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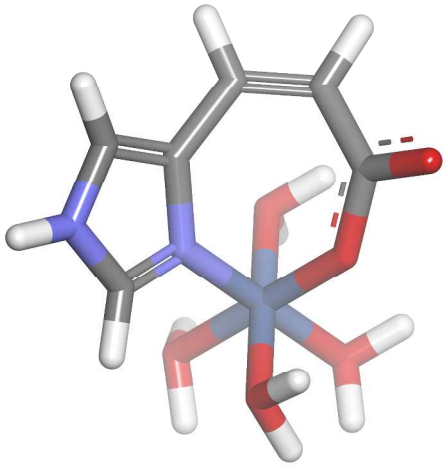

cis-Urocanic acid, a component of human skin, is an efficient ligand for allergenic Ni^{2+} ions, forming high spin complexes.
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ARTICLE TYPE

cis-Urocanic acid as a potential nickel(II) binding molecule in the human skin

Nina Ewa Wezynfeld,[a] Wojciech Goch,[a] Wojciech Bal,[a] and Tomasz Frączyk*[a]

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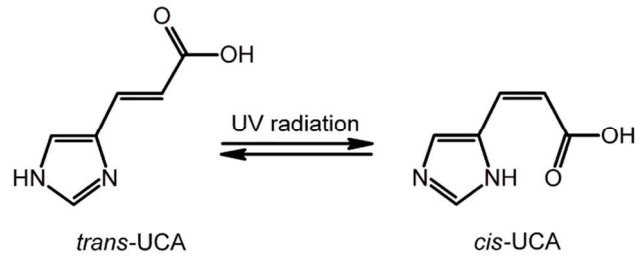
cis-Urocanic acid, a derivative of histidine, is one of essential components of human skin. We found that it can bind nickel(II) ions in a pH-dependent manner, with the dissociation constant in the low millimolar range, as revealed by potentiometry, and confirmed by isothermal titration calorimetry and UV-vis spectroscopy. The binding occurs within the physiological skin pH range. Considering the fact that *cis*-urocanic acid is present in the human skin in concentrations as high as millimolar, this molecule may be a physiologically important player in nickel trafficking in the human organism.

Introduction

Urocanic acid (UCA, 3-(1*H*-imidazol-4-yl)propenoic acid) is present in the uppermost layer of the skin (*stratum corneum*). It is produced by histidine ammonia-lyase (histidase) from histidine as a *trans* isomer (*trans*-UCA, Scheme 1). As urocanate hydratase (urocanase), the enzyme which catabolizes *trans*-UCA, is not present in the skin, *trans*-UCA accumulates in the epidermis to 0.7% dry weight, which means that in live tissue its concentration is millimolar.[1] *trans*-UCA isomerizes to *cis*-urocanic acid (*cis*-UCA) upon exposure to ultraviolet radiation (Scheme 1). The equilibrium concentration for *cis*-UCA reaches 60-70% of total epidermal UCA.[2] Both UCA isomers are removed from the skin in sweat and by the desquamation process, however some UCA is transported to blood and consequently to urine.[3,4] UCA, as a component of Natural Moisturizing Factor (NMF), is involved in the regulation of pH of the skin and skin hydration.[5,6] On the other hand, *cis*-UCA has been shown to play a role in photoimmunosuppresion[7-9] and photocarcinogenesis.[10] The mechanisms of these actions are yet to be elucidated, however.



trans-UCA *cis*-UCA

Scheme 1. Structures of *trans*-UCA and *cis*-UCA.

Allergy to nickel is one of the most common causes of allergic contact dermatitis. It is estimated that 10-15% of women in the general population is sensitive to nickel.[11] The mechanism of this condition has not yet been fully elucidated. One of the important unexplored factors is the first step of delivering Ni(II) to the organism. It is possible that a role in it is played by metal complexes with biomolecules present in the uppermost layers of the skin.

Considering the similarity of *cis*-UCA and histidine we assumed that this molecule can bind Ni(II), as the ability of histidine to form strong Ni(II) complexes is well documented.[12,13] Using UV-vis spectrophotometry, potentiometry and isothermal titration calorimetry (ITC) we proved that *cis*-UCA molecule interacts with Ni(II) ions. We measured stoichiometry, stability constants, enthalpy and entropy of these interactions. Our results suggest a possibility of interaction of *cis*-UCA with Ni(II) in human skin.

