

Ni(II) binding to the 429–460 peptide fragment from human Toll like receptor (hTLR4): a crucial role for nickel-induced contact allergy?[†]

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The FQH₄₃₁SNLKQMSEFSVFLSLRNLIYLDISH₄₅₆TH₄₅₈TR fragment, containing three histidine residues, the conserved H₄₃₁ and the non-conserved H₄₅₆ and H₄₅₈, located from 429 to 460 amino acid residues in the C-terminal portion of human Toll-like-receptor 4 (hTLR4), which is directly activated by nickel, a well known contact allergen, has been tested for Ni(II) binding. The complex formation capability of the 32-amino acid sequence with Ni(II) ions has been followed by potentiometric, UV-Vis, CD, MS and NMR measurements. Ni(II) is able to bind to all three histidines by forming macrocycle complexes at low and physiological pH. From pH 9 on, a 4N diamagnetic species (N_{im}, 3N_{am}[−]) with the participation of an imidazole nitrogen and three deprotonated nitrogens from His₂₈, Ser₂₇ and Ile₂₆ amides from the backbone of the model peptide has been determined. From the NMR results it was possible to determine that His₂₈, which mimics the H₄₅₆ residue in the protein, together with the environment around it, was mainly involved in the binding.

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Introduction

Contact allergy, commonly induced by nickel, is the most frequent cause of contact hypersensitivity in industrialized countries, with 30% of the population being affected.^{1–6}

Recently, it has been reported⁷ that Ni(II) triggered an inflammatory response by directly activating human Toll-like-receptor 4 (hTLR4). The TLR family is one of the best known classes of proteins which senses microbial pathogens and endogenous ligands. Specific activation required distinct sequence motifs that are present in humans but not in mice.

The authors identified the specific region of human TLR4 responsible for nickel responses: the sequence containing three histidine residues, the conserved H₄₃₁, and the non-conserved H₄₅₆ and H₄₅₈, localized in the C-terminus (Fig. 1).

From studies with mutant TLR4 proteins, it seems that the non-conserved sequence motifs in humans were probably required for the recognition of Ni(II) signals by binding it and thus causing inflammation.

As a matter of fact, double mutations of H₄₅₆ and H₄₅₈ decreased the Ni(II) response whereas single substitution had a mild or no effect.

In fact, it has been found that Ni-induced activation is species-specific: nickel activates human TLR4 but not that of mice, which does not contain only those two residues in its sequence indicating that the differences between the Ni(II) responses of mice and humans might depend on sequence variation in TLR4.

In particular, from the structural modelling of putative metal binding sites, it has been proposed that the imidazole side chains of H₄₅₆ and H₄₅₈ histidine residues provide a potential binding site for nickel because they were located at a distance that is optimal to interact with Ni(II) ions, whereas that of H₄₃₁ was further apart.

These results clearly identify nickel as an inorganic activator of the TLR system and they are the first to show direct

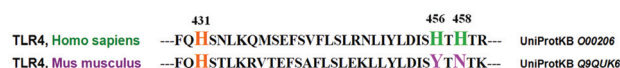


Fig. 1 Sequence comparison of the homologous TLR4 regions in human and mouse species encompassing the Ni(II) responsive sites H₄₃₁, H₄₅₆, and H₄₅₈ of human TLR4 (hTLR4). Conserved histidine residues (H) are orange-coloured; non-conserved histidines green-coloured. The latter, in hTLR4, contains a potential binding site for Ni(II). Reference protein sequences were provided by UniProt Knowledgebase (<http://www.uniprot.org>).

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triggering of pathogen recognition receptors by contact allergens.^{1,2,7}

Following our interest in the study of metal ions binding to relevant fragments in proteins, with particular interest in nickel induced carcinogenesis,^{8–19} we have decided to verify the possibility of metal binding to the 32-FQH₃SNLKQMSEFSVFLSLRNLIYLDISH₂₈TH₃₀TR amino acid peptide as a model of the specific sequence in the protein containing the three histidine residues: the conserved H₄₃₁ (H₃ in the sequence of the peptide model), and the non-conserved H₄₅₆ and H₄₅₈ (H₂₈ and H₃₀, respectively), located from 429 to 460 amino acid residues in the C-terminal portion of human TLR4 protein.

The aim of our work was to support the hypothesis concerning the role of the three histidines in the molecular mechanism of nickel induced contact dermatitis by studying the binding properties of the 32-amino acid minimal model peptide.

Using short protein fragments could provide an easier system to be studied in order to gain structural and chemical information on the metal ion binding properties of the corresponding protein.²⁰

In the present paper, formation equilibria of Ni(II) complexes with the protected peptide, where both ends were blocked by acetylation and amidation to make the fragment a more relevant model of the entire protein, have been investigated.

