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Research Article

Study of peptide–ligand interactions in open-tubular capillary columns covalently modified with porphyrins

The inner surface of fused silica capillaries has been covalently modified with different porphyrins (deuteroporphyrin, complexes of deuteroporphyrin with metal ions Fe(III), Cu(II), Zn(II), Ni(II), and Cu(II)–meso–tetra (carboxyphenyl) porphyrin) and it was applied for the separation of biologically active peptides by open-tubular capillary electrochromatography. Separations were performed in a mobile phase composed of 25 mM potassium phosphate, pH 4.0, 5% v/v ACN and 10 mM hydroquinone. Changes in the effective electrophoretic mobility of peptides were studied concerning porphyrin central metal atom, attachment geometry, and the presence of coordinating or aromatic amino acid residues in the peptide sequence. The results showed that differences in metal core on the porphyrin and the spatial conformation of attached porphyrin result in changes in the analyte interaction with the stationary phase.

1 Introduction

Porphyrins, a group of naturally occurring macrocyclic compounds, have a recognized number of chemical characteristics that can be exploited in analytical chemistry. Porphyrins and their derivatives present different binding capabilities necessary for specific interactions. The tetra-pyrrolic ring exhibits the aromatic character appropriate for π–π stacking interactions, and the porphyrin core forms 1:1 complexes with transition metal ions, which are able to incorporate axial ligands. In addition, they can bind oppositely charged molecules through ionic interactions of their ionogenic groups. Such properties made porphyrin derivatives and their metallo-complexes attractive selective agents in analytical chemistry [1–3]. Particularly with reference to chromatographic and electromigration techniques, various porphyrinic compounds have been applied as ligands for separating different compounds, e.g., carboxylic acids, amino acids, peptides, and nucleotides [4–6].

After mapping the genome of several species, including the human genome, the mapping of the proteome is one of the most ambitious research projects mankind has ever undertaken. Currently, the structure and the function of many proteins are studied through their peptide fragments obtained from enzymatic hydrolysis [7–10]. Thus, the importance of peptide analysis is even increasing and the advances in the field of microseparation techniques are expected to provide a valuable alternative to conventional slab-gel electrophoresis or HPLC, in terms of speed, reagent consumption, and separation efficiency. In this context, capillary electromigration methods have demonstrated a broad applicability in amino acid, peptide, and protein separations by development of different modes of CE techniques [11–19].

The combination of separation principles of CE and HPLC resulted in the rapidly expanded CEC. This powerful technique presents the benefits of high separation efficiency of CE and high selectivity of LC. CEC has successfully been applied to the analysis of a wide range of molecules [20–22]. Among the available different column designs for CEC, open-tubular capillary columns offer some advantages over packed capillaries. In open-tubular CEC (OT-CEC), a thin layer stationary phase is immobilized onto the inner capillary surface, either by physical adsorption or by covalent attachment. The small column diameter allows faster separations with higher electric field strength without significant Joule heating. Moreover, the absence of packing

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Abbreviations: Cu-dP, Cu(II)-deuteroporphyrin; **Cu-TCPP,** Cu(II)–meso–tetra (carboxyphenyl) porphyrin; **dP,** deuteroporphyrin; **Fe-dP,** Fe (III)-deuteroporphyrin; **FS,** fused silica; **HQ,** hydroquinone; **LHRH,** luteinizing hormone releasing hormone; **Ni-dP,** Ni(II)-deuteroporphyrin; **OT-CEC,** open-tubular CEC; **Zn-dP,** Zn(II)-deuteroporphyrin

Colour Online: See the article online to view Fig. 6 in colour.

material and end frits leads to a limited band broadening and avoids flow restriction through the capillary [23].

Relevant performance characteristics of OT-CEC plus the rich variety of possible interactions between porphyrins and peptides can bring an important improvement in the selectivity of the separation system [4]. It is worth noting that the influence of peptide amino acid sequence on peptide–porphyrin interactions and, consequently, the peptide behavior during the separation step results often unpredictable. However, the presence of certain functional groups has demonstrated to condition peptide–porphyrin interactions [6, 24–26].

