Glycerol-based Liposomal Systems

Authors: Julian Smits, Dr. Bernd Albrecht, Lipoid Kosmetik AG, Steinhausen, Switzerland

Abstract

Lecithins, especially phosphatidylcholines, spontaneously form lipid bilayers of spherical (liposomes) or lamellar structures in an aqueous surrounding. Amongst other things, liposomes can be used to encapsulate cosmetic actives to support their penetration into the epidermis. Due to their high water content, liposomal systems need to be preserved by using either conventional preservatives or a self preserving solvent system. As glycerol is the second most used raw material in the cosmetic industry, we have chosen to study a glycerol/water mixture for a new self-preserving liposome-like system. We examined several variations of glycerol-based liposomal preparations regarding their use in formulations, their particle size as well as their physical and microbiological stability. The results show that our novel glycerol-based liposomal preparations have similar properties and functionality as conventional water-based liposomal systems but do not require the addition of any preservative. As a conclusion, we find that glycerol can overtake the role of water for the formation of liposomal products being the foundation for a new generation of liposomes.

Introduction

Liposomal systems have been used in cosmetic applications for decades. Liposomes are defined as "closed vesicles of polar lipid bilayers or lamellae with enclosed aqueous layers and cores, which are able to encapsulate hydrophobic, ambiphilic and hydrophilic substances into their structure."[1] They are used in pharmaceutical, dietetic and cosmetic applications. The main purpose of most of the present applications is the incorporation of substances in order to enable the transportation through biological barriers and to enhance the delivery to certain regions targeted by the application [2, 3, 4]. In a cosmetic context, the epidermis and dermis are the target regions and the *stratum corneum* is the biological barrier that has to be penetrated.

Classical liposomal systems are water-based, which is why they need to be preserved. In order to avoid microbiological spoilage, preservation can, for example, be provided by using either conventional preservatives or self preserving solvent systems. The regulation (EU) 1223/2009 on cosmetic products[5] lists the preservatives currently allowed in cosmetic products. For instance, benzoic acid and its salts, phenoxyethanol, sorbates, parabens or sulphites are used as low dosed additives to the solution that needs to be preserved. As the term implies, self-preserving solvent systems are based on a mixture of two or more solvents, which require no further addition of preservatives. The most prominent example for a self-preserving solvent system would be water/ethanol. Other self-preserving solvent systems are water/propanediol or water/glycerol. In most cases self-preserving solvent systems are characterised by a low water activity (aw) [6, 7].

Following water, glycerol is the second most widely used raw material in the cosmetic industry. It shows excellent solubility in polar solvents, has numerous skin care benefits and, due to its GRAS status, it is very safe to use[8]. In the past, there have been several successful combinations of water, glycerol and lecithins which led to the formation of liposome-like structures but none of the resulting commercialised products were claimed as self preserving[9]. In our attempt to create a self-preserving glycerol-based liposomal system, lecithin/solvent combinations with high amounts of glycerol and low water content led to the desired results.

In order to characterise our novel glycerol-based liposomal system, we have used the following techniques. First, the existence of liposome-like structures was investigated using freeze

fracture electron microscopy. The same method was used in order to show the existence of these structures after incorporation into a cream formulation. Further, the physical and microbiological stability were major aspects to be determined.

Methods

Formulation of Glycerol-based Liposomal Preparations

During the experimental phase, several glycerol-based liposomal preparations have been developed. The first formulation (Liposome HGG, Table 1) was a mixture of lecithins and glycerol, free from water and homogenised by stirring only. Once we had shown Liposome HGG to be a stable system, we replaced the high-grade glycerol with a glycerol diluted with water, considered to remain at a low aw value. Furthermore, a high pressure homogenisation step was introduced to create smaller particle sizes and tocopherol was added as an antioxidant. Several variations (Table 1) of these glycerol-based liposomal

Formulation of Creams

20% of the glycerol-based liposomal preparation Liposome 0093 was incorporated into two cream formulations differing in their method of emulsification. The first formulation (Table 2) contained a phosphate based emulsifier Crodafos, while the other cream formulation (Table 3) was based on SLM

