A Collagen III Amplifier System

Ratan K. Chaudhuri and George Majewski
Rona/EM Industries, Hawthorne, NY, USA
Gilles Guttierez and Mustafa Serrar
Texinfine, Lyon Cedex, France

Key words

liposome, Collagen III, keratinocytes, fibroblast activity, cytokines

Abstract

A unique liposomal system provides age-defying benefits in skin care by modulating the fibroblast phenotype via keratinocytes to induce synthesis of Collagen III.

Ein einzigartiges Liposomensystem hat antiaging Nutzen in der Hautpflege, indem der Fibroblasten-Phenotyp via Keratinozyten moduliert wird, um eine Synthese von Collagen III hervorzurufen.

Un système unique liposomal avec bénéfices rajeunissants dans le maintien de la peau par la modulation du phénotype fibroblaste par l'entremise de kératinocytes pour inciter la syntèse du collagène III.

Sistema liposómico exclusivo que brinda beneficios contra el envejecimiento en productos para la piel mediante modulación del fenotipode los fibrblastos utilizando queratinocitos para inducir la síntesis de colágeno III.

In this article[a], we discuss the design and application of a liposomal system for age-defying skin-care products. This unique system modulates epidermal cytokine production to selectively increase Collagen III. A mechanism for this activity is proposed and supported by in vitro and in vivo data. The proper incorporation of this liposome into skin-rejuvenating products is summarized and the practical benefits are noted.

Changes in the characteristics of human skin during aging are caused by intrinsic factors resulting in alterations of the connective tissue in the dermis. The connective tissue, which is composed of fibroblasts embedded into the extracellular matrix, is also susceptible to alterations by extrinsic factors, such as ultraviolet radiation and environmental pollution.[1]

A major component of the extracellular matrix is collagen. Other constituents found in significant amounts include elastin, proteoglycans, glycoproteins and fibronectin. During the aging process, a thinning of the dermis occurs due to a decrease of both collagen[2] and glycosaminoglycan[3]. The junction between the epidermis and dermis also changes during the aging process, affecting skin integrity. This weak link leaves the skin more vulnerable to mechanical trauma.[4]

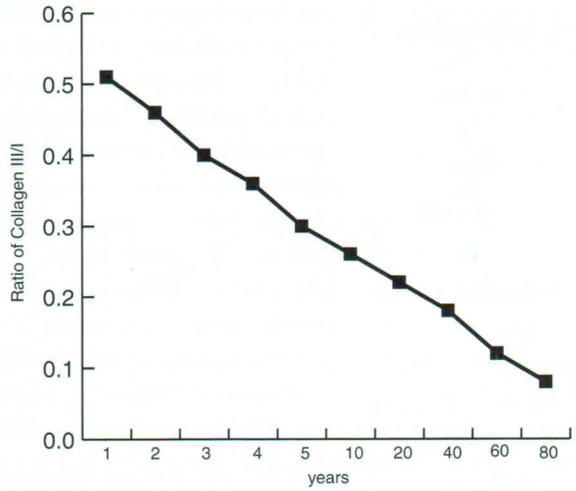

Figure 1. Human skin ratio of Collagen III/Collagen I in relation to age.

Collagen, the most abundant protein in mammals, accounts for about 30% of all proteins. Collagen molecules secreted from fibroblast cells assemble into characteristic fibers to provide functional integrity of such tissues as bone, cartilage, skin and tendon. They constitute a structural framework for other tissues including blood vessels and most organs. This microscopic collagenous mesh that permeates every corner of the human body is responsible for contributing to our physical appearance. This mesh turns out to be extraordinarily important to the way we look and feel. It is the main actor in the extracellular matrix, a support system for the survival of every cell in the body.[5]

In human tissues there are at least 10 genetically and chemically distinct collagen types that have been well-characterized.[6] Several others are currently under study. Collagen I represents about 80% of the dermal collagen in an adult's skin, while Collagen III accounts for about 15%.[7] The remaining 5% is made up of Collagens IV and V. Collagen III is predominant in young skin and during the wound healing process. Thus, Collagen III is also known as a "restructuring" collagen.

