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Microneedle liquid injection system assisted delivery of infection responsive nanoparticles: A promising approach for enhanced site-specific delivery of carvacrol against polymicrobial biofilms-infected wounds

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ABSTRACT

Biofilms present a challenge to wound healing and are among the most feared complications through the course of wound management. Carvacrol (CAR) has manifested its antibiofilm potential against multidrug resistant bacterial biofilms. Herein, infection responsive nanoparticles (NPs) of CAR were developed (particle size: 199 ± 8.21 nm and drug load: 1.35 mg/100 μL) and microneedle liquid injection systems (AdminPen®) of various specifications were investigated as delivery devices to achieve the higher concentrations (in contrast to the concentrations delivered through topical hydrogel) of NPs at the target site. The results exhibited an improved biosafety and antibiofilm activity of CAR after encapsulation into the NPs. *Ex vivo* skin insertion and dermatokinetic studies suggested that AdminPen® 1500 was the most suitable device, as compared to AdminPen® 777 and 1200. Finally, animal studies showed that AdminPen® 1500 delivered around 8.5 times higher concentrations of CAR in the form of NPs as compared with pure CAR from topically applied hydrogel. Moreover, 50% of the delivered NPs from the AdminPen® 1500 were retained at the site of application for 72 h, in contrast to the pure CAR from the hydrogel (5.2% only). Thus, AdminPen® assisted delivery of bacterial enzyme responsive NPs could be an effective approach for enhanced site-specific accumulation of CAR to potentially achieve the prolonged desired antibiofilm effect. However, further *in vivo* efficacy in a diseased model must now be investigated.

1. Introduction

Chronic wounds are a persevering problem influencing physical and mental health, and they represent crucial medical, economic and social burdens (Järbrink et al., 2017). The healing process of wounds, even under the optimum conditions, may result in fibrosis or scars that can further cause multiple problems (Harding et al., 2002; Stadelmann et al., 1998). Commonly, considered as a comorbid condition, chronic wounds represent a silent epidemic that affects a large fraction of the world population (Järbrink et al., 2016). It has been reported that 1% to 2% of the population in developing countries are likely to experience a chronic wound in their lifespan (Gottrup, 2004). Recently, a systematic review and *meta*-analysis reported the pooled prevalence of chronic wounds of 2.21 per 1000 general population (Martinengo et al., 2019). In the US, chronic wounds affect approximately 6.5 million patients, resulting in > 25 billion US$ annual cost for their management (Sen et al., 2009). Annual costs of 13 to 15 billion US$ for global wound care (Walmsley, 2002) mean that management of chronic wounds is a major problem for healthcare systems/professionals. In chronic wounds, infection is the most common reason impeding wound healing, resulting in increased patient morbidity and mortality (Bessa et al., 2015). The occurrence of bacterial biofilms is more crucial in hindering the wound healing process as compared with the more treatable planktonic forms (freely living, unattached cells) of bacteria (Rhoads et al., 2008). Biofilm grown bacteria are 500 times more difficult to kill than their planktonic forms (Donlan and Costerton, 2002; Donlan, 2001).

Biofilms provide physical protection to the enclosed bacterial colonies from an external hostile environment and facilitate their

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communication with each other (quorum sensing), which further results in enhanced virulence and infection (De Kievit and Iglewski, 2000). Additionally, slow growth of bacteria within biofilms may lead to reduced uptake of antibiotics, impairing their effectiveness. Chronic wounds provide favorable conditions to bacteria to produce biofilms, as damaged tissue and proteins (fibronectin and collagen) are present, which facilitate bacterial attachment (Rhoads et al., 2008). The most frequently isolated pathogens from chronic wounds, including *Pseudomonas aeruginosa* and methicillin resistant *Staphylococcus aureus* (MRSA), are typical biofilm formers (Doern et al., 1999). These bacteria are highly invasive in nature and can cause life-threatening infections if not treated properly. These "superbugs" have developed resistance to a wide range of commonly prescribed antibiotics, leading to treatment failure of infected wounds (Poole, 2011; Tong et al., 2015). Therefore, it is required to identify alternative therapeutic strategies to manage the wound infections caused by these multidrug resistant pathogens (Gilmer et al., 2013; Manzoor-ul-Haq et al., 2015).

Carvacrol (CAR), present in many plants' essential oils, has been reported to manifest antimicrobial activity against both the planktonic forms and biofilms of a wide range of multidrug resistant bacteria (Antonia and Teresa, 2012; Marchese et al., 2018). Moreover, it has wound healing potential (Gunal et al., 2014). Although CAR has promising antibiofilm activity, due to its poor skin retention, it is not suitable for direct application, demanding frequent applications. Hence, the establishment of a suitable delivery approach is needed to achieve a prolonged antibiofilm effect at the desired site (Nostro et al., 2015; Scaffaro et al., 2018).

Considering the potential of infection responsive delivery of antimicrobials to prevent the emergence of resistant strains, we have previously developed bacterial enzyme responsive poly (Ɛ-caprolactone) (PCL) based nanoparticles (NPs) of CAR to achieve bacterially-triggered release of CAR. Additionally, encapsulation of CAR in NPs has improved its antimicrobial potential against resistant Gram-negative and Gram-positive bacterial strains (Mir et al., 2019). Moreover, based on the avascular nature of many chronic wounds, local delivery is preferable over systemic drug delivery to accumulate the required concentrations of drug at the target site. Thus, considering the problems associated with conventional dosage forms and the nature of the target site, several dosage forms including hydrogels (Mir et al., 2019) and dissolving microneedles (MNs) (Mir et al., 2020) have been developed by us to enhance site-specific delivery of the established infection responsive NPs. In terms of delivery efficiency, dissolving MN arrays loaded with CAR-PCL NPs were found more suitable in contrast to hydrogels, as MNs successfully delivered 90% of the dose applied as compared to the CAR-PCL NPs loaded hydrogels that were able to deliver only ≈ 15% of the total applied dose after 6 h of application in an *ex vivo* neonatal porcine skin. Thus, hydrogels required longer application times to achieve higher concentrations, which, in turn, can compromise patient compliance and can also lead to inaccurate dosing (due to poor retention of the hydrogel on the skin surface for longer times). Moreover, MNs have been reported to exhibit the potential for successful penetration across hyperkeratotic/necrotic tissue barriers (preventing the need of surgical debridement prior to drug application; a common practice in clinics) helping in direct, rapid and painless delivery of precise doses of drugs into wounds (Caffarel-salvador et al., 2015; Raj et al., 2016; Zaric et al., 2013). Thus, findings of the study (Mir et al., 2020) have proved the hypothesis that dissolving MNs could be a promising strategy to improve the site-specific delivery of CAR-PCL NPs to chronic wounds. However, in order to deliver the concentrations of NPs required producing the desired effect through MNs, several strategies including; (i) increased loading of the NPs into MNs and (ii) various designs/types of MNs need to be explored.

Hence, to address the above mentioned limitation, we have explored for the first time another type of MNs *i.e.* MN liquid injection system to deliver the required concentrations of CAR-PCL NPs to chronic wounds. MN liquid injection systems can offer not only rapid, direct and painless delivery to the chronic wounds but the following additional advantages can also be achieved compared to hypodermic needles: AdminPen® devices are sterile in nature, less chances of microbial penetration, skin trauma is minimized and bleeding is avoided after insertion. Use of MN arrays in contrast to hypodermic needles potentially also prevents the development of skin infections. Needle-phobia from the hypodermic needle is removed, which is experienced by many people and can ultimately reduce adherence (Donnelly et al., 2009).

Herein, we determined the antimicrobial potential of CAR-PCL NPs against biofilms of multidrug resistant pathogens. The MN liquid injection systems of various specifications were investigated to select the optimum one for effective delivery of desired doses of CAR-PCL NPs to biofilm-infected chronic wounds. All three AdminPen® devices were evaluated in terms of their insertion properties and *ex vivo* dermatokinetic profiles, following this, *in vivo* studies and antibacterial effectiveness in an *ex vivo* wound model were performed using the most suitable device. This is the first study reporting the potential of MN liquid injection systems for improvement of site-specific delivery of CAR loaded infection responsive NPs.

2. Materials and methods

2.1. Materials

Carvacrol (> 98% purity), poly(Ɛ-caprolactone)(PCL) (MW 14,000 Da) and Poloxamer® 407 were purchased from Sigma-Aldrich, Hamburg, Germany. AdminPen® devices for liquid injections (AdminPen® 777, AdminPen® 1200 and AdminPen® 1500 microneedle liquid injection systems) were directly purchased from AdminMed, NanoBiosciences LLC, California, USA. Bacterial strains employed in the study including; *Staphylococcus aureus* (NCTC® 10788), *Staphylococcus aureus* [MW2] (ATCC® BAA-1707TM), *Staphylococcus aureus* (ATCC® 33593TM), *Pseudomonas aeruginosa* (ATCC® 9027), and *Pseudomonas aeruginosa* (ATCC® BAA- 47) [PAO1] were directly purchased from LGC Standards, Middlesex, UK. All the other reagents used in the study were of analytical grade.

