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Technical Report

Microbubble-mediated sonoporation for highly efficient transfection of recalcitrant human B- cell lines

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Sonoporation has not been widely explored as a strategy for the transfection of heterologous genes into notoriously difficult-to-transfect mammalian cell lines such as B cells. This technology utilizes ultrasound to create transient pores in the cell membrane, thus allowing the uptake of extraneous DNA into eukaryotic and prokaryotic cells, which is further enhanced by cationic microbubbles. This study investigates the use of sonoporation to deliver a plasmid encoding green fluorescent protein (GFP) into three human B-cell lines (Ramos, Raji, Daudi). A higher transfection efficiency (TE) of >42% was achieved using sonoporation compared with <3% TE using the conventional lipofectamine method for Ramos cells. Upon further antibiotic selection of the transfected population for two weeks, we successfully enriched a stable population of GFP-positive Ramos cells (>70%). Using the same strategy, Raji and Daudi B cells were also successfully transfected and enriched to 67 and 99% GFP-positive cells, respectively. Here, we present sonoporation as a feasible non-viral strategy for stable and highly efficient heterologous transfection of recalcitrant B-cell lines. This is the first demonstration of a non-viral method yielding transfection efficiencies significantly higher (42%) than the best reported values of electroporation (30%) for Ramos B-cell lines.

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1 Introduction

The delivery of heterologous DNA into cells by transfection enables the elucidation and modulation of gene expression for functional analysis and potential medical applications. Gene delivery systems can be classified into viral and non-viral agents [1]. Viral transfection agents have high transfection efficiencies, are able to achieve prolonged stable expression of transgenes and easily engineered [2]. However, lack of site specificity, insertional mutagenesis and safety issues are obstacles to widespread utilization of this technology [2, 3]. Non-viral transfection methods like electroporation and lipid-based systems are safer [1], but require optimization of conditions to achieve high transfection efficiencies [4]. While methods of gene delivery have been widely researched upon for decades, the challenge of developing a balance between a safe and efficient method for transfection is still unmet.

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Abbreviations: GFP, green fluorescent protein; **HEK,** human embryonic kidney; **MBs,** microbubbles; **TE,** transfection efficiency

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Sonoporation has been described as an alternative non-viral transfection method, which combines the use of ultrasound and microbubbles (MBs) to deliver extraneous DNA into eukaryotic and prokaryotic cells [4]. MBs are commercially-manufactured lipid/polymer insoluble gas encapsulated bubbles whose surface can be coupled with the desired transfection agent [5]. On its own, acoustic pressure from ultrasound has been previously documented to result in transient poration of the cell membrane [6, 7], allowing passage of extracellular agents like DNA. When applied with MBs, ultrasound initiates the oscillation of MBs and enhances transfection by lowering the acoustic energy needed for transient poration of the cell membrane [5, 8]. The force and the shear stress generated by the oscillating MBs disrupts and forms transient pores in cellular membranes, facilitating the entry of macromolecules or drugs [3, 5, 6, 9].

Sonoporation has previously been applied to fibroblasts, chondrocytes and CHO cells in vitro for transient and stable transfection [7, 9]. Although proof-of-concept studies were successful, transfection efficiencies varied considerably [9]. It was recognized that optimization of sonoporation conditions was instrumental in increasing transfection efficiencies. There are several parameters in sonoporation that contribute to successful transfection – acoustic power density, exposure duration, carrier frequency, and concentration of MBs and plasmid DNA. The use of cationic MBs serves to encapsulate anionic plasmid DNA and facilitate DNA delivery across the negatively charged cell membrane barrier [4]. Conditions for sonoporation differ between cell types, thus optimization of the process and balancing the different variables is necessary to obtain good transfection efficiencies [8]. Depending on the ultrasound intensity used, either stable or inertial cavitation occurs [3, 7]. Lower ultrasound intensities result in stable MB oscillation and minimal stress to cell membranes, and allows for transient and reversible cell membrane permeability without affecting the integrity of the cell membrane [7, 10]. In contrast, higher ultrasound acoustic energy intensities result in inertial collapse of MBs and permanent structural damage to cellular membranes [3, 7]. Thus, striking a balance between acoustic energy level and MB concentration ensures successful transfection without compromising cell viability.

