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Thermodynamics of binding interactions between divalent copper and chitin fragments by isothermal titration calorimetry (ITC)

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ABSTRACT

Herein we report the degree of binding of chitin fragments to divalent copper cation using isothermal titration calorimetry (ITC). In a previous study we have reported the kinetic and thermodynamic binding constants for chitin polymer interactions with a range of metal cations including copper (Camci-Unal & Pohl, 2009). In order to better determine the molecular basis for metal binding to these carbohydrate polymers, a series of chitin fragments were tested for their ability to bind to divalent copper. Based on ITC data, the binding strength of *N*-acetyl-D-glucosamine (GlcNAc) is weakest among the tested substrates with a binding constant of $3.8 \times 10^3\ M^{-1}$. *N*,*N*′-diacetylchitobiose $(GlcNAc)_2$ and *N*,*N*′,*N*″-triacetylchitotriose $(GlcNAc)_3$ provided binding constants of $5.1 \times 10^3\ M^{-1}$ and $13.3 \times 10^3\ M^{-1}$, respectively. Penta-*N*-acetylchitopentaose $(GlcNAc)_5$ demonstrated the strongest metal interactions with a binding constant of $22.1 \times 10^3\ M^{-1}$. For comparison, a binding value of $24.8 \times 10^3\ M^{-1}$ was found for D-glucosamine, which is the deacetylated analog of GlcNAc, with the divalent copper. All experiments showed enthalpically driven interactions. Free energy of reaction values are all determined to be negative indicating spontaneous reactions. Our results indicate that increasing numbers of GlcNAc residues increase the binding strength towards divalent copper cation. However, the effect of adding sugars to the polymer chain only modestly increases the binding affinity, thereby ruling out any chelation multivalency effects.

1. Introduction

Chitin, obtained primarily from seashells, is known to be the second most abundant natural polysaccharide after cellulose (Cohen-Kupiec & Chet, 1998; McAfee, Gould, Nadeau, & da Costa, 2001; Min et al., 2004; Shahidi, Arachchi, & Jeon, 1999; Tudor, Gryte, & Harris, 2006). This oligosaccharide contains linear beta-1,4-linked *N*-acetyl-D-glucosamine monomer repeating units. Chitosan is a partially deacetylated analog of chitin containing beta-1,4-linked D-glucosamine repeating units (Agboh & Qin, 1997; Ilium, 1998; Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004; Singla & Chawla, 2001). Chitin or chitosan can be used to remove heavy metal cations from industrial wastewater by a cost-effective technique called biosorption (Amuda, Giwa, & Bello, 2007; Kratochvil & Volesky, 1998; Veglio & Beolchini, 1997; Volesky & Holan, 1995; Zhou, Zhang, Zhou, & Guo, 2004). Chitin in particular is reported to chelate heavy metal cations and thereby is useful to treat industrial effluents (Benguella & Benaissa, 2002; Hoshi, Kamada, Inoue, & Matsubara, 1988). It is an inexpensive substrate and commercially accessible. Chitin is also a nontoxic biomaterial which finds use in biomedical applications, especially in tissue engineering. For instance, it is used to deliver drugs, in wound dressings, or as bone substitutes (Khor, 2001, 2002; Khor & Lim, 2003; Shigemasa & Minami, 1996).

Metal cations are involved in numerous biological processes. For example, they act as catalysts in chemical and enzymatic reactions, mediate the oxidation of proteins, interact with nucleotides and DNA, have the potential to induce pathogenesis and carcinogenesis (Kroneck, 2005), are used as chelators in protein purifications or affinity separations (Arnold, 1991), are helpful for targeting and probing the variations on DNA (Barton, 1986), hydrolysis of RNA or enzymes (Dupureur, 2008; Hampel & Cowan, 1997), are present as electrolytes in animals to help osmoregulation (Geldmacher-von Mallinckrodt & Meissner, 1994) and are involved in some metabolic disorders (Volpe, 2008). Given the many processes in which metals are involved, thermodynamic data for a systematic set of metal–ligand interactions is important to predict the binding energetics and thereby help the study of metal cation functions in a range of systems.

