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Enhanced Drug Delivery to the Skin Using Liposomes

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Background: Enhancing drug delivery to the skin has importance in many therapeutic strategies. In particular, the outcome in vascularized composite allotransplantation mainly depends on systemic immunosuppression to prevent and treat episodes of transplant rejection. However, the side effects of systemic immunosuppression may introduce substantial risk to the patient and are weighed against the expected benefits. Successful enhancement of delivery of immunosuppressive agents to the most immunogenic tissues would allow for a reduction in systemic doses, thereby minimizing side effects. Nanoparticle-assisted transport by low temperature–sensitive liposomes (LTSLs) has shown some benefit in anticancer therapy. Our goal was to test whether delivery of a marker agent to the skin could be selectively enhanced.

Methods: In an in vivo model, LTSLs containing doxorubicin (dox) as a marker were administered intravenously to rats that were exposed locally to mild hyperthermia. Skin samples of the hyperthermia treated hind limb were compared with skin of the contralateral normothermia hind limb. Tissue content of dox was quantified both via high-performance liquid chromatography and via histology in skin and liver.

Results: The concentration of dox in hyperthermia-treated skin was significantly elevated over both normothermic skin and liver. ($P < 0.02$).

Conclusions: We show here that delivery of therapeutics to the skin can be targeted and enhanced using LTSLs. Targeting drug delivery with this method may reduce the systemic toxicity seen in a systemic free-drug administration. Development of more hydrophilic immunosuppressants in the future would increase the applicability of this system in the treatment of rejection reactions in vascularized composite allotransplantation. The treatment of other skin condition might be another potential application. *(Plast Reconstr Surg Glob Open 2018;6:e1739; doi: 10.1097/GOX.0000000000001739; Published online 9 July 2018.)*

INTRODUCTION

Many therapies would be greatly enhanced by specific targeting of drugs to the skin. One such example is vascularized composite allotransplantation (VCA), which offers an excellent opportunity for the treatment of large defects that cannot be sufficiently reconstructed with conventional reconstructive methods. Examples of these composite tissue grafts are abdominal wall, hand, arm, or face trans-

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Products and drugs used for this study: Monostearoyl-2-hydroxy-sn-glycero-3-phosphocholine (MSPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (Polyethylene Glycol)2000] (DSPEPEG2000), Genzyme Corporation (Cambridge, MA). Doxorubicin, Bedford Laboratory (Bedford, OH). Rotovap vacuum desiccator, LIPEXTM extruder (Northern Lipids, Burnaby, BC). Nuclepore polycarbonate membrane filters (Whatman PLC, Maidstone, Kent, UK). PD-10 desalting column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Closed-cell extruded polystyrene (Styrofoam) cups (human grade). Digital thermometer (Omega). Waters 2695 HPLC and Waters 2475 fluorescence detector. Fast-Prep, Thermo Savant (doxorubicin extraction). Leica CM1850 microtome. Zeiss AxioSkop II. Metamorph and ImageJ (NIH) software for imaging and analysis.

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plants. Abdominal wall allotransplantation helps cover gaping or nonhealing abdominal wounds.[1–3] Although the technical aspects of VCA have become routine for skilled microsurgeons, the challenge remains in conquering the complex immunogenicity of the diverse tissues.[4–6] Despite the development of several systemic immunosuppressive agents and monoclonal antibodies over the past decades, toxicity of these drugs and concomitant side effects increase the risk to the patient and, justifiably, reduces the use of potential allotransplant cases.[4–6]

Targeting strategies may enhance delivery of therapeutic agents and could achieve effective tissue concentrations locally while keeping the systemic load lower than with a systemic free drug administration. This was successfully demonstrated in cancer therapy using nanoparticle-assisted targeted drug delivery.[7–11] Low temperature–sensitive liposomes (LTSLs) are excellent vehicles for hydrophilic drugs. In the environment of mild hyperthermia (41–42°C), the liposome membrane increases in permeability so that the encapsulated drug is instantly released. Drug and liposome extravasation are further facilitated due to leakiness of the vasculature.[7–11]

Doxorubicin (Dox)–containing LTSLs were chosen for this study because they are a well-established system and because of the known quantitative techniques of dox using its autofluorescent properties. With the skin being a highly immunogenic tissue in CTA, we quantified dox concentrations in the skin as a marker for enhanced delivery.[12–16] Demonstration of enhanced heat-triggered delivery could lead to a therapy that eventually allows reduction of the systemic dose while effectively providing sufficient local drug concentration to prevent episodes of tissue rejection.

