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Solid lipid nanoparticle-based dissolving microneedles: A promising intradermal lymph targeting drug delivery system with potential for enhanced treatment of lymphatic filariasis

Andi Dian Permana\textsuperscript{a,b}, Ismaiel A. Tekko\textsuperscript{a,c}, Maelíosa T.C. McCrudden\textsuperscript{a}, Qonita Kurnia Anjani\textsuperscript{a}, Delly Ramadon\textsuperscript{a,d}, Helen O. McCarthy\textsuperscript{a}, Ryan F. Donnelly\textsuperscript{a,∗}

\textsuperscript{a} School of Pharmacy, Queen’s University Belfast, Medical Biology Centre, 97 Lisburn Road, Belfast, BT9 7BL, UK
\textsuperscript{b} Department of Pharmaceutics, Faculty of Pharmacy, Hasanuddin University, Makassar, Indonesia
\textsuperscript{c} Department of Pharmaceutics and Pharmaceutical Technology, Faculty of Pharmacy, Aleppo University, Aleppo, Syria
\textsuperscript{d} Faculty of Pharmacy, University of Indonesia, West Java, Indonesia

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A B S T R A C T

Conventional oral therapy of lymphatic filariasis drugs is only effective to kill microfilariae in the bloodstream, but is often ineffective to kill adult filarial (macrofilariae) in the complex anatomy of the lymphatic system. The encapsulation of drugs into lipid-based nanoparticles with sizes of < 100 nm, and administration intradermally, could be used to enhance lymphatic uptake. Therefore, we developed an innovative approach, using solid lipid nanoparticles (SLNs) and dissolving microneedles (MNs) to deliver antifilariasis drugs, namely doxycycline, diethylcarbamazine and albendazole, intradermally. The SLNs were prepared from Geleol\textsuperscript{®} and Tween\textsuperscript{®} 80 as a lipid matrix and stabilizer, respectively. The formulations were optimized using a central composite design, producing SLNs with sizes of < 100 nm. Drug release was sustained over 48 h from SLNs, compared to pure drugs. The SLNs were then incorporated into a polymeric hydrogel which was casted to form SLNs-loaded MNs. SLNs-loaded MNs exhibited sufficient mechanical and insertion properties. Importantly, dermatokinetic studies showed that > 40% of drugs were retained in the dermis of excised neonatal porcine skin up to 24 h post-MN application, indicating the high possibility of the SLNs to be taken by the lymphatic system. In in vivo studies, the maximal lymph concentrations of the three drugs in rat, achieved following intradermal delivery, ranged between 4- and 7-fold higher than that recorded after oral administration. Additionally, compared to oral administration, despite the lower plasma C_{max} and organ-distribution, the AUC and relative bioavailability of the three drugs in rat plasma was also higher using our delivery approach. Accordingly, this delivery approach could maximize the drugs concentrations in the lymph system without essentially increasing their plasma concentrations. This could potentially deliver the drugs efficiently to the bloodstream, where the microfilariae reside, while also targeting drug to the lymph nodes, where filarial nematodes reside in infected patients, leading to an effective therapy for lymphatic filariasis.

1. Introduction

Human lymphatic filariasis (LF) is a devastating, neglected tropical disease caused by parasites, namely \textit{Wuchereria bancrofti}, \textit{Brugia malayi} and \textit{Brugia timori}. LF causes damage to the lymphatic system of the patient and causes long-term and permanent disability [1,2]. Adult filarial nematodes can reside in the lymphatic vessels and lymph nodes for several years [3]. Moreover, \textit{Wolbachia}, an endosymbiont bacteria, lives in filarial nematodes, playing a significant role in their growth, development, reproduction and survival [4,5]. In 2017, the World Health Organization (WHO) reported that 51 countries continued to require mass drug administration (MDA) as a LF treatment [1].

Presently, oral administration of a combination of albendazole (ABZ) and diethylcarbamazine (DEC) is the first line chemotherapy in MDA for LF treatment to target the filarial nematodes [2,6]. Administration of doxycycline (DOX) to target the \textit{Wolbachia} results in the death of the adult filarial nematodes [4]. Although the combination in oral administration of these three drugs has macrofilaricidal and microfilaricidal effects and can decrease the viability of microfilariae in the bloodstream, they are less effective at targeting adult nematodes in the...
injection via [12]. Focusing on the practicalities of SLN administration, intradermal delivery of drugs into the lymph capillaries in the underlying interstitial space [12]. Oral administration of these drugs results in inadequate concentrations in the lymphatic system, where the drugs ultimately are targeted [13]. Accordingly, the development of a new delivery platform which effectively targets the lymphatic system, leading to an increase in LF treatment efficacy, is required.

Various delivery systems have been developed for lymphatic targeting purposes, namely polymeric nanoparticles [14], liposomes [15], solid lipid nanoparticles (SLNs) [16], dendrimers [17] and microemulsion (MN) delivery systems [18]. Furthermore, interstitial administration is one of the most feasible routes for lymphatic targeting [19]. With regards to the interstitial administration, some studies have reported that particles with sizes in the range of 10–100 nm are taken up effectively by the lymphatic system. In contrast, while particles of > 100 nm are retained at the administration site, particles with sizes of < 10 nm are taken up by blood capillaries [20]. Addressing these criteria, SLNs have been investigated in a bid to produce small particle sizes with high lipophilicity [21,22]. SLNs are colloidal dispersions of sub-micron size, prepared from solid lipid and stabilized by suitable surfactants [23]. SLNs have benefits over other nanoparticle delivery systems due to the utilization of physiological lipids in their formulation, leading to the documented safe administration of SLNs [24]. To highlight just one study, this nanoparticle approach has successfully delivered mitoxantrone-loaded SLNs to lymph nodes in breast cancer treatment [25].

Taking a lead from these previous research studies, the formulation of antifilariasis drugs loaded into SLNs with particle sizes of < 100 nm could prove a promising system to deliver these drugs to the lymph node, thus improving LF treatment efficacy. In terms of interstitial administration as a potential route for lymphatic targeting, the intradermal route has been reported to be the most suitable route to deliver drugs into the lymph, when compared to other interstitial sites [12]. Focusing on the practicalities of SLN administration, intradermal injection via hypodermic needles could potentially have some inherent problems, such as poor patient compliance, issues surrounding needle phobia and ultimately the production of dangerous biological waste [26]. In addition to this, it has been reported that administration via this route, even when carried out by trained professionals, is not always successful or reliable [27]. Consequently, a new drug delivery system which can deliver the drugs intradermally without causing the aforementioned problems is required. Dissolving MN are a drug delivery system which can by-pass the stratum corneum, the major skin barrier and, hence, are envisioned as novel intradermal drug delivery systems [28]. In addition, in our previous study involving human volunteers, the application of MNs was found to be pain-free [29]. This platform consists of needles < 1 mm in height which penetrate to the dermal microcirculation and cover a greater surface area than single intradermal injection [28,30]. In addition to this, as dissolving MN are self-disabling and are produced from biocompatible and biodegradable polymers, their use does not result in the generation of any biohazardous sharp’s waste [31]. Importantly, intradermal administration of drugs using MNs has been reported to result in higher drug concentrations in the lymph nodes, in comparison with the intravenous and subcutaneous administration [18,32]. Building upon these promising studies, the formulation of antifilariasis and antibiotic drugs into SLNs with particle sizes of < 100 nm, combined with dissolving MNs as an intradermal delivery platform could potentially increase drug concentrations at the infection site in the lymph nodes and, as such, could potentially increase the effectiveness of LF treatment.

In this study, we present, for the first time, the formulation of an antibacterial drug (DOX) and two antifilariasis drugs (DEC and ABZ) into SLNs for intradermal administration using dissolving MNs. As the drugs do not encounter hepatic first-pass metabolism when delivered via the intradermal route [10], albendazole sulfoxide (ABZ-OX), an active metabolite of ABZ after first-pass metabolism, was utilized in this study. A central composite design (CCD) was used for the development and optimization of the drug-loaded SLNs. The optimized SLNs were characterized for their size, polydispersity index, zeta potential, shape, incompatibility and release behavior. The SLNs were formulated into dissolving MNs and the MNs were evaluated for mechanical properties, in vitro dermatokinetic studies, and skin distribution studies. Finally, in order to evaluate lymphatic targeting mediated by this delivery system, in vivo plasma pharmacokinetic, lymphatic pharmacokinetic and biodistribution profiles of the three drugs post-MN administration were compared to oral administration, the conventional administration route utilized in LF treatment. The outcomes of these extensive in vitro and in vivo studies provide evidence for the potential utilization of this innovative drug delivery approach, to maximize the lymphatic system exposure while maintaining the systemic exposure at an appropriate level without the necessity to increase the administered dose. Essentially, this innovative approach could, in time, be used as a significant means of increasing the effectiveness of LF drug delivery.