In this study, changes in the migration/retention behavior of five biologically active peptides were investigated. Peptide effective electrophoretic mobility was studied in capillary columns covalently modified with different porphyrins. Parameters related with reaction conditions, such as the spatial conformation and the amount of fixed porphyrin, were also considered.
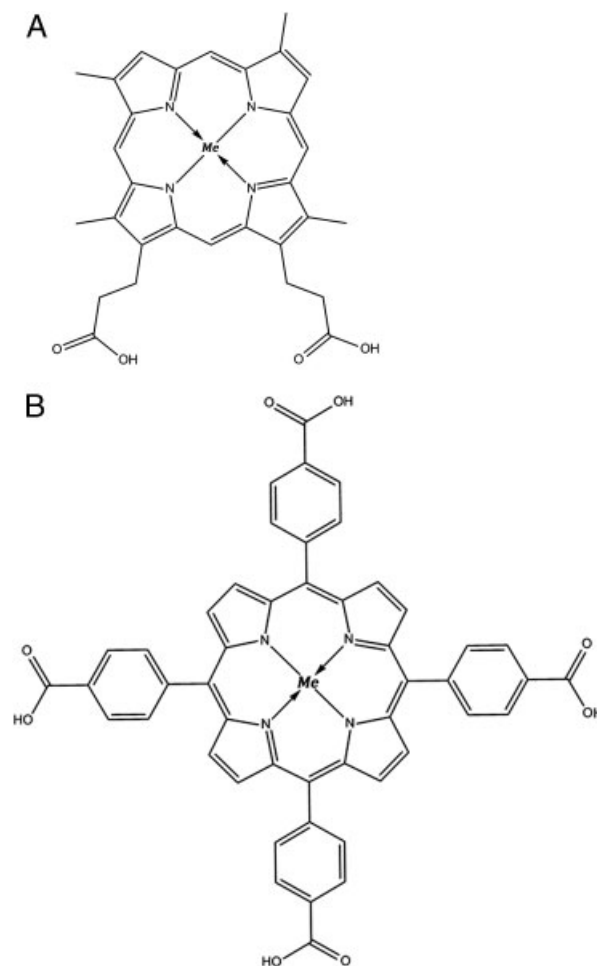
2 Materials and methods

2.1 Chemicals

Disodium hydrogen phosphate, potassium dihydrogen phosphate, sodium hydroxide, phosphoric acid, formic acid, hydroquinone (HQ), ACN, THF, chloroformate, and triethanolamine were purchased from Merck (Darmstadt, Germany). The bioactive synthetic peptides bradykinin, angiotensin I, luteinizing hormone releasing hormone (LHRH), oxytocin, and methionine-enkephalin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Amino acid sequence, M_r and pI of each peptide are listed in Table 1. pI values were estimated from www.embl.de/cgi/pi-wrapper.pi except for oxytocin [27] and LHRH [28]. Deuteroporphyrin (dP), its metal-complexes (Fe (III)-deuteroporphyrin [Fe-dP], Cu(II)-deuteroporphyrin [Cu-dP], Zn(II)-deuteroporphyrin [Zn-dP], Ni(II)-deuteroporphyrin [Ni-dP]), and Cu(II)–meso–tetra (carboxyphenyl) porphyrin (Cu-TCPP) (Fig. 1) were obtained in our laboratory by the resorcinol fusion method, as described by Fischer et al. [29]. Deionized water was purified with an Easy Pure™ UltraPure water system from Barnstead-Thermolyne

Figure 1. Structures porphyrin derivatives: (A) metal-dP, where Me corresponds to Fe(III), Cu(II), Ni(II), Zn(II); (B) meso–tetra (carboxyphenyl) porphyrin, where Me corresponds to Cu(II).

(Dubuque, IA, USA). Nylon membrane filters (0.45 μm) to remove particulate matter were purchased from Micro Separation (Westboro, MA, USA).

2.2 Instrumentation

Separations were performed in a P/ACE MDQ (Beckman Coulter, Brea, CA, USA), equipped with a UV–vis photodiode array detector. Data were processed by 32 Karat™ software (Beckman Coulter). For all experiments, the CE system temperature was held at 25°C. In all cases, UV detection at 214 nm (deuterium lamp) was performed.

