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Note

An investigation into the combination of low frequency ultrasound and liposomes on skin permeability

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ABSTRACT

Antigen application onto skin that has been pre-treated with low frequency ultrasound leads to immunisation, and it was hypothesised that immunisation could be enhanced if antigens were entrapped within liposomes, the latter being known vaccine adjuvants. However, it has been suggested that liposomes can repair skin damage, which could limit antigen permeation and transcutaneous immunisation. The aim of the present work was therefore to investigate the influence of liposome application on subsequent: (i) *in vitro* antigen permeation through, and (ii) *in vivo* barrier properties of, ultrasound-treated skin. Sonication was conducted using either phosphate buffered saline (PBS) or an aqueous solution of sodium dodecyl sulphate (SDS) as the coupling medium, and rats were used as the animal models. Liposome application to sonicated skin reduced antigen penetration and transepidermal water loss (TEWL, used as an indication of skin integrity) when the skin had been sonicated using PBS coupling medium. The influence of liposome was evident within 5 min of its application, and smaller liposomes were more effective at repairing skin disruption caused by sonication. Such skin repair did not, however, take place when the skin had been sonicated in the presence of SDS (which caused greater skin disruption), and changes in *in vitro* antigen permeation and *in vivo* TEWL were negligible. Skin repair by liposomes seems to depend on the extent of the disruption caused by ultrasound application.

The numerous advantages of transdermal drug delivery, such as, simple and non-invasive application, and avoidance of first pass metabolism, has led to investigation into a range of methods of overcoming the considerable barrier properties of the skin, such as, the use of chemicals (e.g. azone and surfactants), physical skin disruptors (e.g. electricity, ultrasound, microneedles, etc.) as well as delivery vehicles (e.g. liposomes and solid lipid nanoparticles). Often, enhancers are used in combination, especially if their mechanisms of action are different, for their synergistic activities and superior enhancement of transdermal delivery. Safety may also be improved if the synergistic effects on transdermal permeability allow a less severe protocol/concentration of the individual enhancers.

The synergistic, or even additive, effects of transdermal enhancers, cannot be assumed, however. For example, combinations of electroporation and liposomes decreased transdermal drug delivery compared to electroporation using the control non-liposomal drug solution (Essa et al., 2003). The reduced drug permeation was thought to be due to repair of the electroporated skin by the liposomal phospholipids (Essa et al., 2003). Combinations of liposomes with iontophoresis were also found to reduce drug permeation (Vutla et al., 1995; Fang et al., 1999), though these combinations were also likely to increase permeation (Kulkarni et al., 1996; Badkar et al., 1999; Fang et al., 1999; Li et al., 2001; Essa et al., 2002, 2004) or to have no significant influence (Vutla et al., 1995). The varied effects of liposome/iontophoresis combination have been related to a number of possible mechanisms which could be operating simultaneously. Increase in drug permeation was assigned to fusion of liposomal phospholipids with the stratum corneum which increased the latter's permeability (Fang et al., 1999; Essa et al., 2002); decreased skin resistance by elastic vesicles (Li et al., 2001); increased deformability of ultradeformable liposomes by electricity which led to their greater skin penetration (Essa et al., 2002); and the influence of drug and liposome charge on their electrophoretic movement in the skin, though charge could also decrease permeation (Essa et al., 2002; Fang et al., 1996). Other mechanisms of permeation retardation include increased liposome stability by the electric field leading to reduced drug release and permeation (Fang et al., 1996), slow drug release from liposomes comprising phospholipids in the liquid crystalline state (Fang et al., 1996), and protection of the entrapped drug from skin metabolism, which led to lower permeation of the larger intact drug molecule (Vutla et al., 1995).

In our laboratory, we are investigating low frequency ultrasound-assisted transcutaneous vaccination (Dahlan et al., 2009). Topical application of low frequency ultrasound increases skin permeability to large, hydrophilic molecules such as proteins (Mitragotri et al., 1995), stimulates the skin's dendritic cells (Tezel

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et al., 2005) and enables transcutaneous immunisation following topical antigen application (Tezel et al., 2005; Dahlan et al., 2009). It was hypothesised that ultrasound-assisted transcutaneous immunisation could be enhanced by liposomal encapsulation of antigens, liposomes being known topical vaccine adjuvants (Vyas et al., 2007). The skin would be pre-treated with ultrasound prior to application of the liposomes, to avoid ultrasonic degradation of susceptible antigens (Dahlan et al., 2005) and of the liposomes. Low frequency ultrasound application to the skin is known to extract skin lipids (Alvarez-Roman et al., 2003), and cause the formation of defects (Wu et al., 1998) and lacunar spaces within the skin (Paliwal et al., 2006), and create highly permeabilised localised transport regions (LTRs) in the skin (Kushner et al., 2004). Thus, liposome application to sonicated skin could allow/enhance liposome/antigen flux into the skin, and consequently enhance transcutaneous immunisation.

