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Metallacrowns as products of the aqueous medium self-assembly of histidinehydroxamic acid-containing polypeptides

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Self-assembly is a widely studied, spontaneous, and reversible phenomenon leading to formation of the ordered structures by non-covalent specific interactions among starting molecules. In this work, a new template for the self-assembly of polypeptides based on peptides containing the C-terminal histidinehydroxamic acid moiety and Cu^{2+} ions is characterized. Two peptide (tripeptide and pentadecapeptide) hydroxamic acid systems were synthesized and their interactions with Cu^{2+} ions were investigated revealing a high stability of the supramolecular assemblies formed. The supramolecular metallacrown-based L_4Cu_5

complexes exist at physiological pH in the presence of Cu^{2+} ions as is evidenced from the spectroscopic methods, ESI mass spectrometry, and physicochemical techniques.

Introduction

Self-assembly is a frequently observed feature of peptides. In the literature, there are numerous examples of peptides forming various structures, including cylindrical micelles, amyloids with catalytic properties, tri- and tetrahelical bundles, nanotubes, and tubular nanostructures[1,2]. Many practical applications of peptide nanostructures, involving tissue engineering and designing new antimicrobial agents biosensors and diagnostic devices, have been also presented.[3]

The concept of the template assembled synthetic proteins (TASP), proposed by Mutter,[4,5,6] was also based on the self-assembly of peptides on a covalent template. An alternative method, which may be used to obtain a system with a well-defined supersecondary structure, is a coordination of metal ions by peptides[7,8]. Moreover, some of these aggregates are known to possess the enzyme-like activity[9,10,11,12,13,14].

The metallacrowns are well known due to their structural analogy to crown ethers[15,16]. Recently the structures and possible applications of these metallamacrocycles have been extensively studied[17,18,19,20,21,22]. As it has been demonstrated, the metallacrowns can be applied in molecular recognition systems and catalyse some organic reactions[23,24]. Their bioactivity has been also demonstrated[25,26,27,28]. Most of these structures are based on low-molecular aminohydroxamic acid derivatives.

According to previous reports, N-benzyloxycarbonylhistidine hydroxamic acids (Z-Hisha) in the presence of Cu^{2+} ions form the multinuclear complexes of the L_4Cu_5 stoichiometry of the metallacrown type[29]. On the other hand, the research on dipeptides with the C-terminal histidinehydroxamic acid demonstrated that at low pH these ligands form

complexes of the 1:1 stoichiometry, whereas above pH 6 the dimeric species can be observed (stoichiometry 2:2)[30]. However, the stability constants of the dimeric form depend on the peptide sequence. Our recent studies on interactions of pentadecapeptides with the C-terminal histidinehydroxamic acid suggested formation of the tetrameric complexes[2]. These molecules were relatively stable in solution and in the gas phase (mass spectrometry measurements). Conformational investigations suggested that formation of those complexes results in the shift of the conformational equilibrium towards helical structures.

Herein we study the interactions of two peptides (a 15-residue peptide [AH]-NHOH: Ac-KALEKALKEALAKLH-NHOH and a short tripeptide Ac-KLH-NHOH) with Cu(II) ions at pH ranging from 2.5 to 9.5. The formation of the supramolecular metallacrown-based Cu_5L_4 assemblies was investigated by modern state-of-the-art spectroscopic and physicochemical techniques. This paper compares the effect of the peptide chains length on the stability constants of the metallacrowns formed. The analysis of this factor has not been described in the literature yet. Such investigations might furnish meaningful information on the stability of metallacrown templates for the design of new supramolecular systems.

Experimental Section

Synthesis and purification of peptides

[AH]-NHOH and Ac-KLH-NHOH were obtained according to the synthetic methods reported previously[31]. The analytical data of Ac-KLH-NHOH are as follows: *m/z*= 454.2837 (calc. *m/z*

= 454.2772 for $C_{20}H_{36}N_7O_6$); R_t = 9.55min, whereas of [AH]-NHOH were described in another paper[31].

All crude products were analysed by the analytical HPLC using a Thermo Separation HPLC system with a UV detection (210 nm) on a Vydac Protein RP C18 column (250 mm x 4.6 mm, 5 μm), with a gradient elution of 0–80% B in A (A = 0.1% TFA in water; B = 0.1% TFA in acetonitrile/H_2O, 4 : 1) over 45 min (flow rate 1 ml/min, rt). Retention times are given as R_t. Preparative reversed-phase HPLC was performed on a Tosoh TSKgel with an ODS-120T column (21.5mm x 300 mm, 10 μm) using the same solvent system, gradient 0.5%/min and a flow rate 7 ml/min.

The potentiometric studies

The titrations of Ac-KLH-NHOH and [AH]-NHOH were carried out in aqueous solution at T = 25 °C under N_2 stream and at the I = 0.1 mol dm^{-3} (KCl). The volume of the sample used for the titration measurements was in the range of 0.9 – 1 ml. NaOH (C_{NaOH} = 0.1 M) was used as a titrant. The ligand concentration was 0.8×10^{-3} M, the metal:ligand ratio was 1:1.1 and 1:2, and pK_w = 13.77.

The pH-metric titrations were performed in 0.1 M KCl on a MOLSPIN pH–meter system using a Mettler Toledo InLab 422 semimicrocombined electrode calibrated with HNO_3[32]. Protonation $\beta_i=[H_iL]/[H^+]^i[L]$, stability constants $\beta_{pqr}=[M_pH_qL_r]/[M]^p[H]^q[L]^r$, and stoichiometry of the complexes were calculated with a HYPERQUAD[33] program. Standard deviations quoted were also computed by HYPERQUAD and referred to the random errors only. The range of pH of each titration for the free ligand and the metal-ligand system was 2.5-9.5.

The spectroscopic measurements

Absorption spectra were recorded on a Varian Carry 50 Bio spectrophotometer, circular dichroism (CD) spectra on a JASCO J-715 spectropolarimeter in the 850–200 nm range, and EPR spectra on a Bruker ESP 300E spectrometer at the X-band frequency (9.4 GHz) at 120 K. The ligand concentration [L] was 0.80×10^{-3} M and the metal:ligand ratio was 0.8:1.

