Review Article **Current Pharma Research**
ISSN: 2230-7842
CPR 3(2), 2013, 836-845.

Recent Advances in Visualization Techniques for Evaluation of Nanocarriers Carries for Enhanced Delivery across Skin.

[1]*Shriya Gupta, [1]Manila Bishnoi, [1]Arvind Sharma, [2]Subheet Jain, [1]Sandeep Arora
[1*]Chitkara School of Pharmacy, Chitkara University, Punjab, India, [2]Department of Pharmaceutical Sciences and Drug Research, Panjabi University, Punjab-147002, India.

Abstract
The use of skin as an alternative route for administering systemically active drugs has attracted considerable interest in recent years. However, the skin provides an excellent barrier, which limits the number of drug molecules suitable for transdermal delivery. Thus, in order to improve cutaneous delivery novel formulations, e.g. microemulsions, liposomes, transferosomes, colloidal polymeric suspensions, or more conventional skin permeation enhancers are targeted passively. The focus of this review was to investigate the local changes in the ultra structure of rat skin in-vitro, in-vivo after topical/transdermal administration of vesicular systems using cryo-scanning microscopy, transmission electron microscopy (TEM), scanning electron microscopy (SEM), confocal scanning laser microscopy (CSLM), fluorescence microscopy and freeze fracture electron microscopy. Despite intensive research, the mechanisms by which vesicular systems deliver drugs into intact skin are not yet fully understood. The objective of this review, therefore, is to evaluate how visualization techniques may contribute to the determination of the mechanisms of diverse skin penetration enhancement strategies.

Key Words
CSLM, Ethosomes, Liposomes, Permeation, SEM, TEM, Transferosomes.

Introduction

Although the skin as a route for drug delivery can offer many advantages, including avoidance of first-pass metabolism, lower fluctuations in plasma drug levels, targeting of the active ingredient for a local effect and good patient compliance[1] the barrier nature of skin makes it difficult for most drugs to penetrate into and permeate through it[2]. During the past decades there has been wide interest in exploring new techniques to increase drug absorption through skin[1,3,5]. Topical delivery of drugs by lipid vesicles has evoked a considerable interest Since the first paper to report the effectiveness of liposomes for skin delivery was published by Mezei and Gulasekharam[4] conflicting results continued to be published concerning their effectiveness, enhancing the controversy of liposomes as dermal

***Corresponding Author:**
shriya.pharmacops@gmail.com

and transdermal drug delivery vehicles[5]. The first therapeutic, using lipid vesicles on the skin, were commercialized shortly before the year 1990, and contained the anti-mycotic agent, econazole. A few other, relatively simple, liposome-based dermal products followed[6,7]. Recently, it became evident that, in most cases, classic liposomes are of little or no value as carriers for transdermal drug delivery as they do not deeply penetrate skin, but rather remain confined to upper layers of the stratum corneum. Intensive research led to the introduction and development[8] over the past 15 years, of a new class of lipid vesicles, the highly deformable (elastic or ultraflexible) liposomes that have been termed Transfersomes®. Several studies have reported that deformable liposomes were able to improve in vitro skin delivery of various drugs[9-13] and to penetrate intact skin, in vivo, transferring therapeutic amounts of drugs[14-16] with efficiency comparable with subcutaneous[15, 17-19]. Ethosome is another novel lipid carrier, recently developed by Touitou et al., showing enhanced skin delivery[20-23] .The aim of this article is to provide an overview of lipid vesicles as carriers for skin delivery of drugs, with special emphasis on recent advances in this field, including the development of deformable liposomes and ethosomes**.** For evaluating the mechanism of better skin permeation of vesicular formulation different visualization techniques e.g. transmission electron microscopy, eosin-hematoxylin staining, fluorescence microscopy and confocal scanning laser microscopy (CSLM) have been used. Often, when used in combination these visualization techniques gave better idea about structure modulation and penetration pathways of vesicles.

