# Binuclear $Cu^{2+}$ complex mediated discrimination between L-glutamate and L-aspartate in water†

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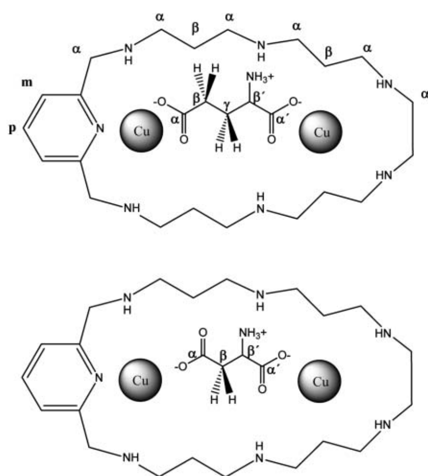
**L-Glutamate and L-aspartate selectivity is achieved by the action of two $Cu^{2+}$ metal ions rightly disposed in a cyclophane-type macrocyclic framework; electrochemical sensing of glutamate has been achieved by adsorption of the copper complexes on graphite electrodes.**

L-Glutamate–L-aspartate discrimination is a key feature of presynaptic receptors.[1] Therefore, over the last years a great deal of research effort has been devoted to individuate the recognition features that operate in aminergic receptors.[2] However, recognition of zwitterionic species in water is still a challenge in host–guest chemistry.[3] In this communication we offer means by which L-glutamate and L-aspartate discrimination can be achieved by the action of metal ions rightly disposed in a macrocyclic framework.

The receptor we deal with is the cyclophane 2,6,10,13,17,21-hexaaza[22]-(2,6)-pyridinophane (**L**) (Scheme 1). **L** can take up six protons in the pH range 2–11, 3.8 being its average protonation degree at pH 7.4.[4] This characteristic facilitates its interaction with oppositely charged species. Additionally, the amino and ammonium groups that **L** has at this pH could contribute to the binding of anions as hydrogen bond donors and/or acceptors while the pyridine aromatic moiety might provide π–ammonium interactions.[5]



**Scheme 1** Discrimination of L-glutamate over L-aspartate by addition of two equivalents of Cu(II) per mole of ligand.

† Electronic supplementary information (ESI) available: tables of NMR data and stability constants, $^1$H NMR and mass spectra and SQWVs. See http://www.rsc.org/suppdata/cc/b5/b503417e/
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A potentiometric study performed in 0.15 mol dm$^{-3}$ $NaClO_4$ shows that in the pH range 2–11, **L** forms adduct species $[H_x\mathbf{L}A]^{(x-2)}$ with L-glutamate and L-aspartate, with $x$ varying from 4 to 8. The values of the stepwise formation constants are in the range *ca.* 2–3 logarithmic units.[6] A plot of the percentage of complexed amino acid per mole of ligand does not show any significant difference between the two amino acids (see lower curves in Fig. 1). However, addition of two equivalents of $Cu^{2+}$ per mole of ligand has a dramatic effect on the selective discrimination of L-glutamate over L-aspartate. pH-metric studies show the formation of mixed metal complexes of $[Cu_2H_x\mathbf{L}A_y]^{(4+x-2y)}$, with $x$ varying from 3 to −1 and $y$ from 1 to 2, which prevail in solution above pH 4 (ref. 7 and ESI†). As shown by the percentages of complexed amino acids, the ternary complexes are much more stable in the case of L-glutamate.

The stoichiometries of the ternary complexes have been confirmed by ESI (positive mode) mass spectroscopy, with peaks detected at *m*/*z* 481.4 and 554.5, attributable to the ionic species $[Cu_2H\mathbf{L}(HGlu)(CF_3SO_3)_2]^{2+}$ and $[Cu_2\mathbf{L}(H_2Glu)_2(CF_3SO_3)_2]^{2+}$. Fig. 1 collects representations of the overall amounts of complexed amino acids in the ternary complexes, showing that the percentage of complexed L-glutamate is much higher than that of L-aspartate. The reason for such behaviour might be that the binuclear $Cu^{2+}$ complexes can lodge better L-glutamate than L-aspartate as a bridging ligand between the metal centers, forming cascade complexes.[8] In order to check these conclusions by independent techniques we have performed paramagnetic $^1$H NMR measurements[9–12] and electrochemical studies.

