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Site-Specific Sonoporation of Human Melanoma Cells at the Cellular Level Using High Lateral-Resolution Ultrasonic Micro-Transducer Arrays

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Abstract

We developed a new instrumental method by which human melanoma cells (LU1205) are sonoporated via radiation pressures exerted by highly-confined ultrasonic waves produced by high lateral-resolution Ultrasonic Micro-Transducer Arrays (UMTAs). The method enables cellular-level site-specific sonoporation within the cell monolayer due to UMTAs and can be applicable in the delivery of drugs and gene products in cellular assays. In this method, cells are seeded on the biochip that employs UMTAs for high spatial resolution and specificity. UMTAs are driven by 30-MHz sinusoidal signals and the resulting radiation pressures induce sonoporation in the targeted cells. The sonoporation degree and the effective lateral resolution of UMTAs are determined by performing fluorescent microscopy and analysis of carboxylic-acid-derivatized CdSe/ZnS quantum dots passively transported into the cells. Models representing the transducer-generated ultrasound radiation pressure, the ultrasound-inflicted cell membrane wound, and the transmembrane transport through the wound are developed to determine the ultrasound-pressure-dependent wound size and enhanced cellular uptake of nanoparticles. Model-based calculations show that the effective wound size and cellular uptake of nanoparticles increase linearly with increasing ultrasound pressure (i.e., at applied radiation pressures of 0.21, 0.29, and 0.40 MPa, the ultrasound-induced initial effective wound radii are 150, 460, and 650 nm, respectively, and the post-sonoporation intracellular quantum-dot concentrations are 7.8, 22.8, and 29.9 nM, respectively) and the threshold pressure required to induce sonoporation in LU1205 cells is ~0.12 MPa.

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Keywords

Biochip; Cell Membrane Wound; Piezoelectric-based Transducer; Quantum Dot; Radiation Pressure; Transmembrane Transport

1. Introduction

Successful gene therapy and delivery of gene products rely on the efficient delivery systems. The lipid bi-layer of cell membranes poses the physical barrier to the transport of large and polar molecules into cells (Stein, 1986). Current methods to enhance the cellular transport across the cell membrane are viruses for gene therapy (Johnson-Saliba and Jans, 2001), electroporation (Canatella and Prausnitz, 2001), ultrasound-mediated plasma membrane permeabilization (sonoporation) (Sundaram et al., 2003), cationic lipids (Brown et al., 2001), and drug conjugates (Fischer et al., 2001). Among these methods, sonoporation has some advantages because the effect of ultrasound on cells is mechanical (Sundaram et al., 2003), rather than chemical and, thus, the enhancement of molecular transport is likely to be broadly applicable to a variety of molecules and cell types.

Research shows that ultrasound-mediated transfer of gene products into the cells is principally due to acoustic cavitations and ultrasound-induced mechanical stresses (Bao et al., 1997). These stresses increase the cell membrane permeability by inducing transient wounds in the cell membrane and facilitate the transfer of DNA/molecules into cells by passive diffusion (Schlicher et al., 2006; Tachibana et al., 1999; Taniyama et al., 2002). While advances in methodologies and transducer designs currently allow the ultrasound to be focused to a large population of cells in the suspension (Kremkau, 1998) for sonoporation, there is yet a lack of technique that allows high lateral resolution to treat small clusters of cells down to the monolayer thickness, which are commonly used in cell-based assays for drug screenings.

In this work, we demonstrated the site-specific sonoporation of human melanoma cells (LU1205) at the cellular level with high spatial specificity and effective lateral resolution using the novel design and technique of ultrasonic micro-transducer arrays (UMTAs). A monolayer of LU1205 cells was grown on the surface of the biochip and the cells seeded above the micro-transducers were selectively sonoporated by the highly-confined ultrasonic beams produced by micro-transducers. The degree of sonoporation is determined from the amount of carboxylic-acid-(surface)-derivatized CdSe/ZnS quantum dots (QDs) transported into the LU1205 cells over a certain period of time following the sonoporation process. FWHM (Full Width at Half Maximum) of the fluorescence spatial profile of the QDs inside the LU1205 cells was used to evaluate the effective lateral resolution of the site-specific sonoporation process with UMTAs. Model-based analyses on the intracellular concentrations of QDs after sonoporation was used to access the size of ultrasound-inflicted wounds in the cell membrane of targeted cells and its dependence on the ultrasound radiation pressure.

The work presented here demonstrates a novel instrumental method that will improve the efficiency, throughputs, multiplexing capability, and robust analysis of micro-fabricated-device-based cellular assay platforms (Fjeldbo et al., 2008; Redmond et al., 2004; Uchimura et al., 2005) by enhancing the delivery of drugs or gene products into the cells using controllable sonoporation. The sonoporation biochip also renders as a subsystem for future large-scale integration with microfluidic components/systems (Curtis et al., 2009) and micro-array transfer printing systems (Hart et al., 2009; Luo et al., 2010; Mishina et al., 2004) to further improve the efficiency and multiplexing in drug discovery research.