Experimental section

Chemicals and reagents

cis-UCA and *trans*-UCA (purity ≥ 98%) were obtained from Sigma-Aldrich. Nickel(II) nitrate hexahydrate, 99.999% trace metal basis, was obtained from Sigma-Aldrich. HEPES was obtained from Carl-Roth. Potassium nitrate(V) was purchased from Merck. Deionized, ultra-pure Milli-Q water was used for sample preparation. In order to prevent UCA isomerisation, all experiments were made with special care to protect samples from ambient light.

UV-vis titrations

Two types of UV-vis pH titrations were performed. In the first one, samples containing 0.1 mM *cis*-UCA were titrated with NaOH in the pH range of 4.7-9.8. In the second type, samples containing 4 mM *cis*-UCA or *trans*-UCA, 2 mM $Ni(NO_3)_2$ and 100 mM KNO_3 were titrated with NaOH in the pH range of 2.6-8.0. The UV-vis spectra were obtained using a Lambda 950 UV/Vis/NIR spectrophotometer (Perkin Elmer) at 25 °C over the spectral range 200-850 nm.

Potentiometric titrations

Potentiometric titration were performed on a 907 Titrando automatic titrator (Metrohm), using a Biotrode combined glass electrode (Metrohm). The electrode was calibrated daily by nitric acid titrations,[14] and 100 mM NaOH (carbon dioxide free) was used as a titrant. Sample volumes of 1.5 mL were used. Samples were prepared by dissolving solid *cis*- or *trans*-UCA in 4 mM HNO_3/96 mM KNO_3 to obtain a 4 mM UCA concentration. The Ni(II) and *cis*-UCA/*trans*-UCA complex formation was studied using samples in which the molar ratio of *cis*-UCA/*trans*-UCA to Ni(II) were: 2.2:1, 3.3:1, 4.4:1 and 6.7:1. All experiments were performed under argon at 25 °C, in the pH range of 2.7-11.0 for titration of *cis*- and *trans*-UCA, 2.7-8.0 for Ni(II) and *cis*-UCA, and 2.7-6.5 for Ni(II) and *trans*-UCA. The collected data were analysed using the SUPERQUAD and HYPERQUAD programs.[15,16] Five titrations were included simultaneously into calculations, separately for protonation and Ni(II) complexation.

ITC titrations

ITC experiments were carried out on the Nano ITC Standard Volume calorimeter (TA Instrument). The sample cell (950 µL) was filled with UCA solution and the reference cell was filled with the Milli-Q water. The syringe (250 µL) was loaded with a Ni(II) solution. The Milli-Q water was degassed under vacuum for 15 min before sample preparation. Furthermore, sample solutions were degassed for 5 min before loading into the cell and the syringe. The cell solutions contained 5 mM *cis*-UCA/*trans*-UCA, 100 mM HEPES and 100 mM KNO_3 at pH 7.4. The syringe solutions contained 25 mM or 50 mM $Ni(NO_3)_2$ and 100 mM KNO_3. The pH values of the cell solution did not change during titrations. The solutions in the cell were stirred at 170 rpm by the syringe. Volumes of titrant injections were 2.57 or 4.00 µL. Intervals between injections were 1000 s to allow complete equilibration of the system. The measurements were performed at 15, 25 or 37 °C for *cis*-UCA and at 25 °C for *trans*-UCA. Background titrations, consisting of the identical syringe solutions and the cell solutions except for *cis*-UCA/*trans*-UCA were subtracted from each experimental titration. The obtained data were analyzed with the SEDPHAT program (version 10.58d) utilizing the global fitting feature.[17] The conditional complex formation constants were calculated for the following equilibria:

