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Biological activities of selected peptides: Skin penetration ability of copper complexes with peptides

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Synopsis

This study concerning the permeability through skin barriers of copper complexes with peptides is an important part of the research on their biological activity. The transport of copper complexes through the skin is essential in treatment of dermatological dysfunctions connected to the deficiency of these elements in the skin. During the last several years, a special interest in transepidermal copper delivery has been observed. This is the reason why copper compounds have been used as active compounds in care cosmetics. Yet, the transport process of copper complexes with tripeptides, glycyl-histidyl-lysine GHK, or γ-glutamyl-cysteinyl-glycine GSH through the stratum corneum has received very little attention in the literature so far.

The penetration ability of GHK-Cu and GSH-Cu through the stratum corneum and the influence of the complexes with tripeptide on the copper ion transport process is the key factor in their cosmetic and pharmaceutical activity. The *in vitro* penetration process was studied in the model system, a Franz diffusion cell with a liposome membrane, where liquid crystalline systems of physicochemical properties similar to the ones of the intercellular cement of stratum corneum were used as a standard model of a skin barrier. The results obtained demonstrated that copper complexes permeate through the membranes modeling the horny lipid layer and showed the influence of peptides on the dynamics of copper ion diffusion.

INTRODUCTION

The existence of metal ions is essential for all living organisms because they are constituents of a large group of enzymes responsible for different physiological processes. Thus, they determine the proper functioning of the whole body, including the skin tissue. One of these essential metals is copper, which, according to its antiradical activity, the potential of regulating the melanogenesis process, and the synthesis of collagen, elastine, and GAGs (1,2), is widely used as a cosmetic ingredient.

Despite the fact that copper is one of the most important metals for normal skin activity and growth, not all of the copper compounds, because of their toxicity, may be used as

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cosmetic ingredients. Therefore, the simplest form of copper, an inorganic salt, cannot be a possible source of delivery of the metal ions to the low layers of skin because of its general toxicity to the organism. This is why other ways of transporting copper to the deep layers of skin tissue have to be found. One of the widely used methods of delivering metal ions into the skin is its complexation with different ligands, among which amino acids and peptides play a main role.

In our investigation, small biological active peptides were used because, beyond their transport potential, they may function as an active ingredient in the cosmetic formulae. Active peptides show cosmetically interesting activities such as stimulation of collagen synthesis, chemotaxis, and antistaining effects (3).

Among many possible natural ligands, GHK and GSH are mainly used due to their properties. Both peptides are intensively investigated because of their existence in the human organism and the different biological effects that they may show. The complexes of GHK and GSH with copper are widely known as protection and repair agents for skin tissue and because of this are often used as cosmetic ingredients.

Originally, GHK-Cu glycyl-histidyl-lysine-Cu(II) was found in human plasma, and all of its properties were drawn on the basis of these investigations. GHK-Cu was isolated from human plasma by Pickart and Thaler in 1973 (4). (see also references 5 and 6). Formerly, GHK peptides found an application in medicine. This peptide was first described as a growth factor for a variety of differentiated cells. What is more, recent data suggest its physiological role is related to the process of wound healing and tissue repair (7–10).

In further studies it was recognized that GHK is endowed with a wide range of more systemic biological activities including angiogenesis (blood vessel formation) (11), acceleration of bone repair (12), and superoxide dismutase-like activity (13). GHK may also have other activities when it is complexed with the Cu metal ion, like the secretion of the tissue inhibitors of metalloproteinase (14).

GSH is the next peptide that was isolated from a human body and enjoyed many researchers' attention. The tripeptide γ-glutamyl-cysteinyl-glycine (GSH) is the major nonenzymatic regulator of intercellular redox homeostasis and is ubiquitously present in all cell types at millimolar concentrations. This cystein-containing tripeptide exists either in a reduced (GSH) or oxidized (GSSG) form, better referred to as glutathione disulfide, and participates in redox reactions by the reversible oxidation of its active thiol (15). Glutathione in the reduced (GSH) and oxidized (GSSG) forms is the main intracellular non-protein thiol that performs the important biological functions involved in active transport of amino acids (γ-glutamyl cycle), operating enzymes (glutathione S-transferase, glutathione peroxidase, and glutathione reductase), complex formation with microelements (Zn^{2+}, Cu^{2+}), and functioning of the redox couple Cu^{2+}–Cu^{+} (16).