Binding interactions of biomolecules can be determined by a powerful technique called isothermal titration calorimetry (ITC)

Abbreviations: G, free energy of binding, cal/mol; H, enthalpy of binding, cal/mol; K, binding constant, M^{-1}; S, entropy of binding, cal/mol K; T, temperature, K.

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Table 1
Complete list of the thermodynamic binding parameters for chitin–metal cation interactions.

Substrate	$K \times 10^{-3}$ (M^{-1})	ΔH (kcal/mol)	ΔS (cal/mol K)	ΔG (kcal/mol)
N-acetyl-D-glucosamine	3.8 ± 0.4	−6.0 ± 0.2	−3.8	−4.87
N,*N*′-diacetylchitobiose	5.1 ± 0.6	−8.4 ± 0.2	−11.3	−5.03
N,*N*′,*N*″-triacetylchitotriose	13.3 ± 1.1	−13.9 ± 0.5	−27.7	−5.64
Penta-*N*-acetylchitopentaose	22.1 ± 0.9	−15.0 ± 0.2	−30.4	−5.94
D-glucosamine	24.8 ± 0.3	−30.2 ± 0.1	−81.2	−5.99

(Cooper, 2003; Dam & Brewer, 2002; Daranas & Turnbull, 2003; Doyle, 1997; Jelesarov & Bosshard, 1999; Ladbury & Chowdhry, 1996; Leavitt & Freire, 2001). This technique requires no substrate immobilization or labeling and allows detection of binding interactions in a computer-controlled fashion at a constant temperature. The injection syringe stirs and titrates the ligand of interest into the macromolecule substrate in the instrument cell. As this is a chemical reaction, heat is either absorbed or evolved during the successive injections. At the end of the experimental run, software is used to integrate the area under these individual titrations to provide the heat of reaction value. One of the powerful aspects of ITC instrument is that it measures the enthalpy of binding directly and provides the binding constant and entropy of binding values as well. The kinetic and thermodynamic binding parameters are calculated by a single ITC experiment along with a reference titration (See Table 1).

In a previous study we have determined the kinetics and thermodynamics of polymeric chitin binding to a range of metal cations (Camci-Unal & Pohl, 2009). In that study, the strength of copper(II) to chitin binding was measured as being $2.91 \times 10^4\ M^{-1}$; this magnitude of binding is often considered to be a moderate-strength binding. Copper(II)–chitin interactions are enthalpically favored and enthalpically driven thermodynamically spontaneous binding reactions. It was unclear from these results, however, if chitin contained specific binding sites in which multiple ligands might chelate the cation and thereby show enhanced metal affinities. The presence of multiple hydroxyl groups and sometimes other functional groups such amines and amides on carbohydrates create multiple potential metal chelation sites (Zheng, Ornstein, & Leary, 1997). Although ITC studies cannot provide direct structural data, such chelation-based multivalency effects should show significantly enhanced binding in a one-site binding model than binding that does not involve multiple chelation sites or enhanced binding as a result of only statistical multivalency effects (Pieters, 2009). In this study we report the first kinetic and thermodynamic binding results for divalent copper