One of the numerous modifications of the extracellular matrix during aging is the significant decrease in collagen synthesis. The ratio of collagen types changes throughout life. Specifically, the age-re-

[a] *This article is adapted from a paper presented by R. Chaudhuri at the HBA Global Expo in New York in 1999.*

lated decrease in the ratio of Collagen III/Collagen I is a dramatic one (Figure 1).[8]

The reduction and alteration of the natural collagen support layer that lies just beneath the skin causes facial lines and wrinkles. Creams and lotions can moisturize or exfoliate the surface of the skin, but they can't diminish the lines or wrinkles that are caused by the reduction and alteration of the underlying collagen support.

In summary, three major changes in skin structure involving collagen occur during the aging process:

- Decrease in collagen biosynthesis by fibroblast cells;
- Relative thinning of extracellular matrix, which becomes more pronounced with reduction in Collagen III than with Collagen I;
- Insolubilization of fibrous collagen leading to loss of skin's biomechanical properties.

Design of a Collagen III Amplifier System

Product concept: Keratinocytes are the skin's "antenna" for the reception of external signals. Fibroblasts are the "machines" that synthesize most of the skin's supportive structures. When these supportive structures are damaged, the fibroblasts start to resynthesize the damaged structures. This requires the transduction of a biological signal from the keratinocytes to the fibroblast. These biological signals are called "cytokines."

Our product concept stems from the basic understanding of "biological signals." The question posed during development of the Collagen III amplifier system was: could the biological system be fooled in such a way that keratinocytes send signals to fibroblasts, which in turn will start synthesizing Collagen III? The answer is yes.

We have developed a unique liposomal system[b] that can mimic the biological system to modulate the fibroblast phenotype via keratinocytes. For the purposes of this article, we'll call this development a Collagen III Amplifier System (CIIIAS). When CIIIAS liposome is added to human keratinocytes, it appears to express mediators that in human fibroblasts specifically induce the synthesis of Collagen III.

Product description: CIIIAS liposome is a suspension of phospholipidic vesicular carriers, in which the external lipophilic wall contains the amphiphilic dipalmitoyl hydroxyproline (DPHP). Its INCI name is water, lecithin, dipalmitoyl hydroxyproline, phenoxyethanol, tall oil sterol, linoleic acid, tocopherol, sodium ascorbate, methylparaben, butylparaben, ethylparaben, propylparaben, mannitol.

CIIIAS liposome selectively amplifies the biosynthesis of Collagen III in human skin. This unique liposome is shaped like a hexagonal pyramid. Its membrane always shows angled structures (Figure 2).

Mode of action: Figure 3 schematically shows the method we have used to demonstrate the activity of CIIIAS liposome. This involves transduction of biological signals from keratinocytes to fibroblasts resulting in selective stimulation of Collagen III expression in human skin fibroblasts.

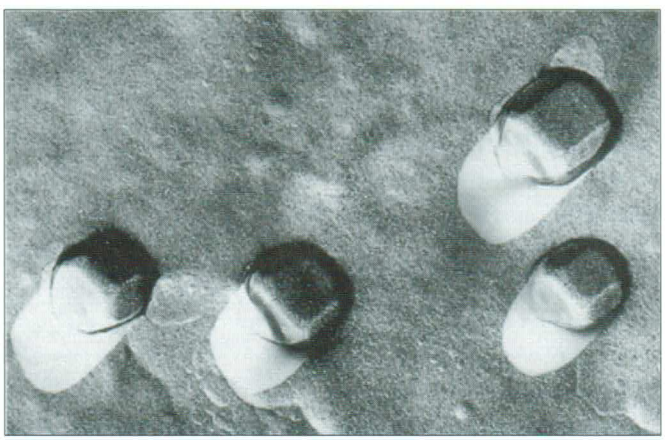

Figure 2. Scanning electron microscopy of CIIIAS liposome.

