ROMANIAN JOURNAL OF INFORMATION SCIENCE AND TECHNOLOGY
Volume **10**, Number 1, 2007, 85–95

Designing of Efficient Lipidic Nanostructures for the Therapy of the Inflammatory Diseases

M. TRIF, M. MOISEI, A. ROSEANU

Institute of Biochemistry, Romanian Academy, Bucharest, Romania
Corresponding author: `trif@biochim.ro`

Abstract. This study focuses on the possible therapeutic utility of lipid nanostructures as drug carriers in the local treatment of inflammatory disorders. The effect of liposomes on the percutaneous permeation of piroxicam, a nonsteroidal anti-inflammatory drug, across the rat skin was investigated in vitro using modified Franz-type diffusion cells. Different liposomal formulations have been prepared and characterized in order to obtain the highest efficiency of the drug entrapment. Their topical performances have been compared with a non liposomal system containing piroxicam. Our results demonstrated that the incorporation of piroxicam in liposomes modified their ability to permeate through skin. Liposome ability to fuse with Stratum Corneum Lipids Liposomes (hSCLLs) as skin model was tested. Resonance energy transfer (RET) assay between N-(7-nitro-2,1,3-benzodiazol-4-yl) (N-NBD), the energy donor and rhodamine, N-(lissamine Rhodamine B sulfonyl), the energy acceptor was developed to monitor the fusion process. The role of the lipid composition on the fusion or lipid mixing properties was investigated in order to obtain suitable formulation for transdermal drug delivery.

Keywords: liposomes, piroxicam, stratum corneum, resonance energy transfer, transdermal drug delivery.

1. Introduction

The lipid nanostructures also known as nanosomes (1) or small unilamellar vesicles (SUV) are vesicles of colloidal structures whose molecular organization is based upon formation of concentric spheres of lipid bilayers, enclosing aqueous compartments (2). They are able to entrap hydrophilic drugs in the large aqueous interior and lipophilic

drugs inserted in the lipid bilayer. Derived from naturally occurring, biodegradable and nontoxic lipids, they are good candidates for local targetting of therapeutic agents to the site of interest while reducing systemic toxicity (3–5).

Piroxicam (Prx), a nonsteroidal anti-inflammatory drug, are commonly used in the treatment of osteoarthritis and rheumatoid arthritis as well as local inflammations (6, 7). Oral therapy using Prx is very efficient, but clinical use is often limited because of the potential to cause adverse effects such irritation and ulceration of gastrointestinal mucosa (8). Transdermal drug delivery (TDD) can be a useful route for drug adminstrations and liposomes have been used to increase the drug penetration rate across the *Stratum Corneum* (SC), the main barrier to percutaneous absorbtion (9–14). Liposome carriers, well known for their potential in topical drug delivery have been chosen to transport Prx molecules through the skin to avoid the side effects associated with oral administration. Phospholipids, being the major component of liposomal system, can easily get integrated with the skin lipids and maintain the desired hydration conditions to improve drug penetration and localisation in the skin layers (10). The incorporation of Prx into lipid bilayers could be an additional benefit in order to influence their fluidity and to increase liposome stability. Studies made by fluoromicrography (14) and confocal laser scanning microscopy (CLSM) (15, 16) suggested that liposomes are not able to penetrate intact through the skin surface. Electron microscopy studies evidenced the adsorbtion and fusion of the vesicle at the surface of SC and the ability of liposomes to modify the ultrastructure of the multilamellar intercellular lipid layers (MILLs) of the SC (10, 17–19). Our previous experiments demonstrated that liposomes are able to modify the fluidity of human SC (hSC) and to facilitate the penetration of hydrophilic and lipophilic agents into the skin (9).

The current study includes the preparation and the characterisation of Prx-loaded liposomes. We have investigated the influence of different lipidic composition on the liposome stability and the drug entrapment efficiency. Percutaneous permeation experiments using rat skin have been carried out *in vitro* using modified Franz-type difussion cells (9) to investigate liposome ability to deliver Prx using topical route. We were also interested in the ability of different liposome formulations to fuse with human Stratum Corneum lipids liposome (hSCLLs) as human skin model. The efficacy of the fusion process has been studied by monitoring the resonance energy transfer (RET) between two fluorophores, N-(7-nitro-2,1,3-benzodiazol-4-yl) (N-NBD), the energy donor and rhodamine, N-(lissamine Rhodamine B sulfonyl), the energy acceptor (20, 21).