K_{NiL}: $Ni^{2+} + UCA \rightleftarrows Ni(UCA)$

K_{NiL_2}: $Ni^{2+} + 2UCA \rightleftarrows Ni(UCA)_2$

Results and discussion

The pH dependence of UV absorption of *cis*-UCA is presented in Fig. 1. The most significant changes, observed at 250 and 290 nm, allowed us to determine the pK_a values for its carboxylate and imidazole groups, given in Table 1. The maximum of absorption blueshifted from 269 nm to 263 nm for pH 1.7-4.7 and back, from 263 nm to 282 nm for pH 4.7-9.8. This shift was not significantly affected for 0.1 mM *cis*-UCA by equimolar Ni(II)†. However, for higher concentrations (4 mM *cis*-UCA and 2 mM Ni(II)), the pH dependent changes were observed in the regions specific for Ni(II) octahedral complexes (~390 nm and ~650 nm) (Fig. 2). Such effects were seen neither for *cis*-UCA nor Ni(II) alone. For the *cis*-UCA/Ni(II) system the absorbance maxima blue-shifted from 391 nm to 383 nm and from 658 nm to 648 nm. The molar absorption for those bands increased along with the increasing pH. This effect is typical for substitutions of oxygen donors in the first coordination sphere of high-spin Ni(II) complexes with nitrogens.[18] Above pH 8.0 a green precipitate appeared in the sample. Dissolution of this precipitate at pH 5 demonstrated that it was composed predominantly of $Ni(OH)_2$, with traces of *cis*-UCA. Only minor changes in the spectra were observed for a sample containing 4 mM *trans*-UCA and 2 mM Ni(II), compared to free *trans*-UCA, and the precipitation occurred at pH 6.5. This whitish precipitate was partially re-dissolved at pH 5. The UV-vis spectra demonstrated that it was composed largely of *trans*-UCA, but also contained significant amount of Ni(II). There was no precipitation of *trans*-UCA seen in the absence of Ni(II). Therefore, most likely the precipitate was composed of Ni(*trans*-UCA) complex neutralized electrostatically by one OH^- ion per Ni(II).

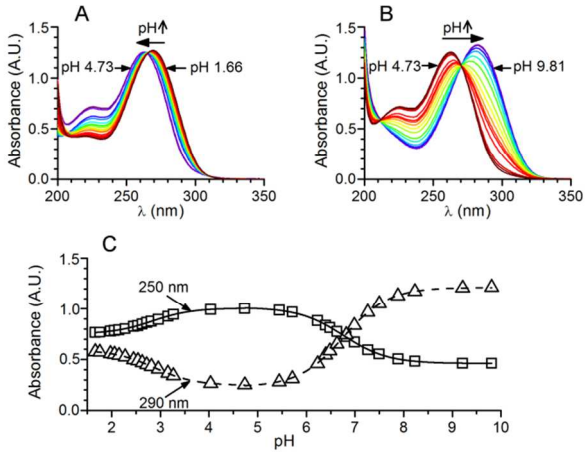


Fig. 1. The pH dependence of UV spectra of 0.1 mM *cis*-UCA in pH ranges of 1.66-4.73 (A) and 4.73-9.81 (B), and absorbance values of 0.1 mM *cis*-UCA at 250 and 290 nm (C).

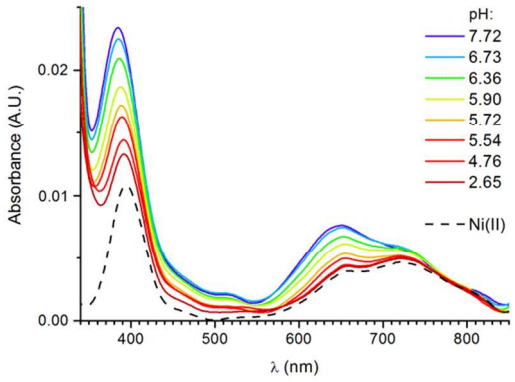


Fig. 2. Selected spectra of Ni(II) (2 mM $Ni(NO_3)_2$, dashed line) and its complexes with *cis*-UCA (4 mM). The pH values are indicated in the graph.

