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HHS Public Access

Author manuscript

*Ther Deliv*. Author manuscript; available in PMC 2016 January 25.

Published in final edited form as:

*Ther Deliv*. 2014 July ; 5(7): 843–857. doi:10.4155/tde.14.32.

# Ultrasound-enhanced transdermal delivery: recent advances and future challenges

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## Abstract

The skin is a formidable diffusion barrier that restricts passive diffusion to small (<500 Da) lipophilic molecules. Methods used to permeabilize this barrier for the purpose of drug delivery are maturing as an alternative to oral drug delivery and hypodermic injections. Ultrasound can reversibly and non-invasively permeabilize the diffusion barrier posed by the skin. This review discusses the mechanisms of ultrasound-permeability enhancement, and presents technological innovations in equipment miniaturization and recent advances in permeabilization capabilities. Additionally, potentially exciting applications, including protein delivery, vaccination, gene therapy and sensing of blood analytes, are discussed. Finally, the future challenges and opportunities associated with the use of ultrasound are discussed. It is stressed that developing ultrasound for suitable applications is key to ensure commercial success.

## Transdermal drug delivery

The skin is an attractive area for the delivery of therapeutic substances due to its prevalence and ease of access. Topical formulations have been used for thousands of years to treat medical conditions. However, the skin is a formidable diffusion barrier. Only small (<500 Da), lipophilic molecules can passively diffuse through the skin. As a result of the barrier function of the skin, passive transdermal delivery has primarily been limited to small molecules where therapeutic benefit can be achieved at low blood concentrations (nanograms of drug per milliliter of blood) [1]. Examples of drugs that have successfully been administered passively via dermal patches and other topical formulations include hormones (e.g., testosterone and estradiol) [2], fentanyl [3], nicotine [4] and nitroglycerin [5,6].

Transdermal drug delivery has many advantages over oral delivery and hypodermic injections. With regard to the former, transdermal delivery can minimize intestinal degradation of drugs by avoiding the low pH and host of the proteases present in the gastrointestinal tract [7]. Additionally, long term oral administration of certain drugs, such

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Financial & competing interests disclosure

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

as non-steroidal anti-inflammatory drugs (NSAID), is associated with gastrointestinal side effects, including abdominal pain, stomach ulcers, or liver and kidney damage [8].

Hypodermic injections pose the risk of unintentional injuries to both the patient and the care provider. Combined with needle-reuse, this increases the transmission of infectious diseases such as hepatitis and HIV, resulting in additional healthcare costs. In 1999, the World Health Organization estimated that 8 to 12 billion injections were administered around the world with unsafe practices leading to 1.3 million deaths. Furthermore, up to half of all injections in developing countries are estimated to be unsafe [9]. In addition to these serious side effects, the pain and discomfort caused by injections leads to decreased patient compliance and needle phobia [10]. The prevalence of needle phobia was recently highlighted by a study which suggested that 3.5% of the entire US population is afraid of needles [11]. Nevertheless, the efficient barrier function posed by the skin often makes needle-based administration the only option.

Because of these serious drawbacks, how-ever, many alternative strategies have been investigated to facilitate drug delivery. These are discussed below.

The focus of this review is on the use of low-frequency sonophoresis for the delivery of drugs and vaccines. Part I of the review discusses mechanisms of skin permeabilization. Part II discusses recent developments and innovations. Part III highlights potential high impact applications, including protein delivery, immunization, gene therapy and sensing of analytes. Finally, we present conclusions and an outlook on future challenges.

## Part I: mechanisms of skin permeabilization

### Skin architecture

The structure of the skin has been thoroughly studied, characterized and is well understood. The skin is a multilayered organ composed of two main layers: the epidermis followed by the dermis (Figure 1) [12]. The outermost layer of the epidermis is the stratum corneum (SC). The SC is approximately 20 μm thick and is comprised of dead, keratin-filled corneocytes that are embedded in a multilayered lipid matrix. It is this ‘bricks and mortar’ structure where the hexagonal corneocytes represent the ‘bricks’ that are embedded in the lipid-’mortar’ that provides the majority of the barrier to diffusion. The entire epidermis ranges in thickness between 100 and 1000 μm. It is composed of keratinocytes (95%), immune-competent dendritic Langerhans cells (LCs), and melanin-producing melanocytes. Keratinocytes are responsible for producing and maintaining the SC, and also participate in immunological and inflammatory processes [13]. The basal membrane separates the epidermis and the dermis. The dermis is primarily composed of collagen and elastic fibers. Interspersed are hair follicles, nerve endings and capillary blood vessels. The thickness of the dermis is between 1 and 2 mm.

Because the SC provides the majority of the diffusion barrier, many methods of transdermal delivery focus on overcoming this skin layer. Such methods include electroporation, iontophoresis, microneedles, various ablation strategies, and the subject of this review,

ultrasound-assisted skin permeabilization, referred to as sonophoresis. All these methods seek to overcome the barrier function of the skin [5,7,14–17].

## Methods of skin permeabilization

Current methods to deliver larger, hydrophilic molecules either act on the drug itself, or on the SC. Methods that act on the drug include special formulations or the application of an electric field, a method known as iontophoresis, to push drug through the skin. Methods that work on the SC include the use of chemical penetration enhancers (CPEs) to attain a moderate amount of permeabilization, or the above-mentioned physical methods to achieve greater permeability.

Methods that act on the drug itself rely on creating a driving force for the drug to diffuse into the SC. These include creating concentration gradients by supersaturating the drug in a reservoir or patch outside the skin, or by the application of an electric field, a method known as iontophoresis [18]. Iontophoresis does not change the structure of the skin and has the greatest effect on charged molecules. Uncharged molecules also experience some enhancement through electroosmotic flow [19]. The advantage of iontophoresis is that the rate of drug delivery correlates with the electrical field that is applied. The electric field depends on the electric current that is applied. However, there are practical limitations on the maximum current that can be applied safely, and as a result, on the rate of delivery [20]. The upper limiting value of current has been suggested to be 0.5 $mA/cm^2$ for iontophoresis [21]. This has tempered enthusiasm for this method.

