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ARTICLE

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Interrelation between HeLa-S3 cell transfection and hemolysis in red blood cell suspension using pulsed ultrasound of various duty cycles

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Abstract We have studied the in vitro transfection of a plasmid DNA with the *lacZ* gene to HeLa-S3 cells and hemolysis in a red blood cell (RBC) suspension under pulsed ultrasound with duty cycles γ of 10, 20 and 30% using a digital sonifier at a frequency of 20 kHz and an intensity of 6.2 W/cm^2 on the surface of a horn tip. Cultured HeLa-S3 cells in suspension were exposed to pulsed ultrasound for an apparent exposure time t' from 0 to 60 s. HeLa-S3 viability decreased as a single exponential function of the total exposure time $t = \gamma t'$ with a common time constant $\tau = 3.8$ s for three duty cycles. Transfection was evaluated by counting the number of β-galactosidase(β-Gal)-positive cells relative to the total number of cells. Pulsed ultrasound provided an enhanced transfer of the β-Gal plasmid to HeLa-S3 cells, 3.4-fold as compared with that in the case of the control. The optimal transfection efficiencies were 0.75, 0.80 and 0.74% near $t = \tau$ with $\gamma = 10$, 20 and 30%, respectively. The number ratio of β-Gal-positive cells to the surviving cells after exposure increased with t' according to a modified logistic equation. The degree of hemolysis also increased exponentially with t' at a time constant $\tau' = \tau_0/\gamma$ for the RBC suspension in physiological saline at a hematocrit concentration of 0.5% with $\tau_0 = 0.9$ s. Thus the total exposure time for the optimal transfection efficiency was τ, that is, nearly four times of τ_0. Hemolysis in the RBC suspension may be a useful model for determining optimal transfection by pulsed ultrasound of various duty cycles.

Keywords Gene transfer · HeLa-S3 cells · Logistic equation · Pulsed ultrasound · Red blood cells

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Introduction

Ultrasound is best known for its imaging capability in diagnostic medicine. However, there have been considerable efforts recently to develop therapeutic uses of ultrasound. Gene therapy will be increasingly important for the treatment of inherited or acquired diseases, such as atherosclerosis and cancer (Finkel and Epstein 1995; Roth and Cristiano 1997). However, its clinical application is hampered by concerns over the safety of viral vectors and the inefficiency of transfection techniques for local gene delivery to a specific tissue or organ. Nonviral gene delivery can be performed by the direct injection of DNA, but such approaches are generally associated with a low transfection efficiency and the transient expression of the gene product (Huber and Pfisterer 2000). Viral vectors significantly increase the efficacy of transfection because of the specific viral machinery that has evolved to introduce foreign DNA into mammalian cells, but viral proteins elicit an immune response within the targeted host or tissue. Recently, significant work has been done to limit or remove the antigenicity of viral vectors such as gutless and lentiviral vectors as well as an adeno-associated virus (Amado and Chen 1999; Yant et al. 2002; Hennig et al. 2004). Several methods have been developed for the delivery of DNA into cells, including electroporation, particle bombardment, calcium phosphate precipitation and liposome-mediated gene transfer (Wyber et al. 1997). Each method has inherent limitations, such as low efficiency, complex protocols or high cost. In particular, many cells are only responsive to one or a few specialized methods. In this respect, mechanical methods are often more versatile as they are based on an increase in cell membrane permeability and are less dependent on cell type.

In in vitro studies, ultrasound has been shown to transiently permeabilize biological membranes, thereby facilitating the delivery of large compounds such as

proteins and DNA into cells and across tissues such as the skin (Mitragotri et al. 1995; Yamashita et al. 1996; Liu et al. 1998). The advantages of ultrasound-mediated DNA delivery are that it is rapid, relatively inexpensive and applicable to a wide range of cell types and tissues. A potentially useful approach to gene delivery could be the use of ultrasound as a physical method of modulating gene transfer. The earliest report on ultrasound-mediated DNA delivery is the introduction of a plasmid carrying the thymidine kinase gene into fibroblasts using pulsed ultrasound from a probe-type sonicator (Fechheimer et al. 1987). Cell transformation was optimal for 30 s of sonication with an output of 2 W and resulted in a 20-fold enhancement of transfection relative to that of the control (Wyber et al. 1997). Huber et al. (1999) compared the transfection efficiencies of shock wave and sinusoidal focused ultrasound using HeLa cells. They showed that focused ultrasound induces up to 80-fold more transfection with a cell survival of 45% than that in the case of the control. Other researchers have also applied ultrasound to HeLa and vascular cells using liposomes or microbubbles for gene delivery (Unger et al. 1997; Lawrie et al. 1999, 2000). Ultrasonic power and duration, ambient temperature and plasmid concentration are critical for achieving maximal transfection efficiency.