B cells are central components of humoral immunity and play a crucial role in the regulation of immune responses against a wide range of pathogens. Upon contact with their antigenic stimuli, B cells exert multiple functions including antibody production, induction of T-cell differentiation and immune memory [11]. These immune responses are mediated by a complex series of overlapping processes, which are still not yet fully understood. To study these processes, gene transfection constitutes an important tool for the elucidation of cell biology and for modulation of immune responses. However, for reasons that remain unknown, B cells are not easily amenable to conventional transfection methods using lipids, calcium phosphate and DEAE-dextran [12, 13]. In this study, we demonstrated the successful use of sonoporation and MBs to introduce genes into human B-cell lines at transfection efficiencies greater than conventional methods. Furthermore, the transfected population can be enriched by an additional 2–3-fold with antibiotic selection and stably propagated.

2 Materials and methods

2.1 Plasmid

The reporter plasmid pEGFP-1, encoding green fluorescent protein (GFP) under the control of the Chinese hamster elongation factor-1α (CHEF1) promoter [14] was used for all experiments (Clontech, Mountain View, CA, USA). The plasmid was transformed and amplified in *Escherichia coli* DH5α competent cells (Invitrogen, Carlsbad, CA, USA) using kanamycin selection for positive transformants. Plasmid DNA from overnight *E. coli* culture grown in LB media (37°C, 220 rpm) was purified using QIAGEN plasmid midi kit (Qiagen, Valencia, CA, USA) as per manufacturer's protocol. Plasmid concentration and purity was assessed by Nano Drop (ND-1000) (Thermo Fisher Scientific, Waltham, MA, USA) at 260 nm. A neomycin-resistance cassette in pEGFP-1 selects for EGFP-stably transfected cells by G418 antibiotics.

2.2 Cell culture

Four cell lines were used in this study. The human embryonic kidney derived 293-HEK adherent cells (Invitrogen) was cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% Fetal bovine serum (FBS) (Thermo Scientific), 0.5% penicillin/streptomycin (Invitrogen) and 1% L-glutamine (Invitrogen). B-cell lines, Ramos (CRL-1596), Raji (CCL-213) and Daudi B were obtained from ATCC and maintained as suspension cultures in RPMI (Invitrogen) supplemented with 10% FBS and 55 μM beta-mercaptoethanol. HEK cells were seeded at a density of 2.3×10^5 cells/mL in a 24-well plate (Nalgene Nunc International, Rochester, NY, USA) 24 h before sonoporation to allow for adhesion. Ramos and Raji cells were seeded at a density of 2.3×10^5/mL on the day of sonoporation in a 24-well plate. All cells were 70–80% confluent before sonoporation. For assessment of cell viability for each sample, trypan blue exclusion was used.

2.3 Microbubble-EGFP conjugation and sonoporation

MBs are cationic delivery agents composed of lipid/polymer microspheres with a decafluorobutane core. Prior to

sonoporation, MBs (Targeson, Catalog No. TS-601, San Diego, CA, USA) of various concentrations ranging from 0 to 11 × 10^7/mL were incubated with 3 μg of pEGFP-1 plasmid for 25 min at room temperature with occasional mixing. After incubation, the entire volume of MB-DNA complexes was added to the seeded cells in the 24-well plate.

Sonitron 2000 (Nepa Gene Co. Ltd, Chiba, Japan) was used in the sonoporation experiments. Ultrasound gel (transmission medium between probe and plate) was applied to an inverted 10 mm transducer probe secured by a retort stand clamp. A 24-well plate containing the seeded cells was held above with contact to the probe. Sonoporation settings for all cell lines are listed in Supporting information, Table S1. Daudi and Raji Cells were sonoporated at a lower exposure time (15 s) as these cells were more sensitive to treatment.

2.4 Lipofectamine and AMAXA nucleofection

Lipofectamine 2000 (Invitrogen) was used in lipofectamine control experiments. Cells were seeded at their respective densities the day before. On the day of transfection, 3 μg of pEGFP-1 was diluted in 50 μL Opti-MEM medium (Invitrogen). Six microliters of lipofectamine was diluted in 50 μL Opti-MEM and incubated at room temperature. After 5 min, the diluted DNA and lipofectamine 2000 were combined, mixed gently and incubated at room temperature for 20 min. The total volume of the lipofectamine-DNA complexes was then added to seeded cells. Electroporation (AMAXA nucleofection) for B-cell lines was performed according to the SG cell line kit (V4XC-3032) (Amaxa, Basel, Switzerland) as per manufacturer's protocol.

