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Heparin-induced circular dichroism of chloroquine

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ABSTRACT

Circular dichroism (CD) and UV/Visible absorption (UV/Vis) spectroscopy techniques were used to investigate the interaction between heparin and chloroquine, an antimalarial drug that has shown potential as an anti-prion agent. CD spectra of *rac*-chloroquine upon addition of heparin provide evidence of glycosaminoglycan (GAG) binding, support recent findings suggesting that interactions between heparin and antimalarial drugs are largely due to electrostatic interactions, and represent the first reported GAG-induced CD signal of a bicyclic, aromatic compound. The association constant ($\sim 10^3\ M^{-1}$) between chloroquine and heparin was calculated from a UV titration curve and provided additional insight into the nature of the association between these two compounds.

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Introduction

Glycosaminoglycans are acidic, linear polysaccharides that are biosynthesized as pieces of larger proteoglycans [1,2]. Heparin, a helical macromolecule, represents the most complex member of this group and is comprised of numerous repeating disaccharide subunits. These subunits are made up of a uronic acid and a glucosamine residue joined via an α-(1 → 4) linkage and are subject to variable patterns of sulfation. This fact, in addition to the presence of carboxylates, makes heparin one of the most negatively charged biomolecules known [3].

Studies indicate that GAGs such as heparin play major roles in the pathogenesis of several prion diseases [4,5]. Prion diseases, like Creutzfeldt–Jakob disease and bovine spongiform encephalopathy (aka. Mad Cow disease), are fatal, neurodegenerative disorders associated with the accumulation of a protease-resistant, abnormal isoform of prion protein (PrP) [6], a protein found in healthy organisms. The pathogenic scrapie isoform (PrP^{Sc}) replicates by distorting the standard conformation of a protease-sensitive, cellular prion protein (PrP^{C}) [7]. Prevailing models support that PrP^{Sc} is then deposited in both neuronal and non-neuronal tissues (e.g. skeletal muscles), leading to a variety of diseases [8]. The mechanism by which these proteins function is still not fully understood and is the subject of intense research [9,10]. It is now thought that tissue metabolism of PrP^{Sc} is directly linked to the local accumulation glycosaminoglycans (GAGs) [5,11]. Indeed, GAGs are now widely considered to be the most important host molecules associated with prion pathogenesis and the formation of cerebral plaques.

Interestingly, several GAGs have also been linked to amyloid pathology [4], which is associated with disorders such as Alzheimer's disease. GAGs are known to be important in the formation of neuritic plaques and are considered major contributors to amyloid β-peptide aggregation [12].

Efforts to develop therapies to combat these protein-conformational diseases have lead to the testing of numerous compounds capable of inhibiting the formation of the PrP^{Sc}. Findings have shown that several anti-malarial compounds (e.g. primaquine, quinacrine, etc.) are effective candidates [13,14]. The action of these drugs has been attributed to protonation at or below physiological pH contributing to significant electrostatic binding with the negatively charged GAG and the disruption of GAG–PrP interactions.

Recent research into the electrostatic interactions between a tricyclic, aromatic antimalarial drug, quinacrine, and GAGs has used CD to monitor the induced optical activity that results upon heparin binding [15]. Support for this finding has been provided by studies investigating similar tricyclic compounds, such as acridine orange [16], methylene blue [17], and their derivatives [18]. These efforts have lead to the suggestion that a tricyclic, aromatic ring system is the minimum structural requirement to observe induced CD activity upon binding to GAGs. Indeed, to the best of our knowledge, no GAG-induced CD activity has ever been reported for a bi- or monocyclic aromatic compound.

In this study, findings are presented that illustrate the occurrence of GAG-induced optical activity of chloroquine, a bicyclic, aromatic drug that has shown potential in inhibiting the formation of the scrapie prion protein [13].

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Materials and methods

Materials. Heparin sodium salt was obtained from Celsus Laboratories (Cincinnati, OH). Racemic chloroquine diphosphate and chondroitin sulfate-B (CS-B) sodium salt were purchased from Sigma–Aldrich (St. Louis, MO). Sodium hydroxide and phosphoric acid were obtained from Fisher Scientific (Fair Lawn, NJ).