Cavitation is the key component of ultrasound-mediated transfection (Kim et al. 1996). In recent experiments, ultrasound was used to increase the transfection efficiency of naked plasmid DNA in skeletal muscles (Taniyama et al. 2002). However, the efficiency of transformation in these early experiments was low. Subsequent studies demonstrated the delivery of viral particles and plasmid DNA into plant cells (Joersbo and Brunstedt 1992). It was found that a single pulse of ultrasound from 0.5 to 1.0 s, using a 20-kHz probe-type sonicator, enhances the uptake of DNA into plant protoplasts.

In in vivo studies, it was demonstrated that the ultrasound transfection method with an Optison (microbubble) enhances the transfection efficiency of naked plasmid DNA. Recently, Lu et al. (2003) have reported that microbubble ultrasound improves the efficiency of gene transduction in skeletal muscles, with reduced tissue damage. The ultrasound-induced microbubble method has been proposed as a new technique for the local delivery of drugs and genes to specific target tissues (Huber and Pfisterer 2000).

In this study, the interrelation between gene transfection to HeLa-S3 cells and hemolysis by pulsed ultrasound is investigated, since sonoporation is part of both phenomena. If we could easily determine the optimal conditions for ultrasound gene transfer, such as ultrasound intensity and duration, it would be much more convenient because generally cell cultures and gene expressions for 7–10 days are required for gene transfection. We determine the optimal conditions for ultrasound gene transfer within 1 h by hemolysis in a very dilute suspension of red blood cells (RBCs).

Materials and methods

The plasmid vector pBK-CMV-*lacZ* (Toyobo, Osaka, Japan) was used for transfection. *Escherichia coli* XL1-Blue MRF' was cultured at 37 °C for 1 day in Luria-Bertani (LB) solid culture medium with 1.5% agar. One selected colony was proliferated in LB liquid culture medium. Competent *E. coli* cells were cultured up to 0.5 OD_{550} by the $CaCl_2$ method (Davis et al. 1983). We placed 100 μL of competent *E. coli* cells and 5 μL of vector DNA into a 2-mL microchip and mixed them. The mixture was incubated in an ice-cold water bath for 30–40 min, then at 42 °C for 45 s, and then in an ice-cold water bath again. 1500 μL of LB liquid culture medium was added to the mixture, which was then incubated for 1 h at 37 °C. Then 20 μL of the mixture was inoculated on LB solid culture medium containing 10 μg/mL kanamycin sulfate and cultured overnight. We selected one or two colonies to culture in LB liquid culture medium overnight. The plasmid DNA from the *E. coli* transformants was prepared with a QIA filter plasmid minikit (Qiagen, Chatsworth, Calif., USA).

HeLa-S3 cells were obtained from the Riken Cell Bank (Riken, Tsukuba, Japan) and cultured in Eagle's minimum essential medium (EMEM, Iwaki, Funabashi, Japan) supplemented with 10% fetal bovine serum (Bio Whittaker, Walkersville, Md., USA) and 100 units/mL penicillin and 100 μg/mL streptomycin (Gibco BRL, Grand Island, NY, USA). Cells were cultured in 25-cm^2 and 75-cm^2 flasks at 37 °C with 5% CO_2 and 100% humidity; the culture medium was changed every 3 days. The cells were washed twice with Dulbecco's PBS(−) containing 0.02% EDTA·4Na, 1 mL of 0.2% trypsin was added, and then incubated for 5–10 min. After the cells separated from the flask bottom, EMEM with 10% fetal bovine serum was added. The cell suspension was centrifuged at 3000 rpm for 5 min and resuspended at 1.0×10^6 cells/mL. 1 mL of cell suspension was added to wells of a 24-well plate with 1 μg of reporter plasmid DNA and soon exposed to ultrasound with insufficient time to settle on non-coated 24-well dishes at room temperature (25 °C), as shown in Fig. 1.

