ORIGINAL RESEARCH

Glycyl-L-Histidyl-L-Liysine-Cu(2+) loaded liposome formulations

Setenay Erdem[1], Murat Türkoğlu[1]

ABSTRACT: Enhancement of collagen synthesis by glycyl-l-histidyl-l-lysine-Cu2+ (GHK-Cu) derivatives is well known. The different activities of GHK-Cu would be of interest for cosmetic applications. Liposomes provide many benefits as topical drug delivery systems. Structure of double layer and lipid composition of liposomes keep the active substance longer in skin and provide regularly release to the deeper skin layers. Our aim in this study was to prepare GHK-Cu loaded liposomes and characterize them to use in a cosmetic formulation. UV spectrophotometric method was used to detect the GHK-Cu in aqueous medium and FTIR spectrums were taken to determine the absorption bands. In stability studies, it was observed that aqueous solutions of GHK-Cu samples maintained their stability at 4oC for 4 months and the FTIR absorption bands of powdered GHK-Cu did not change when stored under the same stability conditions with aqueous samples. Different liposome formulations were prepared by lipid film hydration technique using different kinds of phospholipids (dipalmitoylphosphatidylcholine (DPPC-5911), Epicuron 100H, and Epicuron 200SH). The particle size and shape of liposomes were determined using microscope, SEM, and laser diffraction method. The average particle size was found to be 13μm. In the percent entrapment studies of GHK-Cu in liposomes, it was found that the highest entrapment was achieved with the liposomes prepared with Epicuron 100H. It was concluded that diffusion of GHK-Cu from liposomes prepared with Epicuron 100H was higher and more steady than that of liposomes prepared with DPPC and Epicuron 200SH in diffusion studies where a dialysis tubing was used.

KEY WORDS: Anti-aging; GHK-Cu; Liposomes; Phospholipids

AFFILIATIONS
[1]Marmara Üniversitesi, Eczacılık Fakültesi, Farmasötik Teknoloji Anabilim Dalı, İstanbul, Türkiye

CORRESPONDENCE
Murat Türkoğlu
E-mail:
turkoglu@marmara.edu.tr
murat.trkgl@gmail.com

Received:
March 01, 2010

Accepted:
April 15, 2010

INTRODUCTION

Aging is a complex phenomenon occurs progressively over time. Skin aging is classified as intrinsic aging and photo aging. It emerges physiological, histological and metabolic changes at microscopic level, and wrinkles, dryness, loss of elasticity of skin, and the formation of spots at macroscopic level. Delaying the aging of skin and mitigation of the signs of aging with cosmetic products constitutes one of the most important professions of cosmetic science (1-4).

The use of drug delivery systems in cosmetic preparations have became increasingly common. Especially systems containing liposomes protects active substances from environmental conditions, can mask unpleasant odor and color, and finally provide continued release. At the same time because of their phospholipids structure they minimize the irritations as they are biocompatible with skin. They play an important role in the transfer of the active ingredient through the skin by increasing the skin penetration (5-7).

Peptides have the power to stimulate keratinocytes metabolization in order to increase the amount of extracellular matrix components. Especially copper peptides, pentapeptides and hegzapeptides, are used as cosmetic substances in skin care products to improve skin aging. Glycyl-l-histidyl-l-lysine-Cu^{2+} (GHK), a human peptide and was first discovered approximately 33 years ago, is a unit of feedback mechanism which modulates repairing, remodeling and protection process of tissue. Many copper peptide complexes have tissue protection and tissue restoration ability. Therefore all the concentrations have been focused on a human copper peptide complex known as, glycyl-l-histidyl-l-lysine-Cu^{2+} (GHK-Cu) (8-10).

