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Opto-Acoustic Cell Permeation

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ABSTRACT

Optically generated acoustic waves have been used to temporarily permeate biological cells. This technique may be useful for enhancing transfection of DNA into cells or enhancing the absorption of locally delivered drugs. A diode-pumped frequency-doubled Nd:YAG laser operating at kHz repetition rates was used to produce a series of acoustic pulses. An acoustic wave was formed via thermoelastic expansion by depositing laser radiation into an absorbing dye. Generated pressures were measured with a PVDF hydrophone.

The acoustic waves were transmitted to cultured and plated cells. The cell media contained a selection of normally-impermeable fluorescent-labeled dextran dyes. Following treatment with the opto-acoustic technique, cellular incorporation of dyes, up to 40,000 Molecular Weight, was noted. Control cells that did not receive opto-acoustic treatment had unremarkable dye incorporation. Uptake of dye was quantified via fluorescent microscopic analysis. Trypan Blue membrane exclusion assays and fluorescent labeling assays confirmed the vitality of cells following treatment. This method of enhanced drug delivery has the potential to dramatically reduce required drug dosages and associated side effects and enable revolutionary therapies.

Keywords: acoustic, ultrasound, drug delivery

1. INTRODUCTION

In recent years, significant attention has focused on enhancing the delivery of drugs and genes across cell barriers. We have developed the concept of optically generated ultrasound that could greatly improve drug efficiency, with the potential to decrease dosages and side effects and enable revolutionary new drug therapies. This technique enables ultrasound production at remote sites in the body and offers a new regime of stress wave characteristics. Initial studies of this novel technology are encouraging, warranting further development.

Many other techniques have been used to permeate cell barriers and have shown limited or no success for in vivo applications. One method that shows promise for limited applications is therapeutic transdermal ultrasound. This established method has been shown to increase the efficiency**,** up to 5000 times, of drug transfer across the stratum corneum[1]. This technique has virtually been confined to dermal applications due to poor localization of ultrasound in deep tissues and associated collateral thermal damage[2]. Mounting piezo-electric transducers on the end of catheters is an option but the required size of the transducers would limit applications to large vessels. A recently developed alternative that has shown initial success is laser-induced stress waves that use high energy shock waves to temporarily increase membrane permeability[3,4]. The technique appears to be highly sensitive to dosimetry and thus far drug concentrations have only been increased by 10 times that obtained using simple diffusion.

We have combined aspects of these two mechanisms by using high-frequency optically-produced low-energy stress waves, a technology recently developed at LLNL. Our minimally-invasive optical method of locally generating ultrasound facilitates the delivery of required intensities into remote locations with the potential to significantly increase drug concentration rates. There are many potential applications including: intra-arterial delivery of thrombolytics or restenosis preventing drugs, and localized delivery of photosensitive and cytotoxic drugs for cancer therapy. This procedure could also facilitate the delivery of gene-vectors into target cells for gene therapy.

Optical ultrasound employs a laser coupled to an optical fiber with the distal end of the fiber delivered through a guiding catheter or introducer needle to the target tissue (Figure 1). The laser radiation that emerges from the fiber may be absorbed either by native fluids such as blood, or by an exogenous absorber. Blood has acceptable absorption ($\alpha = 250\ cm^{-1}$) near

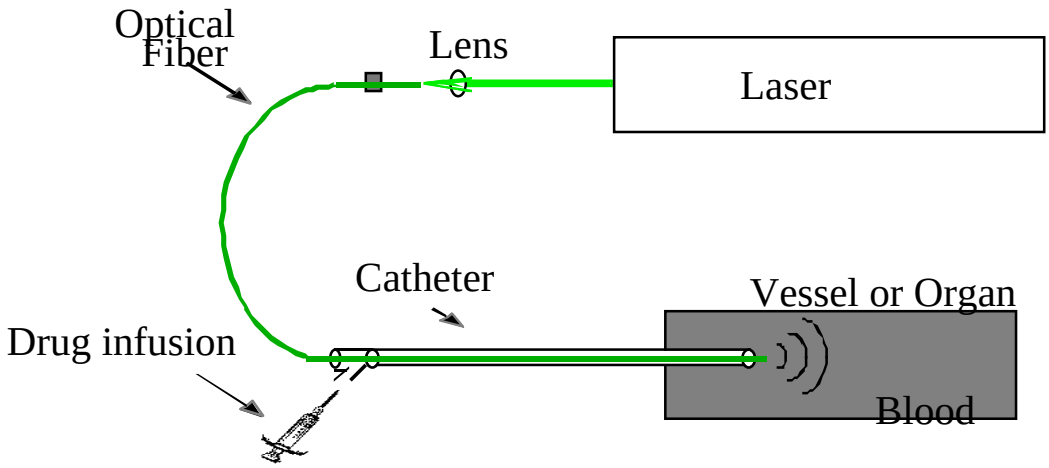


Figure 1. Opto-acoustic ultrasound is created by delivering laser radiation via an optical fiber to an absorber. Acoustic waves are formed in the absorbing material and enhance the uptake of a simultaneously applied drug in surrounding tissues.