Ultrasound was generated using the 20-kHz digital sonifier S-250D (Branson Ultrasonic, Danbury, Conn., USA) at a power of 2 W, that is, at an intensity of 6.2 W/cm^2 on the surface of the tapered microtip with a diameter of 6.4 mm (Fig. 1). The vibration amplitude was 59.5 μm, and the apparent exposure time was 0–60 s (total exposure time 0–18 s) with 10% (the ultrasound exposure on for 0.1 s and off for 0.9 s), 20% (0.2 s on, 0.8 s off) and 30% duty cycles (0.3 s on and 0.7 s off).

For cell viability evaluation, 100 μL of cell suspension was mixed with 0.3% trypan blue (Wako, Japan). Blue (dead) and white (viable) cells were counted under a microscope in an improved Neubauer hemocytometer. Viability was calculated as the ratio of the number of living cells after ultrasonic exposure to that before ultrasonic exposure (ca. 1×10^6 cells/mL). The mean and

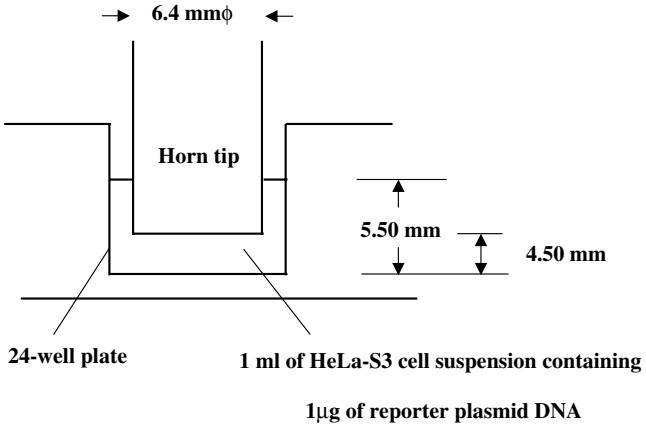

Fig. 1 Ultrasound exposure system with digital sonifier at 20 kHz for HeLa-S3 cells in suspension

standard deviation of the viability were determined from eight independent sets of data.

For the evaluation of transfection efficiency, the cells cultured for 24 h after exposure were washed twice with Dulbecco's PBS(−) and fixed with 1 mL of 2% paraformaldehyde (Wako, Osaka, Japan) and 0.2% glutaraldehyde (Wako) for 5 min at room temperature. The cells were washed three times with Dulbecco's PBS(−) and incubated in a well with 400 μL of X-Gal solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide trihydrate, 2 mM $MgCl_2$, 0.1% Triton X-100 and 0.1% X-Gal; Wako) for 6–9 h at 37 °C. Blue transfected cells in each well were counted under a microscope using 43 sections of 1 mm×1 mm in an adhesive sealing film with a grid (1806-009, Iwaki, Funabashi, Japan). Transfection efficiency and transfection were calculated as the number of transfected cells divided by the total number of cells before and after ultrasound exposure, respectively. The mean and standard deviation were obtained using six independent measurements.

Fresh porcine blood was anti-coagulated with 1 mg/mL K_2EDTA. After the removal of the plasma and buffy coat after 3000 rpm centrifugation for 10 min, the RBCs were washed twice in a physiological saline solution (154 mM NaCl, 3.9 mM K_2HPO_4 and 0.7 mM KH_2PO_4) at pH 7.4. The RBC suspension was combined with a physiological saline solution at a hematocrit H of 0.5%. H was determined by the microhematocrit method. 1 mL of RBC suspension was added to each well of a 24-well plate. After leaving the suspension to stand 1 h at room temperature for the RBCs to settle on the bottom, the RBCs were exposed to ultrasound under the same conditions as those for HeLa-S3 cells. The exposed RBCs transferred into 2-mL microchips were centrifuged at 12,000 rpm for 5 min to measure the optical density (OD_{540}) of the supernatant in 2-mm cuvettes at 540 nm. Hemolysis was defined as the ratio of OD_{540} of the exposed samples to that of the RBC suspension diluted with 0.7 mM KH_2PO_4 buffer solution 80 times from suspension at $H = 40\%$. The mean hemolysis was given by six independent sets of data.