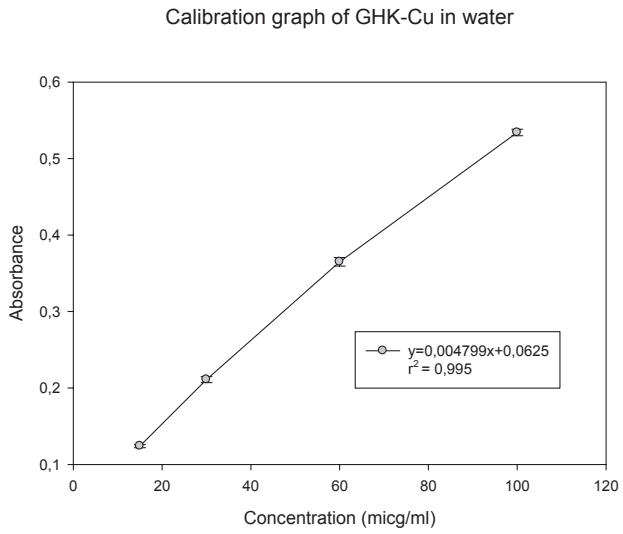

FIGURE 1. Calibration graph of GHK-Cu in water

GHK-Cu has protective and refreshing effects which interest many biological systems such as skin, hair follicles, bone, gastric mucosa and the digestive system. The application of copper peptide complexes as a skin and hair products after dermatological skin renewal procedures such as chemical pealing, laser, and dermo-abrasion has been increasingly common. GHK molecule is found in human blood, saliva, and urine. In plasma, it is associated with the protein albumin, at about 200 ng /ml at age 20 which declines to 80ng/ml by age 60. GHK is generated during proteolytic events after tissue injury and probably during normal tissue turnover. While this is a rare sequence in proteins, it is relatively high in proteins of the extracellular matrix especially in SPARC, a matricellular protein that functions in cellular differentiation and tissue response to injury, known as secreted protein acidic and rich in cysteine, also known as osteoceratin. What makes GHK so unique is its high affinity for copper 2+, rendering it nearly equivalent to the copper transport site on albumin. It can also obtain copper 2+ from albumin. In vivo, about 5 to 20% of GHK converts to the copper complex (glycyl-l-histidyl-l-lysine-Cu^{2+}; GHK-Cu) (11, 12).

This unique tiny molecule as regular constituent of body fluid, GHK has the molecular weight of 340 D, which provides it to approach so closely to the cell membrane receptors and allows it to transfer ionic copper from albumin. Most reported GHK actions use the copper complex but both GHK-Cu and GHK molecules may each have distinct biological actions because of their easy inter-conversion between forms depending on ionic copper availability. Researchers used both GHK and GHK-Cu in their studies. The actual balance of these two molecules depends on the conditions in the physiological milieu of the test system. GHK-Cu is produced in damaged tissue after injury during the proteolysis of the inflamed proteins and extracellular matrix proteins. As a result of a number of cell culture studies, it was stated that the biologically effective level of GHK-Cu should be approximately $1X10^{-9}$ M (13-15).

Chemo-attraction (macrophages, mast cells, capillary cells); Anti-inflammatory (suppresses free radicals, thromboxane formation, release of oxidizing iron, TFG beta-1, TNF alpha,

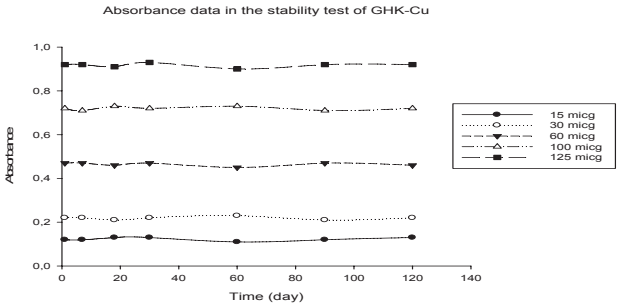

FIGURE 2. Absorbance data in the stability test of different GHK-Cu solutions taken in different time intervals

protein glycation, increases superoxide dismutase and vasodilatation, blocks UV damage to keratinocytes by reactive carbonyl species, aids fibroblast recovery after radiation); Protein expression (increasing collagen, elastin, metalloproteinases, anti-proteases, VEGF,FGF-2, NGF, NT-3, NT-4, erythropoietin); Increases proliferation of fibroblasts and keratinocytes, nerve outgrowth, and angionesis are some of the important biological activities of GHK. Formulations which contains GHK-Cu stimulates wound healing in numerous animal models and in humans, controlled studies on aged skin demonstrated that it tightens loose skin, improves elasticity and firmness, reduce fine lines, wrinkles, photo-damage, and hyperpigmentation. Improving of hair transplant and protection of hepatic tissue from tetrachloromethane poisoning can also be count in the other biological activities of GHK-Cu (16-18).

