Investigation of Sugar-Binding Sites in Ternary Ligand−Copper(II)−Carbohydrate Complexes

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Evaluation of the binding interactions between a template (such as a carbohydrate) and a functional monomer (such as a metal complex) is one of the key steps during the design of molecularly imprinted polymers. Since we are interested in the development of carbohydrate-imprinted polymers as functional enzyme mimics, we have evaluated the binding interactions of several carbohydrates with [(diethylenetriamine)copper(II)] dinitrate (**1**), [*N*-{(2-hydroxyethyl)ethylenediamine}copper(II)] dichloride (**8**), and [{4-(*N*-vinylbenzyl)-diethylenetriamine}copper(II)] diformate (**13**) in aqueous solution. The binding sites of epimeric carbohydrates in complex formation with different copper(II) complexes have been investigated for the first time. The ternary ligand–copper(II)–carbohydrate complexes produced have been characterized by determination of their stoichiometry and their binding strength, by UV/Vis spectroscopy at alkaline pH.

Introduction

The mimicking of carbohydrate recognition by synthetic receptors has attracted much attention, especially in supramolecular chemistry.[1−5] Several attempts to recognize carbohydrates selectively by means of synthetic receptors based on hydrogen bonding, charged interactions, or boronic acids have been made, and the progress achieved was recently reviewed in detail by Davis and Shinkai.[1,6] Hydrogen bonding interactions, which provide the major driving force for sugar binding in apolar organic media, become far less effective in aqueous media.[7] The binding interactions of synthetic receptors with carbohydrates in water should therefore rely on other forces, such as metal coordination.

Functional enzyme mimics can be engineered by the molecular imprinting technique, which is suitable for the development of robust, inexpensive, but highly selective polymers.[8−10] Molecular imprinting involves the preorganization of polymerizable functional monomers around a targeted template (such as a carbohydrate) in solution, forming a monomer−template assembly, followed by cross-linking polymerization to stabilize this arrangement in the three-dimensional polymeric material. The solvent serves as a porogen during the imprinting process and is responsible for the microporosity of the later polymer. During the imprinting process the functional monomers are placed complementarily to the functionality of the template (such as a carbohydrate) inside the polymer matrix. Functional cavities remain after removal of the template, and these enable the matrix to rebind its original template specifically. The preparation of these polymers can only be rationalised with precise knowledge of the binding interaction between the functional monomer and the targeted template (carbohydrate) prior to polymerization.[11,12]

Binding forces applied during the self-organization of functional monomers and carbohydrates as templates include covalent, noncovalent, and coordinative binding interactions. Wulff and co-workers introduced a covalent approach for the separation of racemates of glycosides and free carbohydrates.[13−15] The reversible formation of boronate esters between pairs of hydroxyl groups of the sugar molecule and *p*-vinylphenylboronic acid was used for preorganization of the functional monomer and the carbohydrate. The esters were characterized by common methods, such as ^{1}H and ^{13}C NMR spectroscopy, prior to polymerization. Mosbach and co-workers introduced a noncovalent approach for the investigation of sugar-imprinted polymers. Their study demonstrates the feasibility of noncovalent binding interactions for the preparation of sugar binding polymers, and the binding specificity of the resulting polymer sites.[16] Prior to polymerization, derivatized carbohydrates (templates) were prearranged through hydrogen bonding to methacrylic acid (functional monomer). The complex formation was assumed to be similar to that investigated for the previously reported self-organization of amino acids with methacrylic acid.[17] However, binding sites with high affinity and excellent anomeric and epimeric selectivity suitable for the separation of racemic mixtures were obtained. Arnold and co-workers have also developed a sensing device for glucose, based on an α-methyl-D-glucose-imprinted polymer.[18] Metal coordination was used as the

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binding force between functional monomer and carbohydrate to generate a glucose-specific cavity inside the polymer. Isothermal titration calorimetry was applied in order to study the formation of a ternary triazacyclononane−Cu^{II}−glucose complex in solution at pH = 11.25 [K_{app} = 2.6 (±0.2) × 10^3 M^{-1}] and to study the binding interactions between the triazacyclononane−Cu^{2+} complex and *cis*-1,2- or *trans*-1,2-diols, such as 1,4-anhydroerythritol or 1,4-anhydro-L-threitol. Upon binding of *cis*-diols at high pH (> 9), the metal complex releases protons, which can be easily detected by a pH electrode. However, this sensing device is limited by its operation range, which is not suitable for sensing carbohydrates at physiological pH values.[19]

Taking advantage of the demonstrated recognition capability for *cis*-1,2-diols over *trans*-1,2-diols of metal-coordinated molecularly imprinted polymers, we are aiming to prepare molecularly imprinted polymers as enzyme mimics, that are operational at physiological pH. We have therefore investigated suitable binding interactions and binding sites prior to the imprinting of carbohydrates by metal coordination into polymeric materials. Accordingly, we now report for the first time on the evaluation of the binding sites of several closely related carbohydrates upon chelation to selected copper(II) complexes. We have also characterized the ternary ligand−Cu^{II}−carbohydrate complexes produced in terms of stoichiometry and binding strength.

Results and Discussion

Determination of the Stoichiometry of the Ternary Ligand−Copper(II)−Carbohydrate Complexes

Binding events in light-absorbing metal complexes can easily be observed by UV/Vis spectroscopy, while the application of NMR spectroscopy to the observation and characterization of binding events is widely used only for diamagnetic complexes. Therefore, a direct line of arguments relying on NMR spectroscopy does not appear to be applicable for systems containing paramagnetic copper(II) complexes. Accordingly, the following chain of indirect evidence is given.