2.5 Flow cytometry analysis, antibiotic selection for positively transfected cells and fluorescence microscopy

All cells were harvested 48 h after sonoporation to assess TE. HEK cells were harvested with a quick wash of PBS followed by trypsinization. Cells were then resuspended in 100 μL of 1% BSA/PBS for flow cytometry analysis. Ramos, Raji, and Daudi B cells were harvested without trypsinization and resuspended in 100 μL 1% BSA/PBS for flow cytometry analysis. TE was evaluated on the FACSCalibur System based on the percentage of cells expressing EGFP (Becton Dickinson, Franklin Lakes, NJ, USA).

For positive selection of transfected cells, 24 h after sonoporation, Ramos, Raji, and Daudi B cells were pas-

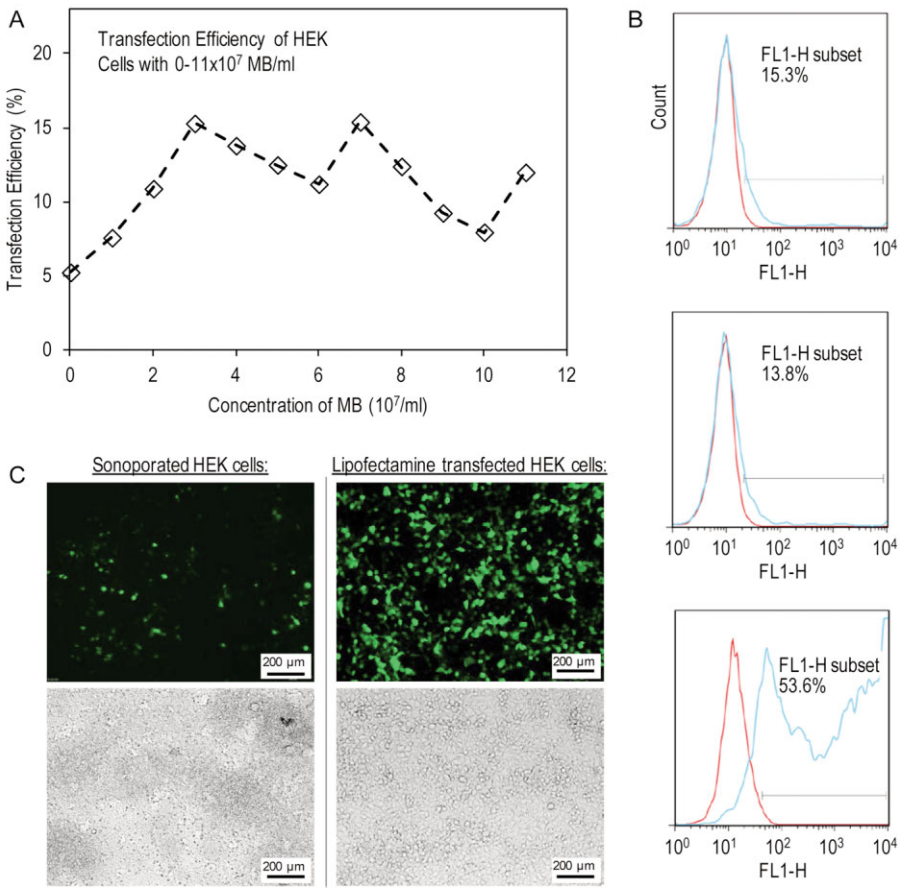

Figure 1. Transfection of HEK cells using sonoporation and lipofectamine. (**A**) HEK cells sonoporated (2 W/cm^2, 15 s, 1 MHz) with 3 μg pEGFP-1 and varying concentrations of microbubbles (0–11 × 10^7 MBs/mL). The values are a representative experiment of three biological replicates. (**B**) FACS analysis was used to determine TE of transfected HEK cells via sonoporation (3 and 4 × 10^7 MBs/mL, Top and Center panel, respectively) and lipofectamine (Bottom panel). TE based on 2% gate. (**C**) Fluorescence and bright-field microscopy of GFP expression in sonoporated and lipofectamine-transfected HEK cells at 3 × 10^7 MBs/mL (Left and Right, resepctively). Scale bar for all microscopy figures is 200 μm.