CPEs increase drug permeation by disrupting protein structure in the corneocytes of the SC, by disorganizing and fluidizing the surrounding lipid bilayers, or by increasing drug solubility in the coupling medium, thereby increasing the drug concentration gradient [22,23]. However, the permeability achieved and the potential for local toxicity and irritation must be balanced, because skin irritation correlates with increasing protein denaturation [24]. In terms of lipid fluidizing effects, Fourier Transform Infrared Spectroscopy was used to measure morphological changes in the SC microenvironment when the skin was exposed to CPEs at different concentrations. This study concluded that lipophilic CPEs have mainly a fluidizing action on the alkyl chains of ceramides, while hydrophilic CPEs decrease the strength of H-bonds between the polar head groups of the lipids [22]. Karande *et al.* developed an *in silico* method for the design of next generation CPEs with increased potency and decreased toxicity for transdermal, dermatological and cosmetic products [24].

Physical permeation enhancers include electroporation, microneedles, skin ablation and ultrasound. Microneedles are thin projectiles with lengths on the order of micrometers, which pierce the SC without reaching the pain sensors in the dermis [5,25]. Electroporation uses high voltage (50–500 V for *in vivo* electroporation of the skin) electric pulses with durations on the order of microseconds to create transient pores that can persist for hours, allowing sustained delivery not just through the skin but also into the cytosol and nucleus of cells. This makes the technology interesting for the delivery of DNA and RNA [26]. Laser, short heat shocks (100 ms to 5 s), or mechanical friction are also methods that have been investigated for their ability to remove the SC for subsequent passive delivery of various

drugs, including small molecules as well as high-molecular weight molecules such as protein antigens and antibodies [27,28].

## Sonophoresis

The use of ultrasound to deliver therapeutics across the skin was first reported in combination with hydrocortisone for the successful treatment of digital polyarthritis in 1954 [29]. Ultrasound is an oscillating pressure wave characterized by a frequency above 20 kHz, the upper limit of human hearing, and an amplitude. Ultrasound is divided into three frequency ranges: low-frequency sonophoresis (LFS; 20–100kHz), therapeutic (0.7–3MHz) and high-frequency sonophoresis (HFS; >3MHz). LFS and HFS have been used for permeabilization of the skin [30]. While ultrasound over all three frequency ranges can enhance skin permeability, the physical mechanisms responsible for enhanced permeation are different in each regime. Initial research focused on HFS, because the skin penetration depth of the ultrasound waves is inversely proportional to the US frequency, thereby limiting its effect on the SC at high frequencies [31]. The typical permeation enhancement achieved with HFS is one-to ten-fold [32]. The enhanced transdermal permeation efficiency of LFS was only discovered later. Mitragotri *et al.* showed that the *in vitro* use of 20 kHz ultrasound (125 $mW/cm^2$, 100 msec pulses applied every second) resulted in up to 1000-times greater permeability for salicylic acid and sucrose across human cadaver skin compared with that achieved with 1 MHz ultrasound (2 $W/cm^2$) [33,34]. PBS was used as the coupling medium in both cases.

LFS was approved by the US FDA for the transdermal delivery of the local anesthetic lidocaine in 2004 [35]. A short LFS pretreatment (15 s) shortened the onset time of local topical anesthesia induced by lidocaine from 60 to 5 min. In a clinical study, no adverse effects were associated with ultrasound. All but two out of 45 subjects rated the ultrasound device sensation as mild or none; the other two rated the ultrasound sensation as uncomfortable but not painful [36].

## Mechanism of ultrasound-assisted skin permeabilization

Machanisms by which ultrasound enhances skin permeability include acoustic cavitation, thermal effects, radiation forces and convection. Convection involves physical agitation of the coupling fluid, which reduces the convective boundary layer that opposes permeant diffusion. It should be noted that only the permeability of therapeutics administered simultaneously with the ultrasound treatment are enhanced by convection. Acoustic radiation force is a phenomenon resulting from the interaction of an acoustic wave with an obstacle placed along its path. This force tends to push the object in the direction of propagation to the regions of maximum ultrasound pressure amplitude.

Absorption of the ultrasound energy and scattering effects in the tissue attenuate the ultrasound waves. The attenuated sound energy is transformed into thermal energy, which heats the exposed tissue. Thermal effects increase with frequency and are most significant at megahertz frequencies. Biological effects in response to elevated temperatures include increased blood flow and the reduction of muscle spasm. However, prolonged exposure to significantly increased temperatures may cause adverse effects in the underlying tissue,

including burns, necrosis and epidermal detachment [37]. To minimize unintended thermal effects, the surface temperature can be controlled by operating the sonicator in a pulsed mode, and by periodically changing the coupling medium during ultrasound treatment. A common practice is to maintain the skin temperature below 40°C [38].

The most important mechanism for skin permeability enhancement is acoustic cavitation [39,40]. Cavitation is the process by which bubbles are created in a fluid when it is acted upon by a force in excess of its tensile strength. Ultrasound propagating through a fluid results in cavitation bubbles. As a result of the oscillating pressure wave, the bubbles oscillate and grow in size over many cycles of the pressure wave. These bubbles can oscillate indefinitely around an average size, a phenomenon known as stable cavitation. Stable cavitation, the oscillation around an equilibrium bubble radius, occurs in response to a relatively low acoustic pressure amplitude (Figure 2) [41]. However, depending on the acoustic pressure, frequency and bubble size, cavitation bubbles can also implode when they are close to liquid– solid interfaces, creating an intense local shockwave, a phenomenon known as inertial or transient cavitation [42,43]. It is inertial cavitation that plays the most important role in ultrasound-assisted skin permeabilization. The size of the bubbles that initially nucleate is inversely dependent on ultrasound frequency. Consequently, at high ultrasound frequencies, the nucleating bubbles are small (the radius is ~3μm at 1 MHz), and can nucleate within the SC, giving rise to some disruption of the ordered SC structure. On the other hand, when using LFS, the nucleated bubbles are too large, and can no longer nucleate within the SC (the radius at 20 kHz is ~150μm, which is much larger than the average SC thickness of 20 μm). Using low ultrasound frequencies, the large bubbles form outside the skin, become unstable and implode violently. Implosion near solid surfaces results in a jet of fluid, referred to as a microjet. When these microjets impinge on the SC, they erode the dead cells and help permeabilize the membrane. Inertial cavitation results in much greater permeability enhancement of the SC than stable cavitation. As a result, in recent years, the focus of sonophoretic research has shifted to LFS (Figure 2) [16,32].