2. Material and methods

2.1. Materials

Doxycycline monohydrate (DOX) and albendazole sulfoxide (ABZ-OX) (purity, ≥ 98%) of analytical grade was purchased from Alfa Aesar (Lancashire, UK). Chlороform, diethylcarbamazine (DEC) (purity, ≥ 98%), albendazole sulfoxide (ABZ-OX) (purity, ≥ 99.9%), poly(vinyl alcohol) PVA (9–10 kDa), PVA (31–50 kDa), sodium hydroxide, sodium laurel sulfate (SLS) and stearic acid were purchased from Sigma-Aldrich (Dorset, UK). Optimal cutting temperature media was obtained from VWR International Ltd. (Leicestershire, UK). Poly(vinylpyrrolidone) PVP (58 kDa) was provided from Ashland (Kidderminster, UK). Gelesol®/glycerol monostearat, Precirol® ATO 5/glycerol disteareate and Compritol® 888 ATO/glycerol dibehenate were kindly gifted by Gattefosse Pvt. Ltd., France. Pluronic® F127 (PF127) was obtained from BASF SE (Ludwigshafen, Germany). Ultrapure water was obtained from a water purification system (Elga PURELAB DV 25, Veolia Water Systems, Ireland). All other reagents were of analytical grade and purchased from standard commercial suppliers.

2.2. Methods

2.2.1. Preparation and optimization of drug-loaded SLNs

2.2.1.1. DOX-loaded SLNs. SLNs containing DOX were prepared using a hot emulsification-ultrasonication technique, as described previously [33] with slight modifications. Firstly, DOX and 100 mg of lipid were dissolved in 20 mL of a mixture of methanol and chloroform (1:1, v/v). The solution was placed in a vacuum rotary evaporator (Rotavapor, Buchi Labortechnik, Switzerland) at 50 °C for 30 min to remove the organic solvent. Following this, the drug embedded lipid was collected and was melted in a glass vial at a temperature 5 °C above the melting point of the lipid. In a separate glass vial, the surfactants were dissolved in distilled water and heated to the same temperature. Following this, the molten lipid-containing drug was then poured into the hot surfactant solution and homogenized using an Ultra-Turrax IKA® T18 basic homogenizer (IKA, Campinas, Brazil) at 15,000 rpm, while maintaining the temperature above the lipid melting point, producing hot, coarse oil-in-water emulsion. This emulsion was immediately sonicated using a probe sonicator (Davidson & Hardy Ltd cooperating with Fisher Scientific, Leicestershire, UK) (an amplitude of 80% with 10 s pulse on and 5 s pulse off) to form the nanoemulsion. The nanoemulsion was then placed directly in an icebox to allow formation of the SLNs.
2.2.1.2. DEC-loaded SLNs and ABZ-OX-loaded SLNs. The double emulsion technique, as outlined previously [34], was applied with slight modifications in order to prepare DEC and ABZ-OX-loaded SLNs separately. Initially, DEC was dissolved in 0.25 mL water and ABZ-OX was dissolved in 0.2 mL of 0.5 M sodium hydroxide. The drug solution was emulsified in 1 mL of chloroform containing 100 mg of lipid and 25 mg of lecithin in the probe sonicator (at an amplitude of 80% with 10 s pulse on and 5 s pulse off) for 1 min, leading to a water-in-oil (W/O) emulsion. This emulsion was again emulsified in water containing stabilizer for DEC-loaded SLNs, and in water containing citric acid and stabilizer for ABZ-OX-loaded SLNs, in the probe sonicator to form a water-in-oil-in-water (W/O/W) emulsion. The SLNs were formed after solvent evaporation by stirring the double emulsion for 6 h at room temperature.

In this study, Geleol®, Precirol® ATO 5, Compritol® 888 ATO and stearic acid were all screened as lipid matrixes. Furthermore, PVA (9–10 kDa), PF127, Tween®80 and SLS were screened as stabilizers. The SLN formulations were optimized using a four-factor, five-level CCD with Design Expert Software version 11 (State-ease, Minneapolis, USA). The drug:lipid ratio, stabilizer concentration, stabilizer volume and sonication time were used as variable factors in the optimization process. Four responses were then recorded, namely particle size, PDI, zeta potential and encapsulation efficiency.

In an attempt to concentrate the SLN dispersions, the resultant formulations were centrifuged at 5000 x g for 30 min at 4 °C in an Amicon® Ultra Centrifugal Device (Millipore Inc, molecular weight cut-off (MWCO) of 12 kDa), resulting in concentrated SLNs. Prior to the lyophilization process, 2.5% w/v of PVP for DOX- and DEC-loaded SLNs and 1% w/v of PVP for ABZ-OX-loaded were added into the concentrated SLNs. These mixtures were pre-frozen at −80 °C for 2 h, then transferred to the freeze dryer for 26 h to produce dry powder particles.

2.2.2. Characterization of drug-loaded SLNs

The determinations of particle sizes, polydispersity index (PDI) and zeta potentials were carried out using a NanoBrook Omni particle sizer and zeta potential analyser (Brookhaven, New York, USA).

The determination of encapsulation efficiencies was carried out by quantifying the amount of the un-encapsulated drugs in each case. In the case of DOX and ABZ-OX, initially, due to their insolubility in water, the un-encapsulated, undissolved drugs were first separated out by centrifugation at 5000 rpm for 30 min at 4 °C. The pellets were then collected. The pellets were dissolved in methanol and diluted as required prior to analysis using the high-performance liquid chromatography (HPLC) method described in the Section 2.2.2.12. In addition to the un-encapsulated undissolved drug, some part of the un-encapsulated drug may well be solubilized in the water phase. Consequently, the amount of soluble un-encapsulated drug was determined by the ultrafiltration method using centrifugal filter tubes with an Amicon® Ultra Centrifugal Device (Millipore Inc, molecular weight cut-off (MWCO) of 12 kDa). Following this step, the amount of drug in the aqueous phase was measured by HPLC, as described in the analytical section. With respect to DEC, due to its high solubility in water, the SLN dispersion was directly placed into centrifugal filter tubes and processed, as described previously in DOX and ABZ-OX-loaded SLNs processes. Encapsulation efficiency of the drug (EE) was calculated using the Equation (1).

\[ EE(\%) = \frac{a - b}{a} \times 100\% \tag{1} \]

Where \( a \) is the amount of drugs added to the formulations and \( b \) is the amount of un-encapsulated drug.

The morphologies of the drug-loaded SLNs were observed using a transmission electron microscope (TEM) (JEM-1400Plus; JEOL, Tokyo, Japan). Fourier transform infrared (FTIR) studies of pure drugs and drug-loaded SLNs were conducted using a FTIR spectrometer (Accurac FT/IR-4100™ Series, Jasco, Essex, UK). Differential scanning calorimetry (DSC) studies of pure drugs, lipids and drug-loaded SLNs were conducted using a differential scanning calorimeter (DSC 2920, TA Instruments, Surry, UK).

2.2.3. In vitro drug release study and mathematical modelling

A dialysis method was used for investigating the in vitro release profile of DOX, DEC and ABZ-OX from SLNs [35,36]. Phosphate buffer saline (PBS) (pH 7.4) was used as a release medium for DOX and DEC-loaded SLNs. To achieve sink conditions, PBS:ethanol (70:30, v/v) was used in the specific case of ABZ-OX-loaded SLNs. The release profiles of pure drug and drug-loaded SLNs were then determined. The pure drug and SLNs dispersion were placed in Spectra-Por®, 12,000–14,000 MWCO dialysis membrane (Spectrum Medical Industries, CA, USA). The bags were suspended in 100 mL of release medium in an orbital shaker at 100 rpm and 37 °C. Aliquots of 1 mL of sample were taken at predetermined time intervals and replaced by 1 mL fresh release medium. To quantify the amount of drug released from SLNs, the samples were then analyzed using HPLC.

The cumulative drug released was fitted to different mathematical kinetic models, namely zero order, first order, Higuchi, Korsmeyer-Peppas and Hixson-Crowell [35,37]. The model parameters were calculated using DDsolver (China Pharmaceutical University, Nanjing, China) [38].

2.2.4. Fabrication of two-layered dissolving MNs containing drugs-loaded SLNs

Various MN formulations based different aqueous gels were prepared using a variety of biocompatible polymers at different concentrations. The various formulation compositions are outlined in Table 1. The preparation of MNs is illustrated schematically in Fig. 1. Firstly, the aqueous formulations of selected polymers were mixed with lyophilized SLNs until homogenous. Then, 100 mg of the blend was dispensed into the silicone MN moulds (needle density of 19 × 19, pyramidal needles; 500 μm height and 300 μm width at base and 300 μm interspacing). A pre-cast dry baseplate (with an area of 1 cm²), fabricated from aqueous gels of 15% w/w PVP (360 kDa) and 1.5% w/w glycerol was placed behind the MN formulations. Following this, the moulds were placed in a positive pressure chamber. To fill the cavity of the molds with the formulations, a pressure of 3–4 bar was applied for 15 min. Finally, the MNs were dried at room temperature for 24 h and were removed from the moulds. The morphology of the MNs was observed using a stereomicroscope (Leica Microsystems, Milton Keynes, UK) and scanning electron microscope (SEM) TM3030 (Hitachi, Krefeld, Germany). In addition to the MNs, needle-free patches were also prepared using the similar formulations. The aqueous blend (100 mg) was placed on the top of a flat silicone sheet. A pre-cast dry baseplate was then placed behind the formulations and these were allowed to dry for 24 h at room temperature.