However, given the reported reduction in transdermal drug permeation with liposome/electroporation combinations and the suggested skin repair by liposomes (Essa et al., 2004), our first aim was to determine whether liposome application to sonicated skin would also lead to repair of ultrasound-induced skin disruption and thereby reduced permeability. Thus, the influence of liposome application on: (i) *in vitro* antigen permeation through sonicated skin, and (ii) *in vivo* barrier properties of sonicated skin, was determined, and is reported in this paper.

Soya phosphatidylcholine (SPC, from Lipoid GmbH, Germany) liposomes were prepared by dissolving SPC (50 mg) in chloroform (5 mL) in a round-bottomed flask, removing the solvent using a rotary evaporator, and rehydrating the resulting lipid film with water (10 mL) in a water bath at 40 °C, with mechanical shaking for 30 min. Subsequently, the liposomes were vortexed for 1 min, and allowed to stand at room temperature for 2 h. This yielded large liposomes whose volume median diameter, measured by laser light diffraction using a Malvern Mastersizer S (Malvern, UK), was found to be 4.56 ± 0.04 μm (span 1.50 ± 0.07 μm, $n = 5$). To determine the influence of liposome size on *in vivo* skin barrier properties, part of the liposome suspension was extruded in a Liposofast 50 extruder (Avestin Inc., Ottawa, Canada) using 200 nm and 100 nm filter membranes (Cyclopore™, Whatman International, UK, each membrane being used 10 times), after which the liposomes were bath sonicated for 3 h. The diameter of the resulting smaller liposomes, measured by photon correlation spectroscopy (PCS) using a Malvern ZetaMaster 2000 (Malvern, UK), was found to be 79 ± 2.3 nm, with a polydispersity index of 0.45 ± 0.02 ($n = 5$). Transmission electron microscopy of the large and small liposomes showed both to consist of multiple layers, though the smaller liposomes had fewer layers (not shown).

The influence of liposomes on *in vitro* antigen permeation through sonicated skin was determined using modified vertical Franz diffusion cells and full-thickness rat skin in a water bath at 37 °C. The donor and receptor compartments were filled with 20 mL of coupling medium (water or 1%, w/v sodium dodecyl sulphate (SDS) aqueous solution) and 4 mL of phosphate buffered saline (PBS), respectively. SDS was included in the coupling medium in some of the experiments, as it has been shown to enhance ultrasound-assisted transdermal drug delivery (Mitragotri et al., 2000; Tezel et al., 2002). Low frequency ultrasound (20 kHz, generated by VCX500 sonicator, Sonics & Material Inc., USA, at 30% amplitude) with a duty cycle of 50% (0.5 s on, 0.5 s off) was applied for a total sonication time of 2 min, with the probe being placed at 5 mm from the skin surface. Subsequently, the coupling medium was discarded, the skin was rinsed and blotted dry, and 4 mL of liposome suspension was applied on the sonicated skin for either 5 or 60 min, after which the liposome suspension was removed, the skin was rinsed, blotted dry and 50 μL of radio-labelled (^{125}I) bovine serum albumin (BSA, model antigen) solution was applied onto the liposome-treated sonicated skin for 24 h. Thereafter, the levels of radioactivity (indicating protein levels, as confirmed by gel electrophoresis, data not shown) in the receptor compartment were measured using a gamma counter (Cobra Packard). Control experiments, i.e. without liposome application were conducted similarly except that liposomes were not applied to sonicated skin.