An advantage of ultrasound over other transdermal delivery technology platforms is the number of parameters that can be tuned to control the desired outcome. Ultrasound parameters that can be tuned include frequency, amplitude, treatment time, duty-cycle, distance between the ultrasound source and the skin, and the composition of the coupling medium. The effect of changing the ultrasound parameters on skin permeability has been extensively studied *in vitro* [38]. The ultrasound frequency is the main tuning parameter that controls the number of pressure cycles per second, and as discussed above, determines the mechanism and extent of skin permeabilization. The ultrasound amplitude is proportional to the displacement of the ultrasound tip during each pressure half cycle and correlates with the ultrasound intensity. The relationship between ultrasound intensity and cavitation is complex. For cavitation, both stable and inertial, to occur there is a minimal negative peak pressure threshold that also depends on ultrasound-frequency, liquid viscosity and liquid surface tension. Above that threshold, cavitation events increase until they reach a maximum, beyond which other effects such as acoustic decoupling, in which the bubbles act to minimize the propagation of the ultrasound, reduce the number of cavitation events observed [38].

The ultrasound duty cycle is the fraction of the treatment time that ultrasound is on. Ultrasound is typically not applied continuously in order to control the heat production, and therefore the rise in temperature experienced by the skin. The distance between the ultrasound horn and the skin also plays an important role in LFS. The pressure amplitude of a point source is inversely proportional to the distance from the skin. An ultrasound source can be considered as a point source when its radius is less than the wavelength of the emitted wave [38]. Finally, the ultrasound coupling medium is a very important variable in sonophoresis. Coupling media used for permeability enhancement are markedly different from those that are used for medical diagnostic purposes. To enhance inertial cavitation, coupling media should have low viscosity, because high viscosity inhibits cavitation. High gas content further increases cavitation. Generally, buffered liquids, such as phosphate-buffered saline (PBS) possessing osmotic pressures that are comparable to those of biological samples, are used. Furthermore, a coupling medium should have an acoustic impedance similar to that of the skin to minimize ultrasound reflection at the coupling medium–skin interface. Recall that acoustic impedence is the product of the speed of sound and the density in $N \cdot s \cdot m^{-3}$. It indicates how much sound pressure is generated by the vibration on molecules in a medium at a given frequency. The simultaneous use of ultrasound and chemical enhancers is discussed in the next section.

## Synergism of low-frequency sonophoresis & chemical enhancers

While CPEs and LFS alone both increase skin permeability, a remarkable synergistic effect is observed when both are used simultaneously. Mitragotri *et. al.* showed in an *in vitro* study on pig skin that the simultaneous use of 1% sodium lauryl sulfate (SLS) in PBS as coupling medium and LFS (20 kHz, 10 $W/cm^2$, 10% pulsed mode over 90 min) resulted in a 200-fold enhancement in mannitol permeability over that of native skin. This should be contrasted with the three- and eight-fold enhancements observed using only SLS or LFS, respectively [44]. This synergism is a result of ultrasound enabling higher concentrations of CPE to enter the SC and enhanced dispersion of CPE in the SC. The authors found a seven-times higher SLS concentration in the skin when ultrasound (20 kHz, 7 $W/cm^2$, 50% duty cycle, 10 min) was applied, compared with soaking the skin for 10 min with a 1% SLS solution *in vitro*. Without ultrasound, CPE diffusion into the SC is limited [45]. This allows for the treatment of more patients in the same period of time and at lower intensities. Furthermore, skin permeability is more predictable and reproducible when SLS is used in combination with LFS, a very desirable feature for clinical applications [46]. As a result, LFS is currently used almost exclusively in combination with SLS. Accordingly, in the following sections, ‘treatment’ refers to the simultaneous use of LFS and a CPE (e.g., SLS).

## Localized transport regions

Another important discovery was the identification of two distinct regions in the skin with different permeabilities resulting from the treatment: localized transport regions (LTRs) and non-localized transport regions (non-LTRs) [47,48]. LTRs are regions in the skin where the SC no longer presents any barrier to diffusion. This results from high local concentrations of CPE as a result of a large number of microjets collapsing over these regions of the skin, (Figure 3). The generation of LTRs is random, therefore, cannot be predicted *a priori*. LTRs are up to 80-fold more permeable than non-LTRs. Non-LTRs are areas in the skin having

lower concentrations of CPE. In these regions, CPE enters the skin through convection. As a result, non-LTRs are more permeable than native skin, but contain less CPE and are less permeable than LTRs [49].

## Part II: recent developments

### Combination of LFS with other physical enhancers

The combination of LFS with other transdermal enhancement platforms, such as microneedles and iontophoresis, has been explored. Synergism between microneedles followed by LFS application has been observed for various molecules, including glycerol, calcein and bovine serum albumin (BSA) [50–52]. A likely explanation for the observed synergism is that the pores induced in skin pretreated with microneedles allow diffusion of CPE from the coupling medium into the skin through the pores in the SC, which makes the subsequent LFS application more effective. In addition, cavitation events within these pores in the skin facilitates additional skin permeabilization. Chen *et al.* developed a hollow microneedle device that emits ultrasound simultaneously [52]. Through the hollow microneedles, the drug is directly injected into the epidermis, while the microneedle device itself emits ultrasound at a frequency of 20 kHz (20% duty cycle, 0.5 W/cm$^2$, 5 min). The authors show up to three-times higher delivery of calcein and bovine serum albumin over either LSF or microneedles alone on pig skin *in vitro*. The advantage of this approach is that both technologies are combined into a single device, which simplifies its use.

Iontophoresis has also been used with LFS. The use of LFS followed by iontophoresis resulted in a synergistic permeabilization profile similar to that resulting from microneedles followed by LFS application discussed above [53,54]. Hikima *et al.* found that to deliver molecules with a molecular weight below 1500 Da, LFS pretreatment at 300 kHz followed by iontophoresis is superior to the simultaneous application of LFS and iontophoresis. This is most likely due to the permeabilization of the SC allowing for a greater iontophoretic flux [53].

With the above in mind, it is clear that combining permeability-enhancement technologies that synergistically increase delivery may be useful to achieve enhanced drug fluxes across the skin. However, the tradeoff lies in the complexity of the required equipment and treatment procedure.