The study has been carried out in aqueous solution and in a wide pH range, at $I = 0.1$ M and $T = 298$ K. Protonation and complex formation constants have been potentiometrically determined; complex formation models and species stoichiometry have been carefully checked by means of MS, UV-Vis absorption and CD spectroscopy.

The effects of peptide titration with Ni(II) ions have been followed by means of mono- and bi-dimensional NMR spectroscopy in order to support the potentiometric results and to gather more details about the metal binding sites, over a wide pH range and at different ligand to metal molar ratios.

Experimental

Peptide synthesis

Peptides were chemically synthesized using solid-phase Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry in an Applied Biosystems Synthesizer.²¹ Peptides were N-terminally acetylated and C-terminally amidated in order to mimic this region of hTLR4 within the full-length protein. Peptides were removed from the resin and deprotected before purification by reverse-phase HPLC. Fractions were collected and analyzed by MALDI-TOF MS. Fractions containing the peptide of the expected molecular weight were then pooled and lyophilized.

UV-Vis and CD measurements

The absorption spectra were recorded in the 230–900 nm range on a Jasco J715 spectropolarimeter (CD) and on a Cary

300 Bio spectrophotometer (UV-Vis); solutions were of similar concentrations to those used in the potentiometric studies; the ligand concentration was 0.5×10^{-3} mol dm⁻³ and the tested Ni(II):ligand molar ratios were 1:1 and 1:2. Absorptivities (ϵ , M⁻¹ cm⁻¹) were calculated at the pH value of maximum concentration of the considered species, as indicated by the potentiometric distribution diagrams. All the used solutions in this study were deaerated.

NMR measurements

NMR experiments were performed using a Bruker Ascend™ 400 MHz spectrometer equipped with a 5 mm automated tuning and a matching broad band probe (BBFO) with z-gradients.

Samples used for NMR experiments were in the range 0.4–2.5 mM and dissolved in (90/10 (v/v) H₂O–D₂O or in D₂O–DMSO-d₆ (dimethyl sulfoxide-d₆) or H₂O–MeOD-d₄ (methanol-d₄) solutions. All NMR experiments were performed at 298 K in 5 mm NMR tubes. 2-D ¹H–¹³C heteronuclear correlation spectra (HSQC) were acquired using a phase-sensitive sequence employing Echo-Antiecho-TPPI gradient selection with a heteronuclear coupling constant $J_{\text{XH}} = 145$ Hz, and shaped pulses for all 180° pulses on the f2 channel with decoupling during acquisition; sensitivity improvement and gradients in back-sweep were also used.^{22–24}

Relaxation delays of 2 s and 90° pulses of about 10 μs were applied for all experiments. Solvent suppression for 1-D and TOCSY experiments was achieved using excitation sculpting with gradients. The spin-lock mixing time of the TOCSY experiment was obtained with MLEV17.²⁵

¹H–¹H TOCSYs were performed using mixing times of 60 ms. ¹H–¹H ROESY spectra were acquired with spin-lock pulses duration in the range 200–250 ms.²⁶

The assignments of the peptide resonances were made by a combination of mono- and bi-dimensional and multinuclear NMR techniques ¹H–¹H TOCSY, ¹H–¹³C HSQC and ¹H–¹H ROESY at different pH values.

All NMR data were processed using the TopSpin (Bruker Instruments) software and analyzed using Sparky 3.11²⁷ and MestRe Nova 6.0.2 (Mestrelab Research S.L.) programs.

Potentiometric measurements

All the potentiometric data were calculated from four titration experiments carried out over the pH range 2.30–11.50 at 298 K in 0.1 M KCl using a total volume of 1.5 mL on a MOLSPIN pH-meter system and a RusselCMAW711 semicombined electrode calibrated in proton concentrations by using HCl. All potentiometric measurements were performed under an argon atmosphere. The purities and exact concentrations of the ligand solutions were determined by the Gran method.²⁸ NaOH was added from a 250 μL micrometer syringe, which was calibrated by both weight titration and standard materials titration. The ligand concentration was 0.5 mM, the Ni(II) to ligand molar ratios were 1:1 and 1:2. Because the complexation of Ni(II) ions with oligopeptide is a very slow process in the amide deprotonation pH range, during potentiometric

experiments long delay time (40–50 s), very small drops of injected base solution (1.3 μL) and small max. drift in delay time (0.03 mV) were chosen. The HYPERQUAD 2008 and SUPERQUAD programs were used to calculate the stability constants.²⁹ Standard deviations were computed using HYPERQUAD 2008 and they refer to random errors only.

