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«Development of Liposomal Carnosine for dermatological and cosmeceutical use»

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Abbreviations (alphabetically)

AGEs: Advanced glycation end products
ALEs: Advanced Lipid Peroxidation end products
BDNF: brain delivered neutrophic factor
CFS: Cerebrospinal fluid
CML: carboxymethyl lysine
CREB: cAMP response element binding protein
D_h: Hydrodynamic diameter
DLS: dynamic light scattering
EE: encapsulation efficiency
EKPA: Ethniko kai Kapodidtriako panepistimio Athinon =National and Kapodistrian university of Athens
ELS: electrophoretic light scattering
FOITS: Fast optical in vivo topometry of human skin
GUVs: giant unilamellar vesicles
HA: hyaluronic acid
HCD: histidine containing peptides
LUVs: Large unilamellar vesicles
MGO: methylglyoxal
MHM: modified heating method
MLVs: multilamellar vesicles
MRS: magnetic resonance spectroscopy
PCS: photon correlation spectrometer
PDI: polydispersity index
RAGE: receptor for advanced glycation end products
RCCs: Reactive Carbonyl Compounds
ROS: Reactive oxygen species
SC: stratum corneum
SUVs: Small unilamellar vesicles
TNF: tumor necrosis factor

Abstract

Carnosine is a dipeptide (β-alanyl- L-histidine) with unique antioxidant and antiaging properties, that exists endogenously in the human body and plays a key role in several physiological aspects of homeostasis. Recently it has been used in several cosmeceutical products to enhance their antiaging and wound healing effect. However, skin penetration of carnosine is rather poor, and research is focusing on finding ways to overcome stratum corneum barrier. Liposomes are a viable and well-studied solution for drug delivery through the skin. Different types of liposomes have been utilized to increase skin penetration of active ingredients.

Three types of carnosine loaded liposomes (2.5% w/w) were developed with Modified Heating Method (MHM) and evaluated in terms of physicochemical stability in a period of 2 months in two different storage scenarios, room temperature and refrigerator. Specifically, a conventional liposome, an ultradeformable liposome (transferosome)with Tween 80 and a ceramide containing liposome (ceramidosome) were studied and then formulated in a neutral gel for dermatological use with the final formula containing 0.5% of liposomal carnosine w/w. Two types of neutral gel were prepared, one containing 0.1% hyaluronic acid and one without. Final formulations of gel were tested to evaluate if liposomes retained their unique properties in the final product

All three types of liposomes prepared with this method exhibit acceptable physicochemical properties consistent with the characteristics of multilamellar vesicles (MLVs) or Large unilamellar vesicles (LUVs) in the range of 300 to 500nm. Z-potential was found in the range of -40mV up to -50mV that correlates to good stability of nanocolloidal system. Polydispersity index showed that all liposomes were homogenous since at day one was found to be 0.295 S. D +/- 0.023 for the conventional liposome, 0.293 S. D +/- 0.015 for the transferosomes and 0.267 S. D +/- 0.027 for the ceramidosomes. Conventional liposomes and transferosomes have been found to have the highest encapsulation efficiency (EE%) at 62.67% and 66.33% respectively, with ceramidosomes having 28.33% carnosine encapsulation. Liposomes that were stored in the refrigerator performed better in terms of stability over time retaining their initial measurements, whereas liposomes stored in room temperature developed microbial growth that affected their pH, their color and resulted in the formation of aggregates that were easily dissolved after mild agitation of the container. Hydrodynamic diameter, dispersity, and EE% were not significantly affected from the storage conditions. When incorporated in the neutral gel only conventional liposomes retained size measurements near to those of pre formulation (341.14 S. D +/- 4.7) but PDI showed a significant rise (0.642 S. D +/- 0.012) when compared to the original liposome. Transferosomes and ceramidosomes exhibit a drop on their size (258.6 S. D +/- 6.3 vs 432.76 S. D +/- 16.03 and 394.10 S. D +/- 10.2 vs 558.37 S. D +/- 22.26 respectively) and significant rise in PDI (0.581 S. D +/- 0.009 vs 0.280 S. D +/- 0.016 and 0.919 S. D +/- 0.140 vs 0.263 S. D +/- 0.039). The addition of hyaluronic acid in the neutral gel did not seem to further affect the physicochemical properties of the liposomes in the final formulation.

1.Introduction

1.1 Physicochemical properties of Carnosine

Carnosine (β-alanyl- L-histidine) is a dipeptide , first discovered along with other histidine containing peptides (HCD , anserine , carnosine and ophidine) by V.S Gulewitch in 1900 in his attempt to identify nitrogen containing not protein compounds in Liebig's meat extract (1).

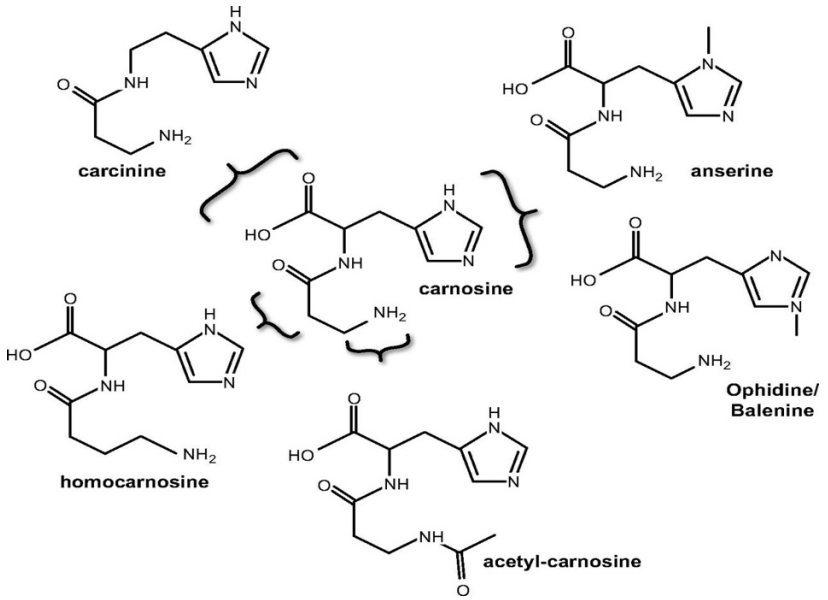


Figure 1 chemical structure of HCD

Carnosine is a very water soluble molecule (1 g in 3,1 ml of water in 25^{o} C) and is characterized by three ionizable groups : the carboxylic group (pK_a 2.76), the amino group of the β- alanine reissue (pK_a 9.32) and the nitrogens of the imidazole ring (pK_a 6.72)(2). At physiological pH carnosine is present in the zwitterionic form. Two tautomeric forms of carnosine's imidazole ring exist in its neutral form, the Nτ protonated (tautomer 1) and the Nπ protonated (tautomer 2). The two tautomers exist in equilibrium in neutral pH and at pH 7 and 9 the tautomer 1 is the most predominant species (75%) and tautomer 2 is energetically less stable in those conditions (3)(4)

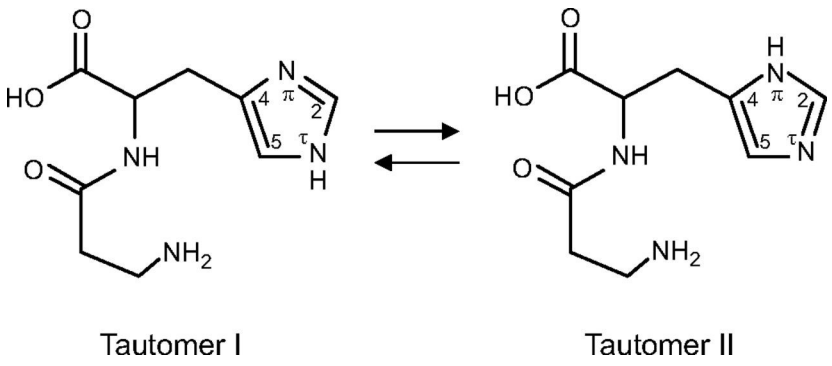


Figure 2. Carnosine's Tautomers

1.2. Role of Carnosine in human organism

Carnosine is endogenously predominant in tissues such as skeletal muscles, nervous system, and the cardiac muscle, which exhibit the most active oxidative metabolism in the human body. In skeletal muscles it is proposed that carnosine plays a role in their contractile function(5) presumably due to the improvement of Ca^{+2} sensitivity of the contractile apparatus,(6) and acts as a pH buffer thus helping in maintaining homeostasis by protecting against acidosis (7). In humans lower levels of muscle carnosine is found in elderly people rather than younger adults (8). Also males tend to have more carnosine in their muscles than female (9)
Carnosine exhibits similar roles in cardiac contractibility. Particularly in a recent study it was proposed that carnosine acts as a diffusible Ca^{+2} /h exchange pump creating functional Ca^{+2} gradients in response to local pH changes in cardiomyocytes(10). Furthermore carnosine has an effect on blood pressure through a variety of vasoactive mechanisms such as the carnosine -histidine – histamine pathway (11),a NO/cGMP mechanism(12) and the modulation of autonomic nervous system(13). In mammalian brain the highest concentration of carnosine is found in the olfactory system. However, homocarnosine's (γ-aminobutyryl derivative of carnosine) concentration on brain is rather high and detectable by proton magnetic resonance spectroscopy (MRS). The fact that these compounds are found predominately in the nervous system is because their components like γ- amibobutyryl are typical for this tissue. Carnosine can penetrate the blood brain barrier but most of carnosine content in brain is attributed to de novo synthesis (9). Little is known about the physiological role of carnosine in the brain , but it's mainly proposed that it somehow modulates neurotransmission and

acts as a homeostatic and protective of the brain through its antioxidant , metal chelating and antiglycative properties (1).The main dietary sources of carnosine for humans is meat and fish ,but it can also be synthesized endogenously in glial cells and myocytes, however vegetarians tend to have less carnosine in their muscles (14) (15) (9)

1.3. Metabolism of carnosine in human body

Carnosine and other HCD are not degraded by regular dipeptases but by a hydrolytic enzyme called carnosinase which is specific to their metabolism. Two types of carnosinase exist , serum carnosinase (CN1) and Tissue carnosinase (CN2)(16,17). CN1 is produced and secreted from the liver into the circulation. Anserine, ophidine and homocarnosine are found to be more resistant to CN1 and to be potent inhibitors of carnosinase towards carnosine as a substrate (1,18). The catalytic rate of CN2 is markedly lower than that of CN1 but carnosine can be still hydrolyzed by it in physiological conditions(1,19). The activity of carnosinase is getting higher with age and this leads to smaller accumulation of carnosine in tissues (9)

Carnosine is transported across cellular membrane mainly through proton-coupled oligopeptide transporters, namely the PEPT1 and PEPT2 (oligopeptide transporters 1 and 2) and PHT1 and PHT2 (peptide/histidine transporter 1 and 2). All these transporters have broad specificity and can transport a high number of other peptides as well .PEPT 1 is located on the intestines and particularly on brush border membrane and plays a significant role on nutritional absorption of carnosine , whereas PEPT2 is localized in the apical membrane of epithelial cells in the renal tubule and is responsible for 90% of the carnosine reuptake from the CSF thus playing a critical role in the homeostasis of carnosine (1,20). On the other hand, little is known for PHT1 and PHT2 transporters.