Instrumentation. All circular dichroism data were collected in quadruplicate using a Jasco (Easton, MD) J-715 spectropolarimeter controlled by Spectra Manager for Windows 95/NT (V. 1.53.00) in a 1.0 cm circular quartz cell. UV–Vis absorption data were collected using a Perkin Elmer (Waltham, MA) Lamda 40 UV–Vis Spectrophotometer controlled by UV WinLab (V. 2.80.03) and a 1.0 cm quartz cell.

Procedures. A 15 mM NaH_2PO_4 buffer containing 0.25 mM chloroquine was used for all experiments. The buffer pH was adjusted as needed using either sodium hydroxide or phosphoric acid. Reported heparin concentrations are based on the molecular weight for the repeating disaccharide subunit (M_w = 665 g/mol).

Results and discussion

Circular dichroism results

The CD spectral behavior of chloroquine was monitored in the presence of increasing concentrations of heparin. Separate buffer solutions (pH 6.0) were prepared for each measurement so that mixing times and drug concentrations could be held constant as the heparin concentration was increased. As seen in Fig. 1, the introduction of heparin into the buffer system quickly gave rise to a significant couplet between 300 and 400 nm with a zero cross-over point at ~345 nm. The induced signal increased in magnitude until a 1:1 ratio was reached between the heparin disaccharides and chloroquine. Testing at higher ratios (e.g. 6:1 ratio) revealed no major change in signal intensity.

Additional research into the nature of the observed induced signal was carried out to account for pH effects. Solutions containing 1.5 mM heparin, 0.25 mM chloroquine, and 15 mM NaH_2PO_4 were prepared fresh at a variety of pH values and given similar mixing times prior to being tested. These findings, shown in Fig. 2, illustrate that the induced signal intensity at 350 nm is subject to large deviation as a function of pH. The maximum intensity for this wavelength was observed at a pH of 6.0 and was noted to decrease by ~20% upon elevating the buffer pH to 7.0, not shown here for graphical clarity.

In contrast to the heparin results, CS-B did not induce an observable induced CD couplet under the tested conditions. This is consistent with previous research that reported the loss of an induced signal for quinacrine between 240 and 310 nm upon substituting heparin for chondroitin sulfate species [15].

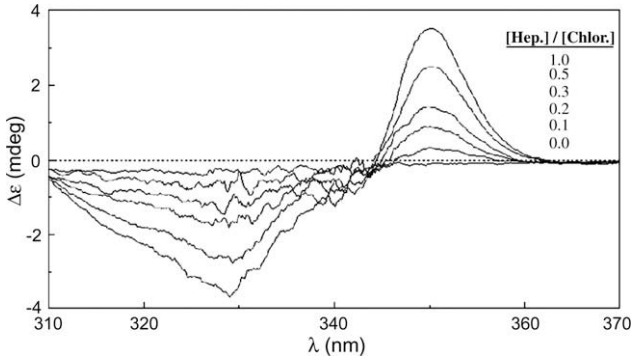

Fig. 1. CD spectra of 0.25 mM *rac*-chloroquine in 15 mM NaH_2PO_4 at pH 6.0 in increasing concentrations of heparin sodium salt.

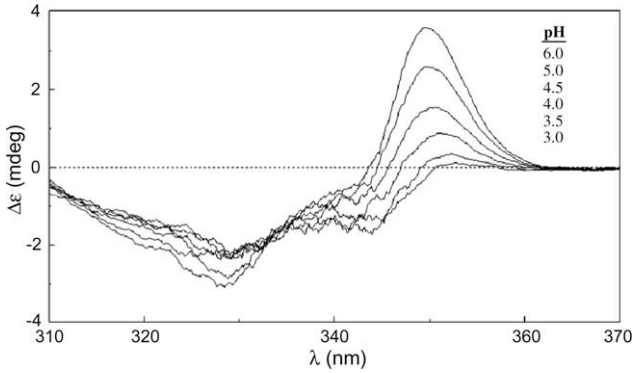

Fig. 2. CD spectra of 0.25 mM chloroquine and 1.5 mM heparin sodium salt in 15 mM at various pH.

