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Ultrasound-enhanced penetration through sclera depends on frequency of sonication and size of macromolecules

Ying Chau [a,b,*], Wai Leung Langston Suen [b], Ho Yan Tse [b], Hoi Sang Wong [b]

[a] Division of Biomedical Engineering, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong
[b] Department of Chemical and Biomolecular Engineering, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong

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ABSTRACT

We previously employed ultrasound as a needleless approach to deliver macromolecules via the transscleral route to the back of the eye in live animals (Suen et al., 2013). Here, we investigated the nature of the ultrasound-enhanced transport through sclera, the outermost barrier in the transscleral route. Thus, the possible role of cavitation from ultrasound was explored; its effect during and after sonication on scleral penetration was measured; and the dependence on the size of macromolecules was determined. We applied ultrasound frequency from 40 kHz to 3 MHz at I_{SATA} (spatial-average-temporal-average intensity) of 0.05 W/cm^2 to fresh rabbit sclera ex vivo. Fluorescent dextran of size 20 kDa to 150 kDa was used as macromolecular probes. We measured the distance of penetration of the probes through the sclera over 30 s during sonication and over 15 min after sonication from cryosectioned tissue images. Deeper penetration in the sclera was observed with decreasing frequency. The presence of stable cavitation was further verified by passive acoustic detection. The effect during sonication increased penetration distance up to 20 fold and was limited to macromolecular probes ≤70 kDa. The effect post sonication increased penetration distance up to 3 fold and attributed to the improved intrascleral transport of macromolecules ≥70 kDa. Post-sonication enhancement diminished gradually in 3 h. As the extent of cavitation increased with decreasing frequency, the trend observed supports the contribution of (stable) cavitation to enhancing transport through sclera. Effect during sonication was attributed to flow associated with acoustic microstreaming. Effect post sonication was attributed to the temporary increase in scleral permeability. Flow-associated effect was more pronounced but only applied to smaller macromolecules.

1. Introduction

Eye diseases that affect the posterior segment of the eye, such as age-related macular degeneration (AMD), can lead to impaired vision and even blindness and have placed a huge healthcare burden on the aging society. In recent years, the approval of new biomolecules (e.g. Macugen®, Lucentis® and Avastin®) offers new treatment options. However, delivery of these large molecules to the eye is a challenging problem. Topical eye drops and oral dosage are ineffective methods for overcoming the delivery barriers of the eye (Geroski and Edelhauser, 2000; Edelhauser and Maren, 1988; Vincent and Lee, 1986). Currently, intravitreal injection is used clinically to place the macromolecular drugs directly near the disease site. Though effective, there is an underlying safety concern especially with frequent and repeated injection. This delivery method may result in an increase in intraocular pressure (Jonas et al., 2003), acute endophthalmitis (Darius et al., 2003), retinal detachment and vitreous hemorrhage (Wu et al., 2008). This has provided the motivation to develop a needleless approach for ocular delivery.

We demonstrated in vivo the feasibility of low intensity and low frequency ultrasound as a non-invasive approach to deliver macromolecules to the back of the eye via the transscleral route. Dextran-T70 (70 kDa) applied on the scleral surface was detected in the vitreous after sonication at 40 kHz (Suen et al., 2013). The barrier function along the transscleral route, which blocks the transport of macromolecules, was restored after 2 weeks. The transscleral route consists of multiple layers of tissues, including the sclera, choroid and retinal pigment epithelium (RPE). We are interested in understanding the mechanism by which ultrasound enhances the transscleral transport by revealing the effect of sonication on each tissue layer. Our investigation starts here with the sclera, the outmost barrier that mainly consists of a matrix of collagen, proteoglycan and elastin (Young, 1985).

In this study, there are three objectives. First, we aim to correlate the enhancement of transport through the sclera with the frequency of ultrasound. Ultrasound frequencies at 40 kHz, 500 kHz, 1 MHz and 3 MHz were tested. We hypothesized that cavitation is important for the enhanced transport through the sclera. It is known that the extent

* Corresponding author at: Room 4353, Academic Building, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong.
E-mail address: ying.chau@ust.hk (Y. Chau).

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of cavitation increases with decreasing frequency (Lavon and Kost, 2004), the results will help to unveil the importance of cavitation, as the extent of cavitation is inversely related to the square root of frequency (Holland and Apfel, 1989). Second, we will examine the effect during and after ultrasound, to gauge separately the contribution of acoustic flow and the changes in scleral permeability. We will measure the duration that the barrier function of the sclera is hampered, which needs to be considered when addressing the safety of ultrasound-mediated delivery. Third, we will investigate how the enhancement depends on the size of macromolecules — by using dextran of 20 kDa to 150 kDa as probes. The latter will also expand the applicability of using ultrasound to deliver therapeutics of a wide range of sizes.

2. Methodology

2.1. Materials and equipment

2.1.1. Materials

Fluorescein isothiocyanate-conjugated dextrans (FITC dextrans with molecular weight of 20 kDa, 70 kDa and 150 kDa) (Sigma, St. Louis, MO) were adopted as model macromolecules. They were dissolved in phosphate-buffered saline solution (PBS, Invitrogen, CA) to prepare 0.1% w/v FITC-dextran solution. Sclera was obtained from New Zealand White rabbits and used fresh for ex vivo experiments. The use of animals was conducted according to the requirements of the Animals (Control of Experiments) Ordinance (Cap. 340) and all relevant legislation and Codes of Practice in Hong Kong. All procedures were approved by the Faulty Committee on the Use of Live Animals in Teaching and Research in The Hong Kong University of Science and Technology (APCF 2012010).