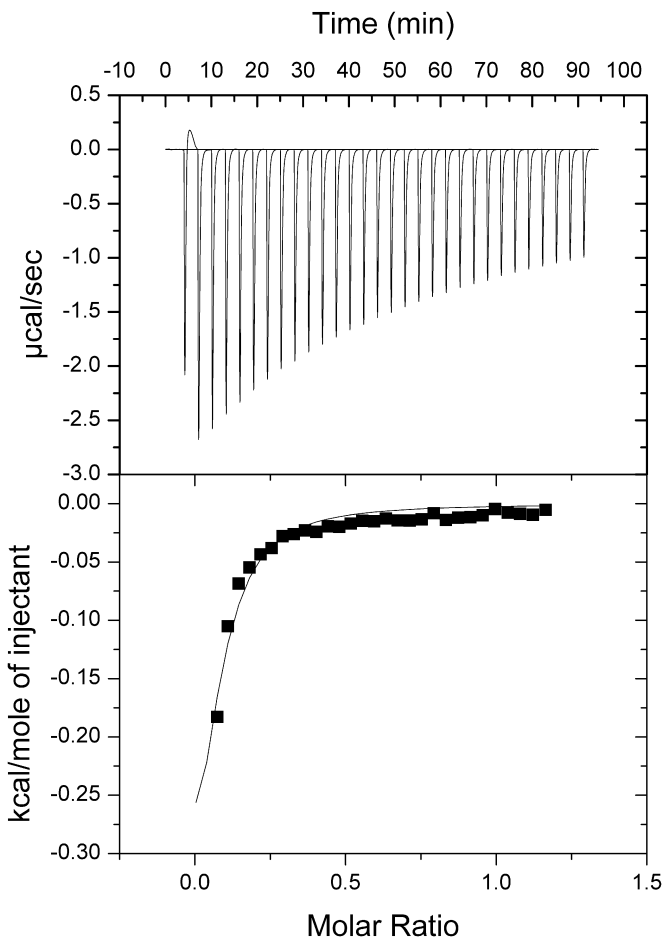


Fig. 1. Binding thermogram and isotherm for *N*-acetyl-D-glucosamine-copper(II) interaction are given at the top and bottom, respectively.

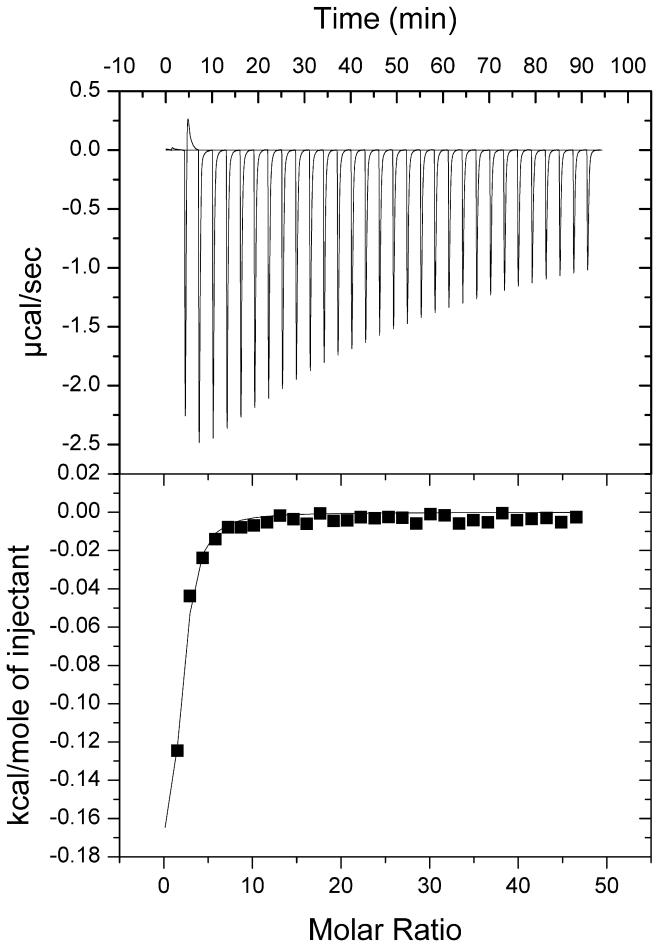


Fig. 2. Binding thermogram and isotherm for *N*,*N*′-diacetylchitobiose-copper(II) interaction are given at the top and bottom, respectively.

with small chitin fragments, namely GlcNAc, $(GlcNAc)_2$, $(GlcNAc)_3$, and $(GlcNAc)_5$ along with D-glucosamine for comparison. This data allows us to draw some conclusions about the nature of the metal–sugar interaction that should aid future computational modeling studies.

2. Experimental

2.1. General methods

N-acetyl-D-glucosamine (GlcNAc), *N*,*N*′-diacetylchitobiose $(GlcNAc)_2$, *N*,*N*′,*N*″-triacetylchitotriose $(GlcNAc)_3$, penta-*N*-acetylchitopentaose $(GlcNAc)_5$ were obtained from Sigma–Aldrich (St. Louis, MO). D-glucosamine hydrochloride was purchased from Ferro Pfanstiehl Laboratories, Inc. (Cleveland, OH). Copper sulfate was obtained from Fisher Scientific (Hanover Park, IL). No further purifications have been utilized for these reagents; they were used as they are received. Nanopure Barnstead E-pure water purification system (18.1 MΩ) was used to provide deionized water for this study.