2.3 Preparation of the OT-CEC columns

The covalent attachment of each porphyrin to the capillary inner surface was performed as described in [30] with minor modifications. Briefly, the porphyrin was activated by dissolving 1 mg with 0.5 mL of chloroformate, under argon

Table 1. Analyzed bioactive peptides and their amino acid sequences, relative molecular masses, M_r, pIs, and effective electrophoretic mobilities, μ_{eff} (10^{-9} m^{2}V^{-1}s^{-1})

Peptide	Amino acid sequence	M_r	pI	μ_{eff}
Bradykinin	RPPGFSPFR	1060.2	12.40	17.6
Angiotensin I	DRVYIHPFHL	1296.5	7.91	16.2
LHRH	Pyr[a)]-HWSYGLRPG	1182.3	7.30	14.8
Oxytocin	CYIQNCPLG	1007.19	7.70	9.63
Methionine-enkephalin	YGGFM	573.7	5.93	1.88

a) Pyr, pyroglutamic acid.

atmosphere, and kept during 20 min in darkness at room temperature. Then, 2 mL of THF and 30 mL of triethanolamine were added, and the solution was degassed with argon. Fused silica (FS) capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 32 cm in length (50 µm id × 365 µm od) were sequentially flushed with water, 1 M NaOH, and water once again, each step for 5 min. Next, they were washed with ethanol and dried with argon. After these steps, the capillaries were filled with the activated porphyrin solution and kept in darkness for 24 h, at room temperature. Finally, the capillaries were washed with THF until a colorless solution was obtained, and last with water.

2.4 Peptide solutions and running conditions

Stock solutions of peptides (1 mg/mL) were prepared by dissolving each synthetic peptide in water, fractionated in aliquots, and frozen at −20°C. Standard solutions were daily prepared by appropriate dilution with water. The mobile phase (BGE) consisted of 25 mM potassium cation (from potassium dihydrogen phosphate), pH 4.0, adjusted with 0.1 M phosphoric acid, 5% v/v ACN and 10 mM HQ, unless otherwise indicated. Peptide solutions were introduced by 5 s at 3.5 kPa, the separation voltage was 12 kV, and the current was 18.5 and 18.0 µA in CZE and OT-CEC experiments, respectively. Before daily work, the capillary columns were washed with BGE for 10 min at 138 kPa. After each run, the capillaries were washed with BGE for 5 min, and finally were flushed with water at the end of the day.

2.5 Determination of metal-dP

The amount of metal-dP bonded to the capillary surface was indirectly assessed by quantification of the metal ion. The modified column was filled with 8 M nitric acid solution, and kept at room temperature for 10 min. Then, about 200 µL of the acidic solution was passed through the capillary. All portions of the acidic solution were collected into a sample microvial and evaporated to dryness in a water bath at 80°C. The residue was reconstituted with 10 µL of 50 mM EDTA solution and the metal-EDTA chelate was quantified by CZE (separation capillary, 75 µm id × 40 cm total length, 31 cm to the detector; running buffer, 40 mM sodium cation [from disodium hydrogen phosphate], pH 8.0, adjusted with 0.1 M hydrochloric acid; sample introduction time, 5 s at 3.5 kPa; applied voltage, 12 kV, current, 72.0 µA).

2.6 Statistical treatment

For data handling and statistical analysis, ANOVA, Tukey, and Student's *t*-tests were applied using the program R v2.11.1 (The R Project for Statistical Computing, University of Auckland, New Zeland). Results that showed no significant difference were confirmed by Student's *t*-test. This procedure was applied to all data from peptide separations.

