Ferrocene–mannose conjugates as electrochemical molecular sensors for concanavalin A lectin

Juan M. Casas-Solvas,[a] Emilia Ortiz-Salmerón,[b] Luís García-Fuentes[b] and Antonio Vargas-Berenguel*[a]

Received 5th June 2008, Accepted 19th August 2008
First published as an Advance Article on the web 29th September 2008
DOI: 10.1039/b809542f

The binding affinity of a series of electroactive glycoconjugates, based on a ferrocene core bearing α-mannose units on one or both of its cyclopentadienyl rings, to lectin Con A was studied by isothermal titration calorimetry (ITC) and voltammetry. Voltammetric measurements were performed by differential pulse adsorptive stripping voltammetry (DPAdSV). Upon complexation of ferrocene–mannose conjugates with Con A, voltammograms showed a decrease of the peak current. Both the monomannosylated ferrocene and the bis(mannosylated) ferrocene derivatives form more stable complexes with Con A than methyl α-D-mannopyranoside. Bis(mannosylated) ferrocene conjugates were found to bind to Con A with enhanced affinity due to the multivalent effect. A comparison of the thermodynamic data obtained by ITC and voltammetry is presented.

Introduction

Molecular recognition involving carbohydrates and cell-surface proteins interactions is key in many biological events such as viral and bacterial infections, cell–cell adhesion, inflammatory and immune response, fertilization, and cancer metastasis.[1,2] The understanding of such phenomena is crucial for shedding light on the molecular mechanisms that govern such biological events. The knowledge of the particular role of carbohydrate units in such biological events is the main aim of glycomics. Inherent in the advance of glycomics, is the challenge to develop synthetic tools that can be used for correlating structure and function, to inhibit, modulate, detect and probe those interactions.[3–5] In this respect, the majority of the carbohydrate–protein interaction detection methods are based on labeled proteins or carbohydrates with a fluorescent or a biotin moiety, so that the recognition event induces a measurable signal.[6] By contrast, there are very few cases of biosensors based on carbohydrates labeled with electroactive moieties triggering electrochemical signals upon binding to a protein specific receptor such as a lectin.[7–9] There are advantages attributed to electrochemical biosensors such as low cost and high sensitivity with relatively simple instrumentation. Also they are more susceptible to miniaturization and well suited for operating in turbid media.[10,11]

In recent years, compounds containing ferrocene and bearing molecular recognition binding sites have received much attention due to the possibility of building redox-switching or sensing molecular or supramolecular systems, which can be controlled through the application of external stimuli.[12–17] In this regard, ferrocene-containing carbohydrates could be of particular interest as their reversible and tunable redox properties could be applied for the development of molecular devices for the detection of carbohydrate–protein interactions, as well as for a redox switchable and tunable control of such interactions. Furthermore, it is well known that many lectins, in addition to the carbohydrate binding sites, possess hydrophobic binding sites, in particular around the site where aglycon would localise upon carbohydrate–lectin complexation.[18,19] Thus, in many cases, aryl glycosides form more stable complexes with lectins than their analogues which do not have hydrophobic substituents, indicating either that the binding pocket itself is hydrophobic or additional hydrophobic sites exist close to the primary carbohydrate binding site. Therefore, one would think that ferrocene, which has a hydrophobic nature and undergoes a fast and reversible one-electron oxidation at readily accessible redox potentials,[16] could be involved in the lectin binding interaction when situated at the aglycon site of the saccharide. On the contrary, its oxidation could lead to a dramatic change in the stability of the lectin–carbohydrate complex. Taking into account such factors, we have reported the synthesis of a series of ferrocene–carbohydrate conjugates and investigated their ability to function as electrochemical molecular sensors against a model receptor such as β-cyclodextrin (β-CD).[20,21] In a further step we have studied the calorimetric and electrochemical behaviour of ferrocene–mannosyl conjugates in the presence of concanavalin A (Con A), a mannose binding lectin. Herein, we report the results obtained.

Results and discussion

Calorimetric binding assays

A series of monovalent (Fc–Man) and divalent (Fc–Man_2) ferrocene-containing mannosyl conjugates having a CH_2S tether, **1** and **2**, CH_2–1,2,3-triazol–CH_2 tether, **3** and **4**, and 1,2,3-triazol–$(CH_2)_2$ tether, **5** (see Chart 1), have been used for the study of the binding affinity with Con A. We first carried out isothermal titration calorimetry measurements. This technique provides direct determination of ΔH, the enthalpy change of binding, and K, the affinity constant. In many cases, it is also possible to obtain n, the stoichiometry (the n values give the [ligand]–[receptor] ratio when the lectin binding sites are fully

[a]*Área de Química Orgánica, Universidad de Almería, Crta. de Sacramento, s/n, 04120, Almería, Spain. E-mail: avargas@ual.es; Fax: (+34) 950-015481*
[b]*Área de Química-Física, Universidad de Almería, Crta. de Sacramento, s/n, 04120, Almería, Spain*