The $^1$H NMR spectrum of the system $Cu^{2+}$–**L** in a 2:1 molar ratio recorded in $D_2O$ at pH = 6.5 shows, in the downfield region, two well resolved hyperfine shifted signals [(a), (b)] and another two [(c), (d)] shifted upfield.

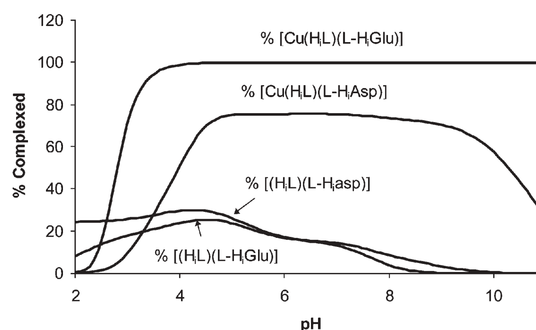


**Fig. 1** Plot of the percentages of complexed glutamate and aspartate by **L** as free receptor and as $Cu^{2+}$ complex.

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The relevant NMR features of these signals are reported in Table 1. Signals (a) and (b) show linewidths, measured at half-height, of around ∼100 Hz, while signals (c) and (d) have linewidths of 1339 Hz. The $T_1$ values are relatively short, varying from <1 ms, in the case of signals (c) and (d), to 4.3 ms for signal (b). These results are consistent with the presence of a spin-coupled binuclear $Cu^{2+}$ complex.[11,13] The signals (c) (−3.2 ppm) and (d) (−9.8 ppm), which integrate for twenty-four protons, were assigned on the basis of the very short $T_1$ and $T_2$ values to the α-$CH_2$ protons closest to the dicopper site (see Scheme 1). As described in a previous paper,[12] α-$CH_2$ protons in spin-coupled $Cu^{2+}$ complexes have similar broad linewidths and very short $T_1$ values. Signal (a), which integrates for eight protons and exhibits a short $T_1$ value (2.1 ms), can be assigned to the β-$CH_2$ protons of the macrocyclic ligand. The other signal, (b), was assigned by exclusion to the protons of the pyridine ring.

When the spectrum of the ternary system $Cu_2$**L**–Glu is recorded in $D_2O$, a new group of signals [(b′) and (d′)] appears (Table 1 and ESI†). These new signals have relatively short $T_1$ values (2.2 and 4.5 ms) and can be assigned to the β- and γ-$CH_2$ methylene groups of the complexed amino acid (see Table 1 and Scheme 1). On the other hand, the integration of the signals in experiments performed with different $Cu_2$**L**:Glu molar ratios show the formation of complexes of 1:1 and 1:2 stoichiometries in agreement with the potentiometric studies .

The $^1$H NMR spectrum of the $Cu_2$**L**–Asp system at pH 6.5 shows a similar pattern of chemical shifts (Table 1). Nevertheless, the integration of signals [(c″) and (d″)] that correspond to the coordinated amino acid is less than in the case of Glu, suggesting that the amount of ternary complexes formed is smaller. Furthermore, variable temperature $^1$H NMR spectra of $Cu_2$**L**, $Cu_2$**L**–Glu and $Cu_2$**L**–Asp, registered from 283 to 323 K, show that all isotropically shifted signals are temperature dependent and follow an anti-Curie behavior typical of antiferromagnetically coupled systems[11] except the signals of the α-$CH_2$ protons of the macrocyclic ligand, which show a Curie behaviour (see Table 1).

In order to check whether the amino acids bind through the amino group and one of the carboxylate groups or through the two carboxylate groups (ligation mode Cu–O–N–Cu or Cu–O–O–Cu), we have recorded the spectrum of the system $Cu_2$**L**–Glu in non-deuterated water. In this case, two new signals at 2.9 and 3.1 ppm appear, indicating the presence of two exchangeable NH protons, which supports the Cu–O–O–Cu ligation mode. Interestingly enough, preliminary studies with the α,ω-diacids succinic and glutaric having, respectively, the same separation between the carboxylate functions as aspartic and glutamic amino acids, yield a similar behaviour, supporting the Cu–O–O–Cu binding mode.

