Biochimica et Biophysica Acta, 1150 (1993) 162–164

BBAMEM 76045

Destabilization of egg lecithin liposomes on the skin after topical application measured by perturbed $\gamma\gamma$ angular correlation spectroscopy (PAC) with ^{111}In

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(Received 11 March 1993)

Key words: Liposome; Perturbed angular correlation spectroscopy; Indium-111; (Skin); (Porcine)

Unilamellar and oligolamellar liposomes were topically applied to the skin of porcine ears. Perturbed angular correlation spectroscopy of ^{111}In-DTPA, initially entrapped into the liposomes, was used to study the release kinetics of the hydrophilic probe. After drying of the liposomal suspensions, a decrease of the time-integrated perturbation factor $\langle G_{22}(\infty)\rangle$ within 30 min indicated destabilization of the liposomes and total marker release in the upper epidermal layers.

Introduction

The skin protects the organism against infections and the loss of water. This is, in addition to the barrier function of the horny cells, due to the complex structures of the intercellular lipids in the horny layer [1,2] which minimize the penetration of particles and hydrophilic molecules. Because of the epidermal barrier, it seems at first unlikely that liposomes as particles with a hydrophilic surface may reach deeper layers of the skin after topical application. Nevertheless, numerous studies have demonstrated their advantage as a possible formulation for a limited number of drugs in dermatology and in some cases even in percutaneous application of bioactive substances (for a review, see Ref. 3).

To elucidate the mechanism of penetration enhancement of drugs by liposomes in the skin, we used perturbed $\gamma\gamma$ angular correlation spectroscopy (PAC), which was introduced into liposome research to measure the integrity of liposomes in situ or even in vivo [4]. The technique is based on the detection of the anisotropic emission of two γ-rays of the radioisotope. This anisotropy is coupled to the direction of the nuclear spin at the moment of the decay. With the first γ-quantum of 173 keV, the initial spin orientation is detected. After a time Δt, the second γ-quantum of 247 keV emitted from the same nucleus reflects the actual spin orientation. If during this time, defined by the nuclear half-life of $8.5 \cdot 10^{-8}$ s, the spin fluctuates due to a motion of the atom, this motion is reflected by the perturbation factor $G_{22}(t)$ and its time integral $\langle G_{22}(\infty)\rangle$.

The two limiting cases of the tumbling rate are a very fast motion of the probe leading to a large time-integrated perturbation factor $\langle G_{22}(\infty)\rangle$ or a low tumbling time in the order of a few nanoseconds, resulting in a strong reduction of $\langle G_{22}(\infty)\rangle$. When encapsulated into liposomes, $^{111}In^{3+}$ has to be chelated by agents like NTA or DTPA, otherwise it would bind to the liposomal membrane. In the inner aqueous compartment, water and buffer ions allow a fast tumbling of the probe. When the liposomal membrane is perturbed, the probe leaks out. If the probe binds to structures like proteins or when surrounding water is lost, then the motion of the probe is strongly reduced. $\langle G_{22}(\infty)\rangle$, which corresponds to the tumbling rate, is therefore a quantitative measure for the liposomal encapsulation of the hydrophilic probe or its hydration.

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Abbreviations: DTPA, diethylenetriaminopentaacetic acid; LUVs, large unilamellar vesicles; NTA, nitrilo-triacetic acid; OLVs, oligolamellar vesicles; PAC, perturbed $\gamma\gamma$ angular correlation spectroscopy; PBS, phosphate-buffered saline.

Materials and Methods

Liposome preparation

Large unilamellar vesicles (LUVs) were prepared by controlled dialysis [5] of mixed micelles of egg lecithin/sodium cholate (1:1.7 mol/mol) 12 h against PBS (pH 7.4), using a highly permeable dialysing membrane with a cutoff of 10 kDa (Diachema, München, Germany). LUVs had a hydrodynamic diameter of 74 nm, as measured by laser photonautocorrelation spectroscopy (Nanosizer, Coulter, Harpenden, UK).