After having an idea about these unique biological activities of GHK-Cu, in this study it is aimed to prepare GHK-Cu loaded liposomes and characterize them to use in a cosmetic gel formulation.

MATERIALS AND METHODS

Materials

Glisil-L-histidil-L-lysine-Cu^{2+} (GHK-Cu) were kindly donated from Dr. Loren Pickart and additional GHK-Cu was kindly donated by Chendu CP Biochem CO. LTD. China. Chemicals, *dipalmitoyl*phosphatidylcholine (DPPC-P5911) and cholesterol (C3292) used in the preparation of liposomes were obtained from Sigma-Aldrich, Germany. Chloroform from Merck, Ger-

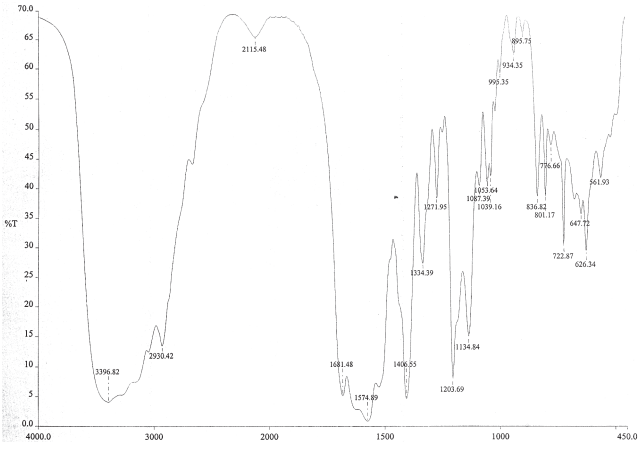

FIGURE 3. FTIR spectrum of powdered GHK-Cu (1st day)

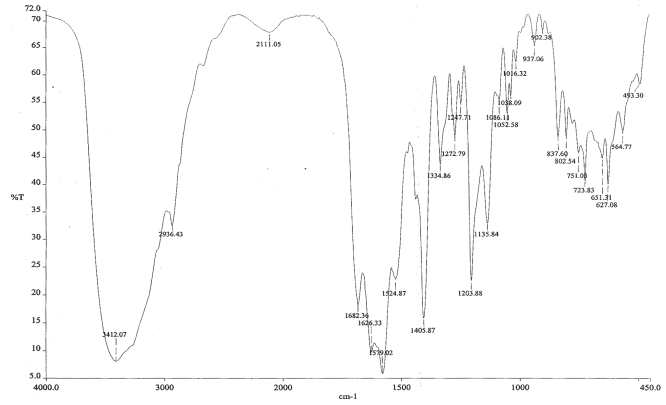

FIGURE 4. FTIR spectrum of powdered GHK-Cu (120th day)

many. Soybean lecithin (Epicuron 100H and Epicuron 200SH) obtained from Cargill, Italy and Carbopol Ultrez 21, Noveon, USA respectively. All other chemicals were, analytical grade

Methods

GHK-Cu assay and stability

A stock solution of GHK-Cu was prepared adding 50 mg of GHK-Cu to 50 ml of water. Dilutions (100, 60, 30, 15 µg/ml) were prepared by mixing GHK-Cu stock solution with water. Absorbance of dilutions was read at 237nm against water and calibration curve was achieved. To evaluate the stability of GHK-Cu in water, solutions at a five different concentration of 15, 30, 60, 100, 125, µg/ml were prepared, then put into 4°C, and followed their decrease of absorbance at 237nm for 4 months. The stability of powdered GHK-Cu was also evaluated using FTIR spectrophotometer. The FTIR spectrums of powdered GHK-Cu were taken at the beginning and the end of stability test and compared whether any differences had been occurred or not.