The possibility of selectively sensing Asp and Glu was investigated using adsorbates and monolayer deposits of the 2:1 $Cu^{2+}$:**L** complexes over glassy carbon electrodes. Adsorbate-modified electrodes were prepared, following literature procedures,[14] by immersion of the bare carbon electrode into aqueous solutions of $CuSO_4 \cdot 5H_2O$ plus **L**·6HBr in a 2:1 ratio at different pH values. Monolayer electrodes were prepared by evaporation of a drop of that solution over the surface of the carbon electrode. Preparation and electrochemical response of self-assembled adsorbates and monolayers have been extensively studied.[15–17] Upon immersion into 0.15 mol $dm^{-3}$ $NaClO_4$ solutions in the pH range 5.5–8.5, the response was similar for both adsorbate-modified and monolayer-modified electrodes, consisting of a well-defined reduction peak at −0.40 V *vs.* AgCl (3 mol $dm^{-3}$ NaCl)/Ag, as shown in Fig. 2a.

The electrochemical response of the modified electrodes was almost unchanged in solutions of aspartic acid (0.2–2.0 mmol $dm^{-3}$); the reduction peak being slightly shifted (20–30 mV) toward more negative potentials and followed by a weak shoulder near to −0.60 V (Fig. 2b). In contrast, their response showed significant changes in the presence of Glu. As can be seen in Fig. 2c, a prominent reduction peak at −0.25 V appears, preceding the reduction peak at −0.40 V. This electrochemistry can be described in terms of the reduction of surface-confined binary $Cu_2$**L** complexes (peak *ca.* −0.40 V) and that of ternary $Cu_2$**L**–Glu or $Cu_2$**L**–Asp ones. An analogous response is observed in ternary $Cu_2$**L**–Glu aqueous solutions. In agreement with potentiometric data, glutamic acid forms much more stable ternary complexes than aspartic acid. Accordingly, the reduction process of surface-confined $Cu_2$**L**–Glu ternary complexes at −0.25 V appears conjointly with the reduction peak at −0.40 V corresponding to binary $Cu_2$**L** complexes. Comparable results were obtained upon immersion of monolayer electrodes into solutions of succinic and glutaric acids.

Again, electrodes modified with 2:1 $Cu^{2+}$:**L** solutions showed significant differences for succinic (Suc) and glutaric (Glr) acids at neutral pH. Here, two well-defined peaks at −0.06 and −0.47 V were obtained upon immersion of monolayer electrodes in solutions of succinic acid (ESI†).

This voltammetric response can be described in terms of the sequential reduction of the surface-confined parent $Cu^{2+}$ complex to $Cu^+$ and Cu metal. In contact with glutaric acid solutions, however, both signals become resolved into separated peaks at −0.08 and −0.12 V, and −0.52 and −0.72 V, respectively, (ESI†).

**Table 1** 400 MHz $^1$H NMR hyperfine-shifted resonances in $D_2O$ at 40 °C and pH = 6.5 for $Cu_2$**L** and for $Cu_2$**L**–Glu and $Cu_2$**L**–Asp complexes, determined for 1:2 molar ratios

| System | Signal | $\delta$ (ppm) | No. protons | Assignment | Temperature dependence | $T_1$ (ms) | $\Delta\nu_{1/2}$ (Hz) | $T_2$ (ms)[a] |
|---|---|---|---|---|---|---|---|---|
| $Cu_2$**L** | a | 9.0 | 8 | β-$CH_2$ | Anti-Curie | 2.1 | 200 | 1.6 |
| | b | 3.1 | 3 | $H_{m,p}$ (Py) | Anti-Curie | 4.3 | 81 | 3.9 |
| | c | −3.2 | 24 | α-$CH_2$ | Curie | <1 | 1339 | 0.24 |
| | d | −9.8 | | | Curie | <1 | 1339 | 0.24 |
| $Cu_2$**L**–Glu | b′ | 5.3[b] | ∼3 × 2 | ββ′-$CH_2$ (Glu) | — | 2.2 | —[c] | —[c] |
| | d′ | 2.5 | ∼2 × 2 | γ-$CH_2$ (Glu) | Anti-Curie | 4.5 | 144 | 2.2 |
| $Cu_2$**L**–Asp | c″ | 3.1 | ∼3 × 2 | ββ′-$CH_2$ (Asp) | Anti-Curie | 3.3 | 192 | 1.7 |
| | d″ | 2.0 | | | Anti-Curie | 2.9 | —[c] | —[c] |

[a] Measured from the line width at half-height. [b] Measured at 288 K. [c] Overlap prevents measurement of this value.