The absorption λ_{max} values of copper complexes were calculated with the help of Billo's empirical equation[34,35]. The theoretical contributions of coordinated moieties to the ligand field are as follows[30]:

$N_{Im} = 0.434,$
$N^{-}_{hydroxamate} = 0.470,$
$O_{carbonyl/water} = 0.294,$
$O_{hydroxamate} = 0.384.$

$$\lambda_{max} = \frac{1000}{\sum_{i-1}^{4} x_i}$$

NMR Spectroscopy

All NMR measurements for the Ac-KLH-NHOH peptide in the absence and presence of Cu^{2+} ions were carried out on a 500 MHz spectrometer at 25 ºC, at the peptide concentration of 3.08 mg/mL, in 99% D_2O at pH (pH reading ±0.20) 6.80, 9.50, and 10.15 .The complete proton resonance assignment of the Ac-KLH-NHOH peptide spin systems (at pH 6.8, without Cu^{2+}) and well separated proton resonances from L_4Cu_5 metallacrown (pH 6.8; 1:0.2 (n:n) L:Cu^{2+}) were accomplished on the basis of 2D ^{1}H-^{1}H TOCSY (20 and 80 ms mixing times) and ^{1}H-^{1}H ROESY (300 ms) spectra. All the spectra were processed by NMRPipe[36] and analyzed with Sparky[37]. The diffusion coefficients (D) and hydrodynamic radii (R_H) were determined from the series of sixteen 1D ^{1}H NMR diffusion-ordered DOSY (Diffusion-Ordered Spectroscopy) experiments (each 32 scans) recorded with a gradient strength changing linearly from 5% to 95%. Gradient calibration was achieved by measuring the diffusion of residual HDO in 99.8% D_2O at 25 ºC.[38,39] For selected signals, their intensities were fitted to the exponential function of $I = I_0\cdot\exp[-D\gamma^2g^2\delta^2(\Delta-\delta/3)]$[40] by a nonlinear least-

square fit of cross-peak heights, where I is the observed intensity, I_0 is the reference intensity (not attenuated signal intensity), D is the diffusion coefficient, γ is the gyromagnetic ratio of the observed nucleus, g is the gradient strength, δ is the length of the gradient, and Δ is the diffusion time[41,42]. The accuracies of diffusion coefficients (D) were determined from the variance-covariance matrices.

Hydrogen-deuterium exchange mass spectrometry

The complex formation was monitored by deuterium–hydrogen exchange of the C_2 imidazole proton. Peptide (2 mg) was dissolved in 0.5 ml of 10 mM $(NH_4)_2CO_3$ (in D_2O). The sample was incubated for 4 days at 60°C. Then the reaction was stopped using 50 µl of HCOOH and the sample was lyophilized. In the next step, the lyophilisate was dissolved in the mixture of H_2O:HCOOH (10:1, v:v) and left for one hour. After that time, the MS spectrum was recorded to confirm the complete exchange of C_2 hydrogen by deuterium. After the measurement the sample was lyophilized.

To determine the kinetics of isotopic exchange, the C_2-deuterated peptide was dissolved in 80 µl of H_2O. Then 10 µl of the stock solution was added to 115 µl of the appropriate buffer and the sample was incubated at 40°C. In the case of metal complexes, $CuSO_4$ was added before incubation ($L:Cu^{2+}$ = 4:5). Aliquots of the incubated sample were collected and added to 100 µl of H_2O:CH_3CN:HCOOH (50:50:0.1, v:v:v).

Mass spectrometry

High resolution mass spectra of pure ligands and metal complexes were recorded on an FT-ion cyclotron resonance (ICR) MS Apex-Qe Ultra 7T instrument equipped with an ESI source. The spectra of products were recorded in solution of H_2O:CH_3CN:HCOOH (50:50:0.1, v:v:v). Argon was used as a collision gas. The instrument was operated in a positive ion mode and calibrated with Tunemix™. The instrumental parameters were as

follows: temperature of the drying gas 200 °C, the potential between the spray needle and the orifice 4.5 kV, source accumulation time 0.5 s, and ion accumulation time 0.5 s.

The measurements of metal complexes were performed in the mixture of methanol and 10 mM aqueous NH_4HCO_3 (1:1, v:v). The registered pH for the sample containing buffer, peptide, and Cu^{2+} ions was 7.7. The concentration of the ligand in the mixtures was 10^{-4} M. The Cu^{2+} ions were added at the ratio of 4:5 ($L:Cu^{2+}$).

Results and Discussion

Results available to date on histidinehydroxamic acid-containing peptides relate to the imidazole derivative of acetic acid, N-α-benzyloxycarbonylhistidine hydroxamic acid, and dipeptide containing histidine hydroxamic acid[29,30,43]. The peptides used in our study have higher molecular weights (they are composed of 3 and 15 amino acid residues). In addition, the N-terminal amino group, which in some Farkas[30] models was the key one in the interactions with Cu^{2+} ions, was protected with the acetyl group. The sequences of the studied peptides (Ac-KLH-NHOH and [AH]-NHOH), as well as of the Farkas model Z-Hisha, are given in **Figure 1**. These two sequences differ in lengths of the polypeptide chains which allows comparison of the influence of the peptide chains interactions on stability of the complex formed. The amphipathic sequence of [AH]-NHOH is based on Mutter's design[4]. This amphipathicity allows formation of a tetrahelical bundle after arrangement of the peptides on the metallacrown template.

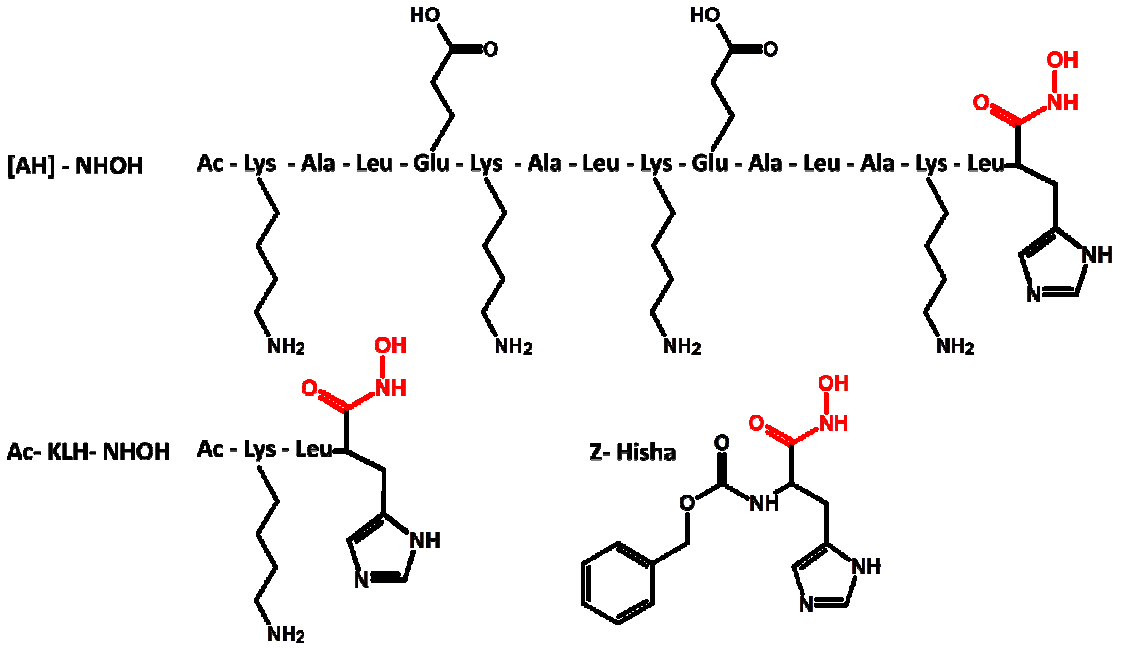


Figure 1. Schematic representations of investigated peptides [AH]-NHOH and Ac-KLH-NHOH in their neutral H_nL form. Z-Hisha – analogous model ligand by Farkas[29].