Potentiometric titrations of *cis*-UCA and *trans*-UCA alone and with Ni(II) ions yielded protonation and Ni(II) complexation constants for both UCA isomers. They are presented in Tables 1 and 2 in the logarithmic form. Protonation constants for both ligands are in a satisfactory accordance with the literature data,

obtained solely by UV-vis (lit.,[19] 3.43 and 5.80 for *trans*-UCA, and 2.7 and 6.65 for *cis*-UCA). The pK_a values determined by potentiometry are also close to those obtained in our UV-vis titrations. The slight difference (Δ = 0.07) in pK_a values for the carboxyl group is probably the result of difference in the ionic strength between these experiments.

Table 1. Logarithmic protonation constants (log β and pK_a) for UCA isomers

Species	*cis*-UCA			*trans*-UCA	
	Log β [a]	pK_a [a]	pK_a [b]	Log β [a]	pK_a [a]
H_2L	9.532(4)	2.79	2.86(4)	9.342(2)	3.51
HL	6.744(2)	6.74	6.76(4)	5.830(1)	5.83

[a] Values determined by potentiometry at 25 °C and I = 0.1 M (KNO_3). Standard deviations on the least significant digits, provided by HYPERQUAD[16] are given in parentheses.

[b] Values determined by revealing the pH dependence of absorbance at 250 and 290 nm at 25 °C for the experiment shown in Fig. 1. Standard deviations on the least significant digits are given in parentheses.

Both *cis*-UCA and *trans*-UCA bind Ni(II) in the 1:1 and 1:2 metal to UCA ratios. The distributions of species for *cis*-UCA/*trans*-UCA and Ni(II), calculated on the basis of potentiometric constants are shown in Fig. 3. It is worth noting that the complex formation occurs in pH range specific for *stratum corneum* (pH 5-6), with the predominance of 1:1 complex, and is even more pronounced for deeper layers of the skin (pH 6-7.4), with higher amounts of 1:2 complex.

On the basis of potentiometric and UV-vis titration data, using the least square method, the spectra of Ni(*cis*-UCA) and Ni(*cis*-UCA)$_2$ complexes were calculated and are shown in Fig. 4. The absorbance maxima for these complexes are 387 (ε/dm^3 mol^{-1} cm^{-1} 10.5) and 658 nm (2.9), and, 380 (15.3) and 640 nm (5.3), respectively. The blueshift of the bands for the latter complex is in agreement with the stronger ligand field effect exerted by the imidazole nitrogen of UCA vs. water molecules.[18]

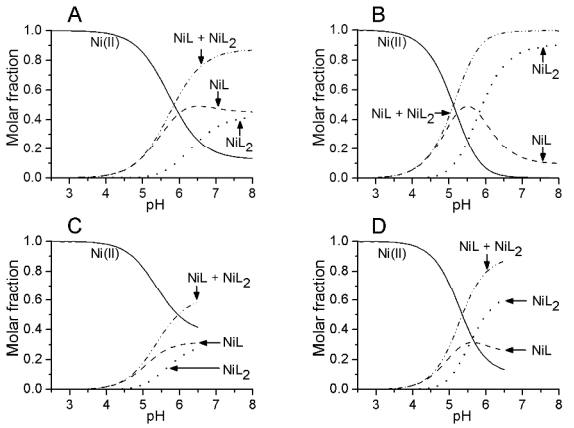


Fig. 3. Species distributions for Ni(II) complexes in a solution of 2 mM Ni(II) and 4 mM *cis*-UCA (A), 1 μM Ni(II) and 14 mM *cis*-UCA (B), 2 mM Ni(II) and 4 mM *trans*-UCA (C) and 1 μM Ni(II) and 6 mM *trans*-UCA (D), based on potentiometric data shown in Tables 1 and 2.

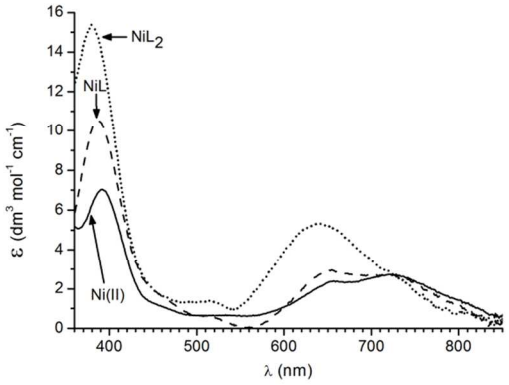


Fig. 4. Spectra of Ni(*cis*-UCA) and Ni(*cis*-UCA)$_2$ complexes, calculated on the basis of potentiometric data shown in Tables 1 and 2, and UV-vis titration data shown in Fig. 2.