3 Results and discussion

3.1 Selected running conditions

Different porphyrins and their derivatives linked to solid supports have been previously used for peptide separation [4, 31, 32]. Particularly when electromigration techniques are employed, a thorough evaluation of separation conditions should be performed. In a previous study, different solution compositions were tested for the separation of the selected bioactive peptides in a Fe-dP-modified capillary column [32]. The same strategy was adopted in the present investigation. Each porphyrin-modified capillary column was tested for the separation of selected peptides at different pHs, ranging from 3.0 to 7.0. Although buffer capacity of phosphate solutions decreases as the pH moves away from the pK_a value, resolution of bradykinin, angiotensin, and LHRH failed at pH lower than 4.0. On the other hand, alkaline pH was not assayed since HQ turns unstable and polymerizes in these conditions. In fact, this reaction even occurs at pH 7.0 coloring the solution after few minutes. HQ was used to prevent pH variation and bubble formation as previously demonstrated [32]. During the application of the separation voltage, BGE electrolysis occurs as follows:

$$\text{Anode}: 2H_2O \Leftrightarrow O_2 + 4H^+ + 4e^-$$

$$\text{Cathode}: 2H_2O + 2e^- \Leftrightarrow H_2 + 2OH^-$$
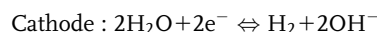
The resulting OH^- and H^+ produced can change the pH in the buffer reservoirs and even in the capillary. Moreover, formation of tiny gas bubbles can be appreciated in both places. The function of the HQ is to alter the electrochemical reaction at the electrode by replacing the oxidation of water with oxidation of more easily oxidizable HQ according to the following equation:

$$HQ \Leftrightarrow p-\text{benzoquinone} + 2H^+ + 2e^-$$
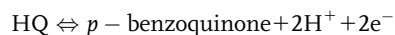
The formation of water-soluble *p*-benzoquinone replaces the formation of oxygen gas in the water electrolysis, and hence leading to bubble reduction.

The addition of ACN modified π–π interactions and possible hydrophobic forces between peptides and porphyrins. All columns allowed best resolution and repeatability for the selected peptides using a 25 mM potassium phosphate solution, pH 4.0, containing 5% v/v ACN and 10 mM HQ, as BGE.

3.2 Peptide separations

The interaction between the metalloporphyrin ligands and the peptides can be established through different binding sites: (i) amino and hydroxyl groups for coordination

binding, (ii) phenyl or hydroxyphenyl moieties for π–π stacking interaction, (iii) amino, hydroxyl, and carbonyl groups for hydrogen bonding, and (iv) methyl groups for hydrophobic interaction. Thus, the various possible sites of porphyrin moiety plus the complexity of peptide structure give place to a wide range of interactions between porphyrin and peptide molecules.

The CZE and OT-CEC peptide separations are shown in Fig. 2. Under acidic pH conditions, all five bioactive peptides possess a positive charge, which made them moving faster than the EOF marker. Migration times of all test mixture components were always longer in porphyrin-modified capillaries than in the bare FS one, being the longest when metal(II)-porphyrins were used as capillary wall modifiers. Additionally, a decrease in the EOF mobility was also observed in all modified capillaries.