Both ligands, as well as their complexes, are water soluble. Above pH 9.5, the stability of histidinehydroxamic acid is limited because of its partial hydrolysis which is supported by the mass spectrometric data (**Figure S2**). Therefore, we analyzed the equilibria in solution up to pH 9.5.

The values of protonation constants for free ligands and the stability constants of the formed complexes obtained by analysis of the potentiometric results are presented in Table 1. The Ac-KLH-NHOH compound is characterized by two protonation constants in the experimental range of pH. The pK_a 6.28 and 8.61 values correspond to deprotonation of imidazole nitrogen and the hydroxamic acid group, respectively. Analysis of the potentiometric results for [AH]-NHOH allowed for the determination of five pK_a protonation constants. Two of them, 6.50 and 8.87, are comparable to the constants found for Ac-KLH-NHOH and correspond to deprotonation of His and the hydroxamic acid group. Next two

constants (3.64 and 4.45) correspond to deprotonation of the carboxyl groups of both glutamic acid residues. The characteristic feature of [AH]-NHOH is the presence of four lysine residues in its structure, however, owing to the experimental conditions, it was possible to obtain only one pK_a protonation constant of lysine with pK=9.86.

Table 1. The dissociation constants and the constants of complex formation of Ac-KLH-NHOH and [AH]-NHOH in solution. T= 25° C, I = 0.1 mol dm^{-3} KCl**.**

Ligands						
	Ac-KLH-NHOH			**[AH]-NHOH**		
Species	**logβ**	***pKa***		**logβ**	***pKa***	
HL	8.61 ± 0.01	8.61	NHOH	9.86 ± 0.03	9.86	N_{Lys}
H_2L	14.88 ± 0.01	6.28	N_{Im}	18.73 ± 0.02	8.87	NHOH
H_3L				25.23 ± 0.03	6.50	N_{Im}
H_4L				29.68 ± 0.03	4.45	COOH
H_5L				33.32 ± 0.03	3.64	COOH
Copper(II) complexes						
	Ac-KLH-NHOH			**[AH]-NHOH**		
Species	**logβ**	***pK_a***		**Species**	**logβ**	***pK_a***
CuLH	14.36 ± 0.01	-		CuLH	19.96 ± 0.02	-
	-			$Cu_5(HL)_4H_{-4}$	73.47 ± 0.10	17.66
	-	-		$Cu_5L_2(HL)_2H_{-4}$	55.81 ± 0.12	18.70
$Cu_5L_4H_{-4}$	36.03 ± 0.04	18.07		$Cu_5L_4H_{-4}$	37.11 ± 0.12	
$Cu_5L_4H_{-6}$	17.96 ± 0.05			$Cu_5L_4H_{-6}$	*	

*The logβ value has not been determined because of ligand intsability at pH>9.5

Both ligands start Cu^{2+} ions coordination around pH 3 by formation of the mononuclear CuHL complex (**Figure 2**), however, analysis of the corrected logβ* value (logβ* = logβ_{CuLHn} – logβ_{HnL}) strongly supports a different coordination mode. In the case of Ac-KLH-NHOH, the comparison of logβ* = (logβ_{CuLH} – logβ_{HL}) = 5.75 with the literature data[29] for analogous complex obtained for Z-Hisha (logβ* = 5.51) suggests the monodentate Cu-N_{imid} coordination

with the hydroxamate group is protonated. For [AH]-NHOH ($\log\beta^* = 10.10$), it is comparable with another Z-Hisha complex ($\log\beta_{CuL} = 10.40$), in imidazole nitrogen as well as the hydroxamate group are coordinated to the Cu(II) ion and the Lys moiety ε-amino group is still protonated.

Above pH 5, both ligands form complexes of the 5:4 stoichiometry – $Cu_5L_4H_n$. These complexes are the most interesting from our point of view, because they may be applied as templates for the self-assembly of peptides. The first complexes formed are CuL_4H_{-4} for Ac-KLH-NHOH and $Cu(HL)_4H_{-4}$ for [AH]-NHOH. Moreover, the appearance of these species strongly influences the spectral properties of the investigated systems.

In the UV-Vis spectroscopy, the d-d band at approx. 641nm ($\varepsilon = 80\ M^{-1}cm^{-1}$) of the Ac-KLH-NHOH complex and at approx. 637 nm ($\varepsilon = 100\ M^{-1}cm^{-1}$) for [AH]-NHOH can be observed (**Figure 3**, **A** and **B**, respectively). The experimental λ_{max} (641 and 637 nm) obtained for both ligands is comparable to the theoretical λ_{max}= 632 nm,[30] calculated for the N_{Im}, $N_{hydroxamate}$, $O_{hydroxamate}$, and $O_{carbonyl}$ chromophores.

Furthermore, the obtained values are in good agreement with the λ_{max} found for metallacrowns presented previously in the literature, with ligands which form a 7-membered ring in the metallacrown structure, for example 640 nm for GABAha[44] or approx. 650 nm for Z-Hisha[30]. More examples of the absorption maxima for 12-metallacrown-4 can be also found in the literature[45,46]. According to the literature data, the coordination model suggests formation of the 7-membered nitrogen (N_{imid}, N_{hydr}) and 5-membered oxygen chelating rings. The band at 641 nm did not change until pH≈9.5 for the Ac-KLH-NHOH complex and pH≈9 for the [AH]-NHOH one, which is in reasonable agreement with the potentiometric titrations. Above this pH, the metallacrown system undergoes decomposition which is also confirmed by NMR spectroscopy. This decomposition is the effect of a slow hydrolysis of the

hydroxamic acid group at alkaline pH (**Figure S2.**) which result in converting hydroxamate into carboxylate.

The CD spectra (**Figures S3 and S4**) recorded at the pH range in which, according to the potentiometric studies, metallacrown is predominant show a similarity to the CD spectra reported by Dallavalle and Tegoni[46] for the metallacrown-type Cu^{2+} complexes of phenylalaninehydroxamic acid (Pheha) and tryptophanehydroxamic acid (Trpha).