Our purpose is to develop a liposome formulation suitable as transdermal drug delivery system.

2. Materials and methods

2.1. Materials

The soy-bean lipid Phospholipon 90 (PL 90) was a product of Nattermann Phospholipids, GmbH, Germany, composed of 97% phosphatidylcholine (PC). Cholesterol

(Chol) was purchased from Sigma Chemicals (St. Louis, Mo). Phosphatidylcholine (PC), dimiristoyl phosphatidylcholine (DMPC), (dipalmitoyl phosphatidylethanolamine), (DPPE), dioleoyl-phosphatidylethanol- amine (DOPE), stearylamine (SA), cholesterol (Chol), were purchased from Sigma Chemicals (St. Louis, Mo). 1,2-dioleoyl-sn-glycero-3-phosphoethanol-amine N-(7-nitro-2,1,3-benzodiazol-4-yl) (N-NBD-PE) and L-α-phosphatidylethanolamine N-(lissamine Rhodamine B sulfonyl (N-Rh-PE) were received from Avanti Polar Lipids (Alabaster, AL, USA). Piroxicam and gel containing piroxicam were provided by Terapia SA, Romania. All other chemicals were of analytical grade and used as purchased.

2.2. Methods

2.2.1. Liposome preparation

Liposomes were prepared using thin film hydration technique (22). A lipid phase was prepared by dissolving different quantities of the Prx, phospholipids and cholesterol (Table 1) in the chloroform-methanol mixture (95:5), in 250 ml round bottom flask. The solvent mixture was removed from the lipid phase by rotary evaporation at 40°C (Laborota 4000, Heidolph 2, Germany), to obtain a thin film of lipids on the wall of the flask. The dry lipid film was hydrated with saline solution and incubated for 5 hours at room temperature to facilitate the annealing process. Small unilamellar vesicles (SUV) were prepared by sonication (at a temperature above the Tc of the lipids) in a bath type sonicator (Grant), for 60 minutes, followed by probe sonication for 5 minutes, using a Branson B-12 probe-type sonicator at 70 W. After sonication, the suspension was allowed to stand at 37°C for 10 min, rapidly cooled in an ice bath and centrifuged for 10 minutes at 10 000 rpm to remove any titanium fragments. SUV were obtained corresponding to a mean diameter of 200 nm following five times extrusion through a special polycarbonate membrane (200 nm) using a Mini-Extruder device (Avanti Polar Lipids). Liposome size was checked by transmission electron microscopy after staining with 1% uranyl acetate.

2.2.2. Drug entrapment studies

Separation of free drug from the prepared liposomes was carried out by gel filtration on Sepharose CL 4B column (saturated with empty liposomes). Appropriate amount of elute was analyzed spectrophotometrically at a λ_{max} = 340 nm (Perkin Elmer UV / VIS Spectrometer) to detect Prx concentrations. Percent drug loading (PDL) for the prepared liposomes with Prx were calculated as:

$$\text{PDL} = (\text{Entrapped drug} / \text{Total drug}) \times 100$$

All the liposomal formulations were monitored for their entrapment efficiency and for their morphological attributes (size and number of bilayers).

2.2.3. In vitro skin permeation studies

The diffusion experiments were performed in non-occlusive conditions, 10 hours at the 37°C using Franz-type diffusion cells (DC) (9). Small pieces of fresh skin obtained

from sacrificed rats were mounted in different DC. Liposomal and nonliposomal Prx formulations were applied uniformly on each piece of rat skin. Aliquots of 1 ml from the receiver compartment of each DC were withdrawn periodically and replaced with same amount of saline solution to maintain the receptor phase volume at constant level. The samples were incubated with 0.1% of Triton X-100 (for liposome solubilisation) and quantified for Prx level.