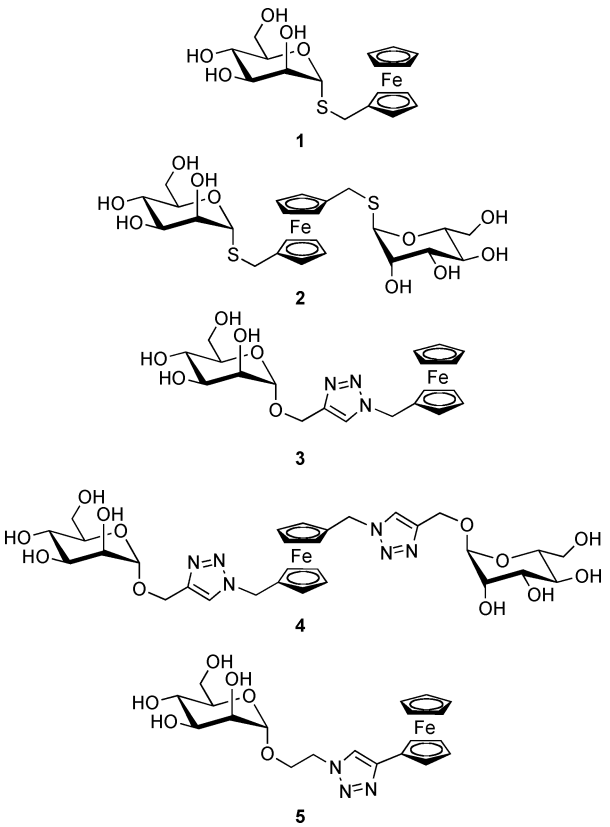

Chart 1 Ferrocene–mannose conjugates **1–5**.

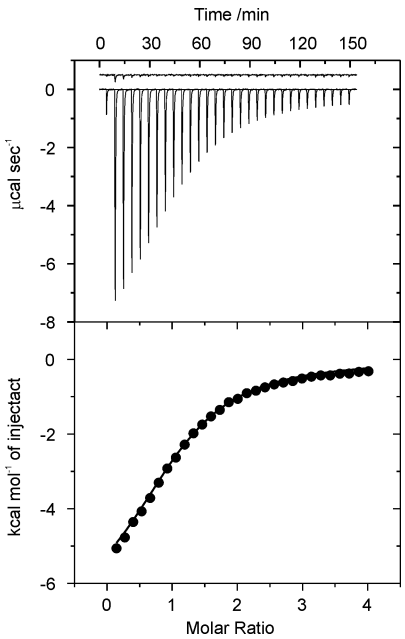

Fig. 1 Titration of Con A (245 µM) with 25 aliquots (10 mm^3 each) of the monovalent mannoside-containing conjugate **3** (5.37 mM) in 20 mM phosphate buffer (pH 7.4) with 20 mM NaCl, 0.1 mM $CaCl_2$ and 0.1 mM $MnCl_2$ at 25 °C. The top panel shows the raw calorimetric data, denoting the amount of generated heat (negative exothermic peaks) following each injection of the conjugate. The area under each peak represents the amount of heat released upon binding of conjugate **3** to the lectin. Note that, as the titration progresses, the area under the peaks gradually becomes smaller due to an increased complexation of the ligand by the protein. This area was integrated and plotted against the molar ratio of the conjugate **3** to Con A. The smooth solid line represents the best fit of the experimental data to a model of *n* equal and independent sites.

saturated). From measurements of K, the free energy of binding, ΔG°, can be calculated and hence the entropy of binding, ΔS°, determined from $\Delta G^\circ = \Delta H - T\Delta S^\circ = -RT \ln K$. The calculation of thermodynamic functions implies the usual approximation of setting standard enthalpies equal to the observed values. ITC data can be fitted using a nonlinear least square algorithm with the three independent variables (n, ΔH and K) for the simplest model based on equal and independent binding sites. In our experiments, the soluble ferrocene–mannosyl conjugate is titrated into a solution containing Con A and the heat released or absorbed during binding is measured as a function of the [glycoconjugate]–[Con A] molar ratio (Fig. 1). The experiments were performed at pH 7.4 at which the lectin is a tetramer. Previous studies on mannose derivatives and Con A interactions have established a single carbohydrate binding site per Con A monomer.[22] ITC experiments showed that the binding interaction between the lectin and the conjugates **1–5** was exothermic. The results of those bindings are shown in Table 1 and Fig. 2. As can be seen, all the Fc–Man–Con A complex formations are enthalpy-driven with an increase in affinity when compared with the binding of methyl α-D-mannopyranoside (Me-α-Man) with Con A. Thus, monovalent Fc–Man conjugates **1**, **3** and **5** bind with K values 1.3 to 2.1 times higher than Me-α-Man to Con A, while divalent Fc–Man_2 conjugates **2** and **4** afforded complexes with the lectin 21 and 33-fold more stable than Me-α-Man, respectively. By analyzing the thermodynamic profiles of the monovalent Fc–Man

Table 1 Thermodynamics and stability constants for the binding of Me-α-Man and ferrocene–mannose conjugates **1–5** to Con A obtained from calorimetric (ITC) and voltammetric (DPAdSV) experiments