2.2. Isothermal titration calorimetry (ITC)

A VP-ITC isothermal titration microcalorimeter (Northampton, MA) was used for thermodynamic binding experiments. Ten millimolar copper sulfate and 0.05 mM or 1 mM range of solutions of small chitin fragments were prepared to be used as the ligand and macromolecules in ITC experiments. All solutions were degassed right before the experimental runs. Copper solutions were added by the ITC syringe (301 μL) and 10 μL titrations were performed into the chitin fragments in the reaction cell (1.4288 mL) at 25 °C and at 180 s intervals utilizing a stir speed of 310 rpm. Blank ITC experiments were done to correct heat of dilution effects. Origin 7.0 (OriginLab Corp., Northampton, MA) was used to analyze the ITC data to determine the binding constant (K) and enthalpy of binding (ΔH) directly from the binding thermograms. From this, the entropy of binding (ΔS) and free energy of binding values are calculated.

3. Results and discussion

3.1. Binding results

Titration of divalent copper into chitin fragments yielded the parameters for binding thermodynamics. In order to evaluate our experimental data, we have used the Origin 7.0. 100-iterations technique to fit the data into a one-site binding model provided with the software. A two-site binding model was also investigated, but was found to not fit any of the acquired data. The software calculated from the binding curves and amount of heat generated the binding constants and enthalpies of the reaction. From that information, the entropies and free energies of binding were extracted.

The binding thermogram and isotherm for the smallest chitin fragment, GlcNAc, are shown in Fig. 1. The binding constant of divalent copper to GlcNAc is determined to be relatively small with

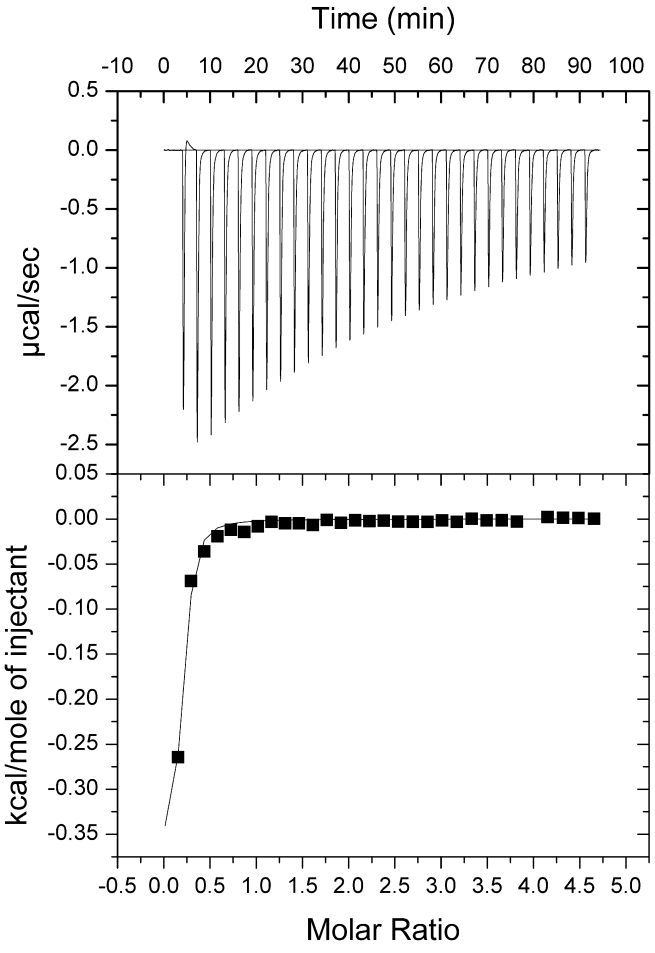


Fig. 3. Binding thermogram and isotherm for *N*,*N*′,*N*″-triacetylchitotriose-copper(II) interaction are given at the top and bottom, respectively.