Identification	Description	INCI
Liposome HGG	Lecithin in high-grade glycerol	Glycerin, Lecithin, Tocopherol
Liposome 0090	Lecithin in a glycerol/water matrix	Glycerin, Water, Lecithin, Tocopherol
Liposome 0093	Lecithin in a glycerol/water matrix plus pear water condensate Water, Lecithin, Tocopherol	Glycerin, *Pyrus Communis* (Pear)
Liposome 0616	Lecithin in a glycerol/water matrix plus collagen hydrolysate	Glycerin, Water, Lecithin, Hydrolysed Collagen, Tocopherol
Liposome 0809	Lecithin in a glycerol/water matrix plus dihydroxyacetone Dihydroxyacetone, Tocopherol	Glycerin, Water, Phosphatidyl- choline,

Table 1. Variations of Glycerol- based Liposomal Preparations

	Ingredient	INCI	%
A	Deionised Water	Aqua/Water	48.80
	Sodium Hydroxide 10%	Aqua/Water, Sodium Hydroxide	1.30
	Keltrol CG-SFT	Xanthan Gum	0.60
B	Sympatens GMS	Glyceryl Stearate	2.00
	Crodafos CES	Cetearyl Alcohol, Dicetyl Phosphate, Ceteth-10 Phosphate	6.50
	Akorex L	Canola Oil, Citric Acid	10.00
	Grape Seed Oil	*Vitis Vinifera* (Grape) Seed Oil	5.00
	Shea Butter Organic	*Butyrospermum Parkii* (Shea) Butter	5.00
	Euxyl PE 9010	Phenoxyethanol, Ethylhexylglycerin	0.80
C	Liposome 0093	Glycerin, Aqua/Water, Lecithin, Tocopherol	20.00

Table 2. Crodafos Cream Formulation

	Ingredient	INCI	%
A	Water	Aqua/Water	28.50
	Glycerol	Glycerin	5.00
	Phospholipon 80 H	Hydrogenated Lecithin	0.50
	Keltrol CG-SFT	Xanthan Gum	0.20
B	SLM 2015	Aqua/Water, Caprylic/Capric Triglyceride, Butylene Glycol, Hydrogenated Phosphatidylcholine	20.00
C	Myritol 318	Caprylic/Capric Triglyceride	15.00
	Grape Seed Oil	*Vitis Vinifera* (Grape) Seed Oil	10.00
	Euxyl PE 9010	Phenoxyethanol, Ethylhexylglycerin	0.80
D	Liposome 0093	Glycerin, Aqua/Water, Lecithin, Tocopherol	20.00

Table 3. SLM Cream Formulation

2015. SLM 2015, which is based on hydrogenated phosphatidylcholines, was used to add lamellar structures to the cream formulation.

The components of phase A except Keltrol were mixed and heated to 85°C. Then Keltrol was added whilst stirring thoroughly. Phase B was heated to 85°C and added to phase A. The mixture was homogenised and cooled down to 35°C under gentle stirring. In the last step the glycerol-based Liposome 0093 was added and the mixture was stirred during cooling down to room temperature.

The components of phase A were mixed and heated to 60°C. Phase B was added successively whilst stirring. The ingredients of phase C were combined and then added to the mixture. The batch was then homogenised using a rotor-stator homogeniser which raised the batch temperature up to 80°C. Glycerol-based Liposome 0093 was added during the cooling phase at 40°C.

Electron Microscopy

Samples were frozen according to the standard jet freeze-fracture method, using liquid propane. After fracturing, samples were shadowed with 1.5nm Platinum/Carbon at an angle of 45° followed by 8nm Carbon at 90° and then examined under the electron microscope (JEM-1011) at an electron accelerating voltage of 100kV. The electron microscopy images were used to determine particle sizes, to look for the formation of liposomal structures and to examine whether these liposomal structures were also present in the cream formulations.

Stability

For stability testing, samples of the glycerol based liposomal preparations were stored at 2-8°C, room temperature and 40°C. Over a time period of eight weeks the following parameters were determined every fourth week: appearance and odour, density at 20°C, pH value of a 20% dilution with water at 25°C, Gardner colour value, refractive index at 20°C and water content.

Microbiological Stability

The microbiological stability was tested as part of the two months stability testing and additionally in a repetitive microbiological challenge test. During the two months stability testing, samples stored at 2-8°C were tested for growth of aerobic mesophilic bacteria, *Escherichia coli*, yeasts and moulds by inoculation of the corresponding culture plates.