2.2. Development and characterization of CAR-PCL NPs

To deliver higher concentrations of CAR-PCL NPs into the skin through AdminPen®, CAR-PCL NPs were prepared by nanoprecipitation method in large volumes using the optimized formula (5.40 mg/mL PCL, 0.5% Poloxamer® 407 and 5 mg/mL CAR.) obtained through design expert software (Mir et al., 2019). Briefly, pre-determined concentration of PCL was dissolved in acetone with mild heating. CAR was also dissolved in acetone in pre-defined concentration. Following this, organic phase was prepared by mixing these two (polymer and drug) solutions. Aqueous phase was prepared by dissolving Poloxamer® 407 in milli-Q water. Finally, the organic phase was added to the aqueous phase at a rate of 0.16 mL/minute under homogenization at 6500 rpm (Silent Crusher M Homogenizer from Heidolph Instruments, Schwabach, Germany). Organic solvent was evaporated at ambient temperature with continuous stirring for 40 min at magnetic stirrer. The resultant formulation was collected by centrifugation (Hermle Labortechnik GmbH, Z-206A, Siemensstr.25 D-78564 Wehingen, Germany) at 13500 rpm (22413 RCF) for 30 min at 4 °C. Supernatant was removed and the resultant pellet was washed twice with milli-Q water and centrifuged for 30 min, as previously described. Pellet was frozen at −80 °C, before lyophilization (Alpha 1–2 LD plus, Christ, Osterode, Germany). Prepared NPs were further evaluated in terms of their size, zeta potential (ZP), polydispersity index (PDI) and drug content following the same protocols detailed in our previously published paper (Mir et al., 2019). Potential of CAR-PCL NPs for bacterially-triggered release of CAR was also reported by us in different release media (Mir et al., 2020). Lyophilized NPs were re-dispersed in purified water (sourced from Elga PURELAB DV 25, Veolia Water Systems, Ireland) to

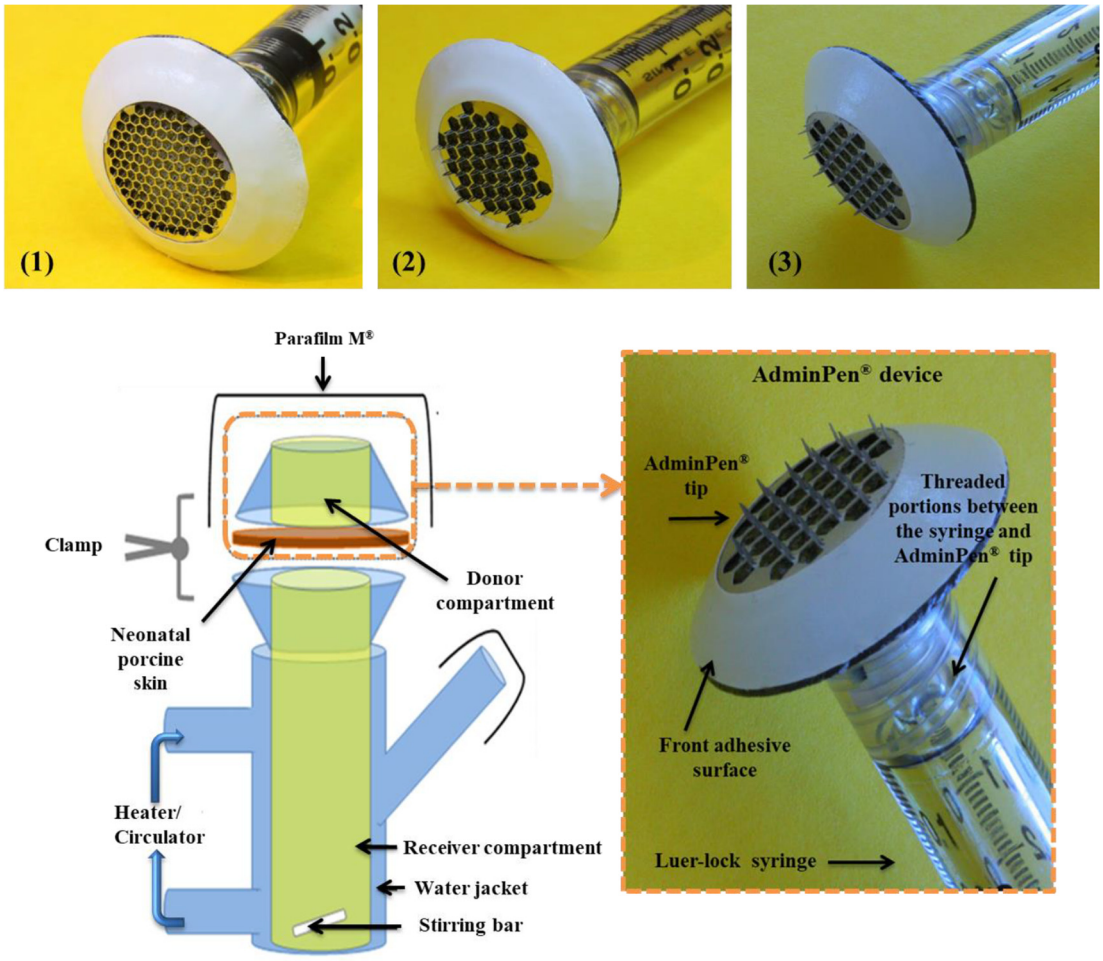

Fig. 1. (A) Images of AdminPen® devices illustrating their particular design; (1) AdminPen® 777, (2) AdminPen® 1200 and (3) AdminPen® 1500. (B) Franz diffusion cell set up used for dermatokinetic and permeation studies.

achieve a formulation with consistency, suitable for administration through AdminPen® device.

2.3. Determination of antibiofilm activity of CAR and CAR-PCL NPs

To estimate the potential of CAR and CAR-PCL NPs to inhibit biofilm-grown bacteria, the biofilms of all the test strains were prepared following the published protocol with slight modifications (Caffarel-salvador et al., 2015). Briefly, bacterial suspensions of all the test strains were prepared to adjust the final concentrations to 2.0×10^5 CFU/mL, following the previously published protocol (Mir et al., 2020). To prepare the biofilms, 100 µL of the prepared bacterial suspensions were added to 96-well plates (Nalgen Nunc International, Rochester, NY, USA), followed by 24 h' incubation at 37 °C. After incubation, the wells were softly washed with sterile phosphate buffered saline (PBS) (pH 7.4) three times to remove any non-adherent bacteria. Following this, 100 µL aliquots of broth (Mueller Hinton broth (MHB) for Gram-positive strains and Luria-Bertani broth (LBB) for Gram-negative strains) contained a specified concentration of CAR-PCL NPs and CAR were added into the wells containing biofilms and then incubated for 24 h at 37 °C. Following the incubation, wells were emptied, softly washed with PBS and 100 µL of sterile PBS was added, followed by sonication in a 150 W ultrasonic bath for 10 min at 50 Hz. Samples from the wells were withdrawn and inoculated on agar plates with suitable dilutions using sterile fresh broth. Plates were analyzed for viable CFUs after 24 h' incubation at 37 °C. A control (without any treatment) was also run under the same conditions. Three replications were performed for the experiment.

2.4. Hemolytic activity of CAR and CAR-PCL NPs

The safety and biocompatibility of novel delivery systems can be estimated by their hemolysis potential. In order to estimate the hemolytic activity of CAR-PCL NPs, *in vitro* hemolytic assay was performed following the previously published method (Yang et al., 2005). For this purpose, erythrocytes were separated from the blood of healthy, female, Sprague–Dawley rats through centrifugation at 2000 relative centrifugal force (RCF) for 10 min, followed by three cycles of washing with PBS by centrifugation at the same conditions. The washed erythrocytes were resuspended in PBS to the final concentration of 10% *v/v*. A volume of 100 µL of the prepared cell suspension was added into 900 µL of the samples containing CAR and CAR-PCL NPs (serially diluted in PBS) in the concentration range of 0.156 to 1.25 mg/L. The mixtures were then incubated at 37 °C for 60 min, followed by centrifugation at 14,000 RCF for 10 min. Finally, the absorbance of the supernatant was measured using UV–Visible spectroscopy (Epoch Microplate Spectrophotometer with Gen5 software, BioTek, Winooski, VT, USA) at 540 nm to estimate the free hemoglobin. PBS and distilled water were run as minimal (negative) and maximal (positive) hemolytic controls respectively. Change in color of serum and plasma was also observed to analyze the hemolysis of the samples. The experiment was performed in triplicate for all the concentrations. The percentage-hemolysis was calculated through Eq. (1):

$$\text{Hemolysis}(\%) = \frac{\text{OD(Testsample)} - \text{OD(Negativecontrol)} \times 100}{\text{OD(positivecontrol)} - \text{OD(Negativecontrol)}} \quad (1)$$

The possible protective effect of nano-encapsulation was determined by comparing the hemolysis induced by CAR-PCL NPs (CN) with that induced by the free CAR (FC) and expressed as hemolysis reduction (HR). Eq. (2) was used to calculate HR (Serrano et al., 2013).

$$\text{Hemolysis Reduction} = \frac{\text{Hemolysis FC} - \text{Hemolysis CN}}{\text{Hemolysis FC}} \times 100 \quad (2)$$

2.5. Evaluation of insertion properties of microneedle liquid injection systems

AdminPen® devices with various specifications were investigated to determine the potential of MN liquid injection systems for delivery of CAR-PCL NPs to the skin. Images of AdminPen® devices illustrating their particular design are provided in Fig. 1 (A). The height and number of the needles on each device was different: AdminPen® 777 had 121, 700 μm-tall MNs, AdminPen® 1200 had 43, 1100 μm-tall MNs, and AdminPen® 1500 had 31, 1400 μm-tall MNs on 1 cm^2 circular microneedle array. AdminPen® devices were investigated for their insertion properties using both Parafilm M® (Bemis Company Inc, Soignies, Belgium) and full thickness neonatal porcine skin as a skin simulant insertion model. In brief, Parafilm M® was folded into twelve layers (≈ 1.52 mm thickness) and placed on dental wax, supported on a rigid base. The AdminPen® device was then carefully inserted into the polymeric layers by applying gentle thumb pressure in a way that the entire front adhesive surface of the AdminPen® device was in complete contact with the Parafilm M® layers. After 15 s of insertion with constant force, the AdminPen® was removed from the Parafilm M® layers and a Leica EZ4 D digital light microscope (Leica Microsystems, Milton Keynes, UK) was used to count the number of microconduits produced. The same protocol was followed to see the insertion in the neonatal porcine skin samples (skin samples were prepared following the reported protocol with skin obtained from stillborn piglets (Permana et al., 2019)).