These spectra are relatively complex and show several peaks and shoulders. The most distinct are: a positive band at approx. 700 nm (713nm for Ac-KLH-NHOH and 690mn for [AH]-NHOH), a positive band at approx. 550 nm (570 nm for Ac-KLH-NHOH and 550 nm for [AH]-NHOH), and a positive band at approx. 400 nm (390 for Ac-KLH-NHOH and 410 for [AH]-NHOH) In addition, in the case of Ac-KLH-NHOH there is a strong negative band at 230 nm which is not observed for [AH]–NHOH because of overlapping with a band at 222 nm assigned to the helical conformation.

According to the interpretation given by Dallavalle and Tegoni[46], these peaks may result from d-d transitions or nonequivalent chromophores in a complex. On the other hand, the metallacrown geometry reported herein is not exactly the same as that of the complex formed by Pheha[46], therefore, our complex should not be directly compared with the Pheha-based metallacrown. In our previous work[2], we reported the CD spectra of [AH]-NHOH which suggest the increase of helicity in this peptides upon addition of Cu^{2+}. In contrast, the measurements performed for Ac-KLH-NHOH did not reveal the CD pattern characteristic for helical structures. The Ac-KLH-NHOH peptide is too short to form α helix, even on the template[4].

The characteristic silent EPR spectrum, recorded at pH = 7.5, when the complex of the formula $Cu_5L_4H_n$ (with $Cu_5L_4H_{-4}$ for AC-KLH-NHOH and $Cu(HL)_4H_{-4}$ for [AH]-NHOH) dominates according to the potentiometric data, strongly supports formation of the

multinuclear Cu^{2+} complexes (**Figure 4**)[17,47]. The electrospray mass spectra obtained for the investigated complexes confirmed the 4:5 ($L:Cu^{2+}$) stoichiometry. As an example we present the mass spectrum of Ac-KLH-NHOH in the presence of Cu^{2+} ions (**Figure 5**). The spectrum is dominated by three peaks corresponding to the same 4:5 ($L:Cu^{2+}$) stoichiometry. The differences between *m/z* ratios of the observed signals result from different protonation levels of the peptide side chains and consequently different charge states (+2, +3, and +4). The experimental values of *m/z* of the observed signals in the MS spectrum (**Table 1)** are the same as the calculated ones supporting the proposed formulas**.** The relative errors do not exceed 10 ppm. On the other hand, the mass spectrum recorded at pH 10.5 do not reveal the system of the 4:5 ($L:Cu^{2+}$) stoichiometry. This result is in good agreement with the NMR data. The MS spectrum measured at pH 10.5 is presented in the Supplementary Information (**Figure S1**). The peaks observed in this spectrum correspond to the inorganic metal cluster (the isotopic pattern does not reveal the presence of carbon in the observed signal). MS data for the complex of [AH]-NHOH and Cu^{2+} obtained at pH 7.5 were presented in our previous work[2] revealing that in this case the metallacrown is also predominant.

Above pH 8.5, formation of further complexes with the 5:4 metal:ligand stoichiometry is observed. Nevertheless, it does not influence the spectral properties of both systems and it supports a proton dissociation from the **ε**-amino groups of Lys moieties (**Figures 3 and 4**).

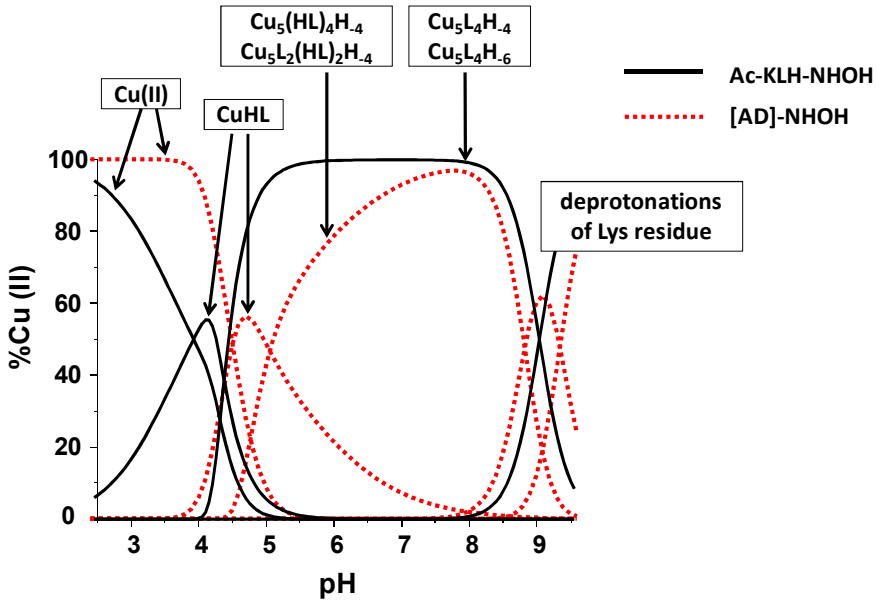


Figure 2. The distribution curves of complexes formed as a function of pH for Ac-KLH-NHOH (solid line) and [AH]-NHOH (dashed line). The metal:ligand molar ratio was 1:1, $C_L = 0.80\times10^{-3}$M, I=0.1 M KCl.