2. Materials and Methods

2.1. The Design and Architecture of UMTA–Biochip

The UMTA–biochip consists of 3×3 arrays of high–aspect–ratio transducers (the height $L = \sim 120\mu\text{m}$ and the cross–sectional area $S = \sim 25\mu\text{m} \times \sim 25\mu\text{m}$) PMN–PT (Lead Magnesium Niobate–Lead Titanate, $\rho = 7,900\text{ kg/m}^3$, $c = \sim 4,600\text{ m/s}$, piezoelectric coefficient $d_{33} = \sim 2,500 \times 10^{-12}\text{ C/N}$) (TRS Technologies, Inc., State College, PA, USA) that produce longitudinal vibrations when an ac electric field is applied (the cross–sectional schematic of UMTA–biochip illustrated in Fig. 1(A)). Micro–transducer pillars (Fig. 1(B)) are laid down on a thin film of silver epoxy coated on the glass substrate, which serves as a common bottom electrode. Epoxy resin is filled in the space between individual micro–transducers in order to dampen or suppress flexural vibrations and to prevent cross–talk between individual micro–transducers. The top surfaces of the micro–transducers are coated with $2000\text{\AA}$ –thick Au films, which serve as top electrodes (Fig. 1(C)). These top Au electrodes are individually connected to their respective bonding pads through Au interconnections. The micro–transducers are encapsulated under a $15\mu\text{m}$ –thick Parylene–C overcoating. The Parylene–C encapsulation is chemical–resistant (Feili et al., 2006), biocompatible (Schmidt et al., 1988), and insulative (Loeb et al., 1977), and has been designed as an acoustic impedance matching layer between the micro–transducer and tissue culture media. Finally, the uppermost of the UMTA–biochip presents $500\text{\AA}$ –thick Au cell–trapping pads, which possess almost the same areas as the cross–sectional areas of micro–transducers and are located above the transducers. In the UMTA–biochip, when the micro–transducer is activated, the maximum peak ultrasound pressure occurs at the end of Fresnel Zone (near field). The near–field length, $NFL = (w_d)^2/4\lambda = (w_d)^2 f/4c$, was calculated to be $\sim 15\mu\text{m}$, given the 30–MHz frequency, $\sim 55\mu\text{m}$ averaged transducers diagonal width (w_d), and the speed of sound in water. As such, the Au cell–trapping pads are vertically separated from the underlying transducers by a $\sim 15\mu\text{m}$ –thick Parylene–C encapsulation layer for the maximum transducer efficiency (Supplementary materials for a brief description of UMTA–biochip fabrication process).

2.2. Cell Seeding on the UMTA–Biochip

Green–Fluorescent–Protein (GFP)–expressing human melanoma (LU1205) cells were kindly provided by Dr. Gavin P. Robertson, Pennsylvania State University Hershey Medical Center, Hershey, PA (Supplementary materials for cell culture protocols). The UMTA–biochip was mounted on the bottom of a glass Petri dish, wire–bonded (with silver epoxy) and sealed (with epoxy resin) for assay test. The assembly was cleaned with ethanol and sterilized under the UV light ($> 30\text{ min}$). The GFP–expressing human melanoma cells (LU1205) were seeded on the surface of the biochip in DMEM with 10% fetal bovine serum and 1% penicillin–streptomycin. The cells were then incubated and grown for 2 days at 37°C and 5% CO_2 (LU1205 cells grown on the surface of UMTA–biochip shown in Fig. 1(D)). After 70–80% monolayer confluency was achieved, the culture was replaced with 110 mL of fresh media (DMEM + 10% FBS, no penicillin–streptomycin) in which 100 nM of carboxylic–acid–derivatized CdSe/ZnS Core/Shell QDs (emission wavelength at 615 nm, hydrodynamic diameter $d_{\text{eff}} \approx 20\text{ nm}$, quantum yield: $> 50\%$, emission spectrum bandwidth (FWHM): $< 25\text{ nm}$, Emission tolerance: $\pm 10\text{ nm}$, and pH stability: 4–10) (Ocean NanoTech, LLC, Springdale, AR, USA) were homogeneously suspended.