The determination of the number of spectroscopic states in an equilibrium gives an insight into the number of complexes formed, and influences the choice of a suitable method to evaluate the binding constant, which strongly depends on the number of absorbing species. The association of a metal complex (M) with a carbohydrate (C) resulting in a ternary ligand−Cu^{II}−carbohydrate complex (MC) is described by Equation (1), its apparent binding constant $pK_{11} = -\log(K_{11}^{-1})$, whereas K_{11} is defined by Equation (2).[20] The number of spectroscopic states does not characterize intramolecular equilibria within ternary metal complexes.

$$M + C \leftrightharpoons MC \quad (1)$$

$$K_{11} = [MC]/([M] \times [C]) \quad (2)$$

When no complex formation between C and M takes place, M remains the only absorbing species in a one-state system, although the number of species (i.e., C and M) in this solution is two. Accordingly, a system containing two absorbing species (i.e., M and MC), refers to a two-state system and also to 1:1 complex formation if the carbohydrate C does not absorb within the selected wavelength range.[20]

If additivity of absorbances is assumed for two solutions k and n at any wavelength pair 1 and 2, Beer's law can be rewritten, resulting in Equations 3:

$$A_{1k} = \varepsilon_{1k}^{M}[M]_k + \varepsilon_{1k}^{MC}[MC]_k \quad (3a)$$

$$A_{1n} = \varepsilon_{1n}^{M}[M]_n + \varepsilon_{1n}^{MC}[MC]_n \quad (3b)$$

$$A_{2k} = \varepsilon_{2k}^{M}[M]_k + \varepsilon_{2k}^{MC}[MC]_k \quad (3c)$$

$$A_{2n} = \varepsilon_{2n}^{M}[M]_n + \varepsilon_{2n}^{MC}[MC]_n \quad (3d)$$

When $[M]_k + [MC]_k = [M]_n + [MC]_n = M_t$ (t = total; M_t is kept constant) and medium-independent spectra can be assumed, then $\varepsilon_{1k}^{M} = \varepsilon_{1n}^{M}$ and $\varepsilon_{1k}^{MC} = \varepsilon_{1n}^{MC}$. It also follows that the ratio of differences of the absorbances is a constant. Plots of $(A_{1k} - A_{1n})$ versus $(A_{2k} - A_{2n})$, $k \neq n$, give straight lines passing through the origin [Equation (4)]. The corresponding system has two spectroscopic states and refers to 1:1 complex formation.[20]

$$(A_{1k} - A_{1n}) = [(\varepsilon_{1k}^{MC} - \varepsilon_{1k}^{M}) \times (\varepsilon_{2k}^{MC} - \varepsilon_{2k}^{M})^{-1}] \times (A_{2k} - A_{2n}) \quad (4)$$

[(Diethylenetriamine)copper(II)] dinitrate — [CuDIEN]-$(NO_3)_2$ (**1**) — and glucose (**2**), galactose (**3**), mannose (**4**), or maltose (**5**) assemble at pH > 10.4 to give 1:1 complexes, resulting in two-state systems. This conclusion is based: (1) on the evaluation of the number of spectroscopic states, and (2) on the method of continuous variation (Job's method).[20] As a representative example for other calculations of numbers of spectroscopic states reported in this

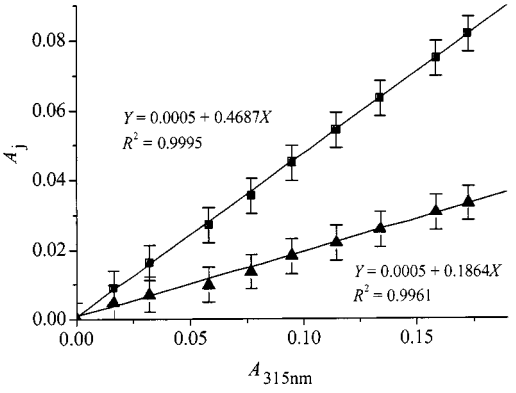


Figure 1. Plot of selected absorbances A_j [j = 325 nm (■) and 335 nm (▲)] over A_{315nm} from titration of [CuDIEN]$(NO_3)_2$ (**1**) with water at pH 12.4, 25 °C (calibration curve)

paper, the stoichiometry of the ternary complex formed from **1** and glucose (**2**) is determined by Equation (4) according to the plots shown in Figure 1 and Figure 2.

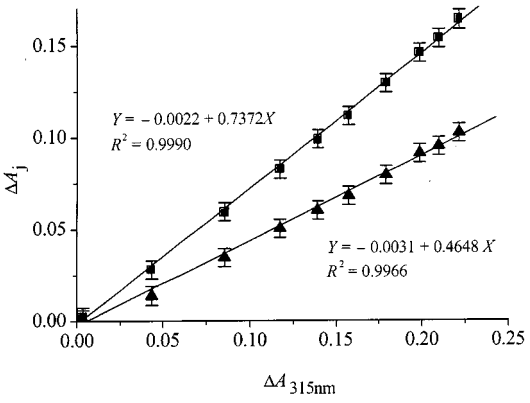


Figure 2. Plot of selected differences in absorbances $\Delta A_j = (A_{j,k} - A_{j,n})$ over $\Delta A_{315nm} = (A_{315nm,k} - A_{315nm,n})$ from titration of $[CuDIEN](NO_3)_2$ (**1**) with glucose (**2**) at j = 325 nm (■) and 335 nm (▲) for solution k containing **1** and **2** and solution n containing **1** at V_t = 2 mL, $[\mathbf{1}]_t$ = 2 mM, t = total, $A_{315nm,\mathbf{1}}$ = 0.0657, $A_{325nm,\mathbf{1}}$ = 0.0354, $A_{335nm,\mathbf{1}}$ = 0.0109, pH = 12.4, 25 °C

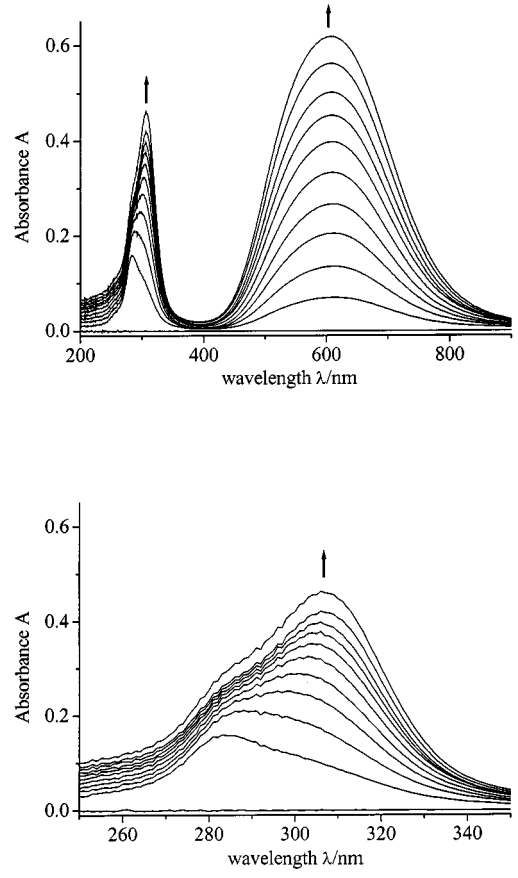


Figure 3. UV/Vis spectra observed during titration of $[CuDIEN](NO_3)_2$ (**1**) with glucose (**2**) at 25 °C in unbuffered, aqueous solution at pH = 12.4 according to Job's method; the concentrations of **1** and **2** were varied from 0−10 mM, while the sums of the total concentrations ($M_t + C_t$ = 10 mM) and the total volume of the solution (V_t = 2 mL) were kept constant