MATERIALS AND METHODS

Preparation and Characterizations of LTSL

Monostearoyl-2-hydroxy-sn-glycero-3-phosphocholine, 1,2-dipalmitoyl-sn- glycero-3-phosphocholine, and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (Polyethylene Glycol)2000] (DSPEPEG2000) were obtained from Genzyme Corporation (Cambridge, Mass.). Doxorubicin (Dox) was obtained from Bedford Laboratory (Bedford, Ohio.). All the other chemicals were obtained from Sigma-Aldrich (St Louis, Mo.).

Liposomes were prepared by hydration of lipid film, followed by extrusion as previously reported.[17,18] Briefly, lipid components (1,2-dipalmitoyl-sn- glycero-3-phosphocholine, monostearoyl-2-hydroxy-sn-glycero-3-phosphocholine, and DSPE-PEG2000) were dissolved in chloroform at a molar ratio of 85.3: 9.7: 5.0. The solvent was evaporated using a Rotovap system and left overnight in a vacuum desiccator. The resulting lipid film was hydrated by a buffer consisting of 300 mM citrate (633 mOsm; pH, 4.0) at 65°C for 25 minutes to yield a final lipid concentration of 50 mg/mL. Liposomes were obtained by extruding the mixture 5 times with a LIPEXTM extruder (Northern Lipids, Burnaby, BC) at 55 °C through 2 stacked Nuclepore polycarbonate membrane filters (Whatman PLC, Maidstone, Kent, United Kingdom) with a nominal pore size of 100 nm. Encapsulation of Dox into the extruded liposomes was carried out using a pH-gradient loading protocol as described by Mayer et al.[19] with a minor modification: exterior pH of the extruded liposomes Dox-Liposome was adjusted to 7.4 by passing through PD-10 desalting column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and the pH adjusted with sodium carbonate solution (500 mM) creating a pH gradient (acidic inside LTSL).[19] The liposomes were incubated with Dox (Dox: lipid weight ratio of 5: 100) at 37°C for 1 hour. The resulting liposomes were stored at 4°C until further use. Particle size of liposomes and Dox release were determined as previously reported.[17,18]

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In Vivo Studies

All animal procedures were approved by the Duke University Institutional Animal Care and Use Committee before initiation. Eleven male Sprague Dawley rats (250–320 g; Charles River, Raleigh, N.C.) were anesthetized with isoflurane via nose cone at 2–3% in oxygen (to effect) an placed on a heating pad to maintain rectal temperature at 37°C. Heparinized saline (20 Units/ml) filled a tail vein catheter. The rat was then placed in a cup of closed-cell extruded polystyrene (Styrofoam) that was customized with a hole to allow for the right hind limb to extend out while the rest of the rat was thermally isolated. The hole was 2–3 mm wider than the hind limb's maximal diameter to prevent constriction and allow good perfusion during the entire experiment. The cup was then placed on a clear acrylic frame over a water bath heated to 41.5–42.0°C. When the heating period began, the right hind limb was submerged into the warm water. Temperature was monitored with 1 digital thermometer (Omega) at the hind limb and 1 analogue thermometer in the center of the water bath and kept within range throughout the entire experiment. Before injection of liposome-dox formulation via the previously inserted tail vein catheter (1.8 mg Dox/ml; 2.5 mg/kg; infusion @ 2.5 ml/Kg body weight), the hind limb was allowed to preheat for 10 minutes. After injection, the hind limb was kept at 41.5–42°C for 60 minutes as previously determined for the Thermally-Sensitive Liposome applications.[7,10,11] Body temperature was monitored with a digital rectal thermometer throughout the experiment and kept at 36.5–37°C with a fan blowing air at the animal if necessary to prevent core temperature to rise above 37°C. The rat was euthanized, while still anesthetized, by intracardiac potassium chloride injection. Skin samples of the heated and unheated hind limb, the dorsum, and liver samples were harvested immediately after sacrificing and snap frozen in liquid nitrogen. An artist's sketch illustrating the experimental setup can be seen in Figure 1.