GSH has many ascribed biological functions for skin, and one of them is implicated in skin lightening. *In vivo* and *in vitro* studies in the literature show the evidence of its involvement in the melanogenic pathway and shed light on its anti-melanogenic effect. The proposed mechanisms of action include the direct inactivation of the enzyme tyrosinase by binding with the copper-containing active site of the enzyme and by mediating the switch mechanism from eumelanin to phaeomelanin production.

In the literature we may find that GSH has an activity of reducing free radicals and peroxides that are responsible for tyrosinase activation, melanin formation, and modulation of the depigmenting abilities of melanocytotoxic agents. This leads to the skin lightening effect of GSH application and a possibility of its usage in the treatment of pigmentary disorders (17–20). Another important issue in skin protectioning is the anti-UV (UVA and UVB) radiation activity of the cosmetic ingredient. Glutathione is one of the ingredients that may play such a role (21–24).

Due to the lack of data concerning the transport of the peptides and their complexes through the skin, we focused our research on this subject. The main goals of our experiments were to prove the ability of copper tripeptide complexes to penetrate the skin, to determine the permeability coefficient for these compounds, and to establish the form of the compound that actually penetrates through the membrane. Our previous study (25) proved that cooper peptides can migrate through the model lipophilic membrane from an aqueous solution (25), which made us continue the investigation of the transport of copper peptide complexes, but this time through an emulsion.

Since most of the cosmetic formulae used as a source of active ingredients, like peptides and their complexes, are O/W emulsions, we used them in our investigations. The *in vitro* penetration process was studied in the model system, a Franz diffusion cell (26–28) with a liposome membrane, where liquid crystalline systems of physicochemical properties similar to the ones of the intercellular cement of stratum corneum were used as a standard model of a skin barrier (29–32).

MATERIALS AND METHODS

TYPES OF APPARATUS

The absorption spectra were recorded using a SPECOL 11 spectrophotometer (Zeiss, Jena, Germany) with 5-mm glass cells. The pH measurements were carried out using an Elmetron ES24 pH meter (Poland).

The reversed-phase liquid chromatographic experiment (RPLC) was performed by a Perkin Elmer binary LC 250 computer-controlled pump (Norwalk, CT) and a Rheodyne model 7125 injection vial with injection loops (20 μl) (Cotati, Rheodyne, CA) with a Perkin Elmer model LC-95 UV/Vis spectrophotometric detector. Peptides were separated on the Hypersil BDS C_{18} analytical column (4.0 × 125 mm) (Agilent Technologies, Wilmington, NC). The acquisition and handling of the data were carried out with a 1020 LC Plus (Perkin Elmer) computer program. The copper peptide complexes were characterized by an ESI mass spectrometer, LC-MSD 1100 (Agilent) with a quadrupole mass analyzer (HP7500A).

REAGENTS

- A stock Cu(II) solution, (1 mg/ml^{-1}) was obtained by dissolution of copper(II) chloride dehydrate (POCH, Gliwice, Poland) in water.
- A GHK-Cu solution (0.01 M) was prepared by dissolution of Prezatide copper acetate (GHK-Cu) (ProCyte Corporation, USA) in water.
- A GSH stock solution (10mg/ml) was prepared by dissolution of glutathione (Sigma-

Aldrich) in water. The solution was diluted in a calibrated flask. The complex of GSH with Cu was molar ratio 2:1 and 1:1.

- A buffer solution (pH 7.4) was prepared by dissolving potassium phosphate (POCH, Gliwice, Poland), and its pH was adjusted to 7.4 by addition of di-sodium hydrogen phosphate dodecahydrate (POCH, Gliwice, Poland). The obtained solution was diluted to 1000 ml with demineralized water.
- A 0.1% biscyclohexanon-oxalyldihydrazone (cuprizon) (Fluka, Buchs, Switzerland) solution was prepared by dissolving 200 mg of cuprizon in 40 ml of hot 50% ethanol. This solution was diluted with ethanol to 200 ml.
- A buffer solution (pH 10.0) was prepared by dissolving ammonium chloride (POCH, Gliwice, Poland) and was adjusted to 10.0 by addition of ammonium (POCH, Gliwice, Poland). The obtained solution was diluted to 1000 ml with demineralized water.
- Trifluoroacetic acid (TFA) solution (0.15%) (Fluka, Buchs, Switzerland) was prepared by dissolving an appropriate amount in distilled water. The solution was diluted in a calibrated flask.
- Components of the model emulsion: 8% glyceryl stearate (Cutina GMS); 20% hexyldecanol, hexyldecyl laurate (Cetiol PGL); 3% emulsifier—Ceteareth-20 (Eumulgin B_2); 0.1% methylchloroisothiazolinone, methylisothiazolinone (Kathon CG); and water—q.s.

