Chlorination and oxidation of heparin and hyaluronan by hypochlorous acid and hypochlorite anions: effect of sulphate groups on reaction pathways and kinetics.

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Abstract

Hypochlorous acid (HOCl), produced in inflammatory conditions by the enzyme myeloperoxidase, and its anion, perchlorite (OCl^-) exist *in vivo* at almost equal concentrations. Their reactions with hyluronan and heparin (as a model for sulphated glycosaminoglycans in the extracellular matrix), have been studied as a function of pH. The major product in these reactions is the chloramide derivative of the glycosaminoglycans. Spectral, chloramide yield and kinetic measurements show sharply contrasting behaviour of heparin and hyaluronan and the data allow the calculation of second-order rate constants for the reactions of both HOCl and OCl^- for all reaction pathways leading to the formation of chloramides and also oxidation products . By comparison with hyaluronan, it can be demonstrated that both N-sulphate and O-sulphate groups in heparin influence the proportions of these pathways in this glycosaminoglycan. Evidence is also given for further oxidation pathways involving a reaction of HOCl with the chloramide product of hyaluronan but not with heparin. The significance of these results for mechanisms of inflammation, particularly for fragmentation of extracellular matrix glycosaminoglycans is discussed.

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 including aggrecan, versican, and tumor necrosis factor alpha stimulated gene 6 (TSG-6) [1,2].

With this central structural function, HA is a particularly important component of the ECM [3, 4], as demonstrated by the fact that 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. The potential mechanisms of oxidative damage in these diseases has been reviewed recently [13].

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. Much of this early work has been reviewed [14].

The focus of HA studies in our laboratories has been to measure 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 polydisperse 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 have been determined [15,16].

The fragmentation of hyaluronan has also been investigated intensively by Davies and co-workers using both electron paramagnetic resonance and sensitive PAGE techniques. The use of the latter technique showed the novel and exciting result that hypochlorite reacts 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]. Using the PAGE technique, similar site-specific reactions were also observed by Davies and co-workers for oxidation of several glycosaminoglycans by peroxynitrous acid, carbonate and hydroxyl radicals [19,20]. Equally significant for the understanding of potential molecular mechanisms involved in inflammation is the demonstration in these studies that hypochlorite species form chloramides when they react with amides [21] and in particular, with glycosaminoglycans [17,18,22]. Here, a substitution reaction occurs where the N-H group becomes an N-Cl group. At sites of

inflammation where myeloperoxidase can form hypochlorous acid, the potential formation of such derivatives may accelerate the fragmentation of glycosaminoglycans within the extracellular matrix. Chloramides are weak oxidising agents and therefore are 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 HA chloramide [18]. It is also a possibility that an N-Cl group presents a more reactive target to strong oxidising agents such as the hydroxyl and carbonate radicals, perhaps leading to efficient site-specific fragmentation.

HOCl has a pKa value of 7.59 [23,25] and therefore at physiological pH there is approximately a 50:50 mixture of the acid and its anion OCl^-. In principle therefore, both species can participate in the substitution reaction to form chloramides. However, most if not all of the fragmentation and kinetic studies on glycosaminoglycan chloramides have been carried at pH 7.4 . In these studies, it is implicit that substitution is the major process. HOCl is also a strong oxidising agent [29]) and there is clearly the potential for it and its anion counterpart to engage in oxidative reactions, particularly in hydrogen abstraction, the dominant mechanism in fragmentation of glycosaminoglycans. It may be therefore that both substitution and oxidation reactions occur simultaneously. It may also be that the N-Cl group itself presents as a more attractive target for oxidation by the hypochlorous species.

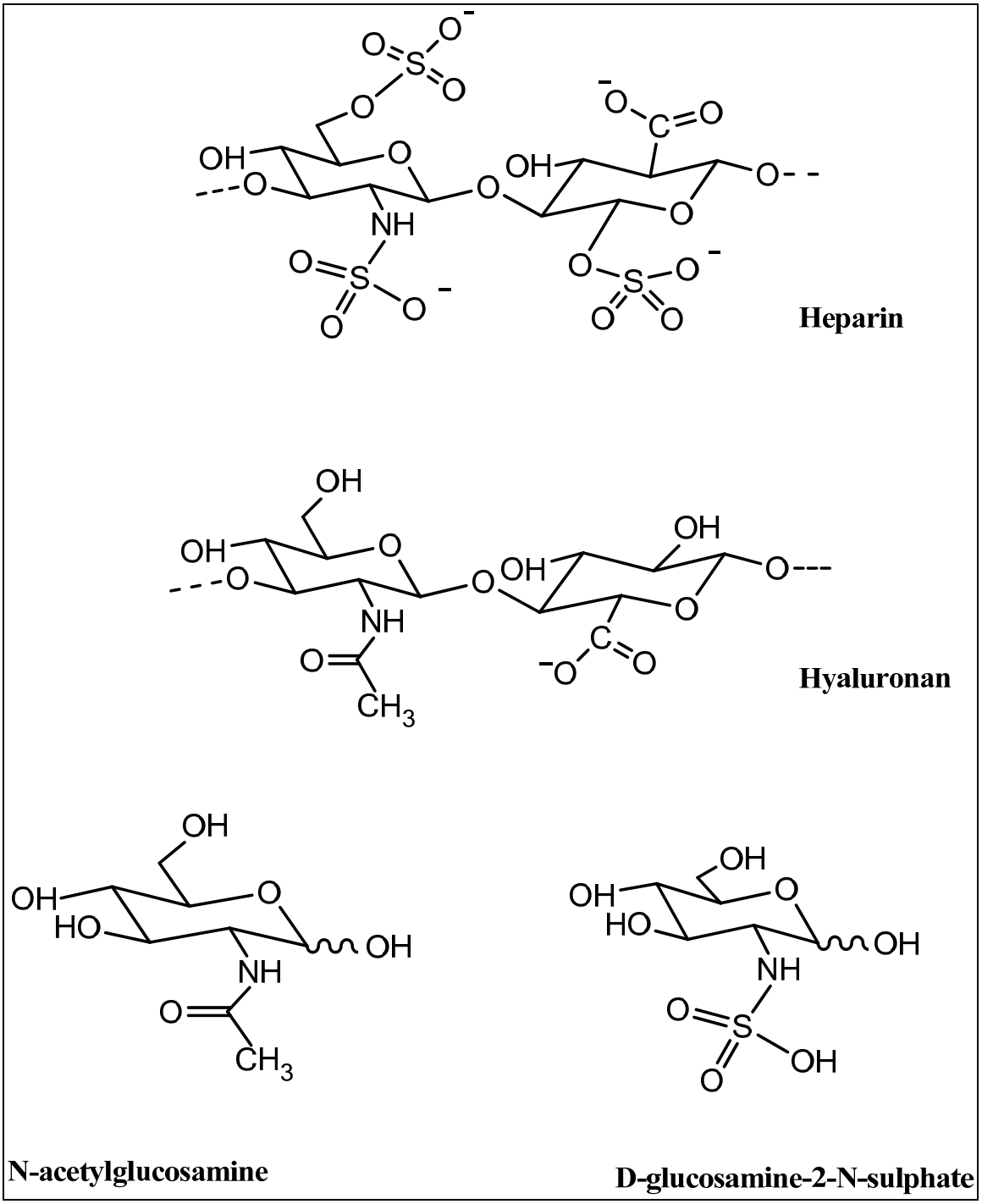


Figure 1 Structures of hyaluronan, heparin, N-acetylglucosmaine and D-glucosamine-2-N-sulphate.

It is the main purpose of this study to investigate both the kinetic reactivity and reaction profiles of the two species , HOCl and OCl^- with both hyaluronan and heparin, the latter being a model for sulphated glycosaminoglycans of the extracellular matrix. Although these two biopolymers have similar structures (see Figure 1), they have large differences in charge density since heparin contains several sulphate groups, on average 2-3 per disaccharide unit. It is anticipated that this study of the reactivity of these contrasting structures will lead to a basis for prediction of the modes of reaction of the hypochlorite species with glycosaminoglycans, particularly to differentiate the response of sulphated and non-sulphated glycosaminoglycans in the extracellular matrix and also to assess whether chloramide formation may accelerate fragmentation via subsequent site-specific oxidation by the hypochlorous species.

Materials and methods

Hyaluronan (45kDa) was provided as a gift by Novozymes. Heparin (porcine) was purchased from Alfa Aesar and Sigma Aldrich. Solutions were prepared in chelex-treated buffers of 0.1 M phosphate (pH 6.5 to 8.5) allowing sufficient time to fully hydrate the glycosaminoglycans. Hypochlorous acid (Sigma) solutions were prepared freshly and the concentrations of stock solutions determined using the extinction coefficient of the hypochlorite ion of 350 M^{-1} cm^{-1} at pH values 10 or above [25].

The reactions of hypochlorous acid with heparin and hyaluronan were investigated by simple mixing experiments conducted at 37°C. Absorption spectrophotometry in the range 200 nm to 400 nm was used to monitor the progress of the reactions. Yields of the respective chloramides were determined using TNB (5-thio-2-nitrobenzoic acid, Sigma

Aldrich) assays after first quenching the reaction with excess taurine. Dilutions of the quenched reactions were selected to match the 35-45 μM concentrations of TNB. After 30 minutes incubation, the absorbance of the TNB thiol oxidation product was measured at 412 nm (extinction coefficient = 13600 M^{-1} cm^{-1} [21]).

Glycosaminoglycan samples were analysed to determine the extent of fragmentation of the biopolymers using the modified Min and Cowman PAGE method [26,27]. 10% and 20% polyacrylamide gels (0.1x16x20 cm) were run using a Bio-Rad Protean II xi multi cell system. 15μl of sample was added to 5μl of loading buffer (10x TBE buffer, 1M tris/0.25M borate/0.01M EDTA) containing 2M sucrose. This 20μl volume was loaded onto gels covered with 1x TBE running buffer. For hyaluronan samples, the gels were run at 125V (20mA) for 20 minutes then at 250V (40mA) for approximately 80 minutes or until the bromophenol blue tracking dye approached the gel bottom. For heparin samples, the gels were run for 7 to 10 hours at 100 V. The gels were immediately transferred to non-stick trays after the run and were soaked with 0.5% alcian blue dissolved in deionised water for 30 minutes in the dark. The gels were then de-stained in deionised water after which they were scanned using a high resolution scanner ,(Epson Perfection V500 Photo flatbed scanner, 6400 dpi x 9600 dpi). The captured images were visualised and digitised using Quantiscan ® software.

The gpc/MALLS system used for the determination of the molecular weight and molecular weight distribution of heparin and hyaluronan comprised a solvent distribution system, Model 6000A (Waters Division of Millipore, USA) connected to a Hemabio linear (10μm) steel column packed with hydrophilic modified HEMA gel. Injection into the gpc/MALLS system was achieved using a manual Rheodyne model 7125 syringe-

loading sample injector equipped with a 1ml sample loop and a concentration detector. The Wyatt Optilab DSP interferometric refractometer operated at 632.8 nm equipped with a 10-mm P100 cell (Wyatt Technology Corp., USA). The DAWN DSP laser light scattering photometer was equipped with a 632.8-nm He/Ne laser (Wyatt Technology Corp.) with 15 detectors calibrated with filtered toluene and normalized with pullulan (23.8 kDa) obtained from Polymer Standards Services. The pullulan was also used to determine the volume delay between detectors. A value of 0.150 was used for the refractive index increment (dn/dc). Data accumulation and analysis was achieved using Wyatt Technology ASTRA 4.9.07software. All measurements were performed at room temperature.