Dox Analysis using High-performance Liquid Chromatography

Dox in rat skin and liver tissue was measured by in-house validated high-performance liquid chromatogra-

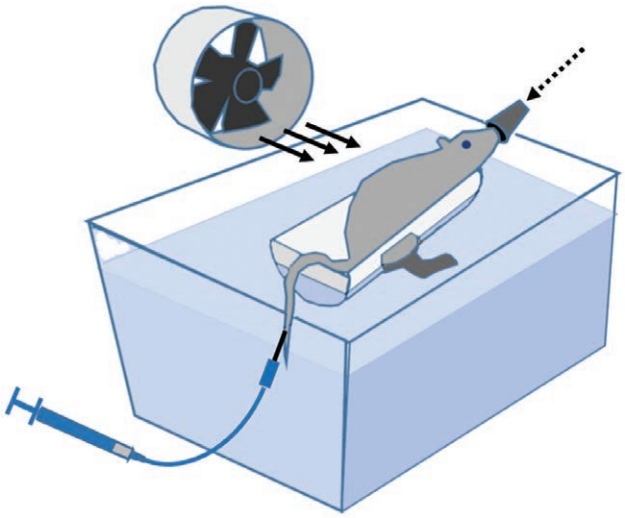

Fig. 1. Sketch of experimental setup. Arrow indicates direction of isoflurane anesthesia.

phy (HPLC)–fluorescence method (Waters 2695 HPLC and Waters 2475 fluorescence detector) by the PK/PD Bioanalytical Core Laboratory, Duke Cancer Institute. Skin samples were cryo-pulverized under liquid nitrogen and ~30 mg of the powder transferred into preweighed 2-mL screw-cap polypropylene vial, and the net weight was recorded. Liver samples were homogenized in a rotary polytetrafluoroethylene pestle/glass vessel homogenizer by addition of 2 mL of water to 1 g of tissue, and 100 µL of the homogenate was transferred to 2-mL screw-cap polypropylene vial. Calibration sample homogenates were prepared in the same manner from dox-free tissue followed by addition of 0.081–10 µg/mL pure dox. The rest of the procedure was similar for both skin and liver. After addition of 10 µL of 5 µg/mL daunorubicin (internal standard), 1.5 mL of chloroform/isopropanol mixture (4/1), and a 4-mm ceramic bead, doxorubicin was extracted by vigorous mixing (Fast-Prep, Thermo Savant, speed 5 for 20 s) followed by centrifugation at 16,000g at 4°C for 5 minutes. The top aqueous and interface "cake" were removed, and 1 mL of the lower organic layer evaporated to dryness under gentle flow of nitrogen for (30 minutes at room temperature). The sample was reconstituted by addition of 50 µL methanol, vortex-mixing, addition of 250 µL mobile phase A (see below), vortex-mixing, and filtered through 0.45 µm nylon filter; 10 µL was injected into LC-MS/MS system. Chromatography conditions: mobile phase A: 0.5% H_3PO_4, 0.5% tetrahydrofuran, 5% acetonitrile; B: acetonitrile/methanol (1/1); gradient elution: 0–12 minutes, 5–45%B, 12–13 minutes, 45–90%B, 13–14 minutes, 90%B, 14–15 minutes, 90–5%B, 15–25 minutes, 5%B; column: Eclipse XDB-C8 4.6×15 cm; column flow: 1.5 mL/min; column temperature: 50°C; autosampler temperature: 4°C. Fluorescence detector: excitation at 480 nm and emission at 550 nm. Study samples were analyzed along with a set of calibration samples and quantified by Waters Empower software. Calibration curves were linear and the lower limit of quantification of the method for skin and liver tissue was 81 ng/mL (15% error criteria).

