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DPPG LIPOSOMES AS PREFERENTIAL VEHICLES FOR "HUMAN-IDENTICAL" CERAMIDES

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Synopsis

Epidermal ceramides are an heterogeneous group of sphingolipids corresponding to the largest polar lipid class among the human epidermal lipids, playing a major role in the regulation of epidermal water dynamics.

However, the incorporation of ceramides in topical formulations, a major goal for the dermatology therapeutical or cosmetological assessment, presents many different and complex problems. These are mainly attributed to the specific physical and chemical properties of these compounds particularly due to their characteristic acyl chains leading to phase separation and crystallisation. Several approaches were chosen to address these problems and to ensure a proper deliver of ceramides within epidermis but, the results obtained are sometimes controversial and often inconclusive .

In the present paper, the authors developed a liposomal formulation choosing DPPG, a negatively charged lipid, to prepare liposomes as a preferential vehicle for "human-identical" ceramides. The results obtained, regarding the formulations stability, seem to suggest good operational conditions for this vehicle which also seems to be particularly suitable for the topical use of ceramides.

Riassunto

Le cerammidi dell'epidermide sono un gruppo eterogeneo di sfingolipidi corrispondenti alla più ampia classe popolare di lipidi tra i lipidi della cute umana, che giocano un ruolo fondamentale nella regolazione delle dinamiche idriche nell'epidermide.

Tuttavia l'inclusione di cerammidi in formulazione topiche, che sarebbero un obiettivo essenziale per la dermatologia terapeutica e per valutazioni cosmetiche, presenta molti problemi differenti e complessi. Questi vengono soprattutto attribuiti alle specifiche proprietà fisiche e chimiche di questi componenti particolarmente dovute alle loro caratteristiche catene di acile che provocano separazione di fase e cristallizzazione.

Diversi approcci sono stati scelti per affrontare questi problemi e assicurare una trasmissione adatta delle cerammidi all'interno dell'epidermide ma i risultati ottenuti sono a volte controversi e spesso inconcludenti.
Nel presente lavoro gli autori hanno messo a punto una formulazione liposomica scegliendo il DPPG, un lipide caricato negativamente, per preparare liposomi come veicolo preferenziale per cerammidi "identiche alle umane".
I risultati ottenuti, per quanto riguarda la stabilità delle formulazioni, sembrano suggerire buone condizioni operative per questo veicolo che sembra essere altresì particolarmente adatto per l'uso topico delle cerammidi.

INTRODUCTION

Ceramides are an heterogeneous group of sphyngolipids which correspond to almost half of the lipid content of human healthy stratum corneum. Ceramides are produced by deglycosylation of the glucosylceramides shortly after their extrusion into the intercellular space. This seems to justify the high ceramide content of the human epidermal cornified layer (7-9).

The recognition of the important functions attributed to ceramides in the preservation of cutaneous barrier integrity (8) their potential therapeutical and/or cosmetological interest lead to a remarkable amount of papers published focusing on their extraction, synthesis and formulation (16, 4, 12, 11, 7) .

Nevertheless, the practical utilisation of ceramides is still a complex matter, involving different limiting factors from their origin (ceramides from vegetal sources are often used without demonstration of its equivalence to the human identical ones), to formulation and stability (12, 13 1) . From a clinical perspective, it is also important to ensure that topically applied ceramides are effectively distributed within epidermis, which stresses the need to ensure formulation stability and, simultaneously, epidermal distribution effectiveness.

Liposomes are formed by phospholipid bi-layers dispersed in aqueous media. These vesicles are able to interact with hydrophobic and hydrophilic substances which will be located inside the vesicle according to their physical-chemical characteristics. The capacity of a substance to electrostatically interact with liposomal bi-layers determines the incorporation efficacy. Water soluble drugs will be included in the liposome's aqueous compartments while hydrophobic drugs will be associated with the bi-layer hydrophobic region (hydrocarbon chains). Other substances may be associated with the membrane through electrostatic interactions. Ceramides will probably interact with liposomes by this mechanism due to their amphiphilic structure with a polar group and a long hydrocarbon chain.

The use of dipalmitoylphosphoglycerol (DPPG) a negatively charged lipid, in the preparation of liposomes may be advantageous since the negative charge on the vesicles surface leads to vesicle-vesicle repulsion. The addition of a charge-inducing agent to the liposomal preparation increase the electrostatic repulsion energy avoiding vesicle aggregation and fusion, increasing the shelf life of the formulation (14).