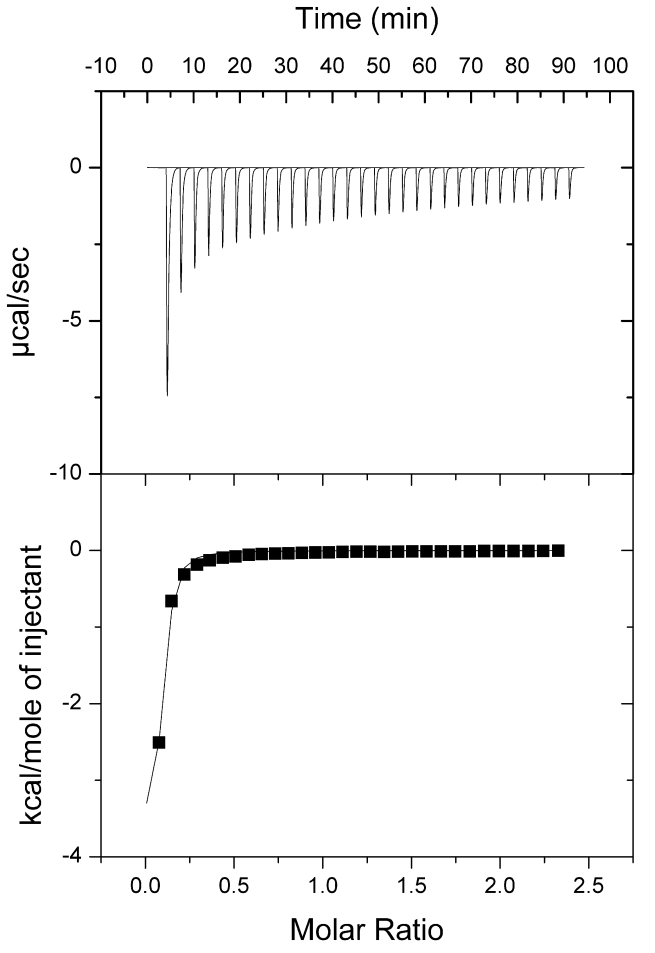


Fig. 4. Binding thermogram and isotherm for penta-*N*-acetylchitopentaose-copper(II) interaction are given at the top and bottom, respectively.

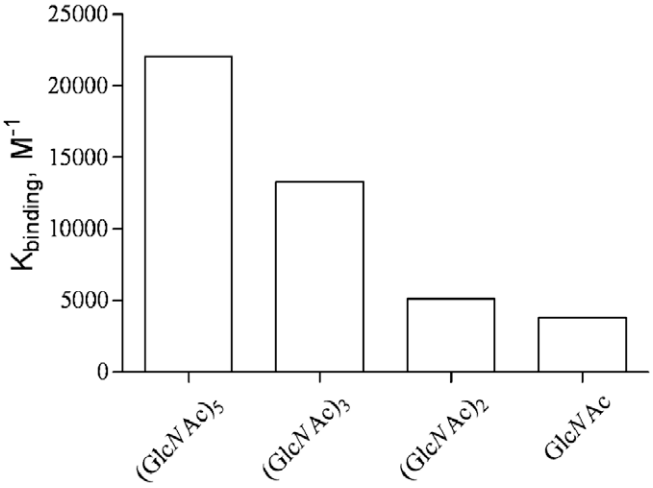


Fig. 5. Binding thermogram and isotherm for D-glucosamine-copper(II) interaction are given at the top and bottom, respectively.

a value of only $3.8 \times 10^3\ M^{-1}$. This value is still large enough to be reliably measured using ITC, however (Velázquez-Campoy & Freire, 2005). When there are two GlcNAc residues, binding increases about 1.5 times giving a value of $5.1 \times 10^3\ M^{-1}$. The trimeric sugar $(GlcNAc)_3$ afforded a binding constant of $13.3 \times 10^3\ M^{-1}$ indicating an approximately 2.5-fold increase. Overall, increasing the number of GlcNAc residues showed increasing strength in binding interactions with divalent copper cation. $(GlcNAc)_5$ provided the tightest binding among the chitin fragments investigated with a K_b of $22.1 \times 10^3\ M^{-1}$. D-glucosamine, the deacetylated form of GlcNAc that forms part of the related polymer chitosan, gave a binding constant of $24.8 \times 10^3\ M^{-1}$ (See Figs. 2–6).