If the system contains only one absorbing species (i.e., M), no complex formation takes place and the slopes of the linearly fitted data for corresponding wavelength pairs in systems containing **1** or **1** and **2** would be identical. This is not observed here; therefore, association between **1** and **2** takes place and at least one ternary ligand−copper(II)−carbohydrate complex is formed (Figures 1 and 2). From the linear fit of the data, the current system can be described as a two-state system, based on 1:1 complex formation.[20]

The method of continuous variation (Job's method) was also applied to demonstrate association between **1** and **2**−**5**. Accordingly, the concentrations of **1** and the carbohydrates were varied, keeping the total concentration of the sugars and the metal complex constant. The titration data of **1** and **2** obtained by Job's method are shown as representative examples in Figures 3−5 and discussed below (for others, see Supporting Information).

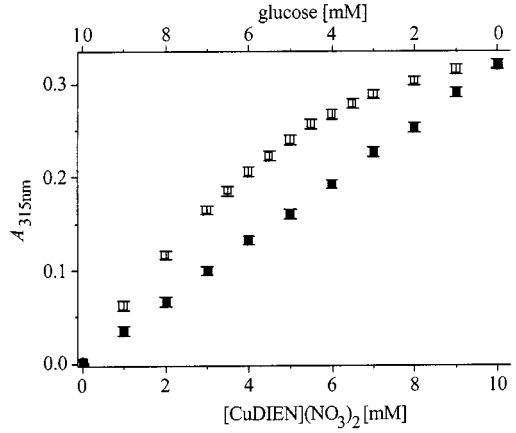


Figure 4. Plot of the absorbance A at 315 nm versus concentration of $[CuDIEN](NO_3)_2$ (**1**), observed during titration of **1** with glucose (**2**) (□) and during titration of **1** with water (■) according to Job's method at pH 12.4, 25 °C

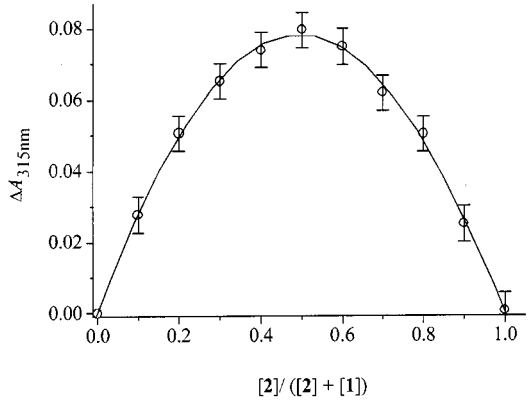


Figure 5. Plot of differences in absorbances ΔA at 315 nm between solutions containing $[CuDIEN](NO_3)_2$ (**1**) and glucose (**2**) and solutions containing pure **1** versus the molar ratio **2**/(**2** + **1**); the maximum is related to the molecular ratio of n and m in $n\mathbf{M} + m\mathbf{C} = \mathbf{M}_n\mathbf{C}_m$ and is defined by $n/(n + m)$; the ratio of $n/(n + m)$ = 0.5 corresponds to n = 1 and m = 1 and refers to 1:1 complex formation of **1** and **2** (Job plot)

There is no evidence for the occurrence of multiple equilibria or other complex formation if a 1:1 molar ratio of the compounds or an excess of ligand is applied. The complex formation is not observable by UV/Vis spectroscopy when the pH value is less than 10.4. In contrast, multiple equilibria are present for the association of glucuronic acid (**6**) with **1** (Figure 6).

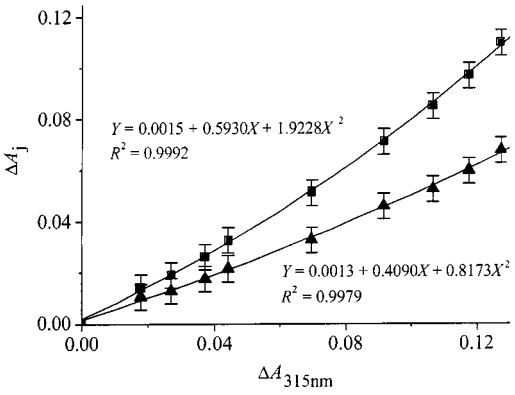


Figure 6. Plot of selected differences in absorbances $\Delta A_j = (A_{j,k} - A_{j,n})$ over $\Delta A_{315nm} = (A_{315nm,k} - A_{315nm,n})$ from titration of $[CuDIEN](NO_3)_2$ (**1**) with glucuronic acid (**6**) at j = 325 nm (■) and 335 nm (▲) for solutions k containing **1** and **6** and solution n containing **1**, V_t = 2 mL, $[\mathbf{1}]_t$ = 2 mM, t = total, $A_{315nm,\mathbf{1}} = 0.0657$, $A_{325nm,\mathbf{1}} = 0.0354$, $A_{335nm,\mathbf{1}} = 0.0109$, pH = 12.4, 25 °C

The nonlinear plot shows complex formation between **1** and **6** and accounts for the presence of more than two spectroscopic states due to multiple equilibria. The carboxylic group at the carbon C^6 of **6**, in contrast to **2**−**5**, offers an additional binding site to copper(II) complexes, which can be used for coordination or, in combination with the hydroxyl group at C^4, chelation to a copper(II) complex.