Oligolamellar vesicles (OLVs) were prepared by extruding [6] a lecithin suspension in PBS three times through a polycarbonate membrane (Nuclepore) with 0.2-μm pores and additionally five times through 0.08 μm. OLVs had a mean diameter of 130 nm and a mean number of lamellae of 2.5, as estimated by the ratio of calculated and found trapping efficiency of a hydrophilic substance [7] like ^{111}In-DTPA.

A part of LUVs and OLVs were pelleted by ultracentrifugation (140 000 $\times g$, 20°C, 2 h) and were resuspended in PBS to yield a high lipid concentration of approx. 120 mmol/l for the following trapping procedure.

Trapping of ^{111}In

10 MBq $^{111}InCl_3$ in HCl (50 mmol/l) (NEN) were purified on anion-exchange gel and dried according to the method of Hwang [4] and dissolved in 10 μl PBS containing 5 mmol/l DTPA. ^{111}In-DTPA was encapsulated into the preformed LUVs or OLVs by detergent-induced liposome loading [7]. 50 μl concentrated liposome suspension were mixed with 7 μl ^{111}In-DTPA and an optimum amount [7] of sodium cholate solution (200 mmol/l) of 5 μl was added and mixed vigorously. The mixture was incubated for 10 min and free ^{111}In-DTPA was then separated from liposomes on a Sepharose 4B-CL column (0.7 × 15 cm) using PBS. Trapping efficiency of ^{111}In-DTPA was approx. 20%. Liposomes were essentially free of detergent and had an unchanged size and lamellarity after the procedure. Trapping was stable with a half-life of the in vitro release of 160 days. Unlabelled and ^{111}In-containing vesicles were mixed to yield an appropriate lipid concentration and radioactivity.

PAC measurement

Ears from freshly-slaughtered pigs were washed and electrically shaved. Ear preparations (including the full dermis and the cartilage) with a size of approx. 4 × 5 cm were fixed on several gauze layers moistened with PBS and horizontally mounted to a thermostated surface. A thermostat temperature of approx. 38°C resulted in a physiologic ear surface temperature of 31°C. On areas of 1 × 2 cm in the middle of the ear preparations, a mixture of unlabeled and ^{111}In-containing liposomes (up to 0.5 mg lipid/cm^2) was applied in a volume of 37.5 μl/cm^2.

PAC was started with application of the liposome suspension, which contained approx. 500 kBq ^{111}In. Measurements were performed using a standard slow-fast four-detector setup with BaF_2 scintillators by recording 12 coincidence spectra simultaneously. These spectra were combined to a time-differential PAC spectrum of $R(t) = A_{22}G_{22}(t)$, covering a time of 500 ns. Each single measurement ran for 6 min. From $R(t)$ values, the time-integrated perturbation factor $\langle G_{22}(\infty)\rangle$ was calculated for measurements between 6 and 180 min.

Liposomes on the skin surface were resuspended in 50 μl of PBS after the incubation and release of ^{111}In was determined on Sepharose 4B-CL using PBS as elution buffer.

As control for the behavior of released ^{111}In, 50 μl ^{111}In-DTPA in PBS, containing the same radioactivity as the labelled liposomes, were topically applied to the skin as shown above and PAC was started.

Results

As shown in Fig. 1, the time-integrated perturbation factor $\langle G_{22}(\infty)\rangle$ of intact unilamellar vesicles (LUVs) has an initial value of approx. 0.7 when vesicles are applied in aqueous buffer to the skin. The drying of the suspension corresponds to a drop of the signal down to a minimum value of approx. 0.3 within 30 min. Oligolamellar vesicles (OLVs) exert an identical spectrum as LUVs in Fig. 1.

After resuspension of OLVs or LUVs from the skin surface in PBS, analysis on Sepharose 4B-CL showed a nearly total release of ^{111}In from the liposomes, independent of their lamellarity.