Visualization Techniques

Transmission electron microscopy / Vesicle skin interaction study

Fig. 1.A-C represents the transmission electron micrographs of phosphate buffer saline (PBS), conventional liposomal and ethosomal formulation treated rat skin. After treatment with ethosomal formulation, areas of lipids with electron dense material were visualized deeper down in the stratum corneum that is fixed only by osmium tetraoxide (OsO4). Since, SC lipids lamellae cannot be fixed by OsO4, it was suggested that the dense material originated from the vesicles. These OsO4 fixed lipid areas containing electron dense material were not observed in PBS and conventional liposomal formulation treated skin (Fig. 2A-B). No ultra structural changes were observed in cell layers below the stratum corneum indicating that rigid liposomal formulation did not induce any changes in the ultrastructure of stratum corneum and accumulated only in the top layer of the skin. These results illustrated that liquid state vesicles might act not only in superficial stratum corneum layers, but may also induce liquid perturbations in deeper layers of the SC, while gel state vesicles interacted only with the outermost layers in the SC. This might explain the difference in drug permeation enhancement between ethosomal and conventional liposomal formulation. In addition,

fusion of conventional liposomal vesicles on top of the stratum corneum might also act as additional barrier for diffusion of drugs and therefore inhibit skin permeation. To support the result of TEM study, Jain *et al.* performed histological studies in order to visualize the changes in the ultrastructure of stratum corneum. The results of eosin-haematoxyline staining study showed that ethosomal formulation affected the ultrastructure of stratum corneum. No change in the ultrastructure of viable tissue (epidermis or dermis) could be observed after treatment with conventional liposomal formulation. TEM photomicrograph of ethosomes revealed the presence of spherical vesicular structures[24]. Jain S. *et al*. prepared Protransferosome gel (PTG) formulations of levonorgestrel and characterized for vesicle shape. Morphological characterization of the protransferosomes as well as existence of their vesicular structure after hydration was confirmed by TEM[25]. Bendas *et al*, prepared the ethosomes of salbutamol sulphate and examination of the prepared formulations by Leica optical microscope and TEM revealed the predominance of spherical-shaped vesicles. As shown in Figure, the vesicles are uniform in size and appear to be multilayered[26].

Scanning electron microscopy-

Jain S *et al.*, prepared ethosomal formulations using lamivudine as model drug and characterized in vitro, ex vivo and in vivo. Transmission electron microscopy, scanning electron microscopy, and fluorescence microscopy were employed to determine the effect of ethosome on ultrastructure of skin[27,28]. The results of SEM and TEM studies together support the hypothesis that the synergistic effect of the combination of ethanol and phospholipid in ethosomes is responsible for its better skin permeability. Y. Hiruta *et al.* investigated the morphology of vesicles by scanning electron microscopy. C16-vesicles were chosen for SEM observation because of their high elasticity and EPC recovery. C16-vesicles after extrusion appeared as particles with a diameter of above 100 nm, like vesicles before extrusion (Fig. 11A, B). High recovery of EPC and SEM observation of vesicle suspensions after extrusion suggest that high elasticity of the bilayers, ultra-deformable vesicles might squeeze themselves and pass through pores much smaller than their own diameter[29].

Confocal scanning laser microscopy (CSLM) study

Light and electron microscopy have been important tools for the analysis of cellular structure, physiology and function of biological tissues. Although transmission electron microscopy (TEM), offers excellent resolution of ultrastructural details (0.1 nm) for skin visualization, it causes damage to the specimen and suffers from fixation and sectioning artefacts. Only small specimen areas can be directly visualized and quantification of a permeating drug molecule is impossible[30,31]. In addition, TEM provides static two-dimensional images that are difficult

