Short thesis for the degree of doctor of philosophy (PhD)

Transition metal complexes of aspartic acid, glutamic acid, histidine and cysteine containing peptides

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Debrecen, 2020

I. INTRODUCTION AND AIM OF WORK

Nowadays, it is obvious that metal ions interact with proteins of living organisms during complex compound formation. Two classes of elements can be separated based on their effect on living organisms: (i) essential and (ii) contaminant elements. The latter category can be divided further: (i) toxic and (ii) indifferent elements. Cu and Zn are definitely essential elements, while Cd is toxic, and the effect of Ni(II) depends on the quality of the studied organism.

Metal ions get into living organisms by being consumed or an external effect (for example industrial work, environmental pollution). They usually interact with oligo- and poly-peptides through their slightly and strongly coordinating side chains by coordinative bond in cells. Aspartic and glutamic acid side chains (carboxyl group) are weak, while histidine (imidazolil group) and cysteine (thiolate group) ones are strong coordination sites for metal ions. Metal ions take part in different processes: (i) forming structure (Zn(II)-finger proteins) (ii) catalysing electron transition in the active site of proteins, furthermore they can participate in harmful processes, for example replacing essential metal ions, oxidative stress, blocking enzymes, forming neurodegeneration. When a metal or its ion accumulates in an organism acutely, later chronic poising appears.

The interaction between the aforementioned side chains, proteins and peptides and metal ions have been studied for decades:
According to the literature data the metal ion selectivity of one, two or more aspartic and glutamic acid containing peptides were less investigated than histidine and cysteine containing ones.
Cysteine- and multicysteine-peptides got into the centre of a lot of publications lately because they can react not only with essential metal ions (Zn(II), Fe(II)/Fe(II)) but also with toxic ones (Cd(II), Pb(II)).

The forming of abnormal structures of such peptid/proteins, which contain histidine residues in different number and position (such as amyloid-β (Alzheimer's disease), prion protein (spongiform encephalopathy), tau protein (tauopathy) are responsible for the evolving of neurodegenerative disorders. Tau protein as well as amyloid-β take part in Alzheimer's disease, which affects the senior part of the population. The systematic solution equilibrium study of tau protein and its fragments contributes to the elucidation the molecular background of these diseases.

Systematic studies related to all the aforementioned research fields in the Bioinorganic Research Group at the University of Debrecen are in progress. I joined these studies and our aims were during my work were:

1. Investigate the Cd(II)-ion bonding affinity of one and more aspartic and glutamic acid containing peptides.
2. The systematic investigation of the small peptides containing one or two cysteinyl residues, which can support the interpretation of the solution equilibrium processes of long peptides. During the studies we focused on, how the number and position of the cysteinyl residues in the sequence of the peptides influence the stoichiometry and stability of their Cd(II) and Zn(II) complexes. Studies were completed with the analysis of Ni(II) complexes.
3. Studies of the model peptides mimicking the 14th and 32th histidine (His14 and His32) binding site of the native tau proteins primarily in the presence of Cu(II). Ni(II) complexes can support the interpretation of the solution equilibrium forms of the Cu(II) complexes, thus, I also investigated Ni(II) complexes in some cases.

II. EXPERIMENTAL METHODS

The p*K* values of the ligands and the stability constants of Cu(II), Zn(II), Ni(II) and Cd(II) complexes were determined by **pH potenciometry**. The measurements were carried out in aqueous media at 298 K temperature, at 0.20 mol/dm^3 ionic strength, in the absence of CO_2, O_3 and O_2 gases. The metal ion/ligand ratio was ranging between 1:4 and 2:1. The stability constants of the metal complexes were determined by PSEQUAD and SUPERQUAD computational programs from experimental data. Based on the calculated stability constants the concentration distribution curves were constructed by MEDUSA program.

Cu(II), Ni(II) and Cd(II) complexes as well as the solution of thiol group containing ligands were studied by **UV and UV-VIS spectroscopy**. The spectra were recorded on Perkin Elmer Lambda 25 double beam spectrophotometer in well closing 1.000 cm cuvettes at varying metal ion/ligand ratios (2:1, 1:1, 1:2, and 1:3) and pH, in the 200-900 nm wavelength range. The ratios and the pH values were chosen according to the potenciometric titrations.