Liposomes containing human-identical ceramides have been described to penetrate and interact with human stratum corneum components (6, 13) despite some difficulties in its technical management (9), while ceramides from other origins, such as yeast derived acylceramides are know to cause aggregation and fusion of liposomes (1).

For the specific purpose of this study which basically consisted in defining a formulation pattern containing human-identical ceramides for topical application, DPPG-liposomes were considered as an appropriate vehicle and tested accordingly.

MATERIAL AND METHODS

Formulation and Ceramide inclusion

Liposomes were prepared containing 0.1% (w/w) of ceramides (kindly supplied by Gist-Brocades-Netherlands). Firstly, 180-200mg of lipids 1,2-Dipalmitoyl-sn-glycero-3-phosphoglycerol, Na (DPPG, Na) were solubilized, with or without ceramides in 5 ml of an appropriate mixture of solvents (chloroform: methanol; 1:1) and this solution was dried under vacuum. The obtained film was then suspended in Phosphate buffer (pH 7.4) with the aid of a sonication probe. The empty liposomes and those containing 0.1% ceramides III, III+IIIb, or VI had an average diameter ranging from 100 to 500nm. Liposomal structure was identified by optical microscopy.

The following liposomal formulations were prepared and characterised:

(A) containing empty liposomes
(B) containing liposomes with 0.1% ceramide IIIs (III+IIIb)
(C) containing liposomes with 0.1% ceramide VI

Characterisation of the liposomal formulations

The formulations were characterised by means of differential scanning calorimetry (DSC) (Perkin-Elmer Thermal Analysis System, UK) and photon correlation spectroscopy (PCS). DSC characterisation basically consisted in submitting liposomes to several temperature cycles (three heating / cooling cycles, ranging from -10°C to 90°C /min) through which DSC profiles changes could be obtained and studied. DSC analysis was performed using a heating rate of 10°C/min and a cooling rate of 20 °C/min.
PSC measurements were carried out using a Malvern (Malvern Zetasizer, UK).

RESULTS AND DISCUSSION

Incorporation of ceramides in liposomes present several difficulties that should be taken into account to achieve a stable formulation. Generally the incorporation capacity of liposomes to hydrophobic molecules increases as the lipid content increases or with the increase of the liposome dimension. In the case of amphiphilic molecules the liposome loading efficiency mainly depends on the molecule affinity for the bilayer, on the density and charge distribution at the liposomal membrane as on the medium ionic strength (14).
It was also considered that ceramide would be incorporated into a lipid bilayer composed of long-acyl chains so that they would be completely surrounded by a hydrophobic environment. DPPG was chosen for this purpose, also taking into account that these phospholipids proved to be up-taken and non-toxic when incorporated in negatively charged liposomes applied in the dorsal pig's skin (13) .
Preparation of liposomes include different steps, involving a lipid hydration step followed by redispersion and reduction of vesicle size. In the present work, sonication was chosen for liposome preparation.
Ceramides may promote vesicle aggregation because these molecules do not necessarily exhibit any sort of preference for a orientation within a membrane so that the formation of membrane-membrane links is a real possibility. We tried to overcome this problem choosing DPPG liposomes which repel one another.
Liposomal formulations were fully controlled and characterised using the DSC calorimetric method. This technique is no time-consuming and allows full assessment of phase separation and formulation's stability (5, 3) .
DSC profiles of empty liposomes and of liposomes containing 0.1% (w/w), III-IIIb (IIIs), and VI are shown in Figures 1, 2, 3. Table I lists the values obtained with the formulations for peak temperature and the amplitude of the reaction involved.
Empty liposomes showed a steady behaviour when submitted to the DSC temperature cycles (Figure 1). The unchanged phase transition temperature between curves 1 and 2 confirmed the good thermal stability of this lipid.
Liposomes containing ceramide III analysed 24 hours after preparation (plots not shown) exhibited phase separation on the first heat cycle. The second and third runs presented a clearly visible phase separation phenomena and peak separation. The peak was characteristic of the DPPG alone, showing that this formulation was unstable and separation occur.
This instability lead us to the preparation of liposomes containing a mixture of ceramide III (III and IIIb; 60:40, containing a total 0.1% w/w). The DSC profile presented by these liposomes is shown in Figure 2.

Table I

Formulation	Runs	Peak (°C)	ΔH (J/g)
Empty Liposomes	1	42.0	3.5
	2	42.2	2.8
Lipoposome + ceramide III+IIIb	1	39.5	0.7
	2	41.4	0.9
Liposome + ceramide VI	1	50.1	4.3
	2	45.8	2.7

Table I. Peak temperatures and ΔH obtained with liposomal formulations (Peak - peak temperature ; ΔH - peak temperature area).