532 nm as do many biocompatable dyes, making standard frequency-doubled Nd:YAG lasers a convenient laser source. These lasers, operating at kHz pulse repetition frequencies are commercially available and fairly robust. If the laser pulse duration is sufficiently short, the absorbed optical energy creates a stress wave in the material through a thermoelastic expansion. There are differences in this method of ultrasound production compared to traditional piezoelectric-produced ultrasound. The opto-acoustic ultrasound produces high frequency (10-200 MHz) acoustic waves with very low duty cycle (< 0.1%). Therapeutic piezoelectic ultrasound typically produces sinusoidally varying ultrasound with 50-100% duty cycling.

Acoustic energy interacts with cells such that passive diffusion of compounds into cells is enhanced. Acoustic waves are postulated to transiently and non-destructively disrupt or disorder the lipid bilayer membrane of cells, causing channels to form, through which compounds can enter the cell[1]. Ultrasound-enhanced drug delivery can increase intracellular concentrations of compounds which normally are impermeable or have low diffusion rates. The required drug dosage can be decreased, resulting in fewer potential side effects. Local drug delivery also decreases drug loss due to liver metabolism and systemic absorption further improving dose efficiency. We have used optically-generated ultrasound to permeate cells in monolayers and allow infusion of a fluorescent dye.

2. MATERIALS AND METHODS

Optically-generated ultrasound was delivered to monolayers of cells. An amount of "impermeant" fluorescent dextran conjugated dye was added to the cell media prior to ultrasound treatment. These dyes have a number of characteristics that make them attractive for study, namely, ease of imaging, low toxicity and availability of different sized molecular complexes. Using fluorescent dextran conjugates of varying weights and sizes, we were be able to probe the limitations of our technique. Dyes were applied at discrete post-irradiation time-points to determine the transient nature of the permeability. Results were observed under a fluorescent microscope and captured on a CCD camera.

Cell cultures (ATCC Manassas, VA) were grown under standard conditions: 37^{o}C, 10% CO_2. Multiple cell types were investigated including: MES-SA (human uterine sarcoma), 769P (human renal carcinoma), NCTC (mouse fibroblast), and NIH3T3 (mouse fibroblast) Cells were allowed to reach near confluency in culture flasks, trypsinized, and subcultured onto glass cover slips coated with poly-L-lysine within culture dishes. Cells were again allowed to grow to near confluency. Immediately prior to experimentation, cell media was removed and replaced with approximately 3 ml of PBS containing fluorescent dye (50 mg/l). Dyes consisted of fluorescently labeled dextran conjugates (Molecular Probes, Eugene, OR), Texas-red or Fluorescein, with molecular weights ranging from 4,000 to 40,000 Da.

Acoustic pressure waves were produced by thermoelastic expansion and vaporization resulting from deposition of laser radiation. The laser used was a diode-pumped frequency-doubled Q-switched Nd:YAG laser (Spectra-Physics T40-Y70, Mountain View, CA) emitting pulses of 532 nm radiation with pulse duration of approximately 90 ns (FWHM). The

repetition rate of the laser, producing required pulse energies, was variable up to 5 kHz. The laser emission was coupled to a 50 μm diameter silica optical fiber and delivered to an absorbing media. Pulse energy exiting the optical fiber ranged from 100 to 350 μJ. The resulting optical power delivered varied up to 500 mW but typically was in the range of 150-300 mW.

Laser radiation exiting the optical fiber was absorbed into a 1% solution (wt/vol) of Amaranth red dye (Sigma Chemical Co., St. Louis, MO) solubilized in distilled water. This dye concentration resulted in an optical absorption coefficient at 532 nm of approximately 900 cm^{-1}. The dye was contained in a segment of Tygon tubing with the distal end sealed with 1 mil thick plastic film (PVC resin). This transducer tip allowed for efficient transmission of acoustic energy from the dye solution through the plastic, and into the surrounding aqueous media. Two small diameter tubes led into the transducer: one tube allowed for passage of the optical fiber and inflow of absorbing dye, the other tube allowed for outflow of dye. Dye was continually pumped during experiments at a rate of 1 ml/min to mitigate thermal effects.

Opto-acoustic treatment was applied to a confined area of cells. The optical-to-acoustic transducer tip was submerged in the cell media. The height above the plated cells was maintained at approximately 1 mm. Treatment was applied for periods of 1, 2, 5, 10, 20, or 30 minutes. Appropriate control groups were run in which samples were prepared identically but received no opto-acoustic treatment.