Cu(II) and Ni(II) complexes are CD active, since they contain chirality centres. CD measurements were performed on a J-810 spectropolarimeter at the Department of Organic Chemistry at the University of Debrecen and at CNR-IBB research center (Consiglio Nazionale Delle Ricerche - Instituto Di Biostrutture e Bioimmagini, Catania, Italy). The pH dependence was recorded in aqueous solution, using in some cases discrete samples, at 2:1, 1:1, 1:2 and 1:3 metal ion/ligandum ratios, at varying pH values, in 0.100 and 1.000 cm cuvettes, in the 200-900 nm wavelength range.

To determine the number of thiolate groups in Cd(II) complexes ^{113}Cd NMR spectroscopy was used. The spectra were recorded on a

Bruker AMX 400 MHz instrument, and for the interpretation of data MestreNova 8.1 software was used.

EPR can be used in the case of molecules that contain unpaired electrons (at paramagnetic molecules). This statement is true for Cu(II), thus its complexes can be investigated by this method too. The binding mode and the quality of the coordinated donor atoms can be concluded from the obtained data through the comparison of the spectral parameters of the complexes.

Measurements were carried out by Giuseppe Pappalardo and his co-workers at CNR-IBB. The measurements were performed on a Bruker Elexsys E500 CW-ESR instrument and the samples contained less than 10% methanol. The temperature was 150 K, and different metal ion/ligand ratios and pH were set.

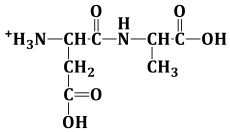
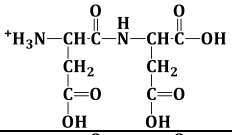
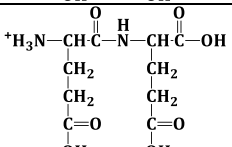
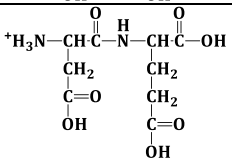
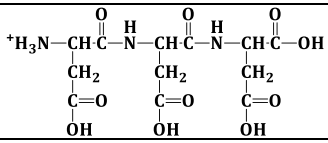
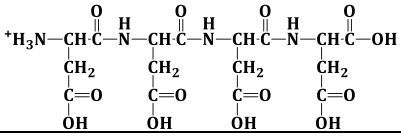
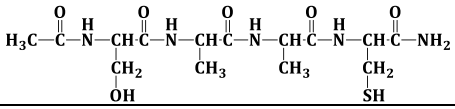
To reinforce the stoichiometry and coordination modes of the complexes MS and MS/MS spectrometry was used. The investigations were also carried out by Giuseppe Pappalardo and his co-workers (in Italy), using a Q Exactive (orbitrap) MS instrument (Thermo Fisher Scientific), in positive mode.

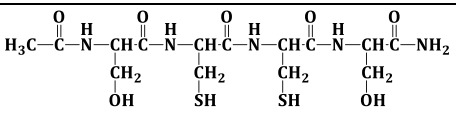
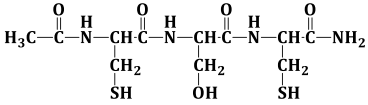
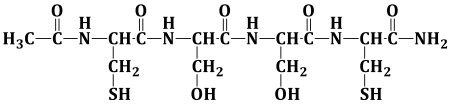
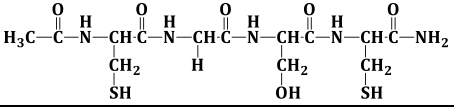
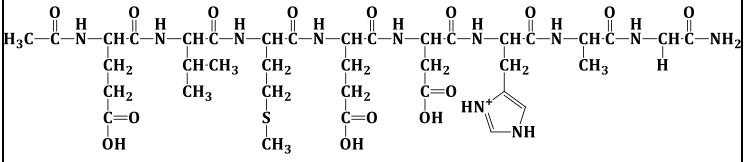
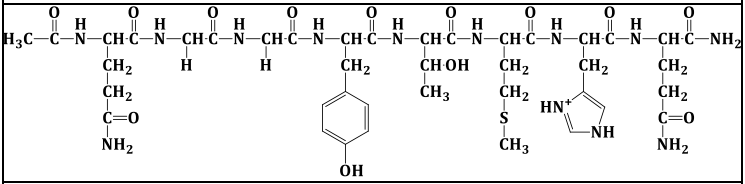
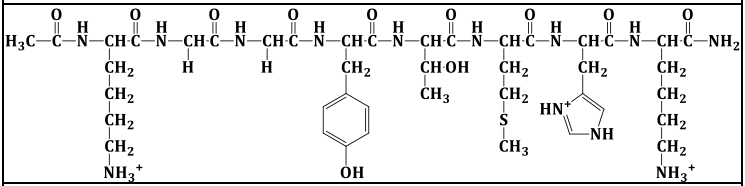
III. STUDIED LIGANDS

