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RESEARCH ARTICLE

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Influence of the modification of the cosmetic peptide Argireline on the affinity toward copper(II) ions

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Argireline (Ac-EEMQRR-NH_2), a well-known neurotransmitter peptide with a potency similar to botulinum neurotoxins, reveals a proven affinity toward Cu(II) ions. We report herein Cu(II) chelating properties of three new Argireline derivatives, namely, AN4 (Ac-EAHRR-NH_2), AN5 (Ac-EEHQRR-NH_2), and AN6 (Ac-EAHQRK-NH_2). Two complementary experimental techniques, i.e., potentiometric titration (PT) and isothermal titration calorimetry (ITC), have been employed to describe the acid–base properties of the investigated peptides as well as the thermodynamic parameters of the Cu(II) complex formation. Additionally, based on density functional theory (DFT) calculations, we propose the most likely structures of the resulting Cu-peptide complexes. Finally, the cytotoxicity of the free peptides and the corresponding Cu(II) complexes was estimated in human skin cells for their possible future cosmetic application. The biological results were subsequently compared with free Argireline, its Cu(II)-complexes, and the previously studied AN2 derivative (EAHQRR).

KEYWORDS
Argireline, complexation process, copper(II) ions, ITC, peptides

1 | INTRODUCTION

In the current cosmetics market, a large range of substances, e.g., fillers, and devices, e.g., laser, are proposed to improve the condition of mature skin, to help fight skin aging. Many of these types of treatments violate the continuity of the epidermis and require specialized equipment and a person authorized to perform them. It also happens that the preparation is highly toxic, e.g., botulinum toxin that has been used for many years to eliminate wrinkles.

A breakthrough in fighting skin aging turned out to be cosmetic products that contain substances claimed to have anti-aging properties. They are much less toxic than popular filler treatments, less expensive, and more easily available. It is commonly known that due to their functions, cosmetic peptides have been divided into three main groups: signal peptides, peptides affecting neurotransmitters, and carrier peptides. Very good and promising results have been achieved with the acetyl hexapeptide-8 named Argireline peptide (Ac-EEMQRR-NH_2). Its action is similar to botox, i.e., it is an inhibitor of neurotransmitters, thus blocking the flow of impulses in the muscles.[1,2] This technology is now quite widely used in cosmetics, and there are new studies on its function and the preparation of new formulas to improve skin condition. Recently, for example, this system was used to design eye patches with microneedles to increase peptide delivery.[3] Nevertheless, it should be noted that peptides entered the cosmeceutical field a long time before Argireline. Already, in the early 70s, Pickart proposed the synthetic tripeptide GHK as a signal peptide enhancing collagen production and acting as a carrier peptide when complexed with Cu(II).[4] Since then, thanks to their versatility, several peptides of cosmeceutical interest have been developed in response to the most frequent and not fully satisfied market requests. However, our group confirmed that Argireline might also be a compound with high chelating properties toward Cu(II) ions. In particular, it may serve as a good model for a system that could stimulate collagen synthesis.[5] Peptides used as cosmetic ingredients with an affinity for the Cu(II) biometal can be used in the fight against skin damage and its excessive pigmentation. It is known that the skin naturally contains

copper, which is important for the production of collagen and elastin, but the amount of copper in the skin decreases with age. Copper-conjugated peptides have the ability to supplement copper deficiency in the skin, which contributes to improving its condition. Additionally, it turns out that copper-chelating peptides can be combined in treatments with retinoids and substances such as hyaluronic acid or vitamin C, which may even increase their effectiveness and increase collagen synthesis in the skin.

Therefore, we report herein affinity studies of Argireline peptide analogs for Cu(II) ions to find the relationship between the structure of the ligands and their chelating properties (Figure 1).[5]

The main purpose of modifying the Argireline sequence in our previous[5] and current studies was to improve peptide complexation properties. The results of previous studies of many research groups have shown that replacing even one amino acid in a peptide sequence can change its physicochemical properties, such as the ability to form complexes with metal ions.[6,7] Therefore, in the first step of our previous research, we implemented one-point or two-point mutations in the Argireline sequence.[5] It has been proven that to obtain a sequence with the ability to bind Cu(II) ions, it is necessary to introduce His into the sequence (H → M), and the presence of two contiguous Arg residues in peptide C-terminal seems to be also important. In the current study, according to the same rationale, we continue to modulate the Argireline peptide sequence, to obtain non-toxic new peptides with high affinity to bind Cu(II) ions. Two structural factors were taken into account for the modification of the original structure of Argireline. First, the pentapeptide ArgirelineAc-EAHRR-NH_2 (AN4) was investigated with the idea in mind that a shorter amino acid sequence will feature a better skin permeability, which is fundamental for topical use. Second, we tested the properties of two different