2.6. Ex vivo skin permeation and dermatokinetic studies

To select the MN liquid injection system, which can deliver the highest concentration of CAR-PCL NPs into the skin, where bacteria can grow and form biofilms, *ex vivo* permeation and dermatokinetic studies were conducted with all three AdminPen® devices. For these investigations, the previously reported method (Mir et al., 2019) was followed with the exception of application/injection method. Importantly, to inject the CAR-PCL NPs through AdminPen®, standard luer-lock syringes (BD Luer-Lock™, Becton, Dickinson and Company, California, USA) of 1 mL capacity were prefilled with CAR-PCL NPs suspension (prepared in Section 2.2). Schematic illustration of the setup used is provided in Fig. 1 (B). The pre-filled syringe was attached to the AdminPen® device, rotating the syringe along the tip of AdminPen® till threaded portions between the syringe and AdminPen® device tip completely interlocked to form a liquid tight seal. While pointing the AdminPen® up, the syringe plunger was moved (0.1 mL up) to remove air from the AdminPen® inter channels, making sure that no liquid was coming to the front surface of the device. After making the device ready for injection, 100 μL of CAR-PCL NPs suspension (equivalent to 1.35 mg of CAR) was carefully and slowly injected (in 30 s) into the skin samples with a constant force. Samples were withdrawn at predefined time points from both the donor compartment (skin samples for dermatokinetic analysis) and receiving compartment (for estimation of permeation of CAR) of the Franz diffusion cell. To collect the required skin samples, biopsy (biopsy punch; 10 mm diameter) (Stiefel, Middlesex, UK) was performed after washing the samples properly with water. The biopsied samples were then heated at 60 °C in a water bath for 2–3 min. Epidermal and dermal layers were separated using tweezers. Following this, stainless steel beads (5 mm diameter) and 500 μL methanol were added to each layer and further subjected to homogenization for 10 min at 50 Hz using Tissue Lyser LT (Qiagen, Ltd., Manchester, UK) for extraction of CAR from the skin. Subsequently, homogenized samples were centrifuged at 14,462 RCF for 15 min and, finally, a sample from the supernatant was diluted with PBS (pH 7.4) prior to quantifying the CAR content employing the analytical method described in Section 2.8.2. One-compartment open model was applied on the data, and the kinetic parameters were determined using PK Solver software for both the epidermis and the dermis. The results of the permeation study were displayed as drug permeated/unit area versus time. Jss was calculated through Equation (3). A comparison of the dermatokinetic profiles of all three systems was made.

$$\text{Jss} = \left(\frac{dQ}{dt}\right)\text{ss} \times 1/A \quad (3)$$

Where: Jss = steady state permeation flux (μg/cm^2/h), (dQ/dt) ss = Concentration of CAR permeating through skin per unit time at a steady state (μg/h), and A = Area of the skin tissue.

2.7. Evaluation of antimicrobial activity in an ex vivo wound model

The potential of NPs to inhibit bacterial burden in an *ex vivo* neonatal porcine skin wound infection model; delivered through MN liquid injection system (AdminPen® 1500) was determined following the already published protocol with slight modifications (Alhusein et al., 2016; Roche et al., 2019). Excised full-thickness neonatal porcine skin was sterilized by immersing in 70% ethanol for 30 min, followed by 20 min drying in a biosafety cabinet. Briefly, burn wounds using red-hot brass knob were created in the sterilized skin pieces and then aseptically placed on trypticase soy agar (TSA) plates. Following this, 100 μL of the bacterial suspensions (2×10^5 CFU/mL) were inoculated to the created wounds and incubated at 37 °C for 5 days to allow the biofilm formation, while the skin samples were transferred aseptically to new TSA plates each day. In order to determine the antibiofilm activity of CAR-PCL NPs and blank PCL NPs administered through AdminPen® 1500, and hydrogel containing free CAR, at 12 h and 24 h post-application, *ex vivo* biofilm-infected skin was homogenized in sterile PBS and cultured onto agar plates at 37 °C for 24 h. The number of viable CFUs was finally counted. Infected wounds without any treatment were used as a positive control and normal uninfected skin was used as a negative control.

2.8. In vivo studies

An *in vivo* experiment was performed to determine the profiles of CAR in plasma and skin following the application of pure CAR in the form of a hydrogel and CAR-PCL NPs administered through AdminPen® 1500. To facilitate the topical application of free CAR, it was loaded into 1% w/v Carbopol® 934 based hydrogel, following the method previously published by us (Mir et al., 2019). All animal's experiments were approved by the Committee of the Biological Research Unit, Queen's University Belfast under Project Licence no. 2794 and Personal Licence no. 1747. All animals experiments were conducted according to the policy of the European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes, executing the principle of the 3 R's (replacement, reduction, and refinement) and that of the Federation of European Laboratory Animal Science Associations. Healthy, BALB/c mice weighing 32.94 ± 5.50 g were acclimatized to laboratory conditions for 7 days before commencing the experiments. Mice were given anesthesia (2–4% *v/v* isoflurane in oxygen) and the hair from their backs was removed using Braun Series 3 Electric Shaver 300 s (Braun, Kronberg im Taunus, Germany) and hair removal cream (Sodalis Srl, Milano, Italy). Skin barrier was then allowed to recover for 24 h before experiment. The mice were divided

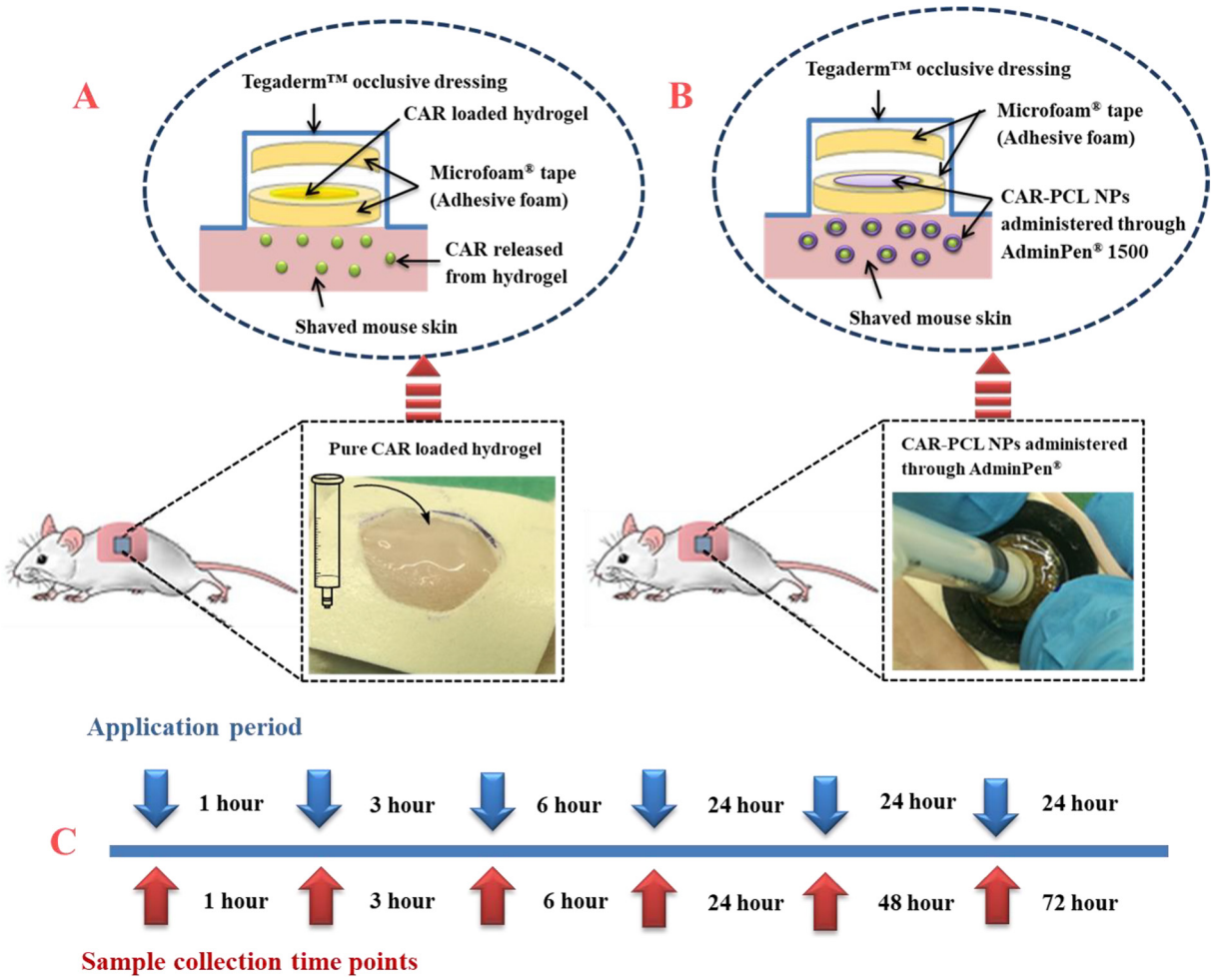

Fig. 2. Schematic representations of the two mice cohorts for the *in vivo* delivery of CAR. In control cohort (A) CAR was applied in the form of hydrogel and held in place with adhesive foam border as illustrated schematically. (B) CAR-PCL NPs administered through AdminPen® 1500. (C) In vivo study plan for application of CAR hydrogel/CAR-PCL NPs and sample collection time points in each mouse group (n = 3).

into two cohorts (n = 18 in each cohort): one for application of pure CAR in the form of hydrogel and the second was for administration of CAR-PCL NPs through AdminPen® 1500. In each cohort, mice were further divided into 6 groups (n = 3 in each group) to study the defined application time points (1, 3, 6, 24, 48 and 72 h). Fig. 2 displays the schematic illustration of the *in vivo* experiment. For the application of hydrogel, a 1 cm^2 circular hole was made into a piece of Microfoam® tape (approximately 1 mm thick) and was then applied on the back of the mouse. CAR loaded hydrogel was prepared to achieve the concentration of 1.35 mg/100 mg, and then 100 mg of hydrogel was applied into the hole of the Microfoam® tape, covered with another piece of Microfoam® tape, and subsequently covered with Tegaderm™ occlusive dressing to prevent the mice from removing the formulation during routine movement.

For administration of CAR-PCL NPs through AdminPen®, a piece of Microfoam® tape with a hole of the same size was applied on the back of the mouse (in order to provide the same surface area for application), and 100 µL of CAR-PCL NPs suspension (equivalent to 1.35 mg of CAR) was carefully injected into the skin, and covered with tape and occlusive dressing in a similar way as mentioned above. The mice were euthanized with carbon dioxide at defined time intervals: 1, 3, 6, 24, 48, and 72 h and blood samples were collected through cardiac puncture. To collect the skin samples, the tape and dressing were removed, the skin from the application site was thoroughly washed with water, the skin was removed using scissors, and further processed for CAR analysis, following the protocol detailed in Section 2.8.1.

2.8.1. Processing and analysis of blood and skin samples

A HPLC-based analytical method was developed and validated using an acid base neutralization approach for quantification of CAR in blood samples. Briefly, blood samples were collected in heparinized tubes and centrifuged at 2000 RCF for 10 min at 4 °C for separation of plasma from the blood. Subsequently, 100 µL of trichloroacetic acid (10% w/v) was added into the separated plasma and vortexed for 15 min. After cold centrifugation of the mixtures at 14,000 RCF, 4 °C for 15 min, the supernatant was transferred to the eppendorf tubes and mixed with 6 µL of 1 N sodium hydroxide for neutralization of the samples. Subsequently, the neutralized samples were centrifuged at 14,000 RCF, at room temperature for 10 min and the supernatant was analyzed using HPLC. The skin samples were collected from the site of application. Collected samples were thoroughly washed with water, finely chopped into thin pieces using scissors and added into 1.5 mL eppendorf tubes with stainless steel beads and 500 µL methanol, and homogenized for 10 min at 50 Hz using Tissue Lyser LT. Subsequently, homogenized skin samples were subjected to centrifugation at 14,462 RCF for 15 min and then, a sample of the supernatant was analyzed for CAR content using HPLC, after making appropriate dilutions with PBS. Data analysis of both the skin and plasma samples was performed using PK Solver software. The non-compartmental kinetic model was applied to calculate the kinetic parameters. The peak plasma concentration (C_{max}), the time of peak concentration (t_{max}), the drug concentration time curve from time zero (t = 0) to the last experimental time point (t = 72 h) (AUC0-72), the mean residence time (MRT) and the mean

half-life ($t_{1/2}$) were all determined.