In the repetitive microbiological challenge test the samples were inoculated with a range of microorganisms (bacteria, yeasts, moulds and spore-forming bacteria) once a week over six weeks. After each inoculation cycle, prior to the next inoculation, streak cultures of the samples were made. The longer the period until the microbial growth appeared, the more effective was the sample's preservation.

Results and Discussion

All preparations and creams could successfully be created according to the methods described above. In the following, three glycerol-based liposomal preparations and four cream formulations were examined using electron microscopy. Figure 1 shows images of Liposomes HGG (1 and 2), Liposomes 0809 (3) and Liposomes 0093 (4).

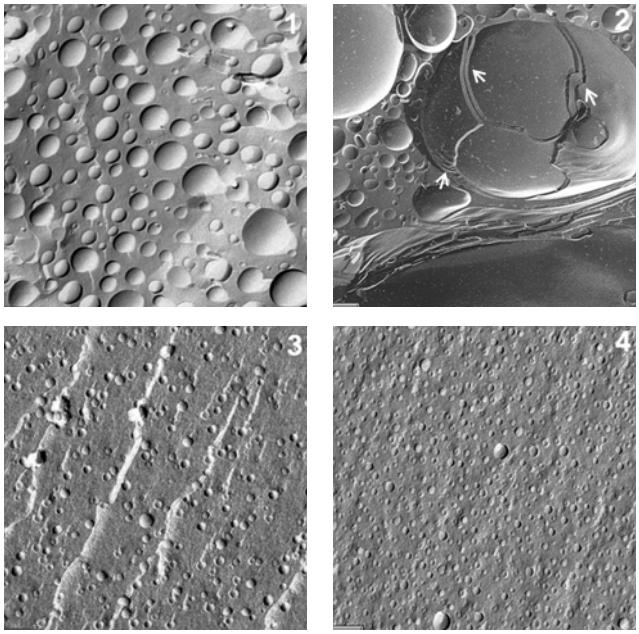

Figure 1. Electron microscopy images of freeze fractures: Liposomes HGG (No. 1 and 2); Liposomes 0809 (No. 3); Liposomes 0093 (No. 4)

The electron microscopy images of the freeze fractures show liposomal structures in all samples examined. Images 1 and 2 (Figure 1) show freeze fractures of Liposome HGG. This sample, a preparation of lecithin in glycerol, was the first to be examined in order to prove the existence of liposome-like structures in a glycerol matrix. Image 1 shows that all particles are clearly separated from one another with no tendencies of association. Particle sizes vary from 15nm to 200nm. In image 2 it is clearly visible that multilamellar vesicle-like structures are present.

The next preparations examined were Liposomes 0809 (image 3) and Liposomes 0093 (image 4). Both sample preparation procedures included homogenisation steps to create a smaller and more homogenous particle distribution. The images 3 and 4 of Figure 1 prove that this measure led to the results intended. As in the high-grade glycerol preparation, all particles in Liposomes 0809 and 0093 are clearly separated from one another and show no tendencies of association. Particle sizes, however, are much smaller and vary between 15nm and 50nm.

In order to prove that the liposome-like structures can still be found after the glycerol-based liposomal preparations have been incorporated into a final formulation, we examined two different cream formulations. Figure 2 compares the Crodafos as well as the SLM 2015 formulation with and without 20% of Liposome 0093.

Liposome-like structures can only be observed in the formulations containing Liposome 0093 (images 2 and 4). There are no such structures in the blank formulations (images 1 and 3). In the Crodafos formulation containing 20% of Liposome 0093

