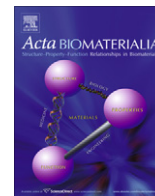


Contents lists available at [SciVerse ScienceDirect](http://www.sciencedirect.com)

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Studies on neutral, cationic and biotinylated cationic microbubbles in enhancing ultrasound-mediated gene delivery in vitro and in vivo

Nikolitsa Nomikou^a, Poonam Tiwari^b, Tanvi Trehan^b, Kriti Gulati^b, Anthony P. McHale^{c,*}

^a Sonidel Ltd., Tonleegee Drive, Dublin 5, Ireland

^b School of Biomedical Sciences, University of Ulster, Coleraine, Co. Derry BT52 1SA, UK

^c School of Pharmacy, University of Ulster, Coleraine, Co. Derry BT52 1SA, UK

ARTICLE INFO

Article history:

Received 4 May 2011

Received in revised form 22 August 2011

Accepted 7 September 2011

Available online xxxx

Keywords:

Microbubbles

Cationic

Neutral

Sonoporation

Gene

ABSTRACT

Ultrasound-mediated gene transfer is emerging as a practical means of facilitating targeted gene expression and is significantly enhanced in the presence of exogenously added microbubbles. This study explores the influence of microbubble surface modifications on their interaction with plasmid DNA and target cells, and the functional consequences of those interactions in terms of ultrasound-mediated gene transfer. Polyethylene glycol-stabilized, lipid-shelled microbubbles with neutral (SDM201), cationic (SDM202) and biotinylated cationic (SDM302) surfaces were compared in terms of their abilities to interact with a luciferase-encoding reporter plasmid DNA and with target cells in vitro. The results demonstrate that the biotinylated cationic microbubble > cationic microbubble > neutral microbubble, in terms of their abilities to interact with target cells and to enhance ultrasound-mediated gene transfer, particularly at low microbubble concentration. The presence of a net positive charge on both cationic microbubbles promoted the formation of microbubble–nucleic acid complexes, although preformation of the complexes prior to addition to target cells inhibited the interaction between the microbubbles and target cells in vitro. The impact of these findings on potential in vitro or ex vivo therapeutic applications of microbubble-enhanced ultrasound-mediated gene transfer is discussed. All three microbubble preparations could be used to facilitate gene transfer in vivo and the potential advantages associated with the use of the cationic microbubbles for targeted gene delivery are discussed.

Crown Copyright © 2011 Published by Elsevier Ltd. on behalf of Acta Materialia Inc. All rights reserved.

1. Introduction

The use of ultrasound as a stimulus for gene transfer offers significant advantage over other gene transfer modalities for application in gene-based therapies exploiting in vitro, ex vivo and in vivo strategies [1–4]. As a physical gene transfer methodology it circumvents many of the problems associated with viral gene transfer and, when compared with other physical gene transfer methods, its non-invasive nature provides significant advantage over alternative approaches. It has been shown that ultrasound-mediated gene transfer is enhanced by exogenously added microbubbles. Currently it is generally accepted that enhanced cell membrane permeabilization is facilitated by a combination of microbubble-induced microstreaming proximal to the target cell membrane resulting from stable cavitation and microbubble-induced microjet formation close to the target cell membrane that results from catastrophic inertial cavitation [4,5]. Microbubbles have been exploited clinically to enhance diagnostic ultrasound imaging and commercially available reagents comprise a significant degree

of heterogeneity with respect to gas and the stabilizing shell composition. Shells are chosen to enhance the stability of the microbubble and may be composed of denatured protein (albumin), phospholipid and polymers [4]. Because these microbubbles can respond to an applied ultrasonic field by physically disintegrating and also by inducing site-specific cell membrane permeabilization, it has been suggested that they may be exploited to enhance gene transfer [6,7].

In light of the above-suggested mechanism by which microbubble-based reagents enhance ultrasound-mediated gene transfer, proximity between the microbubble, the nucleic acid and the target cell membrane would appear to be advantageous. Interestingly, many commercially available microbubble-based reagents that are exploited for diagnostic imaging purposes carry either a net neutral or slightly negative surface charge, and this serves to minimize interactions with cellular or molecular components in plasma [4]. However, if microbubbles are to be used in gene delivery, it would certainly be an advantage for the microbubble to carry a positive surface charge as this would enhance interactions with the negatively charged nucleic acid. Indeed, several studies have described the use of cationic microbubbles for the purposes of enhancing ultrasound-mediated gene transfer and have demonstrated electro-

* Corresponding author. Tel.: +44 2870124616.

E-mail address: ap.mchale@ulster.ac.uk (A.P. McHale).

static interaction between plasmid DNA and the surface of the microbubble [8,9]. Although these cationic microbubbles have been characterized with respect to their abilities to bind nucleic acid and enhance ultrasound-mediated gene transfer, few have been directly compared with neutral microbubbles in terms of their ability to interact with target cell populations and in terms of the impact of those interactions on ultrasound-mediated gene transfer. Here we directly compare cationic and biotinylated cationic microbubble preparations with a neutral microbubble preparation and examine their ability to interact with target cells in the presence and absence of plasmid DNA *in vitro*. We further examine the impact of those interactions on ultrasound-mediated gene transfer. In addition, we compare the ability of those microbubble preparations to facilitate gene transfer *in vivo* following intramuscular injection. The impact of our findings on designing strategies to facilitate microbubble-enhanced, ultrasound-mediated gene transfer for therapeutic purposes is discussed.

2. Materials and methods

2.1. Plasmid DNA and cell culture

The reporter plasmid pCMV-Luc, encoding the firefly luciferase gene under the control of the CMV promoter, was supplied by PlasmidFactory GmbH & Co. (Germany). RIF-1 (radiation-induced fibrosarcoma) cells [10] were employed as an *in vitro* target for gene transfer and expression in these studies because it is a cell line that has previously been characterized in our laboratories in terms of ultrasound-mediated gene transfer [5]. The cell line was maintained in RPMI 1640 tissue culture medium supplemented with glutamine (GibcoBRL, UK) and 10 vol.% foetal bovine serum at 37 °C in a 5% CO₂ humidified atmosphere. When required, single cell suspensions were prepared by treating cell monolayers with a 0.05% (w/v) solution of trypsin containing 0.02% (w/v) ethylenediaminetetraacetic acid in phosphate-buffered saline (PBS). Cells were subsequently harvested and washed in PBS by centrifugation prior to use.