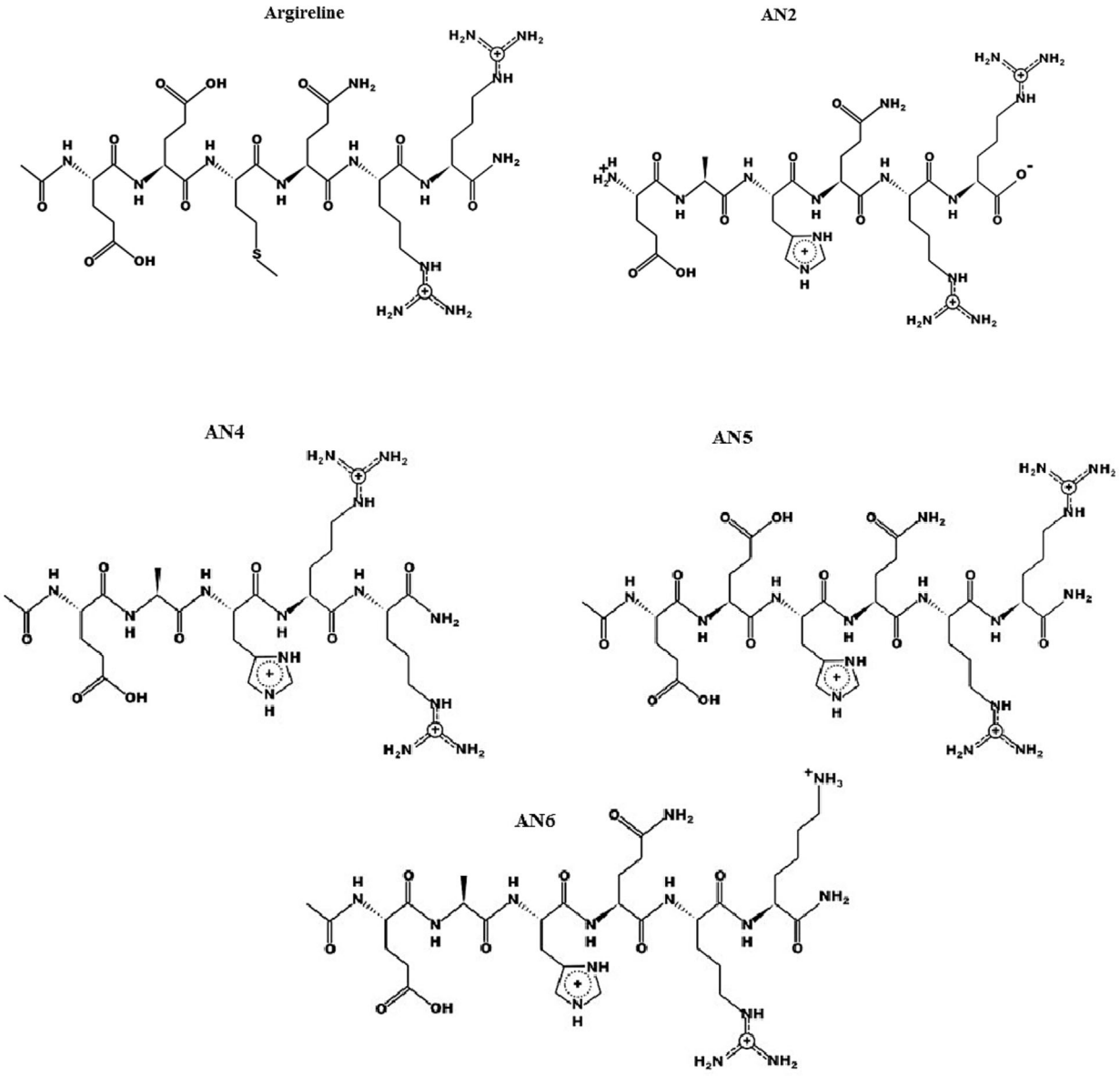

FIGURE 1 Structures of Argireline and the four analogs AN2,[5] AN4, AN5, and AN6 represented in the protonated forms.

hexapeptide analogs of Argireline with one amino acid replaced at position 3 in the original sequence (H → M), that is, Ac-EEHQRR-NH_2 (AN5), or with three amino acids replaced at positions 2, 3, and 6 in the original sequence (A → E, H → M, K → R), that is, Ac-EAHQRK-NH_2 (AN6) (Figure 1).

In this paper, we present the acid–base properties of the investigated peptides as well as the thermodynamics of the Cu(II) complex formation. Furthermore, the coordination mode of Cu(II) ions is discussed based on density functional theory (DFT) calculations.

Since the designed AN4, AN5, and AN6 peptide sequences could potentially be proposed as cosmetics, their cytotoxicity, as well as that of their Cu(II) complexes, was tested. For this purpose, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test was performed on human skin cells. Cytotoxicity of the original Argireline sequence and the previously studied AN2[5] as free sequences as well as of the corresponding Cu(II) ion-bound form were also tested. Separate tests were carried out with Cu(II) ions solution according to the concentrations used as controls. The data obtained provided some insight into the potential use of the novel peptides as cosmetic ingredients.

2 | METHODOLOGY

2.1 | Materials

Fmoc-Rink-Amide resin was purchased from Iris Biotech AG (Marktredwitz, Germany). Peptide grade *N*,*N*-dimethylformamide (DMF), activators *N*,*N*′-diisopropylcarbodiimide (DIC), Oxyma Pure, all Fmoc-L-amino acids, trifluoroacetic acid (TFA), triisopropyl silane (TIS), 1,2-ethanedithiol (EDT), diethyl ether (Et_2O), 2-propanol, and HPLC plus water were purchased from Sigma Aldrich (Milan, Italy). HPLC-grade acetonitrile (ACN) was purchased from Carlo Erba (Milan, Italy). The reagents $Cu(NO_3)_2{\cdot}2.5H_2O$ (≥99.99%) and sodium cacodylate trihydrate (≥98%) (Caco) (Merck) were of analytical grade and were used without further purification.

TABLE 1 Cleavage cocktails used to cleave peptides from resin.

