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Development of Therapeutic Microbubbles for Enhancing Ultrasound-Mediated Gene Delivery

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Abstract

Ultrasound (US)-mediated gene delivery has emerged as a promising non-viral method for safe and selective gene delivery. When enhanced by the cavitation of microbubbles (MBs), US exposure can induce sonoporation that transiently increases cell membrane permeability for localized delivery of DNA. The present study explores the effect of generalizable MB customizations on MB facilitation of gene transfer compared to Definity®, a clinically available contrast agent. These modifications are 1) increased MB shell acyl chain length (RN18) for elevated stability and 2) addition of positive charge on MB (RC5K) for greater DNA associability. The MB types were compared in their ability to facilitate transfection of luciferase and GFP reporter plasmid DNA *in vitro* and *in vivo* under various conditions of US intensity, MB dosage, and pretreatment MB-DNA incubation. The results indicated that both RN18 and RC5K were more efficient than Definity®, and that the cationic RC5K can induce even greater transgene expression by increasing payload capacity with prior DNA incubation without compromising cell viability. These findings could be applied to enhance MB functions in a wide range of therapeutic US/MB gene and drug delivery approach. With further designs, MB customizations have the potential to advance this technology closer to clinical application.

Keywords

ultrasound-mediated gene delivery; microbubbles; nonviral gene transfer; acoustic cavitation; Non-Viral Gene Delivery; Ultrasound; Gene Therapy

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1. Introduction

Ultrasound (US)-mediated gene delivery has emerged as a promising method for targeted and noninvasive gene therapy. Coupled with the use of gas-filled microbubbles (MBs), this gene transfer modality offers significant advantages, including temporal and spatial specificity based on the site of US insonation [1–5] and low risks of immunopathogenesis [6, 7]. The desired US bioeffect is achieved primarily by transient permeabilization of cell membranes, allowing the physical entry of macromolecules into the target tissue [5, 8, 9]. Further, this effect is enhanced by the presence of MBs, which serve as cavitation nuclei that oscillate and collapse under US stimulation. Despite its attractive potential for clinical applications, US-mediated gene delivery has thus far shown limited success compared to viral vectors and other methods [7]. Previous studies by our group and others have explored different ways to improve this technology, including varying drug or DNA dosages [10–15], customizing US systems [5, 12, 16–18] and improving surgical techniques in animal models [16–18]. These investigations suggested that US-mediated gene therapy can be significantly improved for potential therapeutic applications.

Aside from these progresses, development of functional MBs will be an important next step to further enhance the therapeutic potential of this novel technology. Therapeutic US-mediated MB destruction have already been applied in a multitude of ways, including delivery of DNA, siRNA [19–21], drugs [7, 22], stem cells [23] to different cell types such as tumor [24, 25], cardiovascular [26, 27], and skeletal muscle [1, 28], and organs such as liver [16–18], brain [2, 3, 22], and kidney [29, 30]. Given that the effectiveness of MBs depends on several variables such as their acoustic behaviors, size, stability, and compositions [31, 32], it is imperative to generate MBs that are tailored for their specific functions, instead of using generic MBs, such as the commercially available Definity® that is approved as a contrast agent for echocardiographic imaging. Developing a viable MB customization protocol can thus open up a myriad of possibilities for further advancement of US-mediated gene delivery, such as the incorporation of charge [15, 33, 34], functional ligands [35, 36], and polymers [7, 37] for DNA-carrying, cell-targeting, and cargo-protecting MBs.

Our exploration of MB development for gene delivery initiated with a first step of preparing consistent in-house MBs comparable to Definity®. Improved MB synthesis technique has allowed us to successfully generate MBs similar to Definity® in terms of concentration, size distribution, and *in vitro* transfection effectiveness. To further customize MBs for enhanced US-mediated gene delivery, we explored two relatively simple modifications, both of which may improve gene delivery without incorporating toxic or immunogenic substances: 1) increase the MB lipid shell acyl chain length; and 2) addition of positive charge to MB lipid shell. Increasing the phospholipid chain length in the MB shell, from 16 (used in Definity®) to 18 in the present study may help increase the overall MB stability, and resist spontaneous and acoustic dissolution [31]. This could potentially prolong MB half-life and improve MB response when exposed to US [32, 38]. Secondly, a cationic charge on the MB shell surface has several potential advantages. Recent studies have found that cationic MBs can electrostatically couple with anionic DNA, thus protecting it from premature degradation by nucleases while en route to the target location, as well as increasing the genetic payload in

the vicinity of target cells, allowing amplified gene transfer once sonoporation is induced [13, 34, 37, 39]. The purpose of this study was to directly compare the effectiveness of the two customized MBs to that of Definity® and further investigate parameters that can enhance the utility of neutral and cationic MBs in US-mediated gene delivery.