PREPARATION OF THE MEMBRANE

The lipophilic membrane for modeling stratum corneum lipids was prepared by sandwiching 0.125 ml of liposomes (Cerasome) (Lipoid GmbH, Germany) composed of the horny layer lipids. The appropriately thick lipid layer was placed between two membranes (Institute of Chemistry and Nuclear Technique, Poland) of polyester foil (radius, 12 mm; diameter of pores, 0.4 micrometer; thickness, 12 micrometers). The membrane was left for 24 hours to evaporate the water.

EXPERIMENTAL

In vitro membrane permeation experiments were performed using a Franz diffusion cell. The acceptor cell was filled with 15 ml of phosphate buffer (pH 7.4). One gram of a O/W emulsion containing copper complexes with peptides was placed in the donor cell. The available diffusion area between cells was 1.77 cm^2. The contents of the cells were stirred at 1000 rpm by a magnetic stirrer. During the 72 hours of experiments, the water from the emulsion was evaporated. The experiments were conducted at room temperature.

Copper was determined spectrophotometrically at 600 nm. One milliliter of the solution from the acceptor cell (during 72 hours) was transferred into a 10-ml calibrated flask, and 2 ml of 0.1% cuprizon and 2 ml of buffer solution (pH 10.0) were added. The mixture was diluted to 10 ml in a calibration flask, and the absorbance of the solution at 600 nm against a reagent blank was measured (33).

The determination of the total amount of tripeptide in the acceptor cell was carried out by RPLC. A 1-ml sample was carried out from the acceptor cell. A 20-µl portion of this sample was injected onto the column. The flow rate of the eluent (0.15% TFA) was 0.7

ml/min^{-1}, and the eluate was monitored at 200 nm using the UV/Vis detector. The concentration of peptide was determined by measuring the peak area.

Electrospray MS was applied to identify copper complexes present in the acceptor cell. ESI-MS spectra were acquired in the range of 150–1500 μ using 20 ms dwell time and 0.1 μ of step size. The ion spray voltage of 4000 V was applied for positive and negative ion acquisition. The orifice potential was established at 80 V, as the one offering the best signal intensity and causing partial fragmentation of the molecular ion at the peptide bounds (34).

DATA ANALYSIS

To calculate the permeability coefficient, the cumulative amount of copper ions was plotted against the flux (J) of a compound across the membrane, determined at steady state (35). The permeability coefficient of the Cu^{2+} ion in the lipid membrane K_p ($cm \cdot s^{-1}$) was calculated by Fick's first law of diffusion. Figure 1 shows exemplary permeation profiles of ligands and the amount of copper vs time.

RESULTS AND DISCUSSION

The aim of our research was to determine the influence of ligands (peptides) on the permeation process of copper ions. First, our studies confirmed the ability of copper ions to penetrate the model membrane without the determination of a compound form (copper ions or copper complexes). Second, we investigated the concentration of peptides that permeated the membrane. Finally, from the obtained data and ESI-MS results we were able to establish the form in which copper and peptides permeate.

PERMEABILITY COEFFICIENT STUDY

The results introduced in Figure 2 reveal a high influence of complexing agent (GHK or GSH) on the permeability coefficient of copper ions. In all cases, the permeation rates of copper ions were lower than those obtained for complexed copper. For this reason, it may be concluded that the complexing agents (GHK and GSH) accelerate the migration of copper ions through the model membranes. As shown in Figure 2, the influence of peptide complexes on the permeation of copper ions has different levels; the influence of GHK on copper ion penetration was confirmed to be twice as strong as that of GSH.

Research determined the permeation coefficient of peptides from the copper complexes. The concentration of GHK and GSH in the acceptor cell was determined by reversed-phase liquid chromatography (RPLC) with UV-VIS detection. In Figure 3 the comparison of the permeation coefficients of GHK and GSH peptides from the copper complexes is presented. The figure proves that GHK and GHK-Cu have very similar values. What is more, on the basis of Figure 3 the conclusion that tripeptide complexation of copper does not change the K_p value of GHK may be drawn. The GSH values were different: the K_p for GSH was higher than that for the GSH copper complex. Similar properties of the penetration abilities of the GHK peptides confirmed the thesis that the structure and high affinity to the lipid structures of the membrane strongly influence the permeation process. The permeation coefficients of the peptides are significantly lower than those of copper ions.