2.2.4. Determination of Prx retained in skin

The ability of liposomes to increase the drug retention within the skin was investigated by determining the amount of the drug retained in the skin samples employed in permeation studies. At the end of the permeation experiment the small pieces of skin mounted in the DC was removed and gentle cleaned in saline solution and blotted with tissue paper to remove any adhering formulation. Each skin sample was homogenized with chloroform:methanol mixture (2:1) for Prx extraction. The homogenate suspension was carefully filtered and quantified for the drug content.

2.2.5. Human Stratum Corneum (hSC) lipids extraction

Human SC lipids were isolated from plantar *stratum corneum* by extraction in chloroform/methanol and analysed by TLC (21, 23). The human SC liposomes (hSCLLs) included in bilayers the fluorescent markers N-NBD-PE and N-Rh-PE (1 mol % each) were obtained as above.

2.2.6. Resonance Energy Transfer (RET)

Steady-state emission and excitation spectra were obtained by using a Hitachi F 2000 fluorescence spectrophotometer. N-NBD-PE fluorescence measurements of liposomes were made by using an excitation wavelength at 480 nm and an emission wavelength at 530 nm. After each measurement vesicles were disrupted with Triton X-100 (1% final concentration). This treatment eliminated energy transfer and allowed the determination of the concentration of N-NBD-PE from their emission intensity, using direct excitation. The method is based on the fact that the presence of the donor and the acceptor in the same vesicle at high concentrations leads to the quenching of the donor fluorescence energy. Dilution of the probes during fusion with unlabelled vesicles increases the donor fluorescence intensity. Each measurement was repeated at least in triplicate. The experiments were performed at room temperature (23°C). The fluorescence intensity is expressed in arbitrary units.

2.2.7. Liposome–liposome fusion

The extent of intervesicle membrane fusion was determined by fluorescence assay (20, 21). Briefly, 1 μl of the fluorescent liposome suspension was mixed with 10 μl of the unlabeled liposome and incubated for 1 min. The fusion was initiated by addition of Ca^{2+} to 8 mM final concentration and the change in fluorescence intensity was recorded. After fusion, the density of the fluorophores decreased and this caused a decrease in transfer efficiency and an increase of the fluorescence intensity of the donor lipid, N-NBD-PE.

2.2.8. Calculation of transfer efficiency

The efficiency of the resonance energy transfer as a result of fusion between labeled and unlabeled liposomes is calculated from the equation:

$$I(t) = (F_t - F_0) \,/\, (F_{max} - F_0),$$

where F_t represents the fluorescence intensity of the donor labeled vesicles after the addition of unlabelled vesicles, F_0 is the initial fluorescence of the donor in the presence of the acceptor and F_{max} is the maximal level of fluorescence when detergent (Triton X-100) is added and an infinite dilution of the probes is obtained. The fusion efficacy is defined $E = I(t) \times 100\%$ and represents the the percentage of the fusion acquired (20).

3. Results and discussions

3.1. Preparation of drug loaded liposomes

Different liposome-Prx formulations were prepared with varying ratios of drug, PL-90 and Chol in order to obtain the formulation with the highest entrapment efficiency. Table 1 summarizes the influence of drug lipid ratio and the effect of Chol on the percent Prx loading liposomes. In case of Chol free formula (Lipo-Prx1) the percent of drug loading was 54%. An improvement in the entrapment process was noted with the addition of Chol and the increasing of Prx ratio (60% obtained in the case of Lipo-Prx2 and 62% for Lipo-Prx3). It was observed that the subsequent increase of Prx (10 mg/ml) and also the Chol ratio decreased dramatically the percent of drug entrapped (only 32% for Lipo-Prx4).

Table 1. Effect of drug lipid ratio on the Prx entrapment in liposomes

Form. code	Prx:PL90:Chol	Prx_i [mg/ml]	Prx_{en}[mg/ml]	PDL*[%]
Lipo-Prx1	1:70:00	0.5	0.3	60
Lipo-Prx2	2:60:10	1.0	0.6	60
Lipo-Prx3	10:50:20	5.0	3.1	62
Lipo-Prx4	20:40:30	10.0	3.2	32

*(Prx_i – the initial amount of Prx; Prx_e – the amount of Prx entrapped in liposomes; PLD – percent of drug loading).

Based on the above findings liposomal formulation Lipo-Prx3 was selected for further studies.