3.2. Thermodynamic interpretation

All of the experiments gave negative values of enthalpy of reaction; binding of divalent copper to chitin fragments is enthalpically favored. In contrast, all reactions afforded negative values of entropy of reaction. This data suggests that divalent cation–chitin fragment interactions are enthalpically driven at 25 °C. The negative free energy of reaction values show that these binding interactions are all spontaneous reactions. We observed a higher value of enthalpy of reaction for D-glucosamine compared to the other substrates used in this study. Clearly the amine is important for binding to the copper given the approximately 6-fold increase in copper binding in the change from the monosaccharide *N*-acetyl-glucosamine to glucosamine.

3.3. Multivalency effect

Oligosaccharides possess multiple potential binding sites that can lead to multivalency effects that strengthen their binding affinities. However, in this study we did not observe a significant increase in binding toward copper(II) with the addition of additional GlcNAc units to the small chitin polymer fragments. From these experimental ITC binding results, we conclude that multivalency chelation effects are not dominant in copper(II)–chitin fragment interactions. The essentially additive binding affinity increases seen as individual GlcNAc residues are added to the growing chain are only indicative of statistical effects. In other words, the effective local concentration of the metal binding site is increased with the addition of each additional sugar residue, but the addition does not help create a new tighter binding site for the metal cation (See Scheme 1).

3.4. GlcNAc-lectin binding comparison

We would expect a lower binding interaction when a single GlcNAc molecule binds to a plant lectin with hydrogen bonds instead of to a divalent metal cation through an electrostatic interaction. This hypothesis is indeed supported by the data reported by Baines, Lee, Lee, and Freire (1992). These workers titrated GlcNAc into the plant lectin wheat germ agglutinin (WGA) using ITC and obtained a binding constant of $4 \times 10^2\ M^{-1}$. In comparison to our divalent copper-GlcNAc binding data, this number is 9.5-fold smaller — a difference that supports the idea of the reduction in binding strength with a lectin binding partner for Glc*N*Ac.

3.5. Implications

Determination of thermodynamic parameters is crucial to explain the energetics of reactions where biosubstrates are involved. This data also sheds light in studies regarding structure–function