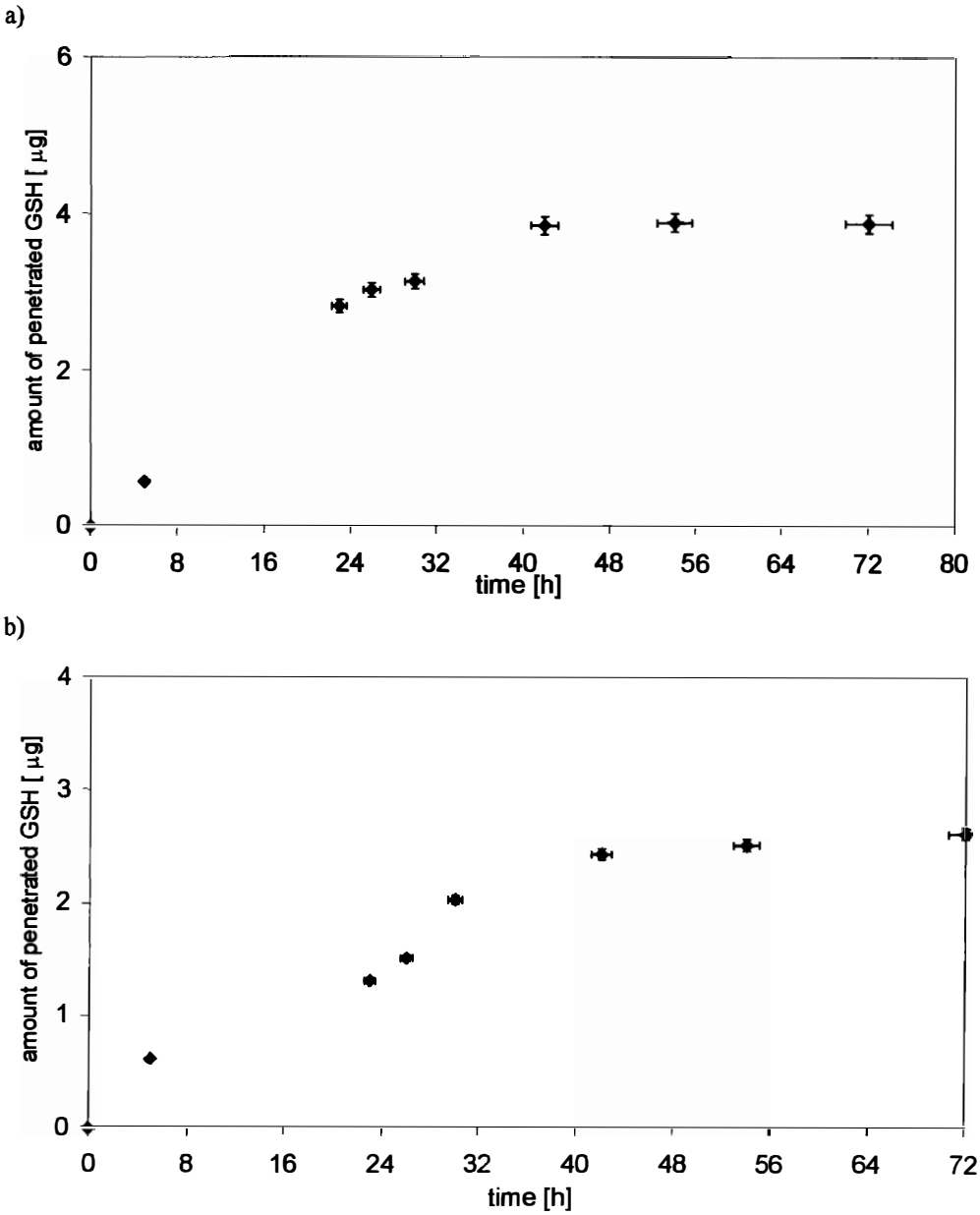

Figure 1. The permeation profiles of peptide GSH from (a) copper complex GSH-Cu and (b) peptide.