2.2.5. Mechanical and insertion properties of MNs

A TA-XT2 Texture Analyser (Stable Microsystems, Haslemere, UK) was used in compression mode to evaluate the mechanical strength of the MNs.

<p>| Table 1 | Composition of the various formulations used to prepare MNs containing drug-loaded SLNs. |</p>
<table>
<thead>
<tr>
<th>Formulation</th>
<th>Composition (% w/w)</th>
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<tbody>
<tr>
<td></td>
<td>Lyophilized SLNs</td>
</tr>
<tr>
<td>A</td>
<td>20</td>
</tr>
<tr>
<td>B</td>
<td>30</td>
</tr>
<tr>
<td>C</td>
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<td>F</td>
<td>40</td>
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the MNs, as described previously [39–41]. To examine the insertion capability of the MNs, Parafilm®M was used as a skin simulant, as documented in a previous study [42]. In addition, an optical coherence tomography (OCT) microscope (Michelson Diagnostics Ltd, Kent, UK) was also utilized to examine the insertion capability of the MNs into both Parafilm®M and full-thickness neonatal porcine skin, collected less than 24 h post-mortem [41]. The imaging software, ImageJ® (National Institutes of Health, Bethesda, USA) was used to determine the height of needles inserted.

2.2.6. Evaluation of the effect of MN formulation on the size and PDI of SLNs

The dry optimized MN formulations containing drug-loaded SLNs were dispersed in water. Following this, the size and PDI of the drug-loaded SLNs were measured using DLS, and were compared to the size and PDI of lyophilized SLNs.

2.2.7. Calculation of theoretical drug content localized to the needles

Initially, the density of each formulation was calculated. To calculate the density of each formulation, flat baseplates were prepared, forming rectangular films. After drying, the dimensions and the mass of the films were determined. These dimensions were used to calculate the volume of the films using Eq. (2):

\[
\text{Volume of film} = \text{Width} \times \text{Length} \times \text{Height} \tag{2}
\]

The density of each formulation was determined using Eq. (3):

\[
\text{Density} = \frac{\text{Mass}}{\text{Volume}} \tag{3}
\]

To calculate the drug amount (mg) in the needle tips, Eq. (4) was used [43]:

\[
\text{Drug in the MN tips: } N \times \left(\frac{h}{a^2}\right) \rho [\text{drug}] \tag{4}
\]

Where N is the number of needle tips (361 tips), a² is the width of base of the tips (0.3² mm), h is the height of the tips (0.5 mm), ρ is the density of the dry formulation, [drug] is the concentration of drugs in dry formulation (mg drug/mg lyophilized SLNs).

2.2.8. In vitro dermatokinetic, skin distribution and deposition studies

These studies were carried out using modified Franz diffusion cells, as outlined in our previous publication [44], using full-thickness neonatal porcine skin. The skin was shaved and pre-equilibrated in PBS pH 7.4, for 30 min prior to commencement of the study and then attached to the Franz diffusion cells using cyanoacrylate glue. PBS (pH 7.4) was used in the receiver compartment. The receiver compartment was stirred at 600 rpm and was thermostatically maintained at 37 ± 1 °C. The donor compartment and sampling arm was sealed using Parafilm®M. The SLN loaded MNs were then inserted into the skin using manual force for 30 s and a cylindrical stainless steel weight of 5.0 g was placed on top to ensure the MNs remained in place for the duration of the experiment.

In the dermatokinetic studies, at predetermined time intervals (0.25 h, 0.5 h, 0.75 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h and 24 h), MNs were removed and the skin was washed three times with deionized water to remove any residual formulations from the surface of the skin. To collect the skin sections, a biopsy punch (5 mm diameter) (Stiefel, Middlesex, UK) was used. The skin was then placed into a 1.5 mL Eppendorf® tube and the tubes were placed in a water bath at 60 °C for 2–3 min. The epidermis layers were then manually detached from the dermis layers using forceps. To extract the drugs from the skin layers, 1 mL methanol was added into the separated layers and each mixture was homogenized for 10 min at 50 Hz, using a Tissue Lyser LT (Qiagen, Ltd, Manchester, UK). The samples were then centrifuged at 14,000 x g for 15 min. The supernatant was collected and analyzed using HPLC. The data obtained were then fitted to a one-compartment open model using PK Solver software (China Pharmaceutical University, Nanjing, China) [45]. The curve of drug concentration versus time was constructed. Finally, Cₘₐₓ, tₘₐₓ and AUC₀-2₄ were determined.

In skin distribution and deposition studies, the time points of 1 h, the dermis tₘₐₓ, determined from the data collected in the dermatokinetic study, and 24 h were selected. The samples were collected using a similar procedure to that outlined in the dermatokinetic study. Initially, the biopsied skin samples were fully immersed in optimal cutting temperature media and were flash frozen in liquid nitrogen. Following this, the frozen skin was sectioned horizontally into 200 μm sections using a Leica CM1900 Cryostat (Leica Microsystems, Nussloch, Germany). This process was performed until the entire skin sample had been sliced. To quantify the amount of drug in the skin sections, 300 μL of methanol was added and each sample was vortexed for 30 min to dissolve the drug. The samples were centrifuged at 14,000 x g for 15 min, supernatants were collected and these were analyzed using HPLC. A control, needle-free patch was used as a comparator in the dermatokinetic, skin distribution and deposition studies.

2.2.9. In vivo studies

2.2.9.1. Evaluation of the plasma and lymph pharmacokinetics, and biodistribution profiles of DOX, DEC and ABZ-OX

The in vivo pharmacokinetic and biodistribution studies were approved by the Committee of the Biological Services Unit, Queen’s University Belfast. The work was carried out under Project License PPL 2794 and Personal
Licenses PIL 1466 and 1890. All experiments were conducted based on the policy of the Federation of European Laboratory Animal Science Associations and the European Convention for the protection of Vertebrate Animals used for experimental and other scientific purposes, implementing of the principles of the 3Rs (replacement, reduction and refinement). Healthy, female Sprague–Dawley rats weighing in the range of 259.87 ± 19.34 g and aged between 10 and 13 weeks, were acclimatized to the laboratory conditions for 1 week prior to the commencement of the experiments. The animals were divided into two cohorts (n = 6 per cohort for the plasma pharmacokinetic study and n = 3 per cohort for the lymph pharmacokinetic and biodistribution study). The first cohort received 1 mL of an oral aqueous suspension in 0.5% w/v carboxymethylcellulose containing 0.8 mg of DOX, 1.1 mg of DEC and 0.94 mg of ABZ-OX. The animals in the second cohort were administered MNs containing drug loaded SLNs. MN patches were applied to the rats as detailed: 1 x MN arrays containing DOX-loaded SLNs; 2 x MN arrays containing DEC-loaded SLNs and 1 x MN array containing ABZ-OX-loaded SLNs. Both cohorts received to a dose corresponding to 3.2 mg/kg of DOX, 4.4 mg/kg of DEC and 3.75 mg/kg of ABZ-OX.

In the second cohort, rats were firstly sedated using gaseous anesthesia (2–4% isoflurane in oxygen) and the hair from their backs was removed using an electric hair clipper and Louis marcel hair removal cream (Sodalis Srl, Milano, Italy). MNs were then secured onto Microfoam® tape and were applied onto the backs of the rats for 30 s using firm finger pressure. To ensure the MN arrays remained securely in place, a small piece of Tegaderm™ (3 M, St Paul, Minnesota, USA), was placed on top of the MNs, and Micropore™ tape (3 M UK Plc, Bracknell, Berkshire, UK) was used to cover the back of the rats. For the plasma pharmacokinetics, blood samples were obtained via tail vein bleeds at predetermined time intervals: 1, 2, 3, 4, 6, 12, 24, 48 and 72 h with a maximum of 200 μL collected at each sampling time point into heparinized tubes. In the lymph pharmacokinetic and biodistribution study, 3 rats were sacrificed from each cohort after drug administration at predetermined time intervals, namely 1, 2, 4, 6, 12, 24, 48 and 72 h for the lymph pharmacokinetic study and 1, 2, 4, 6, 12 and 24 h for the biodistribution study. Following this, various organ samples, namely axillary, iliac and external lumbar lymph nodes, in addition to livers, kidneys and spleens were excised. An illustration of these axillary, iliac and external lumbar lymph nodes, in addition to livers, kidneys and spleens for both cohorts were then analyzed. An illustration of these axillary, iliac and external lumbar lymph nodes, in addition to livers, kidneys and spleens for both cohorts were then analyzed.

Fig. 2. Schematic representation showing the two treatment cohorts investigated in the in vivo experiment. Oral administration of 3.2 mg/kg of DOX, 4.4 mg/kg of DEC and 3.75 mg/kg of ABZ-OX (a). Intradermal application of four MN arrays (1 x MN array containing DOX-loaded SLNs; 2 x MN array containing DEC-loaded SLNs and 1 x MN array containing ABZ-OX-loaded SLNs) to the hairless back of the rats (b). The drug concentrations in plasma, lymph nodes, livers, kidneys and spleens for both cohorts were then analyzed.