Peptide	TFA	TIS	H_2O	EDT
Argireline	91.5%	5%	1%	2.5%
AN4, AN5, AN6	92.5%	5%	2.5%	/

2.2 | Peptide synthesis

All peptides (Argireline, AN4, AN5, and AN6) were prepared by the induction-assisted solid-phase synthesis in a PurePep® Chorus instrument (Gyros Protein Technologies, Uppsala, Sweden), using a Fmoc-Rink-Amide resin (loading: 0.67 mmol/g), with a single coupling protocol, and a 0.1-mmol synthesis scale. Cleavage from the resin was performed according to the peptide sequences, with a mixture of TFA and scavengers as reported in Table 1.

2.3 | Peptide purification

Crude peptides were purified on a Waters 600 HPLC coupled with Waters UV DAD 2487 using a Sepax Bio-C18 reversed-phase column (particle size 5 μm, 10 × 250 mm). Eluents were 0.1% v/v TFA in Milli-Q H_2O (solvent A) and 0.1% v/v TFA in acetonitrile (ACN, solvent B).

2.4 | Peptide characterization

All peptides were analyzed with an Alliance HPLC using a Phenomenex Kinetex C-18 column 2.6 μm (100 × 3.0 mm) working at 0.6 mL/min coupled with a single quadrupole ESI-MS (Micromass ZQ), from Waters (Milan, Italy). The gradient used, the retention times (Rt), and the final HPLC-purity grade are reported in Table 2.

2.5 | Potentiometric titration

Potentiometric titrations were performed at 298.15 K using a microtitration unit (Cerko Lab System, Poland) fitted with a 5-mL syringe (Hamilton) and a pH combined electrode (Schott–Blue Line 16 pH type). A weight calibration method was used for the syringe. The electrode was calibrated according to IUPAC recommendations.[8] The double-distilled water with a conductivity of approximately 0.18 μS/cm was used for the preparation of all aqueous solutions.

All solutions were prepared immediately before measurements. For each titration, a stock solution of the peptide was prepared at a concentration of approximately $c_{peptide} = 0.0015$ mol·L^{-1} and $cHNO_3 = 0.00145$ mol·L^{-1}; 4 mL of this solution was added to the titration vessel. The solutions ($V_o = 4$ mL) were potentiometrically titrated with a standardized 20.7 mM NaOH solution. The

TABLE 2 Analytical characterization of peptides.

Peptide	Sequence	RP-HPLC gradient	Rt (min)	Purity grade (HPLC)	ESI-MS MH^{+1} (*m*/*z*) Calc/found
Argireline	Ac-EEMQRR-NH_2	From 1% to 15% of ACN in 5 min	2.93	>95%	889.43/889.4
AN4	Ac-EAHRR-NH_2	From 1% to 15% of ACN in 5 min	2.46	>95%	709.38/709.4
AN5	Ac-EEHQRR-NH_2	From 1% to 15% of ACN in 5 min	2.56	>95%	895.45/895.5
AN6	Ac-EAHQRK-NH_2	From 1% to 15% of ACN in 5 min	1.26	>95%	809.44/809.4

equilibrium constants were calculated using the STOICHIO program,[9,10] which can handle any model of equilibria and errors in experimental quantities (titrant and titrand concentration, acid contamination of the titrated peptide, parameters E_o and S of electrode equation, etc.).

Peptides AN4 and AN6 were treated as four-functional acids, and four p*K*a values were considered as the quantities to be determined by the STOICHIO[8,9] program as opposed to the peptide AN5, which is a five-functional acid.

2.6 | Isothermal titration calorimetry

All ITC experiments were performed at 298.15 K using an Auto ITC isothermal titration calorimeter (MicroCal, Inc., Northampton, USA). All isothermal titration calorimetry experiments were performed with a 1.4491-mL sample and the reference cells. The reference cell contained distilled water. All details of the measuring devices and the experimental setup were used as previously described.[11,12] All reagents were dissolved directly in a 20 mM buffer solution of Caco. The pH of the buffer solution was adjusted to 6.0 with 0.1 M $HClO_4$. The experiment consisted of injecting 10.02 μL (29 injections, 2 μL for the first injection only) of approximately 0.05 and 0.103 mM buffered solution of Cu^{2+} in Caco into a reaction cell that initially contained 0.786 mM Caco-buffered solution of AN4, AN5, or AN6 peptide. The background titration, consisting of an identical titrant solution but with only a buffer solution in the reaction cell, was removed from each experimental titration to account for the heat of dilution. All the solutions were degassed before the titration. The titrant was injected at 5-min intervals to ensure that the titration peak returned to the baseline before the next injection. Each injection lasted 20 s. For the sake of homogeneous mixing in the cell, the stirrer speed was kept constant at 300 rpm. Calibration of the Auto ITC calorimeter was carried out electrically by using electrically generated heat pulses.

2.7 | Biological tests

2.7.1 | Cell lines and culture conditions

Human dermal fibroblasts, adult (HDFa; Cascade Biologics™, Life Technologies Europe, Bleiswijk, Netherlands), were grown in Dulbecco's modified Eagle medium (DMEM) and immortalized human keratinocytes (HaCaT; CLS Cell Lines Service GmbH, Eppelheim, Germany) in RPMI-1640 medium; media were supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% (v/v) antibiotic/antimycotic solution. Cell culture media and reagents were purchased from Thermo Fisher Scientific, Germany. Cells were cultivated at 37°C in a humidified atmosphere of 5% (v/v) carbon dioxide.