to reconstruct three-dimensionally from serial sections[32]. Conventional light microscopy allows direct examination of viable as well as fixed cells and tissues and dynamic processes can be therefore observed and analysed quantitatively. However, ultrastructural details cannot be obtained because of the relatively low resolution (0.2 mm); moreover, the specimen requires fixation and sectioning, which can lead to artefacts. Localization of a permeating molecule is possible, for example, with a fluorescent[33,34] or radioactive tracer[28], but out-of-focus information can undermine the quality and clarity of the images, especially in thick specimens with overlapping structures. Video image processing (video-enhanced microscopy and videointensified fluorescence microscopy) increases contrast and improves detection, but does not completely circumvent this limitation[35]. This problem can, however, be resolved by the use of confocal laser scanning microscopy (CLSM). CLSM provides valuable additional morphological information to that obtained from conventional microscopy. Simonetti et al.[36] characterized the barrier properties of reconstructed human epidermis by comparing the penetration pathways of fluorescent compounds (nile red and propidium iodide) across its stratum corneum (SC) with those across native tissue. Native and reconstructed epidermis were fixed and processed by embedding in paraffin. The images obtained by CLSM were also normalized to correct for (a) the influence of different vehicles on fluorescence intensity, and (b) the absorption and/or scattering of the exciting and emitted light deeper in the skin. The presence of intensely stained, round structures, suggesting the presence of multiple nuclei, was revealed following topical application of propidium iodide to reconstructed epidermis. In contrast, in hematoxylin–eosin stained tissue, cell nuclei could not be perceived byconventional light microscopy. CLSM is therefore a valuable tool for the rapid evaluation of the quality of reconstructed epidermis. CLSM furthermore allows skin structure to be studied in three dimensions with very high accuracy. Vardaxis et al.[36] combined a fluorescent immunoassay and CLSM to obtain morphological information regarding the epithelial vascular supply, the hair follicle, and the density and arrangement of elastic fibres in the dermis. CLSM, in association with immunofluorescence, provided a clearer and sharper imaging method compared to conventional epifluorescence in studies involving skin injury and dermal healing[37,38]. Assessment of the distribution and abundance of microfibrils at the human dermal–epidermal junction has involved visualization using CLSM in association with an immunofluorescence approach[39]. To obtain three-dimensional information from the skin, it is common practice to acquire a series of optical sections, referred to as a z-series, taken at successive focal planes along the z axis. The principle of z series

acquisition is demonstrated schematically in Fig. 3.

Fluorescence Microscopy-

Penetration ability of nanocarriers can be evaluated using fluorescence microscopy. Godin and Touitou used eosin and haematoxyline staining techniques for histological examination of different skin layers after application of ethosomal formulation and found significant difference between skin biopsies after various treatments. Fluorescence photomicrograph of the skin after a 6 hr application of Rhodamine 123 (lipophilic probe) or 6-CF (hydrophilic probe) loaded liposomal and ethosomal formulation are shown in Fig. 7A-D. Penetration from conventional liposomes was only to upper layer of skin (stratum corneum). Deep penetration from alcohol free liposomes was almost negligible (Fig. 7A, C). In contrast enhanced delivery of 6-CF and Rhodamine 123 in terms of depth and quantity (dermis layer) was observed using the ethosomal carrier (Fig. 7B, D). These results supported the results of skin permeation studies and showed the feasibility of using ethosomal formulation for delivering drugs into the deeper layers of skin or across the skin[40]. Jain S. *et al.* prepared the Proultraflexible Lipid Vesicles of Levonorgestrel and evaluated its penetration ability by using fluorescence microscopy. Six-carboxyflourescein is a hydrophilic fluorescence marker and does not normally get into the deeper layer of the skin when applied in the form of proliposomal formulation (Fig. 8a). However, this dye was transported extensively and reached the dermal layer when applied in the form of PTG formulation (Fig. 8b). The presence of fluorescence marker in the deeper layer of skin shows better skin penetration ability of the PTG formulation[41].

Conclusion

Vesicular systems have been realized as extremely useful carrier systems in various scientific domains. Over the years, vesicular systems have been investigated as a major drug delivery system, due to their flexibility to be tailored for varied desirable purposes. Based on results of reviewed studies, we could support that study / knowledge of various microscopic characteristics play a role in the enhanced skin delivery of drugs by liposomes, ethosomes, and transfersomes.

Acknowledgements

The authors gratefully acknowledge the competent guidance of Dr. Subheet kumar Jain & chitkara educational trust for providing research facilities.

References

1. Williams, A., 1st ed. Pharmaceutical Press. 2003, London.
2. El Maghraby, G.M., Williams, A.C., Barry, B.W., J. Pharm. Pharmacol. 2001b, 53: 1311–1322.
3. Barry, B.W., Eur. J. Pharm. Sci. 2001, 14: 101–114.
4. Mezei, M., Gulasekharam, V., Lotion dosage form. Life Sci. 1980, 26: 1473–1477.