2.2.9.2. Blood and organ samples handling. Blood and organs were processed as described previously [46]. The blood obtained was centrifuged at 3000 x g for 10 min at 4 °C. The plasma was then collected in the supernatants. Harvested organ samples were washed with PBS, pH 7.4 solution and blotted with paper towels before being stored at −80 °C until processing.

2.2.9.3. Sample preparation and analyte extraction. For plasma samples, DOX, DEC, ABZ-OX and (ABZ-ON, a metabolite of ABZ-OX) were extracted using methanol with a one-step protein precipitation method [46]. Methanol (500 μL) was added to an aliquot of 100 μL rat plasma and vortexed for 10 min in a 1.5 mL centrifuge tube. The samples were then centrifuged at 14,000 g at 4 °C for 10 min. The supernatant was removed and placed in a glass culture tube. Subsequently, the methanol was removed under a stream of nitrogen at 35 °C for 30 min using a Zymark TurboVap® LV Evaporator Workstation (McKinley Scientific, Sparta, NJ, USA). The extract was reconstituted in 100 μL mobile phase and this solution was then vortex mixed for 30 s, sonicated for 15 min and centrifuged at 14,000 × g for 10 min at room temperature. The supernatant was collected into an Agilent® HPLC vial and analyzed using HPLC- mass spectrometer.

For organ samples, to 100 mg of organ, methanol (500 μL) was added and the mixture was homogenized at 50 Hz using a Tissue Lyser LT (Qiagen, Ltd, Manchester, UK) for 10 min. The organ homogenates were centrifuged at 14,000 × g for 10 min and were then subjected to the same extraction process outlined above for the plasma samples.

2.2.10. Calculation of pharmacokinetic parameters, relative bioavailability and efficiency of intradermal lymphatic-targeted delivery

Non-compartmental pharmacokinetic analysis of the plasma and lymph concentration profiles was carried out using PK Solver [45]. The curve of drug concentration versus time profiles was created in each case. The maximum drug concentration (Cmax), the time of maximum concentration (tmax), the drug concentration time curve from time zero (t = 0) to the last experimental time point (t = 72 h) (AUC0-72), the drug concentration time curve from time zero (t = 0) to infinity (AUC0-∞), the mean half-life (t1/2) and the mean residence time (MRT) were all calculated.

The relative plasma and lymphatic bioavailabilities (F) of DOX, DEC and ABZ-OX after intradermal delivery of SLNs-loaded MNs, compared to oral administration, were calculated using Eq. (5). The percentage of drug targeting efficiency (DTE) and the direct transport percentage...
(DTP) of lymphatic were calculated using Eqs. (6) and (7) \([47,48]\), respectively.

\[
F = \frac{AUC_{\text{MN}} \times dose_{\text{oral}}}{AUC_{\text{oral}} \times dose_{\text{MN}}} \times 100\% \\
(5)
\]

\[
DTE = \frac{AUC_{\text{MN}} \text{ (lymphatic)} / AUC_{\text{MN}} \text{ (plasma)}}{AUC_{\text{oral}} \text{ (lymphatic)/AUC_{\text{oral}} \text{ (plasma)}}} \\
(6)
\]

\[
DTP = \frac{AUC_{\text{MN}} \text{ (lymphatic)} - AUC_{\text{oral}} \text{(lymphatic) \times AUC_{\text{MN}} \text{ (plasma)}}}{AUC_{\text{MN}} \text{ (lymphatic)}} \times 100\% \\
(7)
\]

Where \(AUC_{\text{MN}}\) is the AUC of plasma or lymphatics from SLNs-loaded MNs administration and \(AUC_{\text{oral}}\) is the AUC of plasma or lymphatic from oral administration of free drugs.

### 2.2.11. Instrumentation and chromatographic condition for analytical method

The concentrations of DOX, DEC and ABZ-OX in all in vitro samples were determined via HPLC (Agilent Technologies 1220 Infinity UK Ltd, Stockport, UK). The separations were carried out using a Phenomenex Luna C18 (ODS1) column \((150 \text{ mm} \times 4.6 \text{ mm i.d. with } 5 \mu \text{m particle size}),\) with the flow rate, the mobile phase and UV detector as presented in Table 2. The injection volume was 25 μL and the analyses were performed at room temperature. The Agilent ChemStation® Software B.02.01 was used to analyze the chromatograms. All analytical methods were validated as per the International Committee on Harmonisation (ICH) 2005.

The analysis of DOX, DEC, ABZ-OX and ABZ-ON in in vivo studies were carried out using series HPLC instrument (Agilent Technologies 1260 Infinity II UK Ltd, Stockport, UK), coupled to a single quadrupole API 6400 mass spectrometer (Agilent Technologies, UK) with electrospray ionization in positive mode \([46]\). The chromatographic separations were performed using an Xselsect CSH™ C18 column \((3.0 \times 150 \text{ mm})\) with particle sizes of 3.5 μm. The detection was performed using single ion monitoring mode, with DOX at m/z of 445.4, DEC at m/z of 200.2, ABZ-OX at m/z of 282.3 and ABZ-ON at m/z of 298.3. A combination of 0.1% v/v formic acid in water (mobile phase 1) and methanol (MeOH) (mobile phase 2) was used as the mobile phase. A gradient condition over 20 min was used to separate the compounds, as detailed in Table 3. The column temperature was maintained at 30 °C and the injection volume for all samples was 10 μL. The HPLC-MS method was validated as per the International Committee on Harmonisation (ICH) 2005 and the US FDA guidelines (2018).

### 2.2.12. Statistical analysis

All data were presented as means ± standard deviation (SD) of the mean. The SD calculation was performed using Microsoft Excel® 2016 (Microsoft Corporation, Redmond, USA). Statistical analysis was carried out using GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA). Where appropriate, an unpaired t-test was performed for comparison of two groups. The Kruskal-Wallis test with post-hoc Dunn's test was performed for comparison of multiple groups. In all cases, \(p < 0.05\) was denoted as a significant difference.

<table>
<thead>
<tr>
<th>Table 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gradient conditions for HPLC-MS mobile phase.</td>
</tr>
<tr>
<td>Time (min)</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>7.5</td>
</tr>
<tr>
<td>14</td>
</tr>
</tbody>
</table>

### 3. Results and discussion

#### 3.1. Preparation and optimization of drug-loaded SLNs

Selected lipids (Geleol®, Precirol® ATO 5, Compritol® 888 ATO and stearic acid) and stabilizer (PF127, PVA (9–10 kDa), SLS and Tween®80) were screened to prepare drug loaded SLNs. In our preliminary study, in the case of all drug-loaded SLNs formulations, SLNs prepared from Geleol® as the lipid component and Tween®80 as the stabilizer, produced the SLNs with the smallest particle size and PDI, in comparison with all other formulations (Figs. S1, S2 and S3). The difference in particle size of all formulations may possibly be caused by the differences in the melting points (MPs) of lipids used and the hydrophilic-lipophilic balance (HLB) value of the stabilizers. The higher MP of lipids results in the higher viscosity of the dispersion medium. Therefore, the efficiency of the homogenization and sonication step in decreasing particle size of melted lipid droplets in the water phase is reduced \([49,50]\). The MPs of Geleol®, Precirol®ATO 5, Compritol®888 ATO and stearic acid are ~55 °C, ~57 °C, 69–74 °C and ~69.3 °C, respectively. Amongst the lipids used, Geleol® has the lowest MP. Therefore, this led to the lowest viscosity in the medium, resulting in the increase of the homogenization and sonication efficiency to produce small particles. In the case of the HLB value of the stabilizer used in SLN formations, the values between 12–16 are considered to be effective to produce a stable oil-in-water (O/W) emulsion \([35]\). The HLB values of the stabilizers are > 24, 18, 44 and 15 for PF127, PVA, SLS and Tween®80, respectively. Accordingly, due to the effective HLB value, the SLNs prepared from Tween®80 produced the smallest particle size, compared to other stabilizers. In addition to this, with respect to the zeta potential measurement, despite the use of Tween®80, PF127 and PVA as non-ionic stabilizers, all formulations exhibited negative charges. These negative charges may be attributed to the anionic nature of the lipid [51].

The amount of drug encapsulated into the SLNs depends on the types of lipid used, as well as the chemical characteristics of the drugs. In our preliminary study, the SLNs prepared from Geleol® as the lipid and Tween®80 as the stabilizer produced the highest encapsulation efficiency compared to other formulations (Figs. S1, S2 and S3). This might be attributed to hydrogen bond formation between two hydrogen bond donor functional groups (hydroxyl group of glyceryls) in Geleol® and hydrogen bond acceptor functional groups of DOX, DEC and ABZ-OX which may form a molecular complex leading to the high solubility of drugs in Geleol® [51]. Consequently, this could improve the amount of drugs encapsulated in the lipid core.