Histology

The frozen rat skin specimens were cryo-sectioned at a 10 µm thickness using the Leica CM1850 microtome. The cryosections were then mounted on Super Frost Plus white microscope slides and stored in -80°C freezer. To evaluate the dox distribution, the slides with the rat skin specimens were fixed in fresh acetone for 15 minutes and then dried for 1 hour under the fume hood. After the drying step, the slides were fluorescently imaged using a Zeiss AxioSkop II using a 20× objective and camera and then stitched together with Metamorph software. Additional cryo-sectioned slides were stained for Hematoxylin & Eosin.

Image Analysis

Acquired images were analyzed using ImageJ (NIH) program. Four different skin regions were chosen within the same image, and the overall grayscale value was measured. The epidermis was excluded from the assessment as it contained many hair follicles, which are brightly auto-fluorescent in the fluorescein emission channel. The average of the 4 grayscale values for each slide was included in a plot, and nonparametric statistical analysis was performed. Figure 2 shows histologic samples of untreated and treated skin.

Statistical Analysis

A Wilcoxon rank sum test was used for comparison of the skin dox concentrations measured by HPLC of right and left hind limbs. A Student's *t* test was used for comparison of normally distributed data of dox concentrations measured by HPLC of the heated skin and the liver. Two-tailed paired *t* test was used to compare gray scale values of the histological images.

RESULTS

All animals were successfully treated with our method. In all cases, the rat circulation and respiration remained stable during the experiments.

LTSLs Characterization

The size of LTSL (n = 3) was 104.6 ± 2.3 nm with polydispersity index 0.02. The amount of dox encapsulated into the LTSL was 1.9 ± 0.05 mg/ml and similar to that reported previously.[17,18] Dox release was monitored using fluorescence dequenching in phosphate buffered saline (PBS) buffer (equivalent to physiological buffer) from 20°C to 55°C at a temperature gradient of 1°C/min (Fig. 3). Dox release starts at ~38°C, and complete release is achieved at ~42°C. Addition of Triton-X100 has resulted in lowering the signal intensity, showing that such surfactant should not be used to indicate complete release in the LTSLs in-vitro release study consistent with 1 of the author previous observation (unpublished data).

Drug Distribution

HPLC was used to quantify dox concentration in skin that was exposed to mild hyperthermia and skin (from the left contralateral side) that was exposed to room temperature. Mean values for the skin of the heated right hind limb was 7,939 ± 3,130 ng/g, for the unheated left side