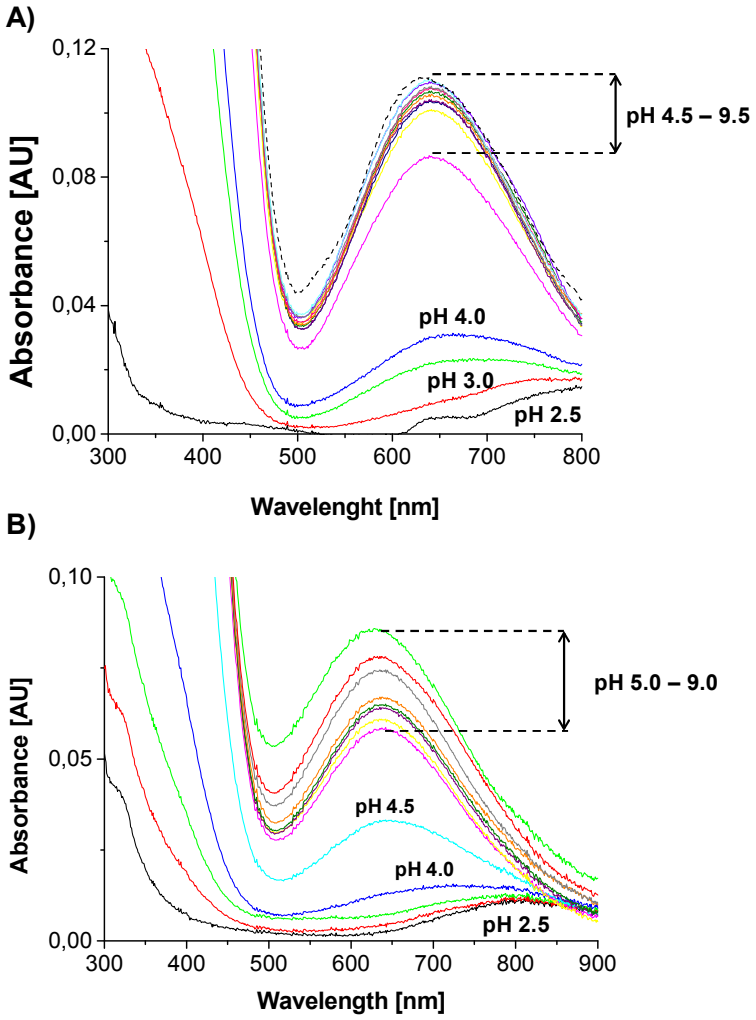


Figure 3. Representative UV-Vis spectra registered for: A) Cu(II) and Ac-KLH-NHOH as a function of pH at the 1:1 metal:ligand ratio: $C_{Cu(II)} = 2.5\times10^{-3}$M; B) Cu(II) and [AH]-NHOH as a function of pH at the 1:1 metal:ligand ratio: $C_{Cu(II)} = 0.80\times10^{-3}$M.

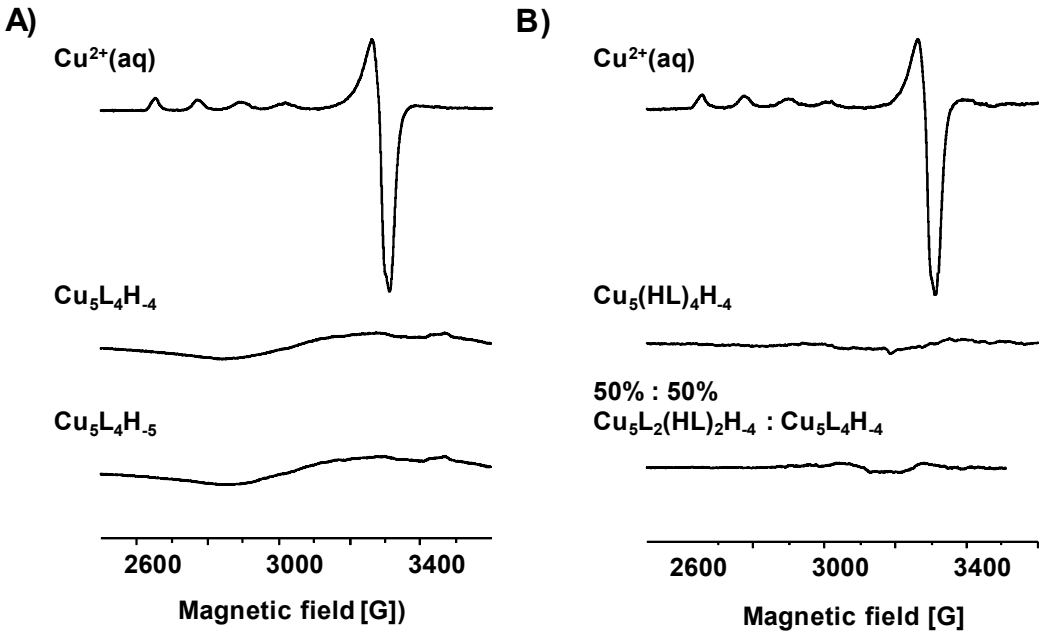


Figure 4. Representative EPR spectra registered for: A) Cu(II) and Ac-KLH-NHOH at the 1:1 metal:ligand ratio, $C_{Cu(II)} = 0.80\times10^{-3}$M; B) Cu(II) and [AH]-NHOH as a function of pH at the 1:1 metal:ligand ratio, $C_{Cu(II)} = 0.80\times10^{-3}$M.

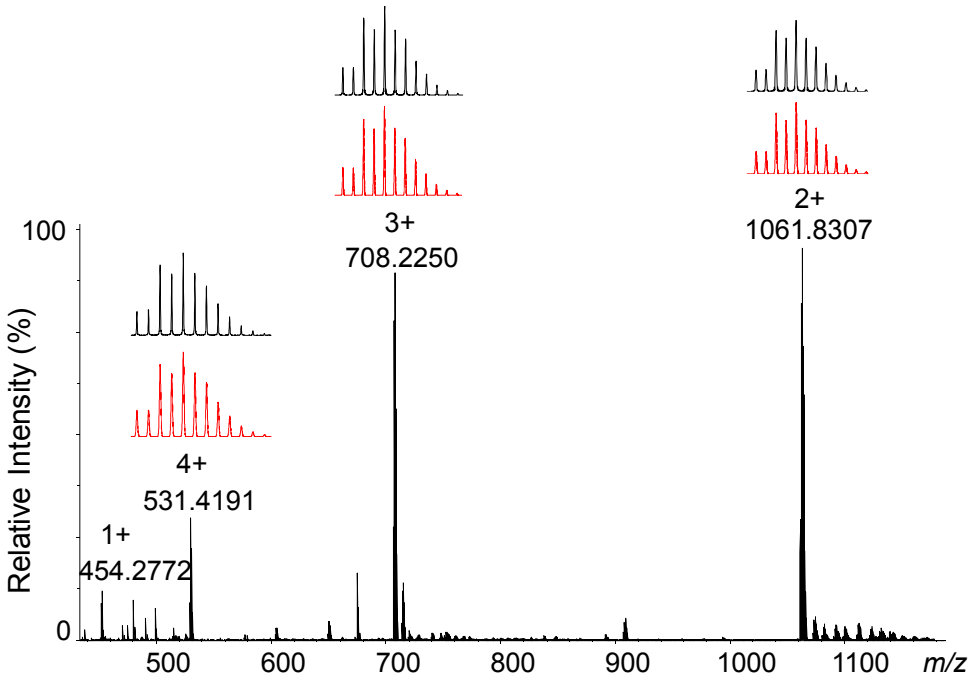


Figure 5. The ESI-MS spectrum of Ac-KLH-NHOH and its complex with copper(II) ions. The spectrum was recorded at pH 7.5. All marked signals (z = 2+, 3+, and 4+) correspond to the metallacrown of the same stoichiometry (Cu_5L_4) but different protonation levels of the amino acid side chains. The calculated isotopic patterns (upper panel) are in good agreement with the experimental patterns (lower panel). The calculated and experimental *m/z* values, as well as the stoichiometry for the observed signals, are presented in the Supplementary information (**Table S1.**)

The NMR investigations carried out on the Ac-KLH-NHOH peptide dissolved in D_2O allowed to assign all amino acid spin systems. The Hα/Cα chemical shifts of the free peptide indicate the predominantly open conformation[48]. After addition of Cu^{2+} ions (0.02 M) equivalent to Ac-KLH-NHOH at pH = 6.8, a new set of peaks from the metallacrown is visible (**Figure 6, A and B**), showing the simultaneous presence of the free peptide and the metallacrown in solution. The resonances of each compound can be assigned to its amino acid residues on the basis of the ^1H-^1H TOCSY (20 and 80 ms mixing times) spectra analysis. The

presence of two well separated sets of peaks for two individual species in solution clearly suggests that this system is characterized by a slow exchange of the peptide between its free and metallacrown-bound forms**.**

Further addition of Cu^{2+} ions up to 0.04 M and 0.2 M results in the proportional increase of the metallacrown concentration, expressed by the corresponding signals intensities, in agreement with the L_4Cu_5 stoichiometry.