2.7.2 | Cell viability assay

Fibroblasts and keratinocytes were seeded to 6×10^3 and 1×10 cells per well of 96-well CELLSTAR cell culture plates (Greiner Bio-One, Frickenhausen, Germany), respectively, and allowed to adhere for 24 h. The growth medium was replaced with a medium containing the test peptides at concentrations ranging from 1.5625 to 100 μM (prepared by a two-fold dilution method); cells were cultured for additional 24 or 48 h. To assess viability, cells were incubated with 1 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma Aldrich, St. Louis, MO, USA) solution in RPMI-1640 medium at 37°C for additional 1–2 h. After removing the MTT solution, the formazan precipitate was dissolved in dimethyl sulfoxide (Sigma Aldrich, St. Louis, MO, USA) and the absorbance was read at 570 nm using VICTOR^3 Multilabel Plate Reader (PerkinElmer, Waltham, MA, USA). Cell viability was calculated relative to control cells (untreated cells, cultured only in a growth medium) and presented as a percentage. One-way ANOVA was conducted to determine statistical significance ($p < 0.05$).

2.8 | Theoretical calculations

Computational methods of theoretical chemistry have been used as a useful tool to predict the structure and stability of the ligands and complexes.[13–19] The study of the molecular orbitals of the 1:1 and 1:2 complexes of Cu^{2+}cations with AN4, AN5, and AN6 has been done on the DFT level of theory with the integral equation formalism for polarizable continuum model (IEFPCM)[20] solvent (water) model introduced upon potential energy surface investigation. The IEFPCM approximation describes a solvent as a homogeneous dielectric medium with electrical permeability (ε) equal to that of a pure solvent, and the cavity size was modeled for a solvent-immersed molecule.[21] The starting structure of the peptide for DFT calculations was generated based on the amino acid sequence after 75 ps simulation at 300 K, without cutoffs using BIO+ implementation of the CHARMM force field. DFT calculations were performed with Gaussian 16 C.01[20] suite of programs using the ωB97X-D[22] long-range corrected hybrid density functional with damped atom-atom dispersion corrections used with double-ζ 6-311G(d,p) basis set. All presented structures were fully optimized. All presented complexes were thermodynamically stable.

3 | RESULTS

3.1 | Potentiometric titration studies

In Table 3, values of estimated dissociation constants of ionizable groups present in AN4, AN5, and AN6 peptides were collected. The AN4 and AN6 peptides were treated as four-functional acids, and four pK_a values were considered as the quantities to be determined by the

TABLE 3 Acid dissociation constants (pK_a) of the peptides (AN4, AN5, and AN6), as obtained by adapting the equilibrium model to potentiometric titration data.

No.	Equilibrium model	AN4 (Ac-EAHRR-NH_2)	AN5 (Ac-EEHQRR-NH_2)	AN6 (Ac-EAHQRK-NH_2)
2	$H_5L = H + H_4L$ $pK_{a\ ref.}$ E = 4.25	-	3.12 (0.26)	-
3	$H_4L = H + H_3L$ $pK_{a\ ref.}$ E = 4.25	4.97 (0.13)	4.57 (0.23)	2.94 (0.09)
4	$H_3L = H + H_2L$ $pK_{a\ ref.}$ H = 6.0	6.43 (0.17)	5.71 (0.19)	5.87 (0.10)
5	$H_2L = H + HL$ ($pK_{a\ ref.}$ R = 12.0) or ($pK_{a\ ref.}$ K = 9)	10.34 (0.14)	8.70(0.23)	8.90 (0.13)
6	$HL = H + L$ ($pK_{a\ ref.}$ R = 12.0)	11.78 (0.16)	11.80 (0.23)	11.39 (0.21)
7	$H_2O = H^+ + OH^-$	14.18 (0.20)	13.80 (0.80)	13.99 (0.19)

Note: The standard deviations are reported in parentheses.

TABLE 4 Conditional thermodynamic parameters of Cu(II) binding to the investigated peptides in 20 mM Caco buffer at pH = 6, 298.15 K.

Parameters	Argireline* (Ac-EEMQRR-NH_2)	AN2* (EAHQRR)	AN4 (Ac-EAHRR-NH_2)	AN5 (Ac-EEHQRR-NH_2)	AN6 (Ac-EAHQRK-NH_2)
N (Cu^{2+}:peptide molar ratio)	0.89 (±0.04)*	1.14 (±0.01)*	1.14 (±0.01)	0.5 (constant)	ND
$\log K_{ITC}$	4.37 (±0.05)*	6.92 (±0.07)*	3.67 (±0.08)	3.93 (±0.11)	ND
ΔG_{ITC} [kcal/mol]	−5.96 (±0.07)*	−9.44 (±0.11)*	−5.04	−5.33	ND
ΔH_{ITC} [kcal/mol]	1.23 (±0.08)*	8.72 (±0.04)*	6.20	6.6	ND
$T\Delta S_{ITC}$ [kcal/mol]	7.19*	18.16*	11.24	11.93	ND

*The marked data were previously reported.[5]
Abbreviation: ND, no data.

STOICHIO program. Peptide AN5 was treated as a five-functional acid and six pK_a values were determined.