Mass spectrometric measurements

High-resolution mass spectra were obtained on a BrukerQ-FTMS spectrometer (Bruker Daltonik, Bremen, Germany), equipped with an Apollo II electrospray ionization source and an ion funnel. The mass spectrometer was operated in the positive ion mode. The instrumental parameters were as follows: scan range m/z 400–4000, dry gas nitrogen, temperature 170 $^{\circ}\text{C}$, ion energy 5 eV. Capillary voltage was optimized to the highest S/N ratio, and it was 4500 V. The small changes of voltage (500 V) did not significantly affect the optimized spectra. The sample (Ni(II) :ligand in a 1:2 stoichiometry, [ligand] = 0.1 mM) was prepared in a 1:1 MeOH–H₂O mixture. Variation of the solvent composition down to 5% of MeOH did not change the species composition. The sample was infused at a flow rate of 3 $\mu\text{L min}^{-1}$. The instrument was calibrated externally with the Tunemix mixture (Bruker Daltonik, Germany) in a quadratic regression mode. Data were processed using the Bruker Compass DataAnalysis 4.0 program. The mass accuracy for the calibration was better than 5 ppm, enabling, together with the true isotopic pattern (using SigmaFit), an unambiguous confirmation of the elemental composition of the obtained complexes.

Results and discussion

Mass spectrometry

Molecular mass of the TLR4 peptide is 3904.47 Da ($\text{C}_{176}\text{H}_{272}\text{N}_{50}\text{O}_{49}\text{S}_1$). Upon the addition of Ni(II) ions to the peptide, only the formation of equimolar species can be observed. The highest intensity of the peptide itself and of its Ni(II) complex was recorded for a +4 charged species, with the m/z ratios 997.27 and 991.25, respectively (Fig. 2). The isotopic distribution of Ni(II) complex is in perfect agreement with the simulated one (Fig. 2, inset).

Protonation equilibria and NMR measurement

The 32-Ac-FQHSNLKQMSEFSVFLSLRNLIYLDISHTHTR-NH₂ amino acid peptide can be considered a H₉L ligand which has nine protonation constants, seven of which we were able to detect by potentiometric measurements.

The first two pK_a values (2.88 and 3.80) correspond to the deprotonation of the carboxylic group of Asp and Glu residues, respectively. The next three pK_a values (4.68, 5.47, and 6.34) arise from the deprotonation of the three imidazole groups of His residues, and the following two (8.89 and 10.45) are the result of the deprotonation of Tyr and Lys side chains. We were unable to detect the pK_a values of the two Arg residues.

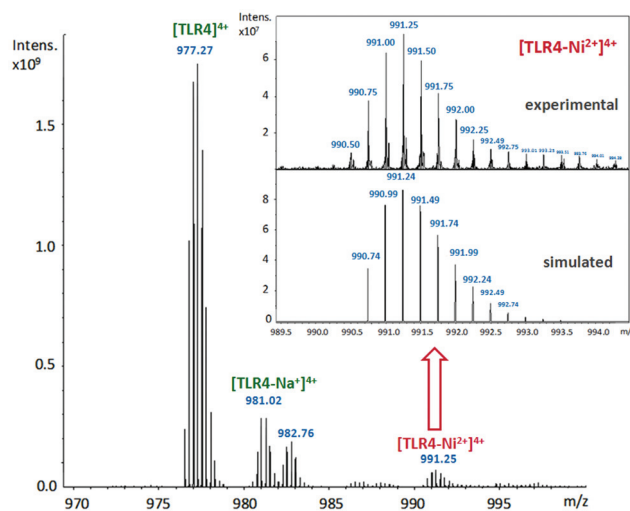


Fig. 2 Q-FTMS spectra of TLR4 32aa peptide– Ni(II) system. Measurements were performed in H₂O–acetonitrile 1:1 solution, pH 7.4. Inset: MS isotopic distribution spectra of a TLR4 32aa peptide– Ni(II) complex together with its simulation.