ESI-MS STUDY

The other significant factor playing a key role in the migration processes is the equilibrium of the complexes in the acceptor solution. Finally, from the obtained data and ESI-MS results, we were able to establish the form in which copper and the peptides permeate. As shown in Figure 2, we could find a lot of molecules in the acceptor cell by ESI-MS study. During the study of the penetration ability of peptides we could find species in acceptor cells (GHK and GSH).

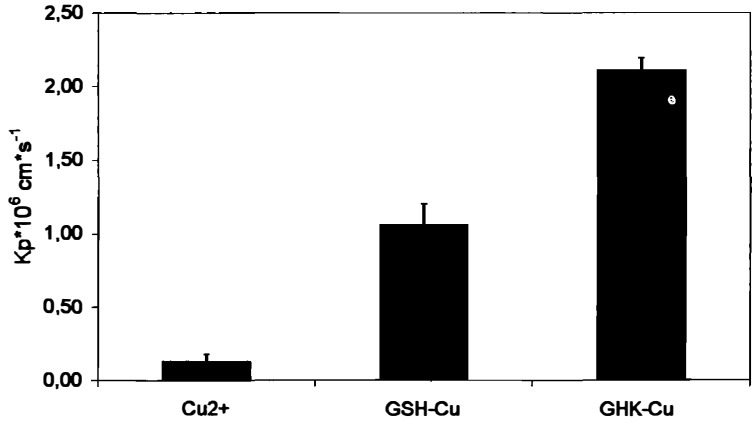

Figure 2. The influence of peptide complexes on the permeability of copper ions.

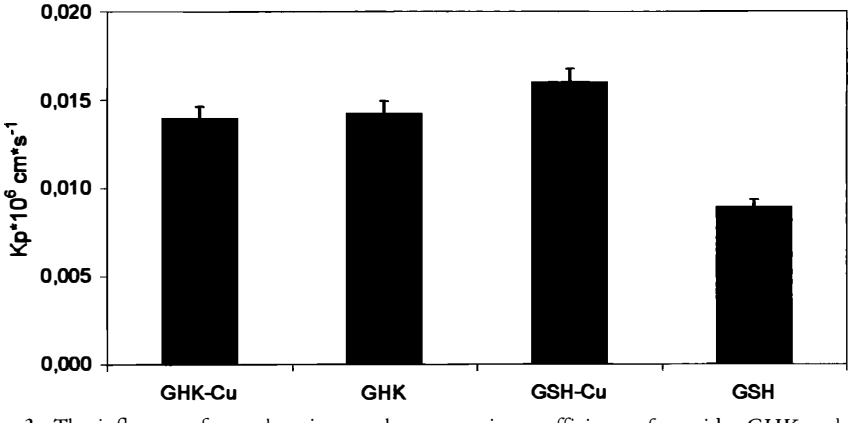

Figure 3. The influence of complexation on the permeation coefficients of peptides GHK and GSH.

The tripeptide GHK was identified by a mass spectrum, in which a signal at *m/z* 339 was registered and identified as a quasi-molecular ion of GHK (Figure 4a). A signal for GSH compound was registered at *m/z* 306 (Figure 4b). ESI-MS study proves that tripeptides GHK and GSH penetrate from the emulsion through the modeling stratum corneum membrane (Table I).

The GHK-Cu complex from the emulsion penetrates in different forms, like tripeptide GHK and GHK-Cu (Figure 5a). The mass spectrum for these studies consists of two signals at *m/z* 339 for GHK and 400 for GHK-Cu. These results are similar to those in our investigations for aquae's solution (25) and show the influence of the structure of the complexes on the penetration ability of the copper tripeptide complexes. This can suggest that GHK-Cu is the most important species in the penetration ability of copper complexes with GHK. It can be confirmed by the fact that the permeability coefficient K_p for copper from the GHK-Cu complex is higher than that for copper ions alone. All the signals were compared to the theoretical profiles.