Based on the results obtained in the screening process, Geleol® and Tween®80 were selected to be carried through into the optimization processes. CCD was used to optimize the formulation of drug loaded SLNs. The representative 3D graphs describing the effect of selected parameters on the particle sizes, PDI, zeta potential and encapsulation...
efficiency are shown in Fig. 3. In the optimization process, the selection requirements for the determination of the optimum formula eliciting minimum particle size, minimum PDI, optimal zeta potential and maximal entrapment efficiency of drug loaded SLNs. The solutions generated by the software were ranked based on the desirability factor. Optimal formulations for DOX, DEC and ABZ-OX were chosen, and the SLNs were produced in three batches. Table 4 presents the optimized formulation, the predicted and actual values of particle sizes, PDI, zeta
potential and encapsulation efficiencies of drug-loaded SLNs. It was found that the bias value was ±15%, implying that this was a successful optimization process.

3.2. Characterizations of drug-loaded SLNs

Fig. 4a–c shows the morphologies of the optimized drug-loaded SLNs observed by TEM. The micrographs illustrated that DOX, DEC, and ABZ-OX-loaded SLNs all exhibited spherical shapes. The size of these SLNs obtained from TEM was in good agreement with those of the particle size analyzer results, which were ~100 nm.

Fig. 4d depicts the FTIR spectra of DOX, DEC, and ABZ-OX and their respective SLN formulations. The specific functional groups of all drugs in FTIR spectra are detailed in Table 5. The results showed that the characteristic groups of DOX, DEC, and ABZ-OX were also found in FTIR spectra of drug-loaded SLNs formulations. Therefore, there were no chemical interactions between any of the excipients used and DOX, DEC or ABZ-OX.

The thermogram profiles (Fig. 4e) exhibited sharp endothermic peaks at 168 °C in DOX, 138 °C in DEC, and 226 °C in ABZ-OX due to the melting points of the drug crystals. The thermogram of Geleol® revealed a sharp peak at 55 °C representing the melting point of the lipid. However, this peak shifted to 51–53 °C in lyophilized SLNs. This may be attributed to the small size (nanometer) and dispersed form of the lipid in the formulation, as well as the presence of other excipients in the formulation [35]. Moreover, the peak of DOX, DEC, and ABZ-OX were not observed in SLNs, indicating the possible change in the physical state of the entrapped drug from crystalline to the amorphous state [50].

3.3. In vitro drug release and mathematical modelling

The in vitro release profiles of DOX, DEC, and ABZ-OX from their SLN formulations, compared to the pure drugs, are summarized in the cumulative percentage release presented in Fig. 4f–h. This study was carried out over 48 h. Dynamic dialysis was selected for separation of free drugs from drug-loaded SLNs. This method has been routinely used in other studies investigating the release profiles of drugs from SLNs [52–54]. The results revealed that the incorporation of the drugs into SLNs led to sustained release patterns of all drugs. In the first 2 h, 11.28 ± 2.7%, 12.15 ± 3.28%, and 9.39 ± 1.78% drugs were released from SLNs of DOX, DEC, and ABZ-OX, respectively. After 48 h, a sustained, slower release of drugs was observed, with 72.18 ± 6.81%, 84.34 ± 8.22%, and 65.35 ± 12.4% of drugs released from SLNs in the case of DOX, DEC, and ABZ-OX, respectively. In contrast to drug-loaded SLNs, pure drugs showed significantly faster and higher release (p < 0.05 in each case) with 98.62 ± 9.24% of DOX and 89.53 ± 8.8% of ABZ-OX released after 48 h. Furthermore, due to its high hydrophilicity, 99.45 ± 9.87% of pure DEC was released within only 3 h. The faster release behavior of the drugs from the SLNs in the initial 2 h might be due to the presence of a small amount of drugs coating the surface of lipid shells [55,56]. This small amount might diffuse into the release medium, resulting in higher release of the drugs [57]. Following the slightly faster release, the slower and sustained release was obtained due to the slow diffusion of drugs from the lipid core [56,58]. Accordingly, the slow release of the drugs from SLNs may lead to lymphatic uptake of the SLNs before release of incorporated drugs into the skin interstitial fluid for subsequent systemic circulation.

In order to investigate the kinetic modeling and release mechanism of drugs from SLNs, the cumulative drug release data were fitted to various kinetic models. Table 6 presents the results of kinetic modeling profiles of all formulations. The most appropriate release model was chosen based on the value of the correlation coefficient of each model. This study showed that the release profiles of all drugs were best fitted with Higuchi and Korsmeyer-Peppas diffusion models. Moreover, the n values were in the range of 0.45–0.89. Accordingly, the release mechanism of drugs from SLNs was non-Fickian (anomalous diffusion). This model describes that the drug release from the formulation matrix depends on the drug diffusion from the matrix, as well as the erosion and the degradation of the lipid core [37,59,60].

3.4. Fabrication of two-layered dissolving MNs containing drug-loaded SLNs

Aqueous blend of different biocompatible polymers was used either alone or in combination to prepare the dissolving MN formulation. In addition, different concentrations of lyophilized SLNs were also utilized to achieve the highest potential drug loading for the system. In this study, PVA (31–50 kDa) and PVP (58 kDa) were selected as the polymers, as indicated in Table 1. In addition, the two-layered MN arrays were prepared. The fabrication of two-layered MN arrays shows numerous benefits. In our preliminary study, MN arrays formulated containing drugs in the needles and baseplates exhibit insufficient mechanical properties, indicated by cracking of the baseplate after mechanical evaluation (data not shown). Therefore, a different baseplate was prepared to support the needles of MN arrays. Additionally, the localization of the drugs into the needles only results in a reduction in drug wastage. Specifically, this approach will ultimately be favorable in scale-up manufacturing processes [61]. In the present study, the drug-loaded SLNs were localized in the MNs needles and the MNs needles were supported by a pre-cast dry baseplate prepared from 15% w/w PVP (360 kDa) and 1.5% w/w glycerol, thus forming a two-layered dissolving MNs. The dry baseplates were chosen to avoid back diffusion of drugs into the baseplate. Our research group has applied this
The results showed that all MNs prepared exhibited homogenous polymer blends with the resultant MNs having sharp needle tips. The morphology of all MNs examined by stereomicroscope are displayed in Fig. 5a–c. Moreover, representative SEM of images of Formulation E (30% w/w of lyophilized SLNs, 15% w/w of PVA and 5% of PVP) are presented in Fig. 5d–f.

Table 5
The distinctive FTIR spectra of DOX, DEC and ABZ-OX.

<table>
<thead>
<tr>
<th>Wavenumber (cm⁻¹)</th>
<th>Functional Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1397</td>
<td>C=O stretching</td>
</tr>
<tr>
<td>1527</td>
<td>Aromatic N–H bending</td>
</tr>
<tr>
<td>1682</td>
<td>C=C stretching</td>
</tr>
<tr>
<td>2917</td>
<td>C–H stretching</td>
</tr>
<tr>
<td>3308</td>
<td>primary N–H group</td>
</tr>
<tr>
<td>3428</td>
<td>primary O–H group</td>
</tr>
<tr>
<td>1265</td>
<td>C–N aromatic stretching</td>
</tr>
<tr>
<td>1407</td>
<td>C–C stretching</td>
</tr>
<tr>
<td>1625</td>
<td>C=O stretching</td>
</tr>
<tr>
<td>3050</td>
<td>C–H stretching</td>
</tr>
<tr>
<td>1027</td>
<td>S=O bonding</td>
</tr>
<tr>
<td>1519</td>
<td>C=N stretching</td>
</tr>
<tr>
<td>1729</td>
<td>–COOH bending</td>
</tr>
<tr>
<td>2972</td>
<td>C–H stretching</td>
</tr>
</tbody>
</table>

Table 6
Kinetic modelling of DOX, DEC and ABZ-OX release from SLN formulations.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>r value of kinetic model</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zero Order</td>
<td>First Order</td>
</tr>
<tr>
<td>DOX SLN</td>
<td>0.8326</td>
<td>0.9044</td>
</tr>
<tr>
<td>DEC SLN</td>
<td>0.8426</td>
<td>0.9262</td>
</tr>
<tr>
<td>ABZ-OX SLN</td>
<td>0.7422</td>
<td>0.8620</td>
</tr>
</tbody>
</table>

preparation method in several studies [41,43,44]. The results showed that all MNs prepared exhibited homogenous polymer blends with the resultant MNs having sharp needle tips. The morphology of all MNs examined by stereomicroscope are displayed in Fig. 5a–c. Moreover, representative SEM of images of Formulation E (30% w/w of lyophilized SLNs, 15% w/w of PVA and 5% of PVP) are presented in Fig. 5d–f.
3.5. Mechanical and insertion properties of MNs

Mechanical strength evaluation has been carried out to determine the capability of MN arrays to resist compression force. The ability of MNs to be well-inserted in the skin is extremely important to its application, because the MNs must penetrate the stratum corneum in order to deliver their payload. The mechanical strength of MN is determined based upon the percentage height reduction of the needles on the arrays after the application of a force of 32 N/array, equitable to manual compression force [42]. The results of this experiment are presented in Fig. 5g. MN prepared using formulations A and B, containing only PVP as a polymer, exhibited the highest needle height reductions (> 30%), representing poor mechanical properties. It has previously been reported that films prepared from PVP alone exhibit poor mechanical strength [62,63]. Formulations C and D, containing only PVA as a polymer, showed needle height reductions of > 10%. MN prepared from Formulation E (30% w/w of lyophilized SLNs; 15% w/w of PVA; 5% w/w of PVP) exhibited height reductions of < 10%, indicating adequate mechanical strength. There was a statistically significant difference (p < 0.05) in the percentage reduction of the MN needle heights for MN prepared using Formulation E, compared to all other formulations. The combination of PVP and PVA in the
formulation could potentially increase the mechanical properties of the formulation, due to the hydrogen-bond interactions between −OH groups of PVA and C−O groups of PVP [63]. Furthermore, the increase of drug loading to 40% w/w (Formulation F) led to a decrease in MN mechanical strength, indicating that the drug loading affected the mechanical properties of MNs. Importantly, it was observed that the baseplate employed to support the MNs did not exhibit any change after compression, implying that the baseplate possessed adequate mechanical strength.