The reproducibility of the liposomal formulation with respect to entrapment efficiency and size was confirmed by preparing the formulation five times. The electron microscopy image of Lipo-Prx3 (Fig. 1) obtained at suitable magnification ($\times$ 25 000) confirms the size and the unilamellar structure of the vesicles (24).

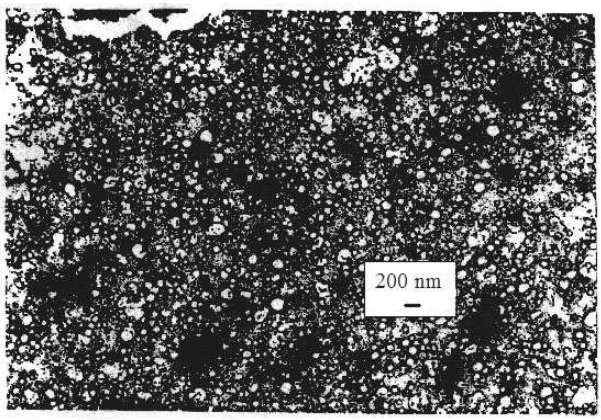

Fig. 1. Electron microscopy image of small unilamellar vesicles entrapped piroxicam; Barr = 200 nm.

3.2. In vitro skin permeation and skin retention studies

The permeation profile of different Prx systems across the rat skin was investigated and the results are shown in Fig. 2. Liposome ability to transport Prx was followed for 10 hours. Comparative studies were performed using Lipo-Prx3 (Table 1), a gel containing the same amount of Prx and an aqueous solution of Prx. The amount of Prx permeated in 10 hours was found to be 97 μg using liposomes as carriers, 65 μg from gel containing Prx and 25 μg in case of aqueous solution. Significant augmentation in the skin permeation of Lipo-Prx has been observed.

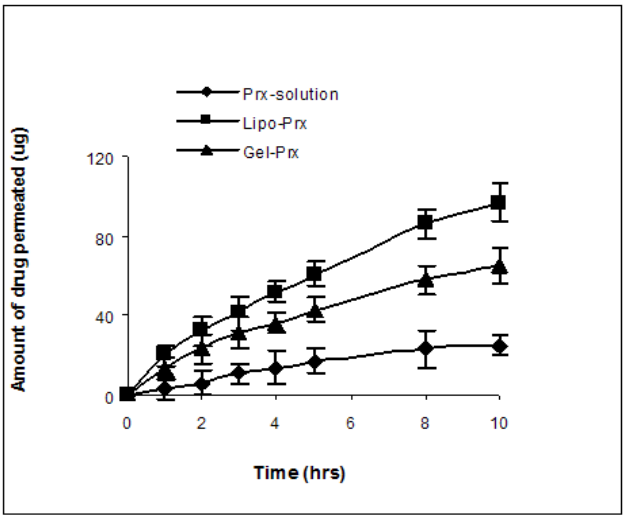

Fig. 2. In vitro permeation profile of different systems containing piroxicam across rat skin.

Higher values of flux obtained with liposomal formulation support our previous data related to the permeation enhancing effect of liposomal lipids on the skin (9).

Liposomal bilayers are able to fuse with multilamellar intercellular lipid layers of the SC altering the phase transition properties of SC lipids and generating a required physico-chemical state of the skin for enhanced permeation of the lipophilic drugs (16, 21). As concerning the amount of Prx retained in skin with its different formulations the data are presented in Fig. 3. Our results have shown that the amount of Prx retained in the skin from the initial amount of 300 μg was considerably higher in the case of liposomal formulation (120 μg) compared to non-liposomal ones (62 μg with gel-Prx and 30 μg with Prx-solution).

Liposomes have not only enhanced the penetration of drug molecules across the skin but also provided a localized depot of the drug in skin. Improved skin permeation of Prx associated with its enhanced retention in the skin using the liposomal formulation can be attributed to the lipo-soluble state of Prx molecules.

