Transfection of cells in suspension by ultrasound cavitation

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A B S T R A C T

Sonoporation holds many promises in developing an efficient, reproducible and permanent gene delivery vector. In this study, we evaluated sonoporation as a method to transfect nucleic acids in suspension cells, including the human follicular lymphoma cell line RL and fresh human Chronic Lymphocytic Leukemia (CLL) cells. RL and CLL cells were exposed to continuous ultrasound waves (445 kHz) in the presence of either plasmid DNA coding for green fluorescent protein (GFP) or fluorescent siRNA directed against BCL2L1. Transfection efficiency and cell viability were assessed using fluorescent microscopy and flow cytometry analysis, respectively. Knock-down of target protein by siRNA was assessed by immunoblotting. Moreover, sonoporation was used to stably transfect RL cells with a plasmid coding for luciferase (pGL3). These cells were then used for the non-invasive monitoring of tumorigenesis in immunodeficient SCID mice. Sonoporation allows a highly efficient transfection of nucleic acid in suspension cells with a low rate of mortality, both in a tumor cell line and in fresh human leukemic cells. It also allowed efficient transfection of BCL2L1 siRNA with efficient reduction of the target protein level. In conclusion, ultrasound cavitation represents an efficient method for the transfection of cells in suspension, including fresh human leukemic cells.

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1. Introduction

Transfection of cells constitutes an essential tool for the understanding of cell biology and therapeutic modulation of gene expression. A variety of DNA delivery methods are being tested in nucleic acid therapy, both to induce expression of a deficient gene or to repress the expression of a target gene. A variety of transfection methods including viral and non-viral vectors have been used to transfect nucleic acids into mammalian cells. Viral vectors such as retroviruses and adenoviruses have been shown to be efficient in transfection [1]. However, these viral vectors present some drawbacks such as lack of site specificity, potentiality for insertional mutagenesis [2], induction of immunological responses and systemic toxicity. Non-viral methods [3] have also been developed such as naked plasmid DNA injection, electroporation, particle bombardment, lipofection and nucleofection. Overall, these methods are less effective for gene transfer than viral vectors, often induce transient gene expression and membranes [10–12], which allow the entrance of large molecules from the surrounding medium into the cell [13–15]. Under optimal conditions, the cell can reseal its membrane and survive its holes without notable damage. The self-sealing mechanism is one of the key factors that determine the transfection efficiency and post-ultrasound cell outcome. It is thought to involve lysosomal exocytosis and Ca2+ release [16]. This delivery of Ca2+ is necessary to avoid the intracellular overload of ions that might trigger many cellular processes such as apoptosis [17] and calcium oscillation [18,19]. Furthermore, several factors, including cellular architecture [20] and sonoporation parameters [21–25] may influence the degree of lines, including a majority of suspended cells have proven hard to transfect. While novel lipofecting agents and nucleofection have contributed to resolving this issue, the transfection of suspension cells remains difficult. The development of an efficient and if possible spatially and temporally targeted DNA delivery method is thus clearly needed.

Sonoporation is a recently developed technology enhancing cell membrane permeability which has been applied to improve the uptake of DNA and drugs by mammalian cells [4]. While the mechanisms of sonoporation are not yet completely understood, several studies have been carried out both in vitro [5,6] and in vivo [7,8] and have shown promising results. It is generally assumed that ultrasound (US)-mediated gene transfer is principally due to acoustic cavitation [6,9]. Sonoporation may increase cell membrane permeability by inducing transient non-lethal perforations in cells and other membranes [10–12], which allow the entrance of large molecules from the surrounding medium into the cell [13–15]. Under optimal conditions, the cell can reseal its membrane and survive its holes without notable damage. The self-sealing mechanism is one of the key factors that determine the transfection efficiency and post-ultrasound cell outcome. It is thought to involve lysosomal exocytosis and Ca2+ release [16]. This delivery of Ca2+ is necessary to avoid the intracellular overload of ions that might trigger many cellular processes such as apoptosis [17] and calcium oscillation [18,19]. Furthermore, several factors, including cellular architecture [20] and sonoporation parameters [21–25] may influence the degree of
membrane permeabilization and cell viability after sonoporation. To date, most cell lines that have been successfully transfected with US have been adherent cells [26–28] whereas only few attempts to porate cells in suspension have been reported [29,30]. These latter attempts were mostly performed using microbubbles known as contrast agents and showed an enhancement in transfection efficiency. Many types of molecules, such as plasmid DNAs [7,12], siRNAs and peptides [30] have been demonstrated to be delivered into cells by US both in vitro and in vivo.