	Calorimetry[a]				Voltammetry[c]	
	n	$K \times 10^{-3}/M^{-1}$	$-\Delta H$/kJ mol^{-1}	$T\Delta S^0$/kJ mol^{-1}	n[d]	$K \times 10^{-3}/M^{-1}$
Me-α-Man[b]	1.00	7.6 ± 0.2	28.5 ± 0.4	−6.3 ± 3.3	—	—
1	1.06 ± 0.33	11.9 ± 2.5	16.3 ± 5.9	7.1 ± 5.9	1.00	11.2 ± 0.1
2	0.53 ± 0.01	161 ± 22	31.8 ± 1.3	−2.1 ± 1.3	0.93 ± 0.04	152 ± 14
3	1.07 ± 0.01	10.2 ± 0.3	29.3 ± 0.4	−6.3 ± 0.4	1.00	8.8 ± 0.1
4	0.39 ± 0.01	247 ± 30	69.5 ± 2.5	−38.9 ± 2.5	0.80 ± 0.04	233 ± 30
5	1.07 ± 0.06	15.9 ± 0.6	31.0 ± 2.1	−7.1 ± 2.1	1.00	16.4 ± 0.1

[a] Determined in 20 mM phosphate buffer (pH 7.4) with 20 mM NaCl, 0.1 mM $CaCl_2$ and 0.1 mM $MnCl_2$ at 25 °C. [b] See ref. 24 [c] Determined in 10 mM TRIS buffer (pH 7.2) with 200 mM NaCl, 0.1 mM $CaCl_2$ and 0.1 mM $MnCl_2$. [d] Variable n was fixed to 1.00 for monovalent conjugates **1**, **3** and **5**.

conjugates, we observe for **3** and **5** ΔH values more negative than the ΔG° values, along with small and negative $T\Delta S^\circ$ values (see Fig. 2). This has been characterised as the typical energetics of protein–carbohydrate associations.[23–26] Interestingly, monovalent conjugate **1** shows a different profile: a ΔH value less negative than the ΔG° value and a positive entropic term upon complexation. Conjugate **1** is an *S*-mannoside that possesses the ferrocenyl moiety located at the shortest distance from the anomeric carbon of the series of monovalent conjugates. This particular structural feature would force the ferrocenyl portion to play a more active role upon the complexation of the conjugate **1** and Con A, perhaps through a higher contribution of the hydrophobic effect to the complex stability as shown by the observed entropic gain. As reported,[18,19,27–30] aryl α-D-mannopyranosides form stronger complexes with Con A than Me-α-Man, mainly due to lesser negative entropic changes as compared with the non-aromatic analogue. This effect has been attributed to a hydrophobic effect caused by the interaction of the aromatic aglycon with tyrosine residues of the lectin.

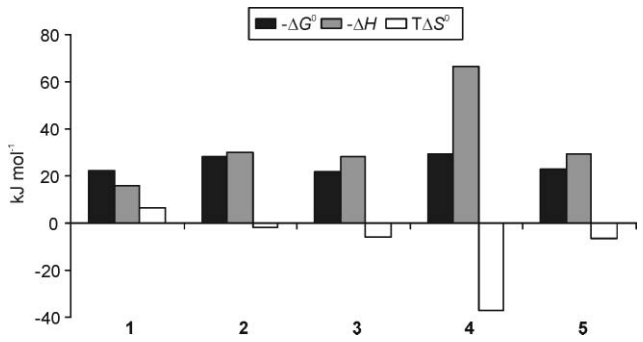

Fig. 2 Free energy ($-\Delta G^0$), enthalpy ($-\Delta H$), and entropy changes ($T\Delta S^0$) for the binding of conjugates **1–5** to Con A in 20 mM phosphate buffer (pH 7.4) with 20 mM NaCl, 0.1 mM $CaCl_2$ and 0.1 mM $MnCl_2$ at 25 °C.

While the *n* value for monovalent glycoconjugates **1**, **3** and **5** binding to Con A is close to 1, in accordance with the expected stoichiometry of one molecule of Fc–Man conjugate per Con A monomer, divalent ferrocenyl glycoconjugates **2** and **4** bind Con A with *n* values of 0.53 and 0.39, respectively. In previous ITC studies, *n* values below one have been associated with binding of a multivalent carbohydrate to a lectin due to the formation of a cross-linked complex between the monovalent lectin and the multivalent carbohydrate.[22] In our case, we expect the formation of complexes by combination of a divalent ligand with a tetravalent receptor leading to *n* values in line with those reported. Therefore, the enhanced affinities shown by **2** and **4** for Con A relative to the monovalent analogues can be attributed to the multivalent effect.[31–36] A closer examination of the ITC data revealed further additional support for the involvement of the multivalent effect in the binding interactions of **2** and **4** with Con A. Thus, in accordance with what has been reported,[22] the ΔH values of the binding of **2** and **4** with Con A are approximately twice the ΔH values of the monovalent analogues **1** and **3**, respectively. As well, divalent conjugates **2** and **4** bind to Con A with $T\Delta S^\circ$ values more negative than those that one would expect by subtracting once the $T\Delta S^\circ$ values of the monovalent analogues **1** and **3**.