UV–Visible absorption results

UV/Visible absorption spectroscopy was used to quantify the extent to which heparin and chloroquine associate under the employed CD conditions. Separate solutions of heparin (2.5 mM) and chloroquine (50 μM) were prepared in 15 mM NaH_2PO_4 at pH 6.0. The heparin solution was then titrated, up to 150 μL, into the chloroquine solution and dilution-corrected changes in the absorbance at 343 nm were recorded as a function of the total heparin concentration. A Benesi–Hildebrand [19] titration curve was plotted, shown in Fig. 3, with this data and used to calculate an association constant of 1100 (±155) M^{-1} at 95% confidence. A similar experiment carried out at pH 3.0 revealed no significant change in the association constant (e.g. 1000 (±270) M^{-1}), despite complete loss of the induced signal at 350 nm. It is important to note that these results represent averaged values, as chloroquine is chiral and racemic chloroquine was used in these studies.

Discussion

To the best of our knowledge, this work represents the first report of GAG-induced CD activity for a bicyclic, aromatic compound. The increased induced CD signal obtained for chloroquine with increasing heparin concentration is particularly interesting in light of a recent reported of a reduction in induced CD signal intensity with increasing heparin concentration for an 81 μM quinacrine solution [15]. In the previous report, the decrease in signal intensity with increasing heparin concentration was attributed to

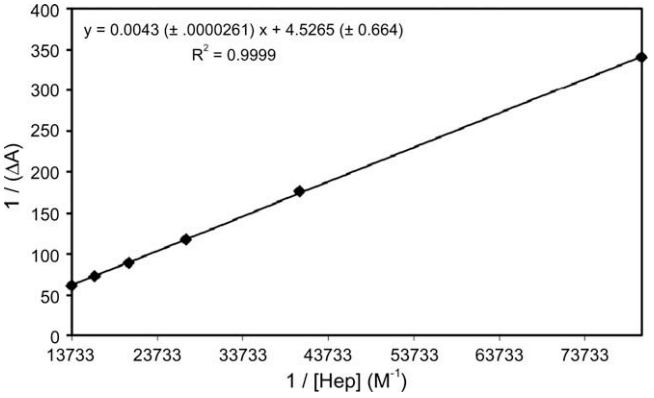

Fig. 3. Benesi–Hildebrand plot (λ = 343 nm) for the determination of heparin–chloroquine association in 15 mM NaH_2PO_4 at pH 6.0.

an increased spacing between the GAG-bound chromophores. The discrepancy between the two studies may be attributable to the significant differences in the heparin binding constants for chloroquine (e.g. $\sim 10^3\ M^{-1}$) vs quinacrine (e.g. $\sim 10^6\ M^{-1}$).

The impact of pH on signal intensity is in agreement with recent investigations concerning ionic strength. These results support electrostatic interactions as a pivotal component in the induction of a CD signal, despite observing essentially no change in the chloroquine–heparin association between pH 3 and 6.

It should be noted that the signal observed in this work might not represent a "true" induced CD spectrum because the two enantiomers are known to be resolved by heparin when it was used as a chiral additive in capillary electrophoresis [20]. Differences in binding may produce nonequivalent spectral shifts for the two enantiomers of the antimalarial drug thereby producing an apparent induced CD spectrum. Research is currently underway to explore the role of these chiral centers in CD-observable GAG-interactions.

The heparin–chloroquine association results provided by UV/Visible absorption spectroscopy are consistent with previous studies that have shown that have shown reduced association of some bicyclic, aromatic species compared with similar tricyclic, aromatic species [15]. As such, these findings support the idea that the association of the aromatic rings with GAG biomolecules may be of great importance to the biological activity and heparin binding of several anti-malarial drugs.

Acknowledgments

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