Based on the cavitation produced by US, a key advantage of this method is its potential for spatial and temporal control. Its specificity resides in combining the capacity of enhancing transfection efficiency with the possibility of restricting the effect of US to the desired area during the desired time. This study was designed to investigate the possibility of delivering nucleic acid stably or transiently with an US device in a human Follicular Lymphoma (FL) cell line (RL) and in Chronic Lymphocytic Leukemia (CLL) cells freshly isolated from patients. Using a 445 kHz transducer, we varied US parameters, the duration of exposure, number of cells and DNA concentrations to optimize nucleic acid delivery with minimal impact on cell viability. We evaluated the possibility of using US to perform transient transfection of plasmid DNA and siRNA, as well as the possibility to obtain stably transfected cells.

2. Materials and methods

2.1. Cell line and culture

2.1.1. In vitro studies

In vitro studies were performed on RL follicular lymphoma cells (obtained from the American Type Culture Collection) and on fresh blood specimens from CLL patients. Patients gave written informed consent after approval of the study protocol by the Institutional Review Board of the Hospices Civils de Lyon. RL follicular lymphoma cells and CLL cells were freshly isolated from patients. Using a 445 kHz transducer, we varied US parameters, the duration of exposure, number of cells and DNA concentrations to optimize nucleic acid delivery with minimal impact on cell viability. We evaluated the possibility of using US to perform transient transfection of plasmid DNA and siRNA, as well as the possibility to obtain stably transfected cells.

2.2. Cavitation device

A 20 mm diameter flat transducer (LT01 EDAP, based on a piezoelectric device) was submerged in a rectangular water bath filled with warm degassed water (20 L; O2 concentration: 3 mg/L; temperature: 37 °C) to 14 mm above the top of the transducer aperture which was in a horizontal position (Fig. 1). The acoustic excitation was a continuous sine-wave at 445 kHz. The signal (generated by a PXI-6711 card, National Instruments) was amplified by a power amplifier (200 W, Adece) before feeding the transducer. The spatial average acoustic intensity (measured using the acoustic balance technique [31]) and the spatial peak acoustic pressure (hydrophone Lipstick GL-0200; SEA) did not exceed 1.7 W/cm2 and 0.46 MPa respectively.

The bottom of each cell-containing well (12-well plates in polystyrene, 20 mm diameter wells, BD Biosciences) was aligned parallel to the transducer at 9 mm from its aperture. Its vertical position was adjusted so that the antinode plan was located at the air/culture medium interface, as described earlier [32]. The attenuation of the ultrasonic beam by the well bottom wall was less than 2%, as shown by Tata et al. [33]. The wells were exposed during time in the range of 30 to 120 s and no significant temperature increase in the medium was observed.

In order to control the bubble activity, a home-made hydrophone (cut-off frequency 10 MHz) [34] realized with a PVDF film (10 mm diameter) moulded in resin (AY103, Araldite) was placed near the ultrasound transducer pointing on the exposed medium volume (Fig. 1). As suggested by Frohly et al. [35], the cavitation index (CI) was defined as the mean of all acoustic spectrum power density points in dB over the range of 0.1 to 7.1 MHz (448 frequency points), normalized by the background noise recorded without transducer excitation. CI is slightly sensitive to harmonic peaks due to the presence of bubbles in medium, and mostly reflects the broadband noise due to inertial cavitation for CI values greater than 6. To perform the control of the bubble activity, regulation system was implemented, fixing CI to a chosen CI setpoint as follows. During sono-irradiation the cavitation signal is saved by an acquisition card (PXI-5620, 14 bit resolution, 60 MHz sampling frequency, National Instruments). These data are transferred into a computer through a data bus (MXI-3 80 Mo/s, National Instruments). The CI value is calculated and compared to the desired CI setpoint. The transducer power is then readjusted by changing the excitation signal amplitude (Fig. 2). The timing is controlled by LABVIEW software (National Instruments) and

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the feedback loop rate was 200 Hz. CI oscillations were less than 10% in the present study.

2.3. Plasmid and siRNA transfection

Fluorescent siRNA directed against BCL2L1 (sense: 5′Alexa Fluor 488–GGG UUU GGA UCU UAG AAG A-3′; antisense: 5′ UCU UCU AAG AUC CAA AGC C-3′) and AllStars Negative siRNA were purchased from Qiagen. The siRNA were suspended in the provided buffer solution and prepared following the manufacturer’s instructions. pEGFP-C2 (BD Biosciences Clontech), pGL3 (Promega) and pcDNA3 (Invitrogen) plasmids were purified using PureLink Hypure Plasmid Filter purification kits following the protocol provided by the manufacturer.