2.3. Site–Specific Cellular Sonoporation with UMTAs

After QD–suspended media was added, micro–transducers were, activated by applying 30–MHz sinusoidal signals (with an Agilent E4422B ESG RF signal generator) for 3 minutes for four different RF powers (low and medium RF powers: 0.5 mW, 1.0 mW, and 2.0 mW; high RF powers: 80.0 mW) (Supplementary materials for the experimental setup). The

schematic of site-specific cellular sonoporation mechanism by UMTAs is illustrated in Fig. 1(E). The sonoporation time and RF powers chosen in this study were much lower than those that result in ultrasound-induced hyperthermia (Liu et al., 2001; Wang et al., 2010). Following the micro-transducer operation, the sonoporated cells were incubated in the QD-suspended media for six more minutes in order to load QDs into the cell by passive diffusion. Next, the cells were gently washed with fresh DMEM (contains no QDs) for 3 times to thoroughly remove the QDs adsorbed to the surface of the cell membrane. Cross-sectional Z-stack images of control cell samples prepared in the Petri dish were taken with the confocal microscope (Olympus Fluoview 1000) in order to determine whether QDs adsorbed on the surface of the cell membrane had been thoroughly removed after washing with fresh DMEM for 3 times (Fig. 2C). Finally, the cells were incubated in DMEM with 10% fetal bovine serum for ~30 minutes for recovery. Trypan Blue Exclusion Assay, which stains non-viable cells that do not possess intact cell membrane with trypan blue, was also performed 2 hours after sonoporation for the highest RF power applied (80–90% viable after 3 minutes of sonoporation).

2.4. Peak Acoustic Radiation Pressure on the Cell Membrane

By design, the peak ultrasonic pressure exerting on the cell membrane is approximately the same as the peak pressure at the end of Fresnel zone in an UMTA-biochip. As high aspect-ratio PMN-PT micro-transducers are embedded in epoxy resin with the piezoelectric coefficients $d_{33} \gg d_{31}$, the flexural vibration modes are dampened, leaving the longitudinal modes as the dominant vibration pattern for the transducer operation. The longitudinal displacement $\hat{\xi}(x, t)$ of the transducer and the maximum peak acoustic radiation pressure $|\hat{p}_{peak}(\omega)|$ exerted by the transducers onto the cell and the fluid column can be derived using the generalized wave equation Eq. (1) with boundary conditions described in Eqs. (2) and (3) (Kinsler et al., 1999; Uchino and Giniewicz, 2003).

$$\frac{\partial^2 \hat{\xi}(x, t)}{\partial x^2} = \frac{\rho}{\gamma} \frac{\partial^2 \hat{\xi}(x, t)}{\partial t^2} \quad (1)$$

$$\text{Fixed end: } \hat{\xi}(0, t) = 0 \quad (2)$$

$$\text{Mass support: } \left. \frac{\partial \hat{\xi}}{\partial x} \right|_{x=L} = -\frac{\hat{f}}{S\gamma} + d_{33}\hat{E} \text{ and } \hat{f} = Z_M S \left. \frac{\partial \hat{\xi}}{\partial t} \right|_{x=L} \quad (3)$$

$$\hat{\xi}(x, t) = \frac{d_{33}|\hat{E}|\gamma \sin(kx)}{2\omega [Z_M \sin(kL) - j\sqrt{\rho\gamma} \cos(kL)]} \text{Exp} \left[j \left(\omega t - \frac{\pi}{2} \right) \right] \quad (4)$$

$$|\hat{p}_{peak}(\omega)| = d_{33}|\hat{E}| \left\{ \left(\frac{1}{\gamma} \right)^2 + \left[\frac{1}{Z_M c} \cot \left(\frac{\omega L}{c} \right) \right]^2 \right\}^{-\frac{1}{2}} \quad (5)$$

where $\hat{E}$ is an applied ac electric field, Z_M is the characteristic acoustic impedance of water, $\hat{f}$ is the force exerted by the micro-transducer. At 30-MHz frequency the computed peak acoustic pressures, $|\hat{p}_{peak}(\omega)|$, are 0.21 MPa, 0.29 MPa, and 0.40 MPa for the applied RF powers of 0.5 mW (0.96 V_{p-p}), 1.0 mW (1.31 V_{p-p}), and 2.0 mW (1.79 V_{p-p}), respectively. Eq. 5 also shows that the peak pressure is maximum at resonance frequencies $f_r = (2n-1)c/4L$ ($n = 1, 2, 3, \dots$) and is minimum (or zero) at anti-resonance frequencies $f_a = mc/2L$ ($m = 1, 2, 3, \dots$) for a particular $\hat{E}$.

2.5. Fluorescence and Analysis

Fluorescence pictures on 3×3 array areas activated (both for Green Fluorescent Protein (GFP) and CdSe/ZnS QDs) were taken with a fluorescent microscope (Olympus BX60) using a water-immersion objective lens (10X, numerical aperture 0.3, working distance 3.3 mm) and white light excitation (mercury lamp). Emission detection at 515 nm-wavelength for GFP and at 615 nm-wavelength for CdSe/ZnS QDs was achieved by setting appropriate filter combinations with a cooled CCD camera (effective image pixel size 90 nm $\times$ 90 nm). The fluorescent intensities of CdSe/ZnS QDs transported into the sonoporated cells were computed by processing with ImageJ software (normalized with the same background intensities). To estimate the intracellular concentration of QDs and to evaluate the lateral resolution of the site-specific cellular sonoporation, the summed fluorescence spatial profile of the QDs transported into the sonoporated cells was obtained and analyzed (Supplementary materials for the algorithm used).