Table 1 Protonation constants at 298 K and $I = 0.10 \text{ mol dm}^{-3}$

Ligand species	Log β	Log K	Residue
LH	10.45 (3)	10.45	Lys
LH ₂	19.34 (4)	8.89	Tyr
LH ₃	25.68 (1)	6.34	His
LH ₄	31.15 (1)	5.47	His
LH ₅	35.83 (6)	4.68	His
LH ₆	39.63 (2)	3.80	Glu
LH ₇	42.51 (2)	2.88	Asp

Table 2 Complex-formation constants and spectrophotometric data for Ni(II) complexes, at 298 K and $I = 0.1 \text{ mol dm}^{-3}$

Ni(II) species	Log β	Log K
NiH_4L	37.62 (1)	
NiH_3L	33.12 (1)	4.50
NiH_2L	28.22 (4)	4.90
NiHL	19.66 (1)	8.56
NiL	10.89 (1)	8.77
NiLH_{-1}	1.53 (7)	9.36
NiLH_{-2}	−8.97 (3)	10.50

The thermodynamic parameters of ligand protonation are reported in Table 1: they are in good agreement with the literature values reported for similar systems.^{8–11,30}

The Ac-FQHSNLKQMSEFSVFLSLRNLIYLDISHTHTR-NH₂ peptide forms equimolar complexes with nickel at the studied 1:1 and 1:2 metal to ligand molar ratios. The complete set of complex-formation constants is reported in Table 2 and the corresponding distribution diagrams are shown in Fig. 3. UV-Vis and CD spectra obtained in the whole range of pH are reported in Fig. 4 and 5, respectively.

The formation of Ni(II) complexes starts before pH 3; in the first species, $[\text{NiH}_4\text{L}]$, maximum formation at pH 4, the metal

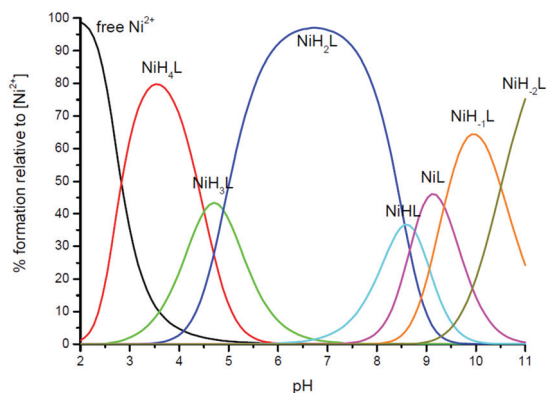


Fig. 3 Representative distribution diagrams. $[\text{Ni(II)}]_{\text{tot}} = 0.5 \text{ mM}$; $\text{Ni(II)} : \text{L}$ ratio = 1 : 1.

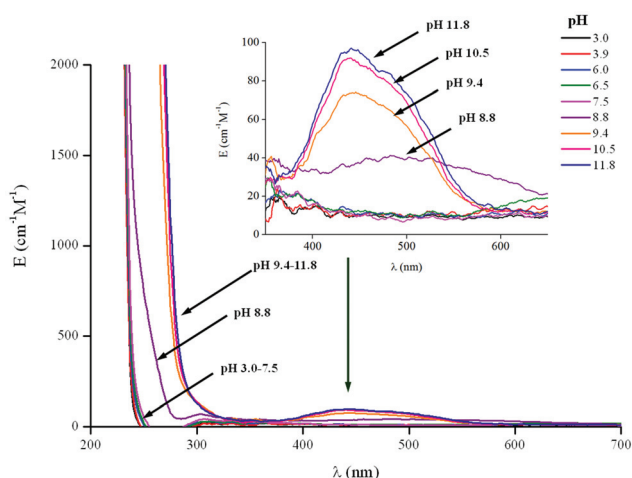


Fig. 4 UV-Vis spectra of TLR4 32aa peptide : Ni(II) system, molar ratio 1 : 1 as a function of the pH.

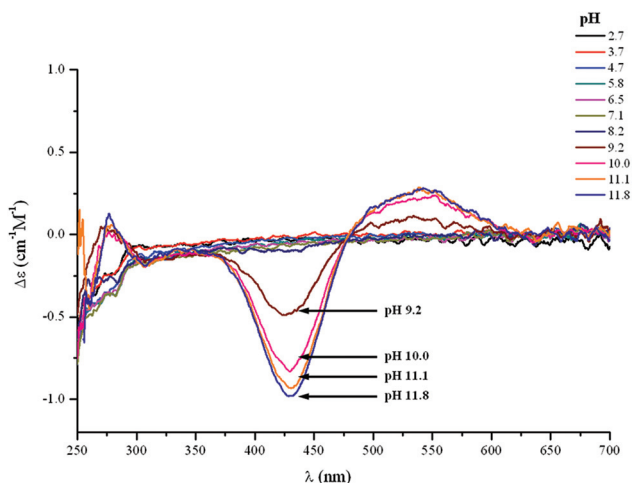


Fig. 5 CD spectra of TLR4 32aa peptide : Ni(II) system, at various pH values, molar ratio 1 : 1.1 ($C_L = 0.6 \text{ mM}$), at 298 K and $I = 0.2 \text{ M}$ (KCl).

ion is most likely linked to the nitrogen of a histidine residue. The aspartic acid residues are deprotonated but they probably do not take part in the binding.