Two ml of cell suspensions (2.10^6 cells/ml in RPMI supplemented with 10% of FCS) were placed in each well of the 12-well plates. pEGFP-C2 was added to the cell suspensions of RL and CLL at a final concentration of 25 µg/ml and BCL2L1 siRNA at 7.5 µg/ml.

Optimal exposure conditions that maximized cell permeability and minimized cell death were identified. CI and US exposure time were optimized for each cell type. All experiments were conducted at 37 °C. Selection of stably transfected cells began after 72 h with continuous exposure to 1.2 mg/ml of G418. Individual clones were screened for luciferase activity and selected clones were injected subcutaneously into three mice.

In conditions where lipofectin (Invitrogen, Cergy Pontoise, France) was mentioned, it was added following the manufacturer’s instructions.

2.4. Analysis of transfection efficiency and cell viability

Twenty-four and forty eight hours after sonoporation of pEGFP-C2 vector, GFP-positive cells were observed using an Olympus IX50 microscope at the excitation wavelength of 488 nm and photographed at Centre Commun de Quantimétrie (Université Claude Bernard Lyon, France).

Cells were also analysed using a fluorescence-activated cell sorter (FACS) (FACS Calibur; Becton, Dickinson and Company, NJ). Results were expressed as a percentage of GFP positive cells using the software CellQuestPro (Becton Dickinson, San Jose, CA). This percentage was calculated on the basis of the total number of cells, including dead cells. However, debris destroyed during sonoporation was not included. The cell suspension was washed twice with PBS. In order to assess cell viability, cells were incubated with 7-amino-actinomycin D (7-AAD, BD Pharmingen) according to the manufacturer’s recommendations for 10 min (10 µl for 1.10^6 cells) prior to the FACS analysis. After incubation, cells were washed with PBS and the pellet was resuspended in 200 µl of PBS. Cells were then transferred into the cytometer where 10,000 events were analyzed for each sample. Fluorescence of GFP and siRNA was detected in FL1 channel while 7-AAD fluorescence was detected in FL3. Furthermore, in order to quench non-specific extracellular fluorescence and to confirm the intracellular delivery of siRNAs, we added Trypan blue (TB) dye (0.2%) (Sigma Aldrich), then we proceeded immediately to flow cytometric analysis.

2.5. Immunoblot analysis

CLL cells transfected with siRNA directed against BCL2L1 or with scrambled control siRNA (negative siRNA control) were incubated for 48 h then lysed as previously described [36]. Briefly, 20 µg of cell lysates were resolved on a 12% SDS-PAGE using an electrophoresis apparatus (Bio-Rad) and transferred onto a polyvinylidene difluoride membrane (Hybond-ECL, Amersham Corp). The membrane was blocked with blocking buffer (LI-COR Biosciences, Germany) for 1 h and subsequently incubated with the primary antibodies directed against Bcl2L1 (clone S18, Santa Cruz) or Bcl2 (clone 124; Dacco, Denmark) overnight at 4 °C. The non-specific binding of antibody was removed by washing with PBS (pH 7.2) containing 0.1% Tween 20 and 5% nonfat, dry milk. The membrane was then incubated with the secondary antibodies (Goat anti mouse IRDye or Goat anti-Rabbit antibody from LI-COR Biosciences, Germany) for 1 h at room temperature. After extensive washing with PBS, membranes were analysed using the Odyssey infrared imaging system (LI-COR Biosciences, Germany). The expression levels of the protein were standardized against the expression level of β-Actin (clone AC-15, Sigma).

2.6. Statistical analysis

The statistical significance of the data was determined with a Student’s t-test. P<0.05, P<0.001 and P<0.0001 indicate a statistically significant (*), highly significant (**) and extremely significant Statistical signifi
difference (**), respectively. Student’s t-test was used to identify differences between US-exposed cells and non-sonoporated cells (NS).

3. Results

3.1. Optimization of ultrasound-mediated transfection in vitro

In order to determine the effect of US intensity on the efficiency of transfection, target population cells were subjected to a variety of parameters. Cell viability was evaluated by 7-AAD uptake. In these experiments, cells in suspension (CLL and RL cells) were exposed to a CI ranging from 12 to 20 and US exposure times ranging from 20 to 100 s. After determining all parameters including cells number per well, medium and FCS volume, plasmids or siRNA quantity, temperature, exposure time and CI, we determined the optimal conditions for each cell type.

The number of cells was set to $4.10^6$ cells per well in 2 ml of RPMI supplemented with 10% of FCS. The plasmid and siRNA concentration was 25 µg/ml and 7.5 µg/ml, respectively and the temperature was set to 37 °C.