Results and Discussion

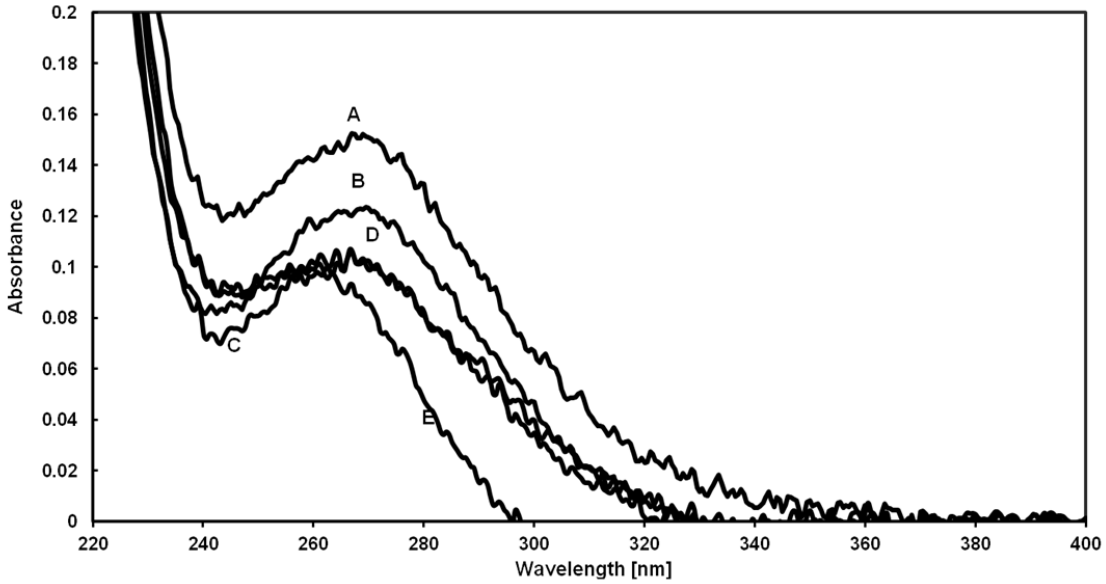
Yields of heparin and hyaluronan chloramides

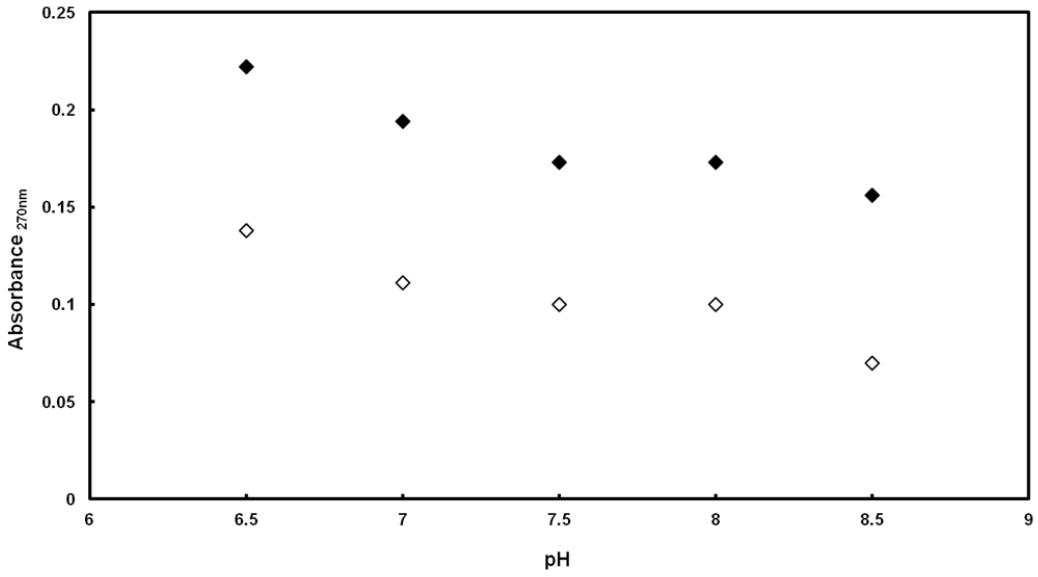
a) 1 mM hypochlorite experiments: In order to minimise the possibility of sequential secondary reactions of hypochlorite with the products of the reaction between hypochlorite and heparin or hyaluronan, relatively low concentrations of 1 mM hypochlorite solutions containing approximately 5 or 10 mM glycosaminoglycan were used to give 1:5 or 1:10 ratios of hypochlorite to glycosaminoglycan (where hypochlorite represents both HOCl and OCl^- species). The concentrations of hyaluronan and heparin were calculated using the molecular mass of the repeating disaccharide units. For heparin, which has some diversity in its structure, the concentrations, unless otherwise stated, were based on a repeat unit of molecular mass of 592. Such solutions at pHs 6.5 to 8.5

were incubated at 37°C for between 180 minutes and 440 minutes (heparin) and up to 600 minutes (hyaluronan) and then quenched by the addition of excess taurine (at concentrations which ensured formation only of taurine monochloramine and not the dichloramine). Changes in the absorbances at 251 nm (the absorption maximum of taurine monochloramine ; $\varepsilon = 429\ M^{-1}\ cm^{-1}$ [21]) and at 292 nm (the absorption maximum of both hypochlorite species [29]) allowed the calculation of unreacted hypochlorite concentrations. A TNB assay for total chloramide and chloramine (from the added taurine) was also undertaken for each reacted solution.

Heparin: Figure 2a shows the absorption spectra measured at pHs 6.5 to 8.5 at the end of the reactions for 5 mM heparin solutions after subtraction of contributions from unreacted heparin and any residual hypochlorite. The presence of an additional absorbing species is seen at 270 nm consistent with that of heparin chloramide as found in a previous study at pH 7.4 [22]. The absorbances at 270 nm show a clear effect of pH with higher values being measured at the lower pHs as shown in Figure 2b. The effect of pH on the % yield of the chloramide , as measured by the TNB assay, is shown in Figure 2c. This also shows a clear pH effect with a 94% yield at pH 6.5 decreasing to 60 % at pH 8.5. On the assumption that most of the absorption at 270 nm is attributable to the chloramide, the TNB assay data at pH 6.5, where a 94% yield of chloramide was measured, can be used to calculate an extinction coefficient for the chloramide at 270nm – here an estimate of 75 +/- 10 $M^{-1}\ cm^{-1}$ was made. From these experiments, it is clear that the reaction of hypochlorite with heparin produces significant concentrations of reaction products other than the chloramide. The high chloramide yield at pH 6.5 (94%) indicates that the reaction of neutral hypochlorous acid to produce the chloramide is faster than any

oxidation processes. The substitution reaction is clearly favoured despite the high oxidation potential of hypochlorous acid (standard reduction potential, 1.49 V) [24]. At pH 8.5, where the negatively charged hypochlorite ion, OCl^- is predominant, it would appear that the $N(H)\text{-}SO_3^-$ group hinders the substitution reaction rate making the possibility of oxidation processes more likely. The effect on the substitution rate constant must be considerable since oxidation rates by hypochlorite ions could be expected to be slower than for hypochlorous acid (standard reduction potential of OCl^- is 0.89 V [24]).





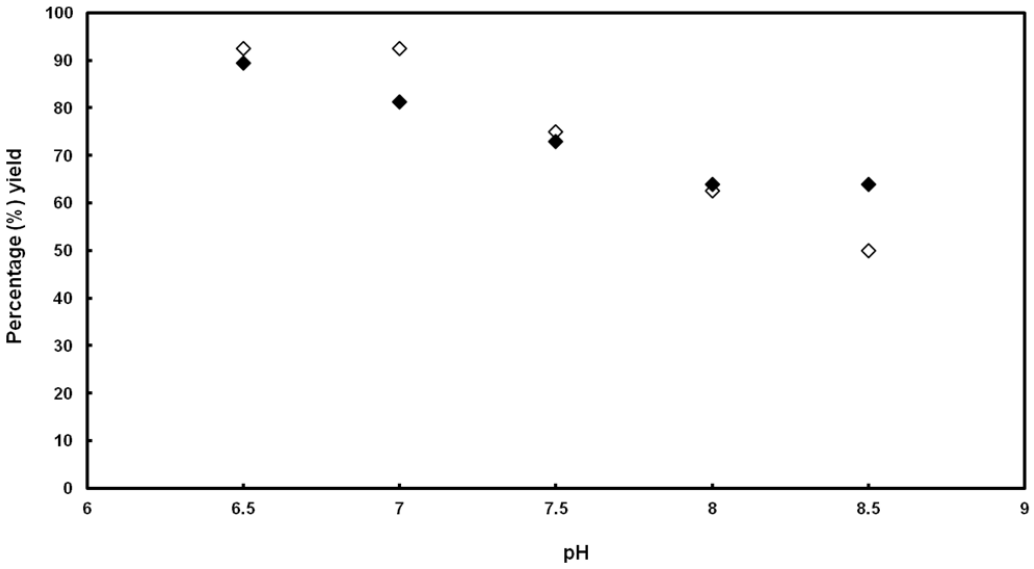


Figure 2 Absorption spectra measured at : **a)** 180 minutes at 37°C of the reaction mixtures of 1 mM hypochlorite and 5 mM heparin at pHs 6.5 to 8.5 . A, pH 6.5 ; B, pH 7.0; C pH 7.5; D, pH 8.0; E, pH 8.5 (2 cm path length; after subtraction of contributions from unreacted heparin and residual hypochlorite) **b)** Absorbance values at 270nm measured as in **a),** ♦ before subtraction of unreacted heparin, ◊, after subtraction of unreacted heparin **; c)** % reaction yields as a function of pH in a 1 mM hypochlorite, 9.67 mM heparin reaction mixture (◊) and in a 5mM hypochlorite and 9.67 mM heparin mixture (♦)

Hyaluronan: In similar experiments with hyaluronan, the spectra at 300 minutes after the start of the reaction of 1 mM HOCl with 10 mM hyaluronan (after subtraction of contributions due to unreacted hyaluronan and hypochlorite) were found to be similar in shape at the five pHs studied (pH 6.5 to 8.5) differing only due to small residual amounts of unreacted hypochlorite. It was clear that the reaction proceeded at a faster rate at higher pHs and so Figure 3 shows only the spectrum at pH 8.5 measured at the end of the reaction. The spectrum shows a broad absorption in the range 240 to 290 nm. At this pH, where a 100% reaction was measured, the extinction coefficient of the chloramide product was calculated as 80 +/- 5 M^{-1} cm^{-1} at 250 nm .

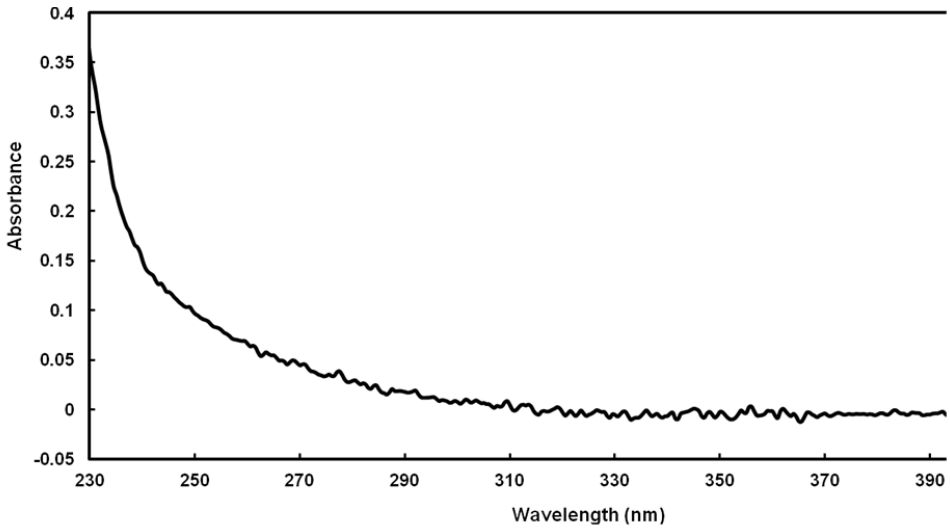


Figure 3 Absorption spectrum measured at 300 minutes of the reaction mixtures at 37°C of 1 mM hypochlorite and 10 mM hyaluronan at pHs 8.5 , (path length = 1 cm; after subtraction of contributions from unreacted hyaluronan and residual hypochlorite.

The effect of pH on the yield of the chloramide measured in TNB assays showed that the yield is close to 100% at all pHs indicating that a simple reaction to form the chloramide occurs with little or no evidence of other reaction pathways, such as those involving oxidation.. This observation is in sharp contrast to the mechanism described above for heparin. In hyaluronan, there are no SO_3^- groups and so the reactions of both hypochlorous acid and its anion, hypochlorite with the uncharged N(H)-acetyl group to form only the chloramide are favoured over oxidation processes.

b) 5mM hypochlorite experiments The effect of pH upon the yields of heparin and hyaluronan chloramides was also investigated by incubating solutions at 37°C of 5mM hypochlorite and approximately 10mM solutions of the glycosaminoglycan in 0.1M phosphate buffers. This facilitated the measurement of both hypochlorite and chloramide

and increased the probability of a secondary reaction of hypochlorite with the chloramide. For heparin, the reactions were monitored for up to 440 minutes. For hyaluronan, the reactions were much slower and were followed for a minimum of 10 hours. For both hyaluronan and heparin, this ensured that the reactions went to at least 80% completion. The reactions were then quenched by the addition of excess taurine. Changes in the asborbances at 251 nm (the absorption maximum of taurine monochloramine) again allowed the calculation of unreacted hypochlorite concentrations. For hyaluronan the absorbance at 292nm can be considered a reliable measure of unreacted HOCl due to the relatively low extinction coefficients of chloramides and other products. A TNB assay for total chloramides was conducted for each reacted solution.