Results

In Table 1 and Fig. 2, the number of viable HeLa-S3 cells considerably declines with ultrasound exposure, and reaches almost zero particularly at 20 and 30% duty cycles for 60 s of the apparent exposure time t'. In the three modes, the viability y at t' decreases in the order of 30, 20 and 10% duty cycles. The solid curves are fitted by:

$$y = A\exp(-\gamma t'/\tau) = A\exp(-t/\tau) \quad (1)$$

where $A = 100\%$, $\gamma = 0.1$, 0.2 or 0.3 corresponding to the percentage of duty cycles, $\tau = 3.8$ s and $t = \gamma t'$ for the time constant and the total exposure time, respectively. Viability as a function of t is represented by a single curve in Eq. (1) in all the three exposure modes.

After ultrasound exposure, β-Gal-positive cells (blue cells) were observed in our experiments, which indicated that the β-Gal plasmid DNA had been successfully

Table 1 Transfection and viability of HeLa-S3 cells exposed by pulsed ultrasound at $\gamma = 10$, 20 and 30% duty cycles

Exposure time t'(s)	Transfected cells (number/43 mm^2)			Viability y(%)			Transfection efficiency w(%)		
	10%	20%	30%	10%	20%	30%	10%	20%	30%
0	116 ± 29	–	–	100 ± 25	–	–	0.24 ± 0.06	–	–
3	–	–	233 ± 30	–	–	75.4 ± 4.6	–	–	0.48 ± 0.04
5	168 ± 36	232 ± 37	311 ± 45	94.4 ± 5.1	77.1 ± 5.4	64.0 ± 7.7	0.35 ± 0.09	0.48 ± 0.09	0.65 ± 0.10
8	–	–	291 ± 59	–	–	54.7 ± 5.5	–	–	0.60 ± 0.10
10	185 ± 42	282 ± 37	352 ± 55	80.9 ± 6.7	59.5 ± 4.9	48.3 ± 5.8	0.38 ± 0.09	0.59 ± 0.09	0.74 ± 0.13
15	217 ± 42	308 ± 42	317 ± 59	70.6 ± 6.2	48.1 ± 4.7	31.7 ± 4.2	0.44 ± 0.09	0.64 ± 0.08	0.66 ± 0.15
20	232 ± 27	384 ± 35	245 ± 54	67.4 ± 4.2	36.4 ± 5.0	23.0 ± 6.8	0.48 ± 0.05	0.80 ± 0.07	0.51 ± 0.12
25	283 ± 44	311 ± 31	219 ± 60	59.2 ± 3.1	29.8 ± 9.5	18.1 ± 8.5	0.58 ± 0.09	0.65 ± 0.05	0.46 ± 0.13
30	298 ± 40	263 ± 56	158 ± 69	50.8 ± 3.8	22.1 ± 3.8	8.6 ± 1.2	0.61 ± 0.09	0.55 ± 0.11	0.33 ± 0.15
35	360 ± 60	197 ± 59	123 ± 50	44.1 ± 7.5	15.6 ± 5.6	5.9 ± 1.1	0.73 ± 0.06	0.41 ± 0.10	0.25 ± 0.10
40	371 ± 72	182 ± 16	114 ± 46	40.3 ± 7.0	14.1 ± 3.8	4.0 ± 1.3	0.75 ± 0.11	0.39 ± 0.04	0.23 ± 0.09
50	328 ± 82	117 ± 22	–	31.0 ± 3.9	8.3 ± 3.2	1.7 ± 0.5	0.68 ± 0.13	0.24 ± 0.04	–
60	317 ± 66	78 ± 36	–	26.7 ± 6.7	3.7 ± 2.0	0.7 ± 0.3	0.66 ± 0.14	0.16 ± 0.07	–

transferred into the HeLa-S3 cells. In Fig. 3 the transfection u, defined by the ratio of the transfected cell number to the viable cell number in Table 1, increases with exposure time, regardless of the mode. The solid curve is given by the logistic equation (Hirano et al. 2004), with modification:

$$u = \frac{B}{1 + [(\beta - t)/(\alpha + t)]^n} \tag{2}$$

where the constants $B = 2.6\%$, $\alpha = 1.2$ s, $\beta = 6.5$ s and $n = 1.4$. The data agree well with the calculation.