In the next complex, $[\text{NiH}_3\text{L}]$, with maximum formation at pH 5, Ni(II) is most likely bound to two imidazole nitrogens from histidine residues. In the third observed complex, $[\text{NiH}_2\text{L}]$, the last histidine is also deprotonated and probably bound to the Ni(II) ion, as it is possible to deduce from the pK_a values (4.5 and 4.9) lower than that of the corresponding pK_a values for the free ligand (6.34, 5.47 and 4.68). $[\text{NiH}_2\text{L}]$ is the predominant species between pH 6 and 8 reaching its maximum formation (90%) at pH 7.

The absence of absorption in the UV-Vis spectra at pH 7 is in agreement with the formation of macrocycle complexes involving N donor atoms from all the three histidine residues.

No significant CD activity was measured for all the species obtained till pH 7. In fact, visible CD was not apparent until the pH was raised over 9.

The lack of appreciable d-d transition CD bands suggests minimal vicinal effects^{15,31} implying that there is no main-chain coordination at physiological pH and below, thus confirming the involvement of imidazole donor atoms in a macro-chelate system.

From potentiometric measurements, by raising the pH, two further protons were released in a rapid sequence leading to the formation of $[\text{NiHL}]$ and $[\text{NiL}]$ species, with maximum formation at pH 8.5 and 9.2, respectively. The protonation constant for $[\text{NiHL}]$, $\text{pK}_a = 8.56$, similar to that observed for the deprotonation of the Tyr residue in the free ligand (8.89), can be attributed to the deprotonation of this residue; the coordination of an oxygen donor atom from the tyrosine residue could not be excluded from the coordination to the metal.

While the multiple histidine binding mode is CD silent, relatively strong CD bands are observed for d-d transitions of tetragonal complexes involving backbone amides and histidine coordination *via* the imidazole ring.

In fact, as it is possible to see in Fig. 5, at a pH above 9 a profound change in the visible CD spectra is evidenced, suggesting a change in the coordination around the nickel atom.

In particular, a band at 275 nm, which is characteristic of $\text{N}_{\text{am}}^- \rightarrow \text{Ni(II)}$ charge transfer transition, is visible. In addition, a positive ellipticity around 525 nm which appears at longer wavelengths than that of the absorption maximum ($\lambda_{\text{max}} = 450 \text{ nm}$), together with a negative one at 430 nm which appears at shorter wavelengths than that of the absorption maximum are visible. They are characteristic of the formation of $\text{Ni(II)}\text{-}4\text{N}$ ($\text{N}_{\text{im}}, 3\text{N}_{\text{am}}^-$) low-spin planar diamagnetic species.

Starting from pH 7.5 on, the appearance of $[\text{NiL}]$ species can derive from the deprotonation of an amide nitrogen from the backbone, $\text{pK}_a = 8.77$, and is visible in the CD spectra from pH 9 on. Above this pH, two further protons are released leading to $[\text{NiH}_1\text{L}]$ and $[\text{NiH}_2\text{L}]$ species; they reach 60 and 70% of the total nickel present at pH 10 ($\text{pK}_a = 9.35$) and at pH 11 ($\text{pK}_a = 10.51$), respectively. Their formation can be attributed to two further deprotonations of amides from the backbone of the

peptide. Above pH 10 a yellow coloured solution, which is characteristic of a diamagnetic planar Ni(II) complex, was observed.

The effect of peptide titration with Ni(II) has been followed by mono- and bi-dimensional multinuclear NMR experiments in order to support the potentiometric results and to gain additional details about the metal binding sites, at different pH values and at different ligand to metal molar ratios.

Although the water solubility of the ligand was not high enough to obtain fully reliable NMR spectra, much information regarding the behaviour of the ligand towards Ni(II) ions has been obtained. In addition, to improve the solubility of the peptide and then the resolution of NMR spectra, DMSO and MeOH solutions of the peptide have also been extensively studied.

Until pH 9 paramagnetic species are mainly present and only at higher pH values does a diamagnetic species start to appear in the NMR spectra, in agreement with the potentiometric results.

In Fig. 6 the aromatic region of 1D spectra obtained below pH 7 and from 1:0.02 to 1:0.1 L:Ni(II) molar ratios in DMSO-D₂O (a) and from 1:0.16 to 1:0.4 L:Ni(II) in MeOH-H₂O at pH 7.6 (b) is reported. In both systems, through a

general overall broadening, the signals mainly affected are those from the three histidine residues; they broadened and tended to disappear by raising the nickel to peptide molar ratio, confirming the presence of paramagnetic complexed species involving all the histidine residues in a macrocycle structure.