To investigate the influence of liposomes on the barrier properties of sonicated skin *in vivo*, experiments were conducted in groups of 5 rats (male Wistar rats, 220–240 g, from Harlan, Oxon, UK) and transepidermal water loss (TEWL) was measured as an indication of the skin's barrier properties. The rats were acclimatized for 1 week prior to experimentation and given food and water *ad libitum*. All animal procedures were approved by The School of Pharmacy's Ethical Review Committee and were performed in accordance with the Animals (Scientific Procedures) Act 1986. Rats (whose abdominal skin had been shaved using electric clippers 24 h previously) were anaesthetised using an intra-peritoneal injection of a mixture of 0.9 ml/kg of ketamine HCl (100 mg/ml) and 0.5 ml/kg of xylazine (20 mg/ml), and were placed on their back. Baseline pre-sonication transepidermal water loss (TEWL) was measured using the condenser-chamber evaporimeter, AquaFlux™ (BIOX Ltd., UK). Subsequently, a flanged cylinder was attached to the abdominal skin using double-sided tape (Tesa, UK), filled with 20 mL of coupling medium (water or 1%, w/v sodium dodecyl sulphate (SDS) aqueous solution) and ultrasound was applied as described above for the *in vitro* permeation studies. Subsequently, the coupling medium was discarded, the sonicated skin was rinsed, blotted dry with tissue paper, and liposome suspension (4 mL) was placed in the flanged cylinder for 5 min, after which liposomes were discarded and the skin was rinsed and blotted dry. The flanged cylinder was removed and transepidermal water loss was measured at (5, 15, 30, 45 and 60) min post-sonication. Once again, control experiments were conducted similarly, except for the liposome application. SPSS 17.0 was used to perform all statistical analyses.

Ultrasound application using PBS coupling medium: The *in vitro* permeation studies showed that liposome application to sonicated skin reduced permeation of the model antigen—by 32% for a 5 min application and 43% for a 60 min application (Fig. 1). The mechanism(s) of such reduction in antigen permeation is thought to include the following: liposome adsorption onto and fusion with the skin surface, with some permeation of the liposomal phospholipids into the skin, especially into the defects and lacunar spaces created by ultrasound, and replenishment of some of the skin lipids extracted during sonication. Thus, some of the ultrasound-induced skin damage would be repaired and the

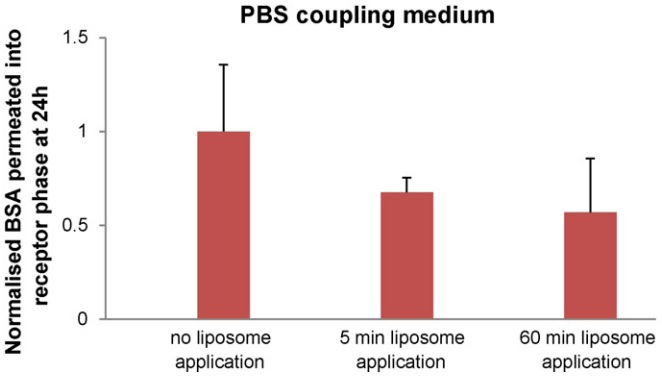

Fig. 1. Change in BSA permeation through sonicated skin following exposure to liposomes for 5 or 60 min. PBS was used as the coupling medium. Means (±SD) of 5 replicates are shown.

ultrasound-induced permeation pathways available to the subsequently applied antigen would be reduced. The large reduction in antigen permeation (Fig. 1) was, however, statistically not significant at an alpha level of 0.05 (ANOVA with post hoc Tukey, $p = 0.06$).

The longer duration of liposome application caused a slightly greater (though statistically not significant; ANOVA with post hoc Tukey, $p = 0.8$) reduction in antigen permeation—by 43% for a 60 min versus 32% for a 5 min application (Fig. 1). Theoretically, a longer 60 min liposome application would allow more time for the penetration of liposomal lipids into the sonicated skin, and greater reduction in antigen permeation could have been expected. The considerable influence of the 5 min liposome application and relatively small further change in permeation with the 60 min application indicates rapid interaction between liposomes and sonicated skin, and consequently, liposome application was limited to 5 min in subsequent experiments.

The reduced antigen permeation *in vitro* was reflected *in vivo* in reduced TEWL, which was significantly lower than that of control sonicated skin unexposed to liposomes (Mixed between-within subjects analysis of variance, $p < 0.05$ for both large and small vesicles, Fig. 2). TEWL is often used as an indication of skin integrity (Nangia et al., 2005), and a lowered TEWL suggests repair of at least some of the ultrasound-induced skin damage by liposomes. The mechanisms of such repair are likely to be the same as described above for the changes in BSA permeation, i.e. adsorption of liposomes on and fusion with skin surface, liposomal lipid penetration into and 'blocking' of ultrasound-induced localised transport regions in sonicated skin. Interestingly, the reduction was consistently greater with the smaller vesicles, though the difference was small and statistically insignificant ($p = 0.3$, Fig. 2). Smaller liposomes are known to be more efficient at penetrating into the skin (Duplessis et al., 1994; Natsuki et al., 1996; Verma et al., 2003), and it is possible that some of the smaller liposomes penetrated intact into the skin, further increasing the skin's lipid content and barrier properties against the movement of water vapour out of the skin. This could have facilitated the return of TEWL to baseline pre-sonication values within 15 min of sonication and liposome application (Fig. 2).