Evaluation of Suitable Ligand−Cu^{II} Complexes for Association with Carbohydrates

We also evaluated whether the reported binding constants of binary, nonpolymerizable Cu^{II}−ligand complexes can be used to interpret their association behavior with carbohydrates. Our idea behind this was: (1) to achieve fast accessibility and screening of potentially useful and structurally different Cu^{II}−ligand complexes, (2) to achieve increased stability of the Cu^{II} complex during the UV/Vis measurement, avoiding polymerization reactions, and (3) to demonstrate the similar association capabilities of structurally related Cu^{II} complexes with and without polymerizable ligands. Accordingly, we picked several tri- and bidentate Cu^{II} complexes with reported binding constants, in order to evaluate their complexation behavior with **2**−**5** (Figure 7).

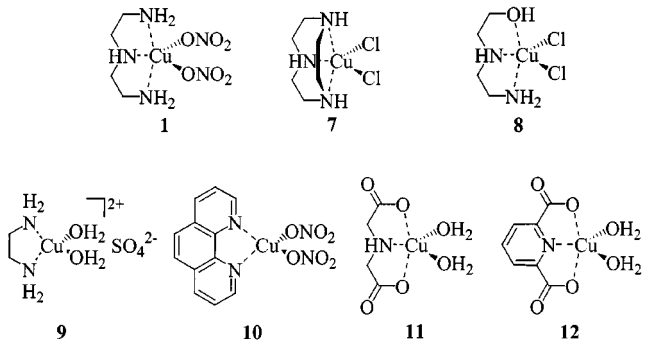


Figure 7. Structures of the copper(II) complexes **1**, **7**−**12**

The Cu^{II} complexes formed from tridentate N- and N,O-ligands **1**, [(triazacyclononane)copper(II)] dichloride $[Cu(TACN)]Cl_2$ (**7**),[18] and *N*-[{(2-hydroxyethyl)ethylenediamine}copper(II)] dichloride CuHEN (**8**), each associate with **2**−**5** with identical stoichiometries (see Supporting Information). In contrast, decomposition of [(ethylenediamine)copper(II)]sulfate (**9**), [(phenanthroline)copper(II)] dinitrate (**10**), copper(II)iminodiacetic acid (**11**), and copper(II)pyridinedicarboxylic acid (**12**) occurs in alkaline solution (pH > 10.4), which prevents the observation of complex formation with **2**−**5**. The formation of a precipitate, which was identified as copper(II) hydroxide, is due to the low chelating ability of the ligands ethylenediamine (EN), iminodiacetic acid (IDA), pyridinedicarboxylic acid (pdca), or phenanthroline (phen), to copper(II) in alkaline solution. Since strong binding of carbohydrates during molecular imprinting requires highly alkaline pHs, only copper(II) complexes stable under these conditions are useful. Surprisingly, *N*-(2-hydroxyethyl)ethylenediamine (HEN) is a suitable ligand for chelating copper(II) in alkaline solution according to our UV measurements, although the reported binding strength of HEN to copper(II) (pK^{Cu} = 10.09) is of the same order of magnitude as those reported for the tridentate IDA (pK^{Cu} = 10.56) or pdca (pK^{Cu} = 9.1), and the bidentate EN (pK^{Cu} = 10.49) or phen (pK^{Cu} = 9.12), while those for copper(II) complexes with tridentate N-ligands — TACN (pK^{Cu} = 15.5) or DIEN (pK^{Cu} = 15.9) — are much higher.[21] The advantage of the copper(II) complex **1** over **7** lies in the low cost of the diethylenetriamine ligand compared to triazacyclononane (1 g of TACN can be purchased from Aldrich for about US $350, whereas 1 g of DIEN costs only about US $0.02), while the binding strength to copper(II) is almost the same (pK^{Cu}_{TACN} = 15.5, pK^{Cu}_{DIEN} = 15.9).[21,22] Accordingly, we chose [{4-(*N*-vinylbenzyl)-diethylenetriamine}copper(II)] diformate $[Cu(styDIEN)](HCOO)_2$ (**13**) as a functional monomer for the preparation of molecularly imprinted polymers after characterization of the stoichiometry of the targeted ternary ligand−Cu^{II}−carbohydrate complexes from **13** and **2**−**5** (see Supporting Information).[23]

As demonstrated for CuHEN, the binding constants of known binary nonpolymerizable Cu^{II}−ligand complexes do not allow a generally valid interpretation of their association behavior with carbohydrates. In contrast, the complexation behavior of the polymerizable Cu^{II} complex **13** can be estimated from the known binding behavior of the corresponding nonpolymerizable Cu^{II} complex **1**.

Investigation of the Binding Sites of Selected Carbohydrates upon Interaction with Copper(II) Complexes

Carbohydrates exist in several equilibrium structures (i.e., α- and β-pyranoses, α- and β-furanoses, and an acyclic

form) and conformers. It is known that both *cis*- and *trans*-diols in six-membered rings can complex to metal ions, but that only *cis*-diols in five-membered rings do.[24] It is also generally accepted that the effectiveness of complexing sites can be listed in descending order: *cis*-diol on a five-membered ring > *cis*-diol on a six-membered ring > *trans*-diol on a six-membered ring.[25] The pyranoses of **2**−**4**, which are the main equilibrium structures in aqueous solution of the saccharides under investigation here,[30] are shown in Figure 8.

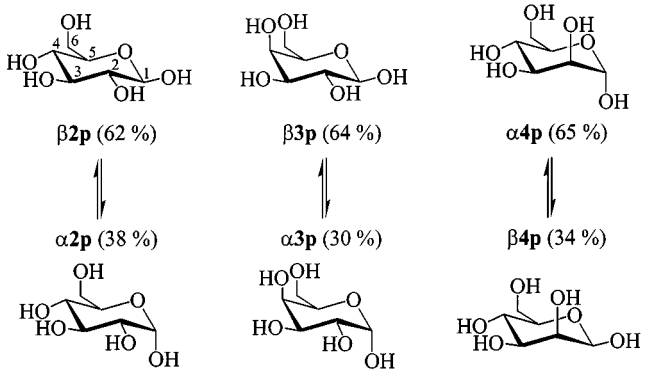
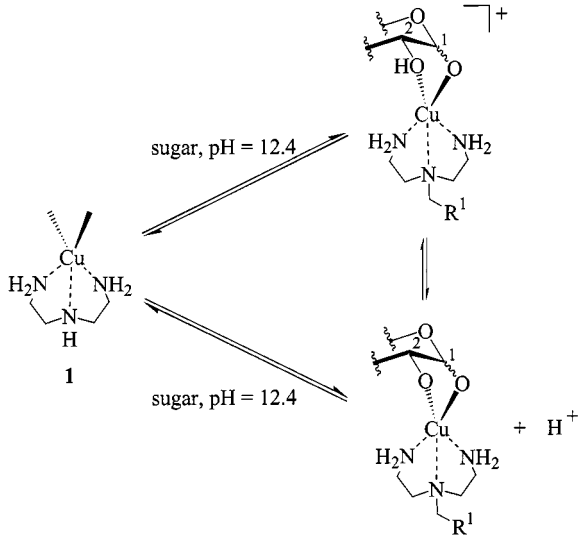