The DOSY measurements performed for the two sets of peaks of two individual forms that are present in solution show that the metallacrown formed moves slower than the free peptide (Figure 6D). In the case of a system of two chemical species (two-site exchange system), which are at the slow-exchange limit, it is possible to observe two spins in exchange that are associated with the individual diffusion coefficients characteristic of the two investigated species when no exchange is present[49].

The diffusion coefficient (D_L) obtained for free Ac-KLH-NHOH alone in solution at pH 6.8 is exactly the same as the one obtained for the free, non-bound peptide in solution with the peptide:Cu^{2+} ratios of 1:0.02, 1:0.04, and 1:0.2. Separate and well resolved signals of the metallacrown (D Ac(Cu_5L_4)), formed upon Cu^{2+} ions addition, have intensities that are proportional to the Cu^{2+} ions amount and are in line with Cu_5L_4 species formation. The diffusion coefficients for the metallacrown were obtained from the Ac signal (approx. 2.19 ppm) intensities collected for samples with the peptide:Cu^{2+} ions ratios 1:0.02, 1:0.04, and 1:0.2, at pH 6.8 (Fig. 6), yielding the same values in agreement with the slow exchange regime.

The increase of pH up to 9.5 and then to 10.15 results in decomposition of the metallacrown because at that time its peaks are no longer observed (**Figure 6C**). At such conditions only one set of peaks is present in the spectrum which suggests a fast exchange of the ligand

between its coordinated and free forms. The observed line broadening of the peaks at higher pH values suggests that Cu^{2+} ions are coordinated then by the imidazole rings of the histidine residues.

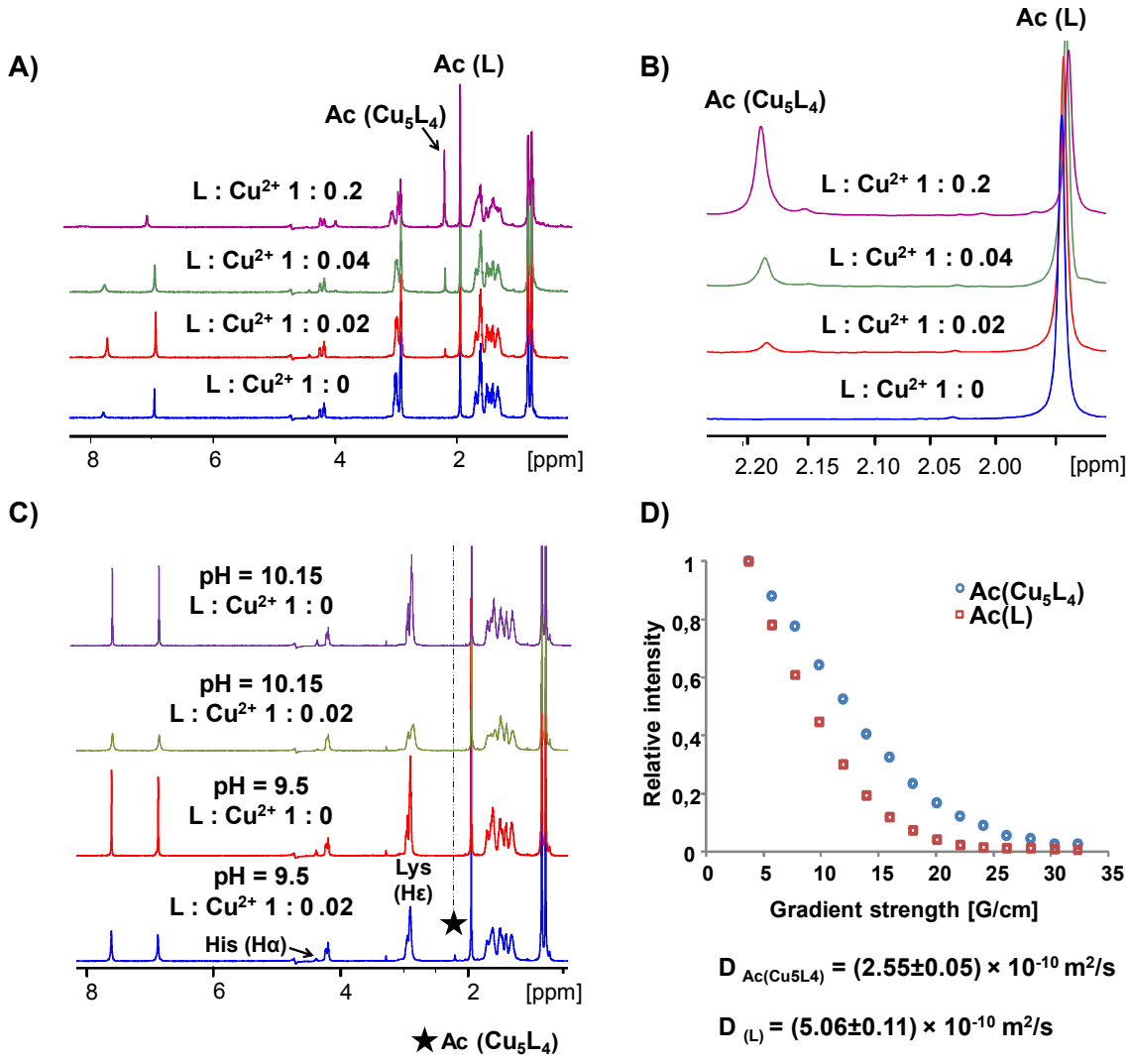
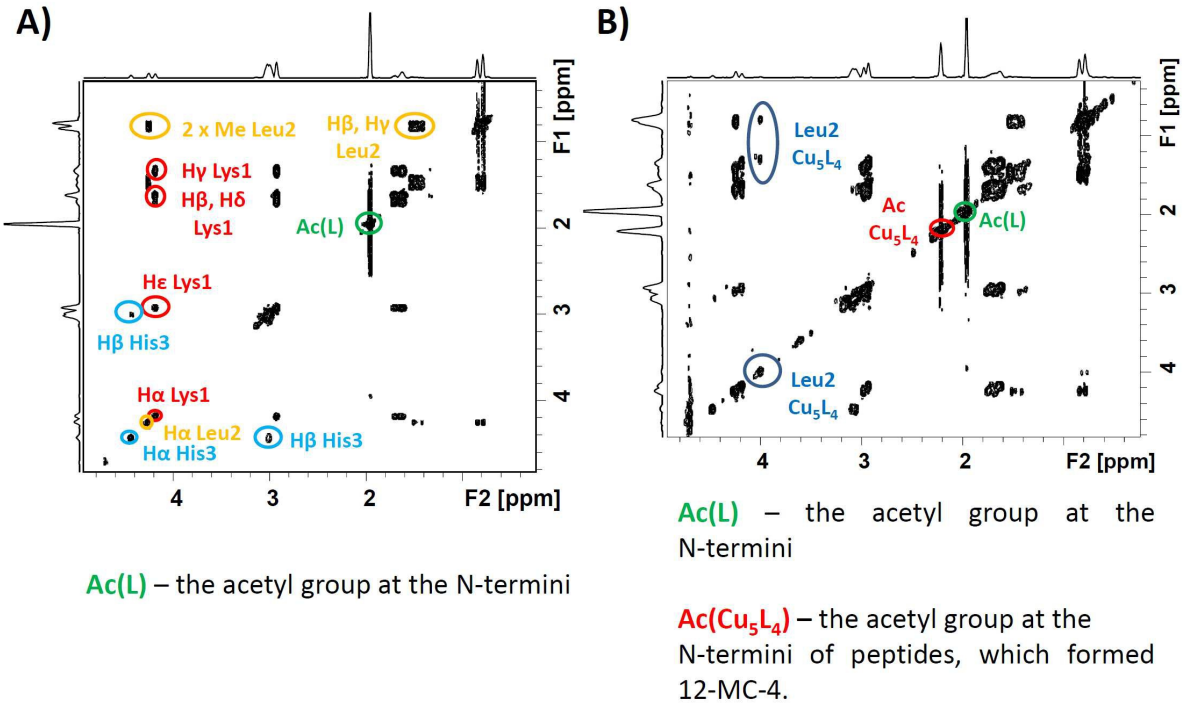