Effective mobilities of all peptides in each porphyrin-modified column were calculated. Relative effective mobilities of studied peptides obtained from CZE and OT-CEC separations are shown in Fig. 3. In order to compare peptide behavior between different columns, effective mobilities were analyzed applying the Tukey's test (Table 2), after checking by ANOVA test that significant differences
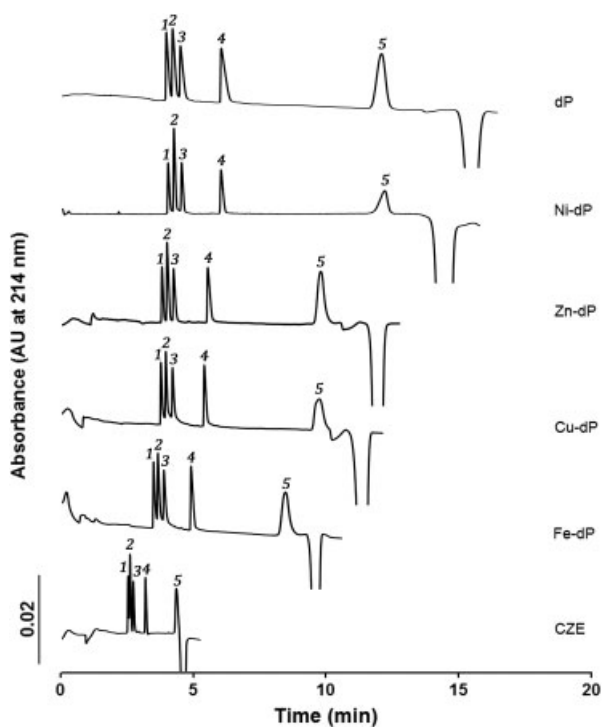
occurred. Figure 4 shows the graph obtained with Tukey's test for methionine-enkephalin. As shown in Fig. 3, the effective electrophoretic mobility of every assayed peptide in all modified columns was lower than in a free capillary in the CZE mode. That implies that one or more of the abovementioned interactions occurs between the peptides and the porphyrin modifiers under the experimental conditions described.
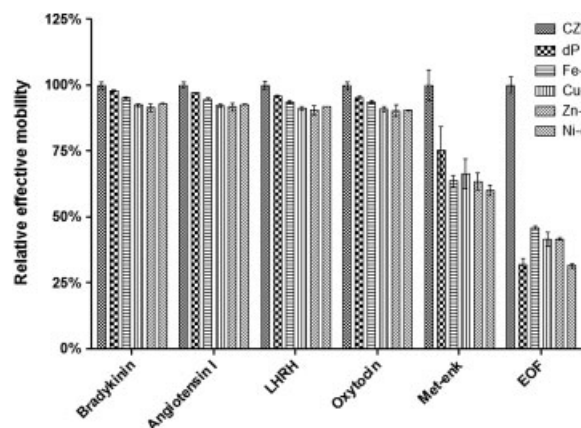
Peptides showed different effective electrophoretic mobility in the Fe-dP-modified column in comparison with all other columns. The only exceptions were oxytocin, which showed a similar behavior in the Fe-dP and the Zn-dP, and methionine-enkephalin which exhibited the same effective electrophoretic mobility in all modified columns.

Peptide effective electrophoretic mobilities were similar in metal(II)-dP modified columns but decreased when compared with the dP (without metal) modified one. This can be explained by the tendency of the bivalent metals to include axial ligands. As expected, we also observed that the effective electrophoretic mobility of the same peptides in metal(II)-dP was lower than in the Fe-dP-modified capillaries, due to the intense electrostatic repulsion of Fe(III)-dP to positively charged peptides.

3.3 The stationary phase

The covalent modification of capillary inner surface was performed under optimized conditions to ensure the maximum reaction yield [26, 28]. The quantity of porphyrin fixed was indirectly estimated by quantification of the metal cation. From these results, we can deduce that metalloporphyrins have been attached in similar amounts regardless of the metal cation (Table 3).

The effect of porphyrin amount attached to the capillary wall on peptide separations was also evaluated. To this end,

Figure 2. Separation of (1) bradykinin, (2) angiotensin I, (3) LHRH, (4) oxytocin, and (5) methionine-enkephalin, 100 μg/mL each, in different modified capillary columns: dP, deuteroporphyrin; Ni-dP, Ni(II)-deuteroporphyrin; Zn-dP, Zn(II)-deuteroporphyrin; Cu-dP, Cu(II)-deuteroporphyrin; Fe-dP, Fe(III)-deuteroporphyrin. Mobile phase (BGE), 25 mM potassium phosphate, pH 4.0, 5% v/v ACN; 10 mM HQ; 12 kV; 18.0–18.5 μA; sample introduction, 5 s at 3.5 kPa; capillary column, 50 μm id × 32 cm in length (21 cm to the detector).
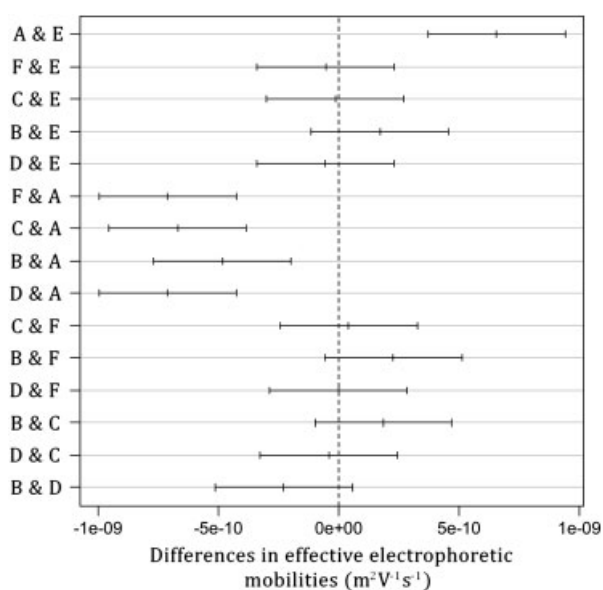