The ESI-MS results for the penetration ability of the GSH-Cu complex show that the compounds that penetrate through the membrane are GSH and a very small amount of

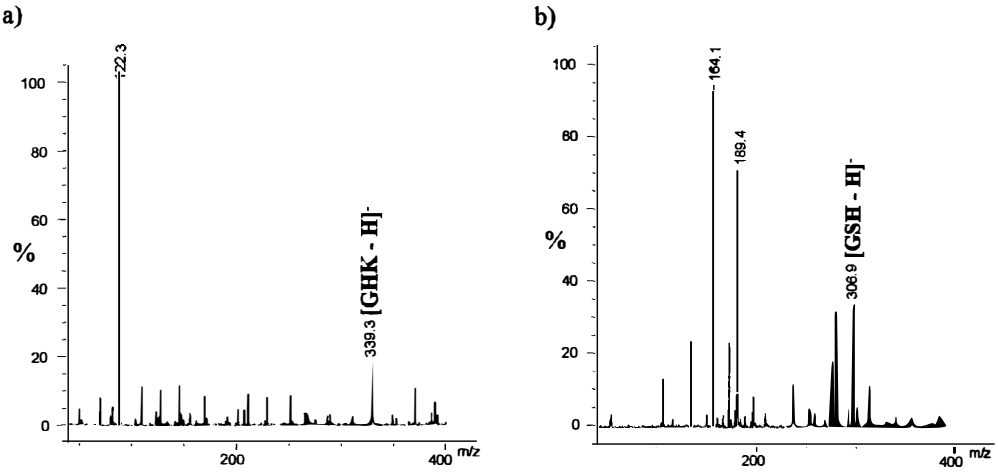

Figure 4. Mass spectra for (a) GHK and (b) GSH from emulsion in acceptor cells.

Table I
Ionized Species Observed in ESI-MS Spectra of GHK-Cu and GSH-Cu Solutions

Positive ion		Negative ion	
m/z	Proposed ion	*m/z*	Proposed ion
341	$[GHK + H]^+$	339	$[GHK + H]^-$
402	$[CuGHK + H]^+$	400	[CuGHK – H]
306	$[GSH - H]^+$	304	$[GSH - H]^-$
369	$[GSH - H]^+$	367	$[GSH - H]^-$

GSH-Cu (Figure 5b). The mass spectrum for these studies consists of two signals at *m/z* 306 for GSH and 369 for GSH-Cu. The participation of the GSH ligand form in the penetration ability of copper shows the important role of these species in the transport process. The ligand influence can be confirmed by the fact that the K_p for copper from these complexes is lower than for copper from the GHK-Cu complex. All signals were compared to the theoretical profiles. This result supports the thesis that copper complexes with bioligands can be formed and penetrate through the model membrane from the emulsion.

CONCLUSIONS

The biological activities of the copper tripeptide complex play an important role in the protection and regeneration of skin tissue, and GHK-Cu and GSH-Cu are a very good copper ion source. The research on copper transport through membrane modeling stratum corneum proved that the tripeptide-copper complex may permeate through a horny layer of epidermis.

GHK significantly participates in the wound-healing process: due to its properties it influences the elasticity and strength of the skin. What's more, GSH play a very

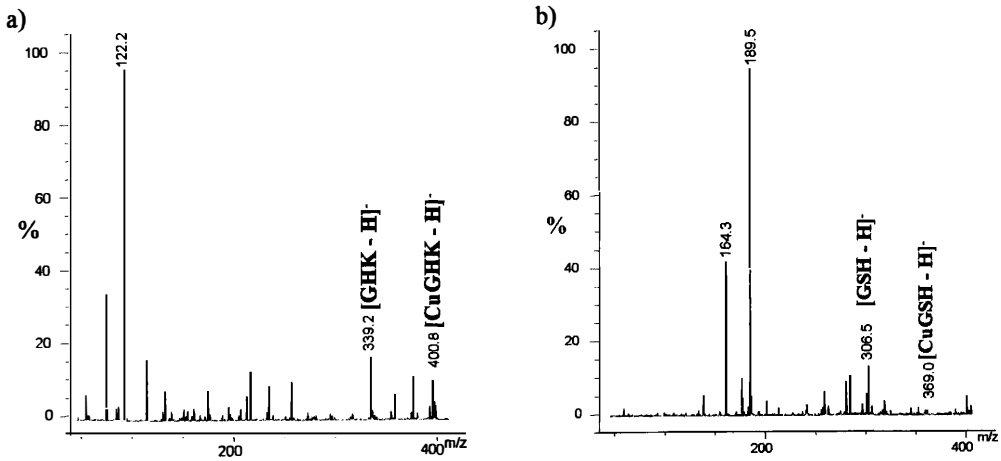

Figure 5. Mass spectra for (a) GHK-Cu and (b) GSH-Cu from emulsion in acceptor cells.

important role in skin lightening. The research proves that the copper complexes might be a good source not only for copper ions but also for peptides. The investigations of the influence of complexing agents on the skin migration rate of copper ions have evidenced their hampering role in this process.