2.2. Microbubbles

Microbubble reagents were provided by SONIDEL Ltd (Ireland). In these studies three microbubble preparations were employed as sonoporation aids with neutral (SDM201), cationic (SDM202) and biotinylated cationic (SDM302) shells, and were prepared by sonication of the relevant lipid mixtures in the presence of a perfluorobutane gas stream. The microbubbles were further stabilized by the inclusion of a polyethylene glycol–lipid conjugate in the shells. The lipid compositions of the microbubble shells are summarized in Table 1. Each preparation was adjusted to a concentration of 1×10^9 microbubbles ml⁻¹ using PBS. The diameter of the microbubbles ranged from 1.4 to 1.7 μm, with >80% of microbubbles in each preparation falling within this range. The ζ-potential for each microbubble preparation, under conditions employed for *in vitro* gene transfer, was measured in OptiMEM at a concentration of 1×10^7 microbubbles ml⁻¹ using a Zetasizer Nano ZS (Mal-

vern, UK) as recommended by the manufacturer. Microbubble suspensions were diluted in PBS and finally mixed with an equal volume of OptiMEM immediately prior to measurement.

2.3. Analysis of the interactions between microbubbles, plasmid DNA and target cells

To study the interaction between the microbubbles and plasmid DNA, 50 μl samples of microbubble suspension (5×10^8 bubbles ml⁻¹) were mixed gently with 20 μl of plasmid solution (0.4 mg ml⁻¹) for a period of 5 min at room temperature. Microbubble preparations were then washed twice with PBS by centrifugation at 500 rpm for 2 min. Microbubbles were dispersed in an original volume of PBS, and a 10 μl aliquot of ethidium homodimer (Sigma) solution (250 μg ml⁻¹) was added to each microbubble preparation. Following a 5 min incubation at room temperature, the microbubble preparations were washed once by centrifugation, from which >90% of the microbubbles were recovered. Just one centrifugation step was found to suffice as no further nucleic acid remained in solution following subsequent centrifugations. Microbubble preparations were examined using a Nikon Eclipse E400 fluorescence microscope (Japan), fitted with a ×60 objective lens and a Nikon G2A filter, facilitating excitation at 518 nm and emission at 605 nm. Photomicrographs were acquired using a digital camera interfaced with a PC operating the Andor™ iQ Standard Video-Meteor II software system (v.1.3) (Andor™ Technology Plc., UK). This software could also be employed to calibrate the objective lens according to the manufacturer's instructions by providing size calibrated size markers on photomicrographs.

In order to study the interaction between microbubbles and target cells, RIF-1 cells were cultured for 6–8 h on a glass slide cover slip to a maximum confluence of 20%. Cover slips were washed with PBS and a 10 μl aliquot of the relevant microbubble suspension (3×10^8 bubbles ml⁻¹) was added to each monolayer, prior to (no premix) or after (premix) the addition of 6 μl of pCMV-Luc plasmid solution (0.4 mg ml⁻¹). Each cover slip was then washed with PBS and placed on a microscope slide. The average number of microbubbles attached to at least 20 individual target cells was determined using a phase contrast setting and white light with the above-described microscope (×40 objective lens).

2.4. Fluorescence-based solid phase assay to examine interaction between microbubbles, plasmid DNA and target cells

In order to study the interactions between microbubbles, plasmid DNA and target cells, RIF-1 cells were added to individual wells of 96-well plates at a concentration of 2×10^4 cells well⁻¹. Twenty-four hours later, the cell growth medium was removed and the cell monolayer was washed with PBS. In cases where the microbubbles were preincubated (premix) with plasmid DNA, 25 μl aliquots of microbubble suspension (3×10^8 bubbles ml⁻¹) were incubated with 6 μl of plasmid solution (0.4 mg ml⁻¹) for 1 min and this mixture was added to each well of the 96-well plate. In cases where no premixing of plasmid DNA and microbubbles occurred, a 25 μl aliquot of microbubble suspension (3×10^8 bub-

Table 1
Percent molar ratio of lipid-based components in each microbubble reagent.

| Microbubble | Surface nature | DSPC | DSTAP | PEG40-stearate | DSPE-PEG2000-Biotin |
|-------------|-----------------------|------|-------|----------------|---------------------|
| SDM201 | Neutral | 51 | 0 | 49 | 0 |
| SDM202 | Cationic | 43 | 8 | 49 | 0 |
| SDM302 | Biotinylated cationic | 42.7 | 8 | 49 | 0.3 |

DSPC: distearoyl phosphatidylcholine; DSTAP: 1,2-distearoyl-3-trimethylammoniumpropane; PEG40-stearate: polyethylene glycol-40 stearate; DSPE-PEG2000-Biotin: Biotin–polyethyleneglycol-2000 distearoylphosphatidylethanolamine.

bles ml^{-1}) was added to each well of the 96-well plate, followed by a similar quantity of the plasmid DNA. After an incubation period of 1 min, cell monolayers were washed twice with PBS and a 6 μl aliquots of ethidium homodimer solution ($250 \mu\text{g ml}^{-1}$) was added to each well. Following a 3 min incubation, monolayers were washed with PBS and finally a 200 μl aliquot of PBS was added to each well. The fluorescence intensity exhibited by each monolayer was measured (from top) using a FLUOstar Omega microplate reader (BMG Labtech, Germany), at an excitation wavelength of 355 nm and an emission wavelength of 590 nm. Fluorescence intensity was expressed as arbitrary units (a.u.) using microbubbles, and ethidium homodimer incubated with cell monolayers in the absence of plasmid DNA was used as the zero reference.

2.5. Ultrasound-mediated gene transfer *in vitro*

Two gene transfer protocols were employed in this study. The first protocol was aimed at determining the influence of premixing the plasmid DNA with each microbubble preparation prior to addition to the target cells on gene transfer. The sequence of additions and quantities of reagents were similar to those described in Section 2.4 above, except that ethidium homodimer was omitted, washing was performed using serum-free OptiMEM (GibcoBRL, UK) and the final volume in each well was adjusted to a total volume of 50 μl using serum-free OptiMEM prior to exposure to ultrasound.