5. Honeywell-Nguyen, P.L., Bouwstra, J.A., Drug Discov. Today: Technol. 2005, 2: 67–74.
6. Cevc, G., Blume, G., Biochim. Biophys. Acta. 2004, 1663: 61–73.
7. Song, Y.K., Kim, C.K., Biomaterials 20006, 27: 271–280.
8. Cevc, G., Blume, G., Biochim. Biophys. Acta. 1992, 1104: 226–232.
9. El Maghraby, G.M., Williams, A.C., Barry, B.W., J.Pharm. Pharmacol. 1999, 51: 1123–1134.
10. El Maghraby, G.M., Williams, A.C., Barry, B.W., J. Pharm. Pharmacol. 2001a, 53: 1069–1077.
11. Trotta, M., Peira, E., Carlotti, M.E., Gallarate, M., Int. J. Pharm. 2004, 270: 119–125.
12. Trotta, M., Peira, E., Debernardi, F., Gallarate, M., Int. J. Pharm. 2002, 241: 319–327.
13. Boinpally, R.R., Zhou, S.L., Poondru, S., Devraj, G., Jasti, B.R., Eur. J. Pharm. Biopharm. 2003, 56: 389–392.
14. Cevc, G., Blume, G., Biochim. Biophys. Acta. 2001, 1514: 191–205.
15. Cevc, G., Blume, G., Biochim. Biophys. Acta. 2003, 1614: 156–164.
16. Cevc, G., Blume, G., Biochim. Biophys. Acta. 2004, 1663: 61–73.
17. Cevc, G., Blume, G., Schatzlein, A., J. Control. Release. 1995, 36: 3–16.
18. Cevc, G., Gebauer, D., Stieber, J., Schatzlein, A., Blume, G., Biochim. Biophys. Acta. 1998, 1368: 201–215.
19. Paul, A., Cevc, G., Bachhawat, B.K., Eur. J. Immunol. 1995, 25: 3521–3524.
20. Touitou, E., Alkabes, M., Dayan, N., Pharm. Res. 1997, 14: 305–306.
21. Touitou, E., Dayan, N., Bergelson, L., Godin, B., Eliaz, M., J. Control. Release. 2001a, 65: 403–418.
22. Ainbinder, D., Touitou, E., Drug Deliv. 2005, 12: 297–303.
23. Paolino, D., Lucania, G., Mardente, D., Alhaique, F., Fresta, M., J. Control. Release. 2005, 106: 99–110.
24. Jain S, Sapre R, Tiwary AK, Jain NK. Proultraflexible Lipid Vesicles for Effective Transdermal Delivery of Levonorgestrel: Development, Characterization, and Performance Evaluation. AAPS PharmSciTech, 2005, 6:3.
25. Jain S. Elastic Vesicles: A Recent Approach for Transdermal/Topical Drug Delivery. Current Drug Delivery. 2006, 3:2.
26. Bendas ER, Tadros MI. Enhanced Transdermal Delivery of Salbutamol Sulfate via Ethosomes. AAPS PharmSciTech, 2007, 8:4.
27. Jain S. Formulation and Evaluation of Ethosomes for Transdermal Delivery of Lamivudine. AAPS PharmSciTech, 2007, 8:4.

28. Jain S, Sapre R, Tiwary AK, Jain NK. Proultraflexible Lipid Vesicles for Effective Transdermal Delivery of Levonorgestrel: Development, Characterization, and Performance Evaluation. AAPS PharmSciTech, 2005, 6:3.

29. Hiruta Y. Journal of Controlled Release. 2006, 113.

30. L.A. Pechtold, H.E. Bodde, H.E. Junginger, H.K. Koerten, J.A. Bouwstra., Pharm. Res. 2001, 18: 1012–1017.

31. B.A. van den Bergh, J. Vroom, H. Gerritsen, H.E. Junginger, J.A. Bouwstra., Biochim. Biophys. Acta. 1999, 1461: 155–173.

32. S.J. Wright, V.E. Centonze, S.A. Stricker, P.J. DeVries, S.W. Paddock, G. Schatten., Academic Press, USA. 1993: 2–43.