It is presumed that carnosine is not directly delivered in the brain, but its action is most likely attributed to brain gut interaction. Specifically, carnosine augments the expression of brain delivered neutrophic factor (BDNF) which supports survival of the neurons and stimulate the growth of new ones. Because BDNF can cross the blood-brain barrier its assumed that it activates the brain-gut interaction.

Furthermore carnosine activates CREB (cAMP response element binding protein) which in terms triggers the synthesis of secretory factors related to brain -gut interaction (9). Also some neural cells express the carnosine transporter PHT2 thereby overcoming the inability of carnosine to transfer across blood brain barrier.(14)

When carnosine enters the body through the oral rout, a small percentage of it, is hydrolyzed by the carnosinase of the enterocyte in the jejunal mucosa. Most of the carnosine ingested is rapidly hydrolyzed in the plasma due to the high activity of the enzyme carnosinase and thus negligible levels of carnosine are detected on the blood stream. Intact carnosine cannot be taken up by muscle because they lack the proper receptors(14)

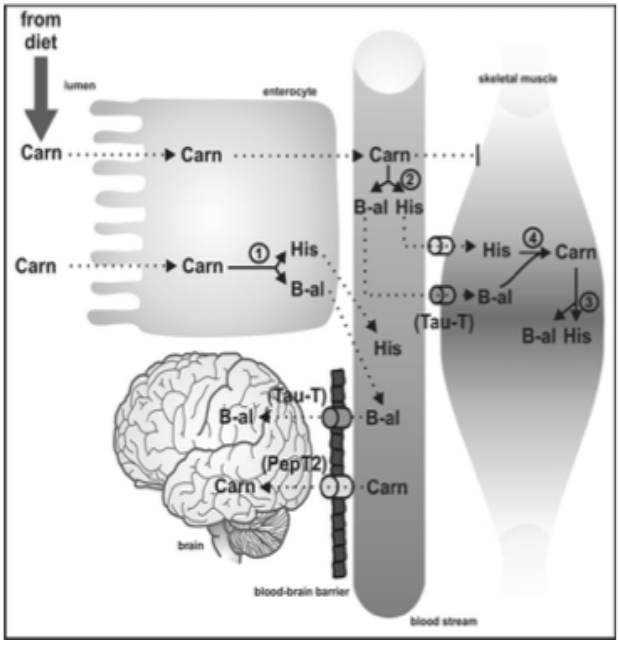


Figure 3.A schematic illustration of carnosine bioavailability from the review article of Sale et al (13)

1.4. General Therapeutic applications of carnosine

Carnosine exhibits significant antioxidant properties as demonstrated in several studies. The antioxidant properties of carnosine are mediated by different mechanisms involving metal ion chelation and scavenging Reactive oxygen species (ROS) and peroxyl radicals (1). Among the most investigated are the Copper and zinc complexes with carnosine due to their biological significance. In particular a zinc and carnosine complex (z-103) was shown to protect gastric mucosa from

experimental ulcerations in vivo (21) and exhibited significant effectiveness against Helicobacter pylori associated gastritis (22).

In several studies in rodents supplemented carnosine has been shown to act as an antioxidant in physiological conditions and in models of induced oxidative damage by xenobiotics. The antioxidant effect exhibited in those studies can be attributed to both the direct scavenging effect towards ROS and by an increase and/or sparing and/or regenerating effect on enzymatic and non-enzymatic antioxidants. This led in some cases to a reduction in tissue damage and functional impairment. There is however no clear understanding of the mechanism in which carnosine potentiates the enzymatic antioxidants .(1)

Carnosine is rapidly glycated by Reactive Carbonyl Compounds (RCCs) such as common sugars with a reactive carbonyl group e.g., glucose, fructose, galactose, ribose and deoxyribose and glycolytic intermediates like the phosphorylated forms of these sugars and thus sparing proteins and lipids from RCC mediated damage. In particular the product of the non-enzymatic reaction of RCC with proteins are called Advanced glycation end products (AGEs) and their reaction product with lipids are called Advanced Lipid Peroxidation end products (ALEs)(8).

The process of the formation of AGEs occurs naturally in normal metabolism but is more widespread in conditions such as oxidative stress, hyperlipidemia, and hyperglycemia. In healthy conditions the human body can inhibit the accumulation of AGEs through several detoxification pathways including glyoxalase enzyme, nuclear factor erythroid 2-related factor, AGE -binding proteins, fructosamine-3-kinase and aldose reductase. During Maillard reaction electrophilic carbonyl groups of glucose and other reactive sugar sugars react with the amino groups of amino acids forming nonstable Schiff base. Further rearrangement leads to a more stable ketoamine (Amadori product). Schiff and Amadori products can react irreversibly with amino acid residues of peptides or proteins and form protein adduct or proteins crosslinks as shown in the figure 4 from the review article of Gkogkoglou et al (23)

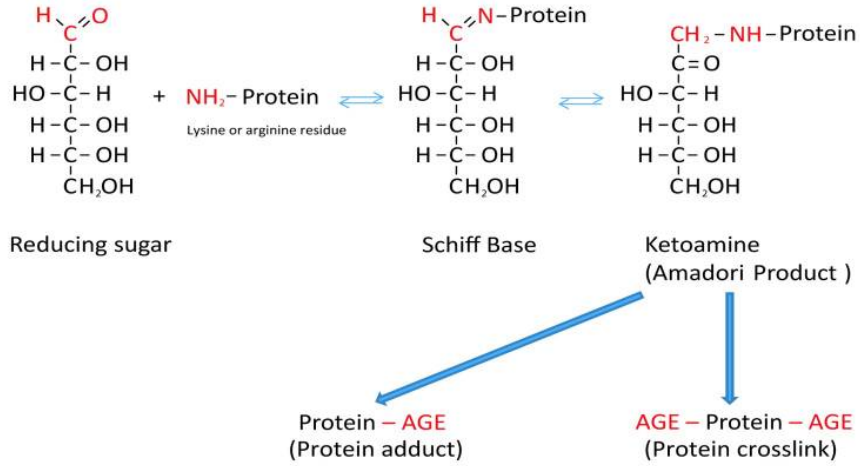


Figure 4. Formation of AGE's

There are multiple suggested mechanisms of action in which carnosine prevents the formation of AGEs. Particularly, Carnosine can act as an electron donor with the ability to neutralize free radicals and thus preventing the production of precursors of AGEs. Its metal chelating ability may also prevent the formation of free radicals due to metal ion capacity to oxidize, by restring the reactivity of metals. Furthermore, Carnosine has been shown to increase antioxidant enzyme activity and levels of glutathione, superoxide dismutase and glutathione peroxidase. This leads to the reduction of TNF-a and the transcription of proinflammatory cytokines that produce free radicals. Carnosine increases the expression of nuclear factor erythroid 2-related factor (Nrf2) which subsequently inhibits lipid peroxidation and upregulates several antioxidant enzymes.

It is also suggested that because Carnosine contains an imidazole ring it possesses Glyoxylase1(GLO) mimetic activity which accounts for its ability to scavenge MGO (15)

Several animal studies suggest that carnosine prevents the intraneuronal accumulation of β- amyloid aggregates or that is possesses inhibitory effect on their formation. In Table 1 a summary of potential causes of neurodegeneration and carnosine's proposed action is provided according to the review article of Chmielewska et al (9)

Table 1. Causes of neurodegeneration and effect of carnosine

Cause of neurodegeneration	Effect of carnosine
Protein misfolding and fibrillation causes the formation of β- amyloid aggregates due to metal ion binding	Carnosine can chelate Cu^{+2} and Zn^{+2}, protecting neurons from lipid peroxidation
Oxidative and nitrosative stress (RNS, ROS)	Decreases the expression of inducible nitric oxide synthase, decreases the secretion of proinflammatory cytokines
Increased AGEs formation cause neurotoxicity by changing the function of proteins, promoting mitochondrial dysfunction and leading to the increase of ROS, that cause cell death	Detoxifies Reactive carbonyl compounds acting as a substrate, forming covalent adducts that are excreted in the urine
Lewy bodies in Parkinson's disease are protein aggregates composed of alpha-synuclein (ASN)	Carnosine decreases ASN misfolding due to its antiglycation properties

1.5. Therapeutic Applications of Carnosine in dermatology

Accumulation of AGEs in the skin may be associated with the skin aging because it has been found to affect extracellular matrix proteins such as collagen, vimentin and elastin. Specifically, glycation of collagen impairs its function due to reduced elasticity and increased stiffness. Those changes promote the appearance of wrinkles. Glycated elastin is found in photo aged skin, which in all probability means that UV radiation stimulates the glycation of elastin. Furthermore, glycated proteins of the extracellular matrix resist degradation by matrix metalloproteinases which in terms leads to their slower removal and replacement by functional newly synthesized proteins. The binding of AGEs with their cell surface receptors RAGE initiates a cascade of signals influencing cell cycle and proliferation, gene expression and inflammation Factors that contribute to the formation and

accumulation of AGEs in the skin are the UV radiation from the sun, smoking and diet. Approximately 10-30% of ingested AGEs are absorbed in the circulation (23)