2.8.2. Analytical method for quantification of CAR

HPLC analysis was carried out using HPLC Agilent Technologies 1220 Infinity Compact LC Series comprising of Agilent degasser, binary pump, auto standard injector and detector (Agilent Technologies UK Ltd, Stockport, UK). The column used was Phenomenex Luna C18 ODS (1) column: 150 mm × 4.60 mm internal diameter, 5 μm packing (Phenomenex, Cheshire). To analyze skin samples, the mobile phase was HPLC grade water: acetonitrile (50:50), with a flow rate of 1 mL/minute, 80 μL of injection volume, and a runtime of 6.5 min. UV detection was performed at a wavelength of 275 nm. Agilent Chemstation® Software B.02.01 was used for the analysis of the chromatograms. To analyze the plasma samples, the same HPLC instrument and chromatographic conditions were used, except the mobile phase *i.e.* HPLC grade water: acetonitrile (70:30), and a runtime of 22 min. The HPLC method was validated per International Conference on Harmonization (ICH) guidelines for Validation of Analytical Procedures Q2 Analytical Validation Revision one (R1) 2005 (ICH, 2005).

2.9. Statistical analysis

All investigations/tests were performed in triplicate and data were presented as a mean ± SD. The results were analyzed by ANOVA and unpaired t-tests to evaluate the statistical significance (defined at $p < 0.05$) using GraphPad Prism® software version 5.03 (GraphPad Software, San Diego, CA, USA).

3. Results and discussion

3.1. Development and characterization of CAR-PCL NPs

The CAR-PCL NPs were developed through a nanoprecipitation approach and evaluated in terms of particles size, ZP and PDI. The CAR-PCL NPs in the form of suspension showed mean particle size of 199 ± 8.21 nm with 0.08 ± 0.006 PDI, demonstrating the homogeneous and monodispersed nature of the formulation. ZP was found to be −16.1 ± 4.21 mV. The final concentration of CAR in the NPs suspension (having consistency suitable for administration through AdminPen® device) was achieved to be 1.35 mg/100 μL (quantified by HPLC), and used in further experiments.

3.2. Determination of antibiofilm activity of CAR and CAR-PCL NPs

All the test strains had the potential to form biofilms. However, Gram-negative strains were found to be stronger biofilm formers in comparison with the Gram-positive test strains, which is in accordance with previously published studies (Živković et al., 2018). The application of CAR in both free form and as CAR-PCL NPs resulted in a significant decrease ($p < 0.0001$) in the viable count for all the Gram-positive and Gram-negative strains tested, in comparison with negative control (no treatment). Importantly, with an increase in concentrations, both free CAR and NPs showed a greater reduction in biofilm grown strains, as can be seen in the Fig. 3, which is in line with the published results, reporting the same pattern (greater antibiofilm activity at higher concentrations) of antibiofilm activity of essential oils (Jafri et al., 2014). Specifically, CAR-PCL NPs and CAR have killed *S.aureus* biofilms at all concentrations tested (Fig. 3 A) as compared to all other test strains (3B-E). Importantly, CAR-PCL NPs resulted in a greater reduction in the viable count of all the biofilms in contrast to free CAR. A similar pattern was observed in the case of inhibition of planktonic forms of the test strains (Mir et al., 2020), indicating the potential of NPs for improved antimicrobial activity in contrast to free CAR. The mean percentage-kill was determined for the various concentrations (0.156 to 1.25 mg/mL) of CAR and NPs in comparison with the positive control (Supplementary Table S1). Kill rates of 88–100% were observed for Gram-positive test strains and 80–99.9% for Gram-negative strains following the application of CAR-PCL NPs and CAR. Moreover, higher kill rates presented by CAR-PCL NPs, even at their respective MIC values, suggested that these developed NPs would be an effective approach against biofilms-infected wounds.

The inhibition of preformed biofilms by CAR and CAR-PCL NPs reported here could be due to several factors acting either individually or synergistically. The antibacterial activity of CAR is usually associated with its hydrophobic nature, interacting with bacterial cytoplasmic membranes and resulting in loss of their integrity. However, CAR has also been reported to have some hydrophilic properties (slightly water soluble; 1.25 mg/mL at 25 °C), that can facilitate its diffusion through the biofilm polysaccharide matrix and further disrupt the microorganism owing to its intrinsic antimicrobial properties (Nostro et al., 2007). This concept is strengthened by the anti-plaque effect of an essential oil-based mouthwash, partly relatable to plaque-permeability potential and rapid killing (Ouhayoun, 2003). Hence, these results have revealed that entrapment of CAR into the NPs had improved its antibiofilm activity, therefore, could potentially be utilized for improved antibiofilm effect.

3.3. Hemolytic activity of CAR and CAR-PCL NPs

Hemolytic activity analysis against mammalian erythrocytes is usually performed to determine the selectivity of antimicrobials for bacteria. Free CAR and CAR-PCL NPs had not displayed any considerable hemolysis (< 5%) at all tested concentrations, as presented in Table 1. However, hemolysis value for NPs (0.78 ± 0.11% at maximum test dose) was lower than free CAR (1.35 ± 0.17% at maximum test dose), that can also be seen from the clearer and more transparent serum or plasma upon treatment with NPs as compared to free CAR (Fig. 4A1, A2). Both CAR and CAR-PCL NPs showed dose dependent hemolysis under test conditions. An average of 41.12 ± 5.45% reduction in hemolysis was observed for the tested concentrations of CAR-PCL NPs in contrast to free CAR. Hemolysis index is regarded as safe when it is < 5% (Zhou et al., 2011), hence, results have indicated that CAR and the NPs would be safe to use at these concentrations.

3.4. Evaluation of insertion properties of microneedle liquid injection systems

All three devices were mechanically strong enough to penetrate the skin showing 100% penetration in the first two layers of Parafilm M® and > 80% in the third layer. AdminPen® 777 (actual height 700 μm) showed > 30% penetration in the fourth layer (≈ 506 μm) and only 11% in the fifth layer. Admin Pen® 1200 (actual height 1100 μm) showed 80% penetration till fifth layer and > 19% in the seventh layer (≈ 886 μm). Admin Pen® 1500 (actual height 1400 μm) showed > 20% in the ninth layer (≈ 1139 μm). Images of the holes produced in the Parafilm M® layers and in the skin are provided in Fig. 4 (C, D: 1–3). Larrañeta *et al.* (Larrañeta et al., 2014), has reported that penetration of MNs is successful if > 20% holes are produced in each layer, therefore, it was considered that AdminPen® 777 penetrated the fourth layer (approximately 72% of the needle height), AdminPen® 1200 penetrated up to 886 μm depth (≈ 80% of needle height) and showed penetration down to 1139 μm (≈ 81% of needle height). Consequently, these results indicated that AdminPen® 1200 and 1500 were comparatively stronger than AdminPen® 777 in addition to being longer and hence, being mechanically strong could facilitate the penetration across hyperkeratotic/necrotic tissue barrier.

3.5. Ex vivo skin permeation and dermatokinetic studies

Dermatokinetic profiles of CAR, following the application of free CAR loaded hydrogel and CAR-PCL NPs loaded hydrogel are previously published by us (Mir et al., 2019). Briefly, free CAR loaded hydrogel