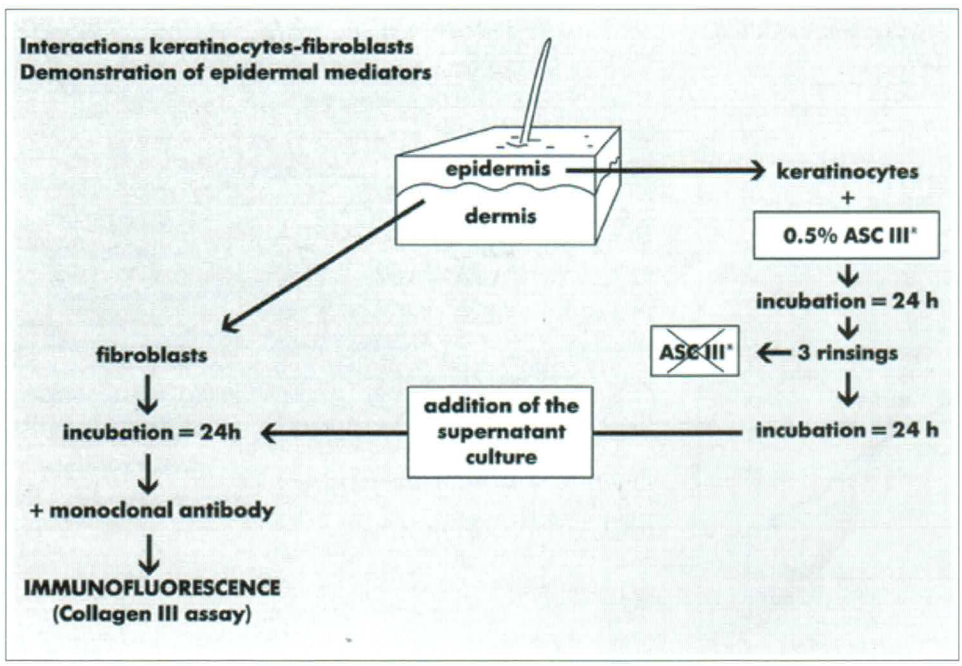

Figure 3. Mechanism of action of CIIIAS liposome.

In this method, the epidermis is separated from the dermis of a normal human skin sample. The epidermal cells (consisting mainly of keratinocytes) are then incubated in the culture media with CIIIAS liposome, stimulating the production of cytokines. The supernatant of the keratinocyte containing the cytokines is collected, filtered, and then added to the fibroblast cultures. If the keratinocytes were stimulated successfully, they would synthesize cytokines, concomitantly enabling the fibroblasts to produce collagen.

[b] *ASC III is a trade mark product of Merck KGaA, Darmstadt, Germany. Rona/EM Industries is an affiliate of Merck KGaA.*

Our data (immunofluorescence staining using a solution of murine antiimmunoglobulin antibodies from rabbits, coupled to fluorescein) shows that CIIIAS liposome appears capable of increasing Collagen III synthesis selectively in elderly fibroblasts by similar degrees as in newborn cells, even though basal levels of collagen synthesis are age-dependent.

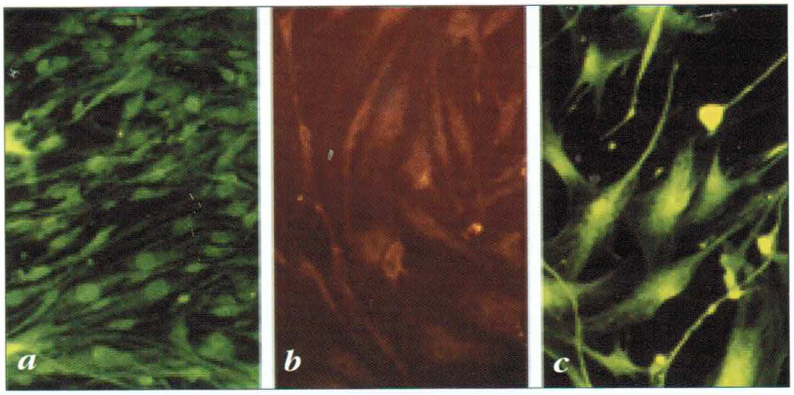

Figure 4. Selective immunostaining of Collagen III in human fibroblasts: a) 4 years old; b) 66 years old; c) 66 years old treated with 0.5% CIIIAS liposome.