Voltammetric studies

In order to evaluate the binding interaction of ferrocene–mannose conjugates **1–5** to Con A by electrochemical methods we carried out adsorptive stripping voltammetry (AdSV) experiments. As mentioned above, conjugates **1–5** have shown their ability to be used as electroactive probes for monitoring binding interactions with a model receptor. The techniques used for those studies were cyclic and differential pulse voltammetry (CV and DPV, respectively). Upon complexation of conjugates **1–5** with β-cyclodextrin, CV and DPV voltammograms showed a shift of the half-wave potential for the ferrocene oxidation to a more positive value as well as a decrease of the peak current. This electrochemical behaviour is due, respectively, to the inclusion of the ferrocene moiety into the hydrophobic cavity of the β-CD and to the decrease of the diffusion coefficient of the complex.[21] It is expected that the "inclusion" of conjugates **1–5** by the lectin Con A would lead to similar behaviour. However, given the large molecular size of the lectin, and hence of the complex, we expect the complex diffusion coefficient to substantially decrease and, as a result, the peak current and consequently the sensitivity of the detection. To overcome the diffusion problem we turned to the use of AdSV, a voltammetric technique that allows a preconcentration of the electroactive species by their adsorption on the working electrode, through a non-electrolytic process, at a constant potential.[37] This is followed by a stripping step in which the preconcentrated species can be quantified by DPV (among other modes). As a result, a sensitive electrochemical measurement can be achieved by using a small amount of lectin. Accumulation voltammetry has been used before for the detection of lectins using a carbohydrate labeled with daunomycin.[7,9] This compound, commonly used as an anticancer drug, is electroactive and strongly absorbs on a carbon electrode surface. However, daunomycin has the disadvantage of being expensive and highly toxic.

For the binding studies, we performed voltammetric measurements using solutions containing ferrocene–mannose conjugates (30 μM) and variable concentrations of Con A (0–90 μM) after incubation for 1 h at room temperature. Some carbohydrates[38–41] as well as Con A[42] were reported to adsorb on a Pt electrode after the application of potentials of around +10 mV. Therefore, we decided to use a Pt electrode as a working electrode, while as a counter electrode we used glassy carbon to which carbohydrates do not seem to be sensitive.[43] We chose to not use phosphate buffer in the voltammetric measurements, as it was used in the ITC experiments, due to its electroactivity on a Pt electrode.[44–46] Instead, we used TRIS buffer (pH 7.2). DPV measurements were performed for each solution after application of a potential of +50 mV for 5 min to promote accumulation on the electrode. DPV voltammograms (Fig. 3 and 4) display a progressive decrease of the peak current with the increase of the Con A concentration while the oxidation potential does not change. This behaviour could be caused by the "encapsulation" of the mannosyl conjugate upon binding to Con A, therefore preventing the oxidation of the ferrocene moiety. In addition, a larger hydrodynamic radius of the complex Fc–Man–Con A is expected to diffuse much more slowly than the free glycoconjugate. Therefore, since the peak current is directly proportional to the square root of the *D*,[47] the smaller the *D* value, the smaller the peak current value.

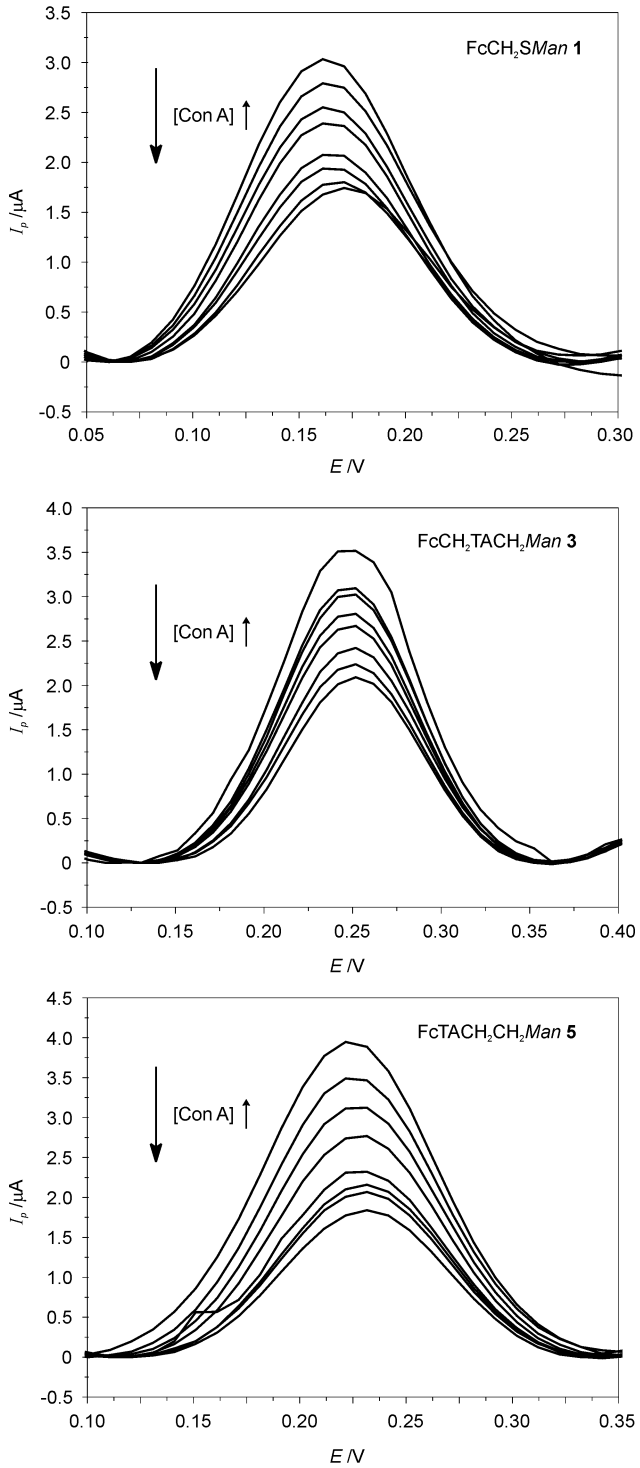

Fig. 3 DPAdSV curves for monovalent ferrocene–mannose conjugates **1**, **3** and **5** (30 μM) in the presence of increasing amounts of Con A ranging from 0 to 90 μM in 10 mM TRIS buffer (pH 7.2) with 200 mM NaCl, 0.1 mM $CaCl_2$ and 0.1 mM $MnCl_2$. A decrease in the current (large arrow) was observed as Con A concentration increased (small arrow).