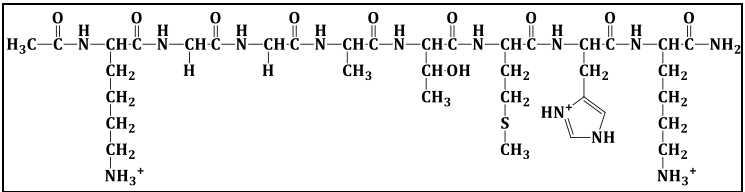
One part of the peptides was obtained from commercial source: aspartic and glutamic acid containing peptides (DA, DD, EE, DE, DDD, DDDD) were produced by Bachem AG. Cysteine derivatives (Ac-SAAC-NH_2, Ac-SCCS-NH_2, Ac-CSC-NH_2, Ac-CSSC-NH_2, Ac-CGSC-NH_2) and Ac-EVMEDHAG-NH_2 were synthesized in the Bioinorganic Research Group at the University of Debrecen, while other tau fragments (Ac-QGGYTMHQ-NH_2, Ac-KGGYTMHK-NH_2, Ac-KGGATMHK-NH_2, Ac-EDHAGTMHQD-NH_2) were produced at CNR-IBB. All the structural and molecular formula of the studied peptides are shown in Table 1.

Microwave-assisted Liberty 1^{TM} (CEM, Matthews, NC) and Fmoc/*t*Bu strategy were used to obtain peptides. The protection of the free N-terminal part was carried out by acetylation, the C-terminal protection was provided by the Rink Amide AM resin. During synthesis the activation of the amino and carboxylic functional groups was achieved by TBTU/HOBt/DIEA strategy. The removal of the resin and the side chain protection groups was carried out by a solution which contains TFA/H_2O/TIS(/DODT). The peptide was yielded from this solution by precipitation in diethyl ether. After filtering or centrifugation the products were lyophilized. The purity of the ligands was verified by HPLC, ESI-MS, ESI-TOF or/and MALDI-TOF techniques. If the compounds contained only peptide and inert contaminant then we used the peptides without any further purification. If purifying was necessary RP-HPLC was used.

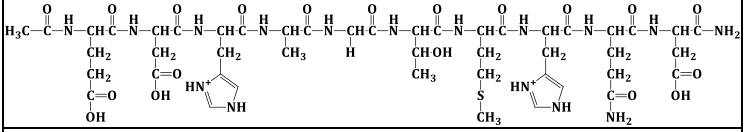
Table 1.: *The structural and molecular formula of the studied peptides*

Structural formula of peptides	**Molecular formula**
	DA
	DD
	EE
	DE
	DDD
	DDDD
	Ac-SAAC-NH_2

	Ac-SCCS-NH_2
	Ac-CSC-NH_2
	Ac-CSSC-NH_2
	Ac-CGSC-NH_2
	
Ac-EVMEDHAG-NH_2, Tau(9-16)	
	
Ac-QGGYTMHQ-NH_2, Tau(26-33)	
	