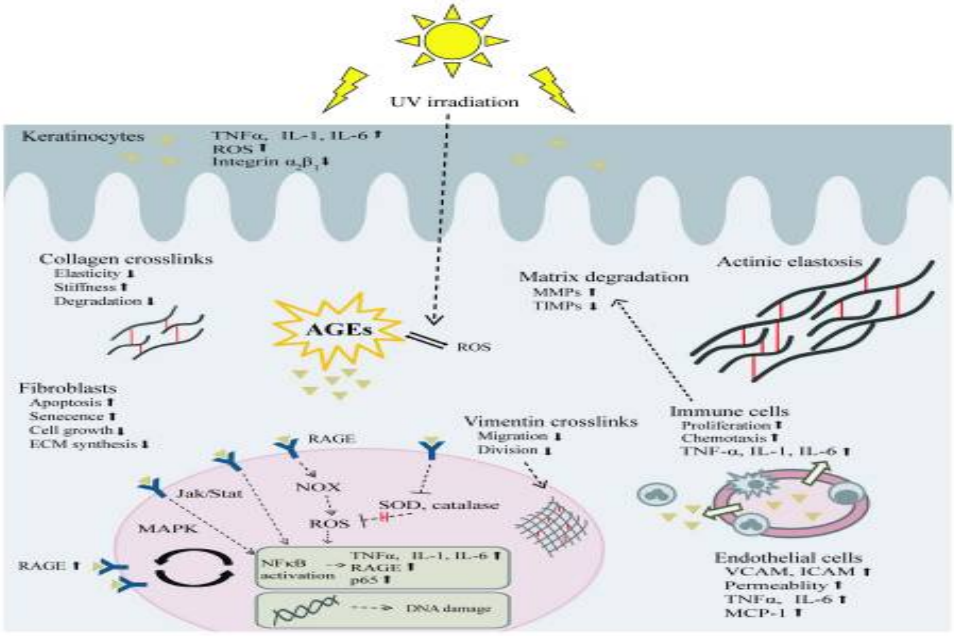


Figure 5. Effect of UV radiation on the formation of AGEs on skin

Carnosine has been shown to protect against the formation of AGEs in skin , particularly carboxymethyl lysine (CML) and pentosidine in an in vitro study in skin explants when applied topically in a concentration of 0.2% in an aqueous solution and in a face cream .The study has shown a reduction of -64% and -41% of CML and pentosidine respectively in epidermis and -48% and -42% in reticular dermis with the use of the carnosine aqueous solution and a reduction of -150% in CML and -122% pentosidine in epidermis and -108% and -136% respectively in reticular dermis. These results suggest that the carnosine carrier plays a significant role in its bioavailability in the skin which in term affect its effectiveness in reducing AGEs (24). In another study a combination of injected carnosine and locally applied carnosine in mice with diabetes mellitus type 2 has shown a remarkable enchantment of wound healing by increasing the expression of growth factors and cytokines genes involved in wound healing. In the same study an in vitro study with human dermal fibroblasts and microvascular endothelial cells showed that carnosine increased cell viability in presence of high glucose. This is especially important because in diabetes numerous factors including impaired angiogenesis , chronic hypoxia , neuropathy , and failure to exhibit the normal sequence of

molecular interaction and signal transduction in addition to an elevated proteolytic environment , delay the wound healing(25)

In a study regarding a night cream formula containing melatonin, carnosine and Helichrysum Italicum extract, it was found that the application of this product significantly improved all markers regarding oxidative stress, hydration and clinical signs of aging. The study was divided into an *ex vivo* study that evaluated the recovery effect of skin explants after exposure to UV, IR and pollution and the second half was a total of four prospective, single centered, open label noncomparative clinical studies that evaluated skin hydration kinetics, comedogenicity, anti-aging efficacy and skin calming effects in subjects with sensitive skin. Authors attributed part of the anti-aging effect of the night cream to the ability of carnosine to limit AGE formation. However complete formulation of the night cream was not provided in the article so it is unknown what the concentration of the cream in carnosine was.(26)

Another research paper concerning two double-blind randomized controlled, split face studies in women 30-70years old with periorbital wrinkles utilized products containing among other ingredients carnosine, showed that usage of one of these products twice a day for two weeks improved smoothness compared to no treatment by evaluating periorbital wrinkles with Fast optical in vivo topometry of human skin (FOITS). The study however does not imply that carnosine alone is responsible for this effect , as the products used contain other active molecules with known anti-aging properties (27). A novel topical product containing traditional physical sunscreens (SPF50), a liposome-encapsulated DNA repair enzymes complex (photolyase, endonuclease and 8 oxoguanine glycosylase) and a potent antioxidant complex (carnosine , atrazine ,ergothionine) was compared to existing products in terms of preventing the formation of cyclobutene pyrimidine dimmers (CPD) , protein carbonylation and 8-oxo-2'-deoxyguanosine (8OHdG) which are molecular markers for skin aging and the development of non-melanoma cancer. The results showed that the synergistic action of those ingredients reduced CPC and PC but not 8OHdG, thus improving the genomic and proteomic integrity of the skin cells after repeated exposure to UVR. Again, in this study carnosine is part of a complex and it was not study on its own (28). The use of topical carnosine

lotions of 0.5% and 1% respectively has been shown to protect effectively from suppressive effects of UVB radiation and contact hypersensitivity caused by the cis urocanic acid on mice. Especially for the effect of the 0.5% carnosine lotion on the UVB, it was not attributed to screening action because carnosine does not absorb in the UVB region. The authors concluded that carnosine demonstrates immunomodulating properties.(29). Nino et al. used a 0.5% carnosine aqueous solution before and after UVB irradiation in twenty healthy volunteers with phototype 2 or 3 resulting a 3.6% reduction of erythema compared to minimal erythemal dose (MED). This is a very interesting study because positive results can only be attributed in the action of carnosine since there were no other excipients in the formula and the erythema reduction is compared to the use of only water among other things. Apparently N- acetylcarnosine water solution of similar concentration (0.5%) demonstrated a 7.3% erythema reduction suggesting that carnosine has poor skin penetration (30). Indeed because carnosine is highly water soluble, although small in size it was not able to penetrate the highly lipophilic stratum corneum (SC)in a study with standard Franz diffusion cells and was only found to penetrate the first layer of stratum corneum . In the same study modified peptide with an attached palmitoyl chain to the terminal NH_2 group exhibited augmented lipophilicity and diffused into the stratum corneum. the epidermal and dermal skin layers(31).

1.6 Overcoming skin barrier challenges

It is apparent that bioavailability plays a key role in the effect that carnosine could facilitate on the skin. An attempt to increase the skin permeation of carnosine was done by forming a magnesium ion complex with carnosine and testing the complex in a gel formulation versus regular carnosine in the same vehicle, in human skin 3 D models. The results obtained by this study support the hypothesis that magnesium can improve the bioavailability of carnosine since it increased the concentration of carnosine in the lower skin layer 60% more than the carnosine alone.(32)
Another way to enhance carnosine penetration was deemed to be the use of 1.2 pentyl glycol in a standard hydro dispersion gel and the formation of micro emulsion which is considered a colloidal carrier system. Carnosine penetration of

those different formulations was tested in *ex vivo* human skin and the results indicated that the addition of 1,2 pentyl glycol significantly improved carnosine transdermal penetration. Specifically there was a sixfold higher carnosine concentration in the stratum corneum and in the viable skin layers .The performance of the microemulsion was underwhelming and authors proposed that the microstructure of the formula might not have been optimized for the hydrophilic properties of the specific dipeptide (33)

1.7. Liposomes as a delivery system

In order to enhance active ingredients permeability through the skin, liposomes are utilized as delivery systems because they possess unique abilities. Due to their vesicular structure liposomes resemble biological membranes. They are composed of amphiphilic molecules usually cholesterol and nontoxic phospholipids in a bilayer conformation. It has been shown that phospholipids tend to self-organize spontaneously to form closed structures when they are hydrated in aqueous solutions. Liposomes are defined as pseudo-spherical vesicles with particle sizes ranging from 30nm to several micrometers. They consist of one or more lipid bilayers surrounding aqueous units in which the hydrophobic moieties interact with each other and are directed inside, and the hydrophilic portions are oriented outside interacting with water. Generally liposomes are classified in large multilamellar vesicles (MLVs) which are essential concentric bilayers separated by aqueous compartments, giant unilamellar vesicles (GUVs) which are typically at the range of 1 μm, Large unilamellar vesicles (LUVs) who range 100nm to 1000 nm , and Small unilamellar vesicles (SUVs)that are smaller than 100 nm (34) (35)

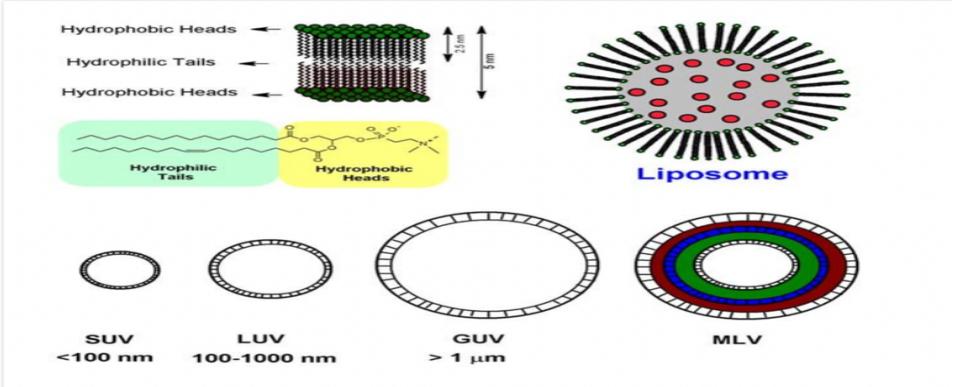


Figure 6. Liposomes basic morphology and types

Besides improving the biodistribution of active ingredients, liposomes are known to make therapeutic compounds more stable, can be used with hydrophilic and hydrophobic molecules , are biocompatible and biodegradable (36).