2.6. Control Experiment: Endocytosis-Driven QD-Uptake

To contrast the enhancement of QD-transport by sonoporation, we used the flow cytometry to quantify the endocytosis-driven QD-uptake (a slow transport process through G-protein coupled receptor-mediated endocytosis (Zhang and Monteiro-Riviere, 2009)) of LU1205 cell as a function of incubation period in QD-suspended media. Six cell samples were prepared and, for all the samples, the cell concentration was made to maintain at $\sim 0.5 \times 10^6$ cells/ml. Then, QD-suspended tissue culture media (100 nM of QD concentration) was evenly introduced into the samples except for one control sample. After 1 hr, 3 hrs, 6 hrs, 12 hrs and 24 hrs of incubation, respectively, samples were washed, lifted off, agitated, and suspended in the new regular media (contains no QDs), and then put into the flow cytometer (Guava PCA-96 System) for fluorescence intensity analysis (five measurements for five different incubation periods). The PCA-96 system shines a 532-nm green laser into the cell suspending media and use two detectors (FSC: forward scattered and SSC: side scattered) to detect excited emission from the total QDs inside the cells. The flow cytometry data were also coupled with time-lapsed fluorescent images (taken with Nikon inverted fluorescence microscope, 10X lens, 1X magnification, white light excitation), and confocal Z-stack microscopic images (taken with Olympus Fluoview 1000, 40X objective lens, and excitation with 543 nm-wavelength laser) in order to confirm and investigate the QD uptake into the cells via endocytosis.

3. Results and Discussion

3.1. Kinetics of Endocytosis-Driven QD-Uptake

Fig. 2(A) and 2(B) show the FSC (Forward Scattered) and SSC (Side Scattered) emission from the QDs inside the LU1205 cells and the normalized QD intensities (normalized with the baseline intensity taken from the control sample) measured with the flow-cytometry from 5 samples at different QD incubation periods. The results coupled with time-lapsed fluorescence images (inset of Fig. 2(B)) show that the endocytosis-driven QD-uptake is significantly slow and detectable amount of QDs is transported into the cells ~ 1 –2 hour after QDs were introduced. Fig. 2(C) shows the Confocal Z-stack image of LU1205 cells after

24-hour of QD (100 nM) incubation (before the imaging was carried out, the cells were gently washed with fresh DMEM, which contains no QDs, for 3 times to ensure that quantum dots adsorbed to the surface of the cell membrane have been thoroughly removed). The Z-stack images show that no or very little QDs were adsorbed to the surface of the cell membrane and the intracellular QD distributions, indicated by the excited emissions from QDs, at different Z-level cross-sections confirm that nanocrystal QDs were taken up by the cells via endocytosis.

3.2. Sonoporation–Enhanced QD–Uptake

Fluorescent microscopic images of GFP–expressing LU1205 cells on the surface of the biochip after ultrasound applications (three different RF powers, 0.5 mW, 1 mW, and 2 mW) is shown in Fig. 3. The Green Fluorescent Protein (GFP) emission, which is used to map the confluency of cell monolayer on the surface of the UMTA–biochip, shows that, after 2–day incubation, LU1205 cells grow homogeneously (~70–80% averaged monolayer confluency) on the surface of the UMTA–biochip having relatively more cells grown on the active areas of 25- μ m transducer arrays due to the present of Au pads (1(A), 2(A), and 3(A) of Fig. 3). The cells were healthy and viable after the sonoporation since high level of GFP expressions (strong GFP emissions) were observed from the ultrasound–treated cells. By comparing the GFP and QD images, it is evident that cellular–level site–specific sonoporation is achieved within the cell monolayer after UMTAs were activated. The red QD emissions from active areas (1(B), 2(B), and 3(B) of Fig. 3) confirm that the ultrasonic pressures generated by the micro–transducers permeabilized the LU1205 cells located above them and enhanced the transport of QDs by passive diffusion within a short time period (<6 minutes). Furthermore, highly–confined ultrasonic beams result in high lateral resolution for the site–specific sonoporation process, which is confirmed by the observation that no or negligible QD uptake occurred for the cells located in the inter–space and surrounding areas. The sum intensity spatial profiles of QD–emissions (1(C), 2(C), and 3(C) of Fig. 3) reveal that the degree of sonoporation and the amount of QD–uptake increase monotonically with the ultrasound radiation pressure, which was correlated to the RF power in the present study. At the applied RF powers of 0.5, 1.0, and 2.0 mW, the averaged sum intensities of QD emissions from the individual micro–transducer active areas are 7.5×10^4 , 2.2×10^5 , and 2.9×10^5 A.U., respectively.