2. Materials and Methods

2.1 Plasmid preparation

Luciferase reporter plasmid pGL4.13 (Promega, Madison, WI) was produced by GenScript Inc. (Piscataway, NJ). pCMV-GFP plasmid was prepared as previously described [40] using Maxiprep (Qiagen, Germantown, MD).

2.2. Microbubble Preparation

Three customized MB formulations were prepared: two neutral (RN16, RN18) and one cationic (RC5K). The lipids used in the MB shells include 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphate (DPPA), 1,2-distearoyl-*sn*-glycero-3-phosphate (DSPA), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000 (MPEG-5000-DPPE), N-(Carbonylmethoxypolyethyleneglycol 5000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (MPEG-5000-DSPE), and 1,2-stearoyl-3-trimethylammonium-propane (DSTAP). All were purchased from Avanti Polar Lipids (Alabaster, AL).

Stock solutions were made by dissolving each lipid in suitable solvent. Aliquots of the stock solution were mixed in a 3 mL serum vial with the total lipid mixture between 1.2 and 1.3 mg. The ratio of lipids used for each MB was based on the formulation that yielded the greatest concentration, stability, and consistency in a series of titration experiments with incrementing lipid molar percentages. After the solvent was removed, the lipid mixtures were re-suspended in 1.5 mL of 1X PBS solution (Cellgro, Manassas, VA) containing 10% glycerol (Sigma-Aldrich, St. Louis, MO) and 10% ethylene glycol (Sigma-Aldrich). The resulting lipid suspensions were then heated to 56 °C and bath-sonicated in a 35-kHz ultrasonic cleaner (VWR International, West Chester, PA) to achieve lipid emulsions. Gas exchange was performed in the capped vials by vacuuming and filling the headspace with 2 mL of octafluoropropane gas (American Gas Group; Toledo, OH). Titration with incrementing gas volume was also performed to determine the optimal gas pressure at which the concentration and stability of activated MBs were greatest. MBs were generated by vigorous agitation of the lipid suspension for 45 seconds using the Vialmix™ (Lantheus Medical Imaging, N. Billerica, MA), yielding an average concentration of $2–5\times10^9$ MBs/mL.

2.3. Microbubble Characterization

MB size distribution was determined using the Multisizer™ 3 (Beckman Coulter, Brea, CA) equipped with a 30 μm aperture tube (0.6–18 μm size range). A 5 μL sample of MBs was diluted in 20 mL ISOTON® II Diluent (Beckman Coulter). All samples were measured three times within 10 minutes after the activation of MBs. MB concentration was determined

using a flow cytometer (BD FACSCalibur; Becton-Dickinson, Franklin Lakes, NJ). 25 μL samples were diluted 1:845 for all measurements. The volumes and counts of MBs obtained from flow cytometer were analyzed on FlowJo Cytometry Analysis software (Tree Star, Inc, Ashland, OR), and were used to calculate the concentration.

2.4. DNA Binding Efficiencies of Microbubbles

Zeta potential measurements were used to characterize the degree of DNA binding for each MB type. 6.4 μg pGL4 was incubated with 10 μL MB for varying durations before diluting in 10 mM NaCl solution (1:100). MB zeta potentials were then measured on a Zetasizer (Malvern Instruments Ltd, Worcestershire, UK) three consecutive times for accuracy. The decrease in MB zeta potential indicates the relative amount of DNA association to MB.

The DNA binding to MBs was further assessed by flow cytometry studies and microscopic analysis. PCMV-GFP plasmid was labeled with Cy™5 using the *Label* IT® Nucleic Acid Labeling Kit (Mirus Bio LLC, Madison, WI) and mixed with MBs at a ratio of 1 μg DNA to 1 μL contrast agents. The mixture was incubated at room temperature for 1 minute then diluted 1:1000 with FACS buffer for data acquisition on the flow cytometer. The percentage of fluorescent MBs and the mean fluorescent intensity (MFI) were determined using FlowJo software.