Ac-KGGYTMHK-NH_2, Tau(26-33)m	



Ac-KGGATMHK-NH_2, Tau(26-33)mA



Ac-EDHAGTMHQD-NH_2, Tau(12-16)(30-34)

IV. NEW SCIENTIFIC ACHIEVEMENTS

4.1. We characterised the Cd(II) binding affinity of peptides contain aspartic and glutamic acid in rising number.

- It was found that the complex formation starts with the coordination of carboxylate groups, which results in the formation of protonated complexes at slightly acidic pH.
- Ammonium group deprotonates in parallel with the rising of the pH and $[NH_2,(COO^-)_x]$ binding mode emerges, which cannot hamper the hydrolysis of the metal ion at the alkaline pH range.
- The rising number of the coordinated carboxylate groups causes a slight stability enhancement of the [CdL] complexes, which is not affected by the rising negative charge of the complexes.
- After comparing these results to the previous results of other metal complexes it can be declared that the peptides are not selective toward Cd(II) ion. (Figure 1)

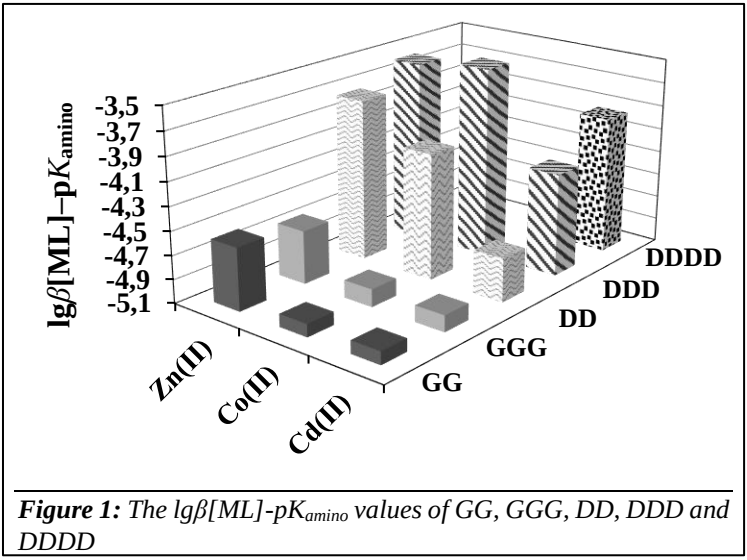


Figure 1: *The lgβ[ML]-pK_{amino} values of GG, GGG, DD, DDD and DDDD*

4.2. We characterised the Cd(II) and Zn(II) complexes of one or two cysteine residue comprising peptides compared to previously studied cysteine containing peptides.

- It was found that stable mono and bis(ligand) complexes are formed, and the stability follows the: Cd(II) > Zn(II).
- It can be concluded from the comparison of the stability of the one cysteine containing peptide with those of Ac-DAAC-NH_2 and Ac-HAAC-NH_2 complexes that the other donor atoms in the peptide side chains enhances the stability of the complexes. In the case of all studied metal ions the stability of complexes changes in the following order: Ac-SAAC-NH_2 < Ac-DAAC-NH_2 < Ac-HAAC-NH_2.
- The stability of the [S^-,S^-] coordinated complexes of the two cysteine containing peptides is the largest when a 12 or 15 membered macrochelate is present.
- If we compare the obtained data to the previously determined results of two terminal donor group containing peptides it can be

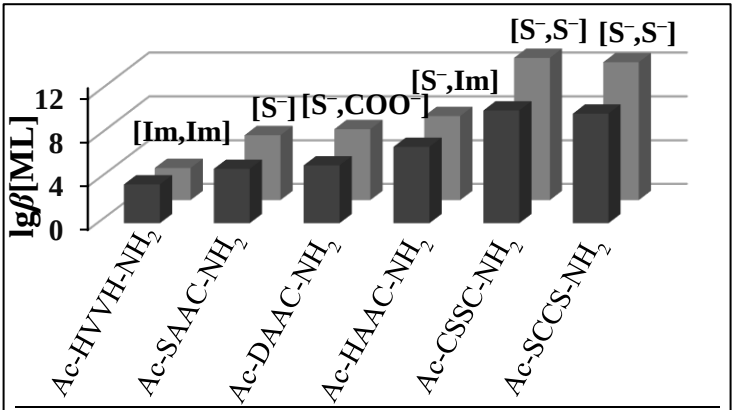


Figure 2: *The stability constants of different binding mode containing peptides*

concluded that the stability of the Cd(II) and Zn(II) complexes, consider the binding mode, varies in the next order: [Im,Im] < [S^-] < [S^-, COO^-] < [S^-,Im] < [S^-,S^-] (Figure 2).

