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Original Study

EPRI Approach on Studying Deep Skin Penetration of Tretinoin-Containing Liposomes

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SUMMARY

Introduction: Tretinoin has a variety of skin-beneficial properties. However, when used at the dosages required to get the best outcomes, it also has significant adverse effects.
Aim: Side-effects caused by tretinoin could be avoided or minimized by integratingit into lecithin liposomes, which have long-term stability and the ability to penetrate into the deep layers of skin.
Material and Methods: Electron paramagnetic resonant spectroscopy imaging (EPRI) was used to study deep skin penetration of hydrogenated lecithin liposomes containing tretinoin, into the porcine skin. To make liposomes EPR-detectable, they were spin-labeled using 16-doxylstearic acid. This paper is a part of an academic (non commercial) study.
Results and Conclusion: EPRI proved to be a powerful technique for tracking the spatial distribution of liposomes in the tissue (skin), with the potential to be applied in pharmaceutical trials. To the best of our knowledge, herein it was used for the first time to track the distribution of tretinoin liposomes through the skin.

Keywords: Liposomes, Tretinoin, EPR Imaging, Encapsulation, Skin Penetration

INTRODUCTION

Ever since 1971, tretinoin (all-*trans* retinoic acid) was widely used to treat various skin conditions such as acne, actinic keratoses, lesions, scaring, and pigmentation [1]. It is still especially used as a remedy for photoaged skin [2]. When prepared in the form of ointment, concentration of tretinoin is commonly 0.05% [1]. However, at this concentration, it exerts severalunwanted effects such as itching, scaling, and erythema. Previous research suggests that in the liposomal form, sufficient treatment effects could be achieved using five to ten times lower concentrations of tretinoin, thus minimizing its side-effects [3]. At the same time, the integration of tretinoin into liposomes protects it from photodegradation. Hydrogenated soy lecithin liposomes containing tretinoin have previously been synthetized [4,5], and their ability to penetrate into deep layers of skin was tested *in vitro* using Franz cells in occlusive conditions. Herein, we have used a different approach for testing tretinoin-containing liposomes by means of spin-labeling and imaging techniques of electron paramagnetic

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resonance imaging (EPRI) spectroscopy. EPR spectroscopy provides higher sensitivity and significantly lower detection limits compared to other spectroscopy methods, and EPRI has an especially important advantage suitable for studies of tissue penetration and the redox status of various biological samples.

AIM

Side-effects caused by tretinoin could be avoided or minimized by integratingit into lecithin liposomes, which have long-term stability and the ability to penetrate into the deep layers of skin.

MATERIAL AND METHODS

Hydrogenated soy lecithin (Emulmetik 320) was purchased from Behawe Naturprodukte (Germany), while 16-doxylstearic acid (16-DS), Na2HPO4 x 12 H2O, citric acid, NaCl, tretinoin, cholesterol, chloroform, methanol and 16-DS were purchased from Sigma Aldrich (Germany). Bovine serum albumin was purchased from Merck (Germany). All of the chemicals were of analytical grade. A small fragment of fresh porcine skin from the ear area has been obtained from the local meat processing facility, upon sacrificing the animal, but before scalding and scraping.

Tretinoin liposomes were synthesized according to the adapted thin film sonication method [4–6]. 50 mg of thin lipid film was formed by mixing hydrogenated soy lecithin, cholesterol, and tretinoin in a 60%:20%:20% (w/w) ratio, respectively, and dissolving them in 2 ml of the mixture of chloroform and methanol (in 4:1 v/v ratio, respectively). Upon the rotary evaporation of the organic solvent under vacuum, the remaining thin film was rehydrated with 3 ml of 50 mM phosphate-citrate buffer (pH 5), followed by 3 series of vigorous vortexing and sonication in 3 min intervals. The formed liposomes were used for further experiments within the same day, and the remaining ones were stored at 4°C. To incorporate the spin label 16-DS into the liposomes, 2 µl of 50 mM solution of 16-DS in ethanol was evaporated to dryness, and 10 µl of liposomes were placed on top of the thin film of the spin probe. Upon that, the solution was vortexed for 3 minutes on low speed, and incubated at room temperature for 20 minutes.