33. T. Chen, R. Langer, J.C. Weaver., J. Invest. Dermatol. Symp. Proc. 1998, 3: 59–165.

34. V. Koprda, M. Harangozo, Z. Kassai., J. Radioanal. Nucl. Chem. 2000, 246: 505–509.

35. O. Simonetti, A.J. Hoogstraate, W. Bialik, J.A. Kempenaar, A.H. Schrijvers, H.E. Bodde´, M. Ponec., Arch. Dermatol. Res. 1995, 287: 465–473

36. N.J. Vardaxis, T.A. Brans, M.E. Boon, R.W. Kreis, L.M. Marres., J. Anat. 1997, 190: 601–611.

37. K.C. Pedley., Digestion.1997, 58: 62–68.

38. D. Salomon, E. Masgrau, S. Vischer, S. Ullrich, E. Dupont, P. Sappino, J.H. Saurat, P. Meda., J. Invest. Dermatol. 1994, 103: 240–247.

39. M. Raghunath, M. Tschodrich-Rotter, T. Sasaki, M. Meuli, M.L. Chu, R. Timpl., J. Invest. Dermatol. 1999, 112: 97–101.

40. Godin B, Touitou E. Mechanisim of Bacitracin Permeation Enhancement through the skin and cellular membrane from an ethosomal carrier. Journal of Controlled Release 2004, 187: 1-15.

41. Jain S, Sapre R, Tiwary AK, Jain NK. AAPS PharmSciTech, 2005, 6: 3.

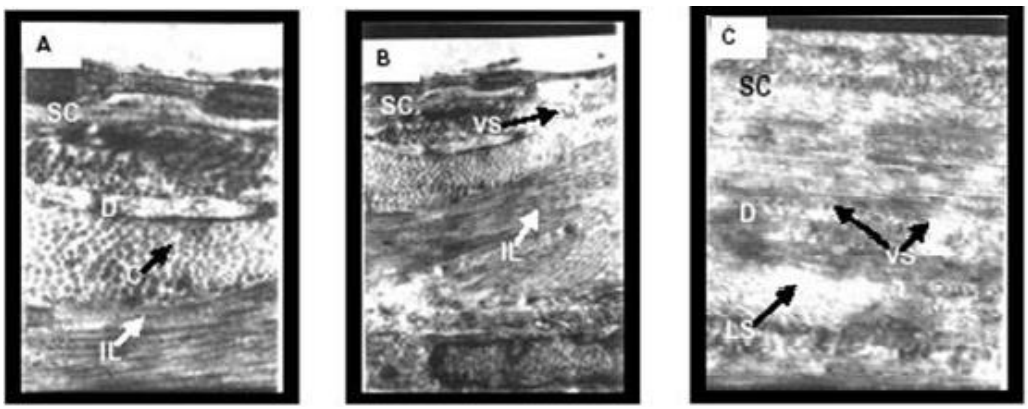

Fig. 1 (A-C): Transmission electron photomicrograph of rat skin treated with PBS (A), with conventional rigid liposomes (B) and ethosomal formulation (C).

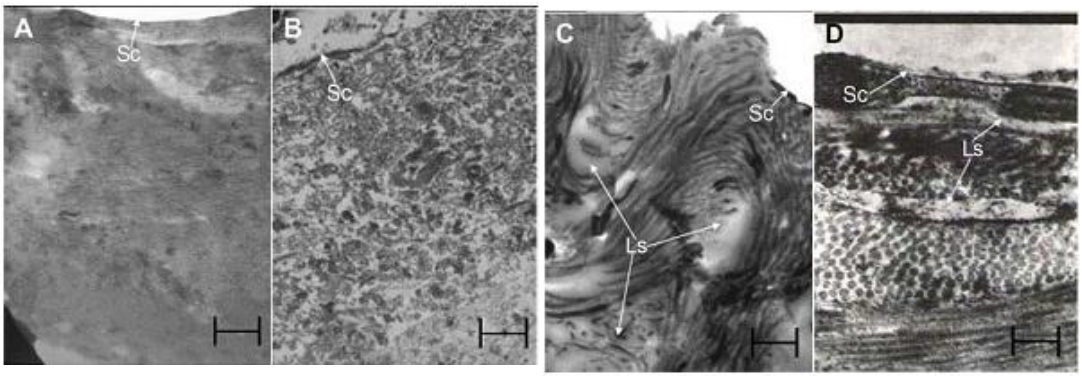

Fig. 2 A, B, C, D: TEM photomicrographs of rat skin treated with (PBS 6.5), 45% hydroethanolic solution, optimized ethosomes (ETH3), or conventional liposomes, respectively.