4.3. We studied how the increasing distance between the cysteine residues influences the stoichiometry of the Ni(II) complexes of the two cysteine containing peptides.

- We stated that the formation of polinuclear complexes is favoured in the presence of Ni(II) ion at slightly acidic and neutral pH. Thiolate-S^- binds to two metal ion, as a bridge ligand, such as in $[Ni_3L_4]^{2-}$ (Figure 3).

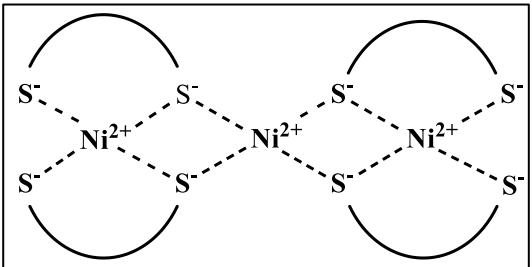


Figure 3: *The schematic figure of* $[Ni_3L_4]^{2-}$ *complex*

These results complete those of similar studies on peptides published earlier, by which only the existence of mononuclear complexes has been established. [1-3]

- It was established that the side chain of the C-terminal cysteine residue is the primary binding site of the metal ion.

[1] M. Rowinska-Zyrek, D. Witkowska, S. Bielinska, W. Kamysz, H. Kozlowski; *Dalton Transactions*, **2011** (40) 5604-5610.
[2] M. Rowinska-Zyrek, D. Witkowska, D. Valesin, W. Kamysz, H. Kozlowski; *Journal of Inorganic Biochemistry*, **2007** (101) 1699-1706.
[3] P. Kolkowska, K. Krzywoszynska, S. Potocki, P. R. Chetana, M. Spodzieja; S. Rodziewicz-Motowidolo, H. Kozlowski; *Dalton Transaction*, **2015** (44) 9887-9900.

- We can state that the increasing of the distance between the cysteine residues in the sequence of the peptides: (i) reduces the tendency to the formation of polinuclear complexes (ii) aside from the anchoring thiol group, the second thiol group hampers the deprotonation and coordination of the previous peptide-NH groups to a lesser extent.
- The comparison of the stability of [ML] complexes the following order can be set: Cd(II) > Pb(II) > Zn(II) > Ni(II), namely, the ligands bind Cd(II) ion the most efficiently. This order is well demonstrated by the concentration distribution diagram (Figure 4), which shows the total ratio of the different metal ion complexes.

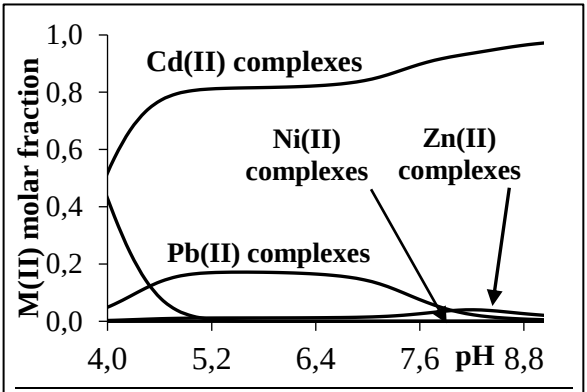


Figure 4: *Concentration distribution diagram of Cd(II):Zn(II):Ni(II):Pb(II):Ac-CSSC-NH$_2$ 1:1:1:1:1 system*

4.4. We investigated the complex formation properties of native fragments and their mutants of tau protein containing His32 and His14 binding sites in the presence of Cu(II) and Ni(II) ion.