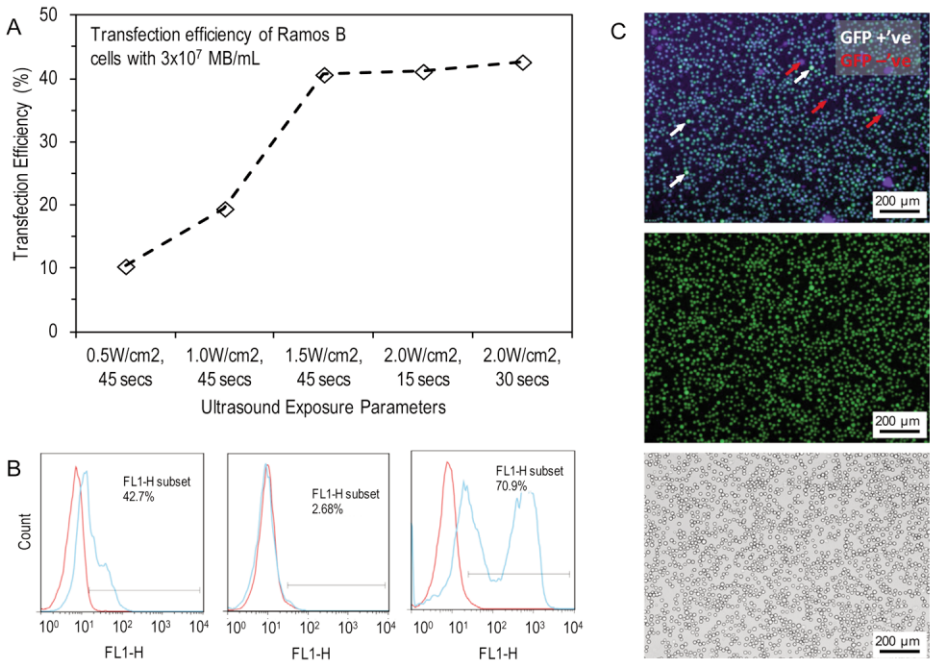

Figure 2. Transfection of Ramos B cells by sonoporation. (**A**) Ramos B cells sonoporated at varying ultrasound exposure parameters with 3 µg pEGFP-1 and 3×10^7 MBs/mL. The values are a representative experiment of three biological replicates. (**B**) FACS analysis used to determine TE for Ramos B cells transfected with EGFP by sonoporation with 3×10^7 MBs/mL (Left panel 42.7%) (Supporting information, Table S1) and lipofectamine (Center panel 2.68%). Transfected population was further enriched by G418 for 3 wk to select for positive clones (Right panel 70.9%). TE was determined based on 2% gate. (**C**) GFP-transfected Ramos cells counterstained with Hoechst dye at 3 wk post-transfection with G418 selection. White arrows represent transfected (GFP +ve) and red arrows represent untransfected cells (Top panel). Corresponding fluorescence and bright-field microscopy of GFP expression in sonoporated Ramos B cells (Middle and Bottom panels) Scale bar for all microscopy figures is 200 µm.

saged with 0.8 mg/mL of Geneticin (G418) (Invitrogen) in RPMI with 10% FBS and 0.1% β-mecaptoethanol and the G418 treatment was continued with every passage of cells for 8 wk after sonoporation. Population of positively transfected cells were assessed via flow cytometry after 7 continuous passages to confirm enrichment. GFP-transfected cells were counterstained (incubated) with Hoechst dye (1:1000 dilution) (Active Motif, Carlsbad, CA, USA) for 25 min before fluorescence microscopy.

2.5.1 Statistical analysis

All values in graphs in Figs. 1 and 2 are a representative experiment of three replicates. Unless otherwise stated, each independent sample consists of one well in a 24-well plate format.