In the second protocol, target cells were plated as described above and a 10 μl aliquot of microbubble preparation, premixed with 6 μl of plasmid DNA (0.1 mg ml^{-1}), was added to each well of the 96-well plate together with 34 μl of serum-free OptiMEM. The ultrasound exposure configuration for both protocols is shown in Fig. 1. Samples were treated with ultrasound using an SP100 sonoprotector (SONIDEL Ltd., Ireland) emitting ultrasound at a frequency of 1 MHz. The transducer had an effective radiating area of 0.8 cm^2 , and a range of calculated ultrasound acoustic pressure outputs from this device has been described previously [5]. Samples were treated for 1 min at an ultrasound intensity/power density of 1.3 W cm^{-2} (spatial average, temporal peak (SATP)), using a 50% duty cycle (100 Hz). For both protocols, following ultrasound treatment, plates were placed in a humidified 5% CO_2 atmosphere at 37°C for 2 h. Plates were then washed once with RPMI 1640 serum-free medium and finally a 150 μl aliquot of RPMI 1640 medium, supplemented with 10 vol.% foetal bovine serum, was added to each well. Plates were then incubated for 24 h in a humidified 5% CO_2 atmosphere prior to analysis for gene expression. Photonic imaging was used to measure expression of the plasmid-encoded luciferase gene. A 70 μl aliquot of growth medium was removed from each well and this was replaced with 50 μl of D-luciferin (BIOSYNTH, Switzerland) solution (4 mg ml^{-1} solution in serum-free RPMI1640). Emitted bioluminescence was detected using a

Xenogen IVIS[®] Lumina imaging system supported by Living Image[®] software version 2.60. Using this software, each well was marked as a region of interest (ROI) and gene expression was expressed as photons emitted per second from each well. The efficiency of gene expression was expressed as photons s^{-1} per 100 viable cells.

2.6. Cell viability assay

Viability of cells was determined using an MTT-based assay. Essentially, culture medium was removed from the cells to be analysed and this was replaced by 50 μl of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide; 2 mg ml^{-1} in serum-free RPMI 1640) per well of a 96-well plate. Plates were incubated at 37°C for a period of 1 h, during which time a purple colour developed as a result of the reduction of the MTT to a formazan product by viable cells. The liquid from each reaction mixture was then removed carefully and the reduced formazan product was dissolved in 200 μl of dimethylsulfoxide. The absorbance at 490 nm was measured using a VERSAmix microplate reader (MDS, USA). Cell viability was determined by comparison with a standard curve constructed using absorbances from known concentrations of cells and the percentage viability of treated cell populations was calculated from the absorbance obtained with untreated populations of cells.

2.7. *In vivo* gene transfer

All animals were treated humanely and in accordance with licensed procedures under the UK Animals (Scientific Procedures) Act 1986. Prior to injection and ultrasound treatment, animals were anaesthetized by inhalation of 2 vol.% isoflurane in an oxygen carrier provided at a flow rate of 10 l min^{-1} . For *in vivo* gene transfer, a 20 μl aliquot of microbubble suspension (5×10^8 bubbles ml^{-1}) was mixed with 20 μl of plasmid DNA solution (1 mg ml^{-1}) and the mixture was injected into the hind leg muscle of 7-week-old C3H/HeN mice. The injection site was then treated with ultrasound at an intensity/power density of 1.3 W cm^{-2} (SATP), at 50% duty cycle, for 1 min, by direct contact with the ultrasound-emitting head. Contact was mediated using an ultrasound contact gel. Twenty-four hours after ultrasound treatment, *in vivo* imaging was performed to assess the functional expression of luciferase. Following induction of anaesthesia (intraperitoneal injection using Hypnorm:Hypnoval), luciferin (100 μl of 40 mg ml^{-1}) was administered by intraperitoneal injection. Photonic imaging to detect the presence of bioluminescence was performed using a Xenogen IVIS[®] Lumina imaging system supported by Living Image[®] software version 2.60. Three animals were employed per treatment group in all *in vivo* experiments, and data were collected from three individual experiments. The software was employed to mark ROIs that were equal in area for each image and the bioluminescence at those regions was expressed in photons s^{-1} .

2.8. Statistical analysis

Statistical analysis of significance was performed using analysis of variance and comparison of data groups was performed using the Tukey MCT in GraphPad Prism version 4.

3. Results and discussion

3.1. Characterization of microbubble preparations

Although a number of studies have described the use of cationic microbubbles to enhance ultrasound-mediated gene delivery, few,

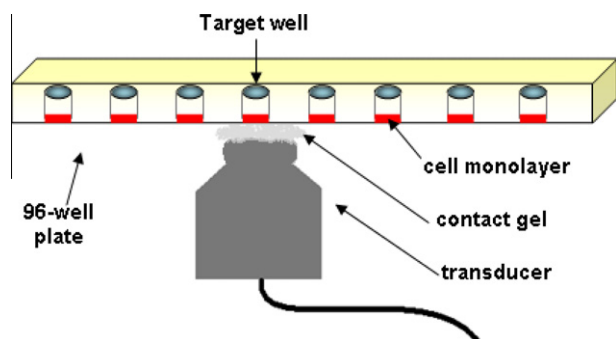


Fig. 1. Ultrasound exposure configuration.

if any, have carried out a comparative analysis with neutral microbubbles in order to identify advantage associated with the use of the former. To this end, we used three lipid-shelled microbubble preparations that were similar with respect to their gas content and with respect to their size. The percentage molar ratios of lipids employed in each formulation are shown in Table 1. In all three cases, over 80% of the microbubbles in each preparation had average sizes ranging from 1.4 to 1.7 μm , and each microbubble preparation had a polyethylene glycol brush on the outer surface to enhance stability. Similarities in size between the microbubble preparations were later confirmed by direct visualization using calibrated microscopy. Microbubble SDM201 was designed as a neutral bubble and the ζ -potential was found to be -0.7 ± 0.5 mV in OptiMEM. Microbubbles SDM202 and SDM302 were slightly cationic in nature, and were found to have ζ -potentials of $+4 \pm 1$ mV and $+5 \pm 1.2$ mV, respectively, when measured in OptiMEM. The latter was chosen because it had a significant buffering capacity. A further reason for choosing it was that it is the medium employed during all gene transfer experiments, and both pH and the presence of ionic character of the suspending medium have previously been shown to significantly influence ζ -potential [11,12]. It is interesting to note that Borden et al. [13] described a lipid-shelled microbubble with a 20% molar ratio of DSTAP and quoted a ζ -potential of approximately 30 mV. Microbubble SDM302 also had a small quantity of biotinylated lipid incorporated into its shell and, as expected, this had no significant effect on the ζ -potential of that microbubble preparation. One reason for incorporating the biotin tag was to include a functionalized microbubble preparation in our study since such preparations could be exploited in future molecular targeting strategies. If such a ligand is to be used for the latter purposes, it would be of value to determine whether or not its inclusion in the microbubble shell would have direct impact on interactions with target cells or on ultrasound-mediated gene transfer.