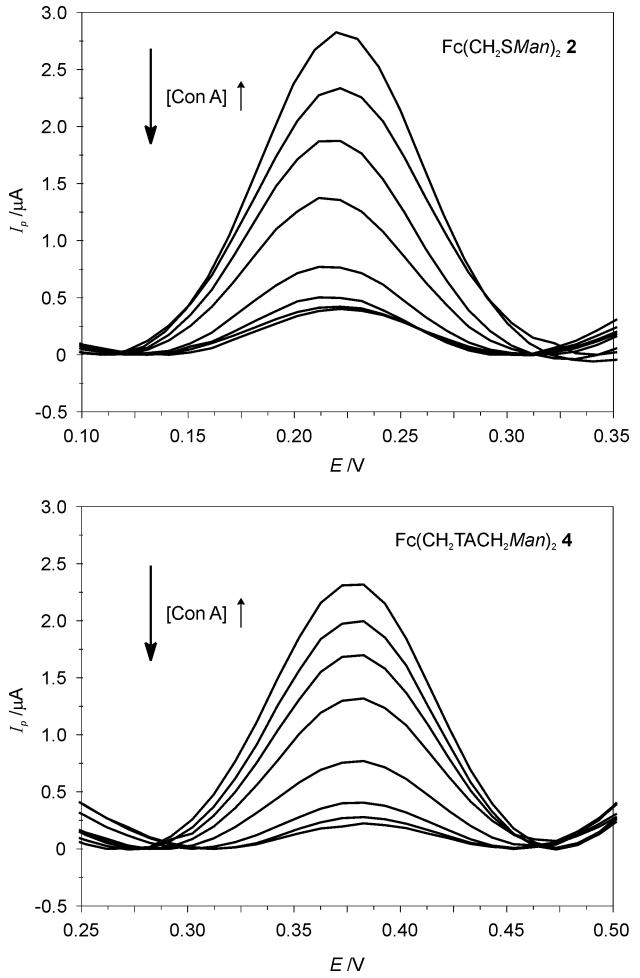

Fig. 4 DPAdSV curves for divalent ferrocene–mannose conjugates **2** and **4** (30 μM) in the presence of increasing amounts of Con A ranging from 0 to 90 μM in 10 mM TRIS buffer (pH 7.2) with 200 mM NaCl, 0.1 mM $CaCl_2$ and 0.1 mM $MnCl_2$. A decrease in the current (large arrow) was observed as Con A concentration increased (small arrow).

As seen in Fig. 5, divalent conjugates $Fc(CH_2SMan)_2$ **2** and $Fc(CH_2TACH_2Man)_2$ **4** show a more marked decrease of the peak current with the increase of the concentration of Con A than that shown by the monovalent conjugates $FcCH_2SMan$ **1**, $FcCH_2TACH_2Man$ **3** and $FcTACH_2CH_2Man$ **5**. In the case of divalent conjugates **2** and **4**, addition of 3 equiv. of Con A leads to a decrease of the peak current of about 86 and 91%, respectively, with values close to 0 μA. By contrast, in the case of monovalent conjugates **1**, **3** and **5**, a more moderate decrease is observed, so that after addition of 3 equiv. of Con A, the peak current dropped 44, 40 and 53%, respectively, to values around 2.0–2.5 μA. ITC data had shown that divalent conjugates **2** and **4** bind to Con A very strongly as compared with the monovalent analogues, forming cross-linked complexes due to the divalency of the ligand. The loss of the electroactivity observed in **2** and **4** when the saturation of the ligand binding sites is reached could be due to an efficient "sequestration" of the electroactive conjugate by the lectin along with a dramatic increase of the hydrodynamic radius of the cross-linked structure of the complex. Monovalent Fc–Man conjugates **1**, **3**, and **5** form with Con A less stable complexes of smaller molecular size than those formed with **2** and **4**.

In order to obtain the K values of the binding interactions from the voltammetric data, we assumed the same model used for the analysis of the ITC experiments. Eqn (1) is the result of the Scatchard linearization of the binding isotherm for the interaction

