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Exploitation of sub-micron cavitation nuclei to enhance ultrasound-mediated transdermal transport and penetration of vaccines

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ABSTRACT

Inertial cavitation mediated by ultrasound has been previously shown to enable skin permeabilisation for transdermal drug and vaccine delivery, by sequentially applying the ultrasound then the therapeutic in liquid form on the skin surface. Using a novel hydrogel dosage form, we demonstrate that the use of sub-micron gas-stabilising polymeric nanoparticles (nanocups) to sustain and promote cavitation activity during simultaneous application of both drug and vaccine results in a significant enhancement of both the dose and penetration of a model vaccine, Ovalbumin (OVA), to depths of 500 μm into porcine skin. The nanocups themselves exceeded the penetration depth of the vaccine (up to 700 μm) due to their small size and capacity to 'self-propel'. *In vivo* murine studies indicated that nanocup-assisted ultrasound transdermal vaccination achieved significantly ($p < 0.05$) higher delivery doses without visible skin damage compared to the use of a chemical penetration enhancer. Transdermal OVA doses of up to 1 μg were achieved in a single 90-second treatment, which was sufficient to trigger an antigen-specific immune response. Furthermore, ultrasound-assisted vaccine delivery in the presence of nanocups demonstrated substantially higher specific anti-OVA IgG antibody levels compared to other transdermal methods. Further optimisation can lead to a viable, safe and non-invasive delivery platform for vaccines with potential use in a primary care setting or personalized self-vaccination at home.

1. Introduction

High rates of infectious disease transmission from needle stick injuries have led to 500,000 deaths from Hep B worldwide in 2004 [1]. Additionally, the emotional trauma and pain associated with injections inhibit patient compliance [2]. Thus, increasing widespread recognition of the disadvantages associated with the use of needles has fuelled research toward non-invasive alternatives. In the context of immunisation, several safe alternative methods of achieving reliable and effective vaccination are under development. Transdermal immunisation (TI) systems in particular have garnered much attention because of the richly immune-cell-populated milieu beneath, ultimately eliciting effective immune responses in both systemic and mucosal compartments [3,4]. Such devices also bypass the hepatic/gastrointestinal metabolism that detrimentally alter immune response profile. This approach involves the application of devices that transiently permeabilise the stratum corneum (SC) in order to enable vaccine delivery. A range of technologies has been proposed and developed in order to overcome the barrier presented by the SC to achieve transcutaneous needle free vaccination, including ballistics-based gene gun devices [5], microneedles [6], iontophoresis [7], chemical enhancers and ultrasound-mediated cavitation [8]. This last approach utilises the shockwaves and microstreaming created by the expansion and violent collapse of gas bubbles in response to ultrasound exposure and is particularly attractive because it can be achieved using safe, low-cost technology already widely available within healthcare settings [9].

The vast majority of studies exploring the use of ultrasound-mediated permeabilisation of the skin have demonstrated that inertial cavitation and related mechanisms are effective in increasing the permeability of the skin to drug and vaccine molecules of around 40 to 70 kDa [8]. Early studies did not use a cavitation nucleation agent, and involved pre-exposure of the skin to ultrasound followed by application of the therapeutic in liquid form on the skin [10–12]. Ultrasound was thus primarily used as a mechanism to enhance skin permeabilisation, rather than the active transport of the therapeutic across the stratum corneum. More recent studies have utilised a simultaneous treatment protocol, whereby through the simultaneous application of drug solution and ultrasound, a higher dose of the drug is driven deeper into the skin [8,10].

Recent work has shown that inertial cavitation nucleated by micron-sized talc particles can also promote the active transport of molecules through a tissue mimicking material (TMM) by simultaneous application of ultrasound and model vaccine [13]. Alternative cavitation nuclei candidates with more clinical translatability include microbubbles

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(widely used as ultrasound contrast agents) and micron sized particles [14]. However, these candidates are of limited utility for transdermal delivery due to their rapid depletion and destruction at the skin surface. Recently, nano-sized cavitation nuclei (nanocups) capable of instigating and promoting inertial cavitation at modest pressure amplitudes and frequencies have been developed [15,16]. These particles mediate sustained cavitation for many minutes, allowing durable exploitation of inertial cavitation mechanisms. Additionally, their size allows for higher concentrations of cavitation nuclei particles and therefore higher intensities of inertial cavitation in a given volume of dosage media.

Nanocups have recently demonstrated enhanced transport of intravenously delivered drugs into tumours [15]. The use of nano-cavitation nuclei in a transdermal delivery system offers exciting potential for vaccine delivery, because they can potentially not only be used to enhance skin permeability but also to mediate the active transport of therapeutics across the stratum corneum without depletion. Here we seek to demonstrate the utility of nanocups for enhanced ultrasound-mediated transdermal vaccine delivery, by embedding them alongside (but not chemically bound to) a model vaccine in a hydrogel formulation that can also serve as a novel dosage form. This approach enables simultaneous application of ultrasound-mediated cavitation with the model vaccine, making it possible to explore the impact of nanoscale cavitation events on both skin permeability and active transport of the therapeutic both *ex vivo* and *in vivo*.