At the present time, the incorporation of copper ions in cosmetics formulations is still very popular in Europe. The beneficial effect of the active substances depends strictly on their skin penetration ability. Our investigations show the possibility of the effective penetration of copper complexes with tripeptide into the stratum corneum, which allows one to exploit the biological activity of these complexes in cosmetics.

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REFERENCES

(1) R. H. Guy, J. J. Hostynek, R. S. Hinz, and C. R. Lorence, *Metals and the Skin* (Marcel Dekker, New York, 1999), pp. 179–189.
(2) M. C. Linder, Copper and genomic stability in mammals, *Mut. Res.,* **475,** 141–152 (2001).
(3) K. Lintner and O. Peschard, Biologically active peptides: From a laboratory bench curiosity to a functional skin care product, *Int. J. Cosmet. Sci.,* **22,** 207–218 (2000).
(4) L. Pickart and M. M. Thaler, Tripeptide in human serum which prolongs survival of normal liver cells and stimulates growth in neoplastic liver, *Nature New Biol.,* **243,** 85–87 (1973).
(5) S. J. Lau and B. Sarkar, The interaction of copper(II) and glycyl-L-histidyl-L-lysine, a growth-modulating tripeptide from plasma, *Biochem J.,* **199,** 649–656 (1981).
(6) C. Conato, R. Gavioli, R. Guerrini, H. Kozlowski, P. Mlynarz, C. Pasti, F. Pulidori, and M. Remelli, Copper complexes of glycyl-histidyl-lysine and two of its synthetic analogues: Chemical behaviour and biological activity, *Biochim. Biophys. Acta,* **1526,** 199–210 (2001).
(7) F. X. Maquart, L. Pickart, M. Laurent, P. Gillery, J. C. Monboisse, and J. P. Borel, Stimulation of