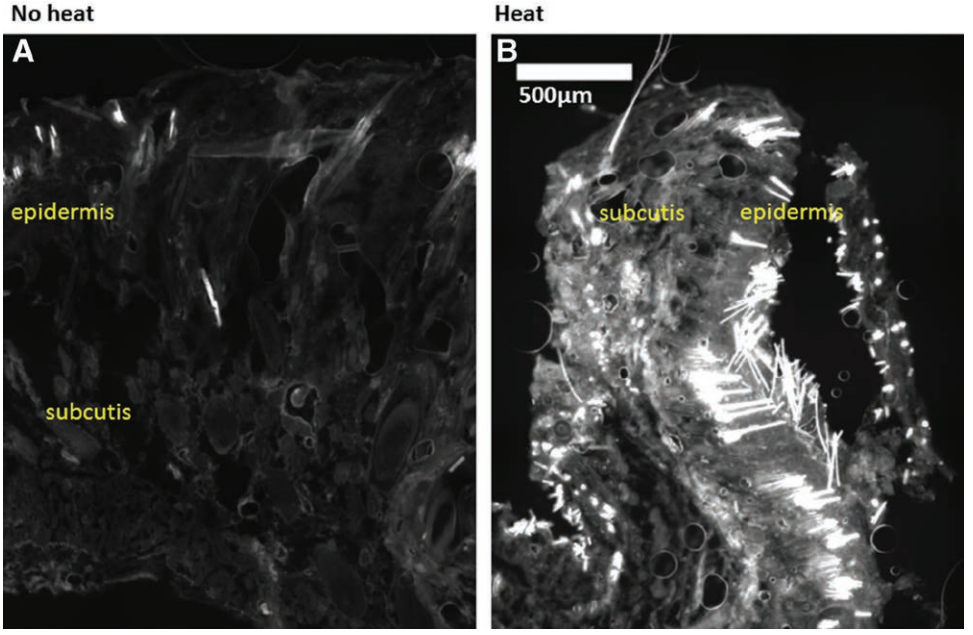

Fig. 2. Histologic samples of untreated (A) vs. treated skin (B). Note that the epidermis was excluded for the gray-scale measurements, as the intense autofluorescence of the hair follicles would have skewed the analysis.

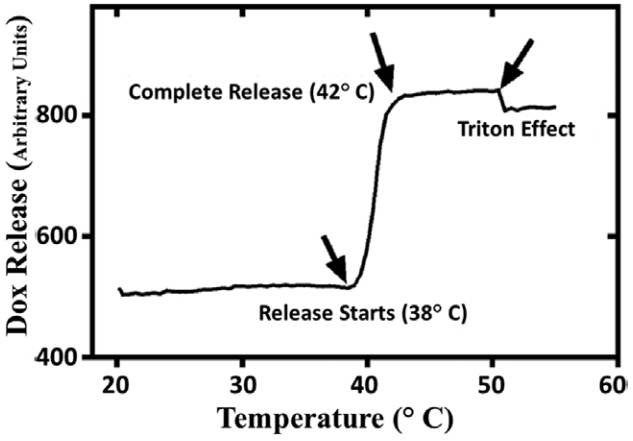

Fig. 3. Release of dox as a function of temperature. The liposomes heated from 20°C to55°C at a rate of 1°C/min.

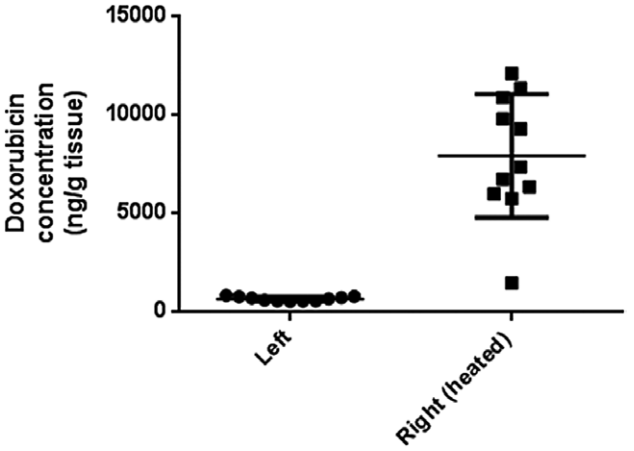

Fig. 4. shows skin dox concentrations measured by HPLC quantification comparing left (unheated) and right (mild hyperthermia at approximately 42°C) side revealing significantly higher tissue levels when treated with hyperthermia ($P = 0.0010$).

675 ± 114 ng/g ($P = 0.0010$; Fig. 4). Dox concentrations in the heated skin were significantly higher when compared with liver sample concentrations of $5{,}450 \pm 1{,}162$ ng/mL ($P < 0.02$).

Histologic image analysis via gray scale values representing free dox distribution in the harvested tissues were significantly higher in the skin treated with mild hyperthermia versus unheated skin ($P < 0.002$; Fig. 5).