Nevertheless, the trend does not hold for very high RF power levels. The site–specific sonoporation result at a RF power of 80.0 mW was shown in Fig. 4. The effective lateral resolution of sonoporation becomes very poor when micro–transducer array is driven at high input RF power (80 mW), where pressure from the side lobes of the ultrasonic beam produced from the micro–transducer becomes sufficiently large to sonoporate cells located on the interspaces between transducer active areas.

3.3. Effective Lateral Resolution of UMTAs for Site–Specific Sonoporation

For ultrasounds generated from the piston–type transducer, the diameter of the beam (or the lateral resolution) at the end of Fresnel zone (or near field) is $BD(-6 \text{ dB}) \approx 0.26w_d(S_F) = 9.1 \mu\text{m}$ (with normalized focal length $S_F = 1$ and diagonal width or aperture width $w_d \approx 35 \mu\text{m}$ for 25- μ m unfocused transducer). For unfocused transducer, the ultrasound pressure is peak at the end of NFL (Near Field Length) and the pressure distribution decreased rapidly with radial distance within the beam with the first diffraction minimum (much lower than the peak) near to the edge of the beam (Lu et al., 1994; Rahim et al., 2006). Since our study has revealed that the degree of sonoporation directly correlates with pressure distribution, the lateral resolution of cellular sonoporation by the transducer array can be approximated as $BD(-6\text{dB})$ when the cells are seeded at the end of the Fresnel zone. However, in practice,

the effective lateral resolution of sonoporation is lower due to the presence of side lobes of the ultrasonic beam produced by radial vibrations in the transducer (Lu et al., 1994).

In the experiment we use the FWHM of summed fluorescence intensity spatial profiles (Fig. 2: 1(C), 2(C), & 3(C)) to estimate the effective lateral resolution of UMTAs for site-specific sonoporation provided that sonoporated cells seeded above the transducers regained their morphology and did not migrate significantly within ~30 minutes during the experiment (FWHM also takes into account of reduced resolution due to cell morphology, spreading, and intracellular QD diffusion after sonoporation). The estimated effective lateral resolution of 25- μm transducers for site-specific sonoporation at the end of Fresnel zone at 30 MHz, $\Delta l_{\text{sono}} = \text{FWHM} \approx 2\sigma \sqrt{2 \ln 2}$, is $18 \pm 3 \mu\text{m}$ (Averaged for all three peak ultrasound pressures). It is, however, noteworthy, that the uncovered resolution of sonoporation with UMTAs only applies to low and medium RF power levels. The effective lateral resolution of sonoporation becomes very poor when micro-transducer array is driven at very high input RF power (80 mW) (Fig. 4), where pressures from side lobes of the ultrasonic beam become large enough to sonoporate cells located on the interspaces.

4. Modeling and Analysis

The purpose of modeling is to 1), determine the size of ultrasound-inflicted membrane wound and the amount of QDs transported into LU1205 cells as a function of radiation pressure applied by UMTAs; and 2), determine the threshold pressure required for sonoporation of LU1205 cells, and 3) compute the UTMA-based-sonoporation-induced QD-transport and QD-uptake enhancement factors. Additionally, the proposed modeling approach provides the theoretical framework guided by observed experimental results in quantifying the bioeffects such as the dynamics of cell membrane permeability changes and enhanced cellular uptake of nanoparticles after the site-specific sonoporation with UMTA. Two models are proposed and developed, which includes the model for ultrasound-inflicted cell membrane wound and that for transmembrane transport of QDs through the wound.

4.1. Ultrasound-Inflicted Wound Model

Plane-progressive acoustic waves exert radiation forces, which are always in the direction of wave propagation, on the incident object (Hasegawa et al., 1993; Mitri, 2006; Nyborg, 2001). Plasma membrane of living cells breaks temporarily when enough mechanical stresses (or forces) are imposed on the membrane (Vlahakis and Hubmayr, 2000; Vlahakis and Hubmayr, 2005). It is conceived that when an adherent cell is subject to mechanical stress due to ultrasonic radiation pressures, the plasma membrane undergoes elastic deformation (i.e., stretching). Above the threshold pressure, stress failure occurs and plasma membrane ruptures resulting in transient wounds in the cell membrane (Fig. 5) (Schlicher et al., 2006).