Several types of liposomes have been developed for topical dermatological use including namely conventional liposomes composed of phospholipids and cholesterol, ultradeformable liposomes or transferosomes composed by phospholipids and surfactant molecules, niosomes primarily composed of nonionic surfactants and phospholipids, ethosomes consisting of phospholipids water and a exhibit several advantages for specific uses (34)

Main theories concerning the way liposomes enhance skin penetration of drugs and active ingredients are the following

1) Free drug mechanism: the drug permeates the skin independently after exiting the vesicles
2) Penetration enhancing mechanism: transdermal delivery is enhanced by lowering the permeability barrier of the skin
3) Vesicle absorption to and/ or fusion with the stratum corneum (SC): the vesicles may absorb to the SC surface with subsequent transfer of the drug directly from vesicles to skin, or vesicles may fuse and mix with the SC lipid matrix thus increasing drug partitioning in the skin.
4) Intact vesicular skin penetration mechanism: large lipid vesicles penetrate the densely packed SC
5) Transappendageal penetration: permeation through the sweat glands and across hair follicles with their associated sebaceous glands

Evidence in the literature suggests that there is not one and unifying theory regarding the way liposomes improve active substances topical delivery through the skin , rather that different types of liposomes loaded with different drugs exhibit different behavior due to different interactions .(37)

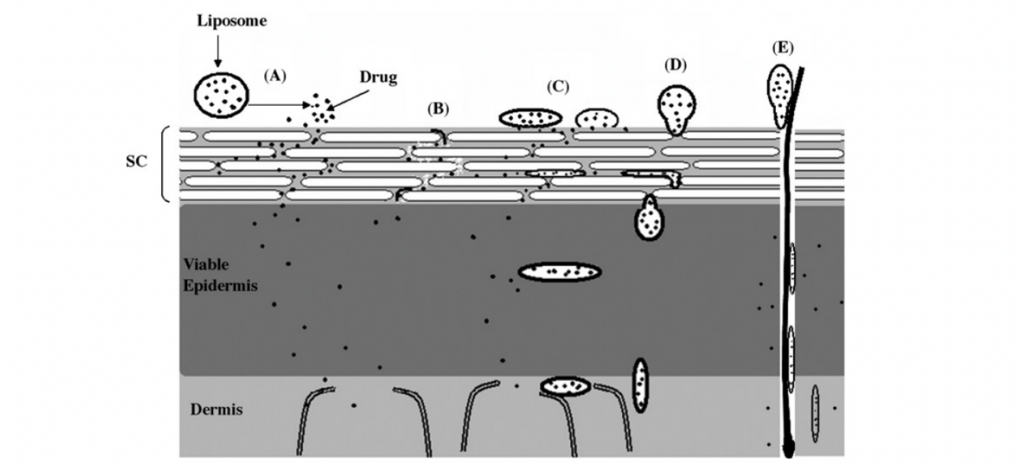


Figure 7.Possible mechanism of cation of liposomes as skin drug delivery systems (A)Free drug mechanism, (B)Penetration enhancement mechanism, (C)vesicle absorption to and/or fusion with the SC, (D) intact vesicles penetration, (E) transappendageal penetration. From article :Liposomes and skin : From drug delivery to model membranes (37)

The physicochemical characteristics of liposomes affect their ability to deliver substances to the skin. Specifically transition temperature (T_m) of the phospholipids forming the liposomes have an impact on the fluidity of the liposomal membranes as well as the passive permeability of water and small molecules from the membrane of the vesicles and thus their stability. Size of the vesicles also play a significant role in liposomes ability to delivery drugs and active substances in the skin. In general vesicles with size greater than 600 nm are unable to reach deeper layers of the skin. Liposomes of approximately 300nm can reach deeper layers of the tissue. Liposomes of 70nm were more promising for topical skin delivery. Surface charge of the liposomes can modify the permeation capacity. Whether anionic or cationic liposomes perform better is rather controversial as studies suggest that both types showed promising results, meaning that surface charge plays a role in the concept of the final formulation and depends on the drug encapsulated in the liposome (34)

1.8. Ultradeformable liposomes

Because elasticity of the bilayer is a key factor for effective skin delivery Ultradeformable liposomes or Transferosomes exhibit great potential as a drug delivery system for skin medications and general active ingredients. Transferosomes were introduced by Cevc et al. and are composed of phospholipids and an edge activator, typical a surfactant. The combination of those two types of

molecules in a single vesicle destabilizes the lipid bilayer and increases their deformability by lowering interfacial tensions, thus creating elastic liposomes. Surfactants used as an edge activator are typically Sodium cholate, Span 60, Span 65, Span 80, Tween 20, Tween 60, and Tween 80.(38)(34)

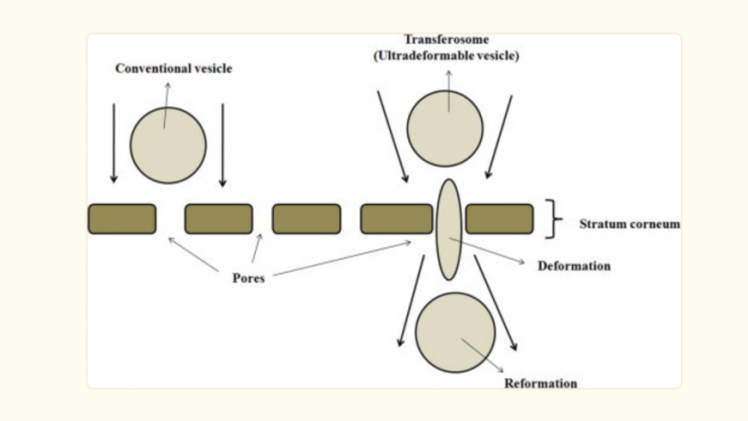


Figure 8. Proposed mechanism of skin penetration of transferosomes(39)

Several studies about transferosomes show that they are a very efficient delivery system. Arora et. al developed a resveratrol loaded ultradeformable vesicular cream by utilizing sodium cholate as a surfactant and various amounts of phospholipids to optimize entrapment efficiency, resulting in higher substance deposition in the skin when compared to a conventional cream. Furthermore resveratrol encapsulated in the vesicular cream has retained its antioxidant activity showing how liposomes protect the active ingredient from the interactions with the environment.(40) Rahman et al developed a formulation containing 0.025% of tretinoin encapsulated in an elastic liposome containing L-α-Lecithin , cholesterol and dicetylphosphate as a surfactant. This formulation generated much lighter erythema than convention tretinoin formulations and improved efficacy on the acneic lesions. In another study coenzyme Q 10 was encapsulated in ultradeformable liposomes containing Tween 80 with alpha lipoic acid that were coated with chitosan. In vitro studies showed that the liposomes were efficient in reducing hydroxyl radicals. Release and permeation studies in rabbit skin indicate that this system promoted sustained delivery and adequate deposition of the active ingredient in the skin (34). Estradiol was formulated in several ultradeformable liposomes containing Span 80 and Tween 80 as edge activators and phosphatidylcholine (PC) compared to liposomes with sodium cholate. All edge

activator at the optimal concentration displayed similar improved skin delivery of estradiol compared to control(41)

1.9. Ceramides and liposomes

Ceramides are a complex group of sphingolipids containing derivatives of sphingosine bases in amide linkage with fatty acids and are one of the components of the lipid matrix of the SC that regulates skin barrier function, cell adhesion and epidermal differentiation. In the SC at least 8 ceramides were identified that differ from each other by the head- group architecture and by the mean of fatty acid chain length. The less dense lipid organization is associated with a reduction of lipid chain length in SC in patients with atopic dermatitis and patients with impaired skin barrier function. (42,43). Numerous skin products utilize ceramides combination to improve skin conditions and liposomes formulations containing ceramides have gained some traction in recent years. Specifically liposomal formulation containing a mixture of ceramide showed promising results in sodium lauryl sulfate induced damage to the skin barrier versus liposomes containing only one type of ceramide. Additionally in vivo studies have shown accelerated rate of repair in stripped and sodium lauryl sulfate damaged skin (43). A liposomal formulation of ceramide/phospholipid mixtures showed stabilizing effect on the barrier function of atopic skin and no result on healthy skin thus implying that such a formulation could have advantageous effects on treating skin conditions where barrier damage is present.(44) However Nanoemulsions were found to incorporate a high percentage of ceramides (48.4% of total lipids by weight) giving a more homogenous nanoparticle distribution and stability over time, whereas solid lipid nanoparticles were stable at much lower concentrations (>10%).These results indicate that Nanoemulsions may be more suitable ceramide carriers than solid lipid nanoparticles(45) Ceramide liposomes loaded with quercetin and rutin were found stable in a hydrogel formulation for over 3 weeks and improved skin permeability of active ingredients significantly when compared to control formula. These results indicate that ceramide liposomes in hydrogel could be used as drug delivery system to enhance skin absorption of those active substances(46).Tokudome et al , developed a liposome containing C8 Ceramide ,

cholesterol ,linoleic acid , and cholesterol sulfate that displayed high membrane fluidity and high fusion activity to SC lipid liposomes and could potentially be used for increasing drugs distribution on the skin.(47)

1.10. Methods of liposome preparation

Liposomes can be prepared using a wide range of methods that are a usually multistep process following those fundamental stages

1) Lipid dissolution in an organic solvent
2) drying of the resultant solution
3) hydration of the dried lipid
4) isolation of the liposomal vesicles
5) quality control assays

The oldest method of liposome preparation is considered to be Film Hydration (Bangham method) which a straightforward and well-studied method but usually leads to production of large particles with no control over the size, displays poor encapsulation efficiency of hydrophilic materials, its time consuming and it poses challenges when it comes to sterilization of the product. Other conventional methods of preparation of liposomes include Reverse phase evaporation method, solvent injection method, detergent removal method and Heating method.

The heating method is the most attractive method for liposome preparation because its organic solvent free, is simple and fast, is a scalable method for industrial use and does not need sterilization thus minimizing formulation complexity. In the heating method, lipids are hydrated for 1 hour and heated for another hour above the transition temperature of the phospholipids in the presence of a hydrating agent such as glycerin or propylene glycol. When cholesterol is a part of the formulation the reaction medium is heated up to 100^{o}C because of its high melting point.(48)

For the purpose of this experiment a patented modified heating method (MHM) was utilized (49)developed in professor Demetzos Lab. This process utilizes in addition to a mechanical shock because of the stirring, a thermal shock caused by the temperature increase in the second step of heating and results in the formation of liposomes with improved physicochemical characteristics and stability.

Liposomes developed with the MHM usually do not require to undergo size reduction processing but, if need be, can be subjected to sonication, homogenization and extrusion. The MHM comprises of the following steps:

1. mixture of amphiphilic lipid and a promoter in a liquid medium of water and a liquid polyol
2. stirring and heating the mixture in a first heating step at 30-80^{o}C
3. stirring and the heating in a second heating step at a temperature 10-50^{o}C higher than the temperature of the first heating step and
4. allowing the mixture to cool down in room temperature.

The temperature in the first heating stage is chosen based on the transition temperature of the mixture of amphiphilic lipids. The stirring speed and temperature in every step are preferable to be kept stable throughout the process. With this method several amphiphilic mixtures in combination with different polyol concentrations in the aqueous medium were tested to develop curcumin loaded liposomes , indicating that this method is very effective and flexible for experimental design and development of stable liposomes (50)

2.Aim of the study

In this study three different liposomes containing 2.5% carnosine will be prepared, namely a conventional liposome, a transferosome containing Tween 80 as an edge activator and a ceramide liposome which for the purposes of this study is going to be referenced as ceramidosome. Specific carnosine concentration was chosen because topical formulations evaluated thus far in the literature contain 0.2%-1% carnosine(24)(26)(27)(28). The liposome will ultimately be incorporated in formula appropriate for topical use with final concentration of 0.5%. Considering all formulation challenges 2.5% carnosine concentration was deemed optimal. Acceptable physicochemical characteristics of liposomes and stability of the formulations for 60 days under room temperature and in a freezer will be measured and evaluated in order to conclude which kind of liposome shows more potential for topical use.