The CI and exposure time were varied to apply to the cell a trade-off between transfection efficiency and cell viability. For RL cells, we used different CI (12, 14, 16 and 20) and different exposure times (20, 30, 40 and 60 s). The percentage of GFP-positive cells, mean fluorescence intensity (MFI) and levels of cytotoxicity were measured on the total population of cells. When applying a high CI for RL cells (e.g., CI of 20), larger fractions of cells were transfected, however, cell viability correspondingly dropped. Histogram (Fig. 3B) showed that CI of 16 is the best CI ensuring good transfection efficiency and low cell mortality ($p<0.05$). RL cells achieved an average of 15% of transfection efficiency detected by flow cytometry and they presented less toxicity (80% of viable cells) (Fig. 3A).

For exposure time optimization, the best irradiation times were 30 and 40 s at CI of 16; however, we sonoporated RL cells during 30 s to minimize cell death (Fig. 3C).

We included the CI of 20 in these two histograms (Fig. 3B and C) to show that cells could achieve highly significant percentage of transfection, however, they suffered an extremely significant level of mortality.

For CLL cells, all CI below 20 did not yield any transfection efficiency when observed by microscopy; therefore, we used the CI at 20, and investigated US exposure time ranging between 20 and 100 s. Fig. 4A shows fluorescent GFP positive cells (green). The transfection efficiency was increased in an exposure time-dependent manner. The longer we exposed cells to US, the more we achieved GFP-positive cells. When cells were sonoporated during 60, 80 and 100 s, we found a significant difference in transfection efficiency calculated as percentage and MFI of GFP-positive cells (Fig. 4B, 4C) compared to non-exposed control; moreover, a statistically significant increase of mortality calculated as percentage and MFI of 7-AAD positive cells when exposure time was increased to 100 s compared to non-exposed cells (Fig. 4B, C).

3.2. Production of stably transfected RL clones

RL cells cotransfected with pGL3 and pcDNA3 plasmids were selected by prolonged exposure to G418. Bioluminescent resistant clones were selected in a 96 well-plates using the Nightowl imaging system immediately after adding D-luciferin into wells as shown in Fig. 5A. These positive clones were then injected subcutaneously into SCID mice. Tumors developed 21 days after implantation of bioluminescent cells, as shown in Fig. 5B. A colour enhanced overlay of the luminescent image over the photographic image demonstrates the location of the implants within the animal. These experiments demonstrate that sonoporation allows stable transfection of RL cells.
3.3. Delivery of BCL2L1 targeted siRNA by sonoporation

After the determination of optimal transfection conditions, we investigated the possibility of using this method to introduce fluorescent siRNAs into fresh CLL cells. BCL2L1, a member of the antiapoptotic BCL2 family members, was chosen as a target.

In Fig. 6A, non-sonoporated cells exposed to siRNA (panel 2) had increased fluorescence in comparison to the control (panel 1), probably due to non-specific binding of siRNA to target cell membranes. However, after exposure to US, we observed a significant increase in the count of fluorescent cells in sonoporated cells in comparison to non-sonoporated cells, confirming that sonoporation had enhanced intracellular penetration of siRNA (panel3).

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We also determined the percentage of transfected cells after quenching the extracellular fluorescence with trypan blue (TB). These experiments were performed along with a lipofectin reagent as a classical control of transfection either alone or combined with siRNAs. Results showed that BCL2L1 siRNA and scramble siRNA are localized intracellularly in cells because they were not quenched with TB; however, when they were combined with lipofectin either with or without sonoporation, significant decrease of the percentage of fluorescent cells were observed confirming that the fluorescence was largely localized extracellularly on cell membranes (Fig. 6B).

Confocal microscopy images further support the interpretation that cell fluorescence has an intracellular localization. Supplementary data includes a series of confocal images confirming the intracellular uptake of BCL2L1 siRNA and scrambled siRNAs.

In order to confirm the inhibition of Bcl2L1 protein by siRNA, total cell lysates (20 µg) were subjected to western blot analysis using specific antibody directed against Bcl2L1 in comparison with a scrambled siRNA. These experiments confirmed a reduction in Bcl2L1 protein in cells exposed to siRNA in comparison to non-exposed control cells and to cells exposed to scrambled siRNA. Moreover, to confirm the specificity of the inhibition of Bcl2L1, we studied the level of Bcl2 protein using Bcl2 antibodies and found no modification of this protein after exposure to siRNA directed against BCL2L1 (Fig. 6C).