Ultrasound application using SDS aqueous solution as coupling medium: Liposome application to skin sonicated in the presence of SDS led to a small (by 12%) and statistically insignificant (Student's *t*-test, $p = 0.2$) reduction in *in vitro* BSA permeation (Fig. 3). This was reflected *in vivo*, where liposome application had a negligible influence on skin TEWL (mixed between-within subjects analysis of variance $p > 0.05$ for both large and small liposomes, Fig. 4). The much greater skin damage caused by the synergistic activities of ultrasound and SDS – reflected in the much higher TEWL values of control sonicated skin in Fig. 4 compared to Fig. 2 – could not be repaired by a 5 min liposome application. It seems that the skin damage caused by the combination of ultrasound and SDS was too great to be repaired by liposome application.

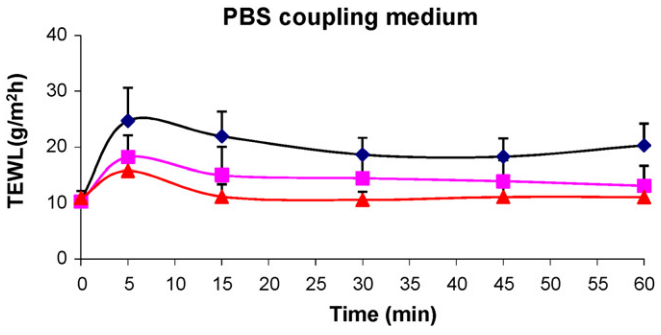

Fig. 2. *In vivo* TEWL from rat skin sonicated using PBS coupling medium that was subsequently: unexposed to liposomes (♦); exposed to large liposomes (■); or exposed to small liposomes (▲), for 5 min. Means (±SD) of 5 rats are shown. TEWL at time 0 represents the baseline pre-sonication values.

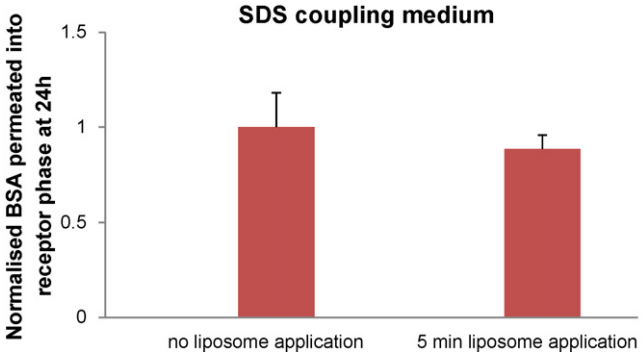

Fig. 3. Change in BSA permeation through sonicated skin following exposure to liposomes for 5 min. SDS solution was used as the coupling medium. Means (±SD) of 5 replicates are shown.

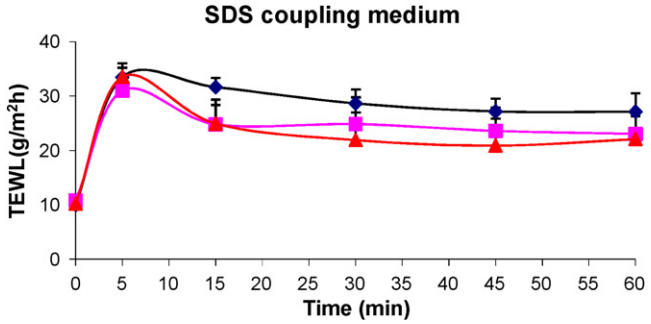

Fig. 4. *In vivo* TEWL from rat skin sonicated using SDS solution as coupling medium that was subsequently: unexposed to liposomes (♦); exposed to large liposomes (■); or exposed to small liposomes (▲), for 5 min. Means (±SD) of 5 rats are shown. TEWL at time 0 represents the baseline pre-sonication values.

These results showing a certain repair of the 'damaged' sonicated skin by liposomes reflect the study by Essa et al. (2003) who suggested repair of electroporated skin by liposomal phospholipids. We found skin repair to occur within minutes of liposome application, and smaller vesicles to be more effective at skin repair, presumably due to a greater permeation of liposomal phospholipids or even of intact vesicles into sonicated skin. Liposomal repair was, however, dependent on the extent of skin damage; greater damage caused by skin sonication in the presence of SDS was not repaired. This suggests that synergistic effects between liposomes and ultrasound for transcutaneous immunisation may be possible.

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