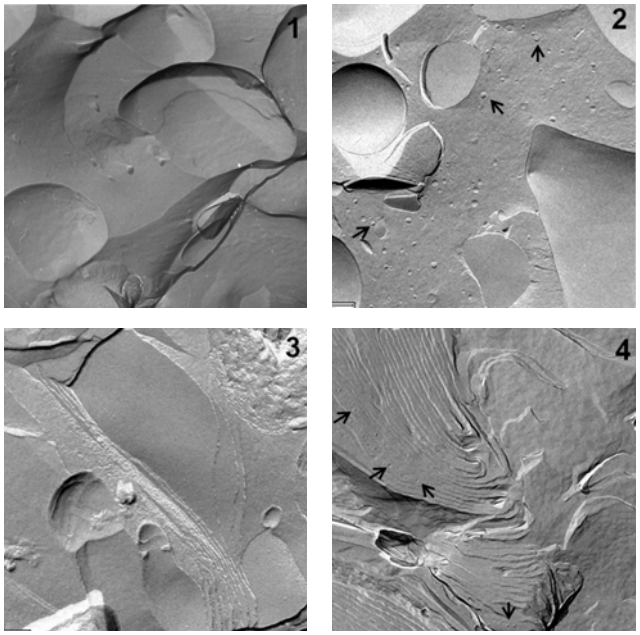

Figure 2. Electron microscopy images of freeze fractures: Crodafos blank formulation (No.1); Crodafos formulation with 20% Liposome 0093 (No. 2); SLM 2015 blank formulation (No. 3); SLM 2015 formulation with 20% Liposome 0093 (No.4).

(image 2), liposome-like structures are only present in the aqueous phase of the formulation. Image 4 shows that the liposome-like structures are also integrated into the lamellar structures of the SLM cream formulation.

The results of the short term stability testing for Liposome 0093 containing pear plant water and Liposome 0809 containing dihydroxyacetone are listed in Tables 4 and 5. For each test series, three samples of each glycerol-based liposomal preparation were stored at 2-8°C, room temperature and 40°C for eight weeks.

The short term stability tests of Liposome 0093 and Liposome 0809 at 2-8°C, room temperature and 40°C, led to the conclusion that both preparations should best be stored at 2–8°C. This finding is similar to other liposomal systems offered on the market. Although most of the parameters remained stable at all three temperatures, Liposome 0809 showed changes in its colouration at room temperature and 40°C, which can be observed in the variation of the Gardner colour values. This may also be related to the nature of the active Dihydroxyacetone which is known to be quite unstable. Since the preparations stored at 2-8°C did not show any dis-

iposome 0093	**t0 - 0 weeks**			**t1 - 4 weeks**			**t2 - 8 weeks**		
	4°C	RT	40°C	4°C	RT	40°C	4°C	RT	40°
Appearance	OK	OK	OK	OK	OK	OK	OK	OK	OK
Odour	OK	OK	OK	OK	OK	OK	OK	OK	OK
Density at 20°C [g/ml]	1.20	1.20	1.20	1.20	1.20	1.20	1.20	1.20	1.20
Gardner colour value	7.6	7.6	7.6	7.6	7.4	7.4	7.6	7.3	7.3
Refractive index at 20°C	1.45	1.45	1.45	1.45	1.46	1.46	1.46	1.46	1.46
pH – value, 20% in H2O at 25°C	6.1	6.1	5.7	6.0	5.9	5.7	6.0	5.8	5.6
Water content [%]	13.8	13.8	13.8	13.7	13.5	13.6	13.8	13.8	13.8
Solubility of 10% in H2O	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear
Solubility of 10% in IPA	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear
Aerobic mesophilic bacteria [cfu/g]	<10			<10			<10		
Yeasts and moulds [cfu/g]	<10			<10			<10		
Escherichia coli	*neg.*			neg.			neg.		

Table 4. Short Term Stability Testing of Liposome 0093

Liposome 0809	**t0 - 0 weeks**			**t1 - 4 weeks**			**t2 - 8 weeks**		
4°C	RT	40°C	4°C	RT	40°C	4°C	RT	40°	
Appearance	OK	OK	OK	OK	OK	OK	OK	OK	OK
Odour	OK	OK	OK	OK	OK	OK	OK	OK	OK
Density at 20°C [g/ml]	1.25	1.25	1.24	0.7	1.8	0.8	1.25	1.25	1.25
Gardner colour value	0.8	0.7	0.8	7.6	7.4	7.4	7.6	7.3	7.3
Refractive index at 20°C	1.46	1.46	1.46	1.46	1.46	1.47	1.46	1.46	1.46
pH – value, 20% in H2O at 25°C	5	4.9	4.9	4.8	4.7	4.5	4.9	4.3	4.4
Water content [%]	13.8	13.7	13.8	13.7	13.8	14.2	13.7	14.1	14.6
Solubility of 10% in H2O	turbid	turbid	turbid	turbid	turbid	turbid	turbid	turbid	turbid
Solubility of 10% in IPA	clear	clear	clear	clear	clear	clear	clear	clear	clear
Aerobic mesophilic bacteria [cfu/g]	<10			<10			<10		
Yeasts and moulds [cfu/g]	<10			<10			<10		
Escherichia coli	*neg.*			neg.			neg.		