For the AN4 peptide, pK_{a1} corresponds to the reactions of deprotonation of glutamic acid (E, Glu) side chain ($pK_{a1} = 4.97$). Compared to a reference value ($pK_{a,ref.}$ ~4.25), the pK_{a1} value only slightly increases. This suggests that the carboxylic function on the Glu side chain is relatively unrestrained, which means that it can be expected to be available during the complexation reaction. The pK_{a1} value (4.97) suggests the engagement of the oxygen atom of this residue in chelating the metal ion. The value of pK_{a2} estimated for AN4 is 6.43, and it corresponds to the reactions of deprotonation of the histidine (H) side chain. Concerning the reference value ($pK_{a,ref.} = 6.3$), this pK_{a2} shows that this residue is also unrestrained (probably available during complexation), and the small change is caused by the very close presence of a strongly positively charged part of the peptide consisting of two arginine residues (R). The $pK_{a3} = 10.34$ and $pK_{a4} = 11.78$ correspond to the reaction of deprotonation of the arginine side chains. Both values are lower than the reference value ($pK_{a,ref.}$ ~12). The decrease in this value is due to the close presence of the adjacent, strongly positively charged R residue. For the AN5 peptide, we can observe a similar situation. Compared to a reference value for Glu ($pK_{a,ref.}$ ~4.25) both pK_a values of the glutamic acids slightly changed ($pK_{a1} = 3.12$ and $pK_{a2} = 4.57$). This shows that both groups are adjacent to the positively charged amino acid side chains. The value of pK_{a3} estimated for AN5 is 5.71 and corresponds to the reactions of deprotonation of the histidine (H) residue. Concerning the reference value ($pK_{a,ref.} = 6.3$), the pK_{a3} shows that this residue is close to a strongly positively charged C-terminal part of the peptide consisting of arginines. The $pK_{a4} = 8.77$ and $pK_{a5} = 11.80$ correspond to the reaction of deprotonation of the arginine residues. The value of the pK_{a4} decreases, and the pK_{a5} is only slightly lower than the reference value ($pK_{a,ref.}$ ~12). The decrease in this value is due to the close presence of the adjacent, strongly positively charged R residue. The $pK_{a1} = 2.94$ estimated for AN6 corresponds to the reactions of deprotonation of the carboxyl group of the glutamic acid side chain (similarly to AN4 and AN5), and in comparison with the reference value, it is lower ($pK_{a,ref.}$ ~4.25). In this case, the decrease of this value results from the close presence of positively charged amino acid residues present in the sequence. The $pK_{a2} = 5.87$ is estimated for the reaction of deprotonation of the imidazole ring in the histidine side chain (H). Concerning the reference value ($pK_{a,ref.} = 6.3$), the pK_{a3} is almost unchanged, which suggests that the histidine side chain is relatively unrestrained. A similar observation was applied to AN4 and AN5. The $pK_{a4} = 8.90$ corresponds to the reaction of deprotonation of the lysine residue and $pK_{a5} = 11.39$ of the arginine residue. The value for lysine residue is significantly lower than the reference value

($pK_{a,refLys}$ ~10.5), while for arginine, it decreased slightly ($pK_{a,refArg}$ ~12). These changes occur because the two residues interact as they are next to each other; one is in the electrostatic field of the other. The PT results show that AN6 is a different system in comparison with AN4 and AN5. The structure of the AN6 peptide is probably more compact, and there is more crowding within the space of the peptide chain. The side chains of the ionized residues (E, K, and R) present in the sequence sense the proximity of the electrostatic, positively charged field, created by neighboring residues.

3.2 | ITC studies

By comparing the binding constants obtained previously using the ITC method for the original Argireline ($\log K_{ITC} = 4.37$) and its derivative AN2 ($\log K_{ITC} = 6.92$), which differs from the original sequence by two amino acids, it can be seen that the introduced substitutions[5] significantly improved the binding affinity of the AN2 peptide for Cu(II) (Table 4, the marked data). It should be noted that the original Argireline sequence is terminally blocked (N-terminal acetamide and C-terminal amide) while the AN2 sequence has both ends free (amino and carboxylic functions). However, our previous results[5] show that the terminal groups, in this case, do not affect the peptide capacity for ligand chelation.

Current studies on the newly designed peptides have shown that shortening or making a very minor substitution in the AN2 sequence diminished this chelating property (Table 2). The elimination of the separator (Gln, Q) between histidine and the very strongly charged positive end consisting of two arginine residues, deteriorates the affinity of the system for Cu(II) by preventing Cu(II) from binding to the rest of the peptide strand for good. Current results show that the Ac-EAHRR-NH_2 sequence (AN4) has a weaker binding affinity for Cu(II) ions compared to AN2 and Argireline. In the Ac-EEHQRR-NH_2 sequence (AN5), replacement of the methionine residue with histidine slightly reduces the complexing properties of the peptide compared to both AN2 and Argireline ($\log K_{ITC\ AN5} = 3.93$ vs $\log K_{ITCArgireline} = 4.37$ and $\log K_{ITC\ AN2} = 6.92$). In addition, as proved by theoretical analysis, AN5 prefers two stoichiometries, i.e., 1:1 and 1:2 (Cu(II)/AN5 peptide), and these two complexes may exist in equilibrium. For the last proposed sequenceAc-EAHQRK-NH_2 (AN6), where lysine residue (K) was introduced in place of the last arginine residue (R), while the N-terminal part of the sequence was left as in the AN2 sequence with the strongest complex-forming properties, ITC results showed that interactions with Cu(II) ions exist, but the