3.2. Interaction of microbubble preparations with plasmid DNA and target cells

In order to examine comparative binding of plasmid DNA to the microbubble preparations, nucleic acid was mixed with each preparation and the microbubbles were subsequently stained with ethidium homodimer. Each microbubble preparation was then examined using fluorescence microscopy. The results obtained

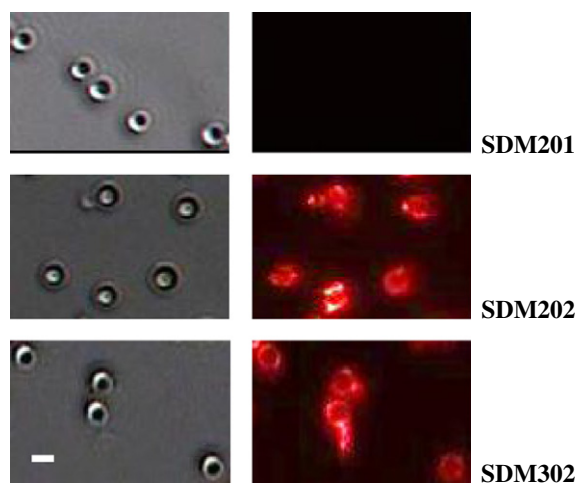


Fig. 2. Photomicrographs of (a) neutral, (b) cationic and (c) biotinylated cationic microbubbles that were exposed to plasmid DNA (pCMV-Luc) (left). Plates on the right were examined using fluorescence microscopy. The size bar represents 1.7 μm .

are shown in Fig. 2. This level of analysis demonstrated that, whilst no nucleic acid was bound to the neutral microbubble SDM201, significant quantities of nucleic acid were associated with the two cationic microbubbles SDM202 and SDM302. From direct observation of fluoromicrographs and from subsequent image analysis, no significant difference was detected in fluorogenic signals from both of these cationic preparations. These data suggest that both microbubbles were binding similar quantities of nucleic acid. If binding of nucleic acid was, as expected, mediated by charge-coupling to the surface of the cationic microbubbles, then these data further suggest that the existence of the biotin tag in the shell of SDM302 had little or no effect on the uptake of nucleic acid.

Since it has previously been suggested that the manner in which microbubbles enhance ultrasound-mediated gene transfer results, at least in part, from ultrasound-induced cavitation effects in the microenvironment of the target cell membrane, proximity between the microbubble, nucleic acid and target cells would be expected to enhance cell “poration” effects and nucleic acid transfer into the target cell [4,5,14]. The use of a cationic microbubble would therefore be expected to enhance gene transfer because of electrostatic interactions with target cells *in vitro*, since the latter usually bear a net negative charge. It was decided, therefore, to examine the interaction between all three microbubbles, nucleic acid and target cells using a novel solid phase fluorogenic assay. This assay was performed on 96-well plates and involved binding of microbubbles to an adhered monolayer of cells. In order to examine the influence of plasmid DNA on the interaction between microbubbles and target cells, it was decided to either pre-mix DNA with the microbubbles prior to addition to target cell monolayers or to add DNA to target cells following addition of microbubbles to those target cells. Any bound DNA was then stained using ethidium homodimer. Fluorescence spectroscopy was then employed to detect the level of interaction between the microbubbles, nucleic acid and target cells. The microbubbles were initially placed in contact with target cells and the nucleic acid was subsequently added. The plates were then washed, and the data obtained following staining with ethidium homodimer are shown in Fig. 3A. The data demonstrate that with the neutral microbubble, SDM201, a relatively low-intensity fluorescent signal was obtained, indicating little interaction between that microbubble, plasmid DNA and target cells. However, with the cationic microbubble, SDM202, a significantly higher fluorogenic signal was obtained, suggesting that the microbubble was mediating retention of DNA on the surface of the target cell monolayer. When the biotinylated cationic microbubble, SDM302, was examined using this assay, the fluorogenic signal obtained following staining was almost double that obtained with the non-biotinylated cationic microbubble (Fig. 3A). This was a surprising result since both microbubbles exhibited similar ζ -potentials and were initially formulated with the same quantity of DSTAP. The results indicate that biotinylation was playing a significant role in enhancing proximity between DNA and the target cells. In overall terms, the results demonstrate that even the slight charge attributed to both cationic microbubbles played a very significant role in mediating the interaction between nucleic acid and the target cell.

In order to determine whether or not preloading of the microbubbles with nucleic acid would have an impact on the overall degree of interaction between nucleic acid and target cells, nucleic acid was preincubated with each microbubble preparation prior to addition to the target cells. These complexes were then incubated with the target cells, and the solid phase was washed and subsequently stained with ethidium homodimer. The results obtained are shown in Fig. 3A. Whilst no statistically significant difference was observed when compared with the neutral microbubble (SDM201) added to cells prior to addition of plasmid

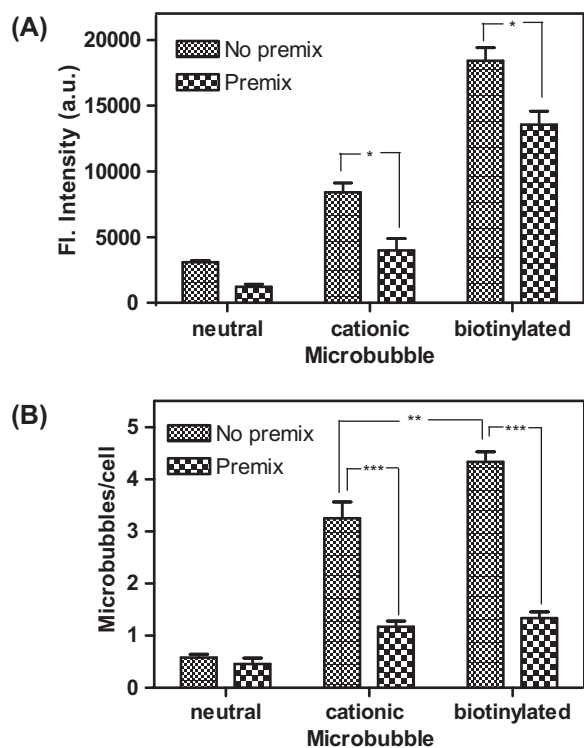


Fig. 3. Interactions between microbubble preparations, plasmid DNA (pCMV-Luc) and adherent RIF-1 cells, using a solid fluorogenic assay (A) and direct counting of microbubble attached to individual cells using microscopy (B). Microbubbles were either premixed with plasmid DNA (Premix) prior to addition to the cell monolayer or added to the monolayer before addition of plasmid DNA (No Premix). Error bars represent \pm SEM (standard error mean), where $n = 5$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and $F = 80$ (A), 56 (B).