Table 5. Short Term Stability Testing of Liposome 0809

Identification	Sterility Control	Inoculation Cycle						Grade
		1	2	3	4	5	6	
Liposome 0090	-	-	-	-	-	-	-	A
Liposome 0809	-	-	-	-	-	-	-	A

Table 6. Repetitive Microbiological Challenge Test

colouration, refrigerator storage is recommended. Also shown in Tables 4 and 5 is the microbiological stability tested at the storage temperature 2-8°C. When streaked out on culture plates, neither preparation caused any microbial growth.

Liposome 0090 and Liposomes 0809 were also tested in the repetitive microbiological challenge test. Both products were given an "A grade" due to no microbial growth after six consecutive inoculations (Table 6). This result complies with the theory that a solution with a low water activity prevents microbial growth. The glycerol-based liposomal preparations can therefore be considered self preserving though being free of conventional preservatives.

Conclusion

In this paper we were able to show the formulation of liposome-like structures in a glycerol/water matrix. It became clear that glycerol can overtake the role of water for the formation of liposomes. Further *in vivo* studies (corneometry) have shown that glycerol-based liposomal preparations lead to a moisturising effect on human skin, which was nearly one third higher than a preparation with glycerol only (publication under preparation). Summing up the results, our novel glycerol-based liposomal preparations show similar properties and functionality as conventional water-based liposomal systems. In addition, preparations of this kind will most probably feature a moisturising effect and will not require further preservation.

References

1. F. Gunstone, Phospholipid technology and applications, The Oily Press Lipid Library Volume 22 (2008)
2. C. Setzer, R. Wajda, E. Hoff, Phospholipids – Natural Components as Functional Additives and Actives in Skin Care Products, Euro Cosmetics 9-2010:15-20 (2010)
3. G. Storm, Liposomes: *Quo vadis*?, Pharmaceutical Science & Technology Today, Vol. 1 Issue 1, 4-1998:19-31 (1998)
4. Van Hoogevest *et al.*, Phospholipids: Natural Functional Ingredients and Actives for Cosmetic Products, SOFW-Journal, 139, 8-2013:9-14 (2013)
5. Regulation (EU) No 1223/2009 of the European Parliament and of the Council of 30th November 2009 on cosmetic products, Annex V, list of preservatives allowed in cosmetic products
6. A. Varvaresou *et al.*, Self-preserving cosmetics, 2009, International Journal of Cosmetic Science, 31, 2009:163–175 (2009)
7. Wallhäusser, Praxis der Sterilisation, Desinfektion, Konservierung. Keimidentifizierung, Betriebshygiene, Georg Thieme Verlag, 35-36 (1988)
8. M. Fevola, Glycerin, Cosmetics & Toiletries Magazine, Vol. 126, No.8, 8-2011:548-551 (2011)
9. Glicerosomes: a new carrier system for topical drug delivery, Enterprise Europe Northern Ireland 2012

Acknowledgements

Freeze fracture electron microscopy images were taken by Dr. Sybille Bayerl, Germering, Germany. The repetitive microbiological challenge test was performed by Schülke & Mayr GmbH, Norderstedt, Germany. The lecithin based liposomal preparations are available from our company.

Authors' Biographies

Julian Smits graduated in 2009 with a Dipl.-Ing. (FH) in Bioengineering at FH Aachen, Jülich Campus - University of Applied Sciences. He wrote his diploma thesis at TU Dresden, Institute of Molecular Cell Physiology and Endocrinology. Julian is currently a member of the R&D group at Lipoid-Kosmetik AG.

Dr. Bernd Albrecht studied chemistry at the University of Bonn, with his main focus on the metabolism of glycosphingolipids and he obtained his Ph.D. in 1996. That same year he joined Lipoid and changed his focus to the other large group of cell membrane lipids: the phospholipids. During the last 17 years, Dr. Albrecht's role has been to promote and sell lecithins and phospholipids to the cosmetic, pharmaceutical and nutraceutical industries.

Dr. Albrecht will now dedicate his efforts completely to cosmetics within the newly formed company Lipoid Kosmetik AG, formerly known as Cosmetochem, which combines lecithins and phosphatidylcholines with natural actives, extracts and additives in a new, enhanced portfolio