The induced wound can be relatively large (>150 nm in radius) and is resealed in an exponential-decay manner with lifetime $\tau_w > 1$ minute via a spontaneous (ATP- and Ca^{2+} -dependent process) healing response involving endogenous vesicle-based membrane resealing (McNeil et al., 2000; Schlicher et al., 2006; Terasaki et al., 1997; Togo et al., 1999; Zarnitsyn et al., 2008; Zhou et al., 2008). In this process, intracellular vesicles are recruited to the site of the wound and their lipid material is used to patch the ruptured membrane. Due to the presence of cytoskeletons (e.g., Spectrin spacing) and their associated proteins, the wound opening becomes sieve-like with nano-size pores as large as 50–70 nm in radius (Zarnitsyn et al., 2008) (See Supplementary Materials). For the wound opening that is much larger than the nano-size pores, the flux density of nanoparticles through the membrane wound weakly depends on the number, size, and distribution of small nano-size

pores within the wound and the transport through the membrane wounds largely depends on the mass transfer along its edges (Sneddon, 1966; Zarnitsyn et al., 2008). We hypothesize that, at higher ultrasound radiation pressures applied to a monolayer of multiple cells, larger and more wounds are created and the induced wounds are estimated as a single large circular opening (i.e., a large single lump of individual wounds) with the circumference equal to the sum of circumferences of individual smaller circular wounds since the mass transfer is mostly along the edges of the wound (See Supplementary Materials). Recent experiment shows that the transient transport of nanoparticles into the cells during the ultrasound application is statistically insignificant in comparison to the post-sonication transport (Zarnitsyn et al., 2008) since QDs are transported predominantly by passive diffusion through the wound in the absence of ultrasound-induced activities. Furthermore, we hypothesized that the effective wound, the porosity and pore size decays exponentially during the resealing process (Zarnitsyn et al., 2008; Zhou et al., 2008) (See Supplementary Materials). As the pore size decreases overtime, the mean effective diffusion time for QDs through the membrane wound (defined as $\tau_{w,eff}$), which largely depends on the hydrodynamic diameter (d_{eff}) of the diffusing molecule, is relatively shorter than the actual lifetime of the wound due to the geometrical hindrance.

4.2. Concentration of Quantum Dots Transported into the Cell

The rate of change of intracellular concentration of QDs $dC_{in}(t)/dt$ after sonoporation process can be obtained from the Eqs. (6) and (7) taking into account the mass balance, mixed boundary values problem, Fick's first law of diffusion through a circular opening, and kinetics of mechanical wound healing or resealing (Schlicher et al., 2006; Zarnitsyn et al., 2008).

$$\frac{dC_{in}(t)}{dt} = \left(\frac{1}{V_{cells}} \right) \left[\frac{h}{D_{pore} S_{pores}} + \frac{1}{4R_{w,eff}^0} e^{-t/\tau_{w,eff}} \left(\frac{1}{D_{out}} + \frac{1}{D_{in}} \right) \right]^{-1} [C_{out} - C_{in}(t)] \quad (6)$$

$$\text{Initial condition: } C_{in}(t_0) = 0 \quad (7)$$

where V_{cells} is the volume of cells occupied above the micro-transducer, h is the thickness of the cell membrane, S_{pores} is the total area of nano-size pores, $\tau_{w,eff}$ is the mean effective diffusion time for QDs through the wound (dependent on the size of the nano-particle being

transported, i.e., d_{eff}), $R_{w,eff}^0$ is the initial effective radius of combined wounds, and t_0 is the lag time following the sonoporation process at which QDs are added. The extracellular QD diffusion coefficients are estimated by Stoke-Einstein equation:

$D_{out} = k_B T / 3\pi\eta d_{eff} = 2.93 \times 10^{-7} \text{ cm}^2/\text{s}$. D_{in}/D_{out} is approximated to be 0.3 (Seksek et al., 1997; Verkman, 2002). For the initial effective radius of combined wounds $R_{w,eff}^0 \gg h$ (or) $S_{pores} \gg h$ diffusion resistance within the wound can be neglected and the total intracellular QD concentration $C_{in}(t)$ can be described by Eq. (8).

$$C_{in}(t) = C_{out} \left\{ 1 - \text{Exp} \left[- \int_{t_0}^t \left(\frac{1}{V_{cells}} \right) \left[\frac{1}{4R_{w,eff}^0 \text{Exp}[-t/\tau_{w,eff}]} \left(\frac{1}{D_{out}} + \frac{1}{D_{in}} \right) \right]^{-1} dt \right] \right\} \quad (8)$$

4.3. Ultrasound–Pressure–Dependent Wound Size

Experimentally, the concentrations of QDs inside the cells can be estimated from the summed fluorescent intensity (Fig. 3(C)) using an empirical conversion factor of $\alpha = 1.0413 \times 10^{-13} \text{ M(A.U.)}^{-1}$, i.e., fluorescence-to-intracellular-QD concentration conversion factor (See supporting materials for the algorithm used to obtain α).

$$C_{in}(t=360\text{s}) = \left\{ \int_{-\infty}^{\infty} \frac{(I_{sum})_{\max}}{3} \text{Exp} \left[-\frac{(x-\mu)^2}{2\sigma^2} \right] dx \right\} \times \alpha \quad (9)$$