### Dual-frequency sonophoresis

An interesting recent advance involves the simultaneous use of HFS and LFS to maximize LTR formation and decrease the required treatment time [55]. Schoellhammer *et. al.* demonstrated enhanced LTR formation through the addition of a 1 MHz horn perpendicular to the 20 kHz low-frequency horn [56]. The authors hypothesized that the high-frequency horn nucleates additional bubbles that can couple to the signal from the low-frequency horn and, as a result, grow and collapse under the influence of the low-frequency horn (Figure 4) [57]. LTR formation exceeding 27% of the treated skin area was reported (Figure 3). This should be contrasted with typical LTR formation of 5 to 10% using LFS alone for comparable treatment times. Additional investigation of this method may allow for the delivery of a much broader class of drugs while decreasing the required treatment time.

### Ultrasound contrast agents for increased transdermal drug delivery

Another interesting approach involves the use of HFS and ultrasound contrast agents (UCAs) in the coupling solution. UCAs are commonly used in contrast-enhanced US imaging of blood perfusion in organs, to measure the blood flow rate in the heart among many other applications [58]. Commercially available UCAs are gas-filled microbubbles and are administered intravenously. UCAs resonate at a specific ultrasound frequency depending on microbubble diameter (typically 1 to 5 μm) and can scatter sound [59]. The ideal resonant frequency for a microbubble is inversely related to the square of its radius. The resonance frequency for phospholipid-coated microbubbles with diameters ranging between 1 to 5 μm is approximately 4 to 0.6 MHz [60]. The bubbles are filled with gas having distinctly different acoustic impendence than blood, because the backscattering echo intensity is proportional to the difference in acoustic impendence at the interface of the bubble with the tissue.

Park *et al.* hypothesized that the addition of specific bubbles with a certain resonance frequency, such as in UCAs, are added to the ultrasound coupling medium, inertial cavitation may be generated more uniformly and predictably over the entire treated area [16].

In two studies, Park *et al.* showed that by adding only 0.1% of the activated Definity® stock solution of UCA to the ultrasound coupling medium, the delivery of glycerol and fluorescently-labeled dextran (4–150 kDa) increased almost twofold compared with the use of HFS alone [61,62].

However, ultrasound frequencies used for medical imaging range from 2 to 10 MHz and, therefore, UCAs developed for these frequencies have a bubble size of 1 to 10 μm, a size that corresponds to their ultrasound resonance frequencies [63]. As discussed above, LFS generally induces up to three orders of magnitude higher skin permeabilization. Accordingly, it would be interesting to investigate if LFS could be enhanced with a contrast agent consisting of bubbles in the resonance region of LFS (20 kHz), which would correspond to a bubble diameter of about 150 μm.

### Miniaturization of the ultrasound equipment

Much of the previous work on ultrasound-mediated drug delivery has been carried out using industrial, off-the-shelf transducers that were intended for different applications, including cell lysis, catalyzing reactions or cleaning. These devices are large, bulky and expensive [64]. As a result, attention has also been paid to device miniaturization and optimization for transdermal drug delivery applications [65].

Particularly interesting designs are the so-called ‘cymbal’ transducers. A cymbal transducer consists of a piezoelectric disk sandwiched between two caps, as shown in Figure 5. The lateral expansion and contraction of the ceramic disk is converted into the axial displacement of the end caps. Taking advantage of the flexural mode of vibration construction, the device thickness can be less than 2 mm, its weight can be less than 3 g, and it can be mass produced for low cost [66]. Multiple cymbal transducers can be connected in parallel for increased efficacy.

More recent advances focusing on optimized designs for reduced power requirements may enable the construction of a fully wearable untethered ultrasound device [68,69]. Additional improvements of this technology could yield clinical prototypes within the next few years.

## Part III: applications spotlight

### Protein delivery

Transdermal delivery of proteins using sonophoresis or other transdermal delivery methods is of exceptional interest. Proteins can only be administered by injection because they cannot withstand the harsh degrading conditions in the gastrointestinal tract. Furthermore, sustained release would be desirable for many biologically active proteins, such as interferons, something that cannot be achieved with a single injection. Proteins such as insulin (6 kDa), interferon gamma (IFN-γ; 17 kDa), and erythropoietin (EPO; 48 kDa) have been successfully delivered *in vitro*, across human cadaver skin, after LFS pretreatment (20 kHz, 100 ms pulses applied every second for 4 h, 225 mW/cm$^2$) [33]. The delivery rates were sufficiently high to deliver these proteins at therapeutically relevant rates. The authors in [33] showed that the blood glucose level of diabetic hairless rats can be controlled by LFS-mediated transdermal delivery of human insulin (20 kHz, 62.6 to 225 mW/cm$^2$, 100 ms pulses applied every second for 1 h, 100 U/ml insulin). Depending on the sonophoresis intensity used (62.5 and 225 mW/cm$^2$), the reduction in blood glucose concentration corresponded to that achieved by subcutaneous injection of 100 mU to 1 U of insulin. In more recent *in vivo* studies, LFS-mediated transdermal delivery of insulin to rabbits (20 kHz, 100 mW/cm$^2$, 20% duty cycle, 60 min) and pigs (20 kHz, 100 mW/cm$^2$, 20% duty cycle, 60 min) was achieved using a cymbal transducer [70,71].

More recently, HFS was used to deliver a monoclonal antibodies, against human papilloma virus (HPV) oncoprotein E6, to different cancer cell lines *in vitro* [72]. Transient cell membrane permeability was induced by a combination of a UCA, DEFINITY®, and high-intensity focused ultrasound (HIFU; 930 kHz, 32 μs pulses with a repetition frequency of 1.5 kHz for a total exposure time of 30 s and 1000 kPa pressure). The combination of HIFU and microbubbles enabled intracellular delivery of the antibody.

The delivery of BSA by a combination of ultrasound and microneedles has been discussed in section II. For the delivery of immunogenic proteins for the purpose of immunization, see the next section. LFS-mediated protein delivery, especially for proteins where only small doses are required, is a potentially impactful area for LFS-assisted drug delivery. When considering potential applications of a delivery system, it is important to ascertain whether the potential medical benefit for patients and revenue to care providers outweighs the cost associated with approval of the delivery system by the authorities.

### Immunization

Immunization is another key application in which LFS may provide a significant advantage over the current state-of-the-art. The mechanisms that generate protective immunity after vaccination or infection with a pathogen are still not fully understood and represent an area of active research. What is known is that the pathogen or antigen has to be taken up by professional antigen presenting cells (APCs), then processed, and finally presented to T

cells. A small fraction of activated T cells will become long-lasting memory T cells that are able to respond quickly to recurring infections [73,74].