- It was established that the histidine residues are able to bind both the Cu(II) and Ni(II) ions, however, the thermodynamic stability of the Ni(II) complexes are significantly lower than that of Cu(II) complexes.
- Histidine residues serve as the primary binding sites for the metal ions, but the carboxylate groups in the sequence of Tau(9-16) peptide enhances the stability of the M(II)-Im coordinated complexes: (i) this phenomenon occurs at slightly acidic pH in the case of Cu(II) ion while (ii) this appears in the physiological pH range in the presence of Ni(II) ion.
- The deprotonation and coordination of peptide-NH donor groups preceding the histidine residue takes place at higher pH range: (i) the process happens in two steps in the case of Cu(II) ion, while (ii) it happens in a cooperative manner in the presence of Ni(II) ion.
- It was stated that the different environments of the two histidine residues causes different CD spectra in the case of Tau(9-16) and Tau(26-33) and the mutants of latter sequences. This phenomenon gives us a chance to distinguish the His14 and His32 binding sites.
- It was concluded, that both metal ions prefer the His32 binding site (Figure 5) at alkaline pH.
- In the Tau(12-16)(30-34) peptide the two histidine containing sequences were connected. This causes differences: (i) the coordination of two imidazolyl group enhances the stability both the Cu(II) and Ni(II) complexes (ii) Tau(12-16)(30-34) can bind two equivalent Cu(II) ion. The spectroscopic investigation of the

equimolar systems reinforced the statement, that His32 of N-terminal part of the tau protein is the primary binding site for both metal ions.

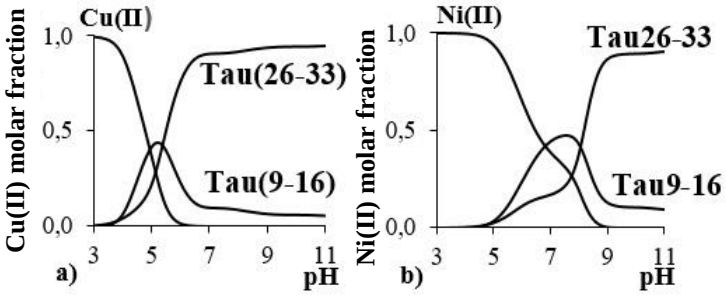


Figure 5: *Concentration distribution curves of Cu(II):Tau(9-16):Tau(26-33) 1:1:1 (a) and Ni(II):Tau(9-16):Tau(26-33) 1:1:1 systems*

V. POSSIBLE APPLICATION OF THE RESULTS

Interaction between various, biologically important metal ions and model peptides of different proteins have been being studied by in vitro circumstances in the Bioinorganic Chemistry Research Group of the University of Debrecen. The achieved results provide scientifically correct bases for the biologically and/or medical investigation in the future.

During my work light is thrown on that $[NH_2,(COO^-)_x]$ (x = 2-5) coordination mode itself is not enough to bind Cd(II) ion selectively, thus the presence of another donor group is also needed in the sequence of the peptides to enhance the selectivity.

Our results show that the CXXC and CXXXC sequences are most capable of the selective binding of Cd(II) at the physiological pH range, thus such peptides can be designed by using them, which may bind Cd(II) selectively. The achievements of the Ni(II) complexes contributes to interpret the spectral properties and structure of metal complexes of larger peptides containing cysteinyl residues in different environment.

A general conclusion cannot be drawn in the case of tau fragments without the coordination chemical knowledge of the manner of other metal binding sites in the protein. However, the different metal ion binding ability of the two N-terminal binding sites contributes to elucidate the molecular background of tauopathies.