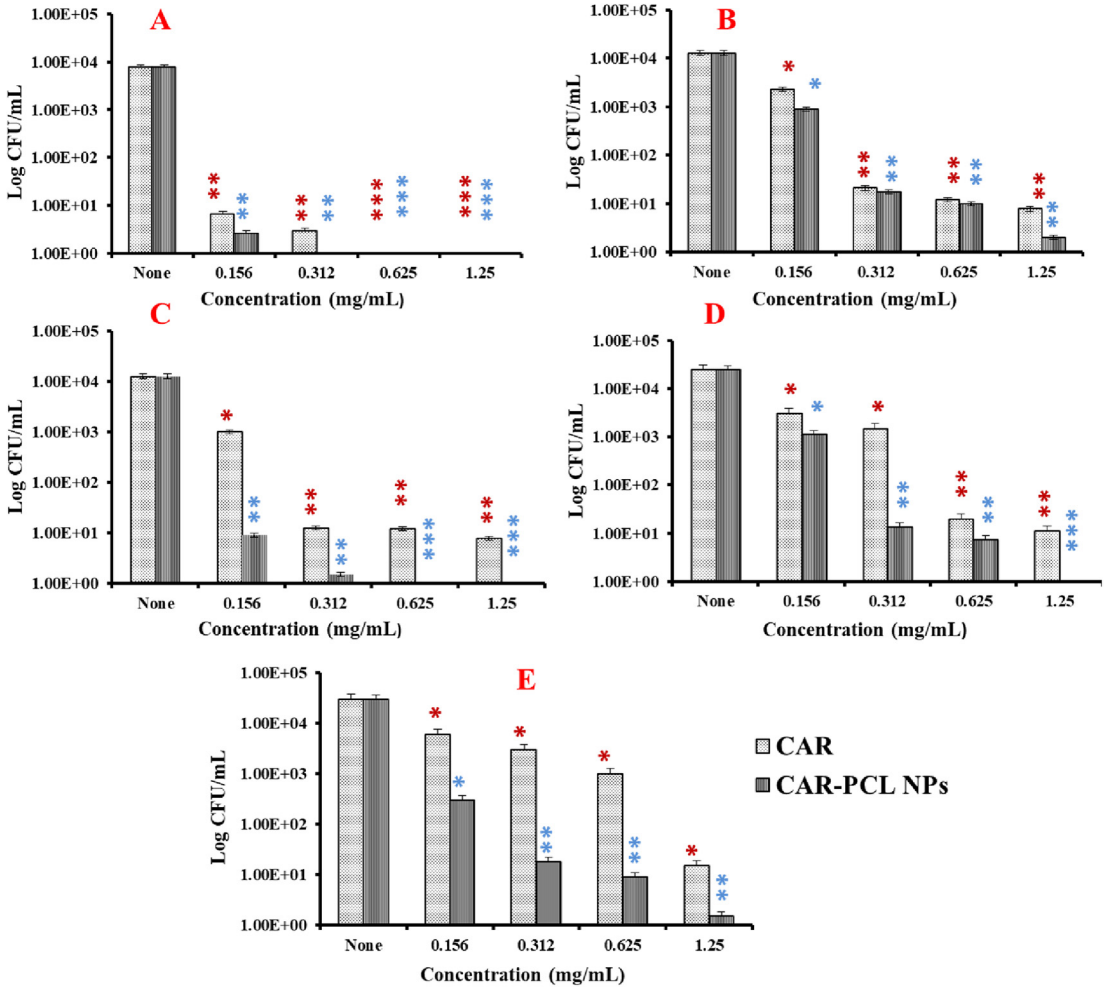

Fig. 3. Effect of exposure to various concentrations of CAR in free form and in the form of NPs on the killing of biofilm grown strains including Gram-positive bacteria (A = *S.aureus; Staphylococcus aureus* (NCTC®10788), (B = MRSA 1; *Staphylococcus aureus* (ATCC®33593™), (C = MRSA 2; *Staphylococcus aureus* [MW2] ATCC®BAA-1707™) and Gram-negative bacteria (D = PA 1; *Pseudomonas aeruginosa* ATCC®9027) and (E = PA 2; *Pseudomonas aeruginosa* [PAO1] ATCC®BAA- 47), (means ± SD., n = 3, *p values in comparison with control (no treatment), * $p < 0.05$, ** $p < 0.005$,***$p < 0.0005$).

and CAR-PCL NPs loaded hydrogel could deliver approximately 15–17% of total applied dose. However, free CAR permeated to the receiver compartment more rapidly in contrast to CAR-PCL NPs. Fig. 5 displays the kinetic profiles of CAR in both the epidermis (A) and dermis (B) layers of the neonatal porcine skin following the delivery of CAR-PCL NPs through all three AdminPen® devices. The results showed that the CAR content in dermal layers obtained using AdminPen® 777 was remarkably lower, as compared with AdminPen® 1200 ($p = 0.033$ for epidermis and $p = 0.0006$ for dermis) and AdminPen® 1500 ($p = 0.0045$ for epidermis and $p = 0.0002$ for dermis). The kinetic profile of AdminPen® 777 displayed that the C_{max} (163.27 ± 11.37 μg/cm^3) was achieved in the epidermis in 2.39 ± 0.32 h, while the maximum concentration of 557.91 ± 62.72 μg/cm^3 in the dermis was achieved in 7.53 ± 0.39 h. With respect to the profile of AdminPen® 1200 and AdminPen® 1500, the value of C_{max} in the epidermis and dermis both, were lower for AdminPen® 1200 in comparison with AdminPen® 1500, but the differences were not statistically significant ($p > 0.05$). Moreover, C_{max} for both AdminPen® 1200 and 1500 in dermal layers was achieved in approximately the same time (t_{max}). The dermatokinetic parameters for all three MNs devices are presented in Table 2.

In comparison to all three AdminPen® devices, CAR-PCL NPs loaded hydrogel showed remarkably lower C_{max} values and higher t_{max} and in both the epidermis (45.53 ± 10.29 μg/cm^3 in 11.40 ± 2.67 h) and

Table 1
Hemolytic activities of CAR and CAR-PCL NPs, (means ± SD., n = 3). * indicates the highest % hemolysis observed.

Sample	Optical density at 540 nm (mean ± S.D)		Hemolysis (% ± S.D)	
Distilled water	0.933 ± 0.001		Positive control	
Phosphate buffer (pH 7.4)	0.039 ± 0.001		Negative control	
	CAR	CAR-PCL NPs	CAR	CAR-PCL NPs
1.25	0.051 ± 0.001	0.046 ± 0.001	* 1.35 ± 0.17	* 0.78 ± 0.11
0.625	0.049 ± 0.002	0.044 ± 0.001	1.11 ± 0.23	0.63 ± 0.17
0.312	0.047 ± 0.002	0.044 ± 0.003	0.89 ± 0.22	0.59 ± 0.34
0.156	0.046 ± 0.003	0.043 ± 0.001	0.78 ± 0.23	0.44 ± 0.11
0.078	0.044 ± 0.002	0.041 ± 0.002	0.55 ± 0.40	0.29 ± 0.23
0.039	0.043 ± 0.001	0.042 ± 0.001	0.44 ± 0.11	0.29 ± 0.17

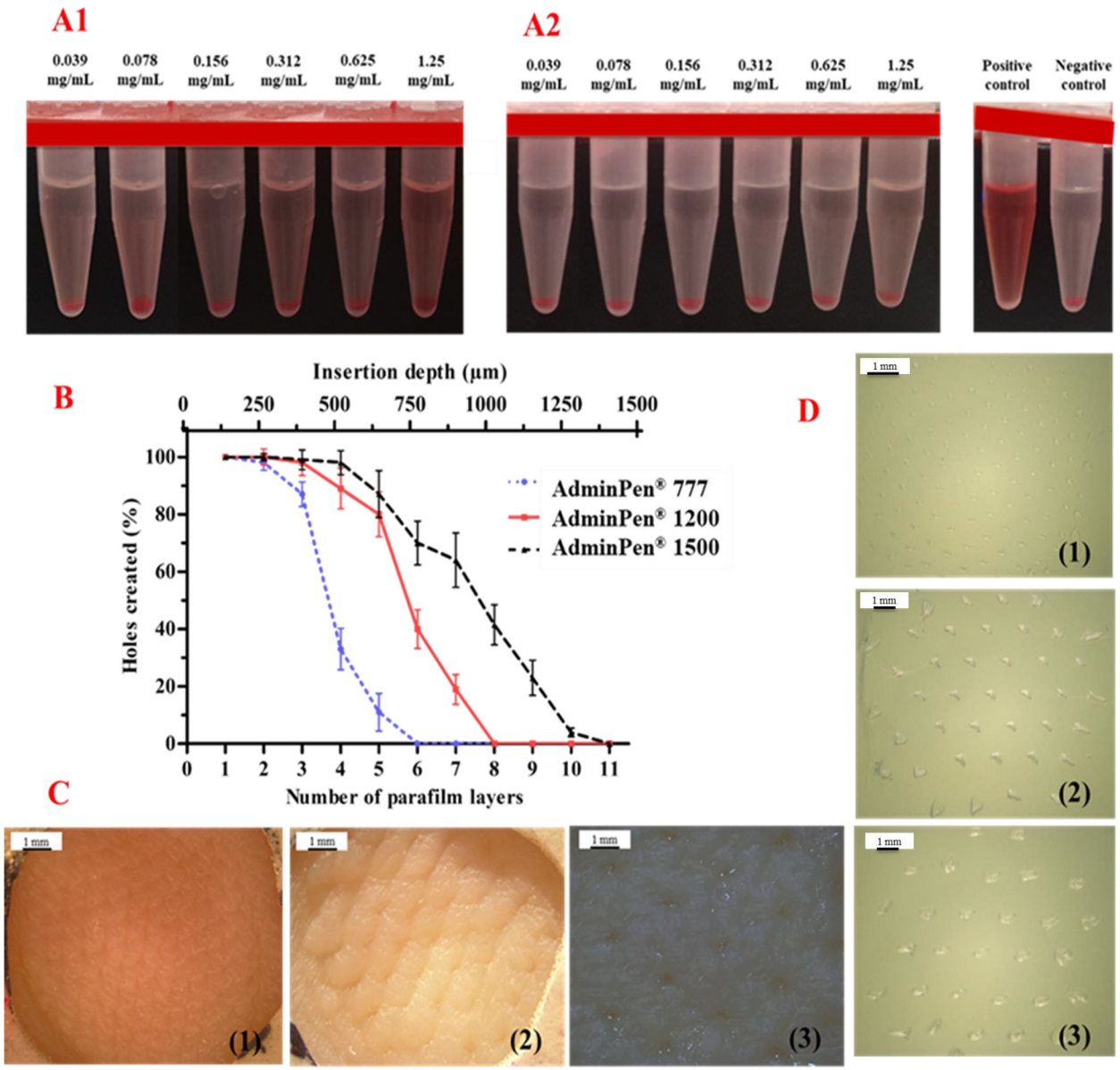

Fig. 4. Determination of hemolytic activity of free CAR (A1) and CAR-PCL NPs (A2) at a concentration range of 0.039 mg/mL to 1.25 mg/mL, by comparing the color of the serum and plasma with that of positive and negative controls. PBS (pH 7.4) and distilled water were included as negative and positive hemolytic controls, respectively. (B) Percentage of microconduits produced in each layer for evaluation of MNs insertion, (C) Digital images of the neonatal porcine skin presenting microconduits created in the skin upon insertion of AdminPen® devices; (1) AdminPen® 777, (2) AdminPen® 1200 and (3) AdminPen® 1500, (D) Digital images displaying insertion of AdminPen® devices in Parafilm M® layers; (1) AdminPen® 777, (2) AdminPen® 1200 and (3) AdminPen® 1500.

the dermis (374.15 ± 31.86 µg/cm^3 in 12.03 ± 1.90 h) (Mir et al., 2019), indicating that AdminPen® devices could potentially use to rapidly deliver higher concentrations as compared with hydrogel. The results have demonstrated that AdminPen® 1200 and AdminPen® 1500 can deliver considerably higher concentrations of CAR-PCL NPs to the skin in contrast to AdminPen® 777 (Table 2). This could be ascribed to their ability to more deeply penetrate the skin, creating deeper microconduits and allowing more liquid to flow from the syringe. The results of the permeation studies are demonstrated in Fig. 5 (C). AdminPen® 777 showed significantly lower permeation ($p = 0.0318$) as compared with AdminPen® 1500. However, AdminPen® 1200 and 1500 did not show any remarkable difference ($p = 0.834$) in CAR permeation.

A percentage comparison between all three AdminPen® devices, in terms of CAR content permeated and the content retained in the skin layers at various time points, is displayed in Fig. 5 (D). Among all three devices, AdminPen® 1500 delivered the highest concentrations of CAR *i.e.* approximately 94% of the total administered dose in comparison with AdminPen® 1200 (83%) and AdminPen® 777 (55%), that is significantly higher than CAR-PCL NPs loaded hydrogel (15% only).