Current vaccinations using injections deliver the antigenic material to immune cell-depleted muscle. This requires addition of adjuvants or viral vectors to induce a robust and long-lived immune response. Both of these additives can help in activating the immune system, but are also responsible for local and systemic side effects of vaccines [75]. Local side effects include erythema, subcutaneous nodules, contact hypersensitivity, granulomatous inflammation and, in some cases, IgE-mediated allergic reactions [76]. Systemic reactions include flu-like symptoms such as nausea, fever or adjuvant arthritis. More severe side effects can result from excessive cytokine liberation and include allergy, anaphylaxis or organ specific toxicity [77]. Furthermore, vaccination with non-replicating antigens cannot elicit a cytotoxic T cell response, also termed Th1 immune response, which is important for clearing intracellular infections and killing cancer cells [78]. The epidermis is populated by a subpopulation of antigen-presenting cells (APCs) known as Langerhans cells (LCs). LCs form a dense network that covers approximately 25% of the skin surface area [79]. The underlying dermis is populated with at least five different types of antigen presenting dendritic cells. This density of APCs make LFS-assisted vaccination very attractive compared with injections into the muscles, which are only sparsely populated with APCs.

It has been shown that immunization with tetanus toxoid after LFS pretreatment elicits a strong antibody response without the need for adjuvants. Mice were pretreated with ultrasound (20 kHz, 2.4 $W/cm^2$, 50% duty cycle, 10 min, PBS containing 1% SLS), and tetanus toxoid (100 μg) was topically applied for 120 min [80]. It was found that serum antibody titers were comparable to those obtained from subcutaneous injection. A second study using LFS (20 kHz, 10% to 20%, 45 s, PBS containing 0.5 to 1% SLS) confirmed these results [81]. A major mechanism for the observed adjuvancy of LFS is likely the release of inflammatory cytokines by keratinocytes, because it is known that keratinocytes release immunomodulatory cytokines in response to a physical insult such as ultrasound. Indeed, other studies, involving disruption of the skin barrier function mechanically, chemically or by dietary restriction, were shown to trigger the release of cytokines such as Il-1, IFN-γ, TNF-α and others [82].

Most importantly, cutaneous immunization has the potential to elicit a cytotoxic T-cell response. Cytokines that are released after skin barrier disruption, especially IFN-γ and TNF-α, are known to induce Th-1 type immune responses [67]. In addition to cytokine release by keratinocytes, there is evidence that simultaneous activation of myriad DC subsets significantly enhances the magnitude, quality and memory of the T-cell response [83,84].

With the above in mind, and because relatively few reports are available in the literature, the use of ultrasound for immunization represents an exciting and rich area for further investigation. Generation of a strong Th-1 type immune response is not only essential to combat intracellular pathogens such as HIV, tuberculosis and hepatitis C, but is also key for cancer immune therapy.

## Sensing

In addition to delivering drugs through the skin, permeabilizing technologies allow for the extraction of interstitial fluid for diagnostic purposes. Non-invasive sensing of glucose, for example, has been studied extensively [85]. The concentration of glucose in the interstitial fluid is proportional to blood-glucose levels [86]. Tight monitoring of blood-glucose levels is crucial in a diabetic state. Misregulation can lead to organ damage from spikes in blood-glucose levels [87]. LFS skin permeation may allow for the detection of glucose in interstitial fluid over an extended period of time.

The first human trial examining the detection of glucose from interstitial fluid using LFS-induced skin permeabilization was carried out in the early 2000s [88]. Interstitial fluid was extracted by the application of vacuum after LFS permeabilization (20 kHz, 10 $W/cm^2$, pulsed 5 s on and 5 s off, 2 min). Glucose values in the extracted interstitial fluid were found to correlate well with blood-glucose values. The study found that the permeability of the skin remained high for up to 15 h, and regained its barrier properties in 24 h.

In a 2008 clinical study, blood glucose levels were measured after skin pretreatment with the FDA-approved LFS device against a skin ablation system [89]. Patients and healthy volunteers were tested in both clinical and outpatient settings. A major innovation relative to the initial study carried out in 2000 was that the glucose levels were measured with a small electrochemical sensor that was placed over the site of LFS pretreatment (55 kHz, 12 W, about 10 s) with a suspension of 0.5% (w/v) Tamsil silica particles in PBS containing 1% (w/v) SLS. The sensor transmitted real-time glucose levels wirelessly to a receiver/monitor. The study concluded that both skin permeabilization methods, LFS and skin ablation, performed equally well. These exciting results indicate the feasibility of creating a portable miniaturized system for applications in humans. However, the peak glucose concentration in the interstitial fluid was found to lag 7 min, on average, behind rises in blood glucose concentrations and 15 min for the sensor described [90]. The upside of this method is that when blood glucose is falling, the glucose concentration measured in the interstitial fluid may fall before the blood glucose concentration, and thereby serve as an early warning for hypoglycemia [91].

More recently, attempts aimed at developing wearable glucose monitoring devices were made. Specifically, a prototype based on a lightweight cymbal transducer array was constructed and tested in pigs *in vivo* (Figure 5) [92]. Four cymbal transducers were connected in a two-by-two pattern, and the biosensor was located in between the array and the skin. The array was operated at a frequency of 20 kHz, 100 $mW/cm^2$, for 20 min. The results obtained showed reliable accuracy comparable to that achieved by measuring blood-glucose levels directly, and demonstrated the feasibility of using cymbal arrays for continuous glucose sensing.

In addition to glucose, LFS-assisted sensing has been shown to be useful for the detection of urea and calcium [88].

### Oligonucleotide delivery

Considerable effort was devoted to investigate the delivery of DNA or RNA via sonoporation. Oligonucleotides (OND) for gene therapy have to be delivered into the cytosol for RNA, or into the nucleus for DNA, of the target cells to be effective. Viral vectors have been successful in overcoming theses barriers. How-ever, there are serious safety concerns about the use of viruses, especially when clinical trials are involved.

Ultrasound, with or without microbubbles, such as UCA, has been shown to induce temporary pores on the cell membrane to allow permeabilization of OND into the cell. Both inertial and stable cavitation, through acoustic sheer forces and microstreeming, have been identified to induce transient membrane permeabilization *in vitro* [93].