Figure 8. Percentage values of the main equilibria structures of glucose (**2**), galactose (**3**), and mannose (**4**) in aqueous solution at pH = 7 at 31 °C (**2**, **3**) and 44 °C (**4**);[30] missing percentage values refer to the furanose and the acyclic carbonyl forms; **p** = pyranose

Subsequently, we firstly determined whether multiple deprotonation of the carbohydrate occurs upon complexation with **1**, **7**, and **8** under the conditions applied, and secondly, which 1,2-diol groups of the carbohydrates **2**−**6** are involved in chelation to **1**, **7**, and **8**.

Multiple deprotonation of metal-bound carbohydrates is a well-known phenomenon in the solid state, particularly when pH values close to 14 are considered. Klüfers et al. reported on a crystal structure of fivefold deprotonated D-mannose as a ligand in homoleptic dinuclear metalates of trivalent iron, vanadium, chromium, aluminium, and gallium.[26] However, the systems under investigation here are clearly different in terms of concentration of the compounds, the kind and charge of the metal ion, and the identity of the metal atom-binding ligand. When the solutions both of the metal complex and of the sugar are adjusted to pH 12.4 prior to mixing, proton release during complex formation is directly related to the single or multiple deprotonation of the carbohydrate in a ternary ligand−copper(II)−carbohydrate complex and observable by a drop in the pH value of the solution.

Since ternary complexes formed both from singly deprotonated and from doubly deprotonated carbohydrate can coexist in equilibrium (Scheme 1), titration experiments were performed at 2, 5 and 10 mM concentrations of **1** with fortyfold, sixteenfold, and eightfold molar excesses of **2** to distinguish both possible binding modes (Figure 9).



Scheme 1. Complex formation of $[Cu(DIEN)](NO_3)_2$ (**1**) with singly and doubly deprotonated carbohydrates at pH 12.4

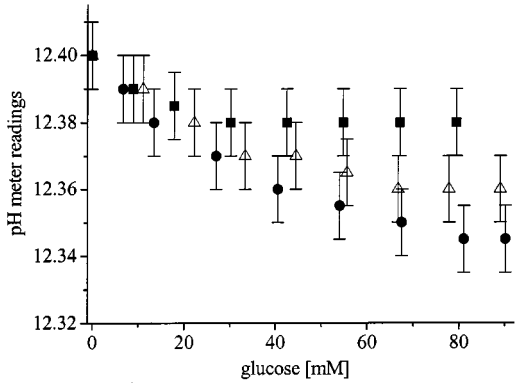
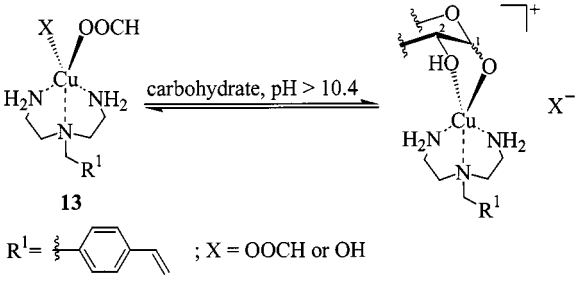


Figure 9. Plot of average pH meter readings from titration experiments of 2 mM (■), 5 mM (▲), and 10 mM (circle) $[CuDIEN](NO_3)_2$ (**1**) solution with up to fortyfold molar excesses of glucose (**2**) at 25 °C; each set of titration was performed three times and the error in the pH reading was within 0.01 units; total concentration of **1** and the total volume of the solution (V_t = 1 mL) were kept constant; nanopure water was sonicated and purged with argon prior to use; stock solutions were separately adjusted to pH 12.4 with $NaOH_{(aq)}$ and kept under argon prior to use

By using the Hendersson−Hasselbach equation and the reported first deprotonation constants of the carbohydrates,[21] we calculated the ratio of singly deprotonated and nondeprotonated sugars in the saccharide stock solution.[27] We further estimated the maximum amount of proton release during the formation of the ternary ligand−copper(II)−carbohydrate complexes for 2, 5, and 10 mM metal ion solution for complete dissociation of **2** into a singly deprotonated saccharide upon binding to the copper(II) complex. The pH value would be expected to drop from 12.40 to 12.38 for 2 mM, from 12.40 to 12.36 for 5 mM, and from 12.40 to 12.32 for 10 mM copper(II) ion solution. In contrast, the pH value of the solution for doubly deprotonated **2** should drop from 12.40 down to 12.34 for 2 mM, from 12.40 to 12.25 for 5 mM, and from 12.40 to 12.03 for 10 mM copper(II) solution. This larger drop is caused by

further dissociation of **2** and additional release of 1 mol proton/mol carbohydrate upon binding to the copper(II) ion. However, the observed changes in pH for the overall solutions of both 5 mM and 10 mM Cu^{II} concentrations correspond to singly deprotonated **2** as the main species in solution in the ternary ligand−copper(II)−carbohydrate complex (Figure 9). The drop in the pH value for the 2 mM solution is close to the experimental error and was not considered.

We therefore propose that complex formation between **1**, **7**, and **8** with **2** at pH = 12.40 takes place with singly deprotonated α-D-glucopyranose (pK_2^H = 12.28)[21] chelating the copper(II) complex through the hydroxyl groups at C^1 and C^2, this being the only equilibrium structure of glucose with a 1,2-*cis* arrangement of hydroxyl groups. The proposed complex formation between **13** and **2**−**5** for aqueous solution at alkaline pH is indicated in Scheme 2.