Taking into consideration, the total CAR content delivered through all three AdminPen® devices, > 70% of CAR was determined in the skin layers even after 24 h of application that could be favorable to avoid frequent applications and hence better patient compliance. As displayed in Fig. 5 (D), at t_{max}, following the administration of CAR-PCL NPs through AdminPen® 777, 12.28 ± 1.32% of total applied CAR was found in the epidermis, 40.78 ± 6.3% in the dermis and only 0.21 ± 0.02% was permeated into the receiver compartment, which after 24 h of application reduced to 9.10 ± 1.62% in the epidermis, 34.57 ± 6.12% in the dermis and 9.40 ± 2.03% permeated. In the case of AdminPen® 1200, at t_{max}, 15.78 ± 2.41% was found in the epidermis, 64.78 ± 13.72% in the dermis and only 0.24 ± 0.03% was permeated, which after 24 h, reduced to 9.03 ± 2.93%,

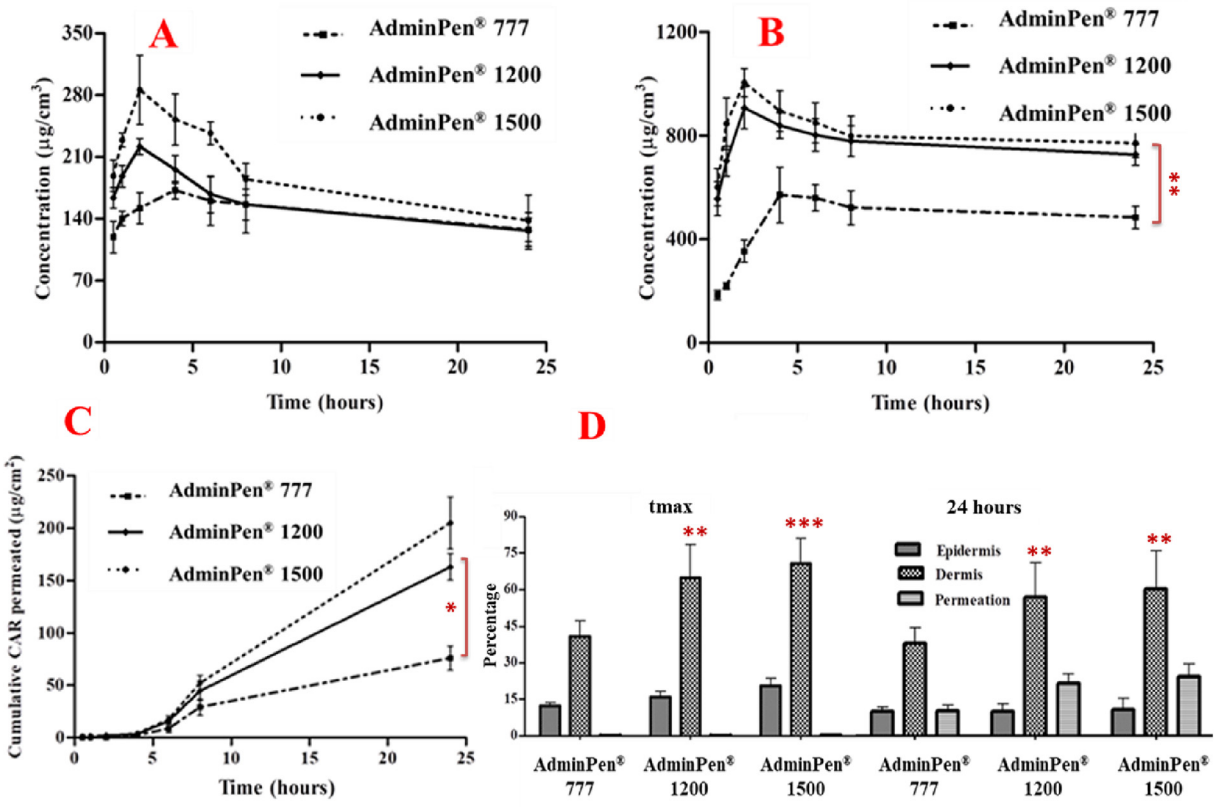

Fig. 5. Kinetic profile of CAR in the epidermis (A) and the dermis (B) followed by the delivery of CAR-PCL NPs through AdminPen® 777, Admin Pen® 1200 and Admin Pen® 1500 (means ± S.D., $n = 3$). (C) Permeation profile of CAR through the skin following the application of CAR-PCL NPs through AdminPen® devices (means ± S.D., $n = 3$), (D) Percentage comparison of CAR retained in the skin layers (considering the total dose applied) and permeated through the skin at t_{max} and 24 h following the administration of CAR-PCL NPs through AdminPen® devices (means ± S.D., $n = 3$). *p values in comparison of AdminPen® 1200 and 1500 with AdminPen® 777, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

Table 2
Dermatokinetic parameters of CAR-PCL NPs delivered through AdminPen® 777, Admin Pen® 1200 and Admin Pen® 1500, (mean ± SD., n = 3). * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, *p values in comparison of AdminPen® 1200 and 1500 with AdminPen® 777.

Dermatokinetic parameters		t_{max} (h)	C_{max} (µg/cm³)	AUC_{0-t} (µg/cm³*h)	$t_{1/2}$ ka (h)	$t_{1/2}$ k10 (h)
AdminPen® 777	Epidermis	2.39 ± 0.32	163.27 ± 11.37	3482.13 ± 302.23	0.31 ± 0.06	68.67 ± 21.35
	Dermis	7.53 ± 0.39	557.91 ± 62.72	11915.32 ± 1187.59	1.30 ± 0.17	69.4 ± 16.63
AdminPen® 1200	Epidermis	1.60 ± 0.43	*	*	0.24 ± 0.05	28.84 ± 9.32
			204.15 ± 8.56	3733.8 ± 384.60		
	Dermis	2.75 ± 0.44	**	**	0.36 ± 0.09	72.03 ± 19.76
			851.32 ± 21.09	18233.26 ± 495.03		
AdminPen® 1500	Epidermis	1.79 ± 0.20	**	**	0.31 ± 0.07	20.30 ± 6.63
			257.96 ± 50.68	4539.74 ± 61.59		
	Dermis	2.43 ± 0.73	***	***	0.32 ± 0.11	75.83 ± 5.43
			916.82 ± 32.68	19678.39 ± 867.86		

51.93 ± 14.13% and 19.7 ± 3.50% respectively. Similarly, AdminPen® 1500 showed CAR retention (in the form of NPs) in the skin layers, at 24 h with 9.85 ± 4.14% in the epidermis, 56.12 ± 14.41% in the dermis and 21.34 ± 4.51% permeation. In contrast, in the case of free CAR loaded hydrogel, after 6 h of application, only 0.2 ± 0.09% of total applied CAR was remained in epidermis, 4.85 ± 1.52% in the dermis and 7.93 ± 2.13% was permeated into receiving compartment, which at 24 h reduced to 0.04 ± 0.01% in the epidermis, 0.15 ± 0.06% in the dermis and 17.73 ± 3.56% showing almost negligible skin retention (Mir et al., 2019). The highest percentage delivery obtained with AdminPen® 1500 suggested its superiority over AdminPen® 777 and 1200. Therefore, AdminPen® 1500 was selected to perform the *in vivo* studies in order to potentially deliver the higher concentrations of CAR-PCL NPs, as compared with AdminPen® 777 and 1200, and hydrogel.

3.6. Evaluation of antimicrobial activity in an ex vivo wound model

Following the biofilm infection in the *ex vivo* skin wound model, the CFU counts displayed a considerable decrease in bacterial biofilm load in treated wounds as compared with untreated wounds (Fig. 6). However, wounds treated with blank PCL NPs were not significantly different from untreated wounds (positive control). In contrast to untreated biofilms (100% viability), both CAR-PCL NPs delivered through AdminPen® 1500 and CAR loaded hydrogel showed > 99% decrease in bacterial load in wounds infected by both Gram-positive and Gram-negative test strains because required effective concentrations of CAR-PCL NPs were available at the desired site. This *ex vivo* model did not display any remarkable difference between the effect of free CAR and CAR-PCL NPs in terms of antibiofilm activity, because firstly, dose of CAR and CAR-PCL NPs applied was above their minimum bactericidal

