferricytochrome c by the superoxide radical observed at 550 nm in the absence or presence of TauCl. a) 0 mM TauCl; b) 1 mM TauCl; c) 2 mM TauCl; d) 4 mM TauCl. All solutions contained 30 mM sodium persulphate, 10 mM sodium formate, 50 μM ferricytochrome c, 5 μM EDTA in 10 mM borate buffer at pH 9.5, and were oxygen saturated.

Discussion

In the present work we have used laser flash photolysis techniques to provide two direct approaches to determine the rate constants for the reaction of superoxide radicals with the chloramides of hyaluronan and heparin, as well as that of the monochloramine of an amino acid, taurine. Measurements of superoxide rate constants are often hindered by the typically low rates of reaction with many substrates in conjunction with the almost diffusion-controlled rates with trace metal aquo ions, such as $\text{Cu}^{2+}_{\text{aq}}$. In this study at pH 9.5, the use of chelex resins and EDTA (to remove ions such as $\text{Fe}^{2+}_{\text{aq}}$, $\text{Fe}^{3+}_{\text{aq}}$ and Cu^{2+} and to make Cu^{2+} species considerably less reactive), resulted in the lifetime of superoxide extending to some 10 s, in line with the established values for superoxide dismutation at this pH [41]. The possibility that the chloramides might bind small amounts of Cu^{2+} and so account for the significant effect of the chloramide and chloramine derivatives on the decay of superoxide seem therefore implausible. The binding of Cu^{2+} to hyaluronan ($K = 3000 \text{ M}^{-1}$ [46]) is relatively weak and would not compete with EDTA. Pulse radiolysis experiments have previously been used to study the effect of pH on the rate of reaction of superoxide with ferricytochrome c, which would be expected to bind Cu^{2+} ions much more strongly than glycosaminoglycans. However, whilst Cu^{2+} ions did increase the rate of decay at pHs between 7 and 8.5, there was no effect at higher pHs where unreactive hydroxo complexes of Cu^{2+} are formed [45]. These considerations argue strongly for the lack of any effect of trace copper ions on the decay of superoxide and conversely, provide an unambiguous demonstration that direct reactions between superoxide radicals and the chloramide / chloramine derivatives are being observed in our laser flash photolysis experiments.

The rate constants for HACl and HepCl, summarised in Table 1, are remarkably similar, indicating perhaps that activation to the transition state is localised at the N-Cl group. Additionally the charge on the glycosaminoglycan has little or no effect on the rate constants. Heparin chloramide, the most-heavily sulphated glycosaminoglycan, has no effect on the reaction of the superoxide anion radical when compared to that with HACl. Glycosaminoglycans however, are not simple ionic species and are strongly associated with their sodium counterions, mitigating any simple kinetic relationships between charged species. The lack of an effect from a sulphate group bound directly to the N atom of the N-Cl group again indicates a high localisation of the transition state in reactions with superoxide. There are no other direct measurements of these rate constants in the literature. However, previous EPR experiments have provided an estimate for the rate constant for reaction of superoxide with taurine chloramines of $5-6 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$ [27]. This value is very close to the values of $3.5-4.0 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$ determined in the current study. In the previous study [27], a thermal source of superoxide, di-(carboxybenzyl) hyponitrite (SOTS-1), was used to generate superoxide. Reaction with the taurine chloramines was demonstrated by the inhibitory effect of superoxide dismutase. In a later study, again using SOTS-1 to react with the chloramides of hyaluronan and chondroitin sulphates, it was concluded, through the inhibitory effect of EDTA, that the superoxide reaction was mediated, at least in part, by Cu^{2+}aq [20], 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 heparin sulphate [47]. This indirect mechanism for reaction of superoxide with glycosaminoglycan chloramides may be in competition with the direct reaction demonstrated in this study. At the