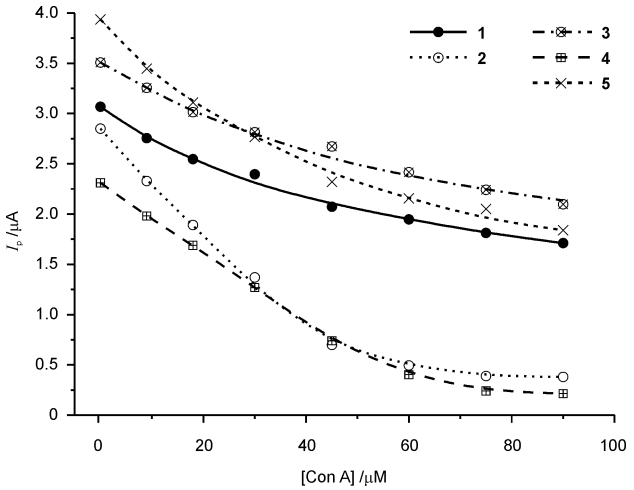

Fig. 5 Graphical plot of peak current (DPAdSV) of ferrocene–mannose conjugates **1–5** (30 μM) *versus* total concentration of Con A (0–90 μM) in 10 mM TRIS buffer (pH 7.2) with 200 mM NaCl, 0.1 mM $CaCl_2$ and 0.1 mM $MnCl_2$. The smooth solid lines are not least square fits.

of the protein with n equal and independent binding sites with a monovalent ligand.[48–51]

$$\frac{[PL]}{[L]} = K(n[P]_0 - [PL]) \quad (1)$$

where K is the affinity constant, [PL] and [L] represent the equilibrium concentration of bound ligand and free ligand, respectively, n is the number of receptor binding sites and $[P]_0$ is the total concentration of the protein. Under the assumption of reversible, diffusion-controlled electron transfer and that the diffusion coefficient of the bound ligand is much lower than the diffusion coefficient of the free ligand, we can make the approximation of $[PL]/[L] = (I_L - I_{PL})/I_{PL}$, where I_L and I_{PL} are the peak currents in the absence and in the presence of protein, respectively.[52–55] If we express [PL] as a function of $[P]_0$, then eqn (1) can be rearranged in the form:

$$\frac{I_L - I_{PL}}{I_{PL}} = nK[P]_0 - \frac{(1 + K[L]_0 + K[P]_0) - \sqrt{\left[-(1 + K[L]_0 + K[P]_0)\right]^2 - 4K^2[L]_0[P]_0}}{2} \quad (2)$$

where $[L]_0$ corresponds to the total concentration of ligand.

The obtained experimental $(I_L - I_{PL})/I_{PL}$ data were plotted *versus* the concentration of Con A monomer (see Fig. 6). A least square fit to eqn (2) provides the data shown in Table 1. Given $n = 1$ for monovalent conjugates **1**, **3** and **5**, the best fit affords K values very similar to those obtained by ITC. Contrary to the linear relation found between [PL]/[L] and the concentration of Con A for monovalent conjugates **1**, **3** and **5**, the best fit calculated for the divalent conjugates **2** and **4** provides an exponential relation. The K values for **2** and **4** obtained from voltammetry were also very close to those obtained by ITC, even though the experiments were performed in a different buffer.[24,29] The other variable obtained for divalent conjugates **2** and **4**, n, shows values below one (0.93 and 0.80, respectively) as expected for a multivalent interaction, but higher than those obtained by ITC.

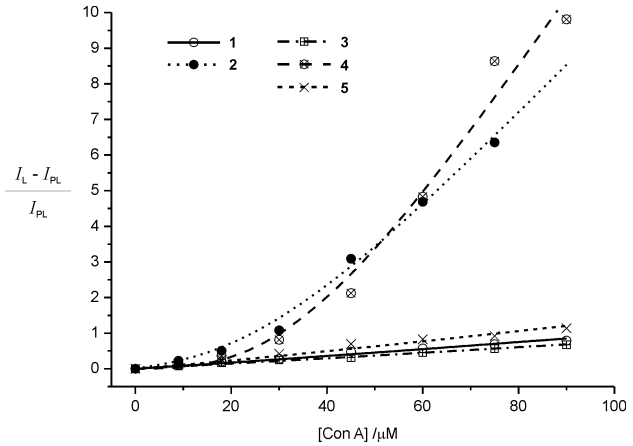

Fig. 6 Graphical plot of $(I_L - I_{PL})/I_{PL}$ data from DPAdSV experiments *versus* total concentration of Con A (0–90 μM) for ferrocene–mannose conjugates **1–5** (30 μM) in 10 mM TRIS buffer (pH 7.2) with 200 mM NaCl, 0.1 mM $CaCl_2$ and 0.1 mM $MnCl_2$. The smooth solid lines represent the best least square fit of the experimental data to eqn (2).

Conclusions

The binding affinity of a series of electroactive conjugates based on a ferrocene core bearing mannose units on one or both of its cyclopentadienyl rings to lectin Con A was studied by isothermal titration calorimetry and voltammetry. Both the monomannosylated ferrocene and the bis(mannosylated) ferrocene derivatives form more stable complexes with Con A than methyl α-D-mannopyranoside. In particular, the divalent conjugates bind to Con A very strongly relative to the monovalent analogues. This enhanced affinity is attributed to the multivalent effect. Both calorimetric and voltammetric measurements provide very similar K values. In addition, the multivalent interaction between Con A and the glycoconjugates can be identified by voltammetry. Therefore, ferrocene–mannose conjugates can be used as electrochemical sensors for the detection of concanavalin A.