DISCUSSION

Targeting therapeutics to the skin could be of great benefit in treating many maladies. Immunosuppression has improved the outcome in transplantation medicine tremendously. Particularly in vascularized composite allotransplantation, maintenance of immunosuppression prevents most episodes of acute rejection.[20,21] However, most patients experience at least 1 episode of acute rejection during the first postoperative year.[20] Aside from noncompliance of the patients, discontinuation of the immunosuppressive maintenance regimen, due to toxic side effects, puts the patient at risk for graft rejection.[20] Acute episodes of rejection are particularly dangerous regarding survival of the delicate graft, often requiring aggressive immunosuppression including high dose corticosteroids.[20] Selective immunosuppression that can be focused on the target area would reduce systemic toxicity while still being effective. This would offer a new option for the treatment of acute rejection and possibly increase applicability of VCA.

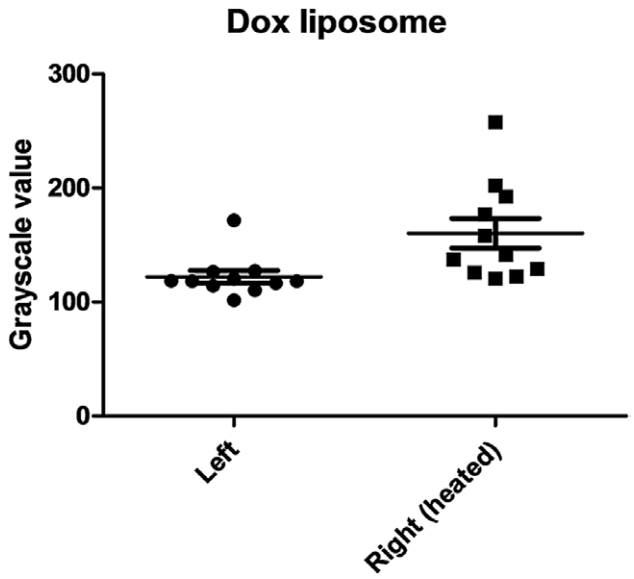

Fig. 5. Frozen rat skin specimens were cryo-sectioned at 10 µm thickness. After drying, the specimens were imaged using a fluorescent camera. Four different skin regions were chosen randomly within the imaged slides and assessed for their gray scale values using the autofluorescent properties of dox in the FITC channel. The average of the 4 grayscale values for each slide was included in the plot, and nonparametric statistical analysis was performed revealing a significantly higher dox accumulation in the dermis of the heated hind limb (right) vs. the left hind limb kept at room temperature ($P = 0.0016$).

One way of targeting immunosuppression is by using vehicles or carriers that transport a desired drug to a preselected area. Then the drug would be released after reaching that area. There are many available carriers, that is, elastin-like peptides, polymersomes, or liposomes that allow transport of a drug through the body via the vascular system. To achieve high tissue concentrations at the targeted tissues while keeping the systemic levels low, the drug must be tightly attached to its carrier at physiologic conditions (systemically inactive), and its release must reliably occur at the specific targeted tissues.

Targeted drug delivery with heat-triggered, LTSLs has shown strong efficacy in cancer therapy and is 1 of the most technologically advanced strategies in the field of focal enhanced drug delivery.[7,8,10,11,22–24] LTSLs are made of a phospholipid (lysolecithin) bilayer similar to mammalian cell membranes. These particles can then be loaded with hydrophilic drugs. Depending on their chemical properties, liposomes can be relatively stable within certain conditions, that is, normal body temperature (37°C), but become highly unstable at sudden change of temperature. Temperature-sensitive liposomes allow for enhancement of drug delivery to tissues that can be mildly heated.

The LTSL-dox formulation used in our experiments showed release in a clinically attainable hyperthermia range, starting at 38°C with maximum release at 42°C (Fig. 3). This formulation offers superior release of the content when compared with other liposome formulations. Early temperature-sensitive liposomes released their payload drug largely at clinically less attainable higher temperatures of 42–45°C. Release from nonthermally sensitive liposomes is not specifically enhanced by hyperthermia.[10] More recently formulated LTSLs release about 50% of their content to the environment under mild hyperthermia. Importantly, the release occurs within only seconds, much faster than older generation liposomes.[7,10,11] In this study, our goal was to show that drug delivery can be enhanced not only to tumors but to the skin, the organ that is most susceptible to rejection after transplantation due to its high immunogenicity.[12,13,20] Clinically substantial release can be reached during first passage, as LTSLs have been shown to release 45% of their content during the first 20 seconds under mild hyperthermia.[7,10,25,26] We achieved an 11.8-fold higher local dox concentration in the skin following mild hyperthermia when compared with the skin of the unheated limb (Figs. 4, 5).