collagen synthesis in fibroblast cultures by the tripeptide-copper complex glycl-L-histidyl-L-lysine-$Cu2^{+}$, *FEB,* 238, 343–346 (1988).
(8) A. Simeon, J. Wegrowski, Y. Bontemps, and F. X. Maquart, Expression of glycosaminoglycans and small proteoglycans in wounds: Modulation by the tripeptide-copper complex glycyl-L-histidyl-L-lysine-$Cu2^{+}$, *J. Invest. Dermatol.,* 115, 962–968 (2000).
(9) L. Pickart, *Biology of Copper Complexes* (Humana Press, Clifton, New Jersey, 1987), pp. 273.
(10) D. Counts, E. Hill, M. Turner-Beatty, M. Grotewiel, S. Fosha-Thomas, and L. Pickart, Effect of Iamin on full thickness wound healing, *FASEB J.,* 6, A1636 (1992).
(11) P. M. Gullino, Microenvironment and angiogenic response, *EXS,* 61, 125–128 (1992).
(12) H. Pohunkova, J. Stehlik, J. Vachal, O. Cech, and M. Adam, Morphological features of bone healing under the effect of collagen-graft-glycosaminoglycan copolymer supplemented with the tripeptide Gly-His-Lys, *Biomaterials,* 17, 1567–1574 (1996).
(13) N. Cotelle, E. Tremolieres, J. L. Bernier, J. P. Catteau, and J. P. Henichart, Redox chemistry of complexes of nickel(II) with some biologically important peptides in the presence of reduced oxygen species: An ESR study, *J. Inorg. Biochem.,* 46, 7–15, (1992).
(14) A. Simeon, H. Emonard, W. Hornebeck, and F. X. Maquart, The tripeptide-copper complex glycyl-L-histidyl-L-lysine-Cu^{2+} stimulates matrix metalloproteinase-2 expression by fibroblast cultures, *Life Sci.,* 67, 2257–2265 (2000).
(15) A. Meister and M. E. Anderson, Glutathione, *Annu. Rev. Biochem.,* 52, 711–760 (1983).
(16) V. G. Shtyrlin, Y. I. Zyavkina, V. S. Ilakin, R. R. Garipov, and A. V. Zakharov, Structure, stability, and ligand exchange of copper(II) complexes with oxidized glutathione, *J. Inorg. Biochem.,* 99, 1335–1346 (2005).
(17) C. D. Villarama and H. I. Maibach, Glutathione as a depigmenting agent: An overview, *Int. J. Cosmet. Sci.,* 27, 147–153 (2005).
(18) T. Yamamura, J. Onishi, and T. Nishiyama, Antimelanogenic activity of hydrocoumarins in cultured normal human melanocytes by stimulating intracellular glutathione synthesis, *Arch. Dermatol. Res.,* 294, 349–359 (2002).
(19) G. Imokawa, Analysis of initial melanogenesis including tyrosinase transfer and melanosome differentiation though interrupted melanization by glutathione, *J. Inv. Dermatol.,* 93, 100–107 (1989).
(20) B. Kasraee, F. Handjani, and F. S. Aslani, Enhancement of the depigmenting effect of hydroquinone and 4-hydroxyanisole by all-trans-retinoic acid (Tretinoin): The impairment of glutathione-dependent cytoprotection?, *Dermatology,* 206, 289–291 (2003).
(21) M. A. Pelissier, N. Savoure, G. Briands, and R. Albrecht, Endogenous glutathione as potential protectant against free radicals in the skin of vitamin A deficient mice, *Food Chem. Toxicol.,* 35, 693–696 (1997).
(22) G. F. Vile and R. M. Tyrrel, UVA radiation-induced oxidative damage to lipids and proteins *in vitro* and in human skin fibroblasts is dependent on iron and singlet oxygen, *Free Rad. Biol. Med.,* 18, 721–730 (1995).
(23) R. Masella, R. Bededetto, R. Vari, C. Filesi, and C. Giovannini, Novel mechanisms of natural antioxidant compounds in biological systems: Involvement of glutathione and glutathione-related enzymes, *J. Nutr. Bio.,* 16, 577–586 (2005).
(24) S. E. Tobi, N. Paul, and T. J. McMillan, Glutathione modulates the level of free radicals produced in UVA-irradiated cells, *J. Photochem. Photobiol. B.,* 57, 102–112 (2000).
(25) L. Mazurowska and M. Mojski, ESI-MS study of the mechanism of glycyl-l-histidyl-l-lysine-Cu(II) complex transport through model membrane of stratum corneum, *Talanta,* 72, 650–654 (2007).
(26) I. J. Bosman, A. L. Lawant, S. R. Aegaart, K. Ensing, and R. A. de Zeeuw, Novel diffusion cell for *in vitro* transdermal permeation, compatible with automated dynamic sampling, *J. Pharm. Biomed. Anal.,* 14, 1015–1023 (1996).
(27) J. Shokri, A. Nokhodchi, and A. Dashbolaghi, The effect of surfactants on the skin penetration of diazepam, *Int. J. Pharm.,* 228, 99–107 (2001).
(28) A. Oborska, J. Arct, M. Mojski, and E. Jaremko, Influence of polyalcohols and surfactants on skin penetration of flavonoids from the emulsion, *J. Appl. Cosmetol.,* 22, 35–42 (2004).
(29) K. Matsuzaki, T. Imaoka, M. Asano, and K. Miyajima, Development of a model membrane system using stratum corneum lipids for estimation of drug skin permeability, *Chem. Pharm. Bull.,* 41, 575–579 (1993).
(30) J. Houk and R. H. Guy, Membrane models for skin penetration studies, *Chem. Rev.,* 88, 455–471 (1988).

(31) M. Ricci, C. Puglia, F. Bonina, C. Di Giovanni, S. Giovagnoli, and C. Rossi, Evaluation of indomethacin percutaneous absorption from nanostructured lipid carriers (NLC): *In vitro* and *in vivo* studies, *J. Pharm. Sci.,* **94,** 1149–1159 (2005).
(32) G. M. M. E. Maghraby, M. Campbell, and B. C. Finnin, Mechanisms of action of novel skin penetration enhancers: Phospholipid versus skin lipid liposomes, *Int. J. Pharm.,* **305,** 90–104 (2005).
(33) L. J. A. Haywood and P. Sutcliffe, Determination of copper in steel, *Analyst,* **81,** 651–655 (1956).
(34) K. Poleć-Pawlak, R. Ruzik, K. Abramski, M. Ciurzyńska, and H. Gawrońska, Cadmium speciation in *Arabidopsis thaliana* as a strategy to study metal accumulation system in plants, *Anal. Chim. Acta,* **540,** 61–70 (2005).
(35) H. Schaefer and T. Redelmeier, *Skin Barrier* (Karger AG, Basel, 1996).