After 2 months' time, the most suitable formulations will be incorporated in a neutral gel for topical use and basic physicochemical characteristics will be evaluated in the final product. To the best of the authors knowledge there is no prior attempt in the literature to produce an active ingredient loaded transferosome and ceramidosome with the MHM.

3.Methods and materials

3.1. materials and organology

For the preparation of liposomes LECIVA -M25P ® (LECITHIN USP-NF) was used as the source of phospholipids, mainly containing phosphatidylcholine 24.73% according to the CoA (Appendices 1)

Carnosine powder and PBS tablets were provided by Doctor's Formula company

According to the CoA of Carnosine (Appendices 2) the Active ingredient conforms with all prerequired standards.

To obtain a 137 mM NaCl, 2.7 mM KCl and 10 mM phosphate buffer solution (pH 7.4 at 25 °C) 1 tablet of PBS was dissolved in 200 ml of distilled water according to the manufacturer (https://www.sigmaaldrich.com/GR/en/product/sigma/79382)

For the rest of the formulation Glycerin liquid by Chemco ® was used as well as TWEEN 80 and a ceramide mixture that were provided by Professor Demetzos lab in the department of Pharmacy of EKPA.

Ceramide's mixture provided for the purposes of this study contained both hydroxy and non-hydroxy fatty acids from a natural, bovine source and was purchased by Matreya LCC.

For the formulation of the gel all materials, namely carboxymethyl cellulose sodium, propylene glycol and methyl paraben. were obtained by own means

For the homogenization of the liposomes an IKA T 18 Digital ultra turrax® homogenizer was used.

To measure the pH of the liposomal suspension Edge HI2020-02 pH meter was utilized

For the evaluation of liposome quality and stability NanoBrook 90plus PALS was used, courtesy of Doctors Formula company

All the raw materials used for the preparation of the samples are compliant with regulation 1223/2009 on cosmetic products

3.2. preparation and evaluation of liposomes

A base liposome without carnosine was developed with the following formula

Table 2. formula of basic empty liposome

Ingredient	Weight
LECIVA ®	5g
Glycerin	10g
PBS WATER	85 g

For the phospholipids to be organized in vesicles a typical concentration of 33μmol of phospholipid and 33μmol of cholesterol in 1.0ml of aqueous phase and 3 ml of solvent is needed .(51) This gives us a basic indication and understanding concerning the phospholipid concertation in order to prepare basic liposomes. Additionally,according to the patent of MHM preferably the total concentration of dispersed amphiphilic lipids and promoters in the liquid medium should range from 5-100 mg/ml(50)(49). Because the carnosine that is intendent to be encapsulated in the liposome is 25 mg / ml and it is water soluble it was deemed necessary to opt for a phospholipidic concertation of at least double of the carnosine in order to obtain a high level of encapsulation rate. For that reason, a concentration of 50 mg/ml of Leciva® was used for the preparation of all liposomes in this experiment The materials were mixed under continuous stirring in 700rpm in a temperature of 60^{o}C for 1 hour according to the MHM.

Measurement of the base liposome showed mean size of 419.58 nm with Std Dev 10.35 and a PDI 0.309 with Std Dev. 0.023. To further decrease the size of the liposomes two separate methods were tested. Part of the basic liposome was placed in the magnetic stirrer plate for another half hour in a temperature of 90^{o}C under 700 rpm. The rest of the basic liposome was homogenized in the IKA homogenizer at 3000rpm for 10, 15 and 20 minutes consecutively and the samples were then measured to evaluate if one of these methods provided smaller liposomes. The experiment was triplicated.

The results of the measurements are shown in the following table 3

Table 3. evaluation of basic liposome

Type of liposome	Mean Eff. Diameter (nm)	Polydispersity
Basic liposome	419.58 S. D +/- 10.35	0.309 S. D +/- 0.023
Basic liposome in 90°C for 30 more minutes	490.34 S. D +/- 8.13	0.277 S. D +/- 0.026
Basic liposome homogenized 10 min	363.30 S. D +/- 7.11	0.296 S. D +/- 0.018
Basic liposome homogenized 15 min	339.16 S. D +/- 5.39	0.275 S. D +/- 0.028
Basic liposome homogenized 20min	358.43 S. D +/-.9.51	0.294 S. D +/- 0.011

Based on these measurements it was found that homogenization of the basic liposome for 15 minutes gave the optimal results in terms of liposome particle size reduction by producing a liposome 19.17% smaller and with 11 % lower polydispersity index and thus was deemed preferable to follow this method throughout the experiment.

For the purposes of this study, three different liposomes were produced in a small scale. All liposomes contained 2.5% carnosine. A basic liposome was produced according to the following formula

Table 4. conventional liposome formula

Carnosine	2.5 g
LECIVA ®	5 g
Glycerin	10 g
PBS water	82.5 g

The ingredients were accurately measured beforehand, and then carnosine was added to the mixture of glycerin and PBS water in room temperature. The mixture was then heated up to 60°C under continuous stirring in a magnetic stirring plate and then the LECIVA ® was gradually added and remained in this temperature under stirring in 700 rpm for 1 hour. The liposomal suspension then was brought up to volume of 100 ml with PBS water to compensate for the loss of water due to the heating procedure and was homogenized in the IKA homogenizer for 15 minutes

under 3000rpm. Three batches of this formula were consecutively produced with the same method. A complete record of actual measurements was kept for each batch. With the use of the pH meter pH was measured and documented at the first day of the production. After the preparation of each batch, 100μl of the suspension was extracted and was diluted with HPLC grade water up to 3 ml final volume and was introduced to NanoBrook 90plus PALS for the evaluation of size, PDI and Z potential.

After documentation of measurements, each batch sample was split in to two bottles, one that was kept in room temperature (24-25^{O}C) and the other one was kept in a suitable freezer in an average temperature of 4^{o}C. Adequate labels with the lot number and information regarding storing conditions were placed in every bottle in order to avoid confusion. Both room and Freezer temperature were monitored throughout the experiment and no extreme values above or below mean temperatures were observed for the duration of the study.

Each sample was then again measured 15 days, 30 days and 60 days after they were originally produced and measurements of pH, liposome size, polydispersity and carnosine content of the liposomes were documented for each sample.

The second liposome containing 0.5% Tween was produced according to the following formula

Table 5. Transferosome formula

CARNOSINE	2.5g
LECIVA ®	4.5 g
Glycerin	10 g
TWEEN 80	0.5g
PBS Water	82.5g

After measurement of the ingredients a mixture of Glycerin, PBS water and Tween 80 was compounded, and carnosine was added until full dissolution under continuous stirring and heat. LECIVA ® was then added in a temperature of 60^{o}C and the suspension was kept under continuous stirring in 700 rpm for 1 hour in the same temperature, followed by homogenization in 3000rpm for 15 minutes. Three batches of the same liposome containing carnosine were produced. The samples

then were evaluated for the same parameters at the first day and then split in to two samples, one to be kept in room temperature and one to be kept in the Freezer under same conditions as the first liposome. Same measurements were made at the same time intervals as the first liposome, namely 15 ,30 and 60 days consecutively for each sample

A third liposome containing 2.5% carnosine was then prepared with the addition of ceramides according to the following formula

Table 6. Ceramidosome formula

Carnosine	2.5 g
LECIVA ®	4.5g
Glycerin	10g
PBS Water	82.5g
Ceramides	0.5g

The same exact procedure was applied in the preparation of this liposome as in the former two liposomes and the evaluation process was also the same.

The developed lipidic vehicles were evaluated in terms of their physicochemical characteristics, by measuring their size (hydrodynamic diameter, Dh), size distribution (polydispersity index, PDI) and zeta potential (z-pot), through dynamic and electrophoretic light scattering (DLS and ELS). To this end, the 100 uL samples that were collected were diluted 30-fold in HPLC-grade H_2O. The measurements were carried out at a detection angle of 90 and at 25°C, in a photon correlation spectrometer (PCS) by measuring the intensity of the scattered light.

3.3 Encapsulation efficiency calculation

The determination of carnosine in each sample was then achieved through derivatization of the amines with o-phthalaldehyde (OPA) and sodium sulfite (Na2SO3) to N-alkyl-1-isoindole sulfonate.

OPA–Na2SO3 solution was prepared combining 25 uL of 0.2 M OPA in methanol and 25 uL of 1M Na2SO3 with 950 uL of 0.1 M sodium borate buffer (pH 9.65).

The liposomes with incorporated carnosine (50 uL samples) were separated from non-incorporated drug molecules by means of size exclusion chromatography (SEC) using a

Sephadex-G75 column and utilizing HPLC-grade water as the mobile phase. Empty nanocarriers were used as controls, of which the absorption was subtracted from that of drug-loaded ones, to eliminate false positive results. The isolated liposomes with incorporated drug molecule were diluted with HPLC-grade water to 1 mL, 1 drop of Triton-X100 was added to lyse the liposomes, samples were diluted 40-fold with HPLC-grade water to 3 mL, 4 drops of the OPA–Na2SO3 solution were added, and the final samples were measured for absorption at 323 nm. Encapsulation efficiency (EE%) was calculated using the following equation:

$$EE = \frac{CARNOSINE\ (AFTER\ COLUMN\ )}{CARNOSINE\ (INITIAL)} X100$$

The amount of carnosine was determined by using the calibration curve:

$$\text{CARNOSINE CONCENTRANTION } \left(\frac{ug}{mL}\right) = \frac{CARNOSINE\ \ ABSORBANCE - 0.0025}{0.0018} (R^2 = 0.9999)$$

CARNOSINE ABSORPTION = 0.086/0.0272

This procedure was repeated in the same intervals as the evaluation of the rest of the physicochemical characteristics of the liposomes in 15,30 and 60 days after the preparation of the original suspension.

3.4 Preparation and evaluation of liposomal gel formulation

To evaluate the stability of the carnosine liposomes in a formula for dermatological use, two differed neutral gels were prepared and the liposome specimens that were deemed more stable after two months, were incorporated by simple mixing in an electric mortar and pestle in low speed for 2 minutes. A 20% concentration w/w of liposome was used in the formulation, resulting in a 0.5% of carnosine in the final preparation. Because in the literature multiple studies evaluate carnosine along other active substances one of the two gel formulation contained Hyaluronic acid (H.A), a very common ingredient in many cosmeceutical formulas. By incorporating the more stable liposome in a simple gel formula by itself and in combination with another active substance we will attempt to evaluate if main physicochemical properties of the liposome were affected in any significant way
The two formulas of gel are listed in the following table

Table 7. Formula of the neutral gel

FORMULA 1		**FORMULA 2**	
Liposome	20 g	Liposome	20 g
Carboxymethyl cellulose sodium	2 g	Carboxymethyl cellulose sodium	2g
Propylene glycol	25 ml	Propylene glycol	15ml
Methyl paraben	0.15g	Methyl paraben	0.15g
Distilled water	Qs 100 ml	Sol. Hyaluronic acid 10%	10 ml
		Distilled water	Qs 100ml

In order to evaluate the physicochemical properties of the liposomes in the gel formulation through dynamic and electrophoretic light scattering (DLS and ELS) 500mg of the gel were diluted with mild agitation in HLPC grade water up to 3 g of final weight. Measurements were carried out at a detection angle of 90 and at 25°C, in a photon correlation spectrometer (PCS) by measuring the intensity of the scattered light.