3 Results

3.1 HEK cells

In an earlier study by Tlaxca et al. [15], a TE of ~70% was achieved when HEK cells were sonoporated in OptiCell® cartridges (Nunc) under their optimized conditions (Supporting information, Table S2). The first objective of our study was to reproduce the observations using our experimental setup in a simpler 24-well plate format. To optimize the efficiency, the concentration of MBs was also titrated from 1 to 11×10^7 MBs/mL. We observed that the maximum TE of 15.3% was obtained with HEK cells at a concentration between 3 and 4×10^7 MBs/mL (Fig. 1A) under our optimized conditions (Supporting information, Table S1). FACS analysis was used to determine TE of transfected HEK cells at $3–4 \times 10^7$ MBs/mL (Fig. 1B, top and center) compared to the lipofectamine method (53.6%) as a positive control (Fig. 1B, bottom). Fluorescence and bright-field microscopy of sonoporated and lipofectamine-treated HEK cells confirmed GFP expression in the transfected cells (Fig. 1C).

3.2 Ramos B cells

Next, we investigated if sonoporation could be applied to human B cells, which are generally known to be harder to transfect [12, 13]. The main difference between B and HEK cells is that HEK cells are cultured in an adherent format whilst B cells are suspension cells. We hypothesized that cells in suspension interact more readily with the oscillating MBs in the medium, facilitating transfection during sonoporation. Using the same MB concentration

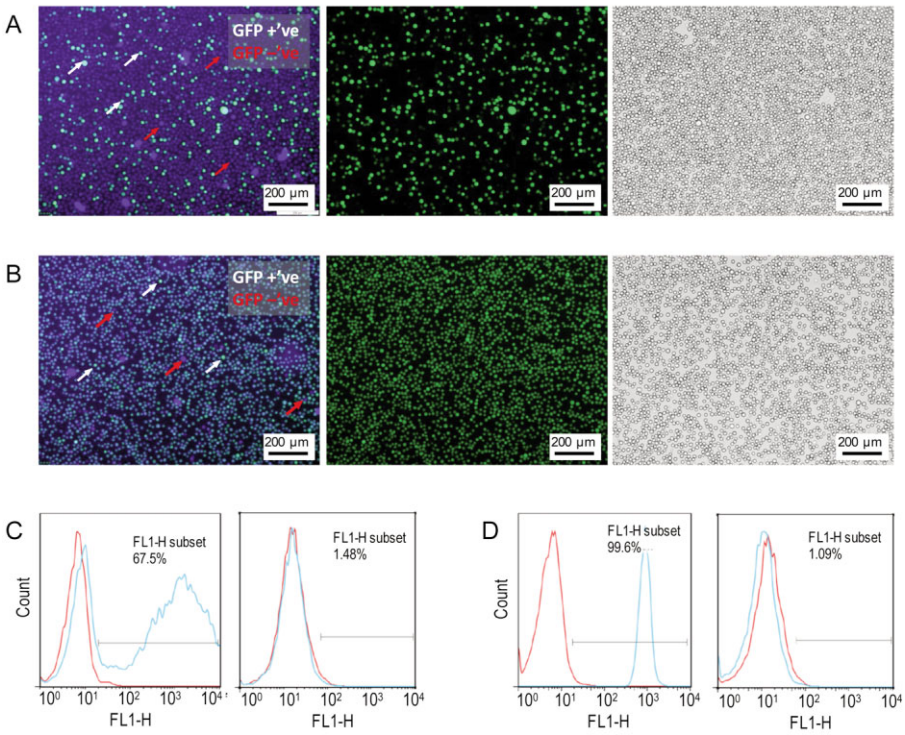

Figure 3. Transfection of Raji and Daudi B cells by sonoporation. Raji and Daudi cells sonoporated (See Supporting Information, Table S1) with 3 µg pEGFP-1 and 3×10^7 MBs/mL (**A** and **B**). GFP-transfected Raji and Daudi cells counter-stained with Hoechst dye at 3 wk post-transfection with G418 selection. White arrows indicate GFP-transfected cells and red indicating non-transfected cells (A and B Left panel). Corresponding GFP expression and bright-field microscopy of sonoporated Raji and Daudi cells (A and B Middle and Right panel). FACS analysis used to determine population of enriched Raji (67.5%) and Daudi (99.6%) B cells, respectively after 2–3 wk post-transfection. Population of enriched cells was determined based on 2% gate. FACS analysis of lipofectamine controls for Raji (1.48%) and Daudi (1.09%), respectively (**C** and **D** Right). Scale bar for all microscopy figures is 200 µm.