Experimental

Materials

Concanavalin A lectin (type VI, lyophilised powder) and all chemicals were used as received without further purification. All solutions were made with pure water (MilliQ, 18.2 MΩ cm). The concentration of the lectin solutions was determined by spectrophotometry ($A^{1\%}_{280\,nm} = 13.7$ for the tetrameric form). Ferrocene–mannose conjugates **1–5** were prepared as previously reported.[20,21]

ITC experiments

The isothermal titration calorimeter was calibrated by known heat pulses as recommended by the manufacturer. The reference cell was filled with MilliQ water. Solutions of the conjugates (1.20–10.27 mM) and Con A (44–245 μM) were prepared in 20 mM phosphate buffer (pH 7.4) with 20 mM NaCl, 0.1 mM $CaCl_2$ and 0.1 mM $MnCl_2$, and degassed for 10 min under vacuum prior to the titration experiments. The sample cell was filled with the protein solution, and 250 mm^3 of each conjugate were injected in 10 mm^3

portions every 5 min. During the titration, the reaction mixture was continuously stirred at 400 rpm. The background titration profiles, under identical experimental conditions, were obtained by injecting each conjugate into pure buffer. The dilution heats were concentration independent and identical to the heat signals detected after saturation was reached. The raw experimental data were presented as the amount of heat produced per second following each injection of conjugate into the Con A solution (corrected for the conjugate heats of dilution) as a function of time. The amount of heat produced per injection was calculated by integration of the area under individual peaks by the Origin software provided with the instrument. The errors are provided by software from the best fit of the experimental data to the model of equal and independent sites, and correspond to the standard deviation in the fitting of the curves.

Electrochemical experiments

The electrodes were carefully cleaned before each experiment. The platinum sheet working electrode (6 × 4 mm, effective area 0.410 ± 0.003 cm^2) was immersed in a 50% v/v H_2SO_4 solution for 5 min. The glassy carbon counter electrode (65 mm, *Ø* 2 mm) was immersed in a 0.1 M HNO_3 solution for 5 min and polished with a basic Al_2O_3–water slurry. Both electrodes were then sonicated in a 1 : 1 : 1 H_2O–MeOH–CH_3CN mixture for 10 min prior to use. The effective area of the working electrode was determined as previously reported.[20,21] A Ag/AgCl (3 M KCl) electrode was used as a reference. Differential pulse adsorptive stripping voltammetric (DPAdSV) experiments were carried out in 10 mM TRIS buffer (pH 7.2) with 200 mM NaCl, 0.1 mM $CaCl_2$ and 0.1 mM $MnCl_2$. Solutions of each conjugate (30 μM) and increasing amounts of Con A varying from 0 to 90 μM were prepared in this buffer and gently shook for 1 h at room temperature. Before each experiment, nitrogen was bubbled for 3 min and an adsorption potential of +50 mV was applied for 5 min. A DPV experiment was then measured between 0 and +700 mV with a scan rate of 5 mV s^{-1}, a step potential of 10 mV, a modulation amplitude of 50 mV, a modulation time of 0.05 s and an interval time of 2 s. The *K* values of the binding interaction of each conjugate to Con A were obtained as described in the text.

Acknowledgements

The authors acknowledge the Spanish Ministry of Education and Science for financial support (Grants BIO2004-02112 and CTQ2007-61207/BQU) and for a scholarship (J.M.C.-S.).