Parafilm®M was used as a validated artificial skin simulant for MN insertion studies, as previously described [42]. The results of the insertion studies were in good agreement with the mechanical strength studies. The insertion capabilities of the MNs prepared using each of six different formulations (A–F) are illustrated in Fig. 6a–f. The results showed that, in the case of each of the drugs, Formulation E inserted into the third layer of Parafilm®M. The mean thickness of a Parafilm®M layer is 126 ± 7 μm, indicating that MNs were inserted up to 378 μm of the total 500 μm height, approximately 75.6% of MN needle. These values was similar to previous studies focused on the insertion of other polymeric MNs into human skin [64]. In contrast, other formulations only penetrated the second layer of Parafilm®M. Leading on from the results of the mechanical and insertion studies, in the case of each of the three drugs under investigation, Formulation E was selected for use in all subsequent experimental evaluations. To visualize the insertion profiles of the MNs, an optical coherence tomography (OCT) was employed using the Parafilm®M skin simulant and full-thickness porcine skin. Previous studies have shown the utility of this technique in examining the insertion capabilities of MN arrays [41,43,64,65]. Fig. 6g–i illustrate the insertion profiles of MNs containing DOX-loaded SLNs (a and b), DEC-loaded SLNs (c and d) and ABZ-OX-loaded SLNs (e and f) following insertion into Parafilm®M film. Representative OCT images of MNs containing DOX-loaded SLNs (g), DEC-loaded SLNs (h) and ABZ-OX-loaded SLNs (i) following insertion into full-thickness porcine skin. The white scale bar represents a length of 1 mm in each case.
3.7. Calculation of theoretical drug content localized to the needles

Prior to the calculation of drug content in the needles, the densities of the formulations were determined. The densities of the formulations were found to be $1.16 \pm 0.14$ mg/mm$^3$, $1.21 \pm 0.12$ mg/mm$^3$, and $1.28 \pm 0.16$ mg/mm$^3$ for DOX, DEC, and ABZ-OX formulations. Upon drying, the amount of drug located in each array was found to be $0.84 \pm 0.02$ mg for DOX, $0.55 \pm 0.001$ mg for DEC, and $0.94 \pm 0.03$ mg for ABZ-OX. These values were subsequently used as the drug dosage in one MN array in in vitro and in vivo studies.

3.8. In vitro dermatokinetic, skin distribution and deposition studies

The primary aim of this study was to deliver the drug-loaded SLNs into the dermis layer, where the initial lymphatic capillaries reside. Therefore, it was crucial to evaluate the kinetic profile of drugs after the MN application to the skin in epidermis and dermis. In order to achieve this, a dermatokinetic study was designed and carried out. Numerous published investigations have been carried out to examine dermatokinetic profiles of a variety of drug delivery systems [67,68]. In the present study, it was found that the drug concentration in the epidermis and dermis layer of the skin after MN application was significantly higher ($p < 0.05$) than those after needle-free patch application. To elaborate on the mechanisms at play, when MNs penetrate the skin, the interstitial fluid is absorbed, hydrating the polymers. Consequently, the hydration and dissolution of the polymers liberates the drug particles, which can then diffuse into the deeper layers of skin. The data presented in Fig. 7a–f illustrate the comparison of epidermal and dermal drug profiles following MN application, as compared to needle-free patch application. The dermatokinetic parameters of DOX, DEC and ABZ-OX in the epidermis and dermis, following application of MNs containing drug-loaded SLNs, in comparison with needle-free patches containing drug-loaded SLNs in the full thickness porcine skin are presented in Tables S1 and S2. The results of the dermatokinetic profiles of DOX-loaded SLNs exhibited that the mean peak drug concentration ($C_{\text{max}}$) in the epidermis and dermis were $192.44 \pm 22.12$ μg/cm$^3$ and $526.56 \pm 59.05$ μg/cm$^3$, respectively. The time of $C_{\text{max}}$ ($t_{\text{max}}$) was observed after $1.52 \pm 0.19$ h in the epidermis and $8.96 \pm 0.92$ h in the dermis after MN application. The area under curve (AUC$_{0-24}$) of DOX in the epidermis and dermis were $2678.77 \pm 273.43$ h.μg/cm$^3$ and $9908.84 \pm 895.87$ h.μg/cm$^3$, respectively. With regards to DEC-loaded SLNs, after MNs application, the $t_{\text{max}}$ was found to be $0.88 \pm 0.09$ h with a concentration of $179.015 \pm 18.32$ μg/cm$^3$. In the dermis layer, $C_{\text{max}}$ was found to be $270.80 \pm 31.04$ μg/cm$^3$ after $3.72 \pm 0.41$ h. The AUC$_{0-24}$ in epidermis and dermis were $1650.77 \pm 181.54$ h.μg/cm$^3$ and $5136.78 \pm 528.82$ h.μg/cm$^3$, respectively. After the administration of MNs containing ABZ-OX-loaded SLNs, $137.16 \pm 14.37$ μg/cm$^3$ was found as the maximum concentration within $1.53 \pm 0.21$ h in the epidermis. The $C_{\text{max}}$ in dermis was $461.97 \pm 37.72$ μg/cm$^3$ within $10.37 \pm 1.43$ h. Furthermore, the AUC$_{0-24}$ of $2463.77 \pm 301.98$ h.μg/cm$^3$ and $9155.15 \pm 953.11$ h.μg/cm$^3$ were found in epidermis and dermis, respectively. Analyzed statistically, the values of $C_{\text{max}}$ and AUC$_{0-24}$ in dermis were found to be significantly higher ($p < 0.05$ each) than in epidermis. Importantly, in all cases, apart from $t_{\text{max}}$, the values of all dermatokinetic parameters in epidermis and dermis of drugs after SLNs loaded MNs application were significantly higher ($p < 0.05$) than needle-free patch application. Bearing in mind the drug amount in MN tips, $46.01 \pm 6.37\%$, $43.05 \pm 5.98\%$ and $58.99 \pm 7.32\%$ of DOX, DEC and ABZ-OX were retained in the dermis after 24 h administration. The results obtained here showed that the combination of drug-loaded SLNs with dissolving MN arrays significantly improved the delivery of those drug particles into the skin, resulting in the retention of the drug particles in the dermis layer, a place replete with lymphatic capillaries [59].

The skin distribution of all drugs in full-thickness porcine skin were then examined. The amount of DOX, DEC and ABZ-OX quantified per cm$^2$ in a range of different depths of skin and after different application times, i.e., 1 h, $t_{\text{max}}$ of dermis dermatokinetic profiles and 24 h, are illustrated in Fig. 7g–i. Drugs were distributed in the tissues up to a depth of 1.5 mm. The results revealed that the drugs were detected in the deeper layers of the skin than the height of MNs (500 μm), indicating the movement of drug particles in the skin. With reference to DOX distribution specifically, drug concentration reached a peak at a depth of 0.9 mm with a concentration of $146.97 \pm 26.6\mu$g/cm$^3$ after 8.9 h. In relation to DEC distribution, after 3.7 h, the drug concentration reached a maximum concentration ($86.90 \pm 11.9\mu$g/cm$^3$) at a depth of 0.7 mm. While in the case of ABZ-OX distribution, the maximum concentration was found at a depth of 0.7 mm after 10.4 h ($146.17 \pm 34.34\mu$g/cm$^3$). On the other hand, negligible in-skin drug concentrations in the case of needle-free patch containing drug-loaded SLNs were found, demonstrating the poor in-skin drug distribution after its administration. The excellent drugs distribution following intradermal administration using the combination of drug-loaded SLN and MNs could hypothetically result in the high possibility of the particles to be taken up by the lymphatic capillaries in dermis layer, leading to high drug concentrations in the lymphatic systems.