The analysis of relation between the complex stability and the ligand basicity for imidazole-type ligands can help reveal whether the chelate effect occurs or the ligand binds the metal ion monodentately by its imidazole nitrogen.[20,21] Specifically, such dependence is linear for ligands with no chelating groups, and for Ni(II) and the ionic strength of 0.1 M is described by the following equation:

$$\log K_{NiL}^{Ni} = 0.225pK_{HL}^{H} + 1.380$$

Such relation is shown in Fig. 5 for 1:1 Ni(II) complexes taken from the literature,[20,22-26] and compared to our data for *cis*-UCA and *trans*-UCA. The stability constant for *trans*-UCA is within the linear correlation observed for ligands interacting only by the imidazole part of the molecule (e.g. imidazole, 4-methylimidazole). It means that only the imidazole nitrogen binds Ni(II) in *trans*-UCA. For *cis*-UCA the stability enhancement is observed, although it is not as large as for e.g. histidine or histamine. It shows that also the carboxylic moiety participates in the interaction of *cis*-UCA with Ni(II). The carboxylate is not so effective in the stabilization of complex as the primary amine in histamine or the secondary amine in 4-(2-methylaminoethyl)imidazole. The Ni(*cis*-UCA) complex is also not so stable as complexes with imidazole-4-acetic acid, which correlates with the higher stability of 6-membered chelates over the 7-membered one which we propose here (Fig 6).

The binding of the second molecule of *cis*-UCA compared to the first one is weaker by 0.58 log units (Log β_{NiL2} − Log β_{NiL}). The nature of this effect is largely statistical, because this difference is only slightly different from the statistical factor of 0.38, resulting from the decreased number of bidentate binding modes for the second ligand molecule (10) vs. the first one (24). The bigger difference is probably due to the repulsion between the carboxyl groups of the two *cis*-UCA molecules.

The analogous statistical factor for the monodentate binding of the second *trans*-UCA molecule is much lower and equal to 0.08 (Log(6/5)). This explains almost the same binding stability of the first and the second *trans*-UCA molecule.

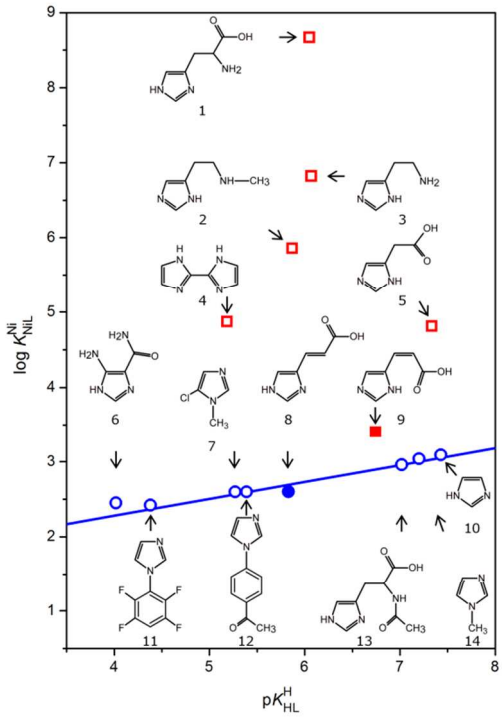


Fig. 5. The relation between the Ni(II) complex stability and the ligand basicity for imidazole-type ligands, shown for 1:1 Ni(II) complexes taken from the literature,[21] and compared to our data for *cis*-UCA and *trans*-UCA. The blue line represents linear dependence for ligands binding Ni(II) monodentately by the imidazole nitrogen (blue circles) described by the equation: $\log K^{Ni}_{NiL} = 0.225pK^{H}_{HL} + 1.380$, valid for the 0.1 M ionic strength.[19,20] Ligands with chelating groups (red squares) show stability enhancement. Data shown are for histidine[22] (1), 4-(2-methylaminoethyl)imidazole[23] (2), histamine[24] (3), 2,2'-biimidazole[25] (4), imidazole-4-acetate[25] (5), 4(5)-aminoimidazole-5(4)-carboxamide[25] (6), 5-chloro-1-methylimidazole[20] (7), *trans*-UCA (8), *cis*-UCA (9), imidazole[20,24] (10), N-(2,3,5,6-tetrafluorophenyl)imidazole[20] (11), 4'-(imidazol-1-yl)acetophenone[20] (12), acetylhistidine[26] (13) and 1-methylimidazole[20] (14).