The transfection efficiency w, the ratio of the transfected cell number to the total cell number before ultrasound exposure (ca. 1×10^3 cells/mm^2), is listed in Table 1 and shown in Fig. 4 for each duty cycle, in which w first increases with t' and then decreases. The optima are $w = 0.75\%$ at $t' = 40$ s, 0.80% at 20 s and 0.74% at 10 s for $\gamma = 10$, 20 and 30%, respectively, in Table 1, that is nearly $t = \tau = 3.8$ s. In Fig. 4, solid curves are calculated using the following equation derived from Eqs. (1) and (2):

$$w = \frac{B\exp(-t/\tau)}{1 + [(\beta - t)/(\alpha + t)]^n} \tag{3}$$

All the transfection efficiencies are also superposed to a single curve with t.

In Fig. 5, the hemolysis z of RBCs in suspension at $H = 0.5\%$ for the three duty cycles is measured at t' values of 0 to 180 s. The solid curves are calculated using:

$$z = A[1 - \exp(-t/\tau')] = A[1 - \exp(-\gamma t/\tau_0)] \tag{4}$$

The time constant τ' defined by the first equation in Eq. (4) is 8.5, 4.8 or 3 s for 10, 20 or 30% duty cycles, respectively. In contrast to the viability in Eq. (1), z in

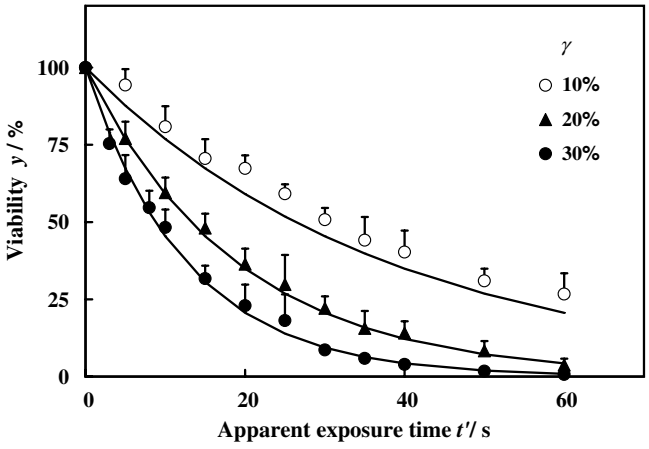

Fig. 2 Viable cell fraction y of HeLa-S3 exposed at $\gamma = 10$, 20 and 30% duty cycles versus apparent exposure time t'; *solid curves* are calculated using $y = 100\%\times\exp(-\gamma t'/3.8\text{ s})$

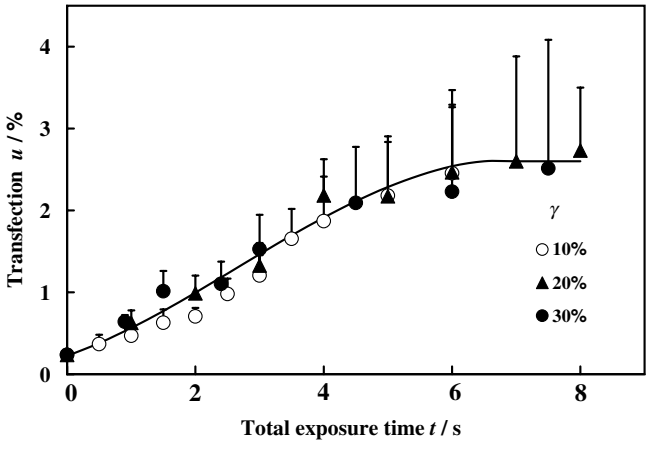

Fig. 3 Transfection u of HeLa-S3 cells exposed at $\gamma = 10$, 20 and 30% duty cycles versus total exposure time t; the *solid curve* was calculated using $u = 2.6\%/\{1 + [(6.5\text{ s} - t)/(1.2\text{ s} + t)]^{1.4}\}$