optimized for HEK cells (3×10^7 MBs/mL), we varied the sonoporation settings for the Ramos B-cell transfection experiments. Trypan blue exclusion and FACS analysis were used to determine the viability and TE, respectively for Ramos B cells transfected with EGFP plasmid by sonoporation. A maximum TE of 42.7% was observed when the cells were sonoporated at 2 W/cm^2 for 30 s exposure time (Fig. 2A). Contrary to HEK cells, the TE for the lipofectamine control was 16-fold lower compared to sonoporation (42.7% vs. 2.68%, Fig. 2B). Visual validation of GFP expression with fluorescence and bright-field microscopy of sonoporated Ramos B cells are shown in Fig. 2C. At these optimal settings, the viability 24 h post-transfection was determined to be 24% (data not shown). This result is comparable to results obtained with AMAXA nucleofection (28% viability). Following sonoporation of the Ramos B cells, the positively transfected cells were further expanded and selected with G418. Figure 2B (right) shows an enrichment of EGFP-positive population from 42.7 to 71% 2 wk post-transfection and selection. This demonstrates that transfected cells can be further enriched and stably propagated for subsequent analysis.

3.3 Raji and Daudi B cells

To demonstrate that this methodology can be applied generically, we extended our study to two additional B-cell lines: Raji and Daudi. Both B-cell lines were sonoporated with 3×10^7 MBs/mL at 2 W/cm^2 for either 15- or 30-s exposure times. As these cells are less robust to physical perturbation, an exposure time of 15 s was found to yield better cell viability for both cell lines 24 h post-transfection (70% for Daudi and 20% for Raji vs. 20–24% by nucleofection for both cell lines) and was hence used for subsequent experiments. Similar to Ramos, Raji, and Daudi cell lines were successfully transfected as observed on fluorescence and bright-field microscopy (Fig. 3A and B). FACS analysis of the sonoporated cells 2–3 wk post-transfection following G418 selection shows an enriched population of transfected Raji (67.5%) and Daudi (99.6%) B cells, while the lipofectamine controls yield lower numbers of GFP-positive cells, i.e. achieving only 1.48 and 1.09% for Raji and Daudi, respectively (Fig. 3C and D).

4 Discussion

Apart from sonoporation, electroporation has been the preferred in vitro non-viral transfection method for difficult-to-transfect cells [16] due to high transfection efficiencies comparable to viral transfection. While DNA transfer in sonoporation is driven by passive diffusion, the driving force in electroporation is electric [4, 9]. The application of high voltages in electroporation may induce tissue damage and affect stability of plasmid DNA during transfection [17]. Electroporation also has limited in vivo applications due to the invasive surgical procedures required in placing electrodes into internal organs [4].

Apart from in vitro applications, sonoporation offers a safe and non-invasive option to existing gene therapy

methods in vivo without the need for surgical intervention. It allows for the administration of microbubbles with therapeutic agents via blood circulation followed by sonoporation in target tissue to facilitate transfection in a localized area [18]. This is especially useful for cancer therapy as microbubbles loaded with a chemotherapeutic agent can respond to an ultrasonic field at a localized site with minimum effect on healthy tissues. On an alternative spectrum, more intense ultrasound inducing cavitational bubbles has been shown to play a role in cell disruption of microbial [19] and tumor cells [20–22]. Examples of cell disruption include the ability to effectively lyse cells, releasing intracellular contents for lab-on-a-chip diagnostics without denaturation of biomarker proteins [19]. Sonoporation has also been employed in cancer chemotherapeutics to target the uptake of drugs in a site-specific manner [23]. In vivo studies have reported successful delivery of therapeutic targets in cancer and tumors, paving the way for sonoporation as a promising technology for gene transfer and other biotechnological applications [20–23]. Numerous studies have optimized sonoporation conditions in cancer therapeutics, achieving transfection efficiencies of 20–50% [24] but transient expression of transgenes have remained an issue [25]. Other sonoporation studies involving various cell types achieve transfection efficiencies from 10 to 50% [7, 9, 26, 27].