Heparin Figure 4a shows the spectra obtained for heparin solutions at pH values 6.5 to 8.5 at 180 minutes (pH 6.5 to 7.5) and at 440 minutes (pH 8.0 and 8.5) after the initiation of the reactions. Contributions to the spectra from unreacted hypochlorite and heparin have been subtracted. The characteristic maximum at 270 nm for the heparin chloramide species [22] is seen clearly at pH 6.5 and, relative to this spectrum, it is seen that increase of pH leads to a decrease in the absorption at 270 nm together with a reduction in the sharpness of the maximum. At pH 6.5, the TNB assay (Figure 2c) showed that at least 90% of hypochlorite species had reacted to form the chloramide of heparin. Using the data at pH 6.5 in Figure 4a, the extinction coefficient of heparin chloramide at 270 nm was determined as 90 +/- 5 M^{-1} cm^{-1}. Adjusting the pH in the pH 6.5 experiment after the reaction was completed through to pH 8.5 produced no detectable change in either absorption maximum or intensity (data not shown), demonstrating that heparin chloramide is not involved in any acid-base equilibria in this pH range. The lack of such

an effect of pH had been also observed for some other chloramines and chloramides [21]. Therefore, the spectral changes in Figure 4a appear to reflect lower yields of heparin chloramide as the pH is increased together with an increasing spectral contribution from other products whose absorption is less intense and without character in this wavelength range. Thus, in Figure 4b, the absorbance at 270nm (after subtraction of small contributions due to unreacted heparin and residual hypochlorite) is seen to decrease from 0.42 to 0.23 as the pH is increased to 8.5. The effect of pH on chloramide yields at the higher hypochlorite concentration is shown in Figure 2c. It is clear that the pH profiles in Figures 4b and 2c are very similar in shape and demonstrate clearly that the yield of heparin chloramide decreases to about 60% value as the pH is increased to 8.5. The 270 nm absorbance data and the % chloramide yield data also show identical decreases, within experimental error, as pH increases to those found in the 1mM hypochlorite/ 5mM heparin experiments (see Figures 2b and 2c). This correlation suggests that hypochlorite reacts with heparin via at least two reaction pathways, one to form the chloramide and the other(s) to form oxidation products. The identical yields of heparin chloramide found for both 1mM and 5mM hypochlorite reactions with 9.67mM heparin at all pHs indicate that reactions which occur subsequently to the formation of the chloramide do not affect the yield within the experimental error limits.

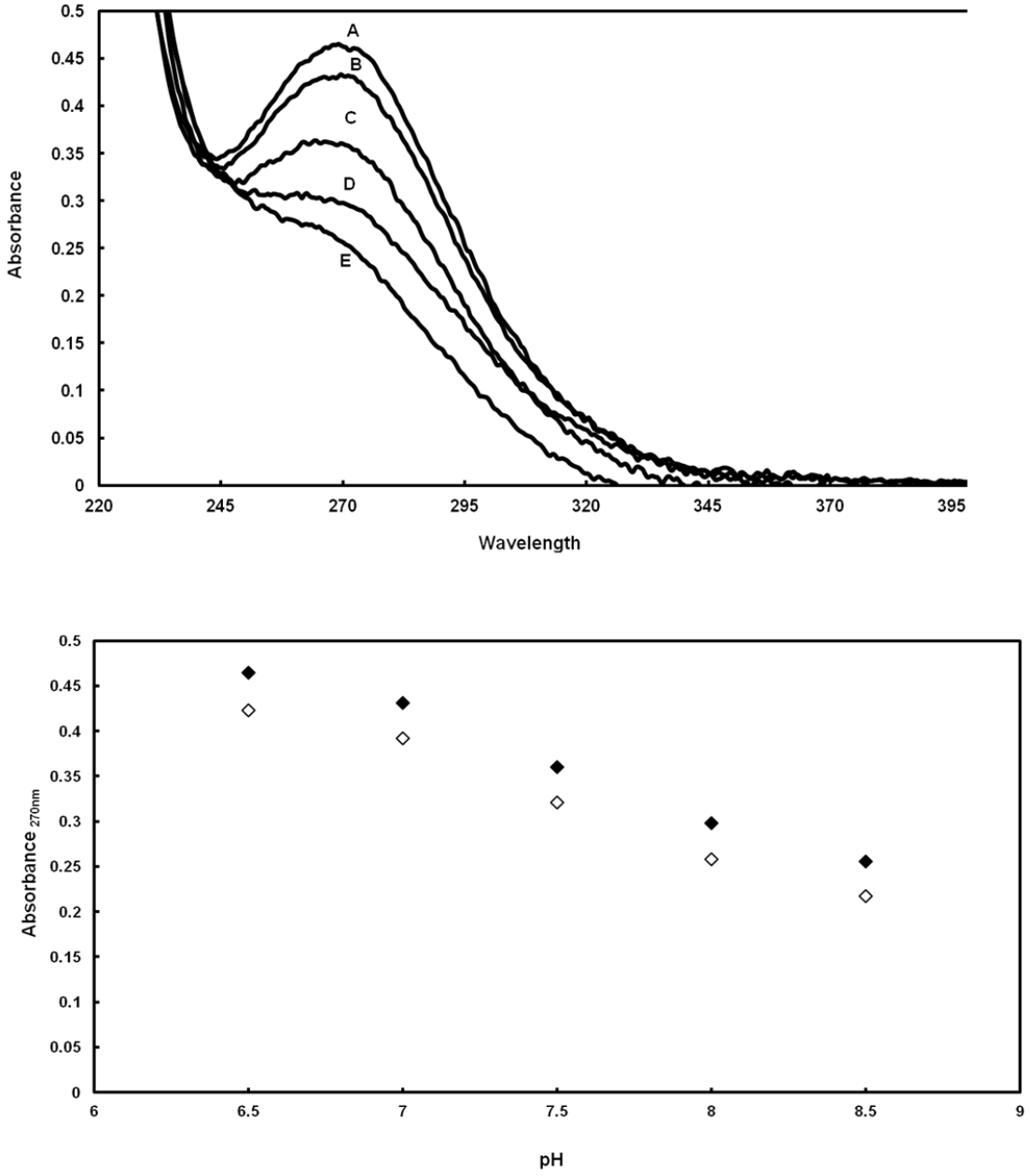
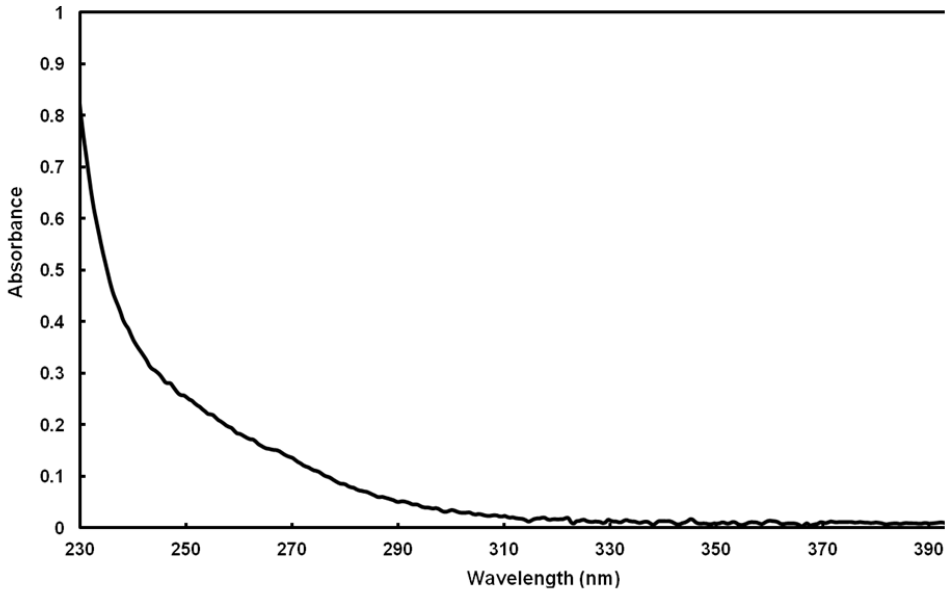


Figure 4 a) Absorption spectra measured at 180 minutes at 37°C for pHs 6.5, 7.0 and 7.5 and at 440 minutes for pHs 8.0 and 8.5 for the reaction mixtures of 5 mM hypochlorite and 9.67 mM heparin A, pH 6.5 ; B, pH 7.0; C pH7.5; D, pH8.0; E, pH8.5 (path length = 1 cm; after subtraction of contributions from unreacted heparin and residual hypochlorite); **b)** Effect of pH on the absorbance at 270nm in figure **4a**.(♦, ◊; before and after subtraction of unreacted heparin contributions respectively).

Hyaluronan The spectra at the end of the reactions in the pH range 6.5 to 8.5 had similar shapes, differing only in minor contributions from residual hypochlorite. Again, the reaction at pH 8.5 proceeded more quickly than other pHs and the spectrum at this pH is accordingly shown in Figure 5a. The apparent extinction coefficient of the chloramide (and other products) at 250 nm at pH 8.5, was thus calculated as 80 +/- 5 M^{-1} cm^{-1} i.e. similar to the value of 75 M^{-1} cm^{-1} found in the 1mM hypochlorite experiment. This similarity may indicate that any non-chloramide products do not absorb significantly at 250 nm. The yields of hyaluronan chloramide at pHs 6.5 to 8.5, determined using the TNB assay are shown in Figure 5b. In contrast to heparin, the yields increase with increase in pH and are much less than the 100% yields found using 1mM hypochlorite and 10mM hyaluronan solutions.



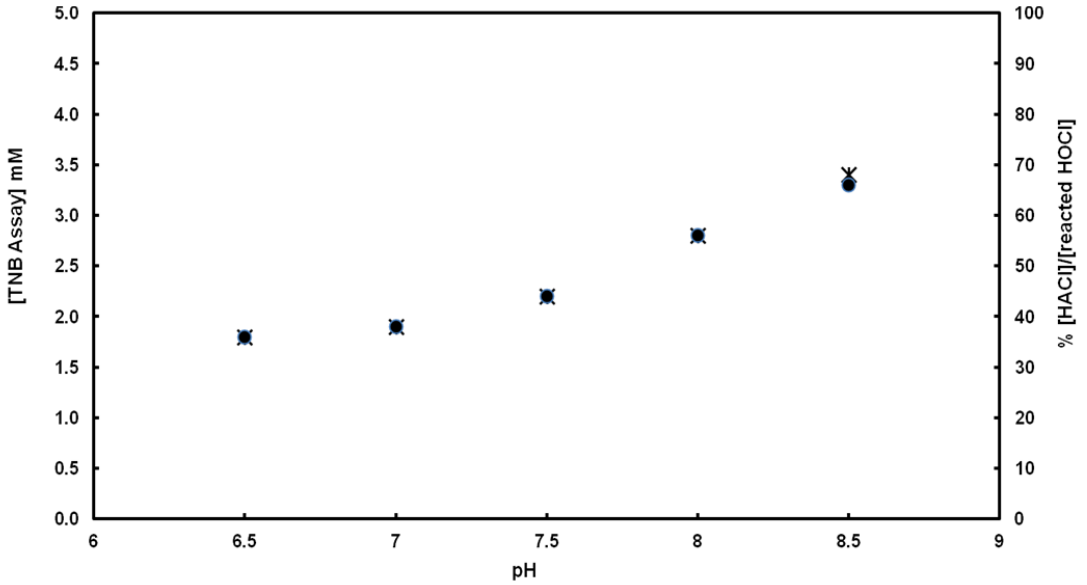


Figure 5 a) Absorption spectra measured at 330 minutes at 37°C for the reaction mixtures of 5 mM hypochlorite and 10 mM hyaluronan at pH 8.5 (contributions to the spectra of unreacted hypochlorite and hyaluronan have been subtracted), 1 cm pathlength ; **b)** Effect of pH on the TNB assays of the solutions in figure **5a.** Also shown are the chloramide yields expressed as a % of the concentration of hypochlorite which has reacted. **x** denotes the simulated yields of chloramide using the denoted k (hypochlorite + HA) values in figure **7c** together with the k (hypochlorite + HACl) values in figure **8b.**

It is clear that at this higher hypochlorite concentration, significant proportions of hypochlorite (35 to 65% as the pH decreases from 8.5 to 6.5) are involved in reactions other than chloramide formation, such as oxidation. However, it has been shown from experiments using 1mM hypochlorite, where reactions of hypochlorite with reaction products are less likely, that hypochlorite reacts with hyaluronan to produce 100% yields of the chloramide. It would appear therefore that the significantly reduced yields of chloramide found at the higher hypochlorite concentration must occur subsequently to chloramide formation. An obvious candidate is the reaction of hypochlorite with the chloramide product.

It is clear from the above experiments with both heparin and hyaluronan that pH has a definite and strong effect on the spectra and yields of the products of the reaction of hypochlorite.

The most probable explanations for the above observations in 5mM hypochlorite solutions containing either 10mM heparin or hyaluronan are: a) the chloramides are sufficiently unstable at 37°C that they decompose during the reaction times studied to obtain the yield data; b) there is some reaction between the product chloramides and hypochlorite occurring over the timescale of yield data measurements; c) the hypochlorite species have different reaction modes depending on the differing proportions of OCl^- and HOCl reactions with glycosaminoglycans, the principal modes being that of substitution to form the chloramides and that of oxidation. These possibilities will now be explored further using data collected in stability, kinetic and fragmentation experiments.

Thermal stability of heparin and hyaluronan chloramides

The stability of approximately 8mM heparin chloramide solutions was monitored, using the TNB assay, for up to 21 hours at 37° C. There was little or no change, at any of the pHs studied, in chloramide concentration . The effect of pH on chloramide yield discussed above is therefore unlikely to arise as a result of thermal instability. In experiments at room temperature and at 4°C, heparin chloramide showed considerable stability with little change after several days at room temperature or even weeks at 4°C.