Preparation of liposome formulations

*Dipalmitoyl*phosphatidylcholine (DPPC-P5911) and Soybean lecithin (Epicuron 100H, and Epicuron 200SH) (Table I and Table III) were used as phospholipids. Cholesterol was used as a stability enhancer during liposome preparation and chloroform was used as a solvent. Briefly 50mg of phospholipids and cholesterol was weighed separately and then dissolved in 10ml chloroform. Chloroform in the lipid solution was evaporated at 57-58°C using rota-vapor under vacuum until thin film layer was occurred. Thin film layer was hydrated with water (with/without GHK-Cu) shaking with sonicator. Obtained liposomes were stored at 4°C just in case all the hydration process was achieved.

Evaluation of particle size and distribution of liposomes

Analysis of particle size and distribution of GHK-Cu loaded liposomes were done at TUBITAK-MAM (The Scientific and Technological Research Council of Turkey-Marmara Research center) Materials Institute. Particle size and distribution was measured using Malvern Mastersizer-X device, uses laser diffraction technique, after addition of distilled water to the liposome suspension and mixed with magnetic stirrer for 5 minutes.

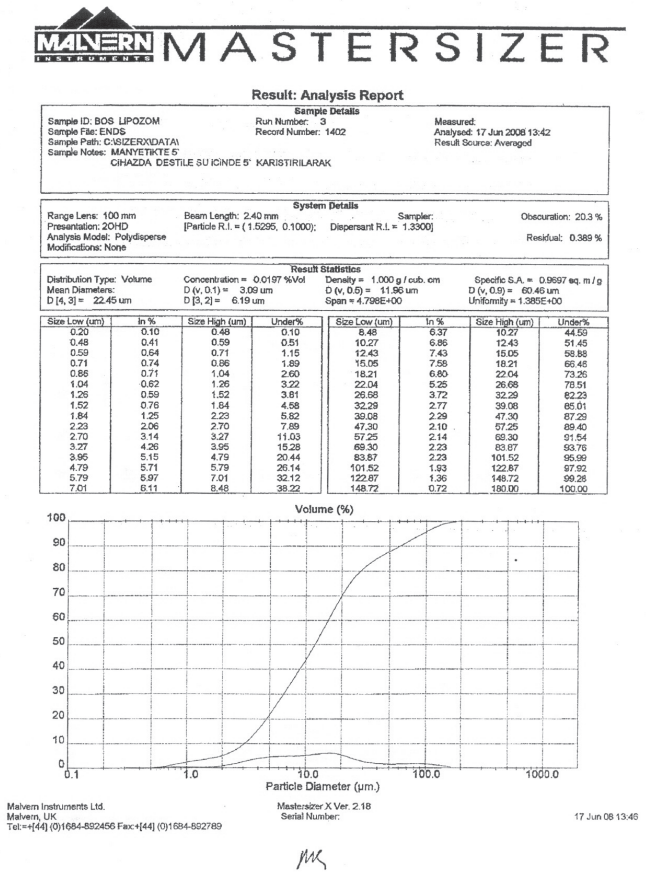

FIGURE 5. Particle distribution analysis data of GHK-Cu loaded liposomes

Evaluation of shapes of liposomes

In order to determine the shape and the number of liposomes layer light microscope (Olympus SZX7, Japan) and SEM microscopy (S-2460N Scanning Electron Microscope, HITACHI Super Scan Elite 20) measurements were done.