Acoustic pressure was measured with a PVDF hydrophone (#TNU001A NTR Systems, Seattle, WA) connected to a 1 GHz digital oscilloscope (Tektronix, Beaverton, OR). The hydrophone was submerged in a water bath and translated with a micrometer for positionally precise measurements along an axis.

Permeability

Determination of opto-acoustic cell permeability was made via fluorescent light microscopy. Following treatment, cells were washed three times with PBS to remove background fluorescence in the media. Cover slips containing the cells were removed from the culture dishes and mounted on glass microscope slides. Cells were observed under an epifluorescent microscope using appropriate excitation and emission filters for each dye. Cells were analyzed within 4 hours of treatment. Fluorescence activity was quantified by capturing an image with a CCD camera and integrating fluorescence intensity over a defined area. Comparisons between treated and non-treated areas were made.

Cell Viability

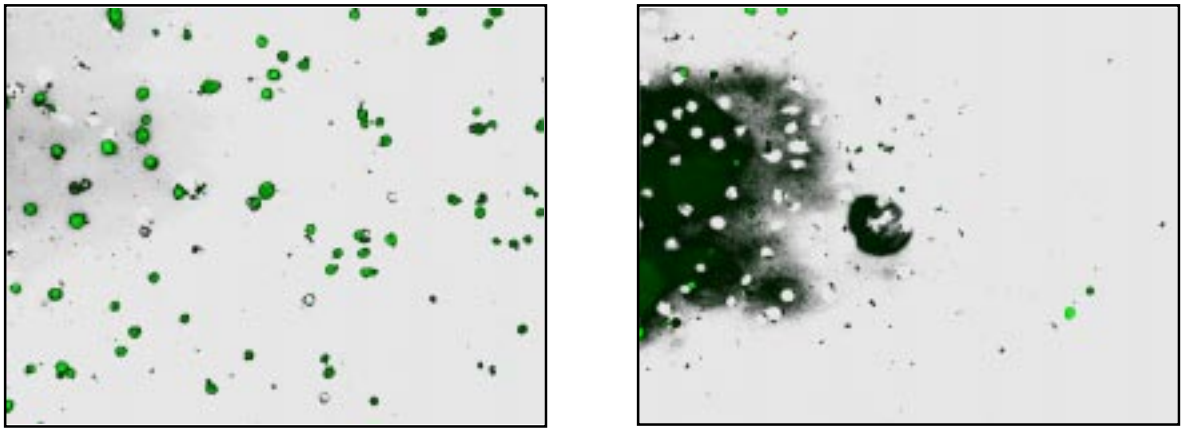
In separate experiments cell viability following optical or piezoelectric ultrasound was analyzed via two methods relying primarily on membrane integrity as a determination of viability. One method used was the Trypan Blue exclusion assay that is well known in the field. The method that we preferred was a two-color fluorescence cell assay (Live/Dead kit, Molecular Probes). Post-ultrasound treatment, the cells were incubated with calcein-AM, which is a 'non-fluorescent' molecule easily taken up into viable cells. Live cells enzymatically cleave the complex into an extremely fluorescent (green), membrane-impermeable calcein molecule. This is done in conjunction with ethidium homodimer-1, a membrane-impermeant dye that only enters cells with compromised membranes, a defining characteristic of dead cells. Once inside the cell, ethidium homodimer-1 binds to the nucleic acids and its fluorescent (red) intensity increases significantly.

3. RESULTS

Successful transient permeation of cells was demonstrated with opto-acoustic ultrasound. Figure 2 shows incorporation of 10 kDa Fluorescein-Dextran dye into MES-SA cells using 100 μJ laser pulses at 5 kHz repetition rate. Compared to control cells, a 14-fold improvement in fluorescence (dye uptake) was achieved. Approximately 50% of opto-acoustic treated cells incorporated dye vs. 6% of control cells. Measurements made with the hydrophone showed that cells experienced pressure magnitudes of approximately 10 bar compressive and nearly equal magnitude in tension. When cells experienced less than these pressures, little cell permeation was seen.

Cell Viability

Cell viability assays demonstrated minimal effect of opto-acoustic ultrasound on short-term viability. Figure 3 shows a representative result 2 hours after treatment with opto-acoustic ultrasound. A small fraction (<4%) of cells are considered dead.



(a) (b)

Figure 2. Incorporation of 40 kDa dye into a) opto-acoustic treated cells and b) control cells. Both figures represent similar numbers of cells.