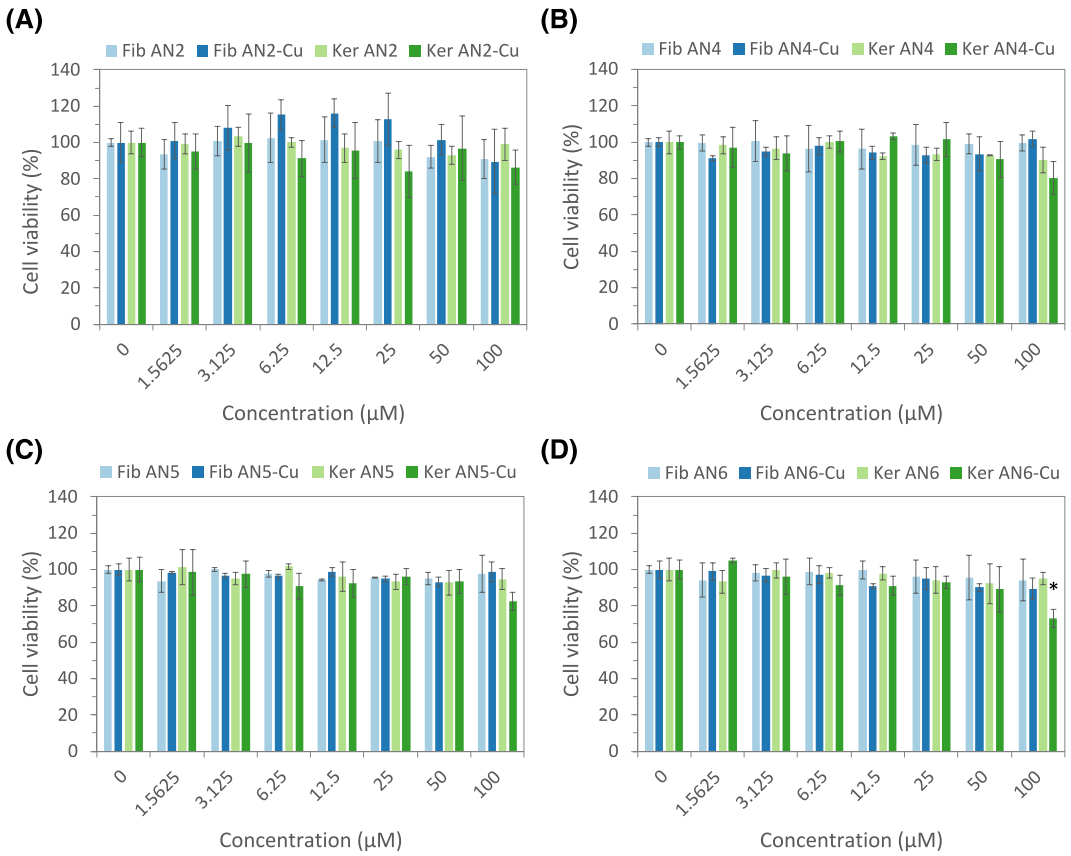

FIGURE 2 Cytotoxicity of AN-series peptides and peptide-Cu^{2+} complexes. Cell viability was assessed for fibroblasts and keratinocytes exposed for 24 h to a range of concentrations of AN2 or AN2-Cu^{2+} complex (A), AN4 or AN4-Cu^{2+} complex (B), AN5 or AN5-Cu^{2+} complex (C), and AN6 or AN6-Cu^{2+} complex (D). Data are presented as mean ± standard deviation of three replicates. Star (*) denotes significance compared to untreated cells (0 μM); one-way ANOVA with Tukey–Kramer HSD post hoc test; $p < 0.05$. Fib, fibroblasts; Ker, keratinocytes.