In Fig. S1 (ESI†) the aromatic region of 1D and ¹H-¹H TOCSY NMR spectra obtained at pH = 10.5 and at 1:0 and 1:1 L:Ni(II) molar ratios in MeOH-H₂O solution is reported.

A shift involving H_{ε1} and H_{δ2} histidine signals (H_α, H_β protons, Fig. S2†) together with a shift of H_δ and H_ε tyrosine protons appears in the spectrum supporting, besides the formation of diamagnetic species, the possible involvement of the tyrosine residue in the coordination. This fact is also suggested by the loss of the degeneration of its aromatic signals whose separation can be explained through a decrease in the conformational freedom of the side chain after coordination.

By raising the pH a gradual change from the paramagnetic species bearing large relaxation effects, to the diamagnetic species in which the remarkable effects are changes in the chemical shifts instead of broadening of the signals has been observed in the NMR experiments carried out in DMSO-D₂O solution. This effect agrees well with the results obtained from potentiometric and spectroscopic (UV-Vis and CD) measurements.

Indeed, by raising the metal molar ratio it is possible to identify, together with a general weak broadening of some signals, several clear shifts regarding the histidine residues; in particular, a large shift of H_{δ2} and H_{ε1} aromatic protons supports the binding of the Ni(II) ion to the imidazole nitrogen of histidines (Fig. S3a, b†). The chemical shift changes, in the order Δδ H_{ε1} ≫ Δδ H_{δ2}, are in good agreement with those obtained for similar systems in which Ni(II) is bound in a 4N (N_{im}, 3N_{am}⁻) low-spin planar diamagnetic coordination mode with a peptide containing a histidine as the metal binding site.^{10–12,14,32–38}

In addition, the complete disappearance of H_N amide resonances from serine S₂₇ and isoleucine I₂₆ residues, that is an indication of the deprotonation of their backbone nitrogens, supports the involvement of the peptide backbone towards the N-terminal end.

Looking at the aliphatic region of the spectra, analogous information can be obtained. In particular, in addition to a diamagnetic shift of H_α signal of histidines, several changes in the electronic environment belonging to threonine T_{29/31} and serine S₂₇ residues have also been detected.

The remaining signals are mainly unaffected and, due to their overlapping, no more information can be gained from the mono-dimensional spectra.

A selection of aromatic (a) and aliphatic (b, c) regions of ¹H-¹³C HSQC spectra for the free peptide and for the Ni(II): peptide system at a 1:0.4 molar ratio, is reported in Fig. S4.†

In the aromatic region only δ₂ and ε₁C-H cross correlations of histidines are clearly affected by the addition of Ni(II) ions,

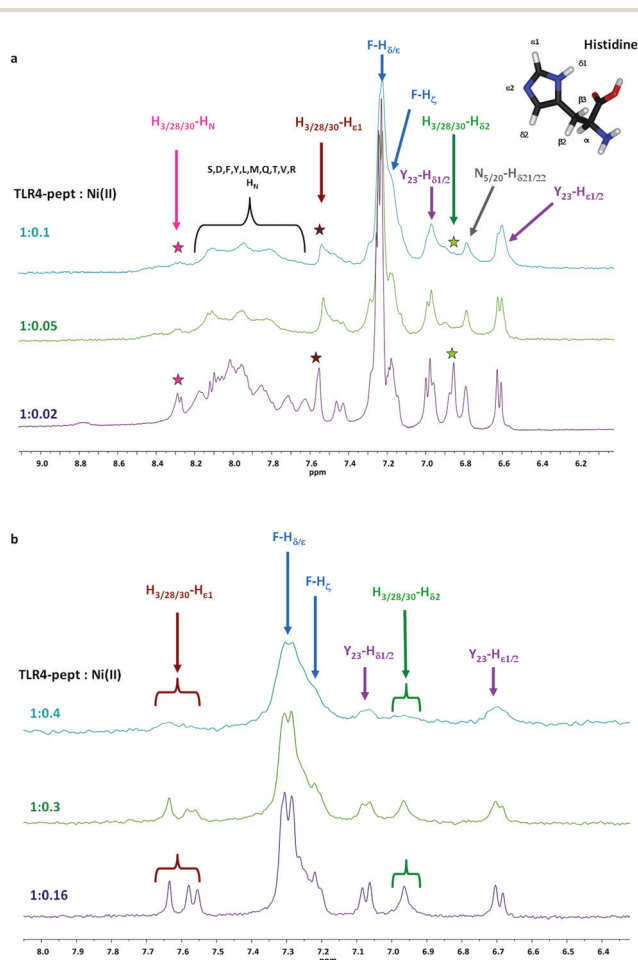


Fig. 6 1D ¹H NMR stacked spectra for the aromatic region of TLR4 32aa peptide, 0.5 mM, *T* = 298 K by increasing amounts of Ni(II) in (a) DMSO-D₂O solution at pH 7 and in (b) MeOD-H₂O at pH 7.6, respectively.