The thermal stability of hyaluronan solutions at 37°C has already been investigated at pH 7.4 [17] where it was shown that 1.6mM solutions showed no degradation over 24 hours. In our studies in the pH range 6.5 to 8.5 using 1mM hypochlorite and 10mM hyaluronan, 100% yields of chloramide were measured at the end of the reaction and at times covering all measurements of yields made throughout this study. This supports the earlier study at pH 7.4 [17]. As with heparin chloramide, hyaluronan chloramide was shown to be remarkably stable at 4°C, showing little or no decay even after 21 days of storage. These stability measurements demonstrate quite clearly that any yields of either heparin or hyaluronan chloramides determined in this study which are less than 100% can not arise from thermal instability.

From the data already presented above, it would appear that the hypochlorite species, HOCl and OCl^-, react with heparin via at least two pathways, one to produce the chloramide and the other to produce oxidation products. The proportions of these pathways at a particular pH are identical, within experimental error, for both 1mM and 5mM hypochlorite solutions; any reaction of hypochlorite with the product heparin chloramide which would be favoured at the higher hypochlorite concentration must be insignificant. As the yield of chloramide has been shown to decrease with increasing in pH from 94% to 60%, HOCl is more effective at producing the chloramide. Conversely, OCl^- is much less effective and produces a significant amount of oxidation (40% at pH 8.5). Since HOCl is the predominant species at pH 6.5 (90%) and OCl^- is predominant at pH 8.5 (90%), the reactions of these species may be summarised as follows:

$$HOCl + Hep \Rightarrow HepCl \quad ; k_1 \qquad (1)$$

$$HOCl + Hep \Rightarrow HepOx ; \ k_2 \quad (2)$$

$$OCl^- + Hep \Rightarrow HepCl ; \ k_3 \quad (3)$$

$$OCl^- + Hep \Rightarrow HepOx ; \ k_4 \quad (4)$$

In sharp contrast, hyaluronan chloramide yields are not affected by pH when 1mM hypochlorite solutions are used, where 100% yields are measured. When 5mM hypochlorite solutions are used however, the yields of chloramide increase with increase in pH. It is clear therefore that there must be an additional reaction pathway at the higher hypochlorite concentration. The most likely one is the reaction of hypochlorite with the product, chloramide. It is also apparent that oxidation of hyaluronan by HOCl is more effective than oxidation by the anion, OCl^- since lower chloramide yields are measured at pH 6.5. These observations can be summarised as follows:

$$HOCl + HA \Rightarrow HACl ; \ k_5 \quad (5)$$

$$HOCl + HA \Rightarrow HAOx ; \ k_6 \quad (6)$$

$$OCl^- + HA \Rightarrow HACl ; \ k_7 \quad (7)$$

$$OCl^- + HA \Rightarrow HAOx ; \ k_8 \quad (8)$$

$$HOCl + HACl \Rightarrow products ; \ k_9 \quad (9)$$

$$OCl^- + HACl \Rightarrow products ; \ k_{10} \quad (10)$$

The contrast between heparin and hyaluronan in these experiments demonstrates clearly the effect of the $N(H)\text{-}SO_3^-$ group in determining the reactivity of heparin towards both chloramide production and oxidation. The negative charge on the sulphate group hinders

particularly the reaction of OCl^-, favouring oxidation over chloramide production despite the low oxidation potential of the hypochlorite anion relative to hypochlorous acid , HOCl (see earlier). In the following section, kinetic data will be presented to support the above mechanisms.

Effect of pH upon reaction kinetics in hypochlorite/ glycosaminoglycan solutions.

Changes in pH in the range 6.5 to 8.5 affect the rates of reaction of hypochlorite with heparin and hyaluronan since the species, HOCl and OCl^-, can be expected to react at different rates [29] and in different modes of reaction, probably to form the chloramides and oxidation products. Where hypochlorite species may also be involved in reactions with these products, particularly with the chloramides as secondary processes, there can also be an effect of pH upon the kinetic reactivity. The latter reaction could for example, account for the lower yields of hyaluronan chloramides obtained in this study at high hypochlorite to hyaluronan concentration ratios. The following experiments were thus performed with a view to calculating the rate constants for these various modes of reaction.

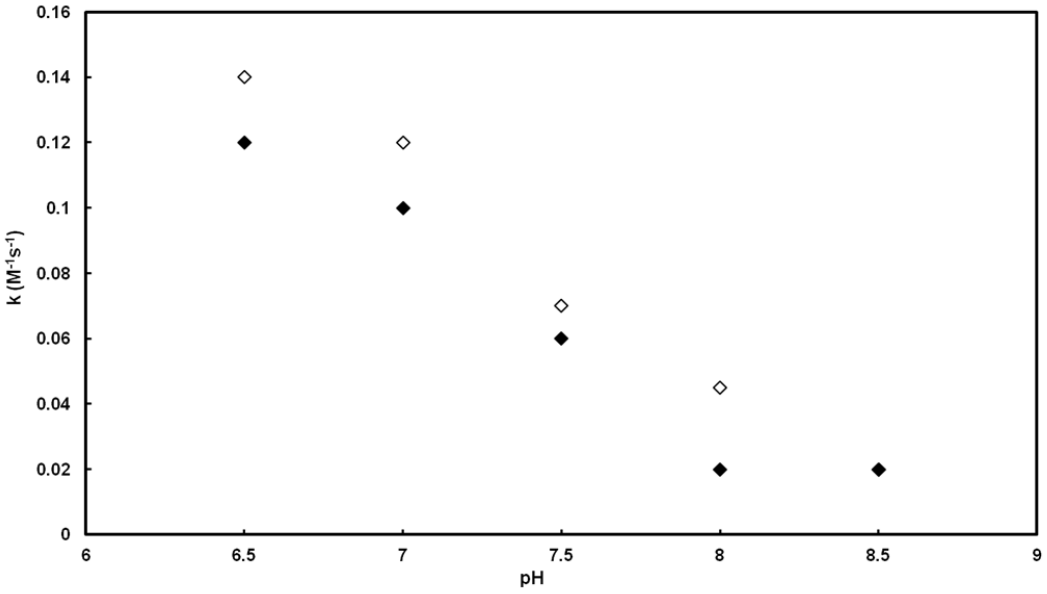
Kinetic studies in 1mM hypochlorite solutions. To minimise the extent of the reaction of hypochlorite with chloramide species, reactions were generally conducted using low hypochlorite concentrations, (1mM) and relatively high glycosaminoglycan concentrations (5 or 10mM). This approach had the additional advantage of being able to analyse the decay of absorbance attributable to hypochlorite species at 292 nm using a 1^{st} order kinetics analysis, as adopted in an earlier study [22]. Kinetic experiments were thus conducted with heparin and hyaluronan by following the decay of hypochlorite species at

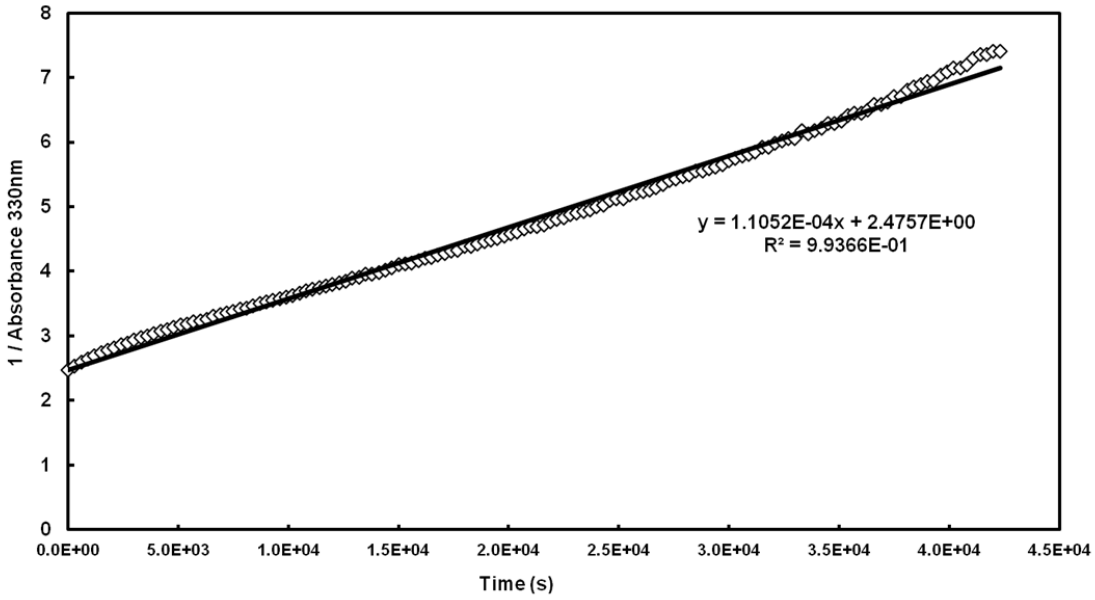
292nm in solutions containing 1 mM hypochlorite reacting with both 5 and 10 mM glycosaminoglycan in buffered solutions.

In the case of heparin, there is a significant residual absorbance at the end of the reaction attributable to the chloramide and perhaps other products. Plots of $\ln(A_t - A_\infty)$ versus time, t , where A_t and A_∞ represent the absorbances at time t and at the end of the reaction respectively, were used and found to be linear. From the slope of such plots, the decay rate constant in s^{-1} was determined and thus the second order rate constants were calculable. At pH 6.5, the extinction coefficients of heparin chloramide and hypochlorite species at 292nm were too similar and it was necessary to use other wavelengths, particularly 240nm, for the kinetic analysis.

In support of the above approach to a pseudo first-order reaction, kinetic simulations were also carried out using the IBM Chemical Kinetics Simulator, IBM N^o 1906. To do this, the extinction coefficients of hypochlorite, heparin and chloramide (+ other products) were needed. At reaction completion, following subtraction of absorbances from unused reactants, the extinction coefficients of the chloramide (and products) were calculated. Both types of kinetic analysis produced the same values of rate constants within experimental error and these are shown in Figure 6a. The second-order rate constants in this figure are based upon the concentration of $N(H)\text{-}SO_3^-$ groups in heparin , using the value of 88.6 per 100 residues as reported elsewhere [28]. The dominant reactivity of this entity was confirmed by reacting 4.9mM hypochlorite with 5mM N-sulphate groups (in heparin) at pH8.5 at room temperature and monitoring the decay of absorbance at 330nm . With almost equal concentrations of reactants and little or no contributions to the absorbance from the products expected at pH8.5, the decay should

follow second-order kinetics as demonstrated by the linear plot of 1/ absorbance v time shown in Figure 6b (the figure also shows the fitted linear plot). The rate constant was determined from the both the slope of the linear plot (= k/l. ε , where ε is the extinction coefficient of hypochlorite species at this pH and l is the path length). The value determined in this way, 0.032 M^{-1} s^{-1}.





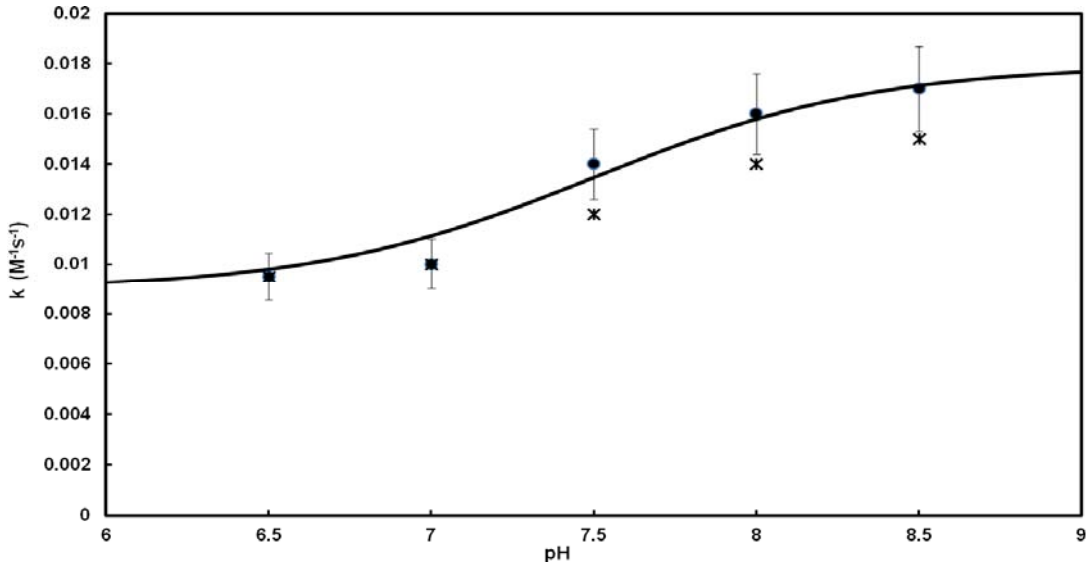


Figure 6 The effect of pH on the second order rate constants for the reaction of hypochlorite and heparin, **a)** for the reaction of 1mM hypochlorite with 9.67 mM heparin (◊) and for the reaction of 5mM hypochlorite with 9.67 mM heparin (♦) (the latter obtained from kinetic simulations, see text); **b)** a plot of 1/OD @ 330nm v time for a 4.9 mM hypochlorite , 5. mM N-sulphate (in heparin) reaction mixture at pH 8.5 at room temperature (also shown is the linear fit for this plot); **c)** the effect of pH on the second order rate constants for the reaction of 1mM hypochlorite with 10 mM hyaluronan (also shown is the fit expected assuming a pKa of hypochlorous acid of 7.6). **x** denotes the rate constant values which, together with the k (hypochlorite + HACl) values in figure **8b,** are used to simulate chloramide yields in figure **5b .**