Scheme 2. Complex formation of [Cu(styDIEN)]$(HCOO)_2$ (**13**) with **2**−**5** at pH =12.40

If this assumption is valid, then the derivatizing carbon atoms C^1 and C^2 should have a strong influence on the binding behavior of glucose to **1**, **7**, and **8**. To obtain confirmation of this, we performed several experiments at pH = 12.40 with **1** as a model for the similar copper(II) complexes **7** and **8**. Firstly, blocking of the hydroxyl group of the anomeric carbon atom C^1 of **2** by using α-D-methylglucopyranoside or β-D-methylglucopyranoside resulted in no detectable binding interactions with **1**. Secondly, removal of the hydroxyl group at C^2 of **2** by using 2-desoxyglucose as ligand resulted in no ternary complex formation. Thirdly, blocking of the hydroxyl group at C^3 by using 3-*O*-methyl-D-glucose (**2a**) as ligand permitted the formation of a 1:1 complex with **1**. These experiments demonstrate that the free hydroxyl groups at carbon atoms C^1 and C^2 of **2** are essential for chelating the copper(II) complex **1** in alkaline solution, while the hydroxyl group at C^3 is not involved in complex formation of **1** and **2** under the provided conditions.

Galactose (**3**) differs from its epimer **2** in the configuration at carbon atom C^4, while the arrangement of the hydroxyl groups at C^1 and C^2 is identical. Complex formation with **3** and **1** can in principle occur through the hydroxyl groups at C^1 and C^2, at C^3 and C^4, or at C^4 and C^6. At pH = 12.40, deprotonation of the hydroxyl group at C^1 takes place (pK_3^H = 12.35)[21] in analogy to **2**, which would be expected to provide stronger and therefore preferable binding interactions with a copper(II) complex than those arising by chelation through the protonated hydroxyl groups at C^3 and C^4, or C^4 and C^6. Under the given conditions, the number of spectroscopic states corresponds to a two-state system and a 1:1 assembly of **3** with **1**. No evidence for multiple equilibria is given by the experimental data when a 1:1 molar ratio between **1** and **3**, or an excess of **3**, is provided during the experiments. Since no binding interactions between **1** and α-methyl-D-galactopyranoside or β-methyl-D-galactopyranoside are detectable, only the hydroxyl groups at the anomeric carbon C^1 and at C^2 of **3** are considered to chelate **1**, in agreement with the observations made for ternary complex formation of **1** with **2**.

Mannose (**4**) differs from its epimer **2** in the configuration at C^2. Chelation of **1** should preferentially be through the *cis*-diol groups at C^1, C^2, and C^3 of **4**. The binding interaction between **4** and **1** at pH = 12.40 was characterized as a two-state system corresponding to a 1:1 molar ratio during complex formation. A protonated hydroxyl group at C^2 of **4** is less likely to act as a donor group for two molecules of **1** at the same time, which is attributable to steric constraints during complexation. Since deprotonation of the hydroxyl group at C^1 of **4** occurs at pH = 12.40 (pK_4^H = 12.08),[21] analogously with **2** and **3**, participation of the deprotonated hydroxyl group at C^1 in chelation has the strongest influence on the complex formation between **1** and **4**. No binding interactions between α-methyl-D-mannopyranoside and **1** were observed even at pH = 12.40. This demonstrates that the deprotonated hydroxyl group at C^1 is necessary for strong binding, and suggests that the hydroxyl groups at C^2 and C^3 of **4** do not contribute significantly to the binding interactions with **1** under the given conditions.

Determination of the Binding Constant of Selected Copper(II) Complexes with Different Carbohydrates

The method of Rose and Drago was applied in order to determine the binding constants of the 1:1 complexes formed from **1**, **8**, and **13** with **2**−**5**.[20] This graphical tech-

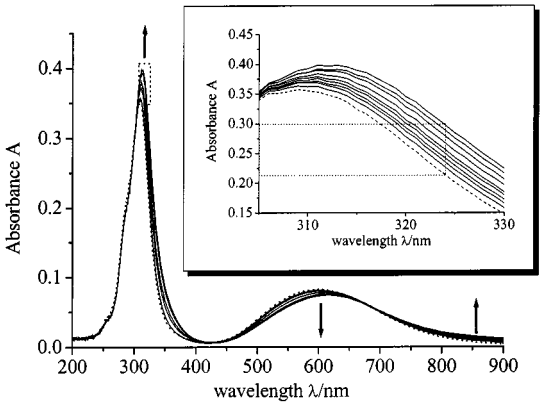


Figure 10. UV/Vis spectra obtained during titration of [Cu(styDIEN)]$(HCOO)_2$ (**13**) with glucose (**2**) at 25 °C in unbuffered, aqueous solution at pH = 12.40; ····· UV/Vis spectrum of **13** (1 mM); —— UV spectra of **13** after addition of **2** (2, 3, 4, 5, 6, 7, 8, 9, and 10 mol equivalents); the total concentration of **13** (M_t = 1 mM) and the total volume of the solution (V_t = 2 mL) were kept constant

nique does not require the assumption that $C = C_t$ made in the Scatchard plot analysis and is only applicable when the total concentration of the copper complex (C_t = 1 mM) and the total volume of the resulting solution (V_t = 2 mL) are kept constant.[28] As representative examples, the UV/Vis spectra obtained during titration of **13** with **2** at 25 °C in unbuffered, aqueous solution at pH 12.4 are depicted in Figure 10.

The binding strengths of the complexes formed from **1** and **6** were determined by nonlinear regression. The obtained apparent binding constants (pK_{11}) from the titration of **1**, **8**, and **13** with **2**−**6** are summarized in Table 1.

Table 1. Apparent binding constants (pK_{11}) for ternary complexes formed from [CuDIEN]$(NO_3)_2$ (**1**), CuHEN (**8**), and [Cu(styDIEN)]$(HCOO)_2$ (**13**) with the carbohydrates **2**−**6** at pH = 12.40

Compd.	pK_{11} [1]	pK_{11} [13]	pK_{11} [8]
2	3.73 ± 0.12	3.37 ± 0.31	3.61 ± 0.17
2a	3.71 ± 0.09	n.d.[a]	n.d.[a]
3	3.70 ± 0.09	3.41 ± 0.43	3.64 ± 0.12
4	3.68 ± 0.12	3.05 ± 0.41	3.38 ± 0.19
5	3.75 ± 0.12	3.41 ± 0.26	3.62 ± 0.09
6	3.48 ± 0.14	n.d.[a]	n.d.[a]
	1.36 ± 0.15	n.d.[a]	n.d.[a]

[a] n.d. = not determined.