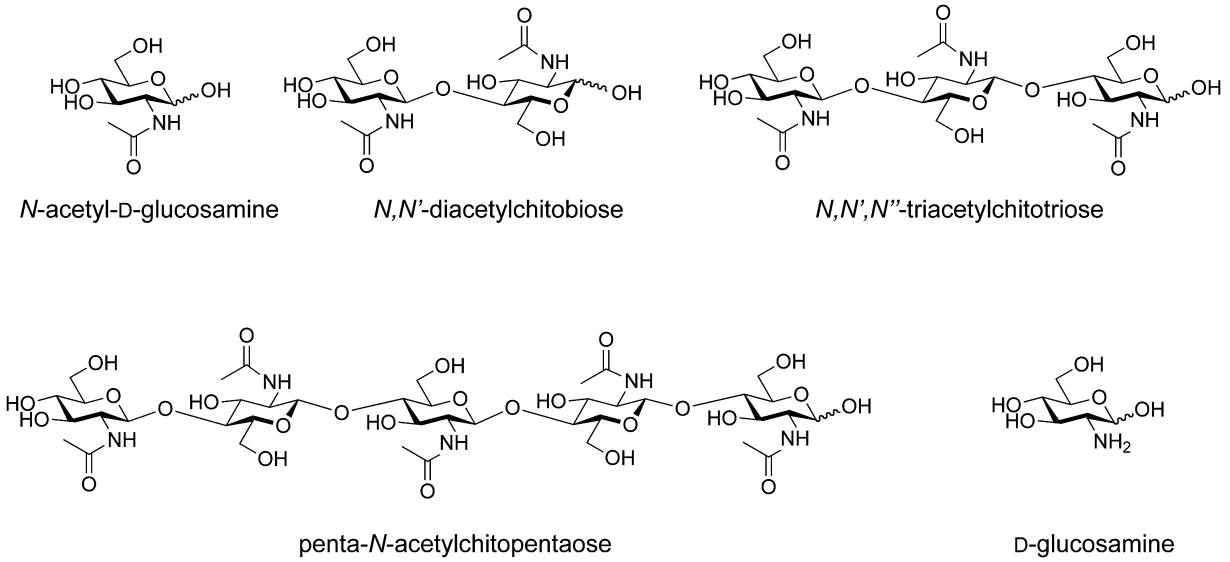
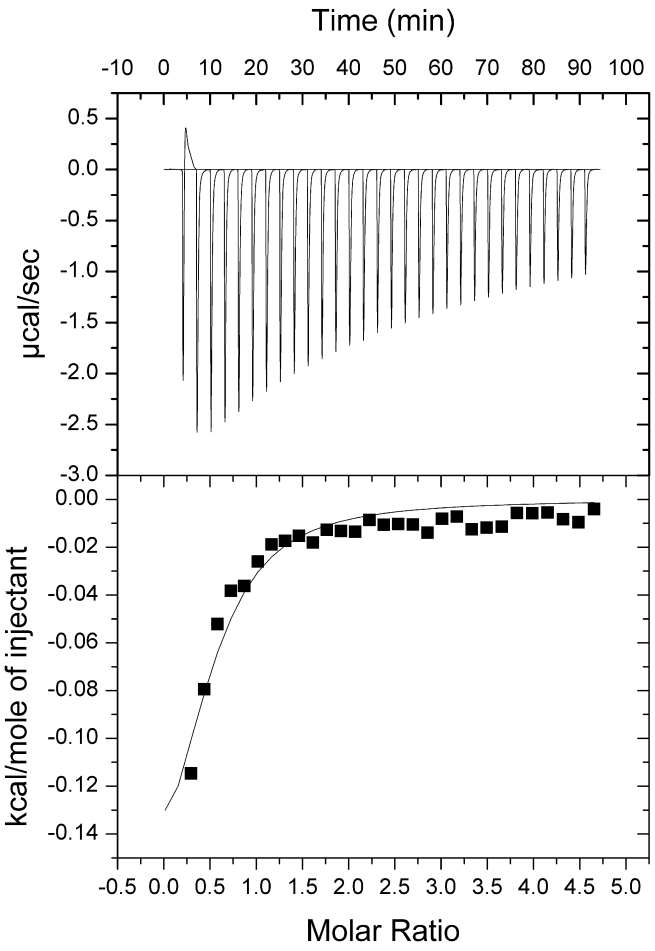


Fig. 6. Chitin-based substrates used in the ITC binding experiments.



Scheme 1. Comparison of binding constants for chitin fragment-divalent copper interactions.

relationships. Such information can foster computational modeling studies to provide reliable predictions for carbohydrate substrates. In addition, knowledge of the affinity of metal cations toward bio-substrate systems could aid in the design of therapeutics as metals act as catalysts and mediators in many biological processes.

4. Conclusion

Chitin is known to have metal binding ability and therefore can be used as a chelating agent for industrial wastewater (Barriada, Herrero, Prada-Rodriıguez, & de Vicente, 2007; Benguella & Benaissa, 2002; Niu & Volesky, 2006; Yang & Zall, 1984). In addition, it is a valuable biopolymer to be used in drug delivery and tissue engineering research (Synowiecki & Al-Khateeb, 2003; Tharanathan & Kittur, 2003). We previously reported the degree of binding of polymeric chitin to divalent copper cation (Camci-Unal & Pohl, 2009) and in this study report binding interactions of much smaller fragments of the polymer with the metal to get some sense of the nature of the metal binding interaction. Enthalpy of reaction, entropy of reaction and free energy of reaction values are all determined to be negative. These interactions are all enthalpically favored, enthalpically driven and spontaneous reactions. The minimum sugar motif for metal binding is found to be only one GlcNAc residue. Given the difference in binding of glucosamine and *N*-acetylglucosamine to copper, the nitrogen is likely involved in the binding of the copper atom with possible additional interactions with the neighboring sugar hydroxyl group. The number of GlcNAc residues appears to boost the degree binding to divalent copper. However, the addition of GlcNAc molecules to the small chitin substrates resulted in only a moderate amount of increase in the binding strength, an increase that points to statistical but not chelation-based multivalency effects. In other words, multiple amide groups are not binding to the same copper atom. This data should serve as a good basis for the development of better computational models of metal binding to carbohydrate substrates and thereby better predicative powers for the design of metal–carbohydrate-based materials and binding partners.

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