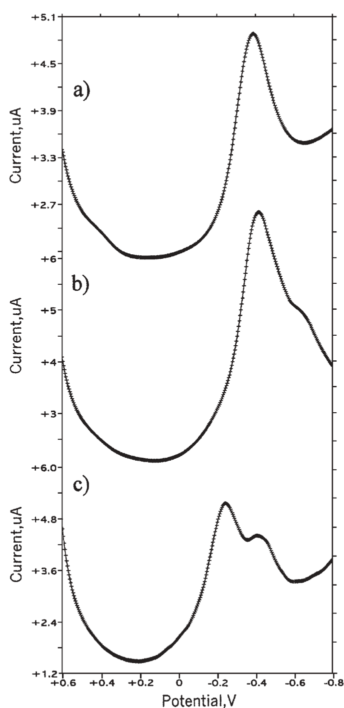


**Fig. 2** SQWVs† at a GCE modified by a deposit obtained *via* evaporation of 50 μL of a $2.0 \times 10^{-3}$ mol dm$^{-3}$ $CuSO_4 \cdot 5H_2O$ plus $1.0 \times 10^{-3}$ mol dm$^{-3}$ **L**·6HBr solution at pH 6.5 immersed into a) 0.15 mol dm$^{-3}$ $NaClO_4$; b) *idem* plus $2.0 \times 10^{-3}$ mol dm$^{-3}$ aspartic acid; c) *idem* plus $2.0 \times 10^{-3}$ mol dm$^{-3}$ glutamic acid, all at pH 7.5. Potential step increment 4 mV; square wave amplitude 25 mV; frequency 5 Hz.

In agreement with the foregoing set of considerations, peak splitting can be attributed to the reduction of binary $Cu_2\mathbf{L}$ and ternary $Cu_2$:**L**–Glr surface-confined complexes.

In conclusion, the systems here presented provide means for discriminating between glutamic and aspartic amino acids by means of their complexation as bridging M–O–O–M ligands to a pyridinophane binuclear $Cu^{2+}$ complex with unsaturated coordination sites and matching dimensions. Moreover, the alteration of the voltammetric response of the $Cu^{2+}$ complex in the presence of glutamic acid permits its electrochemical sensing. Similar arguments can be applied for distinguishing between glutaric and succinic acids.

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6 Potentiometric measurements were carried out at 298.1 K in 0.15 mol dm$^{-3}$ $NaClO_4$. The program HYPERQUAD (P. Gans, A. Sabatini and A. Vacca, *Talanta*, 1996, **43**, 1739) was used to compute the equilibrium constants. Protonation constants for L-glutamate($A^{2-}$): $A^{2-} + H^+ \rightleftharpoons HA^-$, log K = 9.47; $HA^- + H^+ \rightleftharpoons H_2A$, log $K$ = 4.2; $H_2A + H^+ \rightleftharpoons H_3A^+$, log $K$ = 2.32; protonation constants for L-aspartate($A^{2-}$): $A^{2-} + H^+ \rightleftharpoons HA^-$, log $K$ = 9.65; $HA^- + H^+ \rightleftharpoons H_2A$, log $K$ = 3.82; $H_2A + H^+ \rightleftharpoons H_3A^+$, log $K$ = 2.0. System L-glutamate($A^{2-}$)–**L**: $H_3\mathbf{L}^{3+} + HA^- \rightleftharpoons H_4A\mathbf{L}^{2+}$, log $K$ = 1.89(4); $H_4\mathbf{L}^{4+} + AH^- \rightleftharpoons H_5A\mathbf{L}^{3+}$, log $K$ = 2.27(3); $H_5\mathbf{L}^{5+} + AH^- \rightleftharpoons H_6A\mathbf{L}^{4+}$, log $K$ = 2.26(2); $H_6\mathbf{L}^{6+} + AH^- \rightleftharpoons H_7A\mathbf{L}^{5+}$, log $K$ = 2.82(2); $H_6\mathbf{L}^{6+} + H_2A \rightleftharpoons H_8A\mathbf{L}^{6+}$, log $K$ = 2.44(3). System L-aspartate($A^{2-}$)–**L**: $H_4\mathbf{L}^{4+} + AH^- \rightleftharpoons H_5A\mathbf{L}^{3+}$, log $K$ = 2.27(2); $H_5\mathbf{L}^{5+} + AH^- \rightleftharpoons H_6A\mathbf{L}^{4+}$, log $K$ = 2.29(2); $H_6\mathbf{L}^{6+} + AH^- \rightleftharpoons H_7A\mathbf{L}^{5+}$, log $K$ = 2.91(2); $H_6\mathbf{L}^{6+} + H_2A \rightleftharpoons H_8A\mathbf{L}^{6+}$, log $K$ = 2.58(2).