In Vitro Studies

Immunostaining of Collagen III: The selective increase of Collagen III in elderly fibroblasts was observed via selective immunostaining of Collagen III.[9] Qualitative identification of the antibody/Collagen III precipitant was achieved using a solution of murine anti-immunoglobulin antibodies from rabbits coupled to fluorescein. The intensity of the fluorescence is proportional to the content of Collagen III in human fibroblasts.

Figure 4a shows young fibroblasts (4 years old) with a high Collagen III content. Figure 4b shows lower content in older fibroblasts (66 years old). Interestingly, when the same 66-year-old fibroblast culture was treated with CIIIAS liposome, an intense staining was observed, similar to the intensity of 4-year-old fibroblasts, as shown in Figure 4c.

Cell proliferation of fibroblasts: We studied cell proliferation in human fibroblasts.[10] Optical density directly correlates to the number of cells present in the human fibroblast culture. This was recorded over time (Figure 5) using CIIIAS liposome having different levels of DPHP and a control (containing all the ingredients of CIIIAS liposome without the three-dimensional structure). The results for both the control and the CIIIAS liposome do not show cell proliferation. Therefore, the increase in Collagen III biosynthesis in the dermis (after application of CIIIAS liposome) is due to the induction of fibroblast activity and not due to fibroblast proliferation.

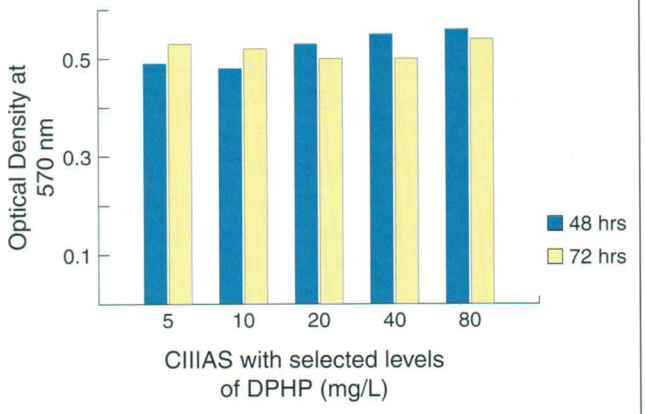

Figure 5. Cell proliferation study in human fibroblasts.

Selective amplification of Collagen III: We studied selective amplification of Collagen III after induction of fibroblasts.[11] Collagen I and III can be simultaneously quantified in human fibroblasts using radioimmunoassay. Figure 6 summarizes the selectivity of results obtained after 48 hrs of incubation using CIIIAS liposome (with two different levels of DPHP) and a mixture of all the ingredients in CIIIAS liposome (reference solution). The results show a ratio of about 60/40 (Collagen III/I) with CIIIAS liposome irrespective of DPHP concentration. The total increase of Collagen I and III was found to be well over 800 mcg/L in a non-dose dependent manner. Meanwhile, the reference solution showed a ratio of about 27/73 (Collagen III/I) with a total increase of Collagen III and I well below 100 mcg/L.

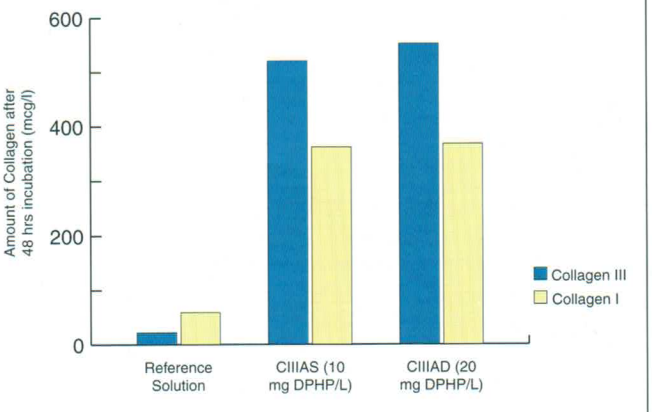

Figure 6. Quantitative determination of Collagen III and I in fibroblast culture.