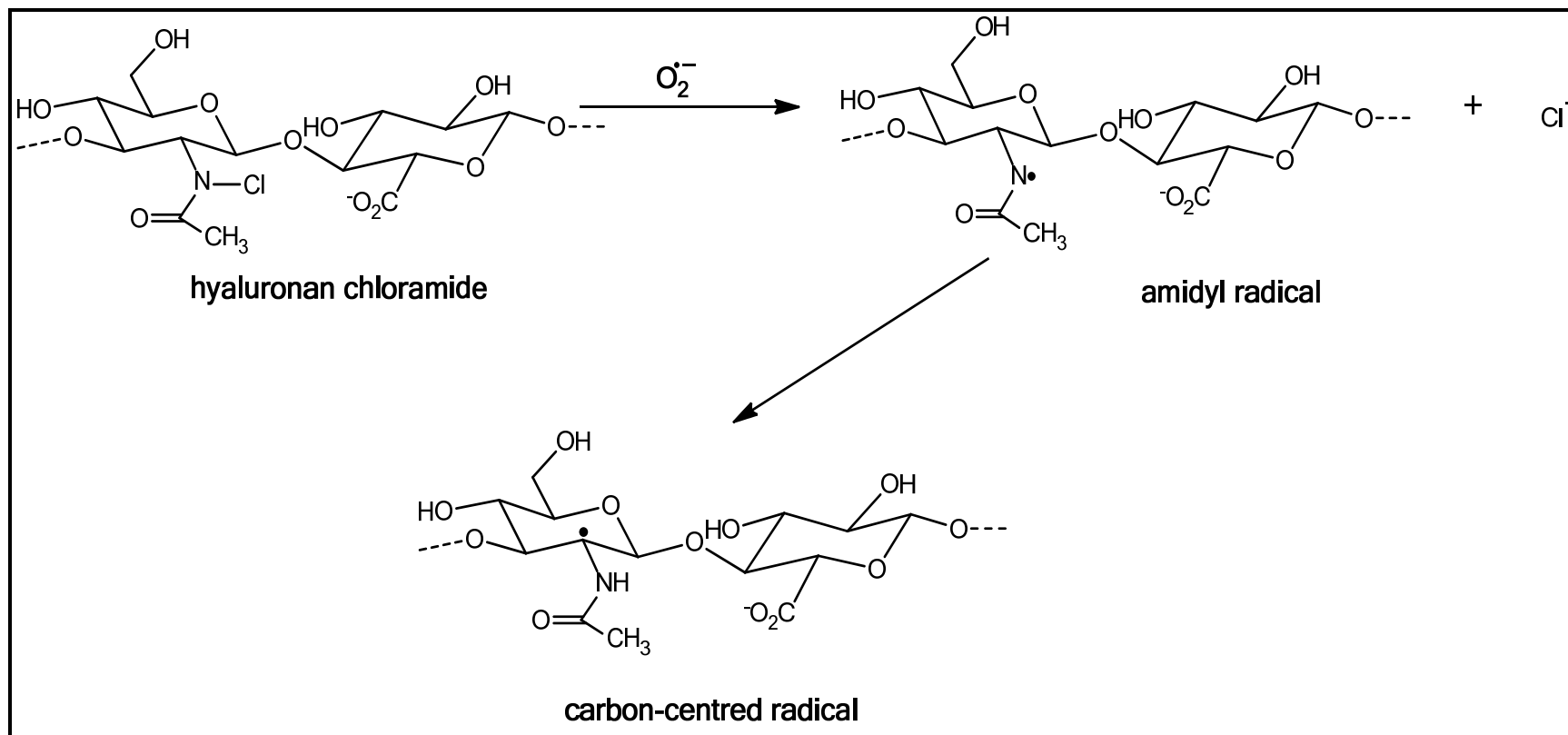
relatively low concentrations of chloramides, 200–300 μM used in the earlier studies [20,27,47], as little as 1 nM of $\text{Cu}^{2+}_{\text{aq}}$ involved in effective redox cycling at pH 7.4 would be the main channel of reaction, since superoxide reacts at diffusion-controlled rates with Cu (I/II) aquo complexes. At the pH of 9.5 and the higher chloramide and chloramine concentrations used in this study, the direct reaction is dominant.

Table 1

R-NCl	k($\text{O}_2^{\cdot-} + \text{R-NCl}$) $\text{M}^{-1}\text{s}^{-1}$
HACl	$2.4 \pm 0.3 \times 10^3$
HepCl	$2.5 \pm 0.3 \times 10^3$
TauCl	$3.8 \pm 0.3 \times 10^2$

Table 1

Second-order rate constants for the reaction of superoxide anion radicals with HACl, HepCl and TauCl



Scheme 1

A mechanism for the fragmentation of HAcI by superoxide anion radicals (see [20]).

The mechanism of the direct superoxide reaction is likely to involve the one-electron reduction at the N-Cl site in which chloride ions are liberated. The one-electron reduction by the strong reducing agent, the hydrated electron, of the simplest chloramine, NH_2Cl , was found to occur at diffusion-controlled rates by pulse radiolysis and assumed to liberate chloride ions [23, 24]. Superoxide radicals are weak reducing agents ($E(\text{O}_2/\text{O}_2^- = -0.33 \text{ V})$ [48] and can be expected to react much more slowly than strong reducing agents. From EPR studies, it has been proposed that both Cu(I) and superoxide radicals transfer an electron to the chloramides, forming nitrogen-centred radicals in the first instance which then undergo a 1, 2 shift that results in C-2 carbon-centred radicals (see Scheme 1 and [20]). The nitrogen-centred radicals are short-lived and only the C-2 radicals can be detected [47]. Pulse radiolysis studies of the reaction of hydroxyl radicals with amides produced carbon-centred radicals with characteristic absorption spectra in the UV/visible region but also did not detect any nitrogen-centred radicals [49]. Although such free radicals may, in principle, be detected in the reaction of superoxide radicals with glycosaminoglycan chloramides, the low rate constant for the reaction makes it very difficult to detect the initial free radical products as they will decay much more quickly than they are formed. It is then the scission of the glycosaminoglycan C-2 radicals which leads to the fragmentation of the biopolymer. It has been estimated that Cu(II)/Cu(I) ions are at least 50% efficient in their reaction in causing fragmentation [47]. There were no similar estimates in the latter work for the reactions of superoxide radicals.

In conclusion, therefore, superoxide radicals have been shown for the first time to react directly with the chloramides and chloramine studied here. There is however no evidence

yet that the direct reaction leads to an electron transfer taking place to produce C-2 radicals and then fragmentation. The occurrence of such a reaction *in vivo* (*i.e.* one not requiring the presence of adventitious metal ions such as Cu(I) or Fe(II)) would be very significant.

Localised excess production of HOCl has been implicated in a number of diseases that involve an acute or chronic inflammatory response (e.g. atherosclerosis, rheumatoid arthritis and asthma) [51,52]. There is evidence that binding of myeloperoxidase to matrix components and cell-surface glycosaminoglycans directs oxidative damage towards these biopolymers [53-55]. At sites of inflammation within the extracellular matrix, activated phagocytes release the haem enzyme myeloperoxidase and produce high concentrations of the superoxide anion and its dismutation product, hydrogen peroxide via an oxidative burst. Myeloperoxidase is a highly basic protein and is known to bind, via electrostatic interactions, to negatively charged materials such as the polyanionic glycosaminoglycans [55]. The combination of site-specific production of chloramides via binding of myeloperoxidase to glycosaminoglycans and the likely selectivity and potential efficiency in causing fragmentation by superoxide radicals in their reactions with extracellular matrix chloramides may be the main channel of fragmentation in inflammation.

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