From Figure 6a, there is a distinct effect of pH on the second order rate constant with values of 0.13 +/- 0.01 M^{-1} s^{-1} at pH 6.5 decreasing to a value of 0.02 +/- 0.05 M^{-1} s^{-1} at pH 8.5. This trend appears to correlate with the pKa of hypochlorous acid which has a pKa of 7.6 [25]. This data can be fitted to the following equation;

$$k_{obs} = (k_a [H^+] + k_b 10^{-pKa}) / ([H^+] + 10^{-pKa}) \qquad (11)$$

where k_{obs} is the second-order rate constant at a particular pH, k_a and k_b are the second-order rate constants for the reaction of HOCl and OCl^- respectively with either heparin or

hyaluronan. This equation is appropriate for a fast equilibrium between HOCl and OCl^- with slower reactions of the two species with either heparin or hyaluronan. Fitting the data yields values for reactions (1) – (4), as follows:

$k_1 + k_2 = 0.15\ M^{-1}\ s^{-1}$ and

$k_3 + k_4 = 0.006\ M^{-1}\ s^{-1}$

The second-order rate constants in Figure 6a are consistent with the earlier value of 0.051 +/- 0.02 $M^{-1}\ s^{-1}$ measured at pH7.4 at 37°C [22].

For hyaluronan, rate constants were determined, as with heparin, by both assuming pseudo first-order kinetics and by simulation. Unlike heparin chloramide however, the extinction coefficient of hyaluronan chloramide at 292 nm was relatively small (15 $M^{-1}\ cm^{-1}$) and this made the simulation process more precise. The resultant rate constants are shown in Figure 6c. Again, in contrast to heparin, increase in pH leads to an increase in the second-order rate constant. Fitting the data in Figure 6c to equation (11), yielded the following values:

$k_5 + k_6 = 0.01\ M^{-1}\ s^{-1}$ and

$k_7 + k_8 = 0.017\ M^{-1}\ s^{-1}$

The second-order rate constants in Figure 6c are consistent with the earlier value of 0.012 +/- 0.03 $M^{-1}\ s^{-1}$ measured at pH 7.4 at 37°C [22].

Kinetic studies in 5mM hypochlorite solutions:

Heparin 5mM hypochlorite was reacted with 9.67mM heparin solutions at pHs between 6.5 and 8.5 and the decay of absorbance followed at 292nm for all pHs with the exception of pH 6.5 where, because of the similarity in extinction coefficients of hypochlorite and heparin chloramide at 292nm, the reaction was studied instead at both 240nm and 310nm. Here, only kinetic simulation of the decay was used to determine the k (hypochlorite + heparin) rate constant, based on the assumption of the single reaction (12) – which is equivalent to reactions (1) –(4):

hypochlorite + heparin => HepCl + products (12)

As in the case of the 1mM hypochlorite experiments, the simulations had to take into account the significant contribution of absorbance of the chloramide (and other products) at 292nm. The apparent extinction coefficient of the chloramide at 292nm decreased with increase in pH due to the increasing contribution of the much weaker absorbance from oxidation products. The apparent extinction coefficient was calculated, as with the 1mM solutions, from the absorbance at the end of the reaction, allowing for any absorbance for small residual amounts of hypochlorite and any unreacted heparin. These extinction coefficients had much smaller experimental errors (+/- 5%) than the ones measured in the 1 mM hypochlorite solutions where contributions from unreacted heparin were a major contribution to final absorbances measured. The simulations to yield the rate constants for reaction (12) for both 1mM and 5mM hypochlorite were carried out using these more reliable values. For both solutions, the values selected were within the errors of each type of experiment. The resultant rate constants are shown in Figure 6a alongside the 1mM hypochlorite rate constants. The similarity in values at each pH indicates that there is little or no need to invoke a reaction between hypochlorite and heparin chloramide.

Hyaluronan Similar experiments were conducted for 10mM hyaluronan solutions containing 5mM hypochlorite. As before, the decay kinetics of hypochlorite were followed at 292nm unlike heparin, the contributions from absorbances of hyaluronan chloramide and products were much smaller. From absorbances at the end of the reactions at pHs from 6.5 to 8.5, there was little or no effect of pH on the absorbances measured. Taking into account that the chloramide yields have already been shown to vary significantly over this pH range it was assumed, that at 292nm, there was little difference in the extinction coefficients of the chloramide and other products. A value of 15 +/- 5 M^{-1} cm^{-1} was therefore estimated for both. Using these values, it was clear that it was not possible to use the value of the rate constant of 9.5 +/- 1.5 x 10^{-3} M^{-1} s^{-1} (measured in 1mM hypochlorite solutions) at pH6.5 to simulate the experimental decay at 292nm seen in 5mM hypochlorite solutions . The slower decay in Figure 7a shows the simulated plot assuming a rate constant of 9.5 x 10^{-3} M^{-1} s^{-1} which is much slower than the actual experimental decay. At pH7, a similar, but much less marked, effect was seen. At higher pHs, the experimental decays could be simulated within experimental error using the rate constants obtained in the 1mM hypochlorite experiments. Further kinetic simulations were therefore carried out at all pHs by considering the following reactions:

hypochlorite + HA => HACl + products (13)

hypochlorite + HACl => products (14)

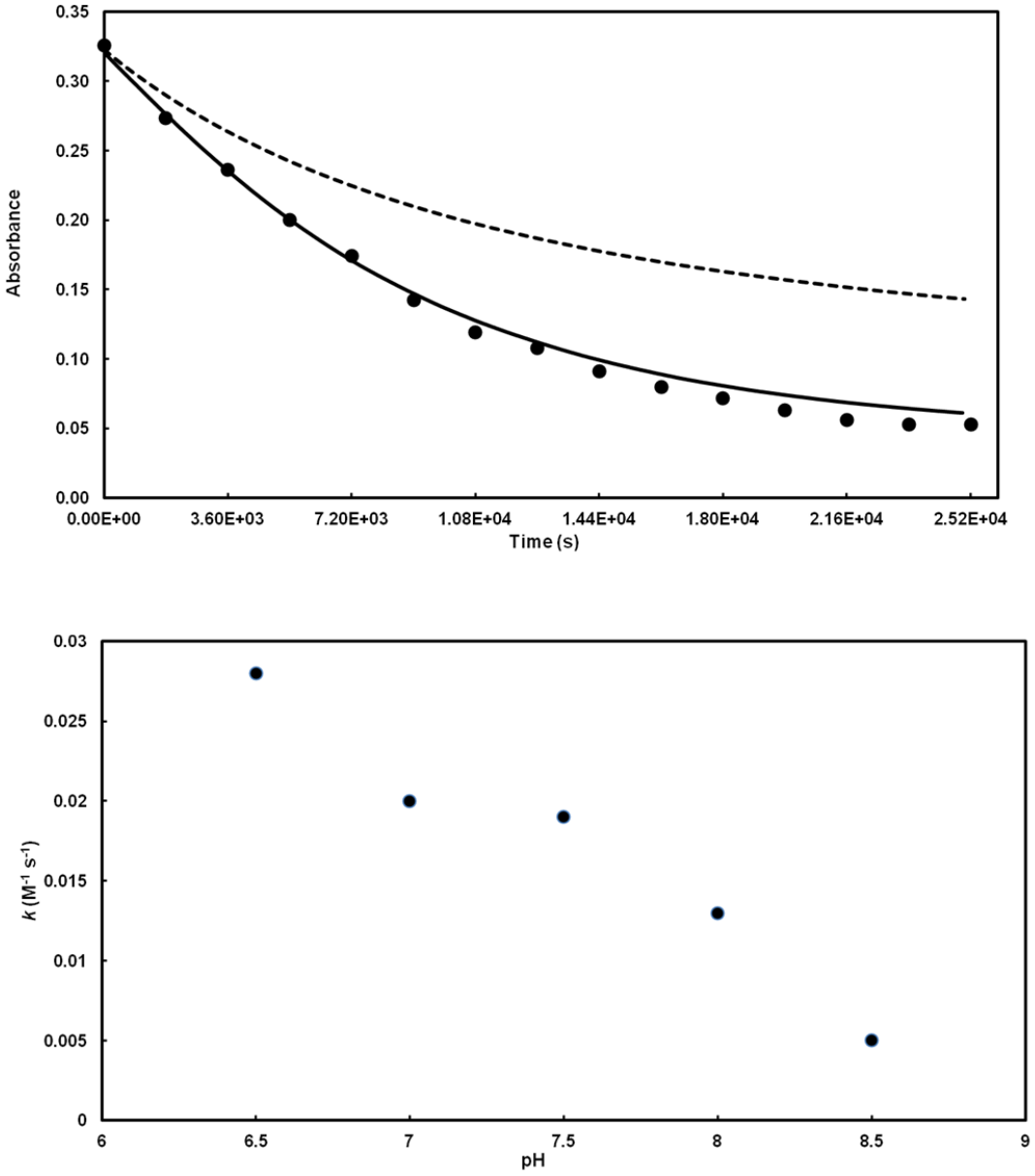


Figure 7 a) The decay of absorbance at 292nm observed in a solution of 5 mM hypochlorite containing 10 mM hyaluronan at pH 6.5. Also shown is the simulated fit for k (hypochlorite + HA) = 9.5 x 10^{-3} M^{-1} s^{-1} and k (hypochlorite + HACl) = 2.8 x 10^{-2} M^{-1} s^{-1} **b)** the effect of pH on k (hypochlorite + HACl), see text.

Reactions (13) and (14) are equivalent to reactions (5) to (10). Figure 7a shows therefore the simulated decay which matches the experimental decay at pH 6.5. In this example,

k(13) is 9.5 x 10^{-3} M^{-1} s^{-1} and k(14) is 2.8 x 10^{-2} M^{-1} s^{-1} . Although there is no analytical solution to equations (13) and (14) to give a decay of absorbance equation which could produce coefficients of correlation between such an equation and the experimental decay, repeat simulations using k(13) values within the experimental error were carried out with a range of k(14) values. Here a reasonable fit was assessed using only simulated decay plots which were within +/- 5 % at the beginning of the decay and +/- 10% at the end of the decay (where smaller absorbances are observed). In this way, k(14) values could be estimated for other pH values. This method was not particularly sensitive unless k(14) is particularly high as at pH6.5 since the reaction of hypochlorite only begins to have an effect on the decay at 292nm when sufficient chloramide has accumulated to react as in reaction (14). The effect on the decay at values of k(14) lower than about 1 x 10^{-2} M^{-1} s^{-1} is only seen towards the end of the decay. For pHs 7.5 to 8.5, values of k(14) ranging from zero to 1 x 10^{-2} M^{-1} s^{-1} would also provide reasonable fits to the experimental decays.

The yields of hyaluronan chloramide in contrast , however, are very sensitive to the values of k(14) as may be expected and as was demonstrated by trial simulations. Figure 7b therefore shows the effect of pH on the k(14) values which fitted both the kinetic decays at 292nm and also the chloramide yields. The fits to the yields are shown in Figure 5b. The k(13) rate constant values used together with rate constants in Figure 7b to predict the chloramide yields were within the experimental error of the values determined in Figure 6c and are shown there as discrete points. Taken together, the k(14) data in Figure 7b provide reasonable fits to both kinetic and yield data obtained in the 5mM hypochlorite solutions.

From the chloramide yield data and kinetic data presented in this study, it is now possible to calculate rate constants for the individual reactions, (1) to (10). Thus, since $k_1 + k_2 = 0.15\ M^{-1}\ s^{-1}$ and $k_3 + k_4 = 0.006\ M^{-1}\ s^{-1}$ and the yields of chloramide are 94 % and 40% respectively for the k_1 and k_3 reaction pathways, it can be calculated, for example, that $k_1 = 0.14\ M^{-1}\ s^{-1}$. Similar calculations can be done for k_1 to k_8 and these are shown in Table 1. Fitting of the data in Figure 7b in equation (11) and assuming the pKa of hypochlorous acid to be 7.4, yields k (HOCl + HACl) and k (OCl^- + HACl) values of $3.0 \times 10^{-2}\ M^{-1}\ s^{-1}$ and $6.0 \times 10^{-3}\ M^{-1}\ s^{-1}$ respectively. These rate constants are also summarised in Table 1.