Evaluation of percent GHK-Cu entrapment of liposomes

Gradual filtration technique and dialysis tubing was used in order to determine the GHK-Cu entrapment of prepared liposomes. Formulations of liposomes are shown in Table I. In order to remove GHK-Cu loaded/unloaded liposomes, prepared liposomes passed through syringe-type filters which have 5µm, 1.2µm, 0.45µm, and 0.2µm pore diameter respectively. The non-encapsulated GHK-Cu was removed by dialysis tubing against water for 24h at 4°C. After the attachment of both GHK-Cu loaded and unloaded liposomes to the filter and dialysis tubing study, absorbance of the clear solution was read at wavelength of 237nm then free GHK-Cu amount was measured using calibration curve. The same process was applied to the hydration solution of liposomes and % GHK-Cu entrapment was achieved after the subtraction of absorbance of filtered /dialyzed solution from the hydration solution.

Evaluation of GHK-Cu diffusion from liposomes

Formulations of liposomes, which are shown in Table III, were prepared using 10ml water containing 150µg GHK-Cu per milliliter shaking with sonicator. Prepared liposomes were placed in dialysis tubing which was stored 4 hour in water in

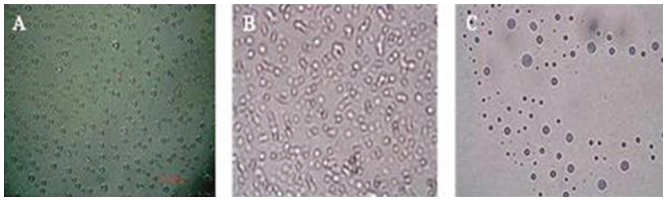

FIGURE 6. a) Light microscope images of liposomes (40X) prepared with Epicuron 100H, b) GHK-CU free liposomes images prepared with Epicuron 100H (40X) c) GHK-Cu loaded liposomes images prepared with Epicuron 100H (40X)

order to totally get rid of glycerol. This dialysis tubing has the ability of making a barrier to the molecules which are larger than 12kD. The non-encapsulated GHK-Cu was removed by dialysis tubing against water for 24h at 4°C. Dialysis tubing was soaked into a beaker containing 30ml water. At the predetermined time intervals, samples were taken from the beaker and evaluated at 237nm. Absorbance of materials which were used in the preparation of liposomes were also read to totally get rid of absorption affects of these materials.

Preparation of gel containing GHK-Cu liposomes

0.5 g Carbopol and 1 g NaOH were weighed. Carbopol was dispersed in 100ml water using a mechanical stirrer. 10 ml liposome suspension containing GHK-Cu was added. 1 g NaOH dissolved in 5 ml of water to make 20% NaOH solution. To make a gel, 6-7 drops of 20% NaOH solution was added to the mixture of Carbopol and liposome. After the gellation of system pH of the gel was measured and set the pH close to the skin pH 5.5. By the help of the light microscope liposomes containing GHK-Cu was observed in the gel (Figure 9).

RESULTS AND DISCUSSION

Results of GHK-Cu assay and stability

UV spectrophotometer was used as an analytical method to determine percent GHK-Cu entrapment of liposomes and GHK-Cu diffusion from the liposomes. 237nm was specified wavelength which shows the maximum absorbance of GHK-Cu in water and this wavelength used in the formation of calibration curve. Using this calibration curve all calculations was made to determine GHK-Cu concentrations throughout in our study (Figure 1). When the stability studies of GHK-Cu taken into consideration it was observed that if kept in dark and air tight place aqueous solution of GHK-Cu complex maintain its stability for 4 months at 4°C (Figure 2). When we look to the FTIR spectrum of the powdered GHK-Cu sample, if stored under the same stability conditions with aqueous sample, it was observed that FTIR absorption bands of GHK-Cu did not change significantly. Stress vibration peaks was seen almost the same (Figure 3, 4).

Results of preparation process of liposome formulations

During the studies, it was observed that phospholipids showed various behaviors in the film formation and hydration. Film layers prepared with Epicuron 200SH and DPPC showed more homogeneous structure than that of Epicuron 100H. On the other hand, the hydration process rate and convenience was better with Epicuron 100H than that of others (Epicuron 200SH and DPPC). Since the used phospholipids were saturated type, only cholesterol was added into the formulation during preparation. To prevent the oxygen exposure film layer was prepared under vacuum at 58°C.