A study by Tlaxca et al. [15] highlighted the importance of titrating microbubble concentration, acoustic power density and plasmid concentration in the transfection of HEK cells [15]. A maximum TE of 70% was observed in HEK cells via sonoporation, statistically comparable to common lipofectamine method. Optimal transfection was observed at 1–2 W/cm^2 and 3–4 × 10^7 MBs/mL without compromising cell viability significantly. On the basis of these findings, we performed HEK sonoporation using an inverted transducer on a 24-well tissue culture plate format. The maximum TE obtained with HEK cells in our study was 17% at 4 × 10^7 MBs/mL with 15 s exposure time. The difference in TE compared with Tlaxca et al. [15] may be attributed to the difference in sonoporation set up and their exposure time being three times longer (45 s). Tlaxa et al's [15] study found that maximum TE without compromising on cell viability was achieved by increasing power intensity up to 2 W/cm^2, MB concentration at 4 × 10^7 MBs/mL and plasmid concentration at 3 μg/mL. Increasing exposure time during sonoporation led to a decrease in cell viability. This reiterates the importance of titrating optimal conditions for sonoporation to achieve significant TE but yet minimizing cell death.

To investigate this further, we evaluated the application of sonoporation for transfection of human B-cell lines – widely known to be recalcitrant to gene transfection [13]. We adopted similar sonoporation conditions as the HEK cells using 3 × 10^7 MBs/mL, 1 MHz and 3 μg/mL plasmid DNA for all B-cell line experiments. We varied exposure time between 30 and 45 s and power intensity between 0.5 and 2 W/cm^2 to optimize sonoporation conditions. Here, we report a maximum TE of 42.7% for Ramos B cells via sonoporation at 2 W/cm^2, 30 s exposure. More importantly, contrary to HEK cells, the TE of B cells in the lipofectamine control was significantly poorer compared to sonoporation (42.7% vs. 2.68%). Our result is comparable to electroporation using Lonza's Amaxa® Cell Line Nucleofector® kit V, which is shown to achieve a 30% TE for Ramos B cells (Product datasheet DCV-1029_2009-05, Cat no. VCA-100). This result is encouraging, demonstrating that under optimized conditions, sonoporation may yield higher TE compared to conventional transfection strategies.

Following sonoporation of the Ramos B cells, the positively transfected cells were further expanded under G418 selection. Examination of the cells 2 wk post-transfection shows an increase in fluorescently transfected cells from 42.7 to 70.9% (Fig. 2B). This demonstrates that transfected cells can be enriched and propagated for subsequent studies. We extended our evaluation to two more human B-cell lines (Daudi and Raji) and observed similar enrichment and propagation of transfected GFP-positive B cells (Fig. 3A and 3B).

We have presented sonoporation as an alternative for transfecting recalcitrant human B cells with high efficiencies. This technology can be potentially applied to other recalcitrant cells, for example, lactic acid bacteria (LAB). LAB is an emerging microbial host for recombinant protein production due to their history of safe use in food, lack of endotoxins and secretion ability [28]. However, a major limiting factor of LAB is low transformation efficiencies with conventional methods [29]. Future studies on sonoporation can be extended to LAB systems with potential impact on biotherapeutics and biotechnology. This in vitro proof of concept study taken together with other in vivo published literature on sonoporation clearly demonstrates the potential use of this technology for clinical and biotechnological applications. To our knowledge, high transfection efficiencies of B cells have not been reported via non-viral means except electroporation. Here, we present sonoporation as an attractive alternative and hypothesize that this technology can be applied at a low cost and at potentially high efficiencies across other hard-to-transfect cell types.

This study has shown the successful transfection and enrichment of positive transfectants in recalcitrant human B cells via MB-mediated sonoporation. A maximum TE of 42.7% for Ramos B cells at 2 W/cm^2, 30 s exposure time was attained. This value is significantly higher than the lipofectamine control (2.68%). The positively transfected Ramos cells can be further expanded under G418 selection to 70.9%. Similar enrichment and propagation of Daudi and Raji B-cell lines were also demonstrated. This technology can be further extended to other cell lines generally resistant to transfection.

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