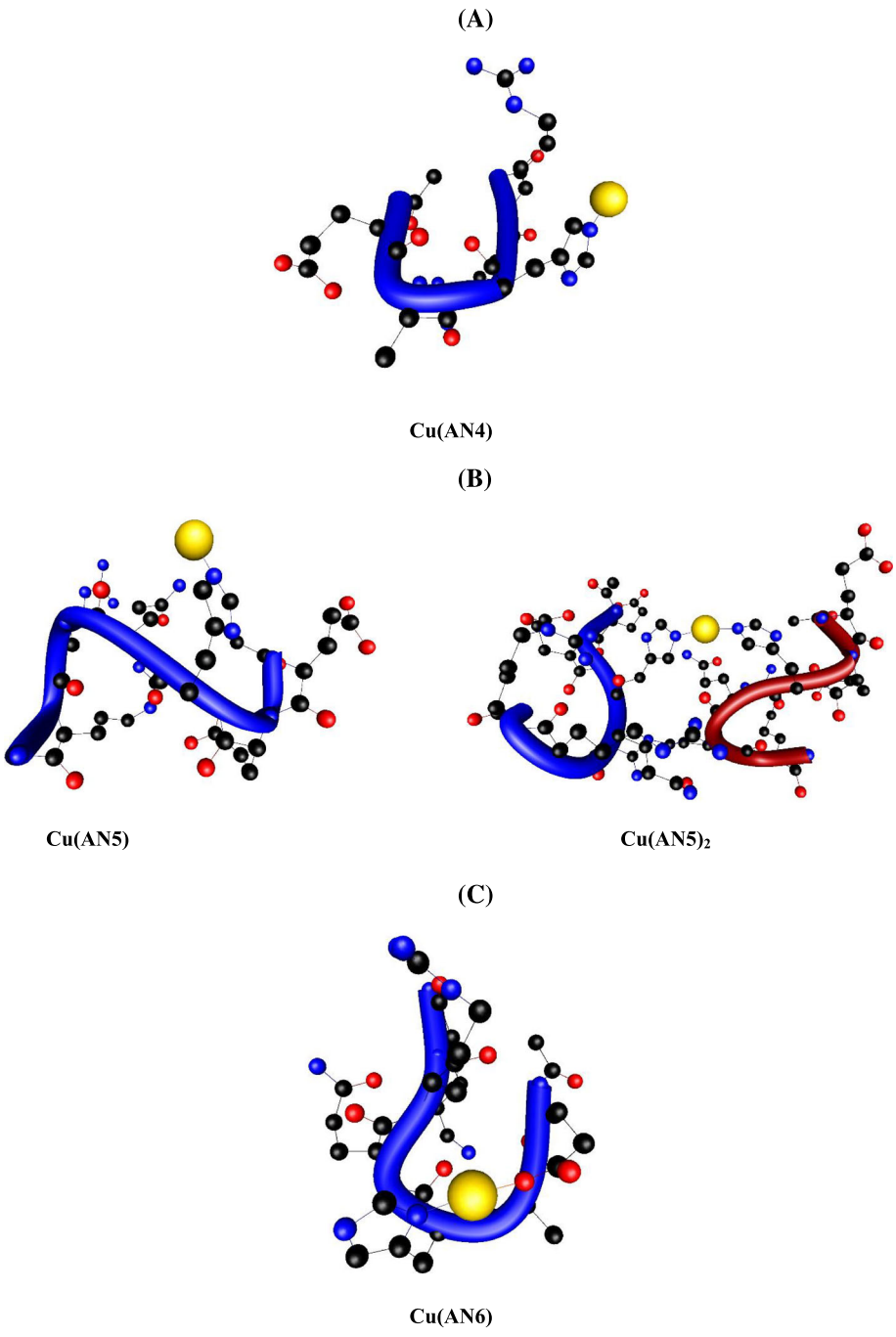

FIGURE 3 The structures of (A) AN4, (B) AN5, and (C) AN6 complexes with Cu^{2+} cation. The tubes follow backbones.

TABLE 5 Metal–ligand distances in Å for AN4, AN5, and AN6 complexes.

	Cu(AN4)	Cu(AN5)	Cu(AN6)	$Cu(AN5)_2$
Cu..N (H^3imidazole A)	1.903 Å	1.910 Å	1.846 Å	1.853 Å
Cu..N (H^3imidazole B)	-	-	-	1.855 Å
Cu..O (E^1 COO^-)	-	-	1.836 Å	-

determination of exact thermodynamic parameters was not possible. Theoretical studies have shown that AN6 is a very specific system because as many as seven hydrogen bonds are present in the copper complex of AN6. This is very surprising information for such a short non-helical system. Once again, it has been proven that the C-terminal part composed of two arginine residues plays a very important role in stabilizing the peptide strand (access of the peptide for Cu(II) is easier).

TABLE 6 Intramolecular hydrogen bonds in the 1:1 complexes.

X	H..PA [Å]	PD..PA [Å]	PD-H..PA [deg]
Cu(AN4)			
N-H..O (Ala^2..Glu^1)	1.661	2.682	163.8
N-H..O (Arg^4..Ac)	1.747	2.767	174.4
N-H..O (His^3..Glu^1)	2.042	2.856	135.6
N-H..O (Arg^4..Ala^2)	1.747	2.768	174.4
Cu(AN5)			
N-H..O (Arg^5..Glu^2)	1.715	2.742	171.5
N-H..O (Arg^5..Glu^2)	1.758	2.786	174.5
N-H..O (Glu^1..Glu^2)	1.910	2.820	146.8
N-H..N (Glu^1..His^3)	1.926	2.940	2.940
N-H..O (Arg^5..Gln^4)	1.907	2.854	153.7
Cu(AN6)			
N-H..O (Glu^1..Glu^1)	1.689	2.709	171.5
N-H..O (Arg^4..Ala^2)	1.710	2.738	172.3
N-H..O (Glu^1..Arg^5)	1.926	2.918	164.4
N-H..O (Arg^5..Lys^6)	1.830	2.841	169.6
N-H..O (Arg^5..Lys^6)	2.020	2.912	145.5
N-H..O (Gln^4..Lys^6)	1.830	2.841	169.6
N-H..O (Lys^6..His^3)	2.069	2.794	126.1

Abbreviations: PA, proton acceptor; PD, proton donor.

TABLE 7 Intermolecular hydrogen bonds in the $Cu(AN5)_2$ complex.

X	H..PA [Å]	PD..PA [Å]	PD-H..PA [deg]
$Cu(AN5)_2$			
O..H-N (Gln^4(A)..Arg^5(B))	1.770	2.779	170.9
N-H..O (Gln^4(A)..Arg^6(B))	1.938	2.893	155.1

Note: A and B letters indicate ligands.

3.3 | Biological tests

3.3.1 | Cytotoxicity of the peptides in skin cells

To evaluate the cytotoxicity of the synthetic peptides (AN-series) and of their complexes with copper, we measured the metabolic activity of human fibroblasts and keratinocytes exposed for 24 h to concentrations in the range of 1.5625–100 μM using the MTT reagent.