Reaction	**Rate constant (M^{-1} s^{-1})**
HOCl + Hep => HepCl	0.14
HOCl + Hep => HepOx	9×10^{-3}
OCl^- + Hep => HepCl	4×10^{-3}
OCl^- + Hep => HepOx	2×10^{-3}
HOCl + HA => HACl	1×10^{-2}
HOCl + HA => HAOx	$< 5 \times 10^{-4}$
OCl^- + HA => HACl	1.7×10^{-2}
OCl^- + HA => HAOx	$< 8 \times 10^{-4}$
HOCl + HACl => products	3×10^{-2}
OCl^- + HACl => products	6×10^{-3}
HOCl + D-Gluc-2-N-SO_3^-	0.6
OCl^- + D-Gluc-2-N-SO_3^-	0.06
HOCl + D-acetylglucosamine	8×10^{-3}
OCl^- + D-acetylglucosamine	3.6×10^{-3}

Table 1 Rate constants of selected reactions of HOCl and OCl^- with heparin, hyaluronan , heparin chloramide, hyaluronan chloramides, N-acetylglucosamine and D-glucosamine-2-N-sulphate.

An alternative approach to investigating the reaction of hypochlorite with heparin and hyaluronan chloramides was also considered. This concerned the prior synthesis of the chloramides and then reacting these with hypochlorite in a subsequent experiment. Heparin chloramide was synthesised in high yield (6.2mM) at pH 6.5 where the chloramide yield is highest and the % oxidation products would be only 6% . The pH of the solution was then adjusted to pH 8.5 where the rate of reaction of hypochlorite with heparin chloramide could be expected to be greatest in that the lowest chloramide yields are measured at this pH, perhaps as a result of a reaction between hypochlorite and heparin chloramide. Simulation of the decay of absorbance in these solutions containing 2 mM hypochlorite was undertaken. Figure 8 shows two such simulations using the extinction coefficients of the chloramide appropriate to pH 6.5 and assuming that the extinction coefficient of the products of any reaction between hypochlorite and heparin chloramide was relatively low compared to the extinction coefficient of hypochlorite. This latter assumption is supported by the effect of pH on the end of reaction spectra at pH8.5, see Figure 2a for example. The slowest simulated decay, where the rate constant for hypochlorite reacting with the chloramide was set as a very low value of 1×10^{-4}, matches the experimental one and demonstrates this reaction is not needed to account for the kinetic decay and indeed, such a reaction is not needed to account for the yields or spectra obtained in 5mM hypochlorite solutions. The second simulated decay shown in Figure 8, which shows a small departure from the fitted plots, assumes that k (hypochlorite + HepCl) = 1×10^{-3} M^{-1} s^{-1} .Thus, the range of the latter rate constant is effectively zero to 1×10^{-3} M^{-1} s^{-1} . Further simulation of the chloramide yields showed that the higher value does not change the chloramide yield that would otherwise be

predicted. All kinetic, spectral and yield data when taken together provide little or no evidence for a significant reaction between hypochlorite and heparin chloramide under the conditions of this study. An upper limit of 1 x 10^{-3} M^{-1} s^{-1} can be estimated for this reaction.

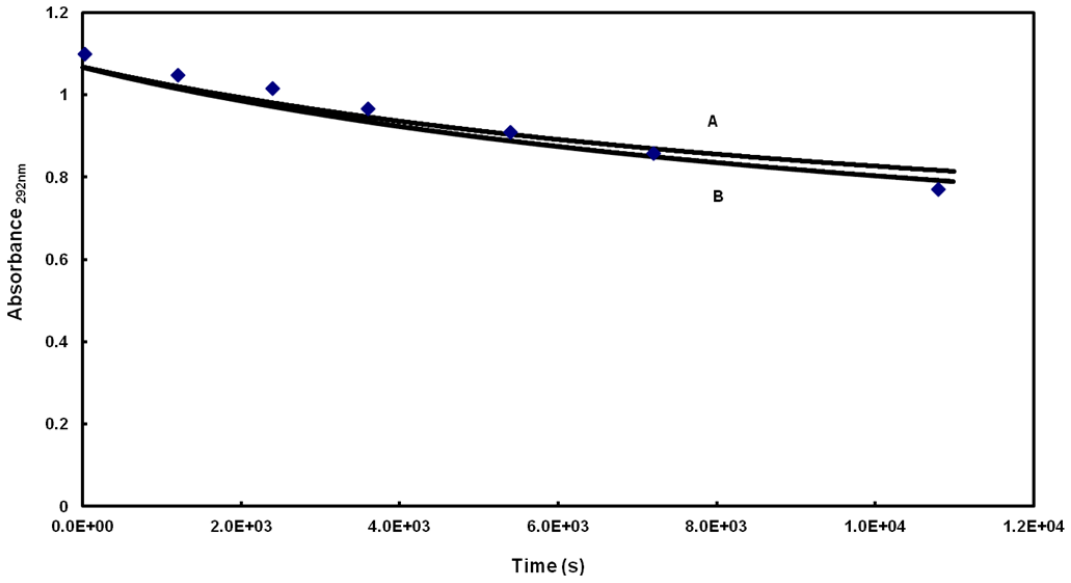


Figure 8 The decay of absorbance at 292 nm in a solution containing 2 mM hypochlorite and 6.2 mM heparin chloramide at pH 8.5 ((♦)experimental data; A; k (hypochlorite + hepCl) = 1 x 10^{-4} M^{-1} s^{-1} ; B; k (hypochlorite + hepCl) = 1 x 10^{-2} M^{-1} s^{-1} ; path length, 1 cm)

For hyaluronan, high yields, close to 100%, of the chloramide could only be obtained in 1 mM hypochlorite solutions containing 10mM hyaluronan. Consideration of the rate constants for the competition of hypochlorite for both hyaluronan and its chloramide indicate that at pH 6.5, where k (hypochlorite + HACl) is highest at 2.8 x 10^{-2} M^{-1} s^{-1}, there would only be an approximately 15 % increase in the initial decay rate of the absorbance at 292nm. A simulation of such an experiment at pH6.5, where solutions

containing 1mM HACl and 9mM hyaluronan are reacted with 2mM hypochlorite showed only small differences in the kinetic decay at 292nm. This is attributed not only to the low concentration of chloramide but also to the relatively low extinction coefficient of hypochlorite at this pH and the fact that the competition produces both an increase in chloramide absorbance as well as a reduction, due to the chloramide reaction with hypochlorite.

Fragmentation yields

An earlier study showed that synthesis of hyaluronan chloramide produced little or no effect on the PAGE scan relative to that of hyaluronan itself. i.e. no fragmentation of the chloramide could be detected [17]. In contrast, in this study ,the effect of pH on yields of the chloramides provides evidence for oxidation pathways which might be expected to produce fragmentation of the glycosaminoglycan chloramides. To investigate this further, PAGE studies were conducted on the potential for fragmentation arising via the synthesis of hyaluronan and heparin chloramides . In these studies, the Rf values of the stained scans have been calibrated against the molecular mass of the glycosaminoglycans using a gpc/MALLS combination (see Materials and Methods).

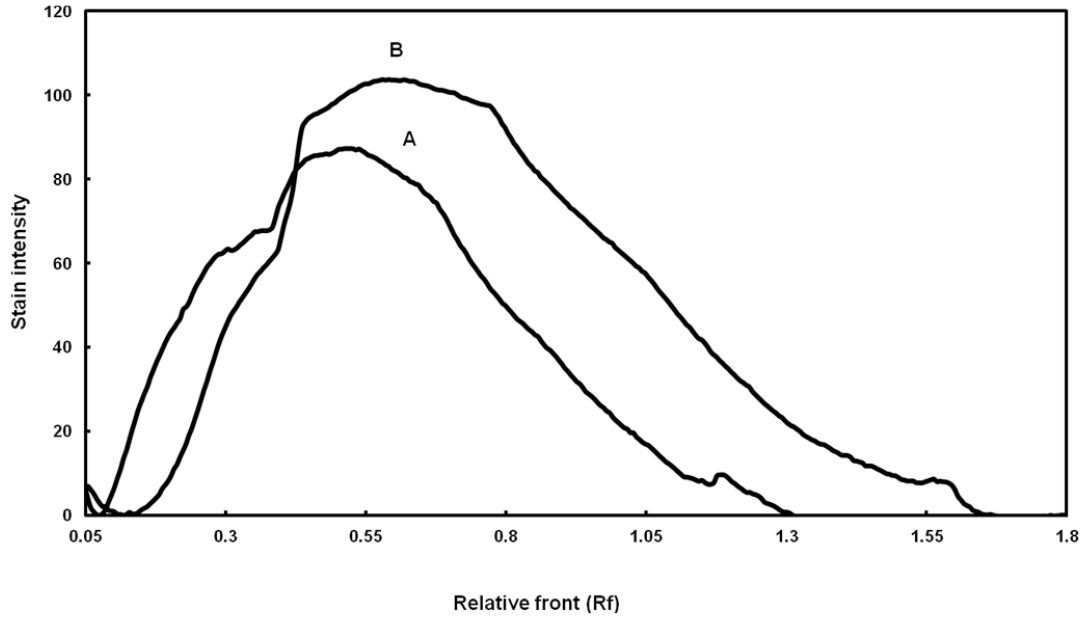
Figures 9a and 9b show scans for heparin at pH 6.5 and hyaluronan at pHs in the range 6.5 to 8.5, (pH did not seem to have a marked effect on heparin as almost identical scans were generated at pH 8.5 (data not shown). In these experiments, the ratios of hypochlorite and heparin are such that the chloramide preparation should be seen as a heavily-substituted heparin polymer - with a parallel scenario envisaged for hyaluronan. The probability of the existence of discrete unsubstituted heparin or hyaluronan

molecules under these conditions would be extremely small. Thus, in Figure 9a, where the heparin chloramide concentration was 6.2mM, approximately 6 out of 10 N(H)-SO_3^- residues in the polymer would be substituted by Cl whereas in the hyaluronan gel experiments (Figure 9b) approximately 1 out of 10 N(H)-acetyl residues would be substituted. The gel scans show clearly that fragmentation occurs in the reactions of hypochlorite with both heparin and hyaluronan. Using the calibrated gels, the number and weight average molecular masses can be calculated for both control samples and the chloramide substituted polymers using the equations;

$$Mn=\Sigma Hi/\Sigma(Hi/Mi) \quad \text{and} \quad Mw=\Sigma(HiMi)/\Sigma Hi$$

where Hi is the intensity of the blue stain in the gels at discrete Mi (molecular mass) values and where Mi has been calibrated using the gpc/MALLS equipment. The number of chain breaks per polymer molecule can then be calculated from the Mn data, as follows:

$$\text{Chain breaks per molecule} = (Mn^0 - Mn^p) / Mn^p$$



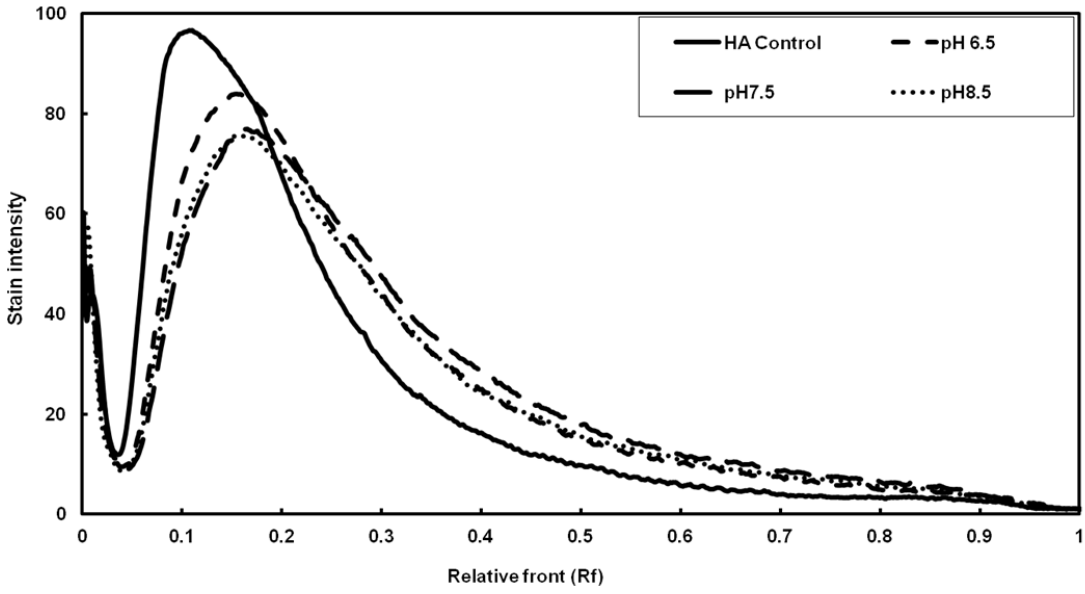


Figure 9 a) PAGE scans of solutions containing 9.67 mM heparin control at pH 6.5 (A) ; heparin chloramide (from reaction of 9.67 mM heparin with 9 mM hypochlorite at pH 6.5) (B), (The gels were run for 8 h 30 minutes at 100V using 20 % polyacrylamide); **b)** PAGE scans of: 10 mM hyaluronan and of 1mM hypochlorite solutions containing 10 mM hyaluronan at pH 6.5 , 7.5 and 8.5. (The gels were run for 4 hours using 20% acrylamide).