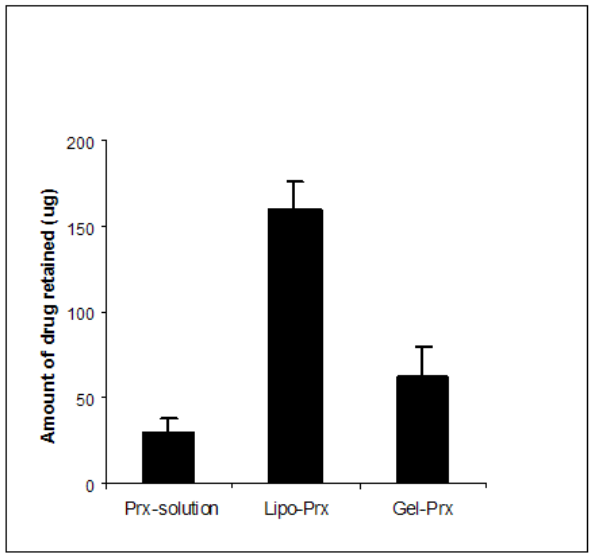

Fig. 3. Amount of piroxicam retained in the rat skin with different formulations.

The phospholipid-rich domains of vesicles might have helped to produce the depot effect for drug molecules.

3.3. Interaction between liposomes and hSCLLs (human skin model)

To investigate the mechanisms of the human skin penetration by various liposome formulations we analyzed by RET assay the fusion of these vesicles with hSCLLs. In these experiments, hSCLLs mimic the SC lipid bilayers of human skin.

The result of RET assay between DPPE:Chol:SA liposome formulation and hSCLLs

are shown in Fig. 4. The fusion efficacy was 26%. SC liposome labeled with two fluorophores were mixed with a ten-fold amount of unlabeled DPPE:Chol:SA vesicles.

Membrane fusion resulted in a dilution of the label and an increase of the fluorescence intensity of the donor from 40% (E_0) to 49% (F_t). In the presence of Triton X-100 (1%) the fluorescence intensity was 74% (F_{max}).

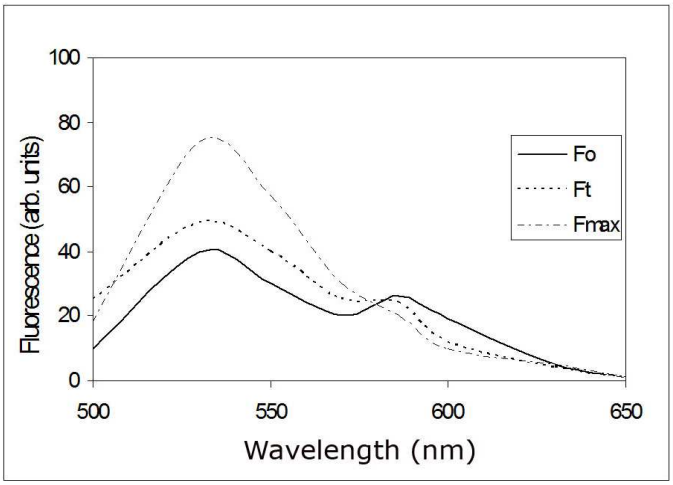

Fig. 4. The fusion efficacy between hSCLLs labeled with two fluorophores (N-NBD-PE / N-Rh-PE) and unlabeled DPPE:Chol:SA (5:5:1) after a RET assay.

3.4. Influence of the liposome composition on the fusion process

The assay carried out with PC:DOPE:Chol showed a better fusion efficacy of 29%, while for hSCLLS it was only 11%. Other liposome formulations showed much less lipid mixing/fusion, the corresponding values being 2% for PC:Chol and 4% for DMPC:Chol (Fig. 5). The RET assay demonstrated that PC:DOPE:Chol, DPPE:Chol:SA and hSCLLs have the ability to mix with SC lipids with different efficacy. A possible mechanism of lipid vesicles penetration can be based on the fusion of liposomes with skin bilayers followed by lipid mixing. These results suggested that liposomes do not penetrate intact into the SC, and this is in agreement with our previous data (9). Liposome containing DPPE or DOPE in the bilayer are able to mix/fuse with SC liposomes (25). DOPE is known to enhance the transdermal delivery of drugs (19, 20) because dioleoyl chains act as penetration enhancers. PE has fusogenic character due to its low degree of hydration, allowing close membrane apposition (26).