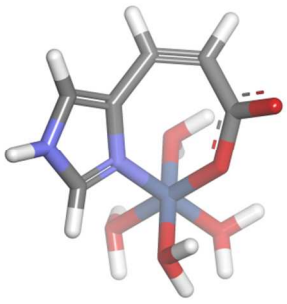


Fig. 6. The proposed structure of the Ni(*cis*-UCA) complex. Shown are Ni(II), *cis*-UCA and four water molecules in an octahedral complex.

ITC experiments were conducted at 15, 25 and 37 °C for *cis*-UCA and at 25 °C for *trans*-UCA, at pH 7.4. The results are presented in Table 2. Temperature influences only slightly the logarithmic stability constants for Ni(*cis*-UCA) and Ni(*cis*-UCA)$_2$ complexes and the binding enthalpy and entropic contribution (-TΔS) for the 1:1 complex. The binding enthalpy for the Ni(*cis*-UCA)$_2$ complex is higher at lower temperatures. On the other hand, the entropic contribution is higher at higher temperatures for the Ni(*cis*-UCA)$_2$ complex. In the result, the decrease of enthalpy at higher temperatures is compensated by the increase of the entropic contribution, which is reflected in Gibbs energy values. The formation of Ni(II) complexes with *cis*-UCA is energetically more favoured compared to *trans*-UCA in the same conditions.

Ni(II) complex formation constants are higher for *cis*-UCA than for *trans*-UCA, as revealed by both potentiometry and ITC. All values obtained for 25 °C by ITC are lower when compared with those obtained by potentiometry at the same temperature. The probable reason for this is the different ionic strength used in ITC (0.15 M) and the presence of HEPES buffer in these samples.

Table 2. Logarithmic Ni(II) complex formation constants for UCA isomers

Species	*cis*-UCA			*trans*-UCA		
	15 °C	25 °C		37 °C	25 °C	
	Log K [a]	Log K [a]	Log β [b]	Log K [a]	Log K [a]	Log β [b]
NiL	3.09(4)	3.09(2)	3.406(4)	3.13(3)	2.53(3)	2.599(7)
NiL_2	5.72(8)	5.61(3)	6.239(4)	5.61(8)	4.04(7)	5.272(7)

[a] Conditional association constant values determined by ITC at 15, 25 and 37 °C, in 0.1 M KNO_3, 0.1 M HEPES and pH 7.4. Standard deviations on the least significant digits, provided by SEDPHAT[17] are given in parentheses.

[b] Values determined by potentiometry at 25 °C and I = 0.1 M (KNO_3). Standard deviations on the least significant digits, provided by HYPERQUAD[16] are given in parentheses.

Table 3. Thermodynamic parameters for Ni(II) complexation determined at pH 7.4 by ITC [a]

	cis-UCA			*trans*-UCA
	15 °C	25 °C	37 °C	25 °C
ΔH_{NiL}	-18.6(0.2)	-18.2(0.1)	-18.4(0.4)	-20.0(0.3)
ΔH_{NiL_2}	-26.7(1.6)	-22.7(0.7)	-18.5(2.4)	-20.5(0.1)
$-T\Delta S_{NiL}$	1.5	0.6	-0.2	5.6
$-T\Delta S_{NiL_2}$	-4.8	-9.4	-15.7	-2.5
ΔG_{NiL}	-17.1	-17.6	-18.6	-14.4
ΔG_{NiL_2}	-31.5	-32.1	-34.2	-23

[a] The values are presented in kJ mol^{-1}. Standard deviations on the least significant digits, provided by SEDPHAT[17] are given in parentheses.