PAC spectra of topically applied ^{111}In-DTPA in

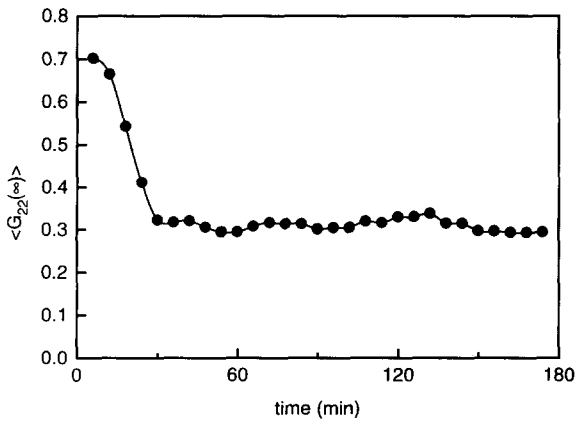

Fig. 1. Motility of the hydrophilic probe ^{111}In-DTPA, initially entrapped into unilamellar eγggr; lecithin vesicles, after topical application to porcine ears, as determined by the time-integrated perturbation factor $\langle G_{22}(\infty)\rangle$ from PAC measurements.

PBS were nearly identical to that in Fig. 1. The decrease in $\langle G_{22}(\infty)\rangle$ occurred only, when the solution dried on the skin. Therefore, a decrease of the signal indicates a reduced probe tumbling by a lack of hydration rather than a significant binding of the probe to the tissue.

In a parallel experiment using LUVs and a higher encapsulated ^{111}In activity, 2 h after liposome application the skin was stripped 4 times using adhesive tapes to remove dried liposomes on the skin surface and the very upper horny layers. The remaining radioactivity of 500 kBq of liposomal material in the skin showed the identical minimal $\langle G_{22}(\infty)\rangle$ value as shown in Fig. 1, indicating only negligible marker entrapment in deeper skin layers.

Discussion

Our PAC data and the recently published skin distribution of fluorescent markers [8] suggest that egg lecithin liposomes disintegrate at the interface to the horny layer, when an aqueous suspension dries on the skin surface. Differences in the stability of unilamellar and oligolamellar liposomes are not significant.

Liposome destabilization is in line with electronmicroscopic investigations, which led to the suggestion that the epidermal barrier in the horny layer constists of intercellular lipid monolayers, which are cross-linked by various ceramide species and cholesterol esters [2]. It seems, therefore, unlikely that liposome particles, which are at least 20 nm in diameter, can penetrate the intact horny layer, either across the corneocytes or along the cross-linked intercellular lipid layers. More probably, as shown by small angle X-ray scattering [9], fluid liposomal and rigid epidermal lipids intermix after fusion of the lipid layers. As shown in the present study, the hydrophilic interior of the liposomes is released during the drying process and a penetration of encapsulated hydrophilic drugs can therefore be excluded.

Some authors have suggested that liposomes reach deeper layers of the skin in their initial form [10]. Electronmicroscopic pictures indeed show multivesicular structures in deeper skin layers after topical application of liposomes. Nevertheless, it is more probable that in deeper epidermal layers multilamellar structures are newly formed by vesiculations of the intercellular lipid layers, which are destabilized after mixing with liposomal lipids.

A pronounced increase in skin penetration by using liposomal formulations is in most cases confined to amphiphilic or hydrophobic drugs [11–13]. Fluidization of skin lipids leads probably to an improved partition of these drugs into the lipidic phases in deeper skin layers.

Further PAC studies will elucidate whether topical application leads to an enhanced skin penetration of intact liposomes or released hydrophilic substances, when drying of the suspension on the skin surface is avoided upon occlusive conditions.

Acknowledgement

This work was supported by grants Schu 800/1–1, De 432/2–1 and La 759/1–1 from the Deutsche Forschungsgemeinschaft.

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