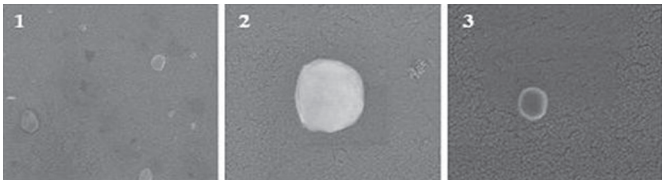

FIGURE 7. SEM images of Liposomes prepared with Epicuron 100H

Results of evaluation of particle size, distribution, and shape of liposomes

It was concluded that the size of the liposomes prepared in our study was between 1-100µm. However, results from Malvern Mastersizer-X showed that (Figure 5) the average size of the liposomes was 13,35µm. The shapes of prepared liposomes were observed with optical light microscope. Obtained images gave an idea about the shape, number of layer, and approximate size of liposomes. To get an effective topical formulation size distribution of the liposomes should be achieved in between 100-800nm. Based on the SEM studies, it was observed that liposome formulations contained small size liposomes like 100 nm. With the SEM images we concluded that all the liposomes have regular structure (Figure 6, 7). To decrease the size of liposomes sonication process and filtering of liposomes from convenient pore diameter filters could be an alternative way. Yalcin and Turkoglu, evaluated the size distributions of liposomes using a Coulter-Counter device in their study (19). In order to evaluate the effect of method to the size distribution of liposomes they filtered the liposome suspension through different size filters such as 0,2 µm, 0,45µm, 1,2 µm, and 5µm. They observed that the size distribution was getting narrower and homogenous with the decrease of pore size of the membrane filter.

Results of evaluation of percent GHK-Cu entrapment of liposomes

In our study, percent GHK-Cu entrapment was tried to calculate using three different liposome formulation (F1, F2, and F3; Table I). Free GHK-Cu was tried to be removed from the medium putting them into the dialysis tubing against water for

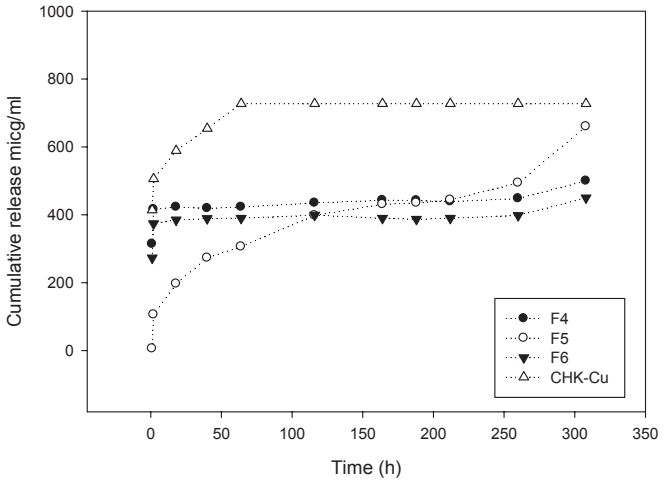

FIGURE 8. Cumulative GHK-Cu diffusion from liposomes

TABLE I. Formulations used in % GHK-Cu entrapment studies of liposomes

Formulation	Phospholipids (mg)	Cholesterol (mg)	Hydration solution	Hydration Type
F 1	Epicuron 200SH (40mg)	10 mg	5 ml 60μg/ml GHK-Cu	Sonicator
F 2	Epicuron 100H (40mg)	10 mg	5 ml 60μg/ml GHK-Cu	Sonicator
F 3	DPPC (25mg)	25 mg	5 ml 60μg/ml GHK-Cu	Sonicator

TABLE II. Data for % GHK-Cu entrapment of liposomes

Formulations	% GHK-Cu entrapment
F 1	6
F 2	33
F 3	27

24h at 4°C. As a result percent GHK-Cu entrapment was found to be 6% for F1; 33% for F2; and 27% for F3 respectively (Table II). When we compare the results the highest efficiency was achieved with F2 made with Epicuron 100H.