HPLC was used for measuring dox tissue concentrations. HPLC has shown to be a reliable method to quantify dox in tissue.[27] However, it does not differentiate between free and encapsulated dox as the liquid–liquid extraction phase in sample preparation brakes down the liposome shell, but only measures total drug-in-tissue concentrations. We harvested liver samples to reflect systemic burden on critical organs, as the liver plays a key role in drug metabolism, that is, via the cytochrome P450 system. We yielded significantly higher drug concentrations in the heated skin when compared with liver ($P < 0.02$, Fig. 6). One can conclude that about half of the concentration of dox in the heated skin is free dox released by the liposomes, as 45% of release occurs within the first 20 seconds[7,10] (see above). Regarding the liver, the percentage of free, nonencapsulated, biochemically active drug should be much lower assuming that only minor leakage of the liposomes occurs at normal core temperature[7,10,11] (Fig. 6).

Histological analysis supports the finding of increased drug delivery in the heated sample. We imaged cryosectioned skin samples of the heated and unheated skin utilizing the autofluorescence of dox in the FITC channel.

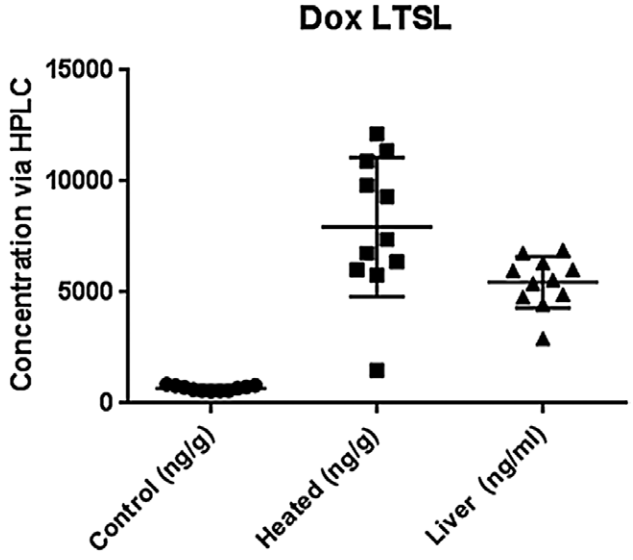

Fig. 6. Comparison of dox concentrations analyzed via HPLC in hyperthermic skin (heated) and the liver ($P = 0.018$).

On these images, free dox can be measured by analyzing randomly chosen areas for their gray scale values.[9] We measured significantly higher dox autofluorescence in sections from the heated skin samples than in nonheated tissue, which is indicative of higher tissue concentrations of free dox ($P < 0.002$; Fig. 5).

Drug delivery to rejecting tissues could be inherently benefitted by the "inflamed state" with its increased temperature, increased perfusion, and increased permeability. The LTSL carrier system presented here would further enhance delivery of a payload therapeutic. However, mass transport could also be impaired if inflammatory cells activate and block perfusion to some capillaries.

The skin was chosen to show that LTSLs lend themselves as a carrier for the delivery of therapeutics to the skin for 2 reasons. The first reason is that the skin's high immunogenicity poses a relevant problem in VCA.[12] We believe that sufficient immunosuppression at the level of the skin can sufficiently protect the allograft tissues from acute rejection. In this study, the skin doxorubicin concentration was increased 11.8-fold under mild hyperthermia (Figs. 4, 5).

The second reason the skin was chosen to target is that it lends itself to external energy-based triggering strategies because of its accessibility. Being at the surface of the body, there is excellent localization and very little dissipation of energy before reaching the site of desired delivery.