Tata *et al.* were the first to use ultrasound (932.7 kHz, 1.67 W/cm$^2$, 24 s) to improve transfection of a plasmid encoding for green-fluorescent protein to prostate cancer cell line LnCap in 1997 [94].

A growing number of research groups have reported ultrasound-assisted gene delivery *in vivo.* For example, optison microspheres in combination with ultrasound (1 MHz, 2.5 W/cm$^2$, 60 s) were used to deliver different pDNA to rat carotid artery *in vivo* and to human aortic endothelial cells *in vitro* [95]. However, only few reports discuss delivering OND across the skin. Tezel *et al.* were the first to report OND delivery across the skin *in vitro* [96]. Radioactively labeled OND were delivered to full-thickness pig skin by simultaneous application of LFS (20 kHz, 2.4 W/cm$^2$, 50% duty cycle, 10 min) and OND. A total amount of 0.5 to 5% of the OND in the donor solution was delivered into the skin.

Tran *et al.* used a cymbal transducer (20 kHz, 20% duty cycle, 15 min) to deliver siRNA targeting $^{V600}$EB-Raf and Akt3 encapsulated in cationic liposomes to melanocytic tumors [97]. The two siRNAs synergistically decreased melanoma development in mice.

Efficient gene delivery to the skin has multiple potential applications, including for wound healing, vitiligo, melanoma, atopic dermatitis or psoriasis [98].

## Conclusions

Transdermal drug delivery is a very active field of research [15]. LFS represents a particularly promising platform among the many available innovative technologies, and offers many benefits compared with traditional hypodermic injections and oral administration. Advantages of LFS include being minimally invasive and pain-free, as well as being able to eliminate the risks and disadvantages associated with needle-based or oral drug delivery. With respect to hypodermic injections, LFS eliminates risk of transmission of blood-borne diseases and needle phobia, and enhances patient compliance. With respect to oral delivery, LFS circumvents the harsh environment in the gastrointestinal tract. The combination of LFS pretreatment with an extended-release transdermal patch could potentially allow for a more uniform drug plasma concentration over extended periods of time. If needed, the treatment can be discontinued by simply removing the patch. This is one benefit over conventional depot injections.

LFS is a physical permeability enhancer that relies on the phenomenon of transient cavitation. These cavitation events erode the SC, the main physical permeation barrier of the skin, to reversibly induce areas of enhanced permeability, known as LTRs. Despite the maturity of this technology, there is still a need for improvements in terms of permeabilities that can be achieved, and the cost and size of the LFS-devices.

Two-frequency ultrasound and the addition of ultrasound contrast agents to the coupling medium are two promising approaches to attain increased permeability. Additionally, cymbal transducers allow for the construction of thin, inexpensive, and mass-produced devices for the delivery of active biomolecules, vaccines, gene therapy and the sensing of blood analytes. The next paradigm will involve translation of these recent advances into medical products for use in the clinic or by patients at home.

## Future perspective

LFS by itself, or in combination with other methods of transdermal drug delivery, represents an important alternative to hypodermic injections and oral delivery, especially for large biomolecules that require small doses, or for small molecules with poor bioavailability. Many drugs have successfully been delivered after LFS-pretreatment, including insulin and other proteins. Reformulation of already approved drugs could be an attractive way for extended patent protection. However, the biggest potential and strength of LFS-assisted drug delivery is in three areas: First, delivery of drug candidates with low oral bioavailability, second, vaccination, and third, delivery of gene therapy to the skin.

Although oral delivery is the most desirable method for patients because of its convenience, only a relatively small number of molecules with particular physical parameters may be delivered via the gastrointestinal tract. In his seminal work, Lipinski found that the oral bioavailability of molecules can be predicted based on several molecular parameters [99]. According to the so-called Lipinski rule, molecules with a molecular weight over 500 Da, a partition coefficient (log P) higher than 5, and more than five hydrogen bond donors or more than ten hydrogen bond acceptors, often have poor oral bioavailability. This rule has proven to be an excellent guiding tool in medicinal chemistry research. In fact, the Lipinsky rule is so useful, that many pharmaceutical companies use high-throughput libraries of molecules that comply with the Lipinski rule of five to screen for new lead candidates. Using these high-throughput libraries has been very successful. However, a large number of potential drug candidates are not included in the screenings. Using an alternative delivery route, without the restrictions associated with oral bioavailability, may help the development of drugs against targets that have been traditionally very difficult for molecules that comply with the Lipinski rule of five. An additional benefit would be the fact that generally many molecules not complying with the Lipinski rule of five do not have patent protection. One such example would be blocking protein–protein interactions. Protein–protein interactions are important in any living cell. They are involved in signal transduction pathways, cell metabolism, muscle contraction and transport [100]. Protein–protein interactions are generally weak and therefore cover larger surface areas. Hence, potent inhibitors are often much larger that 500 Dalton, do not comply with the Lipinski rule of five, and suffer from poor oral bioavailability. The delivery of such large molecules, as they are needed for

blocking protein–protein interactions, is a key area where LFS-assisted drug delivery could have a big impact.

The second area is vaccination. More than 300 years after Edward Jenner's use of cowpox to protect humans from smallpox, we only have vaccines against a small number of diseases. Vaccines against many of today's most serious diseases, such as malaria, HIV and hepatitis C, remain elusive. The mechanisms of vaccination and the complexity of the immune system are gradually being understood. It is now recognized that APCs such as dendritic cells play a pivotal role in the activation of the adaptive arm of the immune system. Because the skin is the first line of defense against foreign pathogens, it is not surprising that such dendritic cells are present at a very high density within the skin. As a result, LFS-assisted vaccination allows delivery of the vaccine directly to the immune-competent dendritic cells. This is followed by cytokine release after skin barrier disruption and provides the necessary immune activation, without the need for additional adjuvants, including the potential for inducing a cytotoxic T-cell response. These exciting attributes of LFS-enhanced vaccination are currently under active investigation to create vaccines that are efficient not only against viral infections, but that may also be useful for cancer immune therapy.

The third area is gene therapy. Therapeutic intervention on a genetic level has the potential to treat many diseases. Viral vector systems are efficient in transferring foreign genetic information to cells. However, safety issues associated with human gene therapy pose concerns. The use of ultrasound and sonoporation, through acoustic cavitation, transiently destabilizes cellular membranes allowing the efficient intracellular delivery of DNA and RNA. Various skin diseases are an ideal target for ultrasound-assisted gene therapy. Ultrasound, in combination with microspheres, could not only enable delivery across the SC, but could also allow intracellular delivery through sonoporation.