Both dioleoyl chains and phosphatidyl-ethanolamine headgroup are esential for hexagonal phase formation and lipid fusion. Thus, lipid composition has an important role in liposome ability to fuse with vesicles obtained from SC lipids (26).

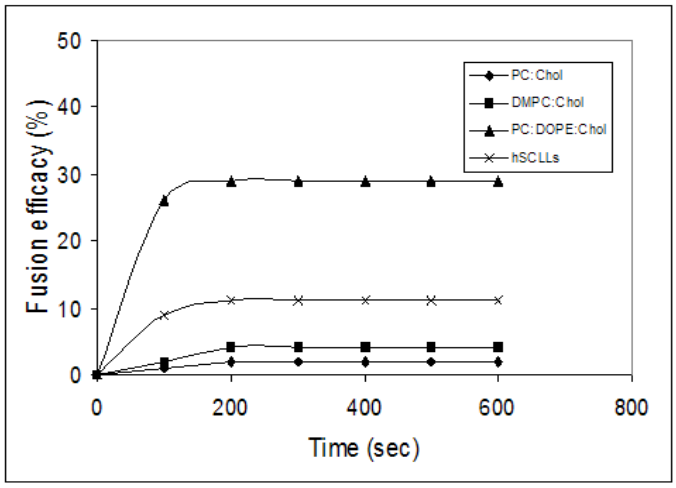

Fig. 5. The effect of the lipid composition on the fusion efficacy (E) of hSCLLs with different liposome formulations. RET assay was performed for each liposome formulation and fusion efficacy was calculated.

4. Conclusions

The results of the present study demonstrated that liposomal formulation of piroxicam with desired characteristics for topical administration could be succesfully obtained. Liposomes facilitated the penetration of Prx molecules across the rat skin and also increased the accumulation of the drug molecules within the skin strata resulting in a depot effect.

Our experiments using hSCLLs as human skin model demonstrated the liposome ability to fuse with this vesicles and the influence of the lipid composition on the fusion efficacy.

Our data suggested that liposome encapsulated anti-inflammatory drugs might be efficient for transdermal route in human therapy.

References

[1] CASTOR, T. P., *Phospholipid nanosomes*, Current Drug Deliver, **2**, pp. 329–340, 2005.

[2] NEW, R. R. C., *Structure of liposomes*, in *Liposomes: A practical Approach. IRC Oxford* (D. Rickwood and B.D. Hames, Eds.), Oxford, New York, Tokyo, pp. 1–31, 1997.

[3] KONIGSBERG, P. J., DEBRICK, J. E., STAERZ, U., *Liposome encapsulated aurothiomalate reduces collagen-induced arthritis in DBA/1J mice*, Biochim Biophys Acta, **1421**, pp. 149–162, 1999.

[4] SRINATH, P., VYAS, S. P., DIWAN, P. V., *Preparation and evaluation of liposomes with indomethacin*, Drug Dev. Ind. Pharm., **26**, pp. 312–321, 2000.

[5] ALLEN, T. M., CULLIS, P. R., *Drug delivery systems: entering the mainstream*, Science, **303**, pp. 1818–1822, 2004.

[6] ROBERTS, M., CROSS, S., *Percutaneous absorption of topically applied NSAIDs and other compounds: role of solute properties, skin physiology and delivery systems*, Inflammopharmacology, **7**, pp. 339–350, 1999.

[7] HADGRAFT, J., PLESSIS, J., GOOSEN, C., *The selection of NSAIDs for dermal delivery*, Int. J. Pharm., **207**, pp. 31–37, 2000.

[8] CORDERO, J., CAMACHO, M., OBACH, R., DOMENENCH, J., VILA, L., *In vitro based index of topical anti-inflammatory activity to compare a series of NSAIDs*, Eur. J. Pharm. Biopharm., **51**, pp. 135–142, 2001.

[9] TRIF, M., ZELLMER, S., ROSEANU, A., MATEI, L., MOTAS, C., LASCH, J., *Stratum corneum permeability modification by liposomes-included hydrophilic and lipophilic agents*, Rev. Roum. Biochim., **36**, pp. 73–78, 1999.

[10] TOUITOU, E., JUNGINGER, H. E., WEINER, N. D., NAGAI, T., MEZEI, M., *Liposomes as carriers for topical and transdermal delivery*, J. Pharm. Sci., **83**, pp. 1189–1203, 1994.