DNA, it was found that the fluorogenic signals obtained with both cationic microbubble preparations were significantly lower than those obtained when nucleic acid was delivered following incubation of microbubbles with target cells. These data demonstrate that, with both cationic preparations, significantly less nucleic acid was associated with the target cells when preformed microbubble–DNA complexes were added to the cells. These results could indicate that the nucleic acid bound to the microbubbles was reducing the net positive charge on the cationic microbubbles, which would negatively impact on the interaction between microbubble complexes and the target cells to yield a lower fluorogenic signal in the solid phase assay. In order to address this issue, cells were exposed to microbubbles prior to and after the addition of plasmid DNA, and the number of microbubbles associated with individual cells was directly counted using microscopy. The results obtained are shown in Fig. 3B and clearly demonstrate that, for both cationic microbubbles, preincubation with nucleic acid had a serious negative impact on binding of microbubbles to cells. Indeed, when nucleic acid was prebound to both of the cationic microbubbles, the degree of binding to target cells was reduced to a value that was only slightly higher than that obtained with the neutral microbubbles. These data are supportive of those in Fig. 3A and suggest that the reduced fluorescence intensity observed with both cationic microbubbles is primarily due to inhibition of interactions between the microbubble–plasmid DNA complexes and the target cell surface. It is highly likely that the binding of nucleic acid to the microbubbles reduces the cationic nature of those microbubbles, thereby reducing charge-coupled interactions with the target cells.

3.3. Impact of microbubble, plasmid DNA and target cell interactions on ultrasound-mediated gene transfer in vitro

Since the above approach demonstrated differential interactions between microbubbles, plasmid DNA and target cells, it was of interest to determine the impact those interactions might have on ultrasound-mediated gene transfer in vitro. To this end, samples were prepared as described above for the studies examining microbubble, plasmid and target cell interactions and, once again, target cells were exposed to either microbubbles that were preincubated with plasmid or to microbubbles followed by the addition of plasmid. The target cell populations were then washed prior to treatment with ultrasound. Twenty-four hours after treatment, 96-well plates were examined using photonic imaging following addition of luciferin in order to detect expression of the transgene-encoded product luciferase. The data are shown in

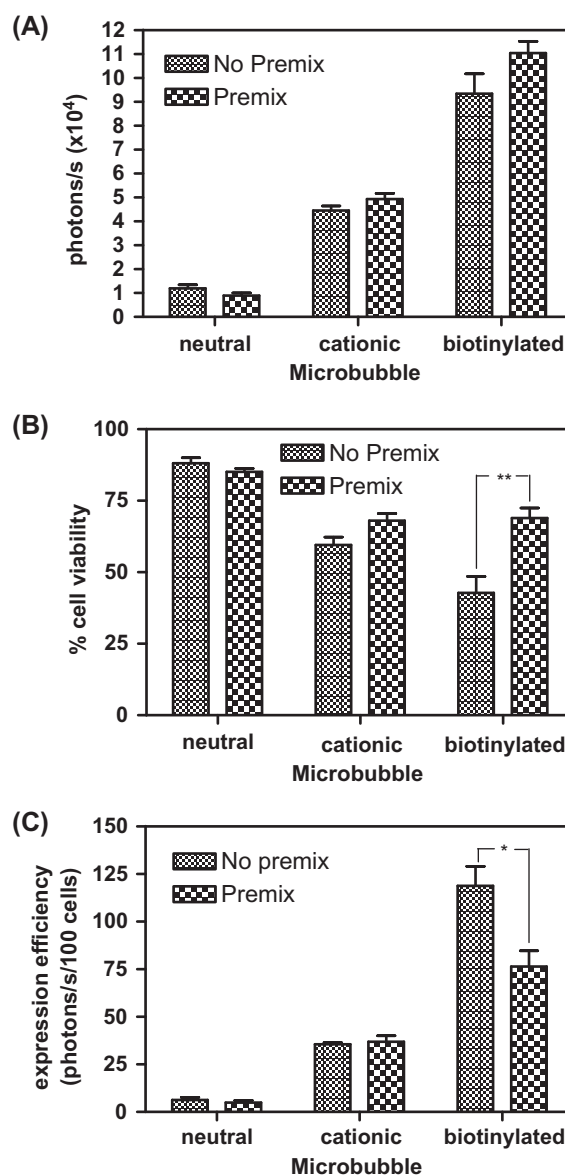


Fig. 4. Analysis of luciferase expression (A) and cell viability (B) following exposure of cells to ultrasound in the presence of each microbubble preparation. Microbubbles were either premixed with plasmid DNA (Premix) prior to addition to the cell monolayer or added to the monolayer before addition of plasmid DNA (No Premix). In (C), gene expression is presented per 100 cells. In all graphs error bars represent \pm SEM, where $n = 5$; * $p < 0.05$, ** $p < 0.01$ and $F = 98$ (A), 26 (B), 62 (C).

Fig. 4A and demonstrated that, when the microbubbles were preincubated with cells prior to the addition of plasmid DNA, the luciferase activity in cells treated with ultrasound in the presence of both cationic microbubbles (SDM202 and SDM302) was significantly higher than that in cells treated with ultrasound in the presence of the neutral microbubble (SDM201). It should be noted that, in controls involving the addition of plasmid alone or in combination with microbubbles in the absence of ultrasound, no gene expression was detected using this method of analysis. Based on the data in Fig. 3A and B, this was to be expected, since both cationic microbubbles would have been retained by charge coupling to target cells. What was unexpected, however, was the observation that, when microbubbles were premixed with plasmid and then used to treat cells, the expression of luciferase was similar to that in cells that were treated with microbubbles and then with plasmid DNA. It has been previously demonstrated that increasing the microbubble concentration can lead to a decrease in cell viability following treatment with ultrasound [5,9,15,16].