Figure 6. The ^{1}H NMR spectra of Ac-KLH-NHOH in the presence of Cu^{2+} ions. A) Spectra measured at pH 6.8 at various metal:ligand ratios; B) enlarged parts of spectra from A showing the appearance of a signal corresponding to the Ac(Cu_5L_4) metallacrown acetyl group (acetyl group protects the N-terminal amino group of the peptide) besides the peak corresponding to the free peptide acetyl group; C) Comparison of the spectra of the complex

of (Ac-KLH-NHOH) and Cu^{2+} at pH 9.5 and 10.15. The dotted line at approx. 2.19 ppm (signal marked as a black star) shows the region where the signal of $Ac(Cu_5L_4)$ appears. At pH 10.15 this signal is not observed. At this pH the signals corresponding to αH (Lys) and αH (His) are broadened what can suggest changes in the coordination sphere and decomposition of 12-metallacrown-4; D). Decay of the signal intensity as a function of increasing gradient strength for the Cu_5L_4 (L:Cu^{2+} 1:0.02) and [AH]-NHOH acetyl groups, at pH 6.8.



Ac(L) – the acetyl group at the N-termini

Ac(L) – the acetyl group at the N-termini

$Ac(Cu_5L_4)$ – the acetyl group at the N-termini of peptides, which formed 12-MC-4.

Figure 7. The 2D [^{1}H,^{1}H] TOCSY spectrum for **(A)** free Ac-KLH-NHOH peptide and for **(B)** metallacrown (12-MC-4) formed upon Cu^{2+} binding (L:Cu^{2+} 1:0.2 (n:n)) at pH = 6.8.

The hydrogen-deuterium exchange (HDX) of the C2-hydrogen of the imidazole ring was monitored using mass spectrometry. These experiments were performed for the Ac-KLH-NHOH peptide and its complex with Cu^{2+} ions. After optimization of the exchange reaction, we measured the back exchange[50] of deuterium to hydrogen (DHX) on the C2 carbon. This approach has many advantages, e.g. elimination of the error associated with the introduction

of additional protons (from buffers) during back-exchange. The preparation of samples is described in the Experimental Section. The deuterium-hydrogen exchange can be analysed as a pseudo-first order process. The kinetics parameter k_i (first-order rate constant) of the DHX reaction can be determined as:

$$\ln Y = \frac{I_M(t)I_{M+1}(t) + I_{M+1}(t)I_{M+1}(0) - I_M(t)I_{M+2}(0)}{I_{M+1}(t)I_{M+1}(0) - I_M(t)I_{M+2}(0)} = k_i t$$

$$lnY = k_i t$$

where I_M (0) and I_{M+1} (0) are the intensities of isotopic peaks with masses M and M+1 after incubation in D_2O. The k_i value is characteristic of the peptide at each pH. Its values were determined as a slope of the lnY(t) function.

Recent paper[51,52] has demonstrated that binding of metal ions influences the hydrogen-deuterium exchange of the imidazole ring C2-hydrogen in histidine-containing peptides. The k values for the peptide at pH 5.2 and its complex at pH 5.2, 7.2, 7.3, 8.2, and 8.3 are equal to 0 within experimental error limits. Therefore, according to the data presented in **Table S2** (supplementary information) the addition of Cu^{2+} ions completely inhibits the isotopic exchange at the pH range in which formation of metallacrowns is postulated. The observed effect is even better pronounced than that observed for binding of zinc ions by histidine residues of metalloproteins[51]. This experiment clearly demonstrates that the histidine imidazole ring is involved in the interactions with Cu^{2+} ions and the equilibrium is almost completely shifted towards complex formation.

The spectroscopic and potentiometric studies as well as ESI-MS spectrometry revealed that for both investigated ligands the metallacrown system is predominant at a relatively broad pH range. To compare the affinity of Ac-KLH-NHOH and [AH]-NHOH towards Cu(II) ions, we calculated the competition curves (**Figure 8B**). According to the presented data, Ac-KLH-

NHOH seems a more efficient ligand for Cu^{2+} ions at the whole pH range. On the basis of literature data[53,54,55] we expect that attaching the amphipathic sequence to the histidinehydroxamic acid will stabilize a complex formed. In addition, our previous studies[2] demonstrated that the interactions of amphipathic peptide aminohydroxamic acids resulted in a shift of the conformational equilibrium to helical structures. The complexation properties of the low-molecular derivative of histidine (**Figure 8A**) (Z-Hisha) have been presented by Farkas[29]. The equilibrium constant reported in the literature indicates that a more stable metallacrown is formed by Z-Hisha ($\log\beta_{Cu5L4H-4}$ = 38.50)[29] than by Ac-KLH-NHOH ($\log\beta_{Cu5L4H-4}$ = 36.03) and [AH]-NHOH ($\log\beta^* = \log\beta_{Cu5(HL)4H-4} - 4x\log\beta_{HL}$ = 34.00). These data show that the stability of metallacrowns formed by ligands containing histidinehydroxamic acid moieties is lower for systems containing a more bulky substituent. The stability order of the examined metallacrowns is presented in **Figure 9.**

The comparison of our results with that obtained by Farkas for short histidinehydroxamic acid moieties containing peptides suggests that the absence of N-teminal α-amino group is essential for the hydroxamate-type Cu complexation and results in metallacrown formation. This result seems unexpected, since many of Cu 12-MC-4 metallacrowns with α-aminohydroxamates have been identified and in solution[45] however it may be explained by excluding the competition of N-terminal amino group with the imidazole nitrogen.