Of note are higher Gibbs energy values for NiL_2 complexes compared with NiL, which is in agreement with absorbance maxima being shifted to shorter wavelengths for Ni(*cis*-UCA)$_2$ complex, reflecting the presence of two nitrogen ligands in NiL_2 complexes.

We showed that both UCA isomers can bind Ni(II) ions with moderate affinity, with a stronger effect visible for *cis*-UCA. Of note is a correlation between concentration values in the *stratum corneum* of *cis*-UCA and Ni(II) ions upon the contact with the nickel-releasing material, which are the highest in this part of the skin. Considering these facts we propose that urocanic acid, especially the *cis* isomer, can be Ni(II) binding molecule in the human skin. Based on the literature[27,28] we estimated concentrations of main components of NMF as 43 mM of serine, 30 mM of glycine, 23 mM of pyroglutamic acid, 18 mM of

alanine, 14 mM of lactic acid, 14 mM of *cis*-UCA and 6 mM of *trans*-UCA. Concentrations of nickel can be estimated taking as the reference the maximum value, allowed by the European Union Nickel Directive (Directive 94/27/EC) for the release of this metal from products intended to come into direct, prolonged contact with skin, being equal to 0.5 μg cm^{-2} per week.[11] Locally this concentration can probably easily reach a micromolar level in the *stratum corneum*, especially because some kinds of jewelry and coins release 100 times more nickel than allowed by the Directive.[29,30] Taking the protonation constants and stability constants of Ni(II) complexes from the literature[26,31,32] and from this paper we calculated the distribution of Ni(II) species for above concentrations of ligands and of 1 μM nickel ions (Fig. 7). At pH 5.6 22% of Ni(II) is complexed with *cis*-UCA, which places this molecule as the second binding compound, after serine. 10% of Ni(II) is in complex with *trans*-UCA. At pH 6.5, still 22% of Ni(II) is bound with *cis*-UCA, after serine. The formation of Ni(II)-UCA complexes can be even more pronounced following the intended use of *cis*-UCA as a component of pharmaceuticals. Topical application of *cis*-UCA has been proposed as a safe and effective way to treat inflammatory skin disorders.[8,9] Deeper investigation is needed as it was shown that *cis*-UCA can form a complex with human serum albumin (HSA),[33] another Ni(II)-binding biomolecule.[34] The general important issue which remains to be resolved is whether *cis*-UCA plays a role of a transporting or rather a buffering agent for Ni(II) ions in the human organism.

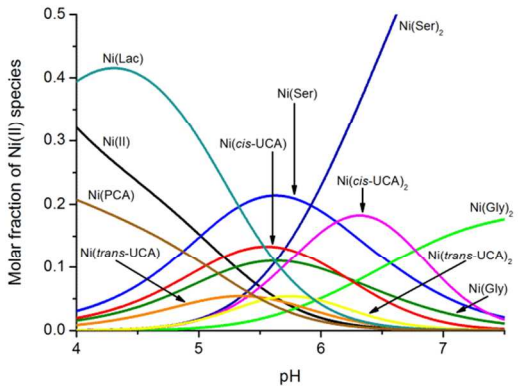


Fig. 7. Ni(II) species distribution simulated for NMF and 1 μM Ni^{2+} ions. The protonation constants and stability constants of Ni(II) complexes were taken from the literature[26,31,32] and from this paper. Concentrations taken for calculations are 43 mM serine, 30 mM glycine, 23 mM pyroglutamic acid (PCA), 18 mM alanine, 14 mM lactic acid (Lac), 14 mM *cis*-UCA and 6 mM *trans*-UCA.[27,28] The binding of Ni(II) by alanine in this conditions is negligible and omitted for clarity.

Conclusions

We proved that *cis*-UCA molecule interacts with Ni(II) ions with moderate stability, the complexes being strong enough to exist in physiological conditions in the outermost layers of the human skin, with consequences for nickel toxicity.

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Notes and references

[a] *Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawińskiego 5a, 02-106 Warsaw, Poland. Fax: +48 22 659 4636; Tel: +48 22 592 5766; E-mail: tfraczyk@ibb.waw.pl*

† Such low concentrations were chosen due to the high absorption coefficients of UCA chromophores.

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