The pK values for the complexes formed from **1**, **8**, and **13** with **2**−**5** are of the same order of magnitude. The ternary complexes formed from **2**, **3**, and **5** with **1**, **8**, or **13** show similar apparent binding constants, while those formed with **4** are smaller in each case. We attribute this to a rearrangement of the ligand−copper(II)−mannose complex when complex formation with the *cis*-1,2-diol at C^2 and C^3 occurs despite the preferential chelation through the hydroxyl groups at C^1 and C^2. Unfortunately, no binding interaction with α-methyl-D-mannopyranoside, which could have provided evidence for complex formation with the hydroxyl groups at C^2 and C^3, was observed by UV spectroscopy at alkaline pH values (see above and Supporting Information).

Since a carbohydrate is mostly deprotonated at a pH value larger than its pK (i.e., pK_2^H = 12.28), the binding strength of the coordinative bond to a metal complex increases with pH.[21] Therefore, the determined binding constants of the ternary complexes formed from **1** and **2** (pK_{11} = 3.73 ± 0.12 at pH = 12.40) or **13** and **2** (pK_{11} = 3.37 ± 0.31 at pH = 12.40) are of the same order of magnitude as those reported by Arnold for **7** and **2**, which were determined by isothermal titration calorimetry (pK_{11} = 2.6 ± 0.1 at pH = 11.25).[18]

In agreement with the number of spectroscopic states, two binding interactions are observed between **1** and **6**. The stronger (pK_{11} = 3.38 ± 0.14) is of the same order of magnitude as that observable between **1** and **2** (pK_{11} = 3.73 ± 0.12) and is due to chelation of **1** through the hydroxyl groups at C^1 and C^2 of **6**. The weaker binding interaction (pK_{11} = 1.36 ± 0.15) is caused by coordination of **1** by the carboxylic group of **6**, which is comparable to that observed for coordination of free copper(II) ions to **6** at neutral pH (pK_{11} = 1.44),[29] in which coordination of **6** is reported to be through the carboxylic group only. A detailed investigation of binding interactions between copper(II) complexes and disaccharides and other carbohydrates providing multiple points of interactions during complex formation is currently underway.

The binding strength of the ternary ligand−copper(II)−carbohydrate complex decreases to some extent when the secondary amino group in the DIEN ligand of **1** is alkylated with a styryl group to yield **13**, but it decreases only slightly when the HEN-based functional monomer **8** is used. Accordingly, the replacement of one amino group by a more weakly electron-donating hydroxyl group influences the stability of complexes formed with carbohydrates less than the alkylation of the secondary amino group in the DIEN-based functional monomer **1**. However, the determined binding strength for a ternary complex consisting of **13** and **2**−**5** [e.g., pK_{11} = 3.37 for complex formation of **13** with **2** (Table 1)] seems to be suitable for imprinting carbohydrates into a polymer matrix.

Determination of the Distribution of Species Participating in Complex Formation

Since different coordinating compounds (species) usually exist in equilibrium structures in aqueous solution, it is important, prior to a planned imprinting process, to examine the total amount of copper(II) bound in the formed ternary ligand−copper(II)−carbohydrate complex. We therefore evaluated the species distribution during complexation of **13** with **2**−**5** from their binding constants, with the SPE computer program.[22] The calculations suggested that only up to 80% of **13** (the overall concentration of which is kept at 2 mM) are bound in a 1:1 complex with **2**−**5** at pH = 12.4. This is not favorable for the planned imprinting process, since about 20% of the functional monomer would remain unbound and would therefore not be able to create specific cavities during polymerization, but would generate randomly distributed, nonspecific binding sites inside the polymer instead. Up to 100% complexation of the copper-containing functional monomer can be achieved by using an excess of carbohydrate, which does not interfere with the 1:1 ratio of complex formation as determined and described earlier.

Investigation of Carbohydrate Stability at Alkaline pH Values

It is known that a Lobry de Bruyn−van Ekenstein rearrangement as well as base-catalyzed fragmentation of aldoses and ketoses may occur under alkaline conditions.[30] Treatment of D-glucose with a 0.035% aqueous sodium hydroxide solution at 35 °C for 100 h results in a mixture containing D-fructose (28%), D-mannose (3%), and unchanged D-glucose (57%).[30] This behavior is not favorable either for carbohydrate chelation to copper(II) complexes, or for a planned imprinting process in which the original template has to be maintained during polymerization. Since we use

even more strongly alkaline conditions (pH = 12.40; i.e., about 0.1% aqueous sodium hydroxide) at 25 °C for strong carbohydrate binding to copper(II) complexes, we investigated the stability of **2**−**4** under these conditions by ^{1}H NMR spectroscopy. A set of ^{1}H NMR spectra recorded for **2** after its exposure to base is depicted in Figure 11 and Figure 12 (for others, see Supporting Information).

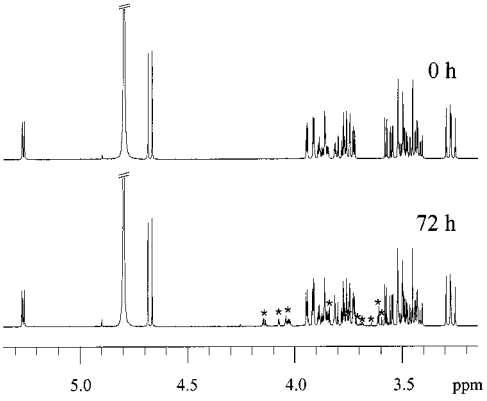


Figure 11. 400.1 MHz ^{1}H NMR spectra (303 K) of glucose (**2**) after exposure to pH = 12.40 at 25 °C for 0 and 72 h

The ^{1}H NMR spectra demonstrate the stability of **2**−**4** at pH = 12.40 for at least 1 h. Additional signals in the recorded ^{1}H NMR spectra occur for the first time only after 3 h. The original carbohydrates **2**, **3**, or **4** are maintained during the UV experiments, which are usually complete in under 20 minutes.