3.4. Statistical analysis

Statistical analysis was performed using the student's t-test at a significance level of 5%. Microsoft's Excel version 16.57(2201101) was used for statistical analysis

4.Results and discussion

4.1Physicochemical properties and colloidal stability of the carnosine liposomes

After the preparation process described in the methods sections, suspensions of carnosine loaded lipidic vehicles were developed. The color of the carnosine suspension was green in all the types of liposomes in day one.

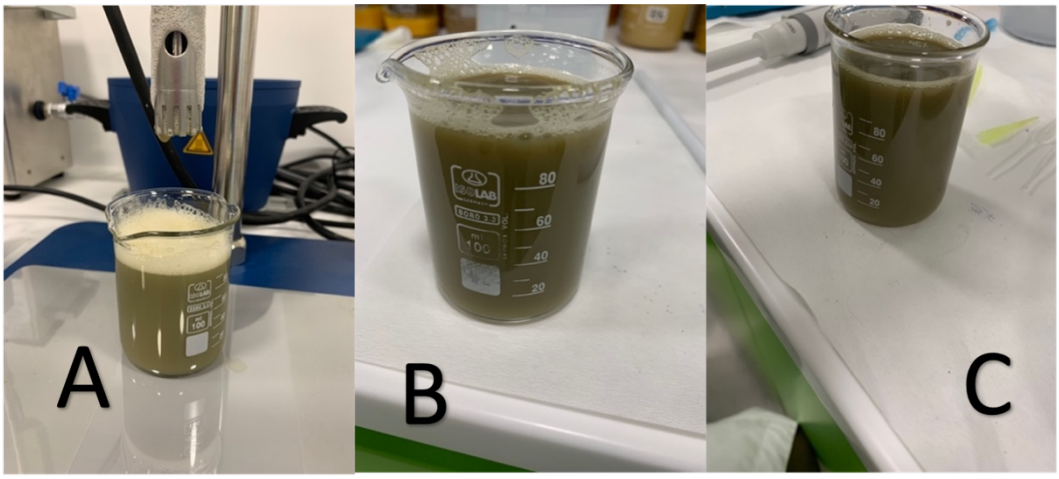


Figure 9. (A)Conventional liposome, (B) transferosome (C) Ceramidosome

Zeta potential of the carnosine liposomes were only measured in the day of the preparation and is shown in the table 8.

Table 8. z-potential values of liposomes

Liposome type	Zpot
Carnosine liposome	-44.13mV std 3.92
Carnosine transferosome	-46.64mV std 4.18
Carnosine ceramidosome	-47.53mV std 2.5

Z-potential is related with the surface charge of the nanoparticle and can affect the liposome's ability to interact with cells which are typically negatively charged. It is also a measure of nanoparticle stability and contributes to their physicochemical characterization. All liposomes prepared for this study show negative charge and are in the range of 40mV up to 50mV which in terms of stability correlates to good stability of the nanocolloidal system. Although skin acts as a negative charged membrane because SC is rich in anionic lipids, it's not clear if cationic liposomes have improved permeability through the epidermis. In fact, anionic liposomes of tretinoin and betamethasone have performed better in tested parameters

compared to positively charged liposomes. In order to evaluate if the type of the charge plays a role in skin permeation of those liposomes further research is needed and conclusions mut not be generalized since study results are controversial and lack consistency.(34,52)

The results in terms of physicochemical properties of all liposomes are presented in tables (9,10,11). The hydrodynamic diameter (Dh) indicates if the method produced systems of small particle size and the polydispersity index (PDI) shows if the systems are homogenous.

Table 9. physicochemical properties of conventional liposome

storage	pH	Size	Pdi
Day1	7.75	356.75 S. D +/- 10.28	0.295 S. D +/- 0.023
refrigerator day 15	7.78	356.77 S. D +/- 13.24	0.291 S. D +/- .021
refrigerator day 30	7.71	341.99 S. D +/- 14.89	0.271 S. D +/- 0.023
refrigerator day 60	7.55	361.47 S. D +/- 6.51	0.273 S. D +/- 0.021
Room temp. day 15	7.58	349.81 S. D +/- 9.08	0.278 S. D +/- 0.018
Room temp. day 30	7.32	323.46 S. D +/- 13.07	0.265 S. D +/- 0.021
Room temp. day 60	6.64	346.96 S. D +/- 6.47	0.283 S. D +/- 0.02

Table 10. physicochemical properties of transferosomes

Storage type	pH	Size	Pdi
Day1	7.56	448.69 S. D +/- 14.40	0.293 S. D +/- 0.015
refrigerator day 15	7.84	408.05 S. D +/- 14.34	0.267 S. D +/- 0.013
refrigerator day 30	7.73	437.70 S. D +/- 13.09	0.279 S. D +/- 0.015
refrigerator day 60	7.73	432.76 S. D +/- 16.03	0.280 S. D +/- 0.016
Room temp. day 15	7.01	409.8 S. D +/- 14.56	0.265 S. D +/- 0.010
Room temp. day 30	5.73	426.05 S. D +/- 8.05	0.259 S. D +/- 0.015

Room temp. day 60	5.78	446.59 S. D +/- 15	0.257 S. D +/- 0.015

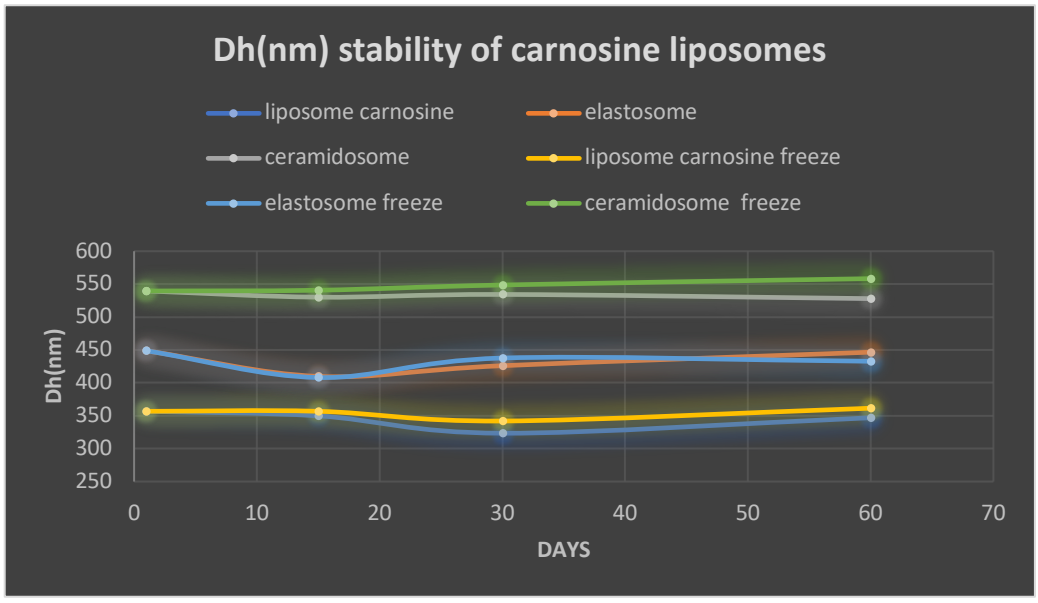
Table 11 physicochemical properties of ceramidosomes

Storage	pH	Size	Pdi
Day1	7.58	539.60 S. D +/- 21.7	0.267 S. D +/- 0.027
refrigerator day 15	7.61	540.72 S. D +/- 23.8	0.262 S. D +/- 0.02
refrigerator day 30	7.77	548.68 S. D +/- 17.36	0.268 S. D +/- 0.022
refrigerator day 60	7.73	558.37 S. D +/- 22.26	0.263 S. D +/- 0.039
Room temp. day 15	7.57	530.17 S. D +/- 16.87	0.265 S. D +/- 0.02
Room temp. 30	6.24	534.32 S. D +/- 33.60	0.269 S. D +/- 0.026
Room temp. 60	5.62	527.97 S. D +/- 15,03	0.250 S. D +/-0,0283

Measurements indicate that all present formulation developed through the MHM are roughly in the range of 300 to 560 nm. Which in case of vesicles corresponds to multilamellar vesicles (MLVs) or large unilamellar vesicles (LUVs).

Figure (10) shows the colloidal stability of the different liposomes in terms of particle size (hydrodynamic diameter D_h) in the 60 days of the experiment

Figure 10. colloidal stability of carnosine liposomes throughout the experiment



In terms of size, conventional liposomes exhibit the best properties in the range of 350 nm, followed by transferosomes with a size of 450nm approximately and ceramidosomes being the largest averaging in 540nm. All systems had very good homogeneity with very similar PDI in the range of 0.250-0.300. All colloidal dispersions retained their size and polydispersity through the experiment regardless of the storage conditions. This indicates that this method of preparation can provide several types of liposomes with acceptable properties. Size plays an important role in skin permeability of the active ingredient, with liposomes of approximately 300 nm being able to reach deeper layers of tissue but smaller sized vehicles (around 70 nm) being optimal for that purpose. (34)