Effect of three-dimensional structure: A series of in vitro tests (selective immunostaining of Collagen III in human fibroblasts) was done to determine the effect of the three-dimensional structure of CIIIAS liposome and its effect on fibroblasts. Results of this work are summarized in Table 1.

This work clearly shows that both DPHP and the three-dimensional structure in CIIIAS liposome are necessary to show selective increase of Collagen III and decrease in collagenase activity.

Comparative studies: In an in vitro comparative study, we incubated cultured human dermal fibroblasts (obtained from a 54-year-old woman, cells at 4th passage) in Dulbecco's modified eagle medium[c] (DMEM) in the presence of CIIIAS liposome (0.2% or vitamin C (50 mg/mL) or retinoic acid (1 mg/mL).[10,12-14] As a control, we used human fibroblast culture in DMEM supplemented

Table 1. Active compound in CIIIAS liposome

Composition	Structure	Effect on fibroblasts
CIIIAS without DPHP	spherical liposome	none
DPHP in ethanol or in a formulation	-	none
Short chain peptides from collagen hydrolysis (PC) in ethanol	-	none
CIIIAS with PC without DPHP	spherical liposome	none
CIIIAS	bipyramidal/angled	increase of Collagen III decrease of collagenase
CIIIAS with PC without DPHP	bipyramidal/angled	decrease of Collagen III increase of collagenase

Table 2. Summary of comparative studies of CIIIAS liposome, retinoic acid and vitamin C*

Effects on fibroblast cells[d]	CIIIAS	Retinoic Acid	Vitamin C
Collagen I amplification[e]	75.0%	67.0%	82.0%
Collagen III amplification[e]	83.0	42.0	67.0
Collagen III/I	1.2	0.7	0.9
Collagenase formation[e]	4.0	63.0	43.0
Net Collagen III increase vs collagenase[f]	44.0	1.4	3.3

*Cell type: Human dermal fibroblasts; 54 year-old woman; incubation time = 72 hr

[d] Coulter Counter method

[e] Selective Immunostaining method

[f] These are calculated by normalizing the data for % amplification of Collagen III and % formation of collagenase, and dividing accordingly.

with 10% fetal calf serum, penicillin and streptomycin. At the start of the experiments, the number of cells was about 50,000/well.

In the cell proliferation assay (Coulter Counter method), no effect was observed compared to the control when cells were treated with CIIIAS liposome. However, retinoic acid causes reduction in cell counts, whereas vitamin C causes an increase in cell counts.

The results (based on fluorescence intensity) showed that both Collagen I expression and Collagen III expression are strongly increased in the presence of each of the three products (Table 2). In the case of retinoic acid and ascorbic acid, Collagen I was altered to a larger extent than Collagen III. However, in the case of the CIIIAS liposome, Collagen III increased more than Collagen I.

Regarding collagenase synthesis, our in vitro studies showed an increase of the enzyme expression in the cultured human dermal fibroblasts with retinoic acid or vitamin C. In contrast, when cells were treated with CIIIAS liposome, the collagenase synthesis was not at all affected resulting in positive net production of Collagen III.

In Vivo Studies

Cutometer study for skin elasticity: This analysis quantifies the degree of elasticity in the upper layers of a subject's skin. In this procedure, skin is sucked into the orifice of a probe using constant vacuum pressure for a set time. Two optical lenses located at the probe orifice measure the depth to which the skin penetrates into the probe.

Two gels—one containing 5% CIIIAS liposome and one without—were applied daily for 27 days on each half of the face (panel size 6, average age 50 years, females with fine wrinkle and dry skin). Measurements were carried out before and after the 27 days of treatment. For a gel containing the CIIIAS liposome, we observed a 35% increase in skin elasticity over the 27 days. Another gel containing no CIIIAS liposome showed only a 10% increase in skin elasticity during the treatment period.