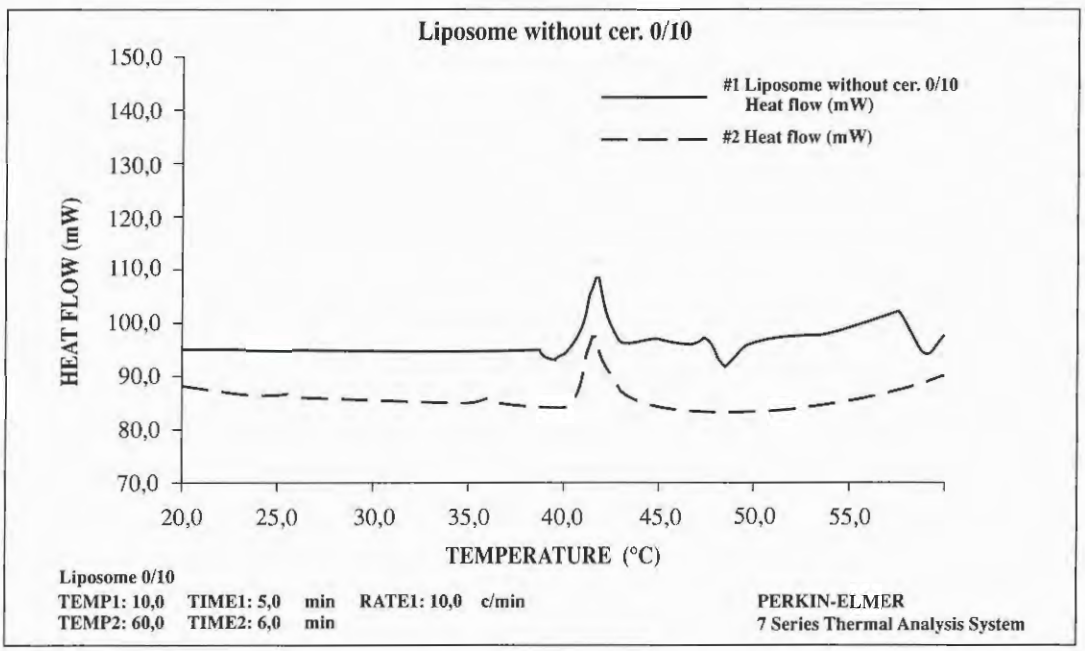

Fig. 1. DSC profile of empty negatively charged liposomes (see text)

Despite the differences in the first thermograms when compared to further runs, probably indicating that a rearrangement has occurred, this liposomes did not showed a clear separation from the lipids glass transition temperature, indicating a strong interaction with DPPG. This is probably due to the fact that ceramide mixture (III and IIIb) has a lower transition temperature probably induced by ceramide IIIb inclusion. The presence of a double bound in the acyl