Polymerization methods commonly used during an imprinting process proceed for about 72 h at 60 °C and so, according to our results, are not applicable to the system described here, especially since even faster decomposition of the carbohydrate at the elevated temperature would be expected. A fast polymerization technique has been developed to prevent the carbohydrates from epimerization, isomerization, or fragmentation during the imprinting process under our conditions. The investigation of the obtained molecularly imprinted polymers is reported elsewhere.[23]

Conclusion

Careful characterization of the binding interactions between functional monomers (such as copper complexes) and targeted templates (such as carbohydrates) prior to polymerization is one of the key steps in the molecular imprinting technique. We therefore report here for the first time on the naturally accessible binding sites in several sugars — namely glucose (**2**), galactose (**3**), mannose (**4**), and maltose (**5**) — upon chelation to copper(II) complexes at alkaline pH values. Under these conditions, only the hydroxyl groups at C^1 and C^2 of the carbohydrates **2**−**5** are involved in formation of ternary ligand−copper(II)−carbohydrate complexes, which were characterized in terms of stoichiometry and binding strength at alkaline pH.

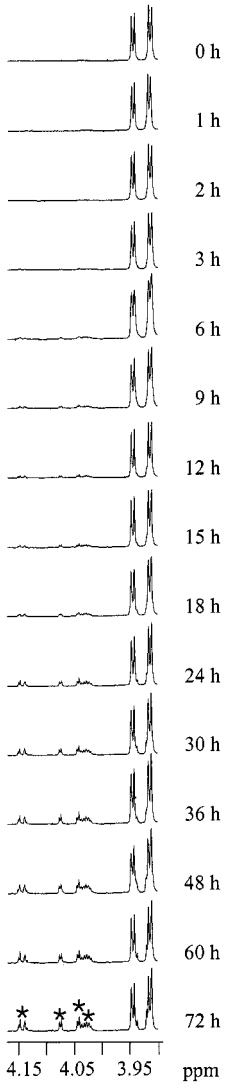


Figure 12. 400.1 MHz ^{1}H NMR signals (303 K) of protons 6a-H and 6b-H of **2** at 303 K after exposure of **2** to pH = 12.4 at 25 °C for 0, 1, 2, 3, 6, 9, 12, 15, 18, 24, 30, 36, 48, 60, and 72 h; the asterisks indicate the formation of new signals

Experimental Section

General Remarks: Methyl β-D-galactopyranoside, β-D-(+)-glucose (**2**), and maltose (**5**) were obtained from Sigma; sodium hydroxide, D-mannose (**4**), methyl α-D-galactopyranoside, methyl α-D-mannopyranoside, methyl β-D-glucopyranoside, and methyl α-D-glucopyranoside were obtained from Fluka; diethylenetriamine, 2-(2-aminoethylamino)ethanol, 3-*O*-methyl-D-glucose (**2a**), and 2-desoxy-D-glucose were obtained from Aldrich; nanopure water, cupric chloride dihydrate, cupric nitrate trihydrate, and D-(+)-galactose (**3**) were obtained from Merck Eurolab. Diethylenetriamine and 2-(2-aminoethylamino)ethanol were distilled prior to use. The nanopure water was sonicated and purged with argon prior to use. All other commercially available reagents were used as received from the supplier.

(Diethylenetriamine)copper(II) dinitrate (**1**),[31] [(1,4,7-triazacyclononane)copper(II)] dichloride (**7**),[32] [{4-(*N*-vinylbenzyl)-diethylenetriamine}copper(II)] diformate (**13**),[23] and [{*N*-(2-

hydroxyethyl)ethylenediamine}copper(II)] dichloride (**8**)[33] were prepared as described.

UV Experiments: All experiments described here were performed on a J & M TIDAS UV spectrophotometer (software SPECTRALYS Version 1.55) with Suprasil® standard cells (200−2000 nm) of 10 mm thickness and 700 μL volume at 25 °C. All experiments were done in unbuffered, degassed, nanopure water, the pH of which was freshly adjusted to pH 12.40 with NaOH for each set of titrations. Typically, 10 mM stock solutions of the appropriate copper complex and 25 mM stock solutions of the carbohydrates were prepared separately and kept at 25 °C. The total concentration of the absorbing copper complex (M_t = 1 mM in the case of **13**; M_t = 2 mM in the case of **1**, **7**, **8**) and the total volume of the resulting solutions (V_t = 2 mL) were kept constant during the titration experiments by addition of an appropriate amount of water. The UV/Vis absorbances of the resulting mixtures were measured immediately, over a range of 200−900 nm. The pH of the resulting solutions did not differ from the solutions prepared initially.

NMR Experiments: All NMR experiments were performed on a Bruker DRX 400 (^{1}H: 400.1 MHz; ^{13}C: 100.6 MHz), D_2O was used as solvent, and residual HDO was used as internal standard [δ = 4.80]. Chemical shifts (δ values) downfield from tetramethylsilane refer to the residual nondeuterated solvent signal.

Typically, **2**−**4** (1 g) was dissolved in H_2O (25 mL) at pH 12.4. The resulting solution was kept for 72 h at 25 °C. After 0, 1, 2, 3, 6, 9, 12, 15, 18, 24, 30, 36, 48, 60, and 72 h, 1 mL of the solution was removed and neutralized with HCl_{aq} (1 N, 30 μL), and the solvents were evaporated to dryness. The residue was taken up in 0.7 mL D_2O and the ^{1}H NMR spectra were recorded at 303 K.

Supporting Information (see footnote, page one) includes Job plots for the demonstration of association of the copper(II) complexes **1**, **13**, and **8** with the carbohydrates **2**−**5** at pH 12.4, 25 °C, and sets of time-dependent ^{1}H NMR spectra (400.1 MHz, D_2O, 303 K) of **3** and **4**.

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[27] $pH = pK_{sugar}{}^{H} + \log([\text{sugar-H}]/[\text{sugar}])$. The calculation suggests that 56.9% of **2**, 52.9% of **3**, and 69.6% of **4** are singly deprotonated in stock solutions at pH 12.4.
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