One of the key objectives of our research was evaluation of the stability of metallacrowns as a framework for peptides self-assembly. We chose the amphipathic sequence which forms a tetrahelical bundle as we demonstrated in a previous paper basing on the CD and NMR measurements[2]. The distinct increase of the helical structures content in the conformational equilibrium demonstrated by circular dichroism can be explained by interactions of leucine moieties in amphipathic sequences. However, surprisingly, these interactions do not result in stabilization of the supramolecular structures formed, expressed as the stability constants. In

our opinion the equilibrium of peptide hydroxamic acids-based metallacrowns formation is controlled by two factors: the interactions of the peptide side chains (hydrophobic or electrostatic interactions and hydrogen bonds) and the steric effects of bulky substituents destabilizing the metallacrown scaffold. In the case of [AD]-NHOH, the destabilizing effect is predominant.

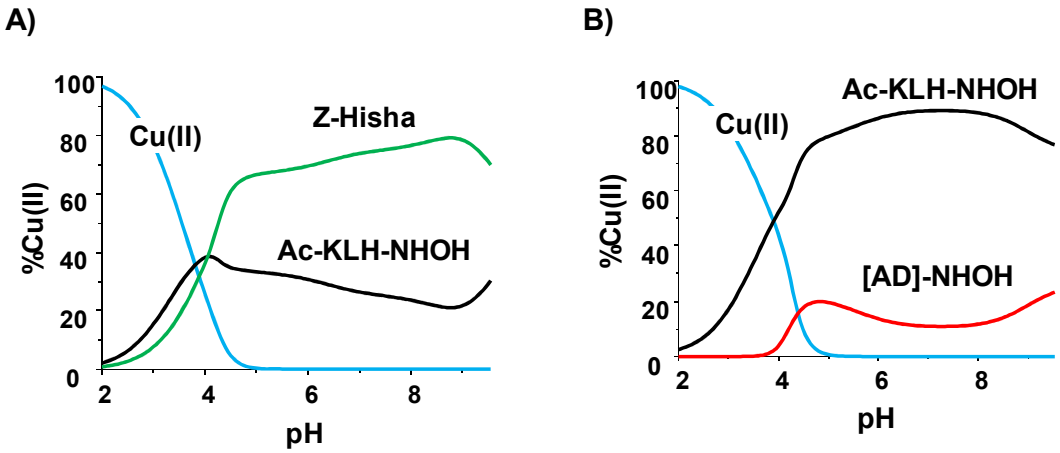


Figure 8. Competition curves for the systems with nL_1:Cu(II):nL_2: A) L_1 - Ac-KLH-NHOH, L_2 - Z-Hisha; B) L_1 - Ac-KLH-NHOH, L_2 - [AH]-NHOH.

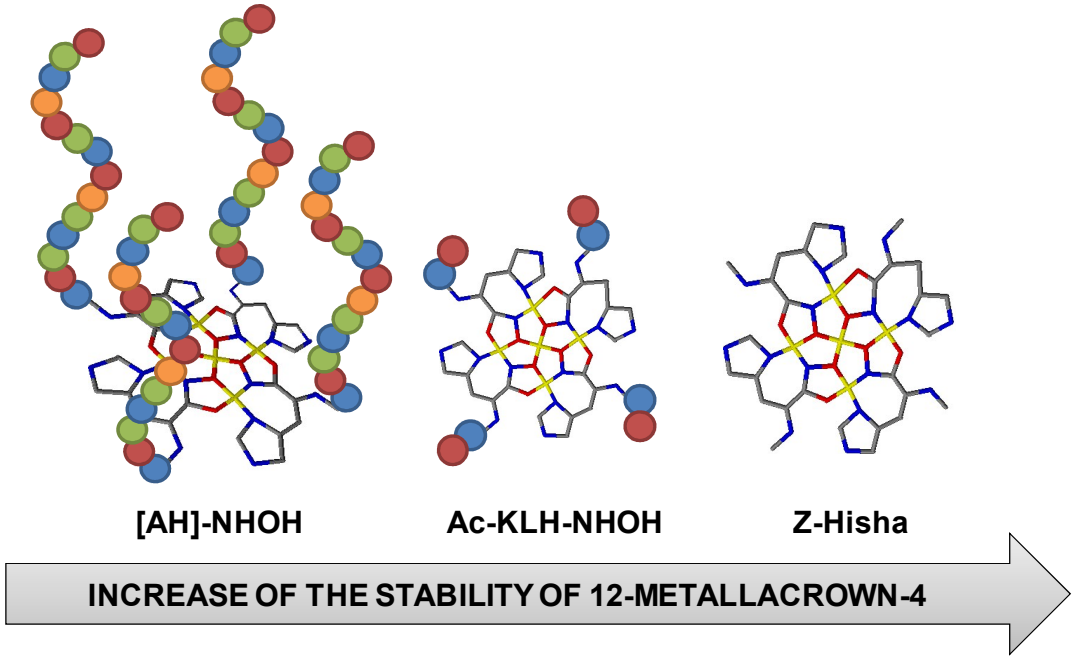


Figure 9. Stability order of various 12-metallacrowns-4 according to the ligand type.

Conclusion

The present studies show that 12-metallacrown-4 is a predominant species at pH 4.5-9.5 for two exanimated ligands: Ac-KLH-NHOH and [AH]-NHOH. The spectral parameters characterizing the complexes formed by both ligands are very similar to those characterizing metallacrowns described previously in the literature. Interpretation of the NMR spectrum of the Ac-KLH-NHOH-Cu(II) complex suggests that the imidazole and hydroxamate moieties are involved in metal binding, while the DOSY data indicate a significant increase of the molecular weight resulting from the interaction of the Ac-KLH-NHOH ligand with Cu^{2+} ions, in agreement with formation of the metallacrown. This paper is a first report on the thermodynamic stabilities of metallacrowns formed by peptidehydroxamic acid systems.

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The interactions of two peptide hydroxamic acids (tripeptide and pentadecapeptide) with copper(II) ions were characterised using spectroscopic methods, ESI mass spectrometry and physicochemical techniques revealing formation of metallacrowns characterized by L_4Cu_5 stoichiometry. The influence of peptide length on metallacrown stability was determined.