Figure 3. Relative effective electrophoretic mobilities of peptides obtained in unmodified and porphyrin-modified capillaries (effective mobility obtained in the unmodified FS capillary was considered as 100%). dP, deuteroporphyrin; Fe-dP, Fe(III)-deuteroporphyrin; Cu-dP, Cu(II)-deuteroporphyrin; Zn-dP, Zn(II)-deuteroporphyrin; Ni-dP, Ni(II)-deuteroporphyrin.

Table 2. Comparison of peptide effective electrophoretic mobility in different modified capillary columns

Peptide	CZE and dP	CZE and Fe-dP	CZE and Cu-dP	CZE and Ni-dP	CZE and Zn-dP	dP and Fe-dP	dP and Cu-dP	dP and Ni-dP	dP and Zn-dP	Fe-dP and Cu-dP	Fe-dP and Ni-dP	Fe-dP and Zn-dP	Cu-dP and Ni-dP	Cu-dP and Zn-dP	Ni-dP and Zn-dP
Bradykinin	Yes[a]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No[b]	No	No
Angiotensin I	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No
LHRH	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No
Oxytocin	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No	No
Methionine-enkephalin	Yes	Yes	Yes	Yes	Yes	No	No	No	No	No	No	No	No	No	No

a) Significant difference, with 5% of confidence level.
b) No significant difference, with 5% of confidence level.

Figure 4. Graph obtained with Tukey's test for methionine-enkephalin effective electrophoretic mobility in unmodified and different porphyrin-modified capillaries. (A) CZE; (B) deuteroporphyrin; (C) Ni(II)-deuteroporphyrin; (D) Zn(II)- deuteroporphyrin; (E) Cu(II)-deuteroporphyrin; (F) Fe (III)- deuteroporphyrin.

Table 3. Amount of metalloporphyrin attached onto the inner capillary surface ($\mu mol/cm^{-2}$)

Column	Metalloporphyrin content
Fe-dP	1.378
Cu-dP	1.048
Ni-dP	1.428
Zn-dP	1.160
Cu-TCPP	1.107

capillary columns were modified using diluted porphyrin solutions. For example, the use of a 0.2 mg/mL of Ni-dP in chloroformate led to a column that contained 3.8 nmol/cm²
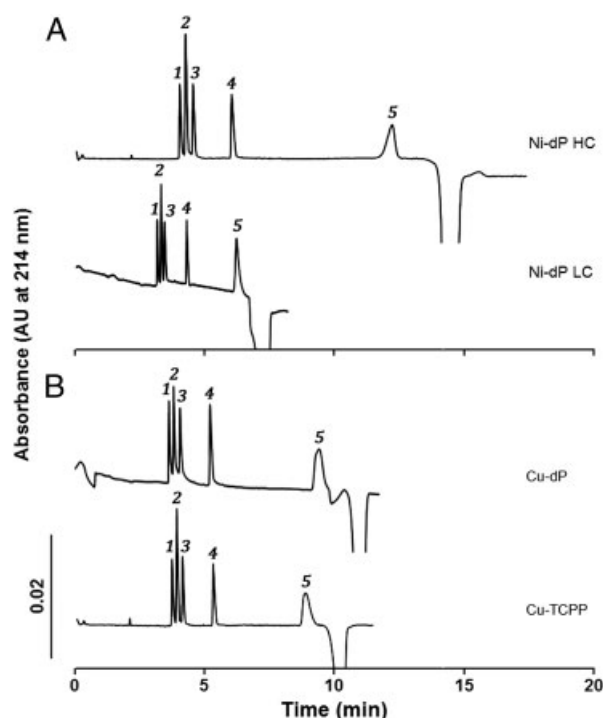