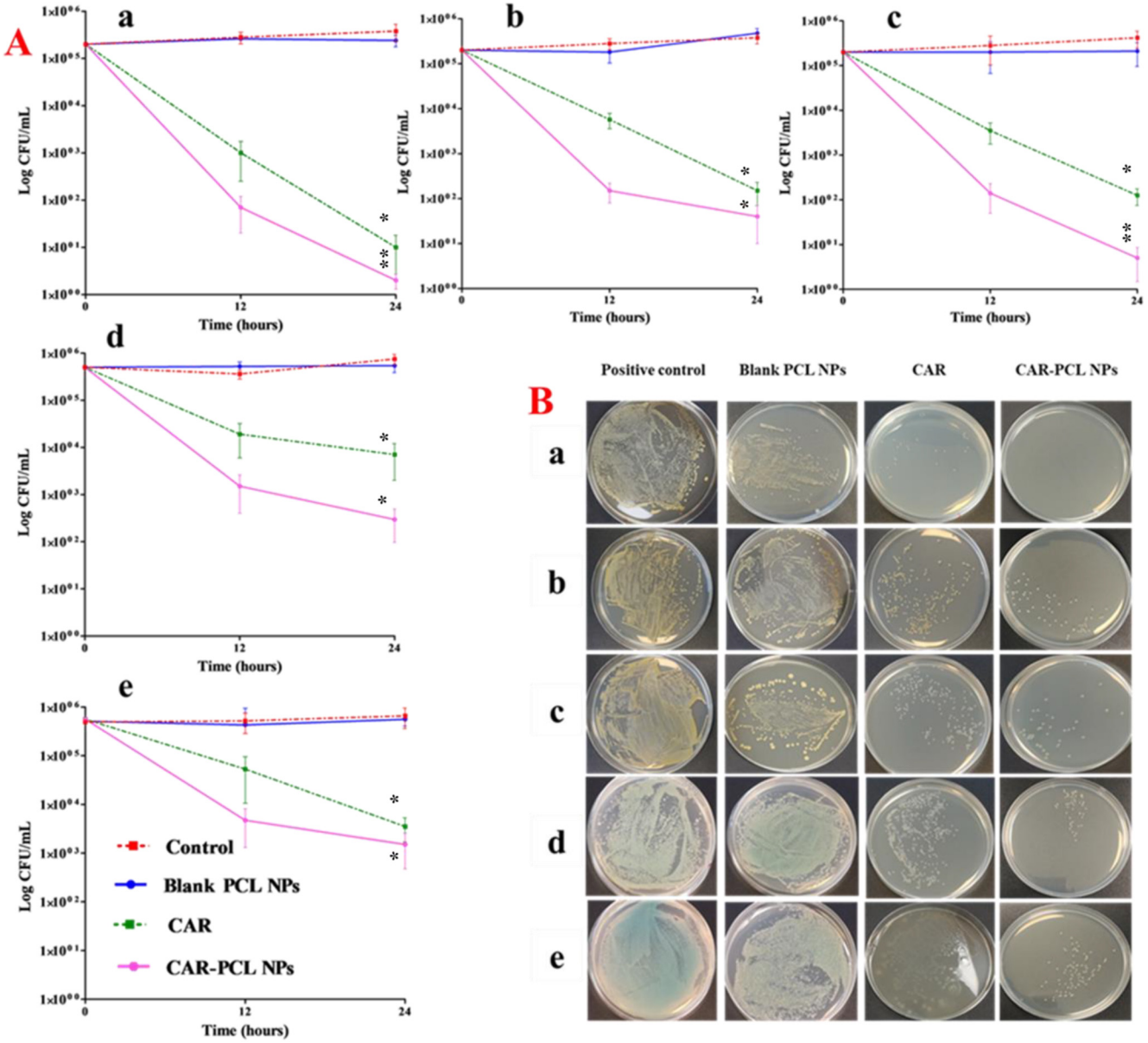

Fig. 6. (A) Bacterial viability (log CFU/mL) in an *ex vivo* biofilm wound model (a) *S.aureus; Staphylococcus aureus* (NCTC®10788), (b) MRSA 1; *Staphylococcus aureus* (ATCC®33593™), (c) MRSA 2; *Staphylococcus aureus* [MW2] ATCC®BAA-1707™), (d) PA 1; *Pseudomonas aeruginosa* ATCC®9027), and (e) PA 2; *Pseudomonas aeruginosa* [PAO1] ATCC®BAA- 47), following the application of free CAR in the form of hydrogel and blank PCL NPs in contrast to CAR-PCL NPs administered through AdminPen® 1500, (means ± S.D., $n = 3$, *p values in comparison with control, * $p < 0.05$, ** $p < 0.01$). At 12 h and 24 h post-application, *ex vivo* biofilm-infected skin was homogenized in sterile PBS and cultured onto agar plates at 37 °C overnight. (B) Photographs of culture plates, depicting *ex vivo* clearance of bacterial biofilms from porcine skin wounds (a) *S.aureus*, (b) MRSA 1, (c) MRSA 2, (d) PA 1, and (e) PA 2, following the treatment with CAR (in the form of hydrogel), blank PCL NPs and CAR-PCL NPs (administered through AdminPen® 1500).

concentrations, secondly, it was a closed system and the applied dose remained available at the site of application (that presumably will not occur in an *in vivo* open model, as also manifested by the results of *in vivo* experiment, showing rapid clearance of free CAR from the site of application in comparison with CAR-PCL NPs). Although, these results manifested the antibiofilm potential of CAR-PCL NPs in *ex vivo* model, however, it is needed to determine their *in vivo* antimicrobial potential.

3.7. In vivo studies

The profile of CAR in the skin following the administration through AdminPen® 1500 was significantly different ($p < 0.0001$) from that of the hydrogel, as can be seen in Fig. 7. The delivered amount of CAR to the skin (≈ 650 μg) using AdminPen® 1500 was approximately 48% of the dose administered, which is around 8.5 times higher than the concentration of CAR delivered (≈ 76 μg) through the CAR loaded hydrogel. Approximately 85% of the delivered CAR from the AdminPen® 1500 was retained at the site of application for 24 h, > 65% for 48 h, and approximately 50% at 72 h. However, < 30% of the delivered CAR from the hydrogel was retained in the skin at 24 h, which was further reduced to 5.2% only at 72 h, as illustrated in Fig. 7A. The kinetic profile of AdminPen® 1500 showed that C_{max} in the skin was achieved to be 674.10 ± 17.15 μg/cm^3 after 1.66 ± 1.15 h. While the highest concentration of 76.92 ± 4.63 μg/cm^3 was achieved in 1.66 ± 0.94 h for CAR loaded hydrogel, which is significantly lower ($p < 0.0001$) than the concentration achieved through AdminPen® 1500. All the kinetic parameters in the skin and plasma were calculated for both the delivery systems (hydrogel and AdminPen® 1500)

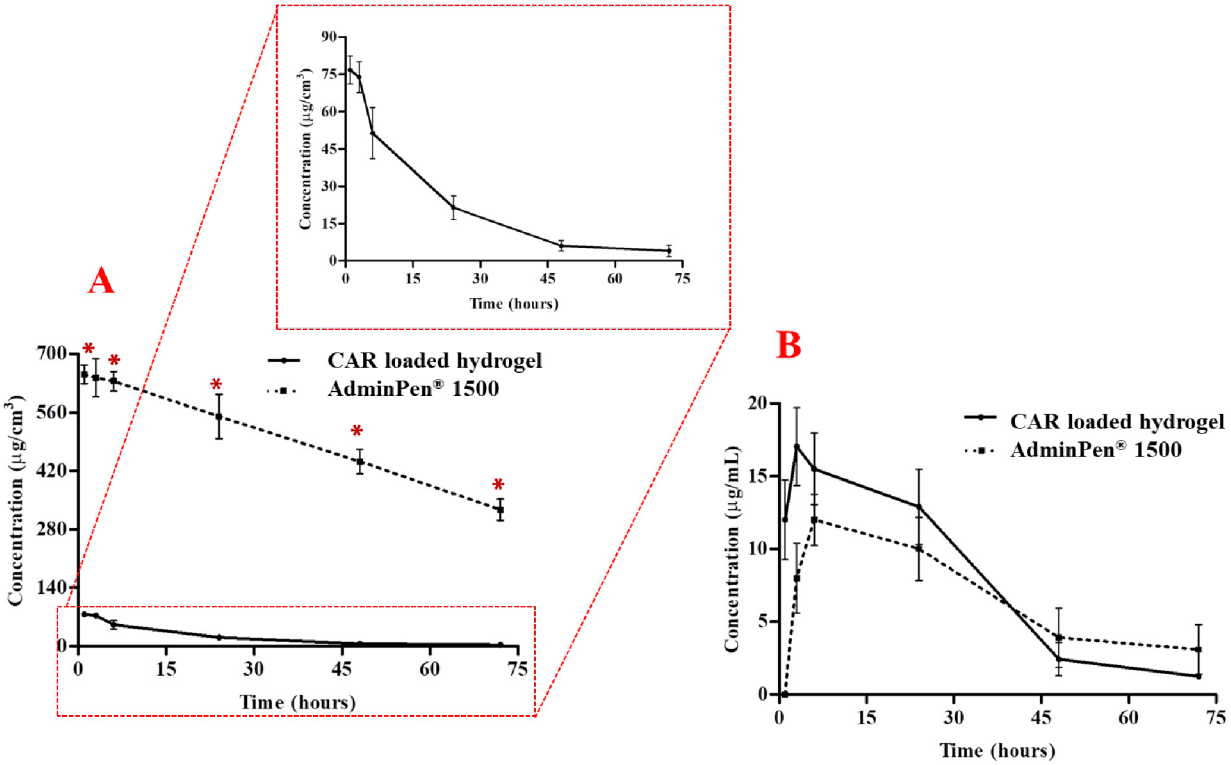

Fig. 7. Kinetic profile in the mice skin (A) following the delivery of CAR-PCL NPs through AdminPen® 1500 as compared to CAR loaded hydrogel (highlighted in a red square) (means ± S.D., n = 3, *p values in comparison with free CAR loaded hydrogel, * p < 0.0001). (B) Kinetic profile of CAR in the mice plasma following the delivery of CAR-PCL NPs through AdminPen® 1500 in contrast to CAR loaded hydrogel (means ± S.D., n = 3).

(Supplementary Table S2). CAR administered through both the hydrogel and AdminPen® 1500, reached the blood, but in very low concentrations. As illustrated in Fig. 7B, C_{max} achieved in case of the hydrogel (17.04 ± 3.21 μg/mL in 4.0 ± 1.73 h) was slightly higher than the C_{max} obtained for AdminPen® 1500 (12.21 ± 3.87 in 6.0 ± 0.02 h), but not statistically different ($p = 0.254$).

Thus, the results clearly indicated the ability of AdminPen® 1500 to deliver higher concentrations of CAR in the form of NPs in contrast to the hydrogel. Concerning the concentration of CAR-PCL NPs delivered is higher than the MIC/MBC values (*in vitro/ex vivo*) of CAR-PCL NPs against both planktonic forms and biofilm grown test strains. Furthermore, the delivered CAR-PCL NPs remained available at the site of application for a longer time that could potentially favor the sustained antimicrobial effect. Very little concentrations of CAR reaching the blood, following administration of CAR-PCL NPs, further confirmed that NPs have potential to be retained in the skin.

4. Conclusion

This study displayed the antibacterial potential of CAR-PCL NPs against biofilms of multidrug resistant pathogens. Biocompatibility studies indicated the biosafety of the designed NPs at desired concentrations. Based on insertion properties and *ex vivo* dermatokinetic studies, AdminPen® 1500 was found to be an optimum one in comparison to AdminPen® 777 and 1200. Moreover, animal studies showed the successful *in vivo* delivery of CAR-PCL NPs to the skin, where bacteria can grow and form biofilms, indicating the suitability of MN liquid injection system for direct delivery of CAR-PCL NPs. The principal benefit of this established site-selective delivery strategy introduced here, as compared with application of pure CAR, depends on the ability of AdminPen® device to directly deliver the NPs to the infected wounds and further on NP's potential for skin retention, which may lead to prolonged desired antibacterial effectiveness circumventing the frequent applications. Hence, it is proved that AdminPen® assisted delivery of infection responsive NPs could be a promising strategy for enhanced site-specific delivery of CAR to the infected chronic wounds. However, further *in vivo* investigations must now be performed in appropriate infection models.

CRediT authorship contribution statement

Maria Mir: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft. **Andi Dian Permana:** Investigation, Methodology, Validation. **Ismaiel A. Tekko:** Investigation, Methodology. **Helen O. McCarthy:** Investigation, Methodology. **Naveed Ahmed:** Writing - review & editing, Visualization. **Asim. ur. Rehman:** Funding acquisition, Resources, Supervision, Writing - review & editing. **Ryan F. Donnelly:** Conceptualization, Resources, Supervision, Project administration, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2020.119643.