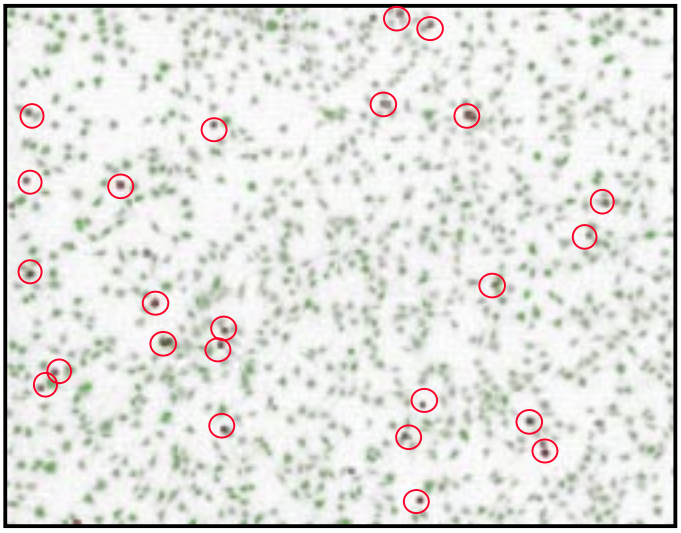


Figure 3. Cell viability assay showing live (green) and dead (red-highlighted with circles) cells 2 hours post-opto-acoustic treatment. Dead cells account for approximately 3.4%.

4. DISCUSSION

We have proven that cells can be transiently permeated with the use of acoustic energy. Others have shown that low frequency continuous wave ultrasound can permeate membrane barriers such as the skin. However, we are the first to use optically generated "ultrasound" to accomplish the same goals. This technique offers the important advantage of remote generation of ultrasound for site specific enhanced drug delivery. There are schemes for delivering therapeutic levels of ultrasound internally, but none have proven practical for remote sites. Opto-acoustic ultrasound has the potential to make this a reality. Further, the waveform characteristics of opto-acoustic ultrasound differ from those of piezoelectric ultrasound and may offer additional advantages.

There are several mechanisms that could be responsible for the transient permeability. Although cavitation with the opto-acoustic technique was not confirmed, the pressures produced are likely capable of water cavitation. Cavitation is thought to disorder the normally highly ordered lipid bilayer comprising the cell membrane, thus forming pores for the infusion of

molecules. Other means for permeation include mechanical stresses and large pressure gradients associated with microjets and microstreaming. The mechanism of action remains to be proven.

Viability assays have demonstrated low levels of cell death, in some instances not much above cell plating efficiency. Acceptable limits of cell death need to be established for particular applications. Likely, one would desire to operate just below this threshold to achieve maximal permeability. Further long term evaluation on cell viability and cell function remain to be performed.

We have explored a range of laser parameters for opto-acoustic enhanced drug delivery and have determined optimal settings within constraints of available lasers. The conversion of optical to mechanical (acoustic) energy is inefficient. It would be advantageous to increase the pressures that the cells are exposed to and there are several ways to exploit this. The easiest method is to increase the laser energy but this can only be accomplished by decreasing the pulse repetition rate as there is limited power available. Further, higher pulse energy beyond that used here may result in optical fiber damage. Another solution is to improve the efficiency of the optical to mechanical conversion which we continue to explore. Factors that affect the conversion at the distal end of the optical fiber include: pulse duration, fiber size, optical penetration depth, thermal and mechanical properties of the absorbing material. Due to the inefficiency of the current configuration, heat accumulated at the fiber tip. We have implemented a system of flowing the absorber past the optical fiber. A flow rate of 1 ml/min was sufficient to alleviate thermal energy and also prevent bleaching of the absorber.

The success of these techniques for transient permeation of cells is encouraging and work in this field will continue. The mechanism of action will be further explored and once elucidated, will be exploited for maximum permeation effect. The use of opto-acoustic ultrasound offers a novel range of acoustic parameters and also enables remote generation of ultrasound. This will make possible the use of drugs that were previously prohibited due to their poor selective uptake and enable exciting new pharmacologic and gene therapies.

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REFERENCES

1. Mitragotri S, Blankschtein D, Langer R. Transdermal drug delivery using low-frequency sonophoresis. Pharmaceutical Research 1996; 13(3): 411-420.
2. Nishioka T, et al. In vivo clot disruption by combination of Dodecafluoropentane and transcutaneous ultrasound. Circulation 1996; 94: 499.
3. Doukas AG, McAuliffe DJ, Flotte TJ. Biological effects of laser-induced shock waves: structural and functional cell damage in vitro. Ultrasound in Med & Bio 1993; 19: 137-146.
4. McAuliffe DJ, Lee S, Flotte TJ, Doukas AG. Stress-wave –assisted transport through the plasma membrane in vitro. Laser in Surgery and Medicine 1997; 20: 216-222.