In addition to the three primary applications of LFS discussed above, potential also lies in sensing of analytes from interstitial fluid. In diabetes, real-time monitoring of blood glucose levels helps patients avoid the side effects of the disease. Other diagnostic-relevant molecules that can be monitored from the interstitial fluid in a non-invasive way, including electrolytes or biomarkers for various diseases, could be of great interest and help develop the LFS-based sensing technology.

The main challenges for the future of ultrasound-assisted drug delivery include the kinetics and quantity of drug that can be delivered transdermally, the size and price of the ultrasound equipment, and long-term safety studies, which still need to be carried out. Specifically, long-term safety of repeated treatment of the same site needs to be confirmed. The innovative delivery modalities involving two-frequency ultrasound and the use of ultrasound contrast agents, will assist in solving the permeabilization issue, while cymbal transducers will enable the low-cost manufacturing of lightweight, wearable devices.

Introducing a new technology to medical practice is always associated with very high costs. The key for the future clinical success of LFS-assisted transdermal delivery modalities will be finding suitable applications, including large molecules to block specific protein–protein interactions, or vaccines for intracellular pathogens or cancer immune therapy.

## Acknowledgments

This work was funded by the National Institutes of Health (Grant# EB-00351, and CA014051). The authors would like to thank the Hope Babette Tang (1983) Histology Facility personnel for preparation of histology slides. Matthias A Oberli gratefully acknowledges a fellowship from the Swiss National Science Foundation (SNSF) grant: PA002_14059.

## Key Terms

| | |
|---|---|
| **Transdermal delivery** | Drugs are applied topically on the skin. This has potentially significant advantages compared with injections or oral administration, such as long-term steady drug concentrations or the circumvention of the first-pass effect. |
| **Low-frequency sonophoresis** | The application of low-frequency ultrasound (20–100 kHz), at acoustic pressure amplitudes which are sufficiently high to induce inertial cavitation, and can enhance skin permeability to a variety of molecules. |
| **Stratum corneum** | Top layer of the skin that is also the main diffusion barrier for drugs. It consists of dead keratin filled corneocytes embedded in lipid bilayers. |
| **Ultrasound** | Oscillating pressure wave characterized by a frequency greater than 20 kHz, which is the upper limit of human hearing. |
| **Acoustic cavitation** | The formation of bubbles in a liquid as a result of acoustic pressure differences. Two forms of acoustic cavitation are possible: stable cavitation, where the bubble oscillates around an equilibrium bubble radius, and inertial or transient cavitation, where the bubbles collapse violently. |
| **Cutaneous immunization** | Delivery of a vaccine to the immune-competent skin dendritic cells. |

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## Executive summary

### Transdermal drug delivery

- The top layer of the skin, the stratum corneum (SC), is a formidable diffusion barrier.
- Compared to oral drug delivery, transdermal drug delivery (TDD) allows circumvention of the first-pass effect and the delivery of drugs with low oral bioavailability without the risk of gastrointestinal side effects.
- Compared with injections, TDD is pain-free, reduces the risk of transmission of blood-borne pathogens, and allows long-term, sustained delivery.

### Low-frequency sonophoresis

- Low-frequency sonophoresis (LFS) is an ultrasound-based method for the non-invasive, pain-free, and reversible permeabilization of the SC.
- The primary mechanism by which LFS permeabilizes the skin is erosion of the SC by inertial acoustic cavitation.
- The first LFS device was approved by the US FDA in 2004 for the topical delivery of the local anesthetic lidocaine.
- LFS and the surfactant sodium lauryl sulfate (SLS) act synergistically and allow higher and reproducible transdermal permeability during shorter treatment times.
- LFS treatment leads to the formation of areas of high skin permeabilization, known as localized transport regions (LTRs).
- After LFS pretreatment, the skin remains permeabilized for about 15 h, and reverts to close to normal in 24 h.

### Recent innovations in LFS-assisted drug delivery

- The combination of LFS with a second high-frequency ultrasound horn can increase skin permeabilization efficiently and reproducibly.
- Ultrasound contrast agents in the coupling medium were shown to increase skin permeabilization.
- Ultrasound equipment was miniaturized. In particular, cymbal transducers allowed the construction of wearable ultrasound devices.

### Protein delivery

- LFS-pretreated skin allows efficient delivery of large molecules including proteins.

### Vaccination

- The skin is an ideal site for vaccination because of the ease of access and the high density of immunosurveillant professional antigen-presenting cells.

- Skin barrier disruption by LFS has an immune stimulatory adjuvant-like effect.
- Targeting the skin dendritic cells may elicit a Th1, cytotoxic T-cell response.

**Gene therapy**

- Delivering DNA and/or RNA into cells is an ultrasound application with many potential disease targets.
- Effective gene therapy delivery to the skin could have application in multiple skin diseases, such as wound healing, vitiligo, melanoma, atopic dermatitis or psoriasis.

**Sensing**

- LFS allows extraction of interstitial fluid for the real-time sensing of glucose and other medically important analytes.
- Wearable prototypes based on cymbal transducers have been tested and hold great promise.

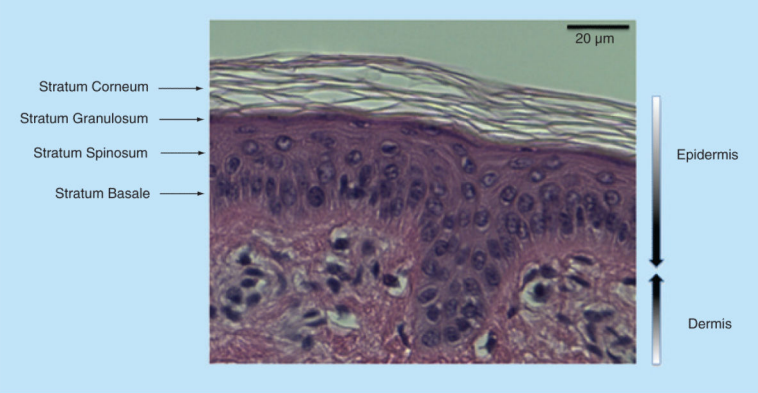


**Figure 1. Hematoxylin and eosin-stained tissue section of native Yorkshire pig skin showing the epidermis and the dermis**

The main diffusion barrier, the stratum corneum, is at the top.