When we examined cell viability in our ultrasound-treated cultures, we obtained the data shown in Fig. 4B. These data demonstrated that, for both the cationic microbubbles, cell viability was reduced below that observed in the samples treated with the neutral microbubbles. No significant difference in cell viability was observed following treatment with the cationic microbubble SDM202, whether this was used as a preformed complex with DNA or added to the cell target prior to addition of nucleic acid. However, with the biotinylated cationic microbubble SDM302, the cell viability was significantly lower in the samples treated with the microbubble prior to the addition of nucleic acid. It was also interesting to note that in Fig. 3A and B these were the samples with the highest number of microbubbles associated with the target cells. For the biotinylated cationic microbubble, SDM302, when differences in cell viability are taken into account and when gene expression was calculated as a function of cell viability, it was found that expression was highest when the microbubbles were placed in contact with the target cells prior to the addition of nucleic acid (Fig. 4C). We suggest that this latter phenomenon is due to the higher number of microbubbles associated with the target cells when the microbubbles are added to those cells prior to addition of the nucleic acid (Fig. 3B). More nucleic acid would be expected to accumulate around the target cells and the enhanced proximity of more microbubbles to target cell membranes would, in turn, enhance the ultrasound-induced sonoporative effects.

As mentioned above, ultrasound-mediated gene transfer offers significant potential for use in gene-based therapies exploiting *in vitro* or *ex vivo* strategies [1–3]. Such strategies involve harvesting cells or tissues from a patient, using gene transfer technology to incorporate a therapeutic gene, and subsequently reintroducing the cells or tissues back into the patient. Indeed, many stem-cell-based therapies depend on such an approach. Being aware of the limitations of the use of cationic microbubbles identified above is important in the design of gene delivery strategies if microbubble-enhanced ultrasound-mediated gene transfer is to be used in such approaches. In practical terms, our observations suggest that, in exploiting ultrasound for such purposes, preformation of the microbubble–DNA complex could be counter-productive if maximum gene transfer efficiency is required.

3.4. The influence of microbubble concentration on gene transfer *in vitro*

From the perspective of controlled gene delivery exploiting an ultrasound-mediated microbubble-enhanced approach systemically or locally to facilitate site-directed gene expression for therapeutic purposes *in vivo*, the ideal sequence of events would be to

load or coat the ultrasound-responsive microbubble-based delivery vehicle with the nucleic acid payload and subsequently administer this complex to the patient. One of the inherent perceived advantages associated with the use of cationic microbubbles is that they can be exploited as a nucleic acid carrier. In any case, separate administration of microbubbles and nucleic acid for *in vivo*-based gene therapies would be impractical and contraindicated by aspects such as reduced nucleic acid stability in circulation [17]. Therefore, in this part of the study the effect of microbubble concentration on the ability of each microbubble preparation to enhance ultrasound-mediated gene transfer using the preformed microbubble–nucleic acid complex was examined. In the case of the neutral microbubble (SDM201), this was preincubated with the plasmid and the mixture was then added to the target cells. Twenty-four hours after ultrasound treatment, gene expression was determined using photonic imaging. The data obtained are shown in Fig. 5A. The results demonstrate that at lower microbubble concentrations ($0.2\text{--}1.0 \times 10^7$ microbubbles ml^{-1}) gene expression is relatively low and in all cases both cationic microbubbles are superior to the neutral microbubble in terms of enhancing ultrasound-mediated gene transfer. In comparative terms, at these lower microbubble concentrations the differences between the cationic microbubble (SDM202) and the biotinylated cationic microbubble (SDM302) are not statistically significant, although the trend indicates that cells treated with the biotinylated cationic microbubble yield a higher level of gene expression. At higher microbubble concentrations ($3\text{--}5 \times 10^7$ microbubbles ml^{-1}) the levels of gene expression increase. Interestingly, at

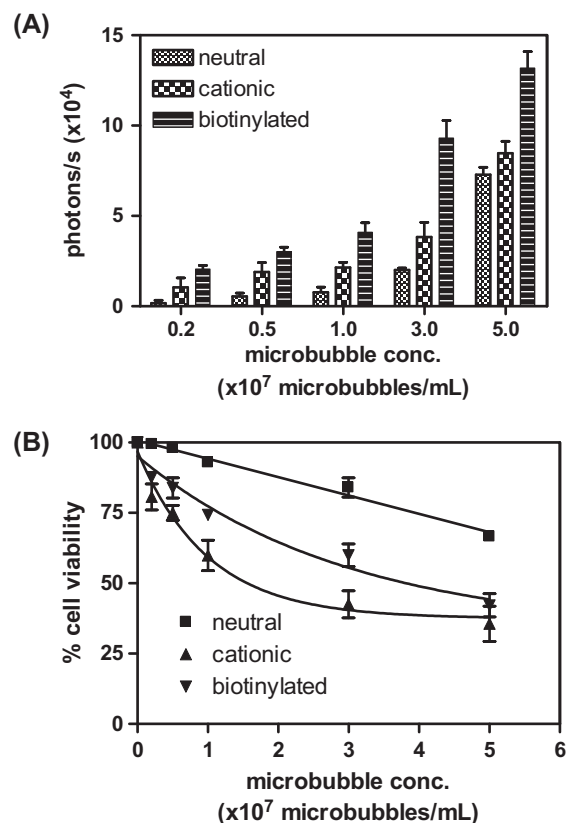


Fig. 5. The effect of microbubble number on luciferase expression (A) and cell viability (B). Each microbubble preparation was premixed with plasmid DNA (pCMV-Luc) prior to incubation with RIF-1 cells. Luciferase expression was detected using bioluminescent imaging. Data obtained with the neutral microbubble were fitted to a linear decay function and data obtained for both the cationic and biotinylated cationic microbubbles were fitted to a non-linear decay function. In A and B, error bars represent \pm SEM, where $n = 5$.

a concentration of 5×10^7 microbubbles ml^{-1} , the neutral microbubble delivers a relatively high level of gene expression, presumably as a result of increased proximity to the target cell membrane which results from the increased number of microbubbles in suspension during ultrasound treatment. Indeed, at higher concentrations, the advantage associated with the use of the cationic microbubble, SDM202 is significantly reduced. As shown above and by others, ultrasound treatment in the presence of microbubbles can lead to a compromise in cell viability [1,7,13,14]. When cell viability was determined in the above-treated cultures, the data shown in Fig. 5B were obtained. With all three microbubbles, cell viability decreased with increasing microbubble concentration. This effect appeared to be more pronounced for both cationic microbubbles. It was noted that, whilst the relationship between increasing microbubble concentration and cell viability appeared to be linear, that relationship for both of the cationic microbubbles appeared to be non-linear. This was supported by the close fit obtained to linear and non-linear decay functions, as shown in Fig. 5B. The latter suggested that, at lower microbubble concentrations, the cationic microbubbles bound to the surface of target cells, and this facilitated a more pronounced impact on cell viability during ultrasound treatment. It is clear from the data presented in our study and those of others that there is an inverse relationship between gene expression and cell viability, and it appears that gene transfer can only exist when some degree of cell damage occurs [1,7,13,14]. This is not unique to sonoporation; others have shown similar effects with electroporation-mediated gene transfer methodologies, where increasing electric field pulse number can have a positive impact on gene expression and a corresponding negative impact on cell viability [18]. It would therefore appear that optimization of sonoporation protocols for gene transfer necessitates identification of an acceptable balance between achieving maximal gene expression and minimal target cell damage. In all of the above studies, the biotinylated microbubble outperformed its cationic counterpart in enhancing ultrasound-mediated gene transfer. Although no precise reason for this is known at present, we are currently exploring the possibility that biotin receptors on the target cells may be mediating this effect [19].