[11] MAESTRELLI, F., GONZALEZ-RODRIGUEZ, M. L., RABASCO, A. M., MURA, P., *Preparation and characterisation of liposomes encapsulating ketoprofen-cyclodextrin complexes for transdermal drug delivery*, Int. J. Pharm., **298**, pp. 55–67, 2005.

[12] BETZ, G., IMBODEN, R., IMANIDIS, G., *Interaction of liposome formulations with human skin in vitro*, Int. J. Pharm., **232**, pp. 243–244, 2002.

[13] HOLICK, M. F., CHIMEH, F. N., RAY, S., *Topical PTH (1-34) is a novel, safe and effective treatment for psoriasis: a randomized self-controlled trial and an open trial*, Br. J. Dermatol., **149**, pp. 370–376, 2003.

[14] WOHLRAB, W., LASCH, J., TAUBE, K. M., WOZNIZK, K. D., *Skin permeation of liposomal incorporated hydrocortisone*, Pharmazie, **44**, pp. 333–335, 1989.

[15] HOFLAND, H. E., BOUWSTRA, J. A., BODDE, H. E., SPIES, F., JUNGINGER, H., *Interactions between liposomes and human stratum corneum in vitro: freeze fracture electron microscopical visualization and small angle X-ray scattering studies*, Br. J. Dermatol., **132**, pp. 853–866, 1995.

[16] ZELLMER, S., PFEIL, W., LASCH, J., *Interaction of Phosphatidylcholine liposomes with the human stratum corneum*, Biochim. Biophys. Acta, **1237**, pp. 176–182, 1995.

[17] ZELLMER, S., REISSIG, D., LASCH, J., *Reconstructed human skin as model for liposome-skin interaction*, J. Control Release, **55**, pp. 271–279, 1998.

[18] SCHUBERT, R., JOOS, M., DEICHER, M., MAGERLE, R., LASCH, J., *Destabilization of egg lecithin liposomes on the skin after topical application measured by perturbed gamma angular correlation spectroscopy (PAC) with* ^{111}In, Biochim. Biophys. Acta, **1150**, pp. 162–164, 1993.

[19] BOUWSTRA, J. A., HONEYWELL-NGUYEN, P. L., *Skin structure and mode of action of vesicles*, Adv. Drug Deliv. Rev., **54**, pp. 541–555, 2002.

[20] STRUCK, D. K., HOEKSTRA, D., PAGANO, R. E., *Use of resonance energy transfer to monitor membrane fusion*, Biochemistry, **20**, pp. 4093–4099, 1981.

[21] TRIF, M., ZELLMER, S., ROSEANU, A., LASCH, J., *Use of fluorescence resonance energy transfer to monitor the fusion between human stratum corneum lipids liposomes and other phospholipid vesicles*, Rom. J. Biochem., **40**, pp. 63–70, 2003.

[22] NEW, R. R. C., *Preparation of liposomes*, in *Liposomes: A practical Approach. IRC Oxford* (D. Rickwood and B.D. Hames, Eds.), Oxford, New York, Tokyo, pp. 33–104, 1997.

[23] ZELLMER, S., LASCH, J., *Individual variation of human plantar stratum corneum lipids, determined by automated multiple development of high-performance thin-layer chromatography plates*, J. Cromatogr. B. Biomed. Sci. Appl., **691**, pp. 321–329, 1997.

[24] TRIF, M., *The use of liposomes as carriers for biological active principles*, PhD thesis, Institute of Biochemistry, Bucharest, p. 89, 1993.

[25] KIRJAVAINEN, M., URTTI, A., SUHONEN, T. M., PARONEN, P., KOSKELA, R. V., KIESVAARA, J., MONKKONEN, J., *Interaction of liposomes with human skin in vitro–the influence of lipid composition and structure*, Biochim. Biophys. Acta, **1304**, pp. 179–189, 1996.

[26] WILSCHUT, J., SCHOLMA, J., STEGMANN, T., *Molecular mechanisms of membrane fusion and applications of membrane fusion techniques*, Adv. Exp. Med. Biol., **238**, pp. 105–126, 1988.