3.9. In vivo studies

3.9.1. Evaluation of the plasma and lymph pharmacokinetics profile of DOX, DEC, ABZ-OX and ABZ-ON

This study was designed as proof of concept for the development of a new drug delivery system to enhance the delivery of antifilarial drugs to the lymphatic systems. In this study, we investigated the plasma and lymphatic pharmacokinetic profiles of the three aforementioned drugs after intradermal administration of drug-loaded SLNs using MN arrays. We compared these results with oral administration of the drugs, the conventional administration route for LF treatment. It has

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Lyophilized SLNs</th>
<th>SLNs loaded MNs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DOX</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Particle size (nm)</td>
<td>98.97 ± 8.69</td>
<td>99.05 ± 7.43</td>
</tr>
<tr>
<td>PDI</td>
<td>0.312 ± 0.02</td>
<td>0.319 ± 0.01</td>
</tr>
<tr>
<td><strong>DEC</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Particle size (nm)</td>
<td>96.92 ± 8.81</td>
<td>98.18 ± 7.29</td>
</tr>
<tr>
<td>PDI</td>
<td>0.264 ± 0.03</td>
<td>0.282 ± 0.02</td>
</tr>
<tr>
<td><strong>ABZ-OX</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Particle size (nm)</td>
<td>95.25 ± 9.26</td>
<td>98.83 ± 8.19</td>
</tr>
<tr>
<td>PDI</td>
<td>0.273 ± 0.02</td>
<td>0.281 ± 0.02</td>
</tr>
</tbody>
</table>

Table 7

The comparison of the particle size and PDI of SLNs in lyophilized form and MN formulations (means ± SD, n = 3).
been reported that ABZ-OX is metabolized to ABZ-ON \[70\]. Therefore, the plasma and lymphatic profiles of ABZ-ON were also studied. This is the first study to use the combination of SLNs with dissolving MNs for facilitated delivery of drugs. Furthermore, this study is the first to investigate the plasma and lymphatic pharmacokinetics of drug uptake, as well as organ biodistribution of drug-loaded SLNs post-intradermal administration via MNs.

In the in vivo study, after 24 h, MNs were removed from the rats and were found to have completely dissolved. Importantly, there were no indications of skin irritation on any of the rats, post-MN removal. All pharmacokinetic profiles of DOX, DEC, ABZ-OX and ABZ-ON after oral and MN-SLNs administration in SD rats are shown in Table S3. The mean plasma concentration and time profiles after oral and MN-SLNs administration of DOX, DEC, ABZ-OX and ABZ-ON are depicted in Fig. 8a–d. With respect to DOX pharmacokinetics in plasma, the $C_{\text{max}}$ was found after 2 h with a concentration of 1.61 ± 0.22 μg/mL after oral administration. In contrast, a $C_{\text{max}}$ of 0.49 ± 0.14 μg/mL was determined 6 h post-MN application. With regards to DEC pharmacokinetics in plasma, the $C_{\text{max}}$ of 1.85 ± 0.39 μg/mL within 3 h and 0.91 ± 0.34 μg/mL within 6 h were found after oral and MN administrations, respectively. In terms of ABZ-OX and ABZ-ON plasma profiles, after oral dosing, the $C_{\text{max}}$ of ABZ-OX and ABZ-ON were found to be 1.27 ± 0.20 μg/mL and 0.09 ± 0.01 μg/mL, respectively. The $t_{\text{max}}$ of ABZ-OX and ABZ-ON were 2 h and 3 h, respectively. After MNs dosing, the $C_{\text{max}}$ of ABZ-OX was 0.43 ± 0.11 μg/mL after 6 h and the $C_{\text{max}}$ of ABZ-ON was 0.03 ± 0.02 μg/mL after 12 h. Additionally, relative bioavailability of DOX, DEC and ABZ-OX DOX plasma after the administration of MNs containing drug-loaded SLNs compared to the oral administration of pure drugs were calculated. The relative bioavailability of these drugs (Fig. 8e) were found to be 150.43 ± 24%, 107.66 ± 19.03% and 111.79 ± 22.58% for DOX, DEC and ABZ-OX, respectively. The results exhibited that the relative bioavailability were all more than 100%, indicating higher bioavailability of these drugs following application of SLNs-loaded MNs in comparison with the oral administration of pure drugs.

Analyzed statistically, the $C_{\text{max}}$ of all drugs after oral dosing were significantly higher ($p < 0.05$) than the $C_{\text{max}}$ post-MN administration. Despite the lower $C_{\text{max}}$ in the MNs cohort, $AUC_{0-72}$, the $t_{\text{1/2}}$, MRT and relative bioavailability of DOX, DEC and ABZ-OX DOX plasma after the administration of MNs containing drug-loaded SLNs compared to the oral administration of pure drugs were determined to be higher than the same values post-oral administration. This may be because the drugs were initially absorbed by the lymphatic system before later reaching the systemic circulation \[16\]. Additionally, the SLN formulations may have sustained the release of the drugs, resulting in the longer $t_{\text{max}}$. It has previously been reported that drugs administered intradermally using MNs result in higher $AUC_{0-72}$, compared to the same agents administrated orally \[71\]. In addition, the $AUC_{0-72}$ and MRT of drug-loaded SLNs were found to be significantly higher, compared to free drug when administrated subcutaneously \[71\]. Therefore, it was not surprising that the combination of drug-loaded SLNs administrated intradermally exhibited higher $AUC_{0-72}$, MRT and relative
bioavailability when compared with oral administration of free drugs. The high AUC\(_{0-72}\) and relative bioavailability of the drugs in the blood and lymph offer two advantages. The combination of MNs and SLNs could be utilized to deliver the drugs efficiently to the bloodstream, where the microfilariae reside; while also targeting drug to the lymph nodes, where filarial nematodes reside in infected patients.

In the lymphatic pharmacokinetic study, three lymph nodes were extracted, namely axillary, iliac and external lumbar nodes. The mean lymph concentration of drugs in axillary, iliac and external lumbar nodes are depicted in Figs. S5 and S6. Following MNs administration, the concentration of all drugs in iliac nodes were significantly higher (\(p < 0.05\)) compared to other two lymph nodes. The axillary lymph nodes are somatic nodes, draining from the skin and underlying musculature \([43]\). Therefore, the concentration of drugs in these lymph nodes post MNs application were higher than the concentration of drugs in iliac and external lumbar nodes, which are visceral nodes, draining from the thoracic, abdominal and pelvic organs. The mean drug concentration in all lymph nodes and time profiles after oral and MN-SLNs administration of DOX, DEC, ABZ-OX and ABZ-ON are shown in Fig. 9a–d. All lymphatic pharmacokinetics profiles of DOX, DEC, ABZ-OX and ABZ-ON after oral and MN-SLNs administration are presented in Table S4. With respect to lymphatic pharmacokinetics, interestingly, the C\(_{\text{max}}\) of DOX, DEC and ABZ-OX after MNs dosing were significantly greater (\(p = 0.0232\)) than the C\(_{\text{max}}\) after oral administration. In contrast, due to avoidance of first-pass metabolism upon intradermal administration, the C\(_{\text{max}}\) of ABZ-ON was significantly lower (\(p = 0.0184\)) than the value after oral dosing. The C\(_{\text{max}}\) of DOX, DEC, ABZ-OX and ABZ-ON after oral dosing were determined to be 0.17 ± 0.03 μg/g, 0.15 ± 0.06 μg/g, 0.07 ± 0.03 μg/g and 0.02 ± 0.01 μg/g, respectively. The C\(_{\text{max}}\) was found after 2 h for DOX and ABZ-ON, and 4 h for DEC and ABZ-ON. In terms of the lymphatic pharmacokinetic profile after MNs administration, the C\(_{\text{max}}\) of DOX was 0.67 ± 0.22 μg/mL, DEC was 0.61 ± 0.20 μg/mL, ABZ-OX was 0.54 ± 0.20 μg/mL and ABZ-ON was 0.007 ± 0.002 μg/mL. Moreover, similar t\(_{\text{max}}\) Values were found in the case of the DOX, DEC and ABZ-OX profiles, which were 4 h. The t\(_{\text{max}}\) of ABZ-ON was 6 h. In addition, the relative bioavailability of DOX, DEC and ABZ-OX in (Fig. 9e) the lymphatics were 639.51 ± 98.43%, 654.46 ± 103.43% and 1340.42 ± 226.65%, respectively, demonstrating that the bioavailability of these drugs following application of SLNs-loaded MNs were higher compared to the oral administration of pure drugs.