30% Bovine serum albumin (BSA) hydrogel was prepared by mixing 30 mg of BSA (fraction V) with 70 µl of deionized water at room temperature. The mixture was vortexed at low speed for 3 minutes. To incorporate the spin label 16-DS into the hydrogel, 2 µl of 50 mM solution of 16-DS in ethanol was evaporated to dryness, and 10 µl of albumin hydrogel was placed on top of the thin film of the spin probe. Upon that, the solution was vortexed for 3 minutes on low speed, and incubated at room temperature for 20 minutes.

To gain insight into the size of liposomes, dynamic light scattering (DLS) method has been used [7]. After being synthesized, 1 ml of liposome suspension was transferred into the disposable square cuvette and placed into the DLS measurement device (Malvern Zetasizer Nano-ZS ZEN3600). Size measurements were conducted at 25°C using non-invasive backscatter (173° scattering angle) in 3 series of 10 accumulations. Consecutively, the same suspension was transferred into the folded capillary cell, and zeta potential was determined at 25°C in 3 measurements of 100 accumulations. To determine their long-term stability, the size and zeta potential of liposomes were measured for up to 7 days.

The ability of liposomes to penetrate through the surface of the skin was tested using electron paramagnetic resonance imaging (EPRI) spectroscopy combined with spin-labeling method [8] and porcine skin as a model. To make liposomes EPR-detectable, they were labeled by 16-doxylstearic acid (16-DS) [6,9]. A section of porcine skin (6x3 mm in size) was covered by thin film formed from 2 µl of liposome suspension, and incubated at room temperature for 15 minutes. Upon that, the skin was thoroughly washed using deionized water, laid onto the EPR tissue cell, and placed into the X-band EPR resonator (Bruker Biospin ELEXSYS-II E540 EPR spectrometer). The 2D EPR image was recorded in ZY-plane under the following conditions: gradient strength=20 G•cm^{-1}, microwave power=10 mW, conversion time=14.65 ms, sweep width=30 G, sweep time=15 s, pixel size=0.125 mm, modulation frequency=100 kHz, modulation amplitude=2 G. Two-dimensional images were reconstructed from raw data using the back-projection algorithm implemented into the Xepr software provided by Bruker.The same procedure was repeated using BSA hydrogel instead of liposome suspension. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

The detected average hydrodynamic diameter of the liposomes measured subsequently to their synthesis was in a narrow range around 104 nm (Figure 1). Based on the literature, these results indicate that the obtained liposomes could penetrate into the deeper layers of skin, since they are ≤ 300 nm[10]. The size of the liposomes did not change significantly, since their average size reached 120 nm and remained stableeven after 7 days. The detected zeta potential of liposomes was -16 mV, and it did not change significantly during the interval of 7 days. Taken altogether, it can be concluded that lecithin liposomes containing tretinoin have long-term stability and uniform size distribution within the range suitable to be used in studies of skin penetration.

Porcine skin treated with 16-DS labeled liposome suspension or 16-DS labeled BSA hydrogel was placed into the X-band EPR resonator in the XY plane, as shown in Figure 2A. The 2D EPR image (EPRI) in YZ plane depicting penetration ofBSA hydrogel orliposomes through the skinis shown in Figure 2B. Signal intensity was color-coded in arbitrary EPRI units as specified by the color bar: yellow to red correlatesto high EPRI signal intensity, while green to blue indicates low to no EPRI signal intensity.