The estimated intracellular concentrations of QDs, which are transported into the cytoplasm of the LU1205 cells (seeded on $25\mu\text{m} \times 25\mu\text{m}$ active area of a transducer), within 6 minutes after sonoporation for applied pressures of 0.21 MPa, 0.29 MPa, and 0.40 MPa are 7.8 ± 2.3 nM, 22.8 ± 1.6 nM, and 29.9 ± 2.5 nM respectively (Fig 6: inset). The estimated ultrasound-induced initial effective wound radius using Eq. 8 (with $t_0 = 0$, $\tau_{w,eff} = 40\text{s}$ for $d_{eff} = 20$ nm (Rahim et al., 2006), $t = 6$ min, and $C_{out} = 100$ nM) are 150 ± 45 nm, 460 ± 50 nm and 650 ± 70 nm for applied pressures of 0.21 MPa, 0.29 MPa, and 0.40 MPa respectively. The initial effective wound size increases in a linear fashion with ultrasound peak pressure (Fig. 6). Extrapolated plot show the threshold ultrasound pressure ($|p_{peak}(30 \text{ MHz})|_{th}$) to induce enhanced QD-transport by sonoporation in LU1205 cells is ~ 0.12 MPa. The threshold pressure is the pressure at which cell membrane undergoes stress failure. Below this threshold, the cell membrane is still intact and may not be permeable enough for QDs to be transported via passive diffusion.

4.4. Sonoporation–Enhanced Transport and QD Uptake

We evaluate the sonoporation-induced transmembrane permeability (or transport)

enhancement factor $\varphi_E \approx \left(\frac{dC_{in}^{sono}}{dt} \right) / \left(\frac{dC_{in}^{endo}}{dt} \right) \Big|_{t=t_0=0}$ which is the ratio of sonoporation-induced transmembrane transport rate of QDs just after the sonoporation process to the rate of endocytosis-driven QD-uptake following the introduction of QD-suspended media. The φ_E also represents the degree of sonoporation by UMTAs. In calculating φ_E , the 3rd order polynomial fit of $C_{in}^{endo}(t)$ is obtained from flow cytometry coupled with fluorescent microscopy (Figs. 2(A) and 2(B), Supplementary materials). The averaged permeability (or transport) enhancement factors via sonoporation for LU1205 cells in this study are 712, 1894, and 2927 for applied pressures of 0.21 MPa, 0.29 MPa, and 0.40 MPa respectively. It is evident from Eq. (8) that φ_E largely depends on ultrasound pressure, the hydrodynamic size of QDs, extracellular concentration, and endocytosis mechanism.

For sonoporation-enhanced total QD uptake by LU1205 cells, the estimated enhancement

factor is evaluated as $v_E = \left(\frac{\Delta C_{in}}{\Delta t_{sono}} \right) / \left(\frac{\Delta C_{in}}{\Delta t_{endo}} \right) = \frac{\Delta t_{endo}}{\Delta t_{sono}}$, which is the ratio of the diffusion time it takes to achieve specified intracellular concentration of QDs via endocytosis to that by sonoporation and takes into account of wound healing kinetics of a particular cell line. The total QD-uptake enhancement factors (averaged) by sonoporation are 86, 200, and 241 for applied pressures of 0.21 MPa, 0.29 MPa, and 0.40 MPa respectively for $\Delta C_{in} = C_{in}^{sono}(t=180 \text{ s})$. Again, Eq. (8) as well as flow cytometry data (Fig. 2) suggests that v_E depends on ultrasound pressure, extracellular concentration, hydrodynamic size of QDs, endocytosis mechanism as well as wound healing kinetics. Furthermore, Eq. (8) can be used to analyze the influence of ultrasound radiation pressure and wound healing kinetics on the sonoporation-enhanced transport process of nanoparticles (e.g. quantum dots) into the cells

(supplementary materials) and will allow us to precisely control and enhance the delivery of drugs or gene products into the cells using UMTA–biochip platforms.

5. Conclusions

In summary, we have successfully demonstrated the site–specific sonoporation at the cellular level using microfabricated high aspect–ratio miniature transducer arrays. Due to very close proximity of cells by chip design and architecture, direct mechanical stresses induced by ultrasounds can create transient wounds in the cell membrane. These stresses are more controllable by adjusting ultrasonic pressures compared to cavitation–induced stresses produced indirectly. The spatial resolution of ultrasound generated for site–specific sonoporation is high, which will be beneficial for cell–based assays. CdSe/ZnS QD uptake is significantly enhanced (an enhanced factor of > 100 at 0.29 MPa for LU1205 cells) due to passive diffusion of QDs compared to the slow QD uptake driven by endocytosis. Model–based calculations and analysis show that sonoporation level determined by the effective plasma membrane wound size is linearly dependent on the ultrasound pressure applied and the threshold pressure required to permeabilize LU1205 cells is ~ 0.12 MPa. The bioinstrumentational method and modeling approaches demonstrated in this study can be extended further for investigating, probing, and characterization of cellular uptake of various therapeutic drugs, gene products, or proteins. Hence, the work represents an important step towards the development of high–throughput cell–based multiplex drug screening, transfection assay, and sensing platforms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