Biopsy studies for CIIIAS efficacy: We used human biopsy studies to evaluate the efficacy of CIIIAS liposome on the skin.[13,14] A study lasting five weeks was conducted. Under local anesthesia, samples of both dermis and epidermis were taken from the face of a 44-year-old male volunteer at the end of week two, three and five. Treatments occurred on a daily basis. Between two and five weeks, the left side of the volunteer's face was treated daily with a cream containing 5% CIIIAS liposome, while the right side was treated with an identical cream without CIIIAS liposome (control). In order to avoid possible cytokine-mediated carry-over effects from the CIIIAS liposome treated left side, skin biopsies were done on the right side (control) first.

Target parameters for measurement of CIIIAS liposome efficacy on skin were the thickness of epidermal keratinocyte layers and relative content of pro-Collagen III. Pro-Collagen III is a precursor of Collagen III and allows one to distinguish between newly formed and pre-existing Collagen III.

Results of immunofluorescence staining of skin show that the untreated skin contained practically no pro-Collagen III at Day 1 (control) and showed a very weak response for the two-week control (without CIIIAS treatment). That was done with treatment of the right side with an identical cream without CIIIAS and evaluation of facial skin after 1 day and two-week intervals. In contrast, the application of CIIIAS resulted in a very significant increase in pro-Collagen III content. This increase in pro-Collagen III content was accompanied by an equally pronounced increase in thickness of epidermal keratinocyte layers, and improvement in dermal/epidermal junction area with reformation of collagen fibers in the dermis (Figure 7).

Formulation Guidelines

CIIIAS liposome is stable at least for 18 months, if unopened and stored at +4 to 35°C. Three-dimensional structure is lost on prolong heating at or temperatures above 35°C or below freezing. However, in formulated products the liposome is stable over a broader temperature range from 40°C to –10°C.

CIIIAS liposome can easily be incorporated into lotions, creams and gels. Ionic surfactants and bivalent cations must be avoided because they could disrupt the liposome structure over time. Water-soluble film formers and silicone polymers should also be avoided because they hinder the reception of

[c] *Purchased from Life Technologies Inc., Rockville, Maryland*

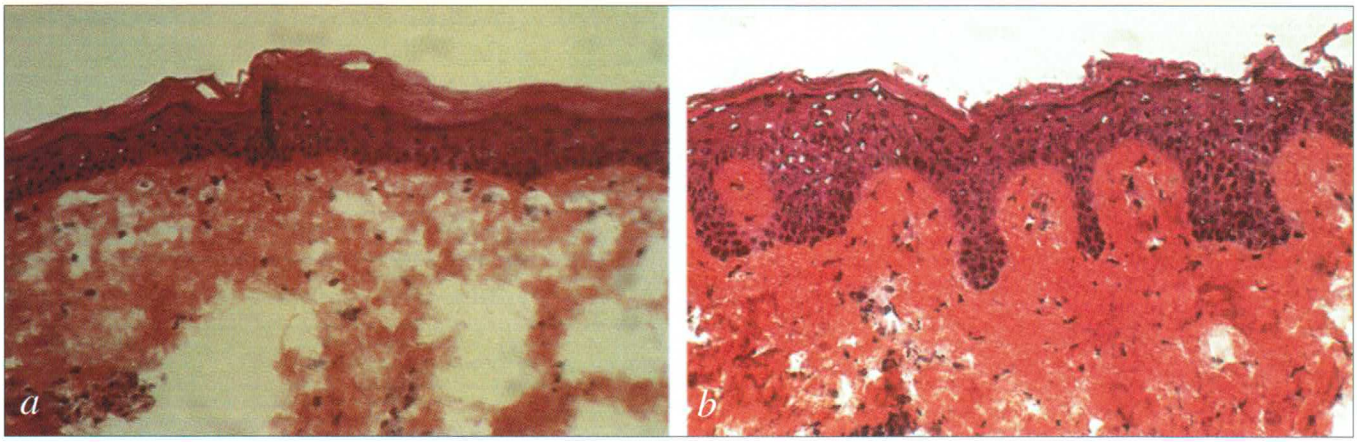

Figure 7. Study of human skin after biopsy: a) before treatment; b) after three weeks of treatment with CIIIAS liposome.

external signals by keratinocytes, resulting in very little production of collagen by fibroblasts. Formulation pH requirements are neutral to slightly acidic. CIIIAS liposome should be added to the formulation after cooling down to 35°C.