Based on Figure 2, it can be observed that spin-labeled liposomes have reached the deepest layers of the skin. In contrast, the same spin label from the BSA hydrogel has reached the same area in drastically lower amount.This proves that liposomes have immensely greater potential in deep skin topical delivery of tretinoin compared to hydrogel, especially keeping in mind its possible interaction with the BSA

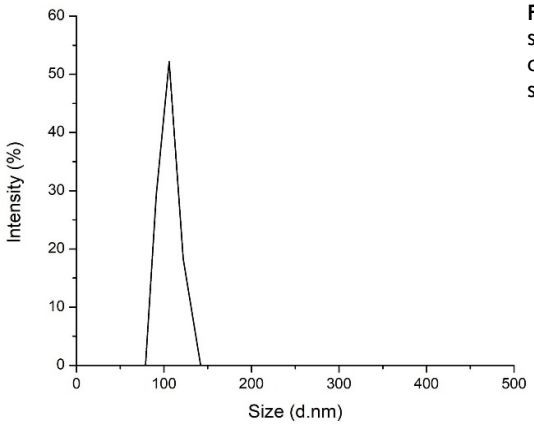

Figure 1. Representative DLS size distribution of tretinoin-containing liposomes by intensity

matrix, which could hamper its beneficial effects. In both cases, penetration through the layers of the upper part of skin was somewhat less pronounced, but this can be ascribed to the combination of capillary forces and gravity. The liposomes have been more concentrated in the center compared to the edges. Due to diffusion, it could be expected that liposomes would, at some point, spread towards the edges of the tissue. To further confirm our results, experiments were performed in triplicate (Figure 3). The same observations could be noticed in all samples.

CONCLUSION

Liposomes containing tretinoin were synthetized using thin film and sonication method. Their size was uniformly distributed around 104 nm, and they exerted long-time stability. Using spin-labeling and EPRI techniques, it was shown that tretinoin-containing liposomes could penetrate into the deep layers of skin. Taken altogether, EPRI proved to be suitable technique for tracking the tissue dis-

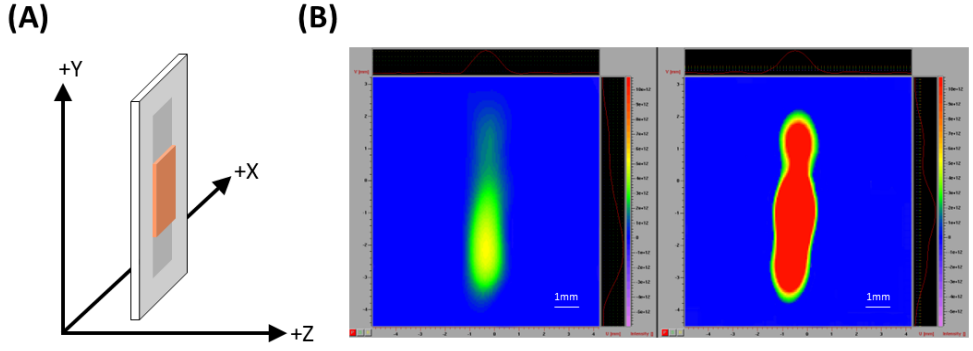

Figure 2. (A) Depiction of the skin tissuesample (orange) placed in X-band EPR resonator in the XY plane; (B) 2D EPRI image of the 16-DS labeled BSA hydrogel (left) and 16-DS labeled tretinoin-containing liposomes' (right) penetration through the porcine skin in the YZ plane