VI. TUDOMÁNYOS KÖZLEMÉNYEK (PUBLICATIONS)

Az értekezés alapját képző közlemények (Articles connected to the thesis) (2):

Tudományos folyóiratban megjelent közlemények (Published articles) (2):

1. M. Lukács, Gy. Szunyog, Á. Grenács, N. Lihi, Cs. Kállay, G. Di Natale, T. Campagna, V. Lanza, G. Tabbi, G. Pappalardo, I. Sóvágó, K. Várnagy, Copper(II) **Coordination Abilities of the Tau Protein's N-Terminus Peptide Fragments: A Combined Potenciometric, Spectroscopic and Mass Spectrometric Study**, *ChemPlusChem*, 84 (2019) 1697-1708. IF: 2,753
2. N. Lihi, M. Lukács, M. Raics, Gy. Szugyog, K. Várnagy, Cs. Kállay, **The effect of carboxylate groups on the complexation of metal ion with oligopeptides – Potentiometric investigation**, *Inorganica Chimica Acta*, 472 (2018) 165-173. IF: 2,433

Az értekezés anyagához kapcsolódó előadások (Lectures connected to the thesis) (7):

1. Lukács Márton, Szunyog Györgyi, Grenács Ágnes, Kállay Csilla, Giuseppe Di Natale, Giuseppe Pappalardo, Cire Gizella, Várnagy Katalin, Sóvágó Imre: **A tau fehérje két kötőhelyét modellező peptidek Cu(II)- és Ni(II)-komplexeinek oldategyensúlyi vizsgálata**, 53. Komplexkémiai Kollokvium, Velence, 2019.05.21.-05.23., E11
2. Lukács Márton, Szunyog Györgyi, Grenács Ágnes, Giuseppe Di Natale, Giuseppe Pappalardo, Cire Gizella, Várnagy Katalin, Sóvágó Imre: **A tau fehérje két kötőhelyét modellező peptidek Cu(II)-komplexeinek oldategyensúlyi vizsgálata**, Tavaszi Szél Konferencia, Debrecen, 2019.05.03.-05.05., 311. oldal
3. Lukács Márton, Szunyog Györgyi, Grenács Ágnes, Giuseppe Di Natale, Giuseppe Pappalardo, Cire Gizella, Várnagy Katalin, Sóvágó Imre: **A tau fehérje két kötőhelyét modellező peptidek Cu(II)-komplexeinek oldategyensúlyi vizsgálata**, I. Fiatal

Kémikusok Fóruma Szimpózium Konferencia, Debrecen, 2019.04.03-04.05., 39. oldal

4. Lukács Márton, Kerekes Zsuzsanna, Várnagy Katalin: **Tau fehérje kötőhelyét modellező peptidek vizsgálata**, Tavasz Szél Konferencia, Győr, 2018. 05.04.-05.06., 203. oldal
5. Lukács Márton, Várnagy Katalin: **Terminálisan védett cisztein tartalmú peptidek oldategyensúlyi és spektroszkópiás vizsgálata**, 51. Komplexkémiai Kollokvium, Balatonvilágos, 2017.05.29-05.31., E11
6. Lukács Márton, Várnagy Katalin: **Terminálisan védett cisztein tartalmú peptidek oldategyensúlyi és spektroszkópiás vizsgálata**, Tavaszi Szél Konferencia, Miskolc, 2017.03.31-04.02., 195. oldal
7. Lukács Márton, Várnagy Katalin: **Terminálisan védett cisztein tartalmú peptidek oldategyensúlyi és spektroszkópiás vizsgálata**, XXXIII. Országos Tudományos Diákköri Konferencia, Miskolc, 2017.03.29-03.31., 16. oldal

Az értekezés alapját képező poszterek (Posters connected to the thesis (5):

1. Lukács Márton, Szunyog Györgyi, Grenács Ágnes, Giuseppe Di Natale, Giuseppe Pappalardo, Várnagy Katalin, Sóvágó Imre: **Metal ion binding ability of the free N-termini of tau protein: appraisal of preference by solution equilibrium study**, International Symposium on Metal Complexes, Hajdúszoboszló (Debrecen), 2019.06.11-06.14., 131-132. oldal
2. Giuseppe Di Natale, Lukács Márton, Valeria Lanza, Giovanni Tabbi, Tiziana Campagna, Kállay Csilla, Giuseppe Pappalardo: **Copper(II) binding within the N-terminal region of the Tau protein: the use of model peptides for the evaluation of metal ion binding preferences**, International Symposium on Metal Complexes, Hajdúszoboszló (Debrecen), 2019.06.11-06.14., 99. oldal
3. Grenács Ágnes, Lihi Norbert, Bodnár Nikolett, Pataki Zsombor, Pálinkás Dóra Csilla, Lukács Márton, Várnagy Katalin: **Effects of polar side chains on the formation of nickel(II), zinc(II) and cadmium(II) complexes of cysteine peptides**,