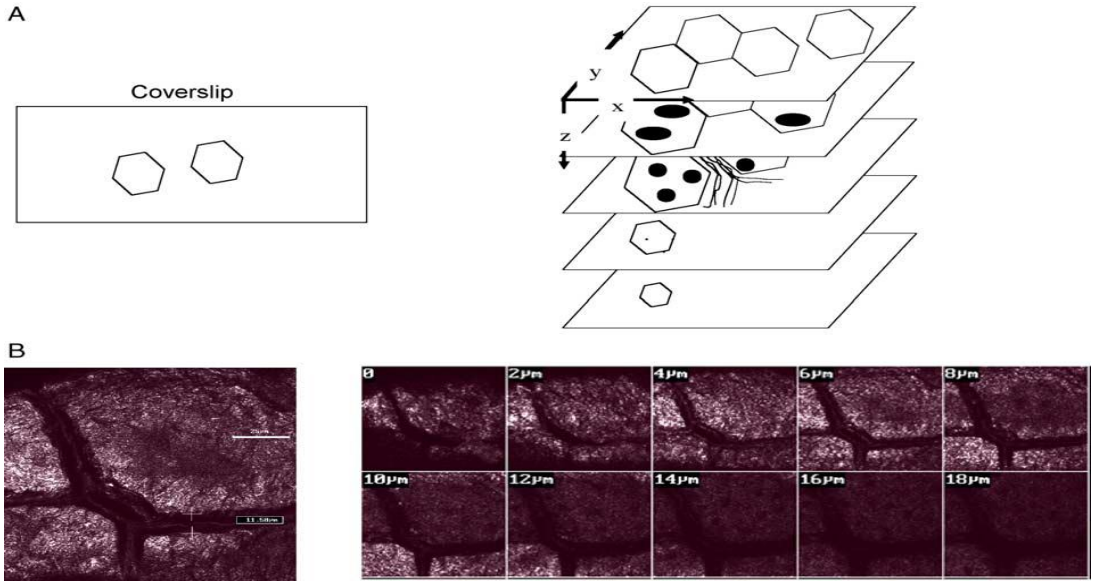

Fig. 3: Confocal optical sectioning I: (A) a schematic of a z-series (sequential xy sections as a function of depth (z)), and (B) confocal images of a z-series through porcine skin.

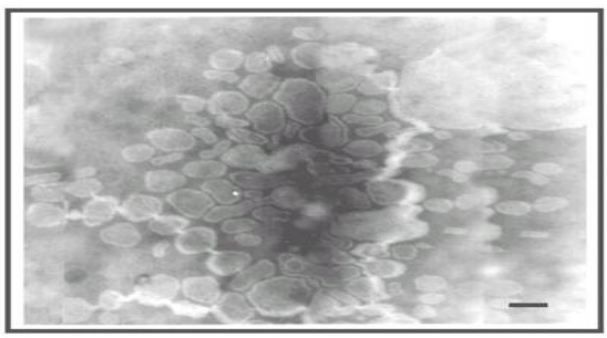

Fig. 4: TEM photomicrograph of PTG formulation after hydration (×1,20,000). Scale bar = 500 nm.