3.5. Comparison of microbubble preparations for in vivo gene transfer

Having demonstrated that all three microbubbles can enhance ultrasound-mediated gene transfer in vitro, it was of interest to compare all three preparations in terms of their ability to enhance ultrasound-mediated gene transfer in vivo. It was decided to initially compare the ability of the microbubbles to enhance gene transfer following direct intramuscular injection because this would preclude dynamic challenges associated with intravenous injection. In addition, and for reasons alluded to in Section 3.2 above, nucleic acid was preincubated with each microbubble preparation prior to injection. It was also decided to employ a microbubble concentration of 2.5×10^8 microbubbles ml^{-1} because this concentration had previously been shown to enhance ultrasound-mediated gene transfer following intramuscular injection with other lipid-based microbubble preparations in our laboratories [5].

Twenty-four hours after injection of microbubble/plasmid mixtures and treatment with ultrasound, gene expression was detected using photonic imaging. The data obtained are summarized in Fig. 6. They demonstrate that, although no statistically significant difference in gene expression was obtained when the neutral and cationic microbubbles were compared, gene expression delivered using the biotinylated cationic microbubble was significantly increased over that obtained with the neutral microbubble. It should be noted that inclusion of controls compris-

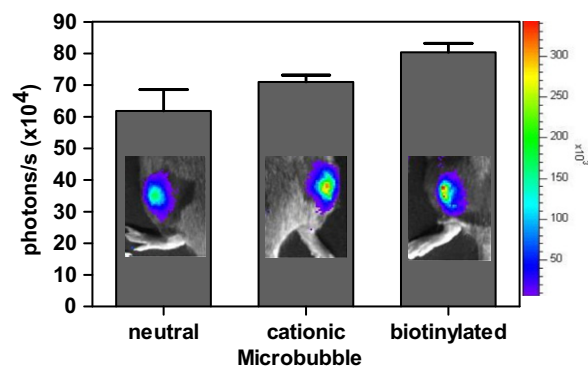


Fig. 6. In vivo microbubble-enhanced ultrasound-mediated gene transfer in mouse hind leg muscle using each microbubble preparation. Luciferase expression was determined using bioluminescent imaging. Typical bioluminescent signals are shown in the inset plates for each microbubble preparation and data on the y-axis refer to the total number of counts in the region of interest. Error bars represent \pm SEM, where $n = 3$.

ing either DNA alone or DNA plus microbubbles, both in the absence of ultrasound, failed to yield gene expression at this level of analysis. Although this was in agreement with our in vitro data, it was somewhat surprising since direct injection of DNA has been shown by others to deliver some level of gene expression [20,21].

Using a plasmid DNA comprising the CMV promoter and luciferase as a reporter gene, Danko et al. [20] demonstrated functional expression of luciferase following direct intramuscular injection into other strains of mice. In addition to using different strains of mice, it should be noted that these authors went to great lengths to extract the reporter gene protein from the target tissue and subsequently assay the activity in vitro, whereas our study involved direct imaging of animals after intravenous injection of luciferin. The lack of a signal in our controls may have been due to pharmacokinetic aspects in terms of luciferin absorption into tissues or, indeed, may be related to the choice of animal strain. In addition, Danko et al. also demonstrated that the level of expression was dependent on the age of the animals, with maximal expression observed in animals that were 2 weeks old; the animals used in our study were 7 weeks old.

Doh et al. [21] demonstrated expression of β -galactosidase activity in mouse muscle following direct intramuscular injection of an expression plasmid encoding the enzyme. They detected the reporter gene 3 h after injection and expression reached a peak at 3–14 days. In our study, we chose data harvested at 24 h because this was the peak obtained following monitoring of the animals for a period of 30 days. Once again, the lack of a signal from our control animals may have resulted from either our choice of animals or the specific nature of the plasmid, although we believe it is most likely to be a result of the sensitivity of the assay method we employed.

Although others have described the use of systemically administered cationic microbubbles to enhance ultrasound-mediated gene transfer in vivo [8,22], we are unaware of any study comparing the effectiveness of neutral and cationic lipid-shelled microbubbles comprising the same gas and the same shell platform in this context. Christiansen et al. [8] compared uptake of nucleic acid by neutral and cationic microbubbles based on the same lipid-shell platform; however, their in vivo studies only employed the cationic microbubble. In their study, although they examined the use of the cationic microbubble to enhance ultrasound-mediated gene transfer in skeletal muscle, their administration of the microbubble-plasmid complex was systemic. Kobulnik et al. [23] demonstrated that ultrasound-mediated delivery of green fluorescent protein and vascular endothelial growth factor-encoding DNA, complexed with cationic microbubbles following intravenous administration, was

as effective as intramuscular delivery in facilitating delivery of the DNA to muscle in rats, although their study did not include a comparison of ultrasound-mediated gene delivery using neutral and cationic microbubbles. From our *in vivo* studies using direct intramuscular injection, the observed benefits associated with the use of cationic microbubbles appear to be minimal. This approach, however, may not be the best means of comparing neutral with cationic microbubbles *in vivo*, since both nucleic acid and DNA would be in relatively close proximity to target cells immediately after intramuscular injection. Nevertheless, we believe that, as a result of their ability to bind and protect nucleic acid-based payloads, their ability to enhance ultrasound-mediated gene transfer and their compatibility with surface modification techniques, the cationic microbubble platform for ultrasound-controlled, site-specific delivery of nucleic acids offers very significant potential for targeted gene-based applications.