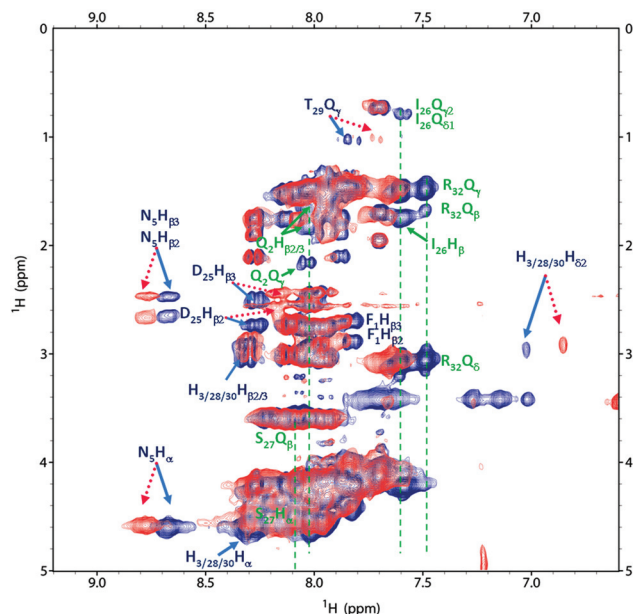


Fig. 7 Selection of aromatic region in the ^1H – ^1H TOCSY NMR spectra for TLR4 32aa peptide, 2.5 mM, $T = 298$ K DMSO– D_2O in the absence (blue) and in the presence (red) of 0.4 equivalent of $\text{Ni}(\text{II})$. Filled blue and dotted red arrows indicate the signals of the peptide in the free and in the bound status, respectively. Green labels indicate the H_N spin system that disappeared following nickel addition.

whereas the resonances of the other residues remain almost unaltered.

In the down-field region of the spectra, C-H_α and C-H_β correlations of histidine residues undergo chemical shift changes, as well as other residues, with the following order of variations: $\Delta\delta \text{C-H}_\alpha\text{-T}_{29} > \text{C-H}_\alpha \text{I}_{26} \approx \text{C-H}_\beta\text{-D}_{25} \approx \text{C-H}_\gamma \text{Q}_2 > \text{C-H}_\alpha\text{-T}_{31}$.

The information obtained from the previous NMR experiments is also confirmed by comparing 2D ^1H – ^1H TOCSY spectra for the peptide before and after the addition of the metal ion (Fig. 7): in addition to the disappearance of H_N spin system for residues close to the histidines and in particular those from serine S_{27} and isoleucine I_{26} close to histidine H_{28} , a remarkable shift of the histidine δ_2 imidazole protons ($\Delta\delta \text{H}_{\delta_2} \approx -0.18$ ppm) is clearly seen.

In addition, arginine R_{32} resonances were also shown to be affected.

Taken together, all the NMR results suggest that the histidine residue H_{28} and the closest peptide backbone towards the N-terminal end of the fragment are primarily involved in the nickel coordination at high pH.

NMR results agree also with the involvement of N_{δ_1} in the coordination towards the formation of five membered chelate rings to give, at high pH, the classical diamagnetic, $4\text{N-Ni}(\text{II})$ species, at least to one histidine site in the peptide fragment. Actually, the comparison of our experimental data with those reported in the literature indicates N_{δ_1} as the donor atom involved in the coordination process.

Finally, from the NMR data we can conclude that the formation of paramagnetic macrocycles, involving all the