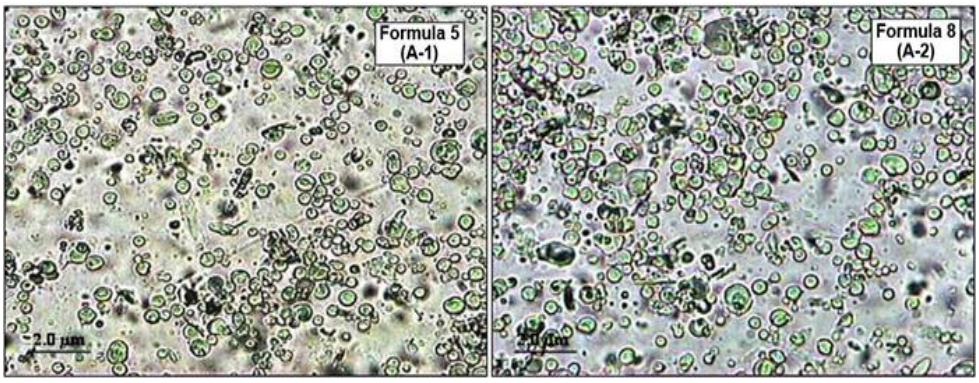

Fig. 5 (A-1, A-2): TEM photomicrograph of salbutamol sulphate loaded ethosomes.

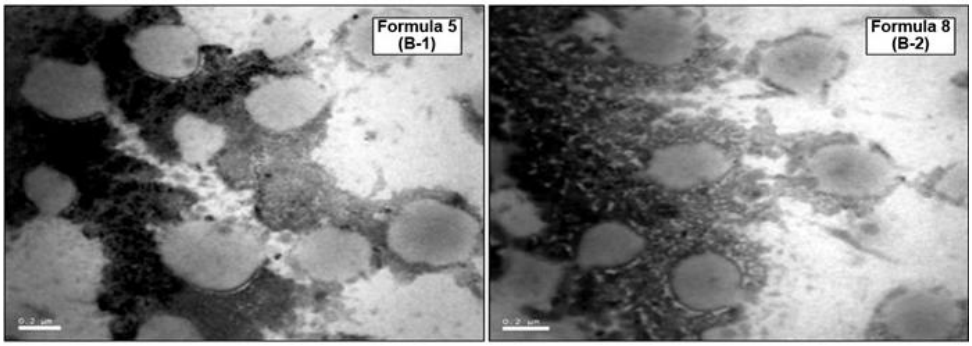

Fig. 6 (B-1, B-2): TEM photomicrograph of salbutamol sulphate loaded ethosomes.

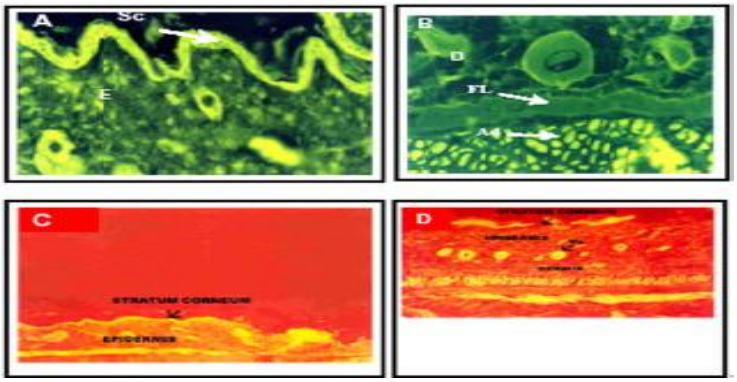

Fig. 7: Fluorescence photomicrograph of rat skin after application of hydrophilic fluorescence probe 6-carboxyfluorescein from (A) Liposomal formulation (x 100); (B) Ethosomal formulation (x100) and Rhodamine 123 from (C) Liposomal formulation; (x 100) (D) Ethosomal formulation (x100).

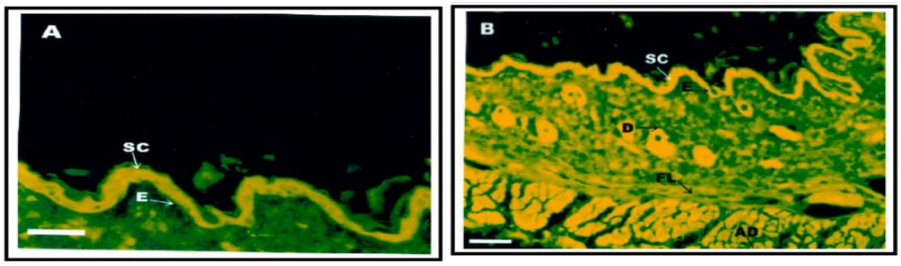

Fig. 8: Penetration of 6-carboxyfluorescein as fluorescence probe after 6-hour application of proliposomal formulation.