The results obtained in this lymphatic pharmacokinetic study...
revealed that the formulation of antifilarisis drugs into SLNs and administered intradermally using MNs led to significantly higher drug concentration in the lymphatic system, the residence site of filarial nematodes, resulting in higher AUC and MRT. The AUC$_{0-72}$ and MRT values of drug-loaded SLNs administered via MNs were significantly greater ($p < 0.05$) than oral administration of free drugs. In this study, interestingly, DOX, DEC and ABZ-OX were detected until 72 h after MNs administration. In contrast, after oral dosing, the drugs were only detected until 24 h after administration. It was previously reported that, following oral administration, drugs must have log $P > 4.7$ to enter the lymphatic [8]. However, DOX, DEC and ABZ-OX all have a log $P$ values of $< 4.7$. Accordingly, it was expected that, following oral administration, the concentration and retention time of drugs in the lymphatics was lower than following the MN administration. After MN

Fig. 9. The mean lymphatic concentrations and time profiles of DOX (a), DEC (b), ABZ-OX (c) and ABZ-ON (d) post-oral administration of pure drugs and post-application of MN containing drug-loaded SLNs. The relative bioavailability of DOX, DEC and ABZ-OX in lymphatic (e) following the administration of MNs containing drug-loaded SLNs compared to the oral administration of pure drugs. The DTE (f) and DTP (g) values of DOX, DEC and ABZ-OX in the lymphatic following the administration of MNs containing drug-loaded SLNs in comparison with the oral administration of pure drugs (means ± SD, $n = 3$).
insertion into the skin, interstitial fluid in the skin dissolves the MNs height. The drug-loaded SLNs would then be liberated from the MN matrices. As the particle size of SLNs are < 100 nm, the SLNs would not be readily taken up by blood capillaries but rather are absorbed by the lymphatic capillaries. Small molecules and macromolecules with sizes of < 10 nm are taken up by the blood and the lymphatic capillary easily [12]. Thereafter, these molecules are mostly carried away from the interstitial and lymphatic capillaries through the blood capillaries. This is because the rate of filtration and reabsorption of fluid across the blood capillaries (20–40 l/day) is higher than lymph flow (2–4 l/day) [66]. It has been postulated that after injection at the interstitial site, larger molecules with sizes of 10–100 nm are absorbed more efficiently by the lymph than the blood capillaries. In contrast, the diffusion of the smaller particles is quicker and facilitates more efficient transport to blood capillaries. The delivery of particles with sizes of > 100 nm interstitially is eventually limited by the dimensions of the water channels in the skin interstitial, which are typically approximately 100 nm in diameter. Therefore, particles with sizes of 10–100 nm would be ideal for lymphatic uptake [72]. Moreover, particles with sizes of > 100 nm are retained at the administration site [20]. Our results were supported by a study investigating the lymphatic targeting of mitoxantrone in mice. In their study, they developed drug-loaded SLNs with a particle size of approximately 79 nm. The lymphatic targeting study showed that drug concentration in axillary lymph nodes after subcutaneous injection of SLNs was approximately two-fold greater in comparison with the oral administration of pure drug solution [35]. In addition to the sizes, due to the higher interstitial pressure and lymph flow rates in the dermis compared to other interstitial sites, the intradermal administration may promote greater lymphatic uptake in comparison with the oral administration of pure drugs. In this study, we evaluated the drug exposure to the lymphatic system following intradermal administration via dissolving MNs compared to the oral route. The values of DTE can vary between -∞ and ∞. The values greater than 1 indicate that the administration can deliver the drug efficiently. With respect to DPT, the values can vary between -∞ and 100%. The values less than zero imply that the administration cannot deliver the drug efficiently to the target [74]. In this study, we evaluated the drug exposure to the lymphatic system following intradermal administration via dissolving MNs compared to the oral route. The values of DTE can vary between -∞ and ∞. The values greater than 1 indicate that the administration can deliver the drug efficiently. With respect to DPT, the values can vary between -∞ and 100%. The values less than zero imply that the administration cannot deliver the drug efficiently to the target [74]. Fig. 9f and 9g show the DTE and the DTP values of DOX, DEC and ABZ-OX in the lymphatic system following the administration of MNs containing drug-loaded SLNs in comparison with the oral administration of pure drugs. In this study, the administration of MNs containing drug-loaded SLNs enhanced the DTE to the lymphatic system by 4.26-fold for DOX, 6.08-fold for DEC and ABZ-OX, respectively, after the MNs dosing, in comparison with the oral dosing. Therefore, skin application of MNs was able to enhance the lymphatic targeting of the drugs of LF treatment when compared with the conventional oral administration.

3.9.2. Evaluation of the biodistribution profiles of DOX, DEC, ABZ-OX and ABZ-ON

The biodistribution profiles of DOX, DEC, ABZ-OX and ABZ-ON after oral and MN dosing are illustrated in Fig. 10. In the liver, the \( C_{\text{max}} \) of DOX was found after 1 h with a concentration of 1115 ± 181.1 ng/g after oral dosing. In terms of MN administration, a lower \( C_{\text{max}} \) was found after 2 h, which was 888 ± 333.3 ng/g. With regards to DEC profile, a \( C_{\text{max}} \) of 1984 ± 856.7 ng/g was observed after 2 h post oral dosing. After MN administration, the \( C_{\text{max}} \) was found after 2 h and the
concentration was 825.5 ± 435.1 ng/g. In terms of ABZ-OX profile, the C\text{max} of ABZ-OX was observed after 1 h with a concentration of 210.8 ± 44.0 ng/g. After 6 h, DEC exhibited a maximum concentration of 490.3 ± 79.65 ng/g after oral administration and 192.4 ± 85.34 ng/g after MN administration. The C\text{max} of ABZ was found after 4 h and 6 h with a concentration of 212.8 ± 90.84 ng/g and 94.6 ± 40.37 ng/g following oral and MNs administration, respectively. For ABZ-OX, the same t\text{max} as ABZ-OX was found, with a C\text{max} of 63.85 ± 27.25 ng/g and 18.68 ± 7.14 ng/g after oral and MN administration, respectively. Analyzed statistically, the C\text{max} of all drugs after MN administration were significantly lower (p < 0.05) than after oral administration.

In the spleen, the C\text{max} of DOX was observed after 2 h with a concentration of 490.3 ± 79.65 ng/g after oral administration. After MN administration, the C\text{max} of DOX was found after 4 h with a concentration of 264.8 ± 43.01 ng/g. In the case of DEC, a C\text{max} of 526.1 ± 224.5 ng/g was determined after 4 h post oral dosing. Following MN administration, a C\text{max} of 265.3 ± 113.2 was found after 6 h. For ABZ-OX, after 4 h, the C\text{max} was found with a concentration of 174.2 ± 50.87 ng/g and 76.4 ± 32.61 ng/g following oral and MN administration, respectively. In the case of ABZ-OX, the C\text{max} was found to be 35.76 ± 15.26 ng/g after 4 h post oral dosing. After MN dosing, the C\text{max} was observed after 12 h with a concentration of 8.22 ± 2.27 ng/g. In comparison, the overall spleen distribution of drug-loaded SLNs administrated intradermally using MNs were significantly lower (p < 0.05) than after oral administration.

Our study showed that the distribution of the drugs in the liver, kidney and spleen was significantly reduced after the intradermal administration of SLNs via dissolving MNs, when compared to the oral administration of pure drugs. Our results are supported by a previous study in our group reporting that the C\text{max} of rhodamine B loaded PLGA nanoparticles in liver, kidney and spleen was lower, compared to free rhodamine B, when administrated via MNs [66]. A similar study was also carried out investigating the organ biodistribution of mitoxantrone loaded SLNs in comparison to free-mitoxantrone administrated subcutaneously. It was found that the C\text{max} and AUC of mitoxantrone in the liver and kidney after free-drug administration were greater than after drug-loaded SLNs administration [25]. The results of the lymphatic pharmacokinetic profile thus indicate that lymphatic uptake of drugs could potentially lead to a decrease in the distribution of the drugs in the organs.

Based on the results presented here, it is clear that the combination of SLNs and MNs could increase the concentration of DOX, DEC and ABZ-OX in the lymphatic system when compared to conventional oral administration. Not only that, but this innovative approach also improved the retention time of the drugs in the lymph nodes, where the filarial nematodes dwell in LF, indicated by the high MRT values. Taken together, the lower biodistribution and C\text{max} in the plasma of the three drugs post SLNs-MN dosing could be highly beneficial as the data indicate that this approach could potentially reduce systemic exposure and the potential side effects of the drugs in specific organs, such as liver, kidney and spleen. Moving forwards, future work including in vivo pharmacodynamic studies must now be performed to evaluate the therapeutic efficacy of this novel approach in animal models of LF.

4. Conclusion

In the present study, we successfully developed DOX, DEC and ABZ-OX-loaded SLNs for LF treatment. The optimizations were carried out using CCD. In the case of all three drug formulations, the optimized drug-loaded SLN was achieved by using Geleol® as a lipid matrix and Tween®80 as a stabilizer resulting in particle sizes of < 100 nm and uniform distribution. Furthermore, the incorporation of drug-loaded SLNs into dissolving MNs resulted in improved delivery and retention times of the drugs in the dermis layer of the skin. Finally, in vivo studies indicated that this combination approach enhanced the relative bioavailability of the three drugs under investigation in plasma and lymph nodes, and decreased the biodistribution of the drugs in liver, kidney and spleen, compared to conventional oral administration. Accordingly, this approach could potentially have significant benefits in the development of more efficacious LF medications, as administration via these vehicles could potentially result in the death of microfilaria in the blood, but also the adult nematodes in the lymph nodes. This was the first study of its kind to combine SLNs with MN-mediated delivery and, as such, a variety of additional experiments must now be considered. For example, studies to determine the dose of drugs required to kill the adult filarial in the lymph nodes must now be carried out. If required, maximizing the dose efficiency of the drugs in the MNs by using different designs/geometries of MNs could lead to the loading of higher amounts of the drugs. Subsequently, pharmacodynamic studies using appropriate infection models to evaluate the efficacy of this system are necessitated. To conclude, before this novel delivery approach can reach clinical practice and achieve patient benefit, usability and acceptability studies should be carried out to inform and ensure maximum impact of the work.

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Appendix A. Supplementary data

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References