Figure 5. Separation of (1) bradykinin, (2) angiotensin I, (3) LHRH, (4) oxytocin, and (5) methionine-enkephalin, 100 µg/mL each one, in (A) Ni-dP HC, Ni(II)-deuteroporphyrin highly modified, and Ni-dP LC, Ni(II)-deuteroporphyrin lowly modified columns; and (B) Cu-dP, Cu(II)-deuteroporphyrin, and Cu-TCPP, Cu(II)–meso–tetra (carboxyphenyl) porphyrin-modified columns. Mobile phase (BGE), 25 mM potassium phosphate, pH 4.0, 5% v/v ACN; 10 mM HQ; 12 kV; 18.0–18.5 µA; sample introduction, 5 s at 3.5 kPa; capillary column, 50 µm id × 32 cm in length (21 cm to the detector).

of Ni(II) cation. In this column, the EOF mobility increased, which was expected, since a smaller number of silanol groups at the inner capillary surface have reacted with the porphyrin (Fig. 5A). Curiously, peptides bradykinin, angiotensin, LHRH, and oxytocin decreased their effective electrophoretic mobilities with respect to those obtained in the Ni-dP highly modified column ($p < 0.05$). It is feasible to

speculate that in these conditions peptides would have a better steric accessibility to ligands which would favor the peptide–porphyrin interaction.

In order to examine the influence of the molecular geometry of the porphyrin, resulting of the attachment mode to the surface, peptide separation was evaluated in two different columns: Cu-dP, and Cu-TCPP. The highly aromatic Cu-TCPP was bonded to the inner capillary surface

through the carboxy phenyl groups (Fig. 1B). Considering Cu-TCPP molecule characteristics, the expectation would be that it is attached by three of the four carboxyl groups. In this format, the immobilized porphyrin structure would be flexible enough to allow Cu(II) coordination with axial ligands [33, 34]. Moreover, the aromatic properties of the porphyrin would be more relevant, whereas the metal ion center would be more exposed in comparison to the stacking
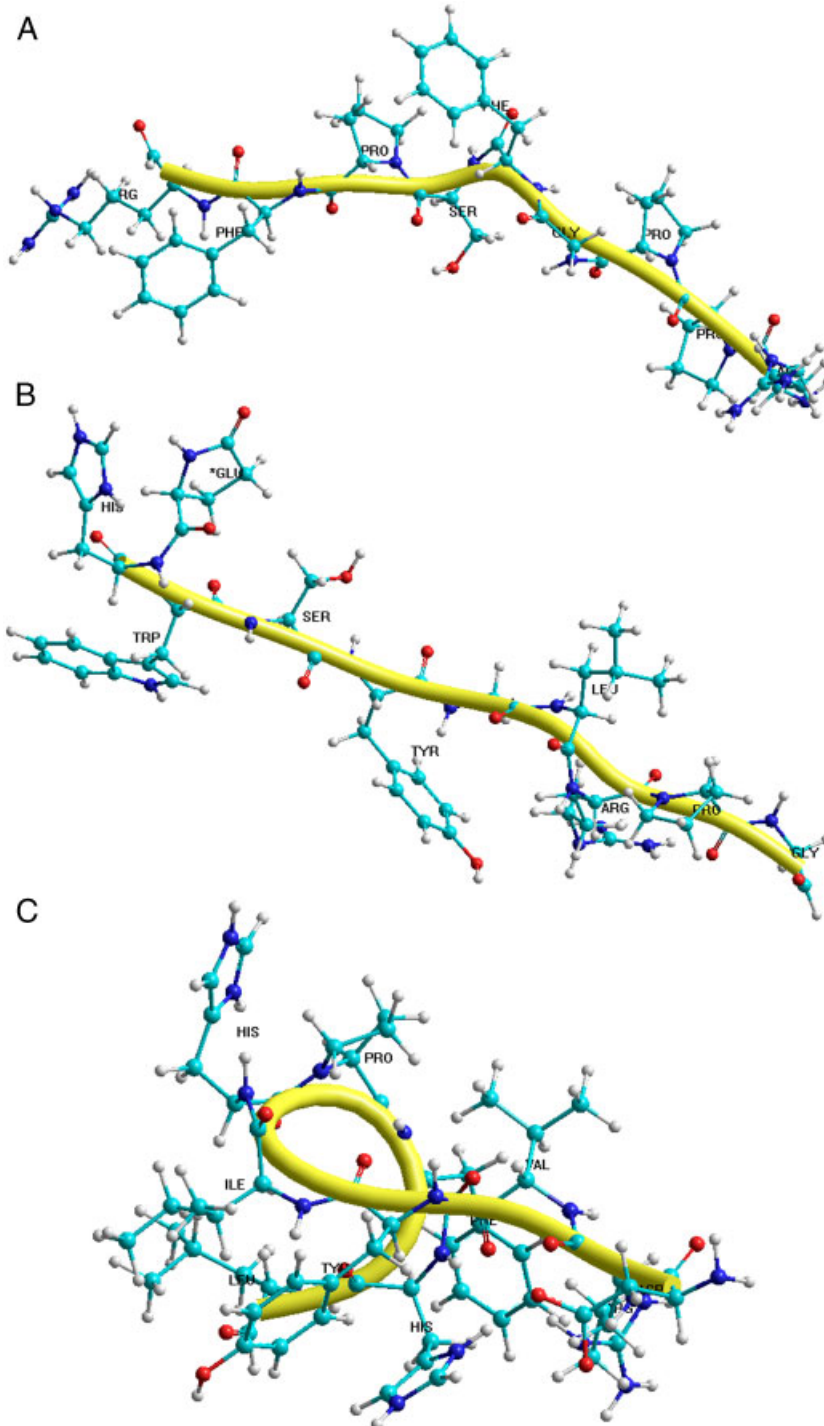
Figure 6. 3-D structure of (A) bradykinin, (B) LHRH, and (C) angiotensin I.