AN2, AN4, and AN5 peptides as well as their complexes with Cu^{2+} at concentrations up to 100 μM did not significantly alter the viability of fibroblasts and keratinocytes. While the AN6 peptide did not affect cell viability too, the Cu(AN6) complex at the highest concentration tested reduced keratinocyte viability to 73% compared to untreated cells, while not affecting fibroblasts (Figure 2). In comparison, cell viability was not significantly affected by Cu^{2+} ions, Argireline, or its complex with copper up to 100 μM (Figures S1 and S2).

3.4 | Theoretical studies

At the DFT level of theory, we have found one 1:1 complex for each ligand with Cu^{2+} cation and one 1:2 complex for the AN5 ligand (Figure 3).

3.4.1 | The 1:1 and 2:1 complexes

All three 1:1 complexes of AN4, AN5, or AN6 with Cu^{2+} cations as well as the $Cu(AN5)_2$ 1:2 complex involve metal–imidazole strong interaction. Bond lengths are similar in the range of 1.85–1.91 Å. The Cu(AN6) complex builds one metal–oxygen supporting interaction where the COO^- group is involved. The 2:1 complex involves two AN5 ligand molecules. Both ligand molecules are connected via His3 side chains with 1.853–1.855 Å metal–ligand bond lengths as presented in Table 5.

All complexes build a relatively rich set of stabilizing intramolecular hydrogen bonds (HB) that involve side chains as well as the backbone. Four HBs have been found in the Cu(AN4) complex, and five HBs were found in the AN5 complex, which is one residue longer. Interestingly, in the Cu(AN6) complex, seven HBs are present which is surprising for such a short non-helical peptide. Note that the introduction of Lys in AN6 is responsible for the observed number of HBs; Lys6 itself builds over 50% of hydrogen bonds present in the Cu(AN6) complex. All intramolecular hydrogen bonds of 1:1 complexes are gathered in Table 6.

The $Cu(AN5)_2$ 1:2 complex builds two additional intermolecular hydrogen interactions as shown in Table 7.

4 | CONCLUSIONS

Our study showed that the AN4, AN5, and AN6 ligands form a 1:1 thermodynamically stable complex with the Cu^{2+} cation. On the other hand, the AN5 ligand can also form a thermodynamically stable complex of a stoichiometry Cu(II)/AN5 equal 2:1. This type of structure is additionally stabilized by two intermolecular hydrogen bonds. It should be noted according to the theoretical study that all tested ligands display a rich network of intramolecular hydrogen bonds where the presence of arginine and lysine residues is responsible for a major number of interactions.

It can be concluded that to improve the binding properties of Argireline toward Cu(II) ions, a modification of the N-terminal part of the sequence should be based on the replacement of the second glutamic acid residue (E) with alanine (A) and the substitution of methionine residue (M) with histidine (H). Also, the C-terminal part of the sequence must always consist of arginine residues (R) placed next to each other, which probably contributes to keeping the histidine side chain exposed, and it does not allow the thread to twist toward the C-termini due to the stiffness of the C-terminal part of the sequence. In all tested systems, the nitrogen atom in the imidazole ring of the histidine side chain participates in the binding of Cu^{2+} ions.

Biological studies showed that all considered compounds are non-toxic to skin cells. Similarly, the previously studied AN2 peptide is also non-toxic to cells. Of the three systems considered, the AN5 sequence has the greatest complex properties. However, compared to the previously studied AN2 sequence, AN5 has a lower affinity for Cu(II) ions. Our previous studies have shown that the terminal groups in the AN2 peptide sequence, even if they are free (N- and C-termini), do not contribute to the binding of Cu(II) ions. It should be noted that both AN4 and AN5 peptide analogs form complexes with Cu(II) ions but do not interact with Mn(II) (data not shown). AN4 and AN5, after the formation of chelates with Cu(II) ions, can potentially deliver this ion to subsequent layers of the skin, thus showing regenerating and rebuilding effects by stimulating tissues to produce collagen. However, as we have proved, the AN2 sequence seems to be the best potential candidate for a Cu(II) ion transporter. In the near future, it would be interesting to evaluate how the modifications proposed for the Argireline peptide that were shown to increase its complexation ability affect its biological function as a neurotransmitter inhibitor. Considering that the maintenance of both functions will be of great interest to the cosmeceutical market, this makes the new peptide sequence even more appealing compared to Argireline itself. Additionally, it should be noted that peptides used in cosmetics immediately stimulate collagen and elastin synthesis and do not irritate the skin-like retinol (vitamin A).[23] Therefore, they are increasingly becoming alternative active ingredients for use in cosmetic preparations, especially for sensitive and dry skin.[24] Despite not yet recognized by the Food and Drug Administration, the term cosmeceutical[25] as a topical agent distributed across a broad spectrum of materials lying somewhere between pure cosmetics and pure drugs should be used.[26] Our study demonstrates the potential for synthesizing safer and more effective alternatives to traditional cosmetic treatments like Botox and retinol. Furthermore, we find it valuable to investigate the potential of combining copper(II) complexes with peptides alongside other cosmetic ingredients, assessing their effectiveness in addressing specific skin conditions. The importance of the complexes studied in our research extends beyond cosmetics, offering potential applications in areas such as wound healing, tissue regeneration, and drug delivery systems. Therefore advancing our understanding of metal–peptide interactions is crucial, as it has the potential to develop novel therapeutic approaches in various medical fields.

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