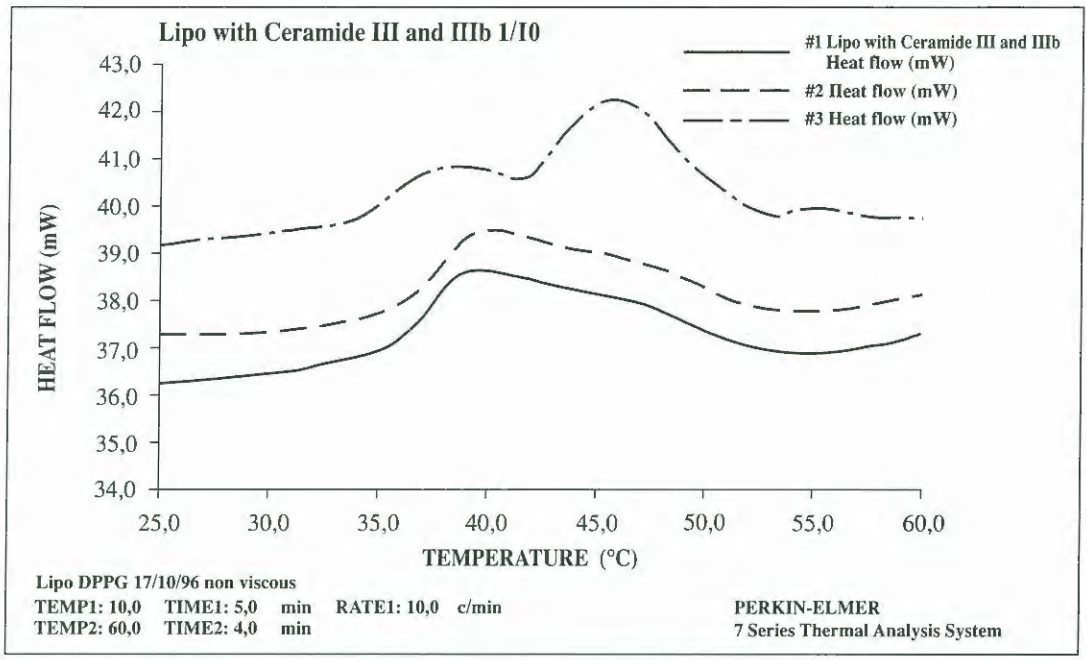

Fig. 2. DSC profile of negatively charged liposomes containing ceramide III - IIIb mixture (see text)

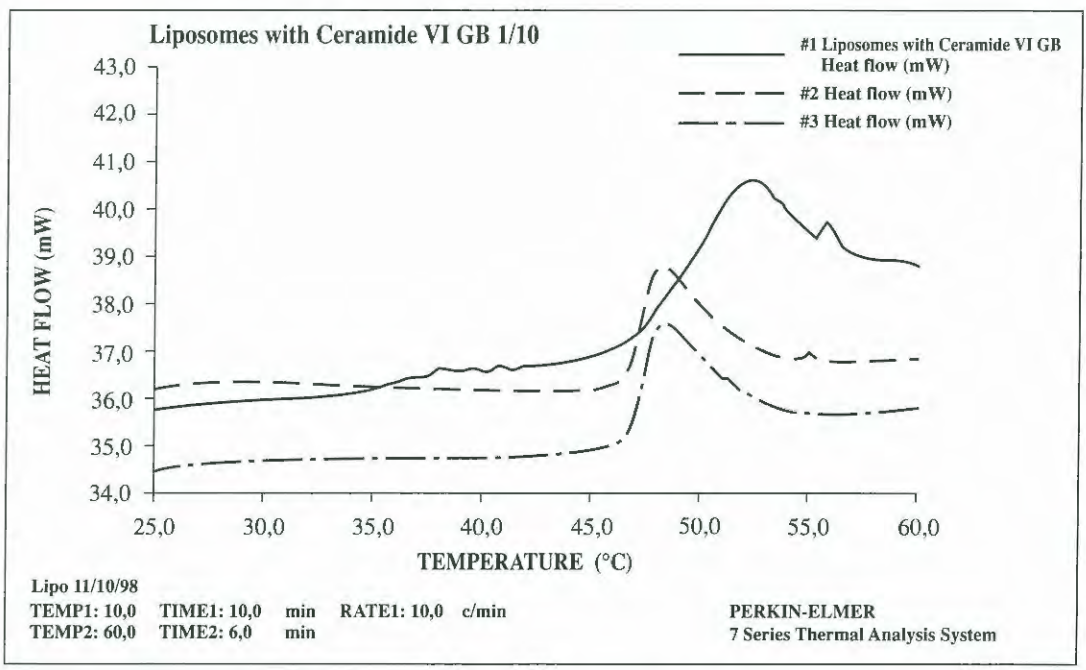

Fig. 3. DSC profile of negatively charged liposomes containing ceramide VI (see text)

chain of ceramide IIIb decreases rotation and system enthalpy, reducing the transition temperature of the lipid mixture (10) .
DSC profile of liposomes containing ceramide VI (0.1% w/w) (Figure 3), clearly showed a slight change in the phase transition temperature comparing curves 1 and 2. These changes probably reflect a reorganisation of the membrane structure. However, subsequently heat cycles did not revealed any major changes and the formulation remained stable. The peak temperature remained at 46°C, far from the DPPG alone (42°C), indicating that molecules of ceramide were interacting with the membrane lipids causing a marked change in the formulation's phase transition temperature.

CONCLUSIONS

The present paper clearly suggests that the incorporation of ceramides in DPPG liposomes is feasible and appears to be an adequate strategy to prevent lipid phase separation, ceramide aggregation and precipitation during storage. The results also suggest that liposomes can be considered as preferential vehicles for ceramides of human origin as in the present case. The preparation methodology chosen allow to easily obtain small vesicles particularly useful for topical formulation purposes.

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