4. Discussion

Introduction of exogenous nucleic acids into mammalian cells represents an essential method for the study of basic cell biology as well as for therapeutic manipulations. While viral-based vectors have proven to be efficient in some cases, these methods are limited by potentially severe side-effects when applied in the clinic. In laboratories, the transfection of suspended cells is often difficult to obtain with currently available methods. Thus there are still large opportunities for the development of reliable, safe, efficient and reproducible transfection methods.

In this study, we determined the feasibility of using sonoporation to transfect a human lymphoma line as well as fresh human leukemic samples. Many approaches have been tested, including techniques developed by ourselves, to enhance the efficiency of nucleic acid transfer into these hard-to-transfect cells. However, our personal experiments with available transfection reagents such as lipofectin did not show significant transfection efficiency for RL cells and CLL cells. Moreover, new alternative methods such as nucleofection have been also tested but yielded low transfection rates in RL cells (data not shown).

The optimization of US parameters represents a major challenge for the application of sonoporation in different cell lines [8]. The effects of US on a population of cells are very heterogeneous [37]. This heterogeneity is mainly due to the random process induced by the cavitation bubble activity; thereby, cells that are located near the bubble explosion are more affected than the distant ones. In mild conditions of US exposure, almost all cells remain viable and only a small percentage of cells showed intracellular uptake. However, in case of strong US exposure, cells showed high transfection efficiency with high mortality rate. In order to obtain a trade-off between high transfection rates and good cell viability, we began our study by optimizing these parameters, including CI values and exposure times.

Our experiments showed that:

- The transfection efficiency was greatest at the intermediate cell concentration studied (4.10⁶ cells/ml). Decreased transfection efficiency at higher cell concentration (8.10⁶ cells/ml) could be...
explained by cells shielding each other from nearby cavitation bubbles and could be also consistent with reduced blast radii of cavitation bubbles [37].

- Increasing the plasmid and siRNAs concentration increased the transfection outcome. The results showed that increasing the concentration of these vectors lead to an increase in the number of transfected cells in both cell types. This observation is consistent with the results of other studies [14].

- High delivery efficiency was depending on CI level. CI was found to largely influence the transfection outcome. As a compromise, we defined the CI that showed the highest transfection efficiency with minimal cell loss. The results presented here show that CI values that achieve the optimal efficiency vary from one cell type to another. Thus, a CI of 16 and 20 were considered to be the optimal CI for RL and CLL cells, respectively, within the limits of the apparatus used.

- Transfection efficiency was increased in a time-dependent manner. The longer we exposed cells to US, the more we achieved GFP-positive cells.

- Increasing temperature from 4 °C or room temperature to body temperature (37 °C) improved the percentage of transfected cells. This was consistent with previous reports which showed that low temperature decreases the membrane fluidity leading to reduced pore formation in cell membrane [38]. Moreover, this temperature may provide the necessary conditions for the cell to resell and survive its membrane disruptions.

- The pore size distribution and their transient existence could also influence the transfection efficiency [15]. In support of this hypothesis, the transfection rates of siRNA were higher than those of pEGFP-C2 and this could be related to the size of the molecule and the distribution of pores (BCL2L1 siRNA is 200 times smaller than pEGFP-C2). Moreover, the lower transfection rates observed as few GFP positive cells could be explained by the instability of naked DNA in the cytoplasm (presence of DNases) [39], the half-life of GFP, and the reduced cell metabolism and growth [40].

- The combination of sonoporation with other methods or reagents, such as lipofectin did not improve the transfection rates. Many studies postulated that the combination of DNA with cationic lipids or polymers could increase the transfection efficiency [41–44]. However, our experiments failed to confirm this hypothesis and we did not find any improvement in transfection when combining these two methods, as compared with lipofection or sonoporation alone. Quenching of the extracellular binding of fluorescent siRNAs on cell membrane demonstrated that the combination of lipofectin and siRNA targeted against BCL2L1 or scramble siRNA did not enhance the transfection efficiency and the fluorescence was largely localized extracellularly on cell membranes (Fig 6B).

- Using this caviation device, we were also able to generate stable transfecants that were then xenografted in mice. This will allow us in the future to study the impact of modifications of gene expression on the leukemic cells growth and response to treatments in vivo.

5. Conclusion

Optimal transfection parameters were achieved for RL and CLL cells using this 445 kHz transducer. We believe that the findings of this study can be used to guide optimization of DNA transfection in other suspension cultures. Sonoporation appears to be a promising method to obtain transient and/or stable transfection of nucleic acids in suspended cells. Further studies exploring this approach in vitro and in vivo are warranted.

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Appendix A. Supplementary data


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