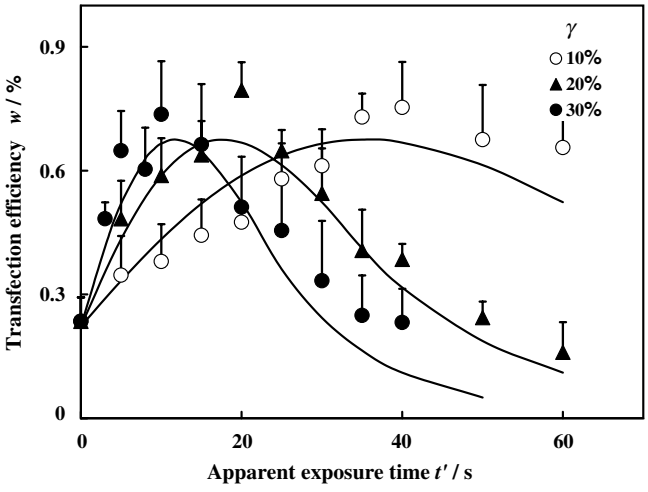

Fig. 4 Transfection efficiency w of HeLa-S3 cells exposed at $\gamma = 10$, 20 and 30% duty cycles versus apparent exposure time t'; *solid curves* were calculated using $w = 2.6\%\times\exp(-\gamma t'/3.8\text{ s})/\{1 + [(6.5\text{ s} - \gamma t')/(1.2\text{ s} + \gamma t')]^{1.4}\}$

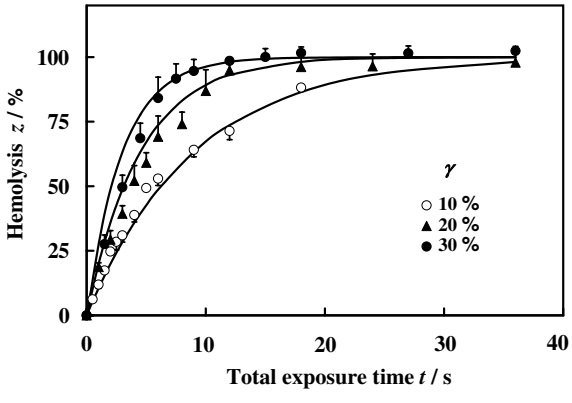

Fig. 5 Hemolysis z of RBCs in suspension at a hematocrit of 0.5% exposed at $\gamma = 10$, 20 and 30% duty cycles versus total exposure time t; *symbols* and *error bars* represent means and standard deviations of three data values, respectively; the *solid curves* were calculated using $z = 100\%\times[1 - \exp(-\gamma t/0.9\text{ s})]$

Eq. (4) increases with γ. Moreover, τ' is inversely proportional to γ in Fig. 6:

$$\tau' = \frac{\tau_0}{\gamma} \tag{5}$$

Here a constant $\tau_0 = 0.9$ s.

We attempted to depict the cell viability y versus the hemolysis z so that they correlated well for $\gamma = 10$ and 20% as $y = 0.9(1-z)$, but the data were markedly far from this line for $\gamma = 30\%$.

Discussion

Gene therapy is a promising tool for the treatment of many diseases. Nevertheless, major obstacles remain to be overcome before its applications can be realized. One of the most critical areas of gene therapy is the design of an appropriate, accurate and effective gene vector that can be applied safely. A few common nonviral or physical in vitro techniques of mediating gene transfer and transgene expression have been reported, with varying degrees of efficiency (Manome et al. 2000). Low-energy ultrasound exposure has been shown to permeabilize plasma membranes and reduce the thickness of an unstirred layer on the cell surface, which should encourage DNA entry into cells (Bao et al. 1997; Lauer et al. 1997). Ultrasound has been proposed as a potential physical method of enhancing gene transfer into cells. In recent experiments, various ultrasound modalities have been used, including burst ultrasound (Tata et al. 1997), continuous wave ultrasound (Kim et al. 1996), shock waves (Lauer et al. 1997) and rotating tubes (Bao et al. 1997) in vitro. In this study, 10, 20 and 30% duty cycles have been used to expose HeLa-S3 cells and RBCs in a dilute suspension.