(A) Cross-sectional schematics of ultrasonic micro-transducer array biochip architecture. (B) SEM image of 3×3 array of micro-transducer pillars (Each pillar has cross-sectional area of $25\mu\text{m} \times 25\mu\text{m}$). (C) Bright field image of top electrodes (Each electrode area: $25\mu\text{m} \times 25\mu\text{m}$) above the micro-transducer array and interconnections. (D) Human melanoma (LU1205) cells growing above the active area presented by a micro-transducer. (E) Schematics of ultrasound-induced site-specific cell membrane permeabilization (sonoporation) at the cellular level. A RF signal is applied across the PMN-PT transducer pillar to generate ultrasonic waves towards the targeted cell(s) above it ($\sim 15\mu\text{m}$ away).

Fig. 2.

(A) Two-parameter histograms obtained by flow cytometry showing endocytosis-driven QD-uptake by LU1205 cells for different QD incubation periods. The amount of QD uptake increases with longer QD incubation period. (B) Dynamic of endocytosis-driven QD uptake by LU1205 cells measured with the flow cytometer. Inset: (1) LU1205 cells in the QD-suspended media; (2), (3), and (4) LU1205 cells showing QD uptake via endocytosis after 2 hrs., 6 hrs., and 12 hrs. of incubation respectively (samples prepared in Petri dishes). (C) Confocal microscopic images of QD distribution inside LU1205 cells. Z-stack images from 1 to 6: (1) the bottom of LU1205 cells; (6) the top of LU1205 cells (A 2- μ m increment in Z-axis between each image). See supplementary materials for 3D deconvolution of Z-stack images). The images were taken after 24 hours of incubation in the tissue culture media that contained 100 nM of carboxylic-acid-derivitized 615-nm CdSe/ZnS QDs.

Fig. 3.

Micro-transducer arrays driven at three different RF powers at 30 MHz for 3 minutes. Each micro-transducer in the array presents $25\mu\text{m} \times 25\mu\text{m}$ active area. RF Powers applied: **(1)** 0.5 mW, **(2)** 1.0 mW, and **(3)** 2.0 mW. **(A)** GFP-expressing LU1205 cells seeded on the surface of UMTA-biochip. **(B)** LU1205 cells located above the micro-transducers (i.e., on the active area) show CdSe/ZnS QD uptake by passive diffusion after 3-minute sonoporation (QDs were transported into the cells within 6 minute post-sonoporation period). The cells located on the interspaces between individual micro-transducers and on the surrounding areas show little or no QD uptake. **(C)** Summed fluorescent intensity spatial profile of QDs transported into LU1205 cells seeded on the active areas along the column 1, 2, and 3 of the 3×3 array. The scale bar is $30\mu\text{m}$.

Fig. 4.

(A) No QD uptake was observed in the areas where micro-transducers are not activated (dashed lines show the location of micro-transducers). (B) QD uptake by LU1205 cells after micro-transducer array (Cross-sectional area: $50\mu\text{m} \times 50\mu\text{m}$) was driven at 80 mW, 30 MHz for 3 minutes. The image shows significant amount of QD uptake by LU1205 cells seeded above the micro-transducer array and interspaces. Higher ultrasound power and larger transducer cross-sectional area result in poor spatial targeting/resolution due to prominent side lobes in ultrasonic beams. The scale bar represents $100\mu\text{m}$.

Fig. 5.

Schematics of acoustic-radiation-pressure-induced cell membrane permeabilization process: (1) Cell membrane in normal steady state before being exerted by ultrasonic radiation pressure; (2) Cell membrane of adherent cell is stretched due to radiation pressure/force exerted by ultrasonic wave resulting in the increase in plasma membrane intermolecular distances. Lipid vesicles from intracellular origin travel to the cell membrane to prevent from membrane rupture; (3) Failure and rupture of the cell membrane when the radiation pressure is beyond the threshold. Transient wound is created in the plasma membrane. Intracellular lipids travel to the wound for repair (membrane patching).

Fig. 6.

Calculated initial effective wound radius after 3-minute sonoporation for three peak ultrasound radiation pressures (0.2, 0.3, and 0.4 MPa). Inset: Estimated intracellular concentrations of QDs after sonoporation (6 minute post-sonoporation QD (100 nM) incubation) for three peak ultrasound pressures.