Despite yielding significantly higher drug levels at the target site, some aspects of this study need to be critically discussed. In the light of VCA, a major limitation is that liposomes are not a suitable carrier system for the immunosuppressants currently used in transplantation medicine. That is, the predominantly lipophilic character of these pharmaceuticals would impede loading and permit greater leakage from the LTSLs due to the LTSLs' propensity to encapsulate hydrophilic chemicals. As we are currently lacking hydrophilic immunosuppressants with an immediate (antirejection) effect on the allografted tissue, alternative vehicles might be more suitable for these immunosuppressants. One example might be the ultrasound-targeted delivery of "lung surfactant microbubbles."[28] Further research might, however, identify new immunosuppressants that could be delivered with the LTSL carrier. Our primary objective was to demonstrate that this carrier system allows for targeting drug delivery to the skin. Doxorubicin was not chosen as a therapeutic, rather as a reliable marker of drug delivery to the tissue based on its unique DNA binding and fluorescent properties.

HPLC is a good way to quantify doxorubicin tissue concentrations. However, this method does not distinguish between free versus encapsulated, nor intra- versus extravascular drug distribution. This makes it more challenging to quantify the true benefit (ie, reduction in overall toxic burden) of LTSL-targeted drug delivery to the skin. Concern could be raised that the differential of drug levels measured via HPLC in target versus nontarget tissue neglects residual drug distribution to other nontarget tissues and wash-out into the systemic circulation with the potential of causing a higher toxicity than estimated. We tried to estimate the potential for reduction of systemic toxicity by comparing tissue concentration of the targeted skin versus the liver (measured via HPLC; Fig. 6). This, again, revealed a significant amount of differential suggesting reduced systemic burden. A study by Hauck et al.[29] demonstrates an immediate decrease in plasma doxorubicin concentration after stopping intravenous infusion. The authors experienced drastically increased doxorubicin clearance of LTSL-encapsulated doxorubicin versus free doxorubicin clearance. Nevertheless, our approach does not ultimately dispel this concern.

To have a second method of measuring the amount of differential between target (hyperthermic) and nontarget (normothermic) tissue concentrations, histologic sampling was performed using the autofluorescent property of doxorubicin (Fig. 5). Histologic analysis revealed a significant, however, lesser amount of differential than measured via HPLC (11.8-fold versus 1.24-fold). It is important to note that a linear relationship between the doxorubicin concentration measured via HPLC and its fluorescent signal cannot be assumed, as different fluorescent properties have been reported for freely dissolved and bound doxorubicin.[30] Although we are not aware of a distinct study that has focused on the change of doxorubicin fluorescence after binding to DNA, in the light of what Shah et al.[30] have reported, it is very likely that fluorescence intensity will change with binding to DNA, which might explain this difference.

Our results indicate the potential for a significant reduction in the overall toxic burden on the organism when comparing LTSL-targeted drug delivery with traditional systemic application (oral/intravenous) and demonstrate that drug delivery specifically to the skin can be targeted and enhanced. With regard to VCA and transplantation medicine in general, the LTSL carrier system is at this point limited to encapsulate currently used "antirejection" agents. These immunosuppressants are predominantly lipophilic. In this study, we have plausibly shown that LTSL delivery can increase bioavailability of drugs to the skin, which was the major scope of this study. In the light of our data, it is likely that this system will be able to increase the area under the curve of potentially newly developed, predominantly hydrophilic, immunosuppressants in the field of VCA or other hydrophilic agents in the treatment of skin conditions.

CONCLUSIONS

LTSLs have a great potential for targeting and enhancing drug delivery to the skin while reducing systemic toxicity, in comparison with a traditional systemic (oral/intravenous) free-drug administration. Their affinity of LTSLs to hydrophilic agents limits at this point their use in VCA and transplantation medicine due to the lipophilic character of currently used immunosuppressants. In the light of our data, it is, however, likely that this system will be able to increase the area under the curve of potentially newly developed, predominantly hydrophilic, immunosuppressants in the field of VCA or other hydrophilic agents in the treatment of other skin conditions.

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