In the present experimental results, cell viability is considerably decreased with exposure time for the three duty cycles (Fig. 2). This might be caused by the thermal effect and/or cavitation after longer ultrasound treatment that induced the inactivation of enzymes and denaturation of some proteins in the HeLa-S3 cells. The collapse and oscillation of cavitation bubbles induce local high temperatures and pressures accompanied by mechanical shear stress and free-radical formation (Kondo and Kano 1988; Garcia et al. 1989). It has been demonstrated that the primary biological effect of acoustic cavitation is cell lysis or molecular transfer through the cell membrane (Gambihler et al. 1994; Miller et al. 1996).

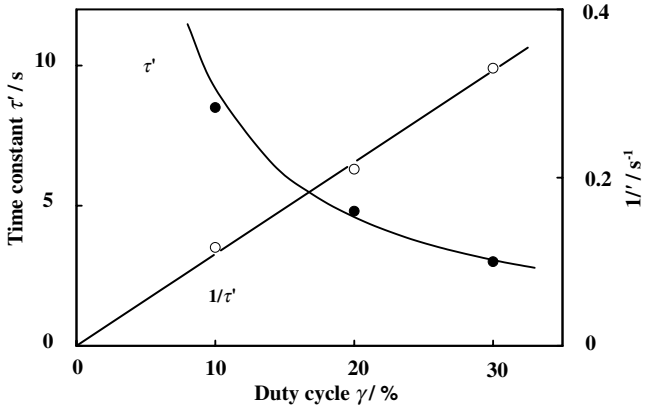

Fig. 6 Time constant τ' for hemolysis of RBCs in suspension at a hematocrit of 0.5% versus duty cycle γ; the *solid curves* were calculated using $\tau' = 0.9$ s/γ

Our results also demonstrate that HeLa-S3 cells are successfully transfected with plasmid DNA in vitro for the three duty cycles (Figs. 3 and 4) for the β-Gal gene-transferred cells stained blue by X-Gal solution. In Fig. 4, there exists a peak of transfection efficiency in each exposure mode. This phenomenon has also been reported in other studies (Bao et al. 1998; Miller et al. 1999). In our experiments, transfection efficiency w decreases with t' owing to the longer ultrasonic exposure that brings about cell death, when the total exposure time $t = \gamma\, t'$ is greater than $\tau = 3.8$ s for each duty cycle γ. The energy released during cavitation would result in plasmid DNA entering the cells and some of the cells surviving to express the plasmid gene (Koch et al. 2000; Frenkel et al. 2002; Ogawa et al. 2002).

In the three modes, the transfection u is overlapped on a single logistic equation against t as in Eq. (2) and Fig. 3. Here, we have a finite transfection even in control without exposure, possibly due to thermal fluctuations, and u appears to be divided into three stages, that is, nontransfected, transition and completely transfected stages, very similar to the dose–response curves in a logistic equation (Hirano et al. 2004). In Fig. 2, the viability y is also represented by a simple exponential equation with the time constant $\tau = 3.8$ s for each duty cycle in Eq. (1). The increase in w is reflected in the intermediate between the nontransfected and transition stages in the logistic equation, as seen in Fig. 4. On the other hand, the decrease corresponds to the late death phase in Fig. 2. The number of dead cells and the extent of the transfection both increase with t in Eqs. (1) and (2). This suggests that the disruption of the permeability of the cell membrane is required for the entry of plasmid DNA into the cells. Cell death of approximately 63% corresponds to the optimum $w = 0.8\%$ at the apparent exposure time t'_0 for the total exposure time $t_0 = \tau = 3.8$ s:

$$t'_0 = \frac{t_0}{\gamma} = \frac{\tau}{\gamma} \tag{6}$$

As seen in Fig. 4, $t'_0 = 38$, 19 and 13 s for $\gamma = 10$, 20 and 30%, respectively.

There are many factors influencing transfection efficiency other than exposure mode, duration and type of ultrasonic system. Kim et al. (1996) have reported that there is an optimal plasmid concentration for gene transfer in mammalian cells. Many studies have shown that ultrasound contrast agents (microbubbles) can markedly enhance transfection efficiency in vitro and in

vivo (Lawrie et al. 2000; Lu et al. 2003). Cationic lipid-mediated gene transfer is also a method for enhancing transfection efficiency by ultrasound (Anwer et al. 2000).