Results of Evaluation of GHK-Cu diffusion from liposomes

GHK-Cu diffusion from prepared formulations (F4, F5, F6; Table III) was investigated using dialysis tubing. To compare the results GHK-Cu solution in water was used as a reference in this study. GHK-Cu diffusion was followed at room temperature after dialyzing the liposomes for 24h at 4°C against water. This process was carried out to get rid of free GHK-Cu molecule from the medium. When we look Figure 8 closely GHK-Cu diffusion from reference solution reached peak level at 64th hour. GHK-Cu diffusion from F4 and F6 reached peak level at 18th hour. Because of burst effect caused by their more hydrophobic structure F4 and F5 released all integrity within few hours. This means formulation F4 and F6 were not able to diffuse GHK-Cu continuously. On the other hand slower diffusion of GHK-Cu from F5 reached 46% at 64th hour, continued and finally reached peak level at 308th hour. We concluded that F5 had an ability of diffusion control. In Figure 8, at the beginning diffusion of GHK-Cu from Liposomes prepared with Epicuron 100H (F5) was lower than the diffusion from that of others, however as time passes it caught, even it passed the diffusion level of F4 and F6. It was suggested that the diffusion of GHK-Cu from F4, F5, and F6 was lower than that of reference solution was related to their percent GHK-Cu entrapment capacity. When we think about the relation between diffusion and phospholipids component of liposomes, GHK-Cu diffusion from liposomes prepared with Epicuron 100H (F5) was higher and continuous than that of liposomes prepared with other phospholipids (Epicuron 200SH, F4; DPPC, F6) (Figure 8). The reason why liposomes prepared with Epicuron 100H behave different from the others is because of its more polar structure than DPPC and Epicuron 200SH. This polar structure gives permission to higher polar molecule entrapment. The results were complied with the data obtained from GHK-Cu entrapment studies. In GHK-Cu entrapment studies the highest entrapment was achieved with liposomes prepared with Epicuron 100H (F2) (Table II).

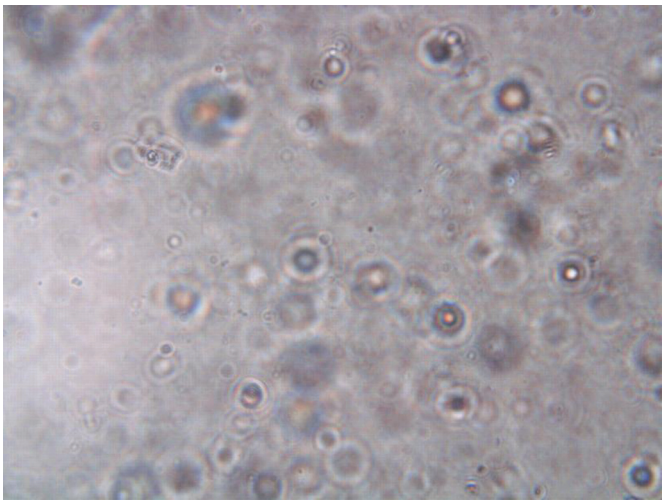

FIGURE 9. Light microscope images of gel formulation containing liposome (100X)

It is well known in the literature that studies with dialysis tubing are generally used in release studies and also separation studies of active agents from liposomes (20, 21). Lipid character of skin puts a barrier for the application of peptide like active agent. In our study we used liposome formulation in order to make more efficient application of hydrophilic macromolecules (protein and peptides) as an active agent through the skin. It is aimed to prepare a GHK-Cu liposome formulation which retards aging and decrease the lesions of aged skin. After the preparation of the GHK-Cu formulation the morphological studies GHK-Cu liposomes and diffusion studies were tried to be evaluated. Finally a hydrogel formulation was tried

TABLE III. Formulations prepared to use in the diffusion of GHK-Cu from dialysis tubing studies

Formulation	Phospholipids (mg)	Colestherol (mg)	Hydration solution	Hydration Type
F4	Epicuron 200SH (40 mg)	10 mg	150μg/ml, 10ml GHK-Cu	Sonicator
F5	Epicuron 100H (40 mg)	10 mg	150μg/ml, 10ml GHK-Cu	Sonicator
F6	DPPC (40 mg)	10 mg	150μg/ml, 10ml GHK-Cu	Sonicator

to be achieved to make a reasonable carrier for topical application of prepared liposomes.