Four formulations are included to demonstrate the ease of incorporation into different skin-care formulations.

Summary

We have described a system that modulates cytokine production to selectively increase Collagen III. The method consists of selecting epidermal cells (mainly keratinocytes) from normal human skin, incubation in culture media with CIIIAS liposome, collection of keratinocyte supernatant culture containing cytokines followed by addition to the fibroblast culture. Data shows CIIIAS liposome is capable of increasing Collagen III synthesis selectively in elderly fibroblasts by similar degrees as in newborn cells, even though basal levels of collagen synthesis are age-dependent.

We performed several in vitro studies, namely, selective immunostaining of Collagen III, cell proliferation study in human fibroblasts, and selective amplification of Collagen III after induction of human fibroblasts. These studies clearly demonstrate selective increase in Collagen III production by the CIIIAS liposome. It is noteworthy that this amplification is the result of fibroblast activity and is not due to fibroblast proliferation. The role of the three-dimensional structure in CIIIAS was also substantiated using an in vitro test method (selective immunostaining of Collagen III in fibroblasts).

From comparative studies of CIIIAS liposome with retinoic acid and vitamin C, we concluded that the net production of collagen III, the desired collagen type, is higher with CIIIAS liposome than from either retinoic acid or vitamin C.

A biopsy study of human facial skin showed the effects of CIIIAS liposome resulting in a very significant increase in pro-Collagen III content accompanied by a pronounced increase of the epidermal keratinocyte layer thickness. It also showed improvement in dermal/epidermal junction area and reformation of collagen fibers in the dermis. Additionally, an in vivo Cutometer study for skin elasticity showed a 35% increase in skin elasticity.

Formula 1. Age-Defying Lotion

A.	Water (aqua), demineralized	qs 79.90% w/w
	Propylene glycol	2.00
	Glycerin	3.00
	Allantoin (Allantoin, Rona)	0.20
	Methylparaben	0.15
B.	Carbomer (Carbopol Ultrez 10, BFGoodrich)	0.20
C.	Caprylic capric triglyceride (Myritol 318, Henkel)	3.00
	Isopropyl myristate (Emerest 2314, Henkel)	3.00
	Cetyl alcohol (and) glyceryl stearate (and) PEG-75 stearate (and) ceteth 20 (and) steareth 20 (Emulium Delta, Gattefosse)	3.50
	PEG-8 (and) tocopherol (and) ascorbyl palmitate (and) ascorbic acid (and) citric acid (Oxynex K Liquid, Rona)	0.10
D.	Triethanolamine, 99%	0.35
E.	Water (aqua) (and) lecithin (and) dipalmitoyl hydroxyproline (and) phenoxyethanol (and) tall oil sterol (and) linoleic acid (and) tocopherol (and) sodium ascorbate (and) methylparaben (and) butylparaben (and) ethylparaben (and) propylparaben (and) mannitol (ASC III, Rona)	4.00
	DMDM hydantoin (Mackstat DM, McIntyre)	0.60
		100.00

Procedure: Combine A; heat to 50°C while stirring until all solids are dissolved. Disperse B in A with a sifter. Heat AB to 65°C. Combine C; heat to 65-70°C while stirring. Add C to AB while stirring. Homogenize ABC. Add D at 55-60°C. Continue homogenizing allowing mixture to cool to 35-40°C. Adjust pH with TEA to 6.8-7.2. When mixture temperature reaches 30-33°C add E and stir gently until mixture is homogeneous. Note: Viscosity 12,000 cps (Brookfield RV 5, 20 rpm) at 23°C

CIIIAS liposome can easily be incorporated at temperatures below 35°C into lotions, creams and gels having a slightly acidic to neutral pH. Ionic surfactants, water-insoluble film formers and silicone polymers should be avoided as they can hinder efficacy.

In summary, skin-care products containing CIIIAS liposome can repair the natural collagen support layer that lies just beneath the skin, resulting in increased skin elasticity and smoothness and reduction of facial lines and wrinkles.