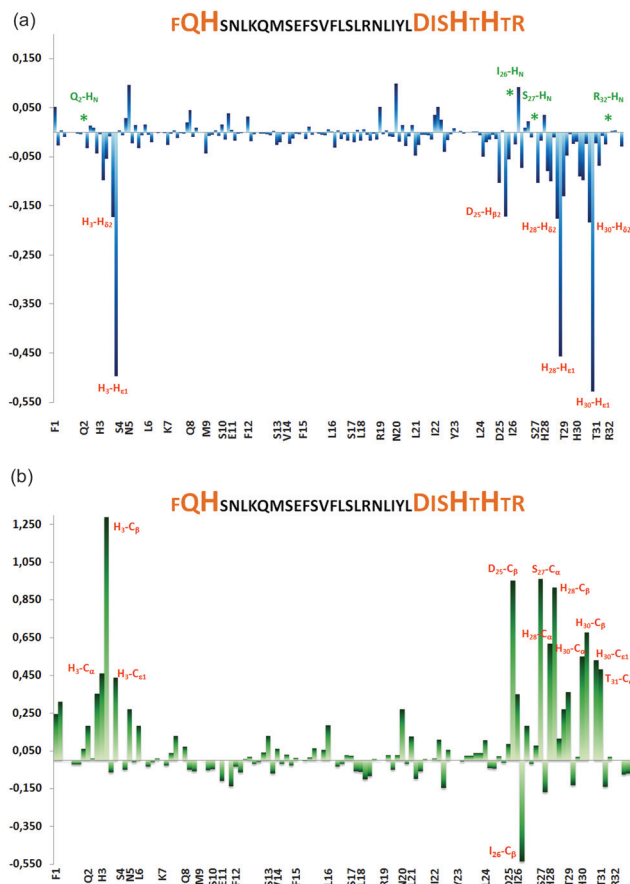


Fig. 8 Plot of the observed ^1H proton (a) and ^{13}C carbon (b) chemical shift changes (ppm) for TLR4 32aa peptide following nickel coordination. The nuclei experiencing the largest chemical-shift perturbation are labeled in orange and the disappearing peaks in green. A visual plot of the chemical shift changes along the peptide sequence gives a simple map of the zone more affected by metal interaction, with a size font of the sequence single letter code directly proportional to the degree of perturbation.

histidine residues as anchoring sites, is evidenced till pH 9; at higher pH the formation of diamagnetic planar species is predominant though in a dynamic equilibrium with some other species involving all the three histidine residues, as it is possible to note in Fig. 8 where the shifts ($\Delta\delta = \delta_{\text{holo}} - \delta_{\text{apo}}$) involving carbon and hydrogen nuclei of TLR4 peptide, induced by adding 0.4 equivalents of $\text{Ni}(\text{II})$, are shown.

It is possible that, under NMR conditions, that is, a low $\text{Ni}(\text{II})$: peptide molar ratio, not all the peptides are involved in the formation of a unique species.

Conclusions

Nickel, which is a widespread environmental and occupational pollutant, when present in Ni-containing jewellery as well as in many other objects, among others in Ni-containing cellular telephones, is known to be able to induce contact allergy.

Progress towards the understanding of the molecular mechanisms of Ni induced allergy has recently been made.

Actually, nickel is not considered to be a classical allergen but a hapten which can lead to contact allergy by binding to proteins and thus inducing the cellular responses which cause inflammation. In that regard, the TLR4 molecule has been identified as the nickel receptor that is the specific binding protein for nickel; the data prove that the unique sequence motif of human TLR4 dictates the species difference for recognition of Ni(II) ions by human and mouse cells.

We focused on the specific region of human TLR4 which is believed to be responsible of the Ni(II) response. Here, we investigated the possible involvement of TLR4 in nickel binding by studying the coordination abilities of the specific fragment by potentiometric and spectroscopic methods.

Metal complexes with peptides containing multi-histidine residues have striking coordination abilities and can mimic the structure of various multi-histidine metal binding sites in protein.

The presence of two or three imidazoles within the peptide sequence allows metal ions to form a poly-imidazole macrocycles binding mode, which dominates at physiological pH and below.

Although it was not possible to distinguish among the three different histidines, the disappearance of the H_N spin system together with the shifts evidenced for serine S_{27} and isoleucine I_{26} side chains suggest that the histidine mostly involved in the coordination, at high pH, could be H_{28} , the residue that is located in the environment required for the recognition of Ni(II) signals.

The signal disappearance is the direct result of metal binding to histidine H_{28} first, though an additional involvement of the environment around histidine H_{31} is also supported by the disappearance of some arginine R_{32} resonances.

In conclusion, on the basis of our results, though only a minimal model fragment has been studied here, Ni(II) ions are shown to bind particularly at histidine residues which are located in the region required for the recognition of nickel signals, though in a dynamic equilibrium among different species which can also involve the other histidine residues.

If these binding events can be demonstrated *in vivo*, possible strategies could be studied for interfering with nickel sensitivity.

We believe that our study could give an additional clue to the understanding of the role of metal ions binding in crucial multi-histidine proteins.

Further work is in progress in order to take into consideration the behaviour of the human TLR4-human MD2 dimer complex towards Ni(II) binding.

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