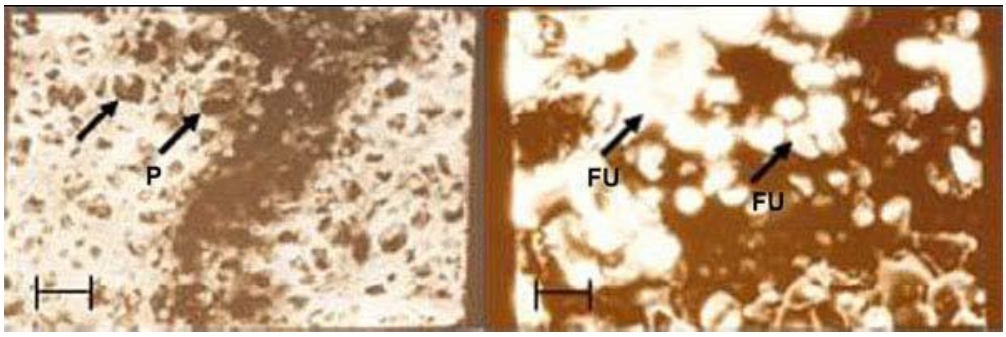

Fig. 9: Scanning electron microscope photomicrograph of membrane surface treated with (A) ethosomal and (B) conventional liposomal formulation.

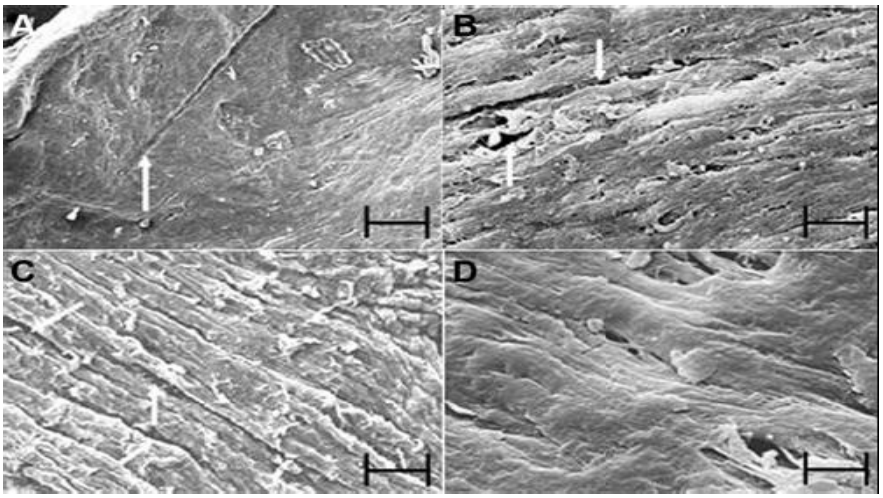

Fig. 10: Scanning electron microscope photomicrograph of rat skin treated with (A) phosphate buffer solution, (B) 45% hydroethanolic solution, (C) ethosomes, and (D) and liposomes. Scale bar =100 nm.

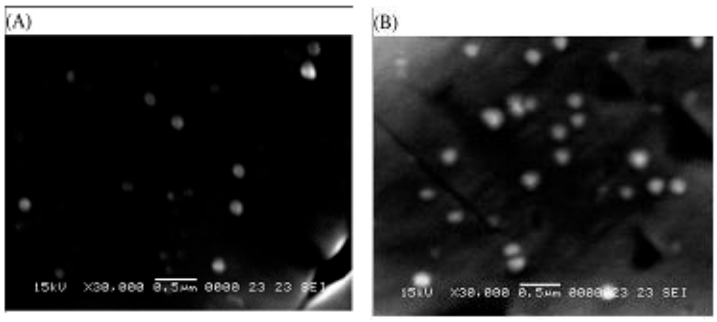

Fig. 11: Scanning electron micrographs of C16-vesicles before (A) and after extrusion (B) through a polycarbonate membrane with a pore size of 50 nm. Scale bar=0.5 μm.

******