arrangement already described in the metallo-dP-modified columns [35]. Furthermore, the presence of phenyl groups in the Cu-TCPP reinforces the aromatic character of the porphyrin molecule. Taking these into account, we expected the effective electrophoretic mobility of studied peptides to vary since all of them have at least one aromatic residue (for amino acid sequence of each peptide, see Table 1). On the other hand, bradykinin, angiotensin, and LHRH bear strong ligands to the metal ion, i.e. amino acid residues for coordination with copper ion such as histidine, arginine, tryptophan, phenylalanine, and tyrosine. Figure 5B shows the separation of the five peptides in the two columns. Interestingly, we observed that only bradykinin and angiotensin showed significant difference in their effective electrophoretic mobility. This fact could be related to the presence of a tryptophan residue which is excellent to coordinate copper through the nitrogen in the pyrrole cycle [36]. Such property, combined with the proximity of the histidine and the aromatic character, would explain the strong peptide interaction with the Cu(II) porphyrins. In addition, the fairly straight conformation of LHRH (Fig. 6) would favor the peptide–porphyrin interaction in the stacking arrangement.

In the case of methionine-enkephalin, which showed a similar behavior in all modified columns, it can conclude that working at a pH value near to pI, the interactions between the peptide and the capillary coating cannot be made evident. In this sense, we are planning additional studies comparing small peptides of similar relative M_r and pI.

4   Concluding remarks

This investigation showed that the chemical modification of the capillary inner surface with all tested porphyrins produced a decrease in the EOF mobility and peptide effective electrophoretic mobility as compared with the bare FS capillary column. In addition, the presence of different metal ions in porphyrin structure affected the peptide migration/retention behavior. The electrophoretic separation of the analyzed peptides contributed significantly to their resulting resolution as follows from the fact that the elution order of peptides in OT-CEC mode was identical with their migration order in the CZE mode. The better resolution of peptides by OT-CEC was achieved not only because of their interaction with dP and metal-dP complexes but also due to the lower EOF in the dP- and metal-dP-coated FS capillaries. Finally, the spatial conformation resulting from the attachment conditions applied seems to be an important factor that affects peptide–ligand interactions.

The authors have declared no conflict of interest.

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