The concentration of chain breaks (M) is then given by :

[Chain breaks] = chain breaks per molecule x [polymer control]

This data is summarised in Table 2.

System	Mn (kDa)	Mw (kDa)	CB/molecule	[CB]
Hep control, pH 6.5	13.3	14.8	Not applicable	Not applicable
Hep control, pH 8.5	13.3	14.8	Not applicable	Not applicable
HepCl, pH 6.5	12.2	13.2	0.09	41 μM
HepCl, pH 8.5	12.2	13.2	0.09	41 μM
HA control	43.4	57.0	Not applicable	Not applicable
HACl, pH 6.5	37.1	50.7	.17	1.6 μM
HACl, pH 7.5	35.6	50.1	.22	2.0 μM
HACl, pH 8.5	37.0	51.8	.17	1.6 μM

Table 2 Mn , Mw and chain break yield data for heparin and hyaluronan chloramides.

The concentration of chain breaks found in the heparin chloramide - containing solutions was calculated as 41μM at both pHs 6.5 and 8.5 , equivalent to only 0.4% of the reacted hypochlorite. So although oxidation processes consume between about 6% (at pH6.5) and 40% (at pH8.5) of the hypochlorite, it would appear that oxidation does not lead to any significant fragmentation of heparin, in contrast to high efficiencies of fragmentation found when oxidising free radicals react with hyaluronan, as previously observed [16].

The concentration of chain breaks determined in the preparation of hyaluronan chloramide solutions, although low at 1.6-2.0μM, was easily detected in the PAGE scans . In spite of the fact that the yields of chloramide were found to be 100% in these 1mM hypochlorite solutions at any pH in the range 6.5 to 8.5, these chain break data indicate that a very small percentage, 0.16-0.20 %, of hypochlorite is actually used to produce fragmentation. This small amount would not be detected in a reduction of the TNB yield, being well within the detection error limits of the assay. It may be that this small proportion of hypochlorite does react via an oxidative pathway with very low rate constants (limits given in Table 1); An equally plausible possibility is perhaps the reaction of hypochlorite with impurities such as residual copper ions, despite the attempt to remove such ions using ion exchange resins.

In conclusion, PAGE is a very sensitive method to measure fragmentation of glycosaminglycans , however, the yields are low and it is clear that the oxidation of heparin and hyaluronan does not lead to significant fragmentation.

Reaction of hypochlorite with model monosaccharides

In order to confirm the effect of pH on yields, spectra and kinetic reactivity already determined for heparin and hyaluronan D-glucosamine-2-N-sulphate and N-acetylglucosamine were selected as models for the hypochlorite-reactive monosaccharide units in heparin and hyaluronan respectively. Thus, 1mM hypochlorite was reacted with 5 mM D-glucosamine-2-N-sulphate at pHs in the range 6.5 to 8.5. At the end of these reactions, when there was little or no residual hypochlorite, the chloramide yields were measured using the TNB assay. In contrast to heparin, 100% yields were found at all pHs examined. It is immediately clear therefore that the reactivity in heparin, where oxidation processes compete with chloramide formation, is influenced by either the presence of adjacent monosaccharide groups or, more likely, by the presence of sulphate groups in the N-sulphated residue. The yield of chloramide of only 60% in the reaction of the hypochlorite anion, OCl^-, suggests that the presence of other sulphate groups in the most commonly-occurring disaccharide, the 2-O-sulphated ioduronic acid, 6-O-sulphated, N-sulphated glucosamine provides an additional electrostatic effect preventing a 100% chloramide yield as found in the model compound. The 3-dimensional structure of heparin [31,32] shows that the 6-O-sulphate group is closer to the N-sulphate group than is the 2-O-sulphate group in the ioduronic acid moiety and is therefore likely to produce a greater negative electrostatic effect on the rate of reaction of OCl^- with the N-sulphate group. This effect is clearly sufficient to allow an oxidation step to be preferred in 40% of the OCl^- reaction with heparin at pH8.5, in contrast to no oxidation in the D-glucosamine-2-N-sulphate model compound.

Confirmation of 100% chloramide yields found using TNB data at pHs 6.5 to 8.5 was obtained from the end of reaction spectra where identical spectra attributable to the

chloramide were observed at each pH with a maximum of 270nm and an extinction coefficient of 110 +/- 5 M^{-1} cm^{-1}. Figure 10a shows a representative spectrum at pH 7.0.

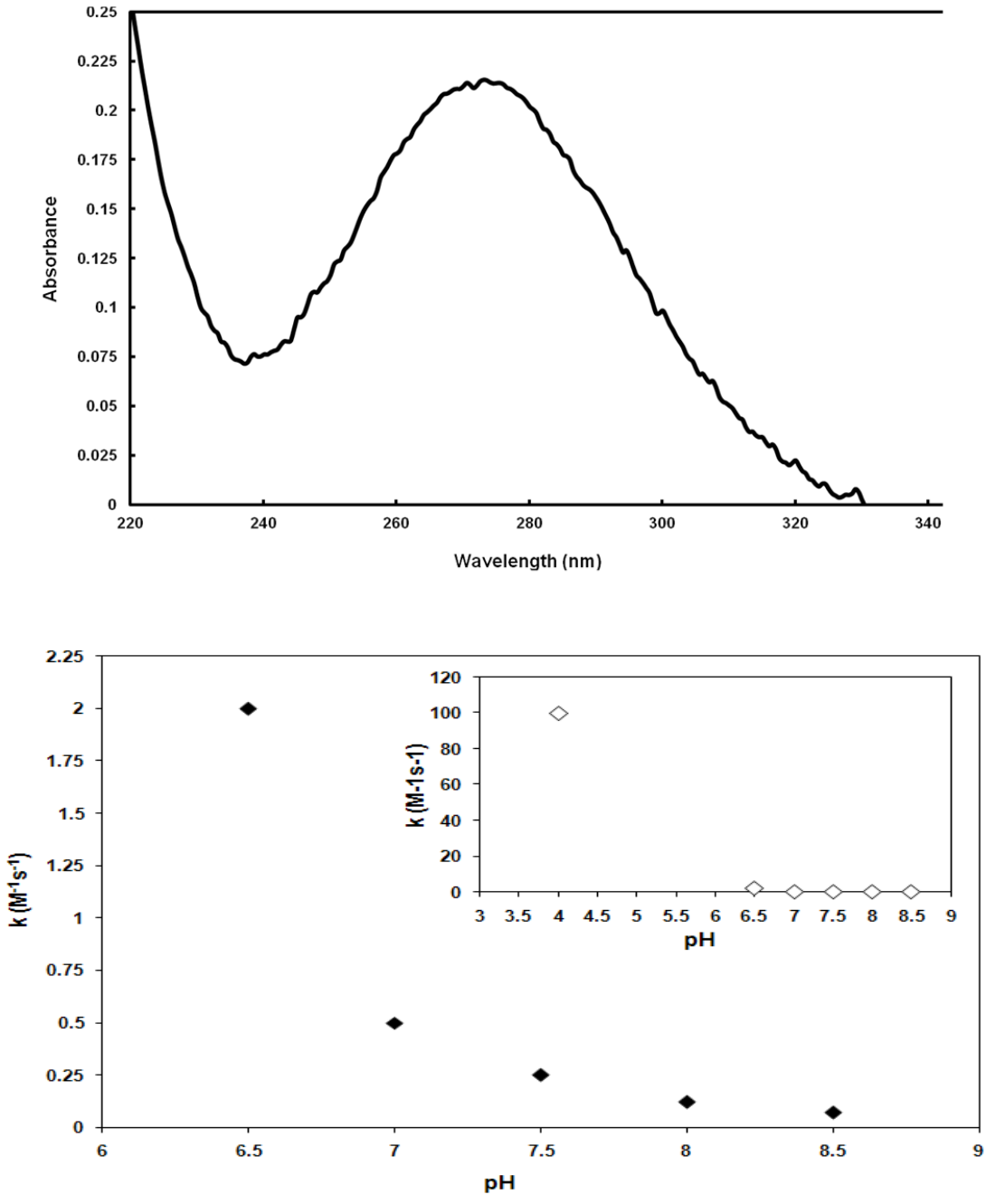


Figure 10 a) The absorption spectrum of D-glucosamine-N-sulphate chloramide measured at 200 minutes in a reaction mixture containing 1 mM hypochlorite and 5 mM

D-glucosamine-N-sulphate (path length 2 cm); **b)** the effect of pH on k (hypochlorite + D-glucosamine-N-sulphate) .

The rate constants for the reaction of 1mM hypochlorite with 5mM D-glucosamine-2-N-sulphate were determined by simulation at 240nm, 292nm and at 330nm. As before, the kinetic simulations required the extinction coefficients of the reactants and products. For this model, where the monosaccharide has no detectable absorption at these wavelengths, the process was more straightforward. The simulations at 330nm were yet more straightforward in that the chloramide did not absorb significantly. The effect of pH on the rate constants is shown in Figure 10b. From the latter, the rate constant at pH 7.5 was estimated as 0.25 +/- 0.01 M^{-1} s^{-1}. This can be compared the value of 0.91 +/- 0.13 M^{-1} s^{-1} found at pH7.4 in an earlier study [22]. There is no apparent reason for this discrepancy – however the difference is relatively large. Figure 10b also shows an unusual effect of pH not seen elsewhere in this study in that the rate constants cannot be fitted to equation (11) which takes account of the rapid equilibrium between HOCl and OCl^- . It is clear that the rate constant of 2.0 +/- 0.1 M^{-1} s^{-1} found at pH 6.5 is much higher than the value of about 0.5 M^{-1} s^{-1} which would otherwise be expected. An additional experiment using lower concentrations of both hypochlorite and heparin (both 1mM) was therefore carried out at pH4.0 and a kinetic simulation at 240 nm was performed. As the inset in Figure 10b shows, a very high rate constant of 100 +/- 10 M^{-1} s^{-1} was estimated. It was confirmed that the chloramide was still being produced in 100% yield. At pH4, the predominant species is still hypochlorous acid and thus it cannot be this which is responsible for the high rate constant. There is also no carboxylate function in D-glucosamine-2-N-sulphate and there is therefore no pKa in the model compound which could account for this pH

effect, the $N\text{-}SO_3^-$ being too acidic. By elimination therefore the candidate appears to be chlorine itself or instead an acid-catalysed reaction of hypochlorous acid. Evidence for the latter type of reaction has not been substantiated for reactions of aqueous chlorine systems containing organics [29] and so reactions of Cl_{2aq} are usually proposed instead. At pH4, there is however little Cl_{2aq}; at low chloride ion concentration, the equilibrium constant of 5.1×10^{-4} M^2 [29] would indicate a $[Cl_2aq] / [HOCl]$ of about 2×10^{-5}. This low concentration of chlorine at pH4 implies that the rate constant at a much lower pH where only Cl_{2aq} would exist, would be several orders of magnitude higher than the value of 100 M^{-1} s^{-1} found at pH4.0 in this study . In general, the kinetics of reactions of chlorine and related species have received considerable attention largely because of interest in water treatment but also because of the potential reactions of HOCl and OCl^- reactions *in vivo*. The review of kinetics and mechanisms by Deborde and von Gunten [29] confirms this level of interest. Their review gives a substantial amount of kinetic data on reactions with amines and amides which lead to chlorination and are generally much faster than reactions with other categories of organics. Such studies are focussed on hypochlorous acid and the hypochlorite anion, with reactions of hypochlorous acid being faster. Some kinetic data is available in the review which shows even faster reactions for Cl_{2aq}. In the case of the secondary amide, N-methylpropanamide, the respective rate constants for reactions of Cl_{2aq}, HOCl and OCl^- were 147, 1.7×10^{-2} and 9.2×10^{-3} M^{-1} s^{-1} [30]. This latter findings provides some support therefore for the high rate constant estimated in this study for reactions of hypochlorite with D-glucosamine-2-N-sulphate at pH 4, and so support for a very high rate constant ($>>10^2$ M^{-1} s^{-1}) for the reaction of Cl_{2aq} with D-glucosamine-2-N-sulphate. From the data presented, and using equation

(11), the rate constants for the reaction of HOCl and OCl^- with D-glucosamine-2-N-sulphate could be estimated as 0.6 +/- 0.1 and 0.05 +/- 0.005 M^{-1} s^{-1} respectively.