References

Alhusein, N., Blagbrough, I.S., Beeton, M.L., Bolhuis, A., De Bank, P.A., 2016. Electrospun zein/PCL fibrous matrices release tetracycline in a controlled manner killing *Staphylococcus aureus* both in biofilms and *ex vivo* on pig skin, and are compatible with human skin cells. Pharm. Res. 33, 237–246.

Antonia, N., Teresa, P., 2012. Antimicrobial activity of carvacrol: current progress and future prospectives. Recent Pat. Antiinfect. Drug Discov. 7, 28–35.

Bessa, L.J., Fazii, P., Di Giulio, M., Cellini, L., 2015. Bacterial isolates from infected wounds and their antibiotic susceptibility pattern: some remarks about wound infection. Int. Wound J. 12, 47–52.

Caffarel-salvador, E., Kearney, M., Mairs, R., Gallo, L., Stewart, S.A., Brady, A.J., Donnelly, R.F., 2015. Methylene blue-loaded dissolving microneedles: potential use in photodynamic antimicrobial chemotherapy of infected wounds. Pharmaceutics. 7, 397–412.

De Kievit, T.R., Iglewski, B.H., 2000. Bacterial quorum sensing in pathogenic relationships. Infect. Immun. 68, 4839–4849.

Doern, G.V., Jones, R.N., Pfaller, M.A., Kugler, K.C., Beach, M.L., 1999. Bacterial pathogens isolated from patients with skin and soft tissue infections: frequency of occurrence and antimicrobial susceptibility patterns from the SENTRY Antimicrobial Surveillance Program (United States and Canada, 1997). Diagn. Microbiol. Infect. Dis. 34, 65–72.

Donlan, R.M., Costerton, J.W., 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin. Microbiol. Rev. 15, 167–193.

Donlan, R.M., 2001. Biofilm formation: a clinically relevant microbiological process. Clin. Infect. Dis. 33, 1387–1392.

Donnelly, R.F., Singh, T.R.R., Tunney, M.M., Morrow, D.I., McCarron, P.A., O'Mahony, C., Woolfson, A.D., 2009. Microneedle arrays allow lower microbial penetration than hypodermic needles *in vitro*. Pharm. Res. 26, 2513–2522.

Gilmer, D.B., Schmitz, J.E., Euler, C.W., Fischetti, V.A., 2013. Novel bacteriophage lysin with broad lytic activity protects against mixed infection by *Streptococcus pyogenes* and methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents. Chemother. 57, 2743–2750.

Gottrup, F., 2004. A specialized wound-healing center concept: importance of a multidisciplinary department structure and surgical treatment facilities in the treatment of chronic wounds. Am. J. Surg. 187, 38S–43S.

Gunal, M.Y., Heper, A.O., Zaloglu, N., 2014. The effects of topical carvacrol application on wound healing process in male rats. Pharmacogn. J. 6, 10–14.

Harding, K.G., Morris, H.L., Petal, G.K., 2002. Healing chronic wounds. Br. Med. J. 324, 160–163.

Ouhayoun, J.P., 2003. Penetrating the plaque biofilm: impact of essential oil mouthwash. J. Cinical Periodontol. 30, 10–12.

Jafri, H., Husain, F.M., Ahmad, I., 2014. Antibacterial and antibiofilm activity of some essential oils and compounds against clinical strains of *Staphylococcus aureus*. J. Biomed. Ther. Sci. 1, 65–71.

Järbrink, K., Ni, G., Sönnergren, H., Schmidtchen, A., Pang, C., Bajpai, R., Car, J., 2017. The humanistic and economic burden of chronic wounds: a protocol for a systematic review. Syst. Rev. 6, 15–21.

Järbrink, K., Ni, G., Sönnergren, H., Schmidtchen, A., Pang, C., Bajpai, R., Car, J., 2016. Prevalence and incidence of chronic wounds and related complications: a protocol for a systematic review. Syst. Rev. 5, 1–6.

Larrañeta, E., Moore, J., Vicente-pérez, E.M., González-vázquez, P., Lutton, R., Woolfson, A.D., Donnelly, R.F., 2014. A proposed model membrane and test method for microneedle insertion studies. Int. J. Pharm. 472, 65–73.

Manzoor-ul-Haq, R.V., Singh, D., Singh, A.K., Ninganagouda, S., Hiremath, J., 2015. Dried mushroom *Agaricus bisporus* mediated synthesis of silver nanoparticles from Bandipora District (Jammu and Kashmir) and their efficacy against methicillin resistant *Staphylococcus aureus* (MRSA) strains. Nanosci. Nanotechnol. Int. J. 5, 1–8.

Marchese, A., Arciola, C.R., Coppo, E., Barbieri, R., Barreca, D., Chebaibi, S., Sobarzo-Sánchez, E., Nabavi, S.F., Nabavi, S.M., Daglia, M., 2018. The natural plant compound carvacrol as an antimicrobial and anti-biofilm agent: mechanisms, synergies and bio-inspired anti-infective materials. Biofouling. 34, 630–656.

Martinengo, L., Olsson, M., Bajpai, R., Soljak, M., Upton, Z., Schmidtchen, A., Car, J., Järbrink, K., 2019. Prevalence of chronic wounds in the general population: systematic review and meta-analysis of observational studies. Ann. Epidemiol. 29, 8–15.

Mir, M., Ahmed, N., Permana, A.D., Rodgers, A.M., Donnelly, R.F., Rehman, A., 2019. Enhancement in site-specific delivery of carvacrol against methicillin resistant staphylococcus aureus induced skin infections using enzyme responsive nanoparticles : a proof of concept study. Pharmaceutics. 11, 606.

M. Mir A.D. Permana N. Ahmed G.M. Khan A. Rehman ur, Donnelly, R.F., Enhancement in site-specific delivery of carvacrol for potential treatment of infected wounds using infection responsive nanoparticles loaded into dissolving microneedles: a proof of concept study Eur. J. Pharm. Biopharm. 147 2020 57 68.

Nostro, A., Roccaro, A.S., Bisignano, G., Marino, A., Cannatelli, M.A., Pizzimenti, F.C., Cioni, P.L., Procopio, F., Blanco, A.R., 2007. Effects of oregano, carvacrol and thymol on *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. J. Med. Microbiol. 56, 519–523.

Nostro, A., Scaffaro, R., Botta, L., Filocamo, A., Marino, A., Bisignano, G., Scaffaro, R., Botta, L., Filocamo, A., Marino, A., Bisignano, G., 2015. Effect of temperature on the release of carvacrol and cinnamaldehyde incorporated into polymeric systems to control growth and biofilms of *Escherichia coli* and *Staphylococcus aureus*. Biofouling. 31, 639–649.

Permana, A.D., Mccrudden, T.C., Donnelly, R.F., 2019. Enhanced intradermal delivery of nanosuspensions of antifilariasis drugs using dissolving microneedles : a proof of concept study. Pharmaceutics. 11, 1–22.

Poole, K., 2011. Pseudomonas aeruginosa: resistance to the max. Front. Microbiol. 65, 1–13.

Raj, R., Singh, T., Tekko, I., Mcavoy, K., Donnelly, R.F., Mcmillian, H., Jones, D., 2016. Minimally invasive microneedles for ocular drug delivery. Expert. Opin. Drug. Deliv. 5247, 525–537.

Rhoads, D.D., Wolcott, R.D., Percival, S.L., 2008. Biofilms in wounds: management strategies. J. Wound Care 17, 502–508.

Roche, E.D., Woodmansey, E.J., Yang, Q., Gibson, D.J., Zhang, H., Schultz, G.S., 2019. Cadexomer iodine effectively reduces bacterial biofilm in porcine wounds *ex vivo* and *in vivo*. Int. Wound J. 16, 674–683.

Scaffaro, R., Lopresti, F., D'Arrigo, M., Marino, A., Nostro, A., 2018. Efficacy of poly (lactic acid)/ carvacrol electrospun membranes against *Staphylococcus aureus* and *Candida albicans* in single and mixed cultures. Appl. Microbiol. Biotechnol. 102, 4171–4181.

Sen, C.K., Gordillo, G.M., Roy, S., Kirsner, R., Lambert, L., Hunt, T.K., Gottrup, F., Gurtner, G.C., Longaker, M.T., 2009. Human skin wounds: A major and snowballing threat to public health and the economy. Wound Repair Regen. 17, 763–771.

Serrano, D.R., Hernández, L., Fleire, L., González-Alvarez, I., Montoya, A., Ballesteros, M.P., Dea-Ayuela, M.A., Miró, G., Bolás-Fernández, F., Torrado, J.J., 2013. Hemolytic and pharmacokinetic studies of liposomal and particulate amphotericin B formulations. Int. J. Pharm. 447, 38–46.

Stadelmann, W.K., Digenis, A.G., Tobin, G.R., 1998. Physiology and healing dynamics of chronic cutaneous wounds. Am. J. Surg. 176, 26S–38S.

Tong, S.Y., Davis, J.S., Eichenberger, E., Holland, T.L., Fowler, V.G., 2015. *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management. Clin. Microbiol. Rev. 28, 603–661.

Živković, V., Kurevija, T., Haršanji, D.I., Bogdan, M., Tomić, P.M., Talapko, J., Drenjančević, D., 2018. To Biofilm or Not to Biofilm ? Southeast. Eur. Med. J. 2, 12–19.

Walmsley, S., 2002. Advances in wound management. Clin. Reports. 5, 5–7.

Yang, Z.G., Sun, H.X., Fang, W.H., 2005. Haemolytic activities and adjuvant effect of *Astragalus membranaceus* saponins (AMS) on the immune responses to ovalbumin in mice. Vaccine. 23, 5196–5203.

Zaric, M., Lyubomska, O., Touzelet, O., Poux, C., Al-zahrani, S., Fay, F., Wallace, L., Terhorst, D., Malissen, B., Henri, S., Power, U.F., Scott, C.J., Donnelly, R.F., Kissenpfennig, A., 2013. Skin dendritic cell targeting via microneedle arrays laden with co-glycolide nanoparticles induces efficient antitumor and antiviral. ACS Nano 7, 2042–2055.

Zhou, H.Y., Zhang, Y.P., Zhang, W.F., Chen, X.G., 2011. Biocompatibility and characteristics of injectable chitosan-based thermosensitive hydrogel for drug delivery. Carbohydr. Polym. 83, 1643–1651.