Defects of Biolayers Generated by Ultrasound

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Abstract: Transdermal drug delivery is handicapped by the low skin permeability caused by highly ordered structure of lipid bilayers in the outer human skin layer. It has been reported that ultrasound can increase the permeability of human skin. The enhancement was attributed to acoustic cavitation but the underlying physical mechanism is not fully understood. As a model, dipalmitoylphosphatidylcholine (DPPC) lipid bilayers are insonicated by ultrasound of two sub-megahertz frequencies (168 kHz and 707 kHz). The free field spatial peak pressure amplitudes of both are measured to be 6 x 10^5 Pa. Bilayer defects, which have average diameters of tens to hundreds of nanometers and can be detected by an atomic force microscope, are generated within less than 0.5 minute. The number of the defects grows with time. The defect growth rate at the 168 kHz frequency is about 3.5 times that at the 707 kHz frequency.

INTRODUCTION

Development of technologies for enhancing the transport of small as well as large molecules across the skin (transdermal drug delivery) is an important avenue of drug delivery research. The low skin permeability is mainly caused by the stratum corneum (SC), the outer-most skin layer. The SC is usually about 15 μm thick. It consists of keratinocytes which are dead cells and filled with keratin fibers surrounded by lipid bilayers (1). The bilayers have highly ordered structure that makes the SC impermeable.

Over the last two decades, ultrasound has been applied to enhance transdermal drug delivery. This method is called sonophoresis. Experimental work was reported in 1986 by Kost et al (2). They used 1.5 MHz ultrasound of pressure amplitude 1 x 10^5 Pa and increased transdermal permeation of mannitol and physostigmine across rat skin in vivo by up to 15-fold. The sonophoresis work reported before 1995 used primarily frequencies in the megahertz range and was limited to small molecules.

Recently, it has been shown that low frequency (20 kHz) ultrasonic tonebursts (duty cycle = 10% and pressure amplitude = 2.6 x 10^5 Pa) can increase the permeability of human skin to drugs, including high molecular weight proteins such as insulin, γ-interferon (given to enhance the immune response of patients suffering from AIDS, cancer or any viral infection), and erythropoietin (given to patients suffering from severe anemia to enhance the process by which the red blood cells are formed) by several orders of magnitude (3). Acoustic cavitation was proposed as the possible cause of the enhancement.

One possible scenario of the enhancement is that ultrasound introduces defects that are devoid (holes) of lipid molecules in bilayer. When the number of defects grows and their sizes become large enough to allow the passage of some drug molecules through otherwise orderly bound bilayers, the permeability of the skin to the drug is significantly increased. However, to our knowledge there has been no direct microscopic experimental evidence that ultrasound of moderate pressure amplitude in the order of submerge Pascal can disorganize bilayers. Using supported dipalmitoylphosphatidylcholine (DPPC) lipid bilayers and an atomic force microscope (AFM), we were able to show that defects of average sizes between tens to hundreds of nanometers were created and grew with time when the bilayers were insonified by 168 kHz ultrasound, the spatial peak pressure amplitude of which was measured to be 6 x 10^5 Pa in a free-field condition.

EXPERIMENTAL METHOD AND RESULTS

An atomic force microscope (A NanoScope E AFM, Digital Instruments, Santa Barbara, CA), equipped with oxide-sharpened Si_N4 tips with a nominal spring constant of 0.06 N/m was used to exam the specimen used in this work. All images were obtained under a probe force of about 0.5 nN in the contact mode, at a pixel number of 512 x 512, and with a scanning line speed of about 5 Hz. The piezo scanner used had a scanning range of 10 μm.

Each prepared bilayer was first examined by AFM and then irradiated with ultrasound and examined by AFM again to detect the creation of bilayer defects after insonification. The total period of the above series was less than 2 days for each bilayer used. It had been shown previously that during a two-day period natural occurrence of bilayer defects due to the lipid loss is negligible. In other words, the bilayers are quite stable in a period longer than two days. Thus, the creation of bilayer defects in our experiments should be the result of insonification.
FIGURE 1. Five typical DPPC bilayers imaged by AFM in water. (a) the original bilayer (the two arrow points to two very small bilayer defects, about 40 nm in diameter) and (b) to (e) under different insonification (168 kHz, SPPA=6x10^5 Pa) time periods (b): 0.5 min; (c): 1 min; (d): 1.5 min; and (e): 2 min. Image size for each is 2 μm x 2 μm. Dark regions in these images are bilayer defects (holes), noting that in (e) defects are large.

The lipids were obtained from Avanti Polar Lipids (Alabaster, AL) and used without further purification. The bilayer was prepared as described elsewhere (4). Briefly, a droplet of lipids dissolved in chloroform was placed in a glass culture tube and dried under nitrogen for about two hours. The bottom of the tube was then coated by a film of dried lipids. About 1 ml 20 mM NaCl was placed in the culture tube and the tube was sealed after flushing the air with nitrogen gas. The concentration of lipids in the lipid suspension was about 0.5 mg/ml. The sealed tube was sonicated in an ultrasonic cleaning bath until the lipid suspension became clear. The above procedure broke large vesicles into smaller ones with the radius of curvature below the wavelength of visible light. A droplet (0.05 - 0.2 ml) of the sonicated lipid suspension was applied to freshly cleaved thin mica and the sample was incubated at 4°C overnight. Afterwards, the sample was heated to 55°C for 30 min. A supported bilayer on mica was formed and residual vesicles were removed by solution exchange. During the above operation, the piece of mica was always immersed in solution without being directly exposed to air. The dimension of the bilayer samples is about 3 mm x 5 mm. The sizes of mica are slightly larger than those of the bilayer sample. The thickness of the mica is less than 0.2 mm. The prepared specimen then was mounted facing vertically upward in a glass container filled with distilled water. Absorbers were present to minimize the standing-wave formation. Temperature of the water in the container was 25 °C. A 55 mm diameter unfocused PZT ultrasonic transducer whose fundamental resonance frequency is 168 kHz was placed facing downward above the specimen. The distance between the specimen and the front face of the transducer was about 1 cm. The thickness of the bilayer prepared was about 6 nm measured by AFM. The specimen was insonified continuously for the desired time. The spatial peak pressure amplitudes (SPPA) of the sound field in situ was determined using a calibrated pvdf needle hydrophone, which has a 0.6 mm diameter sensing element.

Figure 1 contains five typical AFM images; one is for an original bilayer before insonification (Fig. 1a) and the rest are bilayers after insonification (Fig. 1 b-e) by 168 kHz ultrasound for different time periods. The creation and the growth of bilayer defects of tens to hundreds of nanometers can be observed from these images. In short time scales (< 2 min), the decrease in bilayer coverage is accompanied with relatively uniform increase of the number of defects. A similar experiment was repeated using 707 kHz ultrasound of the same spatial peak pressure amplitude. The decrease of bilayer coverage is similar to the case with 168 kHz insonification, but the time required is much longer.

Figure 2 summarizes the two cases corresponding to the two different frequencies. The results are obtained with several specimens at each frequency. The large standard deviations in both cases may have resulted from the intrinsic random nature of the creation of bilayer defects. Despite this randomness, it clearly shows that the defect growth-rate is much slower for 707 kHz than for the 168 kHz case.

REFERENCES