2.1.2. Ultrasound device and calibration

Ultrasound transducers were powered by a function generator (GFG-8216A; GW Instek, CA, USA) connected to an amplifier (Model 7500 Amplifier; Krohn-hite, MA, USA) to emit unfocused ultrasound. The input signal was monitored by an oscilloscope (DS1002B Oscilloscope; Tektronix, Beaverton, OR, USA) connected in parallel to the amplifier. Ultrasound transducers emitting four different frequency ultrasound: 40 kHz (Ultrasound transducer; Beijing Cheng-cheng Weiye Science and Technology Co., Ltd., Beijing, CN), 500 kHz (Precision Acoustics Ltd., Dorset, UK), 1 MHz and 3 MHz (BTL-4000; BTL Industries Inc., Clark, NJ, USA) were used.

The driving frequencies of the transducers listed above (which is the same as the pulse repetition frequency of a continuous wave) were measured respectively by a membrane hydrophone (HMB-0500, Onda Corp., Sunnyvale, CA, USA) connected to the oscilloscope. The hydrophone was placed 26 mm away from the transducers to measure the center frequency. The center frequency was confirmed to be 40 kHz, 500 kHz, 1 MHz and 3 MHz respectively.

The spatial average temporal average ultrasound intensity was calibrated by the calorimetric method (Zderic et al., 2002). Briefly, 15 ml of water was put into a plastic test tube with cotton wrap around as an insulating layer and was sonicated by the ultrasound device for 5 min. The temperature rise was measured by a thermocouple (206-3738, RS Components Ltd., Hong Kong). The thermocouple was placed on in the middle of the test tube for temperature measurement. The ultrasound intensity was calculated by the following equation:

$$I = \frac{m_{water}\ C_{p,water}}{A}\frac{\Delta T}{\Delta t} \quad (1)$$

where

I is the average intensity (W/cm^2)
m_{water} is the mass of water (g)
$C_{p,water}$ is the specific heat capacity of water ($J\,g^{-1}\,°C^{-1}$)
ΔT is the change in temperature (°C)
A is the cross-sectional area of the transducer probe (cm^2)
Δt is the time for the calorimetric test (s)

2.2. Ex vivo ultrasound experiments

Fresh sclera from the posterior segment of the rabbit eye was mounted on a Franz diffusion cell with a contact area of 0.238 mm^2 at room temperature (20 ± 2 °C) with the orbital side facing the donor chamber and the uveal side facing the receiver chamber. An ultrasound absorber AptFlex F28 (Precision Acoustics, United Kingdom) was placed at the bottom of the receiver chamber. 1 ml FITC-dextran solution was placed in the donor chamber and 4 ml phosphate-buffered saline in the receiver chamber. Ultrasound at different frequencies (40 kHz, 500 kHz, 1 MHz and 3 MHz), with a spatial average temporal average intensity (I_{SATA}) of 0.05 W/cm^2 was applied for 30 s about 26 mm directly above the sclera. The sclera was removed from the set-up for cryosectioning 15 min after ultrasound application. In another study to isolate the contribution of ultrasound-induced flow (or acoustic microtreaming), the sclera was cryosectioned immediately after sonication. In the control experiments, no ultrasound was applied. The scleral surface temperature before and after ultrasound application was measured by a thermocouple (206-3738, RS Components Ltd., Hong Kong).

To estimate the duration of the effect of ultrasound on the scleral structure, ultrasound (at 40 kHz) was applied on the sclera using the same setting as above except that the donor chamber was initially filled with PBS. A lag time ranging from 15 min to 3 h was spent prior to transferring the sclera to a Franz cell containing FITC-dextran solution on the donor side. Dextran was then allowed to diffuse through the sclera for 15 min before the tissue was removed for cryosectioning.

2.3. Measurement and comparison of the transscleral penetration distance

The sclera tissues were cryosectioned into 10 μm thick sections using a cryostat (CM1850, Leica, Wetzlar, Germany). The sectioned images were then examined under a fluorescence microscope (Olympus; Center Valley, PA, USA; Model BX 41). The image of the sclera was captured under optical mode, and FITC-Dextran penetration into the sclera was captured under the fluorescence mode (bandpass filter: 460–490 nm, dichroic mirror: 505 nm, barrier filter: 515 nm, exposure time: 1000 ms). The pictures from these two modes were merged, allowing the simultaneous visualization of the ocular structures and the protein penetration. The thickness of the sclera and the penetration distance of FITC-Dextran were obtained using the software SPOT® (Diagnostic Instruments, Inc.; MI, USA; Version 4.6). Penetration distance was measured based on the green color of the fluorescence image. Same microscope setting and exposure time were used in capturing images from different experiments in order to obtain comparable contrast in the fluorescence images. The black background was used to define the baseline where green-colored FITC-Dextran has not permeated across the sclera. The penetration distance is defined as the distance from the sclera surface to the deepest depth at which the green color intensity is just above the baseline. Experiments of each condition were repeated at least 3 times ($n \geq 3$). In each trial, the average penetration distance was obtained from 12 sclera sections with a minimum of 20 measurements per sclera. Comparison between different conditions was performed by two-tailed *t*-test and was considered statistically significant when $p < 0.05$.

The transscleral penetration distance of dextran during the 15 min contact time ($L_{15\ m}$) was obtained by subtracting the penetration distance of dextran after 30 s insonation ($L_{30\ s}$) from the penetration distance of dextran after 30 s insonation and 15 min contact time ($L_{30\ s\ 15\ m}$):

$$L_{15\ m} = L_{30\ s\ 15\ m} - L_{30\ s}$$