References

1 R. A. Dwek, *Chem. Rev.*, 1996, **96**, 683–720.
2 *Essentials of glycobiology*, ed. A. Varki, R. Cummings, J. Esko, H. Freeze, G. Hart and J. Marth, Cold Spring Harbor Laboratory Press, New York, 1999.
3 C. R. Bertozzi and L. L. Kiessling, *Science*, 2001, **291**, 2357–2364.
4 R. Raman, S. Raguram, G. Venkataraman, J. C. Paulson and R. Sasisekharan, *Nat. Methods*, 2005, **2**, 817–824.
5 M. S. Timmer, B. L. Stocker and P. H. Seeberger, *Curr. Opin. Chem. Biol.*, 2007, **11**, 59–65.
6 R. Jelinek and S. Kolusheva, *Chem. Rev.*, 2004, **104**, 5987–6015.
7 K. Sugawara, H. Kuramitz, T. Kaneko, S. Hoshi, K. Akatsuka and S. Tanaka, *Anal. Sci.*, 2001, **17**, 21–25.
8 K. Sugawara, T. Shirotori, G. Hirabayashi, H. Kuramitz and S. Tanaka, *J. Electroanal. Chem.*, 2004, **568**, 7–12.
9 K. Sugawara, G. Hirabayashi, N. Kamiya and H. Kuramitz, *Talanta*, 2006, **68**, 1176–1181.
10 M. Mehrvar and M. Abdi, *Anal. Sci.*, 2004, **20**, 1113–1126.
11 B. Pejcic, R. De Marco and G. Parkinson, *Analyst*, 2006, **131**, 1079–1090.
12 A. E. Kaifer, *Acc. Chem. Res.*, 1999, **32**, 62–71.
13 A. Harada, *Acc. Chem. Res.*, 2001, **34**, 456–464.
14 J. H. R. Tucker and S. R. Collinson, *Chem. Soc. Rev.*, 2002, **31**, 147–156.
15 P. D. Beer and E. J. Hayes, *Coord. Chem. Rev.*, 2003, **240**, 167–189.
16 R. van Staveren and N. Metzler-Nolte, *Chem. Rev.*, 2004, **104**, 5931–5985.
17 F. Hapiot, S. Tilloy and E. Monflier, *Chem. Rev.*, 2006, **106**, 767–781.
18 H. Lis and N. Sharon, *Chem. Rev.*, 1998, **98**, 637–674.
19 S. S. Komath, M. Kavitha and M. J. Swamy, *Org. Biomol. Chem.*, 2006, **4**, 973–988.
20 J. M. Casas-Solvas, A. Vargas-Berenguel, L. F. Capitán-Vallvey and F. Santoyo-González, *Org. Lett.*, 2004, **6**, 3687–3690.
21 J. M. Casas-Solvas, E. Ortiz-Salmerón, J. J. Giménez-Martínez, L. García-Fuentes, L. F. Capitán-Vallvey, F. Santoyo-González and A. Vargas-Berenguel, *Chem.–Eur. J.*, 2008, DOI: 10.1002/chem.200800927.
22 T. K. Dam and C. F. Brewer, *Chem. Rev.*, 2002, **102**, 387–429.
23 E. J. Toone, *Curr. Opin. Struct. Biol.*, 1994, **4**, 719–728.
24 M. C. Chervenak and E. J. Toone, *Biochemistry*, 1995, **34**, 5685–5695.
25 A. Cooper, *Curr. Opin. Chem. Biol.*, 1999, **3**, 557–563.
26 A. Cooper, *Biophys. Chem.*, 2005, **115**, 89–97.
27 F. G. Loontiens, J. P. van Wauwe, R. de Gussem and C. K. de Bruyne, *Carbohydr. Res.*, 1973, **30**, 51–62.
28 J. P. van Wauwe, F. G. Loontiens, H. A. Carchon and C. K. de Bruyne, *Carbohydr. Res.*, 1973, **30**, 249–256.
29 G. R. Munske, H. Krakauer and J. A. Magnuson, *Arch. Biochem. Biophys.*, 1984, **233**, 582–587.
30 P. N. Kanellopoulos, K. Pavlou, A. Perrakis, B. Agianian, C. E. Vorgias, C. Mavrommatis, M. Soufi, P. A. Tucker and S. J. Hamodrakas, *J. Struct. Biol.*, 1996, **116**, 345–355.
31 *Neoglycoconjugates. Preparation and applications*, ed. Y. C. Lee and R. T. Lee, Academic Press, San Diego, 1994.
32 Y. C. Lee and R. T. Lee, *Acc. Chem. Res.*, 1995, **28**, 321–327.
33 R. T. Lee and Y. C. Lee, *Glycoconjugate J.*, 2000, **17**, 543–551.
34 S. M. Dimick, S. C. Powell, S. A. McMahon, D. N. Moothoo, J. H. Naismith and E. J. Toone, *J. Am. Chem. Soc.*, 1999, **121**, 10286–10296.
35 J. J. Lundquist and E. J. Toone, *Chem. Rev.*, 2002, **102**, 555–578.
36 S. K. Choi, *Synthetic Multivalent Molecules: Concepts and Biomedical Applications*, Wiley-Interscience, New York, 2004.
37 R. Kalvoda and M. Kopanica, *Pure Appl. Chem.*, 1989, **61**, 97–112.
38 S. Ernst and J. Heitbaum, *J. Electroanal. Chem.*, 1979, **100**, 173–183.
39 Y. B. Vassilyev, O. A. Khazova and N. N. Nikolaeva, *J. Electroanal. Chem.*, 1985, **196**, 105–125.
40 S. Park, H. Boo and T. D. Chung, *Anal. Chim. Acta*, 2006, **556**, 46–57.
41 M. I. Manzanares, V. Solis and R. H. de Rossi, *J. Electroanal. Chem.*, 1997, **430**, 163–168.
42 J. Rodriguez Flores, R. O'Kennedy and M. R. Smyth, *Analyst*, 1988, **113**, 525–526.
43 R. P. Deo and J. Wang, *Electrochem. Commun.*, 2004, **6**, 284–287.
44 M. A. Habib and J. O'M. Bockris, *J. Electrochem. Soc.*, 1985, **132**, 108–114.
45 V. B. Paulissen and C. Korzeniewski, *J. Electroanal. Chem.*, 1990, **290**, 181–189.
46 F. C. Nart and T. Iwasita, *Electrochim. Acta*, 1992, **37**, 385–391.
47 A. J. Bard and L. R. Faulkner, *Electrochemical Methods. Fundamentals and Applications*, John Wiley & Sons, New York, 2nd edn, 2001, pp. 286–293.
48 G. Scatchard, *Ann. N. Y. Acad. Sci.*, 1949, **51**, 660–672.
49 I. M. Klotz and D. L. Hunston, *Biochemistry*, 1971, **10**, 3065–3069.
50 H. G. Weder, J. Schildknecht, R. A. Lutz and P. Kessenlring, *Eur. J. Biochem.*, 1974, **42**, 475–481.
51 K. Zierler, *Trends Biochem. Sci.*, 1989, **14**, 314–317.
52 M. T. Carter, M. Rodriguez and A. J. Bard, *J. Am. Chem. Soc.*, 1989, **111**, 8901–8911.
53 M. Aslanoglu, C. J. Isaac, A. Houlton and B. R. Horrocks, *Analyst*, 2000, **125**, 1791–1798.
54 M. S. Ibrahim, *Anal. Chim. Acta*, 2001, **443**, 63–72.
55 M. S. Ibrahim, M. M. Kamal and Y. M. Temerk, *Anal. Bioanal. Chem.*, 2003, **375**, 1024–1030.