International Symposium on Metal Complexes, Hajdúszoboszló (Debrecen), 2019.06.11-06.14., 112. oldal

4. Lukács Márton, Szunyog Györgyi, Lihi Norbert, Grenács Ágnes, Giuseppe Di Natale, Giuseppe Pappalardo, Várnagy Katalin: **Copper(II) complexes of peptides mimicking the Tau protein binding sites**, 35th International Conference on Solution Chemistry, Szeged, 2018.08.26.-08.30., 91. oldal
5. Lukács Márton, Várnagy Katalin: **Cadmium(II) and zinc(II) complexes of terminally protected tetrapeptides containing cysteinyl residue**, 13th European Biological Inorganic Chemistry Conference Budapest, 2016.08.28.-09.01., 161. oldal

Az értekezés alapját képező egyéb publikációk (Other related publication) (1):

1. Lukács Márton, Szunyog Györgyi, Grenács Ágnes, Giuseppe Di Natale, Giuseppe Pappalardo, Várnagy Katalin, Sóvágó Imre: **A tau fehérje két kötőhelyét modellező peptidek Cu(II) komplexeinek oldategyensúlyi vizsgálata**, I. Fiatal Kémikusok Fóruma konferencia kiadvány, Debrecen, 2019.04.03.-04.05., 97-102., 97-101. oldal

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Registry number: DEENK/244/2020.PL
Subject: PhD Publikációs Lista

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List of publications related to the dissertation

Foreign language scientific articles in international journals (2)

1. **Lukács, M.**, Szunyog, G., Grenács, Á., Lihi, N., Kállay, C., Di Natale, G., Campagna, T., Lanza, V., Tabbi, G., Pappalardo, G., Sóvágó, I., Várnagy, K.: Copper(II) Coordination Abilities of the Tau Protein's N-Terminus Peptide Fragments: A Combined Potentiometric, Spectroscopic and Mass Spectrometric Study.
   ChemPlusChem. 84 (11), 1697-1708, 2019. ISSN: 2192-6506.
   DOI: http://dx.doi.org/10.1002/cplu.201900504
   IF: 2.753
2. Lihi, N., **Lukács, M.**, Hadháziné Raics, M., Szunyog, G., Várnagy, K., Kállay, C.: The effect of carboxylate groups on the complexation of metal ion with oligopeptides - Potentiometric investigation.
   Inorg. Chim. Acta. 472, 165-173, 2018. ISSN: 0020-1693.
   DOI: http://dx.doi.org/10.1016/j.ica.2017.07.032
   IF: 2.433

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List of other publications

Foreign language scientific articles in international journals (2)

3. Balogh, B. D., Bihari, Z., Buglyó, P., Csire, G., Kerekes, Z., **Lukács, M.**, Sóvágó, I., Várnagy, K.: Metal binding selectivity of an N-terminally free multihistidine peptide HAVAHHH-NH. *New J. Chem. 43* (2), 907-916, 2019. ISSN: 1144-0546.
   DOI: http://dx.doi.org/10.1039/C8NJ04538K
   IF: 3.288

4. Lihi, N., **Lukács, M.**, Szűcs, D., Várnagy, K., Sóvágó, I.: Nickel(II), zinc(II) and cadmium(II) complexes of peptides containing separate aspartyl and cysteinyl residues. *Polyhedron. 133*, 364-373, 2017. ISSN: 0277-5387.
   DOI: http://dx.doi.org/10.1016/j.poly.2017.05.044
   IF: 2.067

Total IF of journals (all publications): 10,541
Total IF of journals (publications related to the dissertation): 5,186

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

26 August, 2020

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