4. Conclusions

In this study we sought to compare the abilities of neutral, cationic and biotinylated cationic microbubbles to enhance ultrasound mediated gene transfer using both *in vitro* and *in vivo* analytical approaches. When compared with neutral microbubbles, use of both cationic microbubbles enhanced the association between nucleic acid and target cells. However, preformation of a microbubble–nucleic acid complex with the cationic microbubbles significantly reduced the binding of those complexes to target cells *in vitro*, which in turn reduced ultrasound-mediated gene transfer. This would have a significant impact on the design of protocols for either *in vitro* or *ex vivo* therapeutic approaches exploiting ultrasound-mediated gene transfer. Further comparative studies with the three microbubble preparations demonstrated that the advantage associated with the use of the cationic microbubbles was reduced at higher overall microbubble concentration, although the biotinylated cationic microbubble consistently delivered the highest levels of gene expression. This was further confirmed *in vivo*. In overall terms, the results demonstrate a significant advantage associated with the use of cationic microbubbles in enhancing ultrasound-mediated gene transfer *in vitro* and *in vivo*, although the observations described herein will impact significantly on the design of therapeutic protocols to maximize the practical exploitation of that advantage.

Disclosure

Dr. N. Nomikou is Research and Development Manager at Sonidel Ltd., Dublin, Ireland.

Acknowledgements

This study was partially funded by a Fusion Project Award administered by InterTradeIreland. The authors wish to acknowledge the assistance and advice received from Dr. G. McKerr in relation to the studies involving microscopy and image analysis.

Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figures 1, 2 and 6, are difficult to interpret in black and white. The full colour images can

be found in the on-line version, at [doi:10.1016/j.actbio.2011.09.010](https://doi.org/10.1016/j.actbio.2011.09.010).

References

- [1] Akowuah EF, Gray C, Lawrie A, Sheridan PJ, Su C-H, Bettinger T, et al. Ultrasound-mediated delivery of TIMP-3 plasmid DNA into saphenous vein leads to increased lumen size in a porcine interposition graft model. *Gene Ther* 2005;12:1154–7.
- [2] Isaka I. Gene therapy targeting kidney diseases: routes and vehicles. *Clin Exp Nephrol* 2006;10:229–35.
- [3] Ghali S, Dempsey MP, Jones DM, Grogan RH, Butler PE, Gurtner GC. Plastic surgical delivery systems for targeted gene therapy. *Ann Plast Surg* 2008;60:323–32.
- [4] Nomikou N, McHale AP. Exploiting ultrasound-mediated effects in delivering targeted, site-specific cancer therapy. *Cancer Lett* 2010;296:133–43.
- [5] Li YS, Davidson E, Reid CN, McHale AP. Optimising ultrasound-mediated gene transfer (sonoporation) *in vitro* and prolonged expression of a transgene *in vivo*: potential applications for gene therapy of cancer. *Cancer Lett* 2009;273:62–9.
- [6] Hernot S, Klivanov AL. Microbubbles in ultrasound-triggered drug and gene delivery. *Adv Drug Del Rev* 2008;60:1153–66.
- [7] Suzuki R, Oda Y, Utoguchi N, Maruyama L. Progress in the development of ultrasound-mediated gene delivery systems utilizing nano- and microbubbles. *J Control Rel* 2011;149:36–41.
- [8] Christiansen JP, French BA, Klivanov AL, Jaul S, Linder JR. Targeted tissue transfection with ultrasound destruction of plasmid-bearing cationic microbubbles. *Ultrasound Med Biol* 2003;29:1759–67.
- [9] Tlaxca JL, Anderson CR, Klivanov AL, Lowrey B, Hossack JA, Alexander JS, et al. Analysis of *in vitro* transfection by sonoporation using cationic and neutral microbubbles. *Ultrasound Med Biol* 2010;36:1907–18.
- [10] Nomikou N, McHale AP. Electrokinetic dispersion of a cancer chemotherapeutic drug for the treatment of solid tumours. *Cancer Lett* 2009;279:202–8.
- [11] Takahashi M. ζ Potential of microbubbles in aqueous solutions: electrical properties of the gaswater interface. *J Phys Chem B* 2005;109:21858–64.
- [12] Oliveira C, Rubio J. Zeta potential of single and polymer-coated microbubbles using an adapted microelectrophoresis technique. *Int J Mineral Process* 2011;98:118–23.
- [13] Borden MA, Caskey CF, Little E, Gillies RJ, Ferrara KW. DNA and polylysine adsorption and multilayer construction onto cationic lipid-coated microbubbles. *Langmuir* 2007;23:9401–8.
- [14] Judo N, Okada K, Yamamoto K. Sonoporation by single-shot pulsed ultrasound with microbubbles adjacent to cells. *Biophys J* 2009;96:4866–76.
- [15] Rahim A, Taylor SL, Bush NL, ter Haar DR, Bamber JC, Porter CD. Physical parameters affecting ultrasound/microbubble-mediated gene delivery efficiency *in vitro*. *Ultrasound Med Biol* 2006;32:1269–79.
- [16] Li T, Tachibana K, Kuroki M, Kuroki M. Gene transfer with echo-enhanced contrast agents: Comparison between Albunex, Optison and Levovist in mice – initial results. *Radiology* 2003;229:423–8.
- [17] Houk BE, Hochhaus G, Hughes JA. Kinetic modelling of plasmid DNA degradation in rat plasma. *AAPS Pharmsci* 1999;9:1–6 [article 9].
- [18] Yao S, Rana S, Liu D, Wise GE. Improvement of electroporation to delivery plasmid DNA into dental follicle cells. *Biotechnol J* 2009;4:1488–96.
- [19] Yellepeddi VK, Kumar A, Palakurthi S. Biotinylated poly(amido)amine (PAMAM) dendrimers as carriers for drug delivery to ovarian cancer cells *in vitro*. *Anticancer Res* 2009;29:2933–44.
- [20] Danko I, Williams P, Herweijer H, Zhang G, Latendresse JS, Bock I, et al. High expression of naked plasmid DNA in muscles of young rodents. *Hum Mol Gen* 1997;6:1435–43.
- [21] Doh SG, Vahlsing HL, Hartikka J, Liang X, Manthorpe M. Spatial–temporal patterns of gene expression in mouse skeletal muscle after injection of LacZ plasmid DNA. *Gene Ther* 1997;4:648–63.
- [22] Haag P, Frauscher F, Gradl J, Seitz A, Schafer G, Lindner JR, et al. Microbubble-enhanced ultrasound to deliver an antisense oligonucleotide targeting the human androgen receptor into prostate tumours. *J Steroid Biochem Mol Biol* 2006;102:103–13.
- [23] Kobulnik J, Kuliszewski MA, Steward DJ, Lindner JR, Leong-Poi H. Comparison of gene delivery techniques for therapeutic angiogenesis. *J Am Coll Cardiol* 2009;54:1735–42.