As a result, having lots of activity such as skin reconstruction, cell renewal and anti-aging we strongly believe that using natural tripeptide-Cu complex (GHK-Cu) in liposome formulation has much more effective than that of other GHK-Cu formulation like lotions, ointments, solutions and etc. We think that development of modified liposomes as a hydrophilic molecule carrier has an important potential for cosmetic science. The development in this area could be possible with the correlation of invitro/invivo studies. It is promising that the design of GHK-Cu peptide liposomes gives a light to the preparation of topical and transdermal application of other peptide and protein type molecules.

ACKNOWLEDGEMENTS

This study was supported by the Scientific Research Projects Unit of Marmara University (Project No: SAG-C-YLP-060308-0030). Authors would like to thank to Dr. Loren Pickart of the Skin Biology, WA, USA and Chendu CP Biochem CO., LTD., China for kind donations of GHK-Cu^{2+} , and acknowledge the generous donations of Cargill, Italy.

Glisil-L-Histidil-L-Lisin-Bakır(II) peptid içeren lipozom formülasyonları

ÖZET: Glisil-l-histidil-l-lisin-Cu2+ (GHK-Cu) türevlerinin kolajen sentezini arttırdığı bilinmektedir. GHK-Cu'ın farklı aktiviteleri, kozmetik uygulamalar açısından ilgi çekmektedir. Lipozomlar topikal ilaç taşıyıcılar olarak bir çok fayda sağlamaktadırlar. Çift tabaka yapısı ve lipit bileşimi, etkin maddenin deride daha uzun süre tutulmasını ve devamlı bir şekilde, derinin derin tabakalarına salınmasını sağlamaktadır. Bu çalışmada, GHK-Cu yüklenmiş lipozomlar hazırlamayı ve bir kozmetik formülasyonda kullanmak üzere lipozomları karakterize etmeyi amaçladık. İlk olarak, GHK-Cu'ın miktar tayini için UV Spektrometre kullanılarak kalibrasyon eğrisi hazırlanıp, absorbsiyon bantlarının belirlenmesi için FTIR spektrumu çekildi. İçinde farklı miktarlarda GHK-Cu bulunan örneklerde 4 ay boyunca + 4ºC'de yapılan stabilite çalışmalarında GHK-Cu'ın sudaki çözeltisinin bozulmadığı saptandı. Aynı şartlarda saklanan toz halindeki GHK-Cu örneğinde yapılan FTIR spektrumu çalışmalarında maddenin yapısal olarak bozulmadığı spektrum bantları karşılaştırılarak görüldü. Lipit film hidrasyonu metoduyla farklı fosfolipitler (Dipalmitoilfosfatidilkolin (DPPC-5911), Epicuron 100H ve Epicuron 200SH), içeren lipozomlar hazırlandı (F1-F6), partikül boyutları ve şekilleri invert mikroskop, SEM ve lazer ışığı kırınım yöntemi kullanılarak değerlendirildi ve ortalama lipozom büyüklüğünün 13.35μm olarak saptandı. Lipozomlar tarafından tutulan GHK-Cu yüzdesi değerlendirildiğinde en yüksek GHK-Cu tutulmasını Epicuron100H fosfolipit'i ile hazırlanan lipozomların gerçekleştirdiği saptanmıştır. Diyaliz tübü kullanılarak yapılan difüzyon çalışmlarında fosfolipit olarak Epicuron 100H kullanılarak hazırlanan lipozomlarda GHK-Cu difüzyonunun DPPC ve Epicuron 200SH ile hazırlanan lipozomlara göre daha kararlı ve daha yüksek olduğu sonucuna varılmıştır.

ANAHTAR KELİMELER: Anti-aging, GHK-Cu, Lipozom, Kozmetik

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