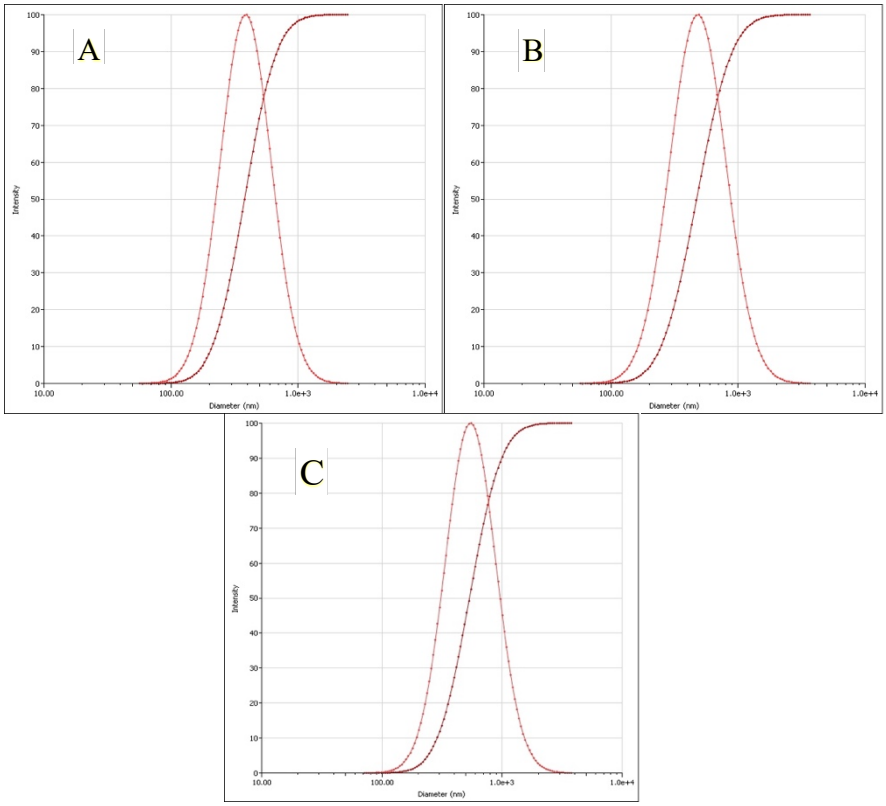


Figure 11 . DLS distribution per intensity. The x axis represents the particle size and the y axis the light scattering intensity (A)conventional liposome (B) Transferosome (C) Ceramidosome

Conventional liposomes prepared with this method are very close to the range of desirable size and is a positive predictive factor for further investigation in a future permeability study. Furthermore, carnosine loaded conventional liposomes have approximately the same size as empty liposomes produced with the same method which indicates that carnosine does not affect basic physicochemical properties of phospholipids organization in vesicles. However as indicated previously

transferosomes and ceramidosomes possess unique abilities because of their ingredients and slight increase in their size does not necessarily mean that those two types of liposomes will show poorer permeability properties than smaller conventional liposomes.

It was observed that samples that were kept in room temperature retained their original color whereas samples that were refrigerated showed a slight change in their color as indicated by the picture (11). This was observed in all types of liposomes after 30 days

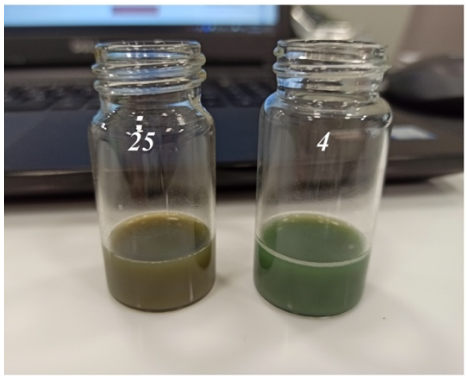


Figure 12. color of transferosome in day 60 at 25^{O} C and 4^{O} C storage temperature

Conventional liposomes had a small decrease in the pH around the day 60 in the samples that were stored in room temperature and liposomes B and C showed a bigger drop in pH from day 30 and forth, even though physicochemical properties were rather unchanged throughout the experiment. Furthermore, transferosome and Ceramidosome kept in room temperature developed a characteristic odor, and their color was slightly changed towards to a browner shade than the original. Aggregates were observed in the bottom of the bottles that those samples were kept in storage in room temperature although they were dissolved when the bottle was shaken lightly. This was attributed in bacterial or fungal growth because none of the formulas contained preservatives. The sift of the pH to the more acidic range presumably because of the bacteria or fungal growth may also have affected the color of the suspension because in pH areas of around 6 zwitterionic form prevails (4).

Statistical analysis verified that the difference in size between conventional liposome and transferosome and conventional liposome and ceramidosome is

significant (p=0.012< 0.05 and p= 0.0078<0.05 respectively) but found no significant differences in z pot and PDI between all types of liposomes prepared. It was deemed for that reason that the samples that were stored at the refrigerator were more stable in all aspects and that is the reason only samples that were refrigerated were used in the preparation of the final formulation

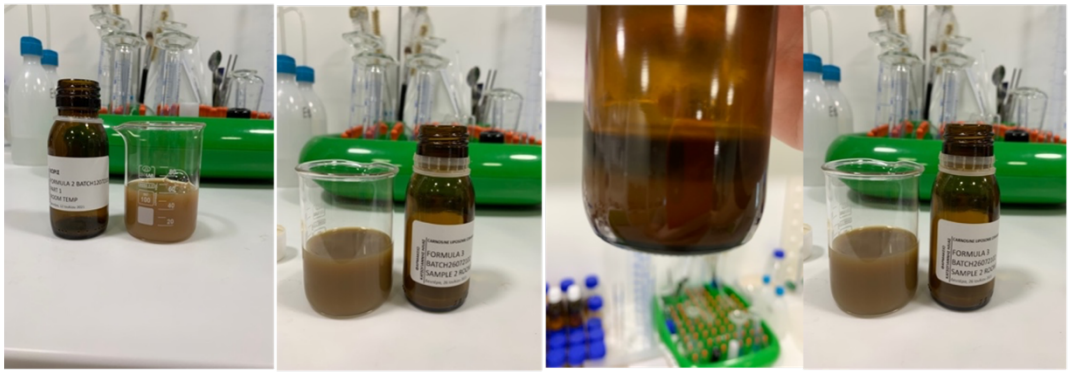


Figure 13. sings of discoloration and aggregation in colloidal dispersions stored in room temperatures in day 60

4.2. Encapsulation efficiency (EE%) of the carnosine liposomes

Tables (12-13-14) show the EE% of each liposome and the stability depending on the storage conditions in terms of temperature throughout the days of the experiment. Transferosomes exhibit the best EE% overall and it was relatively stable in both storage conditions up to 60 days. Conventional Liposomes were the second best in terms of EE% but the samples that were refrigerated showed a significant drop compared to those that were stored at room temperature although carnosine incorporation was relatively high anyway. A formulation change in the conventional liposomes may be appropriate because storage conditions seem to affect the stability of the liposome and the EE% of carnosine. The drop of the Ph and the growth of microbes in the conventional liposome when stored at room temperature can be addressed by the addition of preservative. This will probably result in the preparation of a stable liposome in room temperature that exhibits the best carnosine retention throughout the 60 days period, although this hypothesis must be verified in future experiments. Ceramidosomes were found to have the lowest EE% from the three formulations but maintained carnosine incorporation well up to 60 days regardless of the storage conditions. Low EE% of carnosine in ceramidosomes may be attributed in that hydrophilicity of carnosine or the fact that ceramidosomes exhibit a tighter self-organization because of higher lipid

content and lipidic interactions between phospholipids and ceramides. Whether low percentage of encapsulated carnosine in that type of liposomes will have an impact on skin permeability is not clear at this stage of the experiment. Penetrating enhancement mechanism suggests that lipids act as penetration enhancers ''altering the lipid structure of the SC reducing its packaging and weakening its barrier function'' (34). Because ceramides are part of the lipid matrix of the epidermis their affinity to the skin barrier may lead to enhanced penetration of the non-encapsulated carnosine, but further testing of this hypothesis is essential.(46) Figure (14) presents the EE% of all types of liposomes in all storage conditions from day 1 to day 60

Table 12. EE% of conventional liposomes

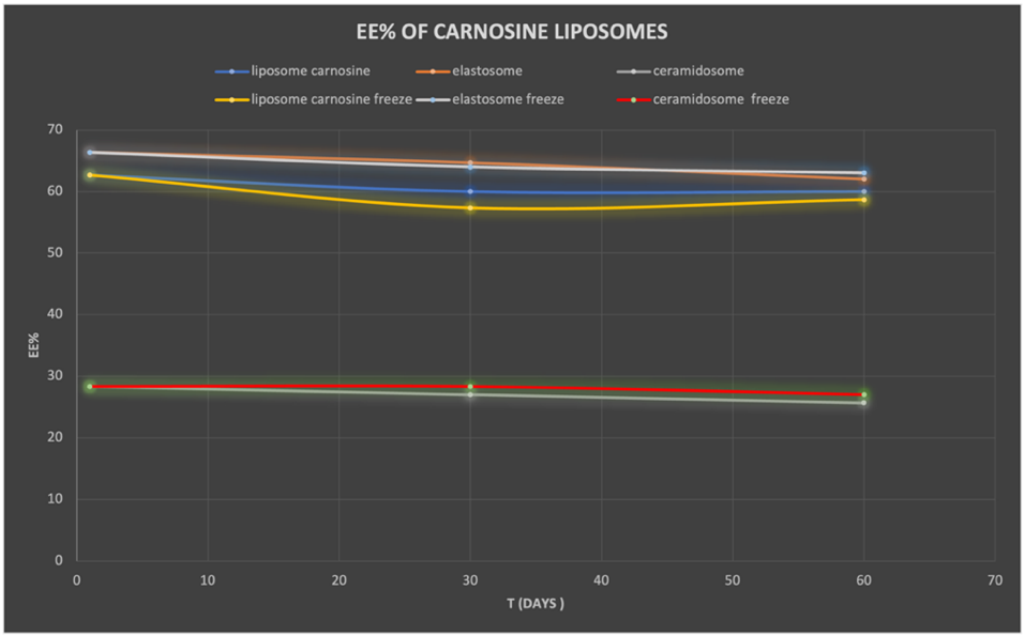
Storage type	Carnosine incorporation
Day1	62.67%
refrigerator day 30	57.33%
refrigerator day 60	58.66%
Room temp. day 30	60%
Room temp. day 60	60%

Table 14.EE% of transferosome

Storage type	Carnosine incorporation
Day1	66.33%
refrigerator day 30	64%
refrigerator day 60	63%
Room temp. day 30	64.66%
Room temp. day 60	62%

Table 13. EE% of ceramidosome	
Storage type	**Carnosine incorporation**
Day1	28,33%
refrigerator day 30	28.33%
refrigerator day 60	27%
Room temp. day 30	27%
Room temp. day 60	25,67%

Figure 14. EE% of carnosine for all types of liposomes throughout the experiment



4.3. Stability of liposomes in gel formulation

Table (15) presents basic physicochemical characteristics of liposomes when incorporated in the two neutral gel formulation with and without Hyaluronic acid (H.A), namely size (Dh) and polydispersity (PDI). By comparing the two types of gels, we observe that the existence of the H.A in the formula does not affect significantly the size of the liposomes. Conventional liposomes in the neutral gel retain a similar particle size compared to the refrigerated sample of the day 60 that was used to formulate the gel (341.14nm and 348.14 nm vs 361.47nm) but it seems that polydispersity of the vesicles in the gel medium is worse (0.642 and 0.651 vs 0.273)

Table 15. Physicochemical characteristics of liposomes in Neutral gel

TYPE OF GEL	**SIZE**	PDI
CONVENTIONAL LIPOSOME GEL	341.14 S. D +/- 4.7	0.642 S. D +/- 0.012
CONV. LIPOSOME GEL + H. A	348.14 S. D +/- 8.1	0.651 S. D +/- 0.023
TRANSFEROSOME GEL	258.6 S. D +/- 6.3	0.581 S. D +/- 0.009
TRANSFEROSOME GEL +H. A	275.5 S. D +/- 11	0.704 S. D +/- 0.201
CERAMIDOSOME GEL	394.10 S. D +/- 10.2	0.919 S. D +/- 0.140
CERAMIDOSOME GEL + H. A	382.2 S. D +/- 11.1	0.814 S. D +/- 0.161

On the other hand, transferosome size is reduced when incorporated in the gel formula when compared with the refrigerated sample used to prepare the formula (258.6 nm and 275.5 nm vs 437.26nm). This phenomenon may be attributed to the fact the activating agent of the transferosome as a surfactant may interact with gel matrix thus leaving the bilayer lipid membrane resulting in liposome size reduction. If that is the case, transferosome may lose its deformability which means that it may not exhibit enhanced permeation in the skin tissue. This hypothesis should be

tested in a permeability study. A possible solution to this issue is the application of a different medium for incorporating the transferosome that does not affect the edge activator or redesigning the transferosomes with different surfactants as edge activators.