2. Methods and materials

2.1. Ex vivo skin source

Porcine skin was chosen as a model for human skin based on its similarities to human skin in terms of structure, thickness and cellular composition [17]. Furthermore, it has similar characteristic impedance ensuring comparable transmission of ultrasound into the skin [18,19]. Experiments were performed on full thickness porcine skin harvested from the medial thigh of the animals immediately after abattoir sacrifice (female Landrace pigs, weighing 40–50 kg). Hair on the surface of the skin was trimmed. Shaving and the use of hair removal chemicals were not performed to prevent affecting the integrity of the SC. Skin was then cut into smaller (50 × 50 mm) pieces, wrapped in aluminium foil and stored in a 4 °C fridge for no longer than 3 days. Prior to an experiment, skin was mounted in a Franz diffusion cell shown in Supplementary Fig. S1.

2.2. Nanocup (NC) cavitation nuclei formulation and characterisation

Nanocups (NCs) are gas-stabilising solid 'cup' shaped polymeric nanoparticles [15]. Briefly they are manufactured in a seed polymerisation reaction and are composed of a copolymer of hydroxyethylmethyl methacrylate (HEMA) and methyl methacrylate (MMA) formed around and linked to a poly(styrene-co-divinylbenzene) (PS:DVB) copolymer core (10:1:6 v/v/v ratio of MMA:HEMA:DVB formed around a 300 nm diameter PS bead). Dynamic light scattering measured the diameter of NCs to be 460 nm ± 24 nm and the zeta potential as -15 mV ± 1 mV (n = 10).

2.3. Ex vivo coupling gel formulation

Before formulation within an ultrasound coupling gel [20], model vaccine and NCs were fluorescently labelled to enable tracking of their delivery through the skin. OVA (A5503, Sigma-Aldrich, UK) was conjugated with FITC dye using the Fluorotag FITC conjugation kit (FITC1, Sigma-Aldrich, UK). FITC-OVA was then added to each gel (±NC) at a concentration of 1.5 mg/mL in the gel. NC were labelled with anthracene, (excitation peak 362 nm (UV))/emission peak 407 nm (blue), to give fluorescent nanocups (F-NCs), and were added to the FITC-OVA laden gel at a concentration of 5×10^9 NC/mL.

SDS PAGE, fluorescence spectroscopy (Luminescence Spectrometer LS 50B, Perkin Elmer, USA) and dynamic light scattering (Zetasizer Nano ZS, laser wavelength 633 nm; Malvern Instruments, UK) were employed to verify OVA stability before and after ultrasound exposure in the presence and absence of NCs.

2.4. Therapeutic ultrasound setup, exposure parameters and acoustic cavitation detection

A focused ultrasound (FUS) transducer operating at 256 kHz at pressure amplitudes not exceeding 1.5 MPa was used to initiate cavitation in the coupling gel formulation. The FUS system is shown in Supplementary Fig. S1, and described in the legend to Supplementary Fig. S1 and in Ref. [13]. Acoustic emissions associated with acoustic cavitation were recorded throughout exposure using a 5 MHz passive cavitation detector confocally and co-axially aligned with the FUS transducer [20].

2.5. Electrical resistivity measurement

The comparison of the electrical conductivity of the skin is the current gold standard in detection and monitoring of skin permeability *in vitro* [21]. In order to determine permeability increases in the skin as a result of each treatment condition, the electrical resistance of the skin was used to determine conductivity enhancement ratios, as described in Tezel et al. [22]. Measurements were taken with PBS in both the donor and receiver compartments prior to and immediately following each treatment [23].

2.6. Multi-photon microscopy

Skin samples were imaged using multi-photon microscopy (MPM) to assess penetration of fluorescently labelled OVA and NCs. Immediately following ultrasound exposure, the treated skin samples were coated with fluorescent red 2 μm diameter latex beads (SpheroTech, USA) to demarcate the skin surface. These 2 μm beads were chosen because they were too large to diffuse through the skin. Thus the beads acted as markers for the skin surface to allow visualisation and quantification of the penetration depths of both FITC-OVA and nanocups in the skin. A modified BioRad Radiance 2100 MP Multiphoton Microscope (Zeiss; Jena, Germany), was used for 3D imaging. Near infrared (NIR) laser beams (λ = 800 nm) were obtained from a tunable 76 MHz femtosecond pulsed Ti:sapphire laser (Mira 900-F, Coherent, Ely, UK) pumped by a 10 W multiline argon ion laser (Verdi; Coherent). A Nikon S Fluor 20× objective (NA = 0.75) was used for all images. The emission wavelengths of the filters selected were: 495 nm (blue), 525 nm (green), and 595 nm (red). 3 dimensional, 3 colour images were obtained of XY dimension 389 × 389 μm up to 1.5 mm deep (z-slice resolution 1.95 μm) into each skin sample. 3D representations were generated from the data acquired using Imaris software (Bitplane, USA). Data was post processed using Matlab software (Mathworks, USA). A custom written algorithm obtained an extrapolated skin surface from the red beads, and then measured the depths in each column of the 3D image between this skin surface and every voxel containing FITC-OVA (stained green) or F-NC (stained blue) to obtain a distribution of depths. The depth distribution was weighted according to the fluorescence intensity of each voxel analysed, with a view to giving the intensity distribution of the FITC-OVA and F-NCs in each measured area of the skin. Positive control samples were not imaged due to the denaturation of FITC-OVA by sodium dodecyl sulphate (SDS), rendering it non-fluorescent.

2.7. In vivo study design

Studies were performed in line with UK Home Office legislation. 40 five-week-old BALB/c female mice were obtained from the Biomedical Services Unit at the John Radcliffe Hospital (Oxford, UK). Mice were divided into two 20 mice treatment groups consisting of a Delivery and