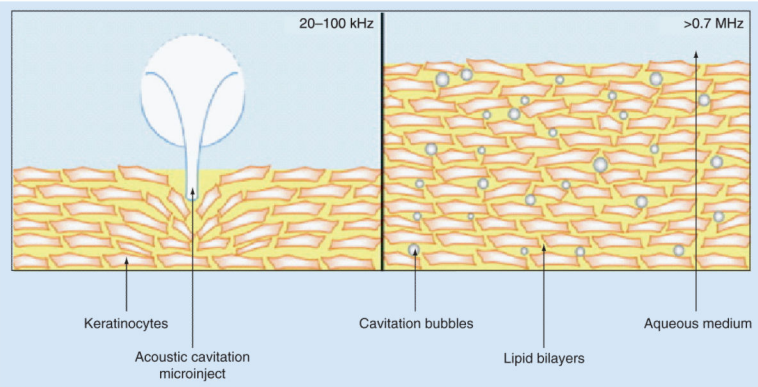


**Figure 2. Different proposed mechanisms of skin permeabilization of low-frequency sonophoresis on the left, and of high-frequency sonophoresis on the right**

In low-frequency sonophoresis, inertial cavitation outside the skin erodes the stratum corneum (SC), while in high-frequency sonophoresis, cavitation within the skin disorganizes the ordered SC structure. Figure reproduced with permission from [32].

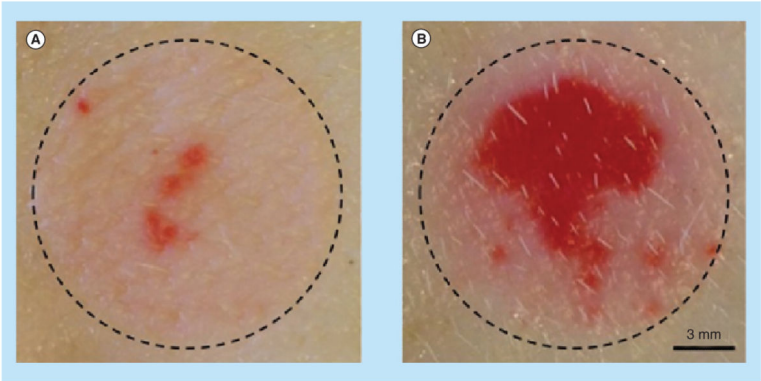


**Figure 3. Localized transport region formation**

**(A)** As a result of single-frequency (20 kHz) or **(B)** dual-frequency (20 kHz + 1 MHz) ultrasound treatment for the same time. The dotted line indicates the area of the skin that was treated with ultrasound and exposed to the dye allura red for the purpose of visualizing the localized transport regions.

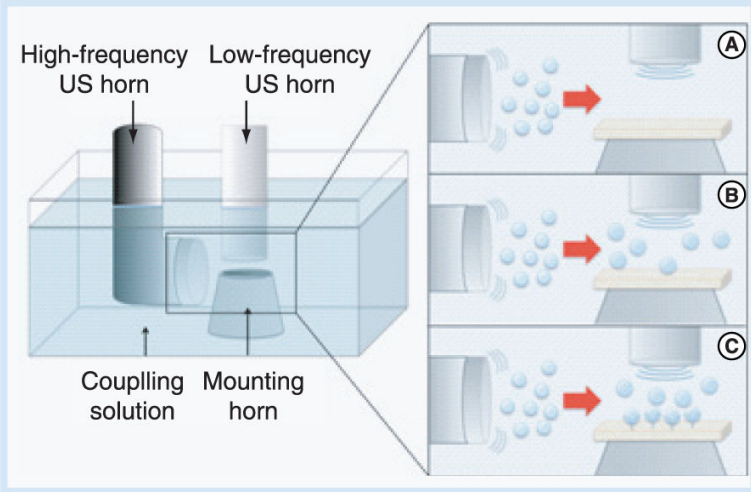


**Figure 4. Experimental setup allowing for the simultaneous application of dual-frequency US**

The authors hypothesized that the cavitation bubbles nucleated by the high-frequency US horn implode under the influence of the low-frequency US horn, increasing the number of microjets impinging upon the skin.

Reproduced with permission from [49].

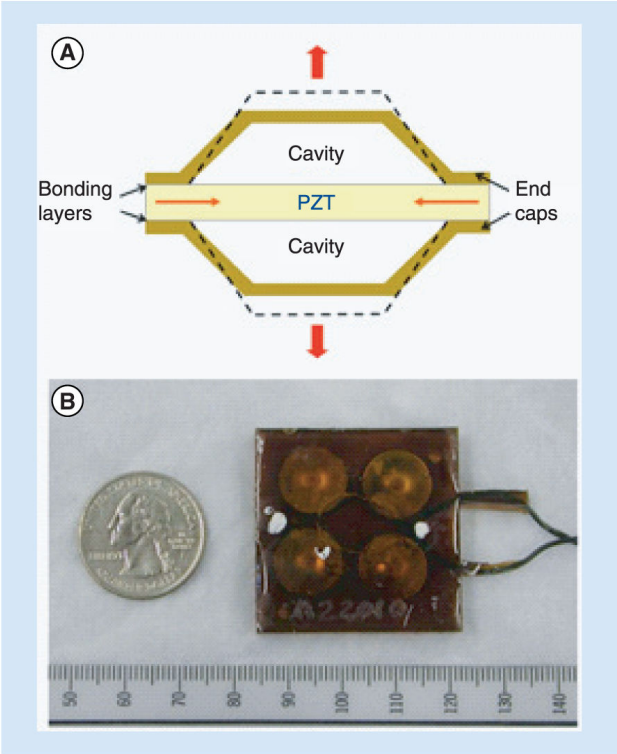


**Figure 5. A cymbal transducer**

**(A)** The resonance frequency of a cymbal transducer depends on the geometry. A ceramic piezoelectric disk is sandwiched between two brass caps. Displacement of the ceramic disk is converted into the axial displacement of the end caps. The dashed lines represent the flexing of the end caps, and the arrows indicate the motion. **(B)** For glucose sensing; a lightweight cymbal array was constructed using four cymbal transducers, which were connected in a 2 Â × 2 pattern and encased in a polymer. The electrochemical glucose sensor is placed in the center of the array.

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