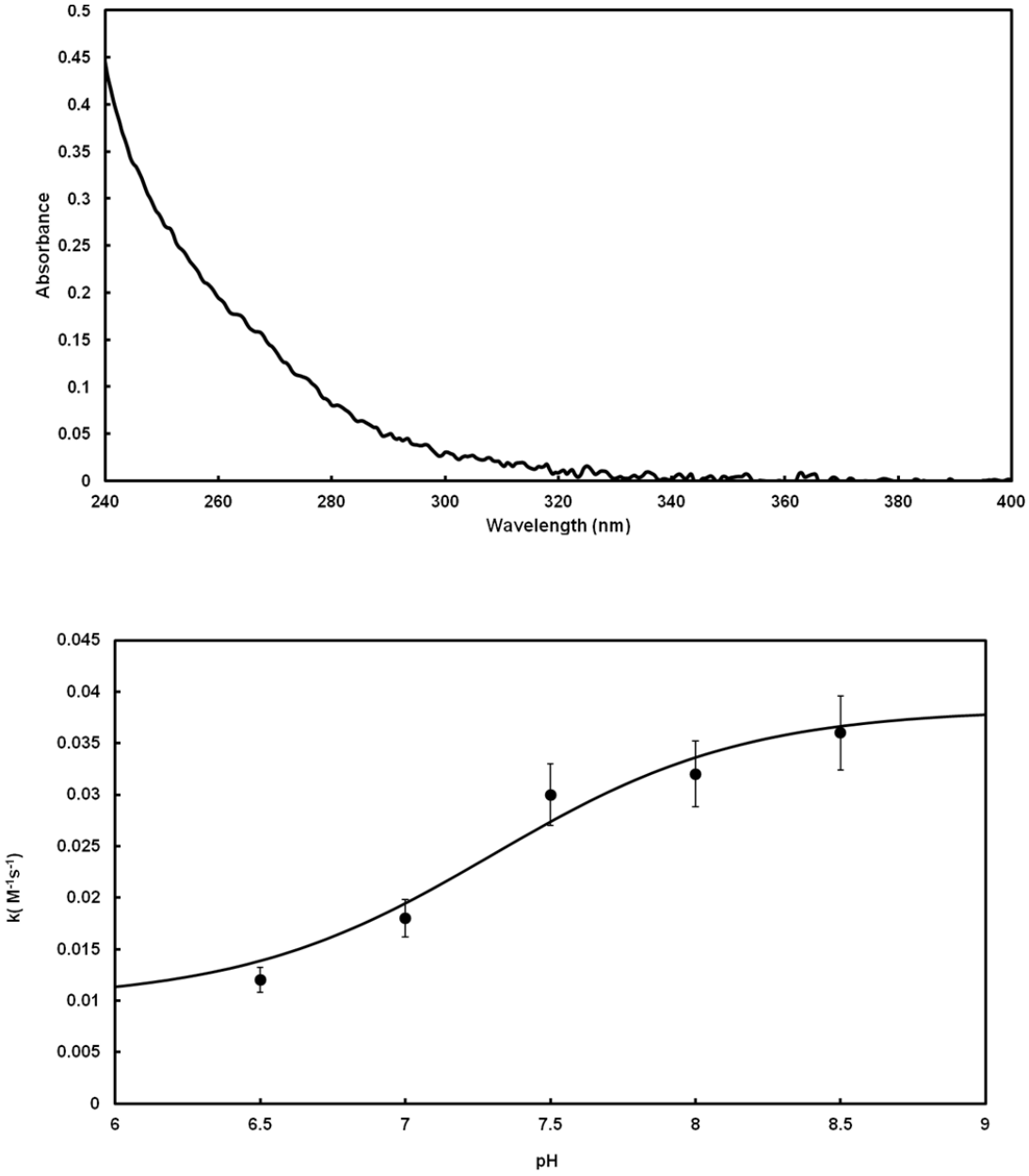


Figure 11 a) The spectrum of N-acetylglucosamine chloramide at pH 8.5 (measured at 200 minutes in a reaction mixture containing 1 mM hypochlorite and 10 mM N-acetylglucosamine (1 cm path length); **b)** the effect of pH on k (hypochlorite + N-acetylglucosamine). Also shown is the fit to the pKa of hypochlorous acid of 7.4

For 1mM hypochlorite reacting with the HA monosaccharide model, 10mM N-acetylglucosamine, the reaction to form the chloramide was 100% efficient at all pHs, as assessed by the TNB assay. This finding supports the 100% chloramide yields found in 1mM hypochlorite solutions containing 10mM hyaluronan. Figure 11a shows the spectrum of the product chloramide at the end of the reaction at pH8.5 under the same conditions. At 250nm, the extinction coefficient of the chloramide was estimated as 135 +/- 10 $M^{-1}cm^{-1}$. Kinetic measurements of the reaction were again performed using the simulation procedure, this was more straightforward than with hyaluronan since the model compound had no detectable absorption at 292 nm. As with hyaluronan itself, increase of pH increases the rate constant – as seen in Figure 11b. At pH7.5, a value of 0.03 +/- .003 M^{-1} s^{-1} was estimated and this can be compared to the value of 0.064 +/- 0.003 found in an earlier study [22]. Again as in the case of D-glucosamine-2-N-sulphate, the heparin model compound, this rate constant estimated in the current study is significantly lower than in the earlier study. There is no apparent reason for this difference. Unlike heparin, there was no increase in the rate constant as the pH was decreased from 6.5 to 4.0, a result that was repeated with hyaluronan itself. It thus appears that Cl_{2aq} does not compete with HOCl to chlorinate the N-acetyl group. From the data presented, and using equation (11), the rate constants for the reaction of HOCl and OCl^- could be estimated as 8.0 +/- 0.08 x 10^{-3} and 3.6 x 10^{-2} M^{-1} s^{-1} respectively.

Major features of the spectral, yields and kinetic studies:

From this detailed study of the effect of pH on spectra, yields and kinetic reactivity for reactions of hypochlorite with heparin, hyaluronan and related monosaccharide model compounds, a number of interesting features emerge.

The spectral and yield measurements show clear differences between the two glycosaminoglycans, heparin and hyaluronan. At low concentrations of hypochlorite, N-Cl yields are 100% for hyaluronan whereas for heparin at both high and low hypochlorite concentrations, there are significant competitive oxidation pathways at all pHs. Thus, unlike heparin, the electrostatically neutral acetyl-N-H group does not provide any effect on the efficiency of N-Cl production in the direct reaction between hypochlorite and hyaluronan. In contrast, the presence of the $N(H)\text{-}SO_3^-$ in heparin provides a negative electrostatic effect on the rate of reaction which produces the chloramide allowing significant proportions of hypochlorite, up to 40%, to participate in oxidation pathways. The PAGE studies show that such oxidation only produces fragmentation at very low levels for both glycosaminoglycans (e.g. 0.4 % in heparin chloramide) unlike the high efficiencies measured in H-abstraction from CH(OH) groups in studies of free radicals reacting with, for example, hyaluronan [16]. The effect of the $N(H)SO_3^-$ group on the probability of oxidation competing with chlorination however is not seen in the model, D-glucosamine-2-N-sulphate providing some evidence of an additional negative electrostatic effect in heparin, probably from the nearest 6-O-sulphate group in the glucosamine-N-sulphate moiety. At high hypochlorite concentrations, oxidation becomes effective at all pHs for both heparin and hyaluronan with much reduced yields of N-Cl (more effective at low pH for hyaluronan and more effective at high pH for heparin). Evidence has also been presented for oxidation of the hyaluronan chloramide product by hypochlorous acid which is seen as the only mode of reduction of N-Cl yields at high hypochlorite concentration.

The data obtained on yields of N-Cl and competing oxidative reactions has allowed the estimation of many individual rate constants as shown in Table 1. A major feature here is that HOCl chlorinates heparin much faster than does OCl^- . In contrast, the opposite is found for hyaluronan. Both trends are also supported by studies of the model compound. One explanation of these opposing trends can be gleaned from the studies of Thomm and Wayman on reactions of hypochlorite with aliphatic secondary amides [30]. There, it was proposed that N-H forms a hydrogen bond with O^- (in OCl^-) and that electron withdrawing groups weaken the N-H bond making kinetic reactivity of OCl^- greater than that of HOCl. This is supported by the rate constants for reaction of HOCl and OCl^- with N-methylformamide (1.7×10^{-3} and 0.21 M^{-1} s^{-1} respectively) and with N-methylacetamide (1.7×10^{-3} and 1.8×10^{-2} M^{-1} s^{-1} respectively) [30]. With N-methylpropanamide however, the reverse is found with OCl^- (7×10^{-2} and 9.2×10^{-3} M^{-1} s^{-1} respectively [30]). Thus, it would appear that the electron-withdrawing effect of the N-sulphate on the N-H bond in heparin is much weaker than for the N-acetyl group in hyaluronan. With regard to oxidation, the kinetic data show that the oxidation by HOCl occurs, as expected from its greater oxidation potential, much faster than for OCl^-.

Finally, both kinetic and yield data have been used to provide evidence for an effective reaction between HOCl and hyaluronan chloramide, indeed this reaction is almost three times faster than the reaction of HOCl with hyaluronan itself. This is likely to be an oxidation reaction and as such may present an alternative target to reactive oxidative species in inflammatory processes *in vivo*. However, there was little or no evidence for such a reaction in heparin. It may be that the weak electron-withdrawing effect of the N-

sulphate group relative to that of the N-acetyl group makes removal of the Cl group in N(SO_3^-) less likely.

Conclusions

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) [36,43] .There is evidence that binding of myeloperoxidase to matrix components and cell-surface glycosaminoglycans directs oxidative damage towards these biopolymers[33,37,38].

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 [33]. In perlecan, for example, it binds to the heparin sulphate side-chains of this proteoglycan [34]. Myeloperoxidase reacts with hydrogen peroxide to form a mixture of hypochlorous acid and its anion hypochlorite. Developments in the understanding of the biochemistry of myeloperoxidase, the oxidants that this enzyme generates, and the use of inhibitors to inhibit such damage have been reviewed recently [35].

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

[39,40], with 2.5–5 mM HOCl produced at sites of inflammation [41]. It is known that hypochlorous species , in particular HOCl, react rapidly at rates up to 10^8 M^{-1} s^{-1} with amino acids and peptides, with cysteine and methionine being amongst the most reactive [29, 42] . In a homogeneous distribution of HOCl and the heparan sulphate proteoglycans, glypican-1 and perlecan it has been estimated that most of the reactions occur at the protein core for ratios of HOCl : proteoglycan ranging from < 1 up to 200. For glypican-1, as the ratio increases between 24 and 200, there is an increasing reaction at $GlcNH_2$ residues in the heparan sulphate moiety [42]. Similarly, in the extracellular matrix, where hyaluronan has a central structural role, it can also be anticipated that HOCl will react preferentially with neighbouring proteins. There is thus little evidence for a direct reaction between hypochlorous species and reactive N-H groups in hyaluronan and heavily-sulphated glycosaminoglycans such as heparan sulphate. However, such a theoretical model does not take into account accessibility of the reactive N-H groups in proteins and glycosaminoglycans in an extracellular environment. Perhaps more importantly, it does not take into account the possibility of localized chlorination and oxidation reactions when the cationic myeloperoxidase itself is localized preferentially at glycosaminoglycan moieties in the extracellular matrix. Thus, it is difficult to exclude the direct formation of chloramides at the NHAc and $NHSO_3^-$ groups in hyaluronan and heparin respectively. At the lowered pHs at inflammation sites, HOCl (and not OCl^-) will be the major reacting species.

The main purpose of this study was to investigate both the kinetic reactivity and reaction profiles of the two species, HOCl and OCl^- with hyaluronan and with heparin, the latter being the most-heavily sulphated glycosaminoglycan. Hyaluronan is a significant

component of the extracellular matrix and is unusual in that it is not found *in vivo* as a proteoglycan. 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. This study was designed to provide a basis provide a basis for prediction of the modes of reaction of the hypochlorite species with glycosaminoglycans, particularly to differentiate the response of sulphated and non-sulphated glycosaminoglycans in the extracellular matrix and also to assess whether chloramide formation may accelerate fragmentation via subsequent site-specific oxidation by the hypochlorous species.

This study has shown that charge density in glycosaminoglycans has a major effect in determining the mode of reaction of hypochlorite species and emphasises the dominant role of the N-sulphated group. In the case of the non-sulphated and lightly-charged hyaluronan, this study shows that both oxidative damage (but little fragmentation) and chloramide formation are significant modes of reaction for hypochlorite species. At high levels of hypochlorite found at sites of inflammation, initial chloramide formation will be followed by further reaction with HOCl leading to fragmentation. For heparin, chloramide formation by HOCl at $NHSO_3^-$ is an efficient process with little oxidation (< 5%) seen. Unlike hyaluronan, there is no evidence of subsequent reactions of the chloramide HOCl which would lead to fragmentation of the glycosaminoglycan. Reactions of OCl^- with heparin are directly influenced by the N-sulphate group with lower yields of chloramide and a correspondingly higher yield of oxidation. In whatever way chloramides of glycosaminoglycans are formed *in vivo*, they are likely to be more susceptible to attack by both reactive oxidative species than the parent biopolymers and

particularly by reducing species, including superoxide radicals, with high probability of site-specific reactions leading to efficient fragmentation of these important biopolymers of the extracellular matrix.

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