Hemolysis means the leakage of macromolecular hemoglobin through membrane pores. As seen in Fig. 5 and Eq. (4), the hemolysis z in ultrasound exposure exponentially increases with the total exposure time t, but does not overlap with a single curve against the total exposure time t for $\gamma = 10$, 20 and 30%, in contrast to the viability versus t that is independent of γ as in Eq. (1). However, against a new time $t' = \gamma t = \gamma^2 t'$, z superimposes on a single curve with another time constant $\tau_0 = 0.9$ s since $\tau_0 = \gamma\tau'$ from Eq. (5), so that we have $z = A[1-\exp(-t'/\tau_0)]$ in Eq. (4), very similar to that in the case of the viability in Eq. (1). The transfection efficiency is optimum at $t_0 = \tau = 3.8$ s, that is nearly four times greater than $\tau_0 = 0.9$ s, which corresponds to τ' near $\gamma = 25\%$ in the hemolysis, as seen in Fig. 6. Cavitation around the RBC membrane would be the major factor leading to hemolysis or cell lysis (Daniels et al. 1995; Miller et al. 1996).

Ultrasonic exposure might induce some loosening of cytoskeletal and membrane structures in RBC suspensions at $H = 1\%$ (Kawai and Iino 2003). RBC suspensions at $H = 0.5\%$ have also been measured for hemolysis under ultrasound exposure (Miller et al. 1991; Carstensen et al. 1993). Assuming a HeLa cell radius $r = 10$ μm, the volume fraction $\phi = (4\pi/3)r^3 n$ is calculated to be 0.4% for the number concentration $n = 1\times10^6$ cells/mL. We have selected $H = 0.5\%$ because it is close to $\phi = 0.4\%$ in HeLa cells and also most researchers have used $H = 0.5\%$ in RBC suspensions.

Hemolysis in vitro usually occurs within 3–5 min under ultrasonic exposure, in contrast to the viability of HeLa-S3 cells observed 24 h after ultrasound exposure. Moreover, cell size may also be important in ultrasound-induced hemolysis, that is, the larger the cells, the greater the extent of sonolysis (Miller and Battaglia 2003). Porcine erythrocytes have a radius $r' = 2.93$ μm$\approx$3 μm on average for 30 RBCs (Dobashi et al. 1987), so that HeLa cells with a three times larger radius may be damaged more by ultrasound than RBCs. On the other hand, RBCs have neither nuclei nor any organelles nor cell adhesion molecules (CAMs). The cytoskeletal framework and membrane structure of RBCs seem to be affected by ultrasound exposure, while CAMs in HeLa cells may be damaged since we have observed no changes in floating dead HeLa cells both soon and 24 h after the exposure under a microscope. However, more experiments would be necessary for quantitative arguments on different susceptibilities to ultrasound between RBCs and HeLa cells. In Fig. 6 the inverse of τ' passes the origin and increases linearly with γ, which means that $\gamma = 0$ for no ultrasound without hemolysis and the more frequent ultrasounds lyse more easily. At present, there are no studies of the interrelation between hemolysis and gene transfer. It may be concluded that we determined the conditions of optimal transfection under pulsed ultrasound from cell lysis, corresponding to 63% hemolysis in a dilute suspension of RBCs at $H = 0.5\%$ and 25% duty cycle.

In summary, our experiments indicate that pulsed ultrasound with various duty-cycle-mediated gene transfections in HeLa-S3 cells is described by simple equations, including a logistic equation, as well as a single exponential for the viability against the total exposure time t more universally, regardless of the duty cycle γ. The transfection efficiency is optimal at the total exposure time t_0 equal to a time constant $\tau = 3.8$ s in terms of viability. Hemolysis in RBC suspension at $H = 0.5\%$ is also represented by a single exponential with a time constant $\tau' = 0.9$ s/γ inversely proportional to γ. The optimal exposure in transfection might be predicted by $t_0 = \tau \approx \tau'$ at $\gamma = 25\%$. Hemolysis in very dilute RBC suspension seems to be a useful model for validating and determining the optimal conditions in gene transfection by ultrasound with other modes and/or for other cells.

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