7 System $Cu^{2+}$–L-glutamate($A^{2-}$)–**L**: $M\mathbf{L}^{2+} + A^{2-} \rightleftharpoons M\mathbf{L}A$, log $K$ = 8.92(7), $M\mathbf{L}H^{3+} + A^{2-} \rightleftharpoons M\mathbf{L}AH^+$, log $K$ = 9.14 (7); $M\mathbf{L}H^{3+} + AH^- \rightleftharpoons M\mathbf{L}AH_2^{2+}$, log $K$ = 8.73(6); $M\mathbf{L}H_2^{4+} + AH^- \rightleftharpoons M\mathbf{L}AH_3^{3+}$, log $K$ = 7.84(4); $M\mathbf{L}H_3^{5+} + AH^- \rightleftharpoons M\mathbf{L}AH_4^{4+}$, log $K$ = 7.56(3), $M\mathbf{L}H_4^{6+} + AH^- \rightleftharpoons M\mathbf{L}AH_5^{5+}$, log $K$ = 5.94(6); $M\mathbf{L}H_3^{5+} + H_2A \rightleftharpoons M\mathbf{L}AH_5^{5+}$, log $K$ = 6.44(6); $M_2\mathbf{L}^{4+} + AH^- \rightleftharpoons M_2\mathbf{L}AH^{3+}$, log $K$ = 8.41(5); $M_2\mathbf{L}H^{5+} + AH^- \rightleftharpoons M_2\mathbf{L}AH_2^{4+}$, log $K$ = 9.04(5); $M_2\mathbf{L}H^{5+} + H_2A \rightleftharpoons M_2\mathbf{L}AH_3^{5+}$, log $K$ = 8.09(2); $M_2\mathbf{L}^{4+} + 2A^{4-} \rightleftharpoons M_2\mathbf{L}A_2$, log $K$ = 14.04(2); $M_2\mathbf{L}AH^{3+} + A^{2-} \rightleftharpoons M_2\mathbf{L}A_2H^+$, log $K$ = 5.77(7); $M_2\mathbf{L}A_2 + H_2O \rightleftharpoons M_2\mathbf{L}A_2(OH)^- + H^+$, log $K$ = 10.44(1); $M_2\mathbf{L}(OH)^{3+} + 2A^{4-} \rightleftharpoons M_2\mathbf{L}A_2(OH)^-$, log $K$ = 11.37 (9). System $Cu^{2+}$–L-aspartate($A^{2-}$)–**L**: $M\mathbf{L}H_2^{4+} + AH^- \rightleftharpoons M\mathbf{L}AH_3^{3+}$, log $K$ = 2.50(1); $M\mathbf{L}H_3^{5+} + AH^- \rightleftharpoons M\mathbf{L}AH_4^{4+}$, log $K$ = 3.54(1); $M\mathbf{L}H_4^{6+} + AH^- \rightleftharpoons M\mathbf{L}AH_5^{5+}$, log $K$ = 3.43(8), $M\mathbf{L}H_4^{6+} + H_2A \rightleftharpoons M\mathbf{L}AH_6^{6+}$, log $K$ = 3.06(2), $M_2\mathbf{L}^{4+} + AH^- \rightleftharpoons M_2\mathbf{L}AH^{3+}$, log $K$ = 4.15(8); $M_2\mathbf{L}H^{5+} + AH^- \rightleftharpoons M_2\mathbf{L}AH_2^{4+}$, log $K$ = 5.09(7); $M_2\mathbf{L}H^{5+} + AH_2 \rightleftharpoons M_2\mathbf{L}AH_3^{5+}$, log $K$ = 5.30(2); $M_2\mathbf{L}^{4+} + 2A^{4-} \rightleftharpoons M_2\mathbf{L}A_2$, log $K$ = 8.88(1); $M_2\mathbf{L}AH^{3+} + A^{2-} \rightleftharpoons M_2\mathbf{L}A_2H^+$, log $K$ = 5.04(9).


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