The same phenomenon is observed in ceramidosome size in the gel formulation when compared to ceramidosome sample kept in the refrigerator for 60 days. There is a significant drop in size in both gel formulations (394.10 nm and 382.2 nm vs 558.37 nm).

Polydispersity index of the lipidic vesicles was dramatically affected by the incorporation of the liposomes in the neutral gel with or without the H.A resulting in less homogeneous dispersions, although the presence of H.A does not seem to affect PDI in the final formulation. This however may not affect the ability of the liposomes to penetrate SC and their distribution in the skin tissue

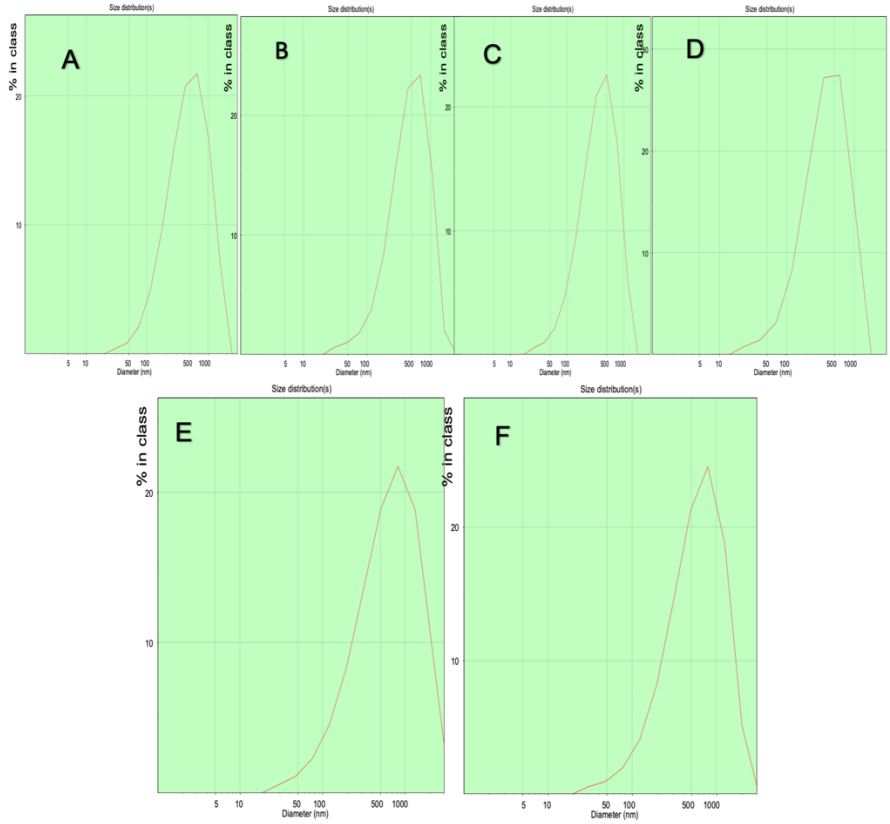


Figure 15. DLS distribution per intensity. The x axis represents the particle size and the y axis the light scattering intensity

(A) conventional liposome in gel (B)conventional liposome in gel with H.A (C)Transferosomes in gel (D)Transferosomes in gel with H.A (E) Ceramidosomes in gel (F) Ceramidosomes in gel with H.A

5.Conclusions

In this study three different types of liposomes for dermatological use were prepared by a modified heating method and evaluated in their original form and in a skin gel formulation for stability. This method produced liposomes with acceptable physicochemical characteristics compatible with those of MLVs or large ULVs. Furthermore, those physicochemical properties could, according to existing theories in the literature, lead to the enhanced permeation of carnosine. Stability of the liposomes was an issue only when liposomes were stored at room temperature and was attributed to microbial growth because an antimicrobial preservative was not added to the original formula of the liposomes. However, size and polydispersity of the liposomes were not significantly affected by the microbial growth. If the preservation issue is addressed liposomes could perhaps be stored in room temperature as well which could potentially result in a more manageable scale up procedure and handling of the products in terms of cost and complexity. Further testing is required to evaluate the stability of a new formula containing several preservatives, but this should preferably be done when is decided which of the three liposomes possess the best qualities overall. Carnosine was incorporated well in conventional liposomes and transferosomes despite its hydrophilicity but remained in low EE% in ceramidosomes. This does not necessarily mean that ceramidosomes will exhibit less carnosine permeability in the skin tissue but further research in Franz diffusion cells is needed to correlate both EE% and size to the desirable effect, which is enhanced penetration of carnosine in dermis layer where it can express its beneficial action.

The incorporation of all types of carnosine liposomes in a simple neutral gel formulation with or without H.A resulted in a rise of the PDI of the liposomes and in the case of transferosomes and ceramidosomes in a drop of their size when compared to the PDI and size of the liposomes used for the gel. The existence of H.A in the gel formula does not affect the physicochemical properties of the liposome in the gel.

In order to determine which liposome type is more suitable for topical use of carnosine as an antiaging and antioxidative agent for the skin, further research is needed. Skin permeation studies will also help us draw some conclusion on how several parameters of the liposomes and the final cosmeceutical formulation affect tissue distribution of carnosine.

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Appendices 1

CERTIFICATE OF ANALYSIS

Product Name:	**L-CARNOSINE**	
Batch Number	L-CAR. 2011051	
Date of Manufacture	05/11/2020	
Re-Test Date	04/11/2022	
CAS No	305-84-0	
Molecular Formula	$C_9H_{14}N_4O_3$	
EEC No	206-169-9	
Source	β – alanine	
Suitable for Vegetarians	Suitable	
Suitable for Vegans	Suitable	
Test	**Specification**	**Result**
Appearance	Powder	Conforms
Colour	White/off white	Conforms
Odour	Odourless	Conforms
Flavour	Characteristic	Conforms
Identification	Positive	Conforms
Assay (as Dried Substance)	99.0% - 101.0% (HPLC)	100.2%
Sieve Analysis	100% pass through 80 mesh	Conforms
Ash	0.1% Max	0.04%
Loss on Drying	1.0% Max	0.07%
Bulk Density	≥ 0.20 g/ml	0.30g/ml
pH (in 2% water)	7.5-8.5	8.3
Heavy Metals	Max 10ppm	Conforms
Lead (Pb)	Max 3ppm	Conforms
Cadmium (Cd)	Max 1ppm	Conforms
Arsenic (As)	Max 1ppm	Conforms
Mercury (Hg)	Max 0.1ppm	Conforms
Total Plate Count (TVC)	Max 10,000cfu/g	<10cfu/g
Yeast & Moulds	Max 1000cfu/g	20cfu/g
E.Coli	Negative	Negative
Salmonella	Negative	Negative

1 of 2

GMO Status	Non-GMO	Non GMO
Irradiation Status	Non Irradiated	Non Irradiated
TSE/BSE Status	TSE/BSE Free	TSE/BSE Free

This material conforms to the USP and French Arrêté 26 September 2016 list of substances authorised in food supplements Standard.

This material is to be stored in a tightly sealed bag/container and to be kept in a cool place away from moisture and direct sunlight.

Please note that surveillance testing may mean that not all the parameters stated on this specification are tested for every batch.

We confirm that the information above is sourced from the original manufacturers/suppliers Batch Certificate of Analysis.

To be used as per local legislation.

2 of 2

Appendices 2

VAV LIPIDS PRIVATE LIMITED

Plot C-76, Mirjole Industrial Area, MIDC, Ratnagiri-415 639, Maharashtra, India.
Phone : 02352-229035
C.I.No.: U24100MH2009PTC190024

CERTIFICATE OF ANALYSIS

NAME OF PRODUCT	LECIVA-M25P (LECITHIN USP-NF)	AR. NO.	FP2006072
BATCH NO.	20/LECIVA-M25P/006	BATCH SIZE	50.15 kg
MFG.DATE	JUN 2020	EXP.DATE.	MAY 2022
DATE OF ANALYSIS	12/06/2020	DATE OF RELEASE	24/06/2020

SR NO.	TEST	SPECIFICATION	RESULT
01	Description	Light yellow powder	Complies
02	Solubility	Practically insoluble in water but it readily hydrates to form emulsions, Sparingly soluble in absolute ethanol. practically insoluble in acetone.	Complies
03	Identification Test		
	By TLC	The Rf value of the spot for phosphatidylcholine, from the sample solution corresponds to those from standard solution A or working standard.	Complies
04	pH (0.05 % w/v)	Between 4.5 to 7.5	6.48
05	Acetone Insoluble Substances	Not less than 97 % w/w	97.71
06	Hexane insoluble substances	Not more than 1.0% w/w	0.07
07	Lead	Not more than 10 ppm	0.33
08	Moisture (By KF)	Not more than 2.0 %	1.39
09	Assay by HPLC (Content of Phospholipid)		
	Phosphatidylcholine	Between 20 to 25%	24.73

	PREPARED BY	CHECKED BY	APPROVED BY
NAME	Sanket Chalke	Sambhaji Dewarde	V.R.Rao
DESIGNATION	Sr. Executive QC	Sr. Executive QA/QC	Manager QA/QC
SIGNATURE			
DATE	24/06/2020	24/06/2020	24/06/2020

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