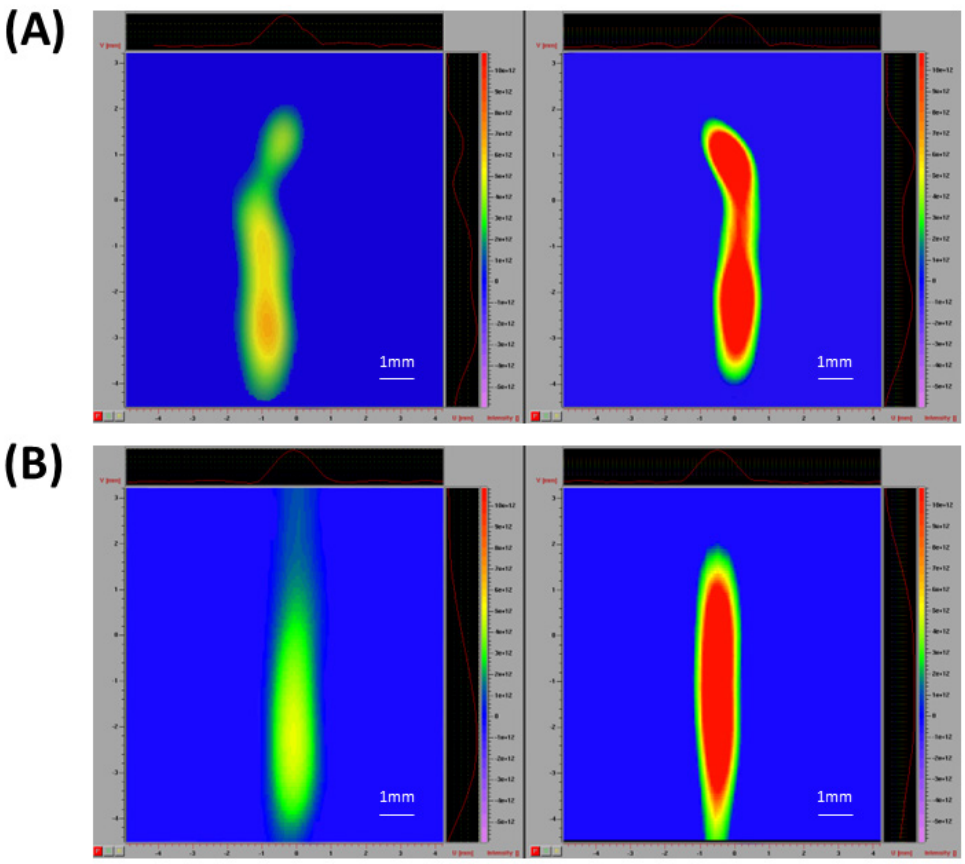

Figure 3. Two additional replications of the experiment: 2D EPRI image of 16-DS labeled BSA hydrogel (left) and 16-DS labeled tretinoin-containing liposomes' (right) penetration through the porcine skin in the YZ plane.

tribution of liposomes, and encapsulation of tretinoin into the liposomes could be useful in avoiding its side-effects, at the same time improving its tissue penetration ability.

CONFLICT OF INTEREST

All authors declare no conflict of interest.

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EPRI pristup u izučavanju prodiranja tretinoinskih lipozoma kroz duboke slojeve kože

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KRATAK SADRŽAJ

Uvod: Tretinoin poseduje raznolika, po kožu korisna svojstva. Međutim, kada se primenjuje u dozama neophodnim za postizanje najboljih ishoda terapija, ispoljavaju se značajni neželjeni efekti.
Cilj: Neželjeni efekti upotrebe tretinoina mogu se izbeći ili umanjiti njegovom integracijom u lecitinske lipozome, koji poseduju stabilnost tokom dužeg vremenskog perioda, kao i sposobnost prodiranja u duboke slojeve kože.
Materijal i metode: Imidžing elektronskom paramagnetnom rezonantnom spektroskopijom (EPRI) upotrebljen je za praćenje prodiranja lipozoma od hidrogenizovanog lecitina koji sadrže tretinoin u duboke slojeve svinjske kože. Kako bi lipozome bilo moguće detektovati EPR-om, spinski su obeleženi pomoću 16-doksilstearinske kiseline.
Rezultati i zaključci: Potvrđeno je da je EPRI značajna tehnika za praćenje prostorne raspodele lipozoma u tkivu (koži), i da se potencijalno može upotrebiti i u farmaceutskim studijama. Koliko je autorima poznato, ovo je prvi rad u kome je EPRI upotrebljena za praćenje raspodele lipozoma koji sadrže tretinoin kroz kožu.

Ključne reči: lipozomi, tretinoin, EPR imidžing, enkapsulacija, prodornost kroz kožu

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