To quantify the amount of DNA binding to MBs, 32 μg of pGL4 was mixed with 50 μL of MBs in a microcentrifuge tube to allow DNA binding. After incubating for 15 minutes at room temperature, the solution was diluted to a final volume of 500 μL with TE buffer (Qiagen, Germantown, MD), and spun at 200*g* (1500 rpm on a tabletop centrifuge) for 8 minutes to separate the MBs from the solution containing the unbound DNA. A sample of the solution from the bottom of the tube was collected and filtered through a 0.45 μm filter (Millipore, Billerica, MA). The absorbance of the filtered solution was then measured on a Nanodrop (Nanodrop, Wilmington, DE) at λ=260 nm to determine the concentration of unbound DNA, which was used to extrapolate the amount of DNA bound to each MB.

2.5. Microbubble Destruction Efficiency

To assess the cavitation efficiencies of the MBs, the different types of MBs were exposed to US in a setup identical to that of the *in vitro* transfection. A flow cytometer was used to measure the MB concentrations before and after 10 seconds of $2 W/cm^2$ US exposure, giving rise to the calculation of the extent of MB destruction.

2.6. *In Vitro* US-mediated Gene Delivery

Human embryonic kidney 293T cells (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagles medium (DMEM) (Mediatech, Inc, Manassas, VA) containing 10% fetal bovine serum (FBS) (Atlanta Biologicals Inc, Lawrenceville, GA), 1% MEM nonessential amino acids, 1% Penicillin/Streptomycin, and 1% L-Glutamine. Twenty four hours before transfection, cells were seeded at 4×10^5 cells per well on 6-well plates.

Due to the buoyancy of gas-filled MBs in media, an inverted setup (Figure 1) was used to allow better interaction between MBs and cells. The slight attenuation of US intensity across

the polystyrene layer of the culture plate appears to be consistent among experiments (~87% US efficiency was obtained compared to the control; see Supplemental 1 for details [41–43]). During transfection, the culture media was first removed, and a sterilized US-absorbing silicone stopper (Plasticoid Company, Elkton, MD) was placed on each well. 3.4 mL of pre-mixed media containing 1.9 μg/mL plasmid DNA and 0.3% (v/v) MBs was loaded into each well through one of the needle ports in the stopper. US was generated from Sonitron 2000 sonoporator (Artison Corp, Inola, OK), which was scanned from above through a thin layer of conducting gel to cover the whole well area at a constant rate of 1 cycle per 3 seconds. Cells in each well were insonated for 3 minutes at US intensities between 1 and 5 W/cm^2 at 1 MHz, 20% duty cycle, and 100 Hz pulse repetition frequency (PRF). Culture media was changed after four hours of incubation at 37°C.

2.7. Transgene Expression

The transgene expression in 293T cells was evaluated 48 hours after US-mediated transfection. Cells transfected with Lipofectamine 2000 (Invitrogen, Grand Island, NY) were used as positive controls. GFP expression after transfection with pCMV-GFP plasmid was visualized by fluorescence microscopy and quantified using a FACSCalibur Flow Cytometer. Luciferase expression from pGL4-transfected cells was quantified by using the Luciferase Assay System (Promega) and the light produced from the oxidation of luciferin was measured using Victor 3 luminometer (Perkin-Elmer, Wellesley, MA). Luciferase expression is defined as relative light units per mg protein (RLU/mg).

2.8. Cell Viability

Forty eight hours after transfection experiments, the culture medium was removed and the MTT reagent (Invitrogen) was added to each well. After two hours of incubation at 37 °C, 0.04 N HCl in isopropanol was added and the absorbance of each well was measured at 560 nm in an absorbance plate reader. The viability of the cells was determined by comparing the absorbance of US-treated cells to that of DNA-only treated cells.

2.9. *In Vivo* US-mediated Gene Delivery

All procedures were performed according to the guidelines for animal care of both the National Institutes of Health and Seattle Children's Research Institute. More detailed description of the surgical procedure is mentioned previously [16]. Briefly, six-week old SAS SD male rats were obtained from Charles River Laboratories, Inc. (Wilmington, MA), and were kept in a specific pathogen-free (SPF) vivarium for at least three days for acclimation. Rats were anesthetized by continuous inhalation of isofluorane (2–3%). A midline incision was made to access the right medial liver lobe, and a 24-ga catheter is inserted into a portal vein branch leading to the target lobe. 150 μg pGL4 was added to 1.3x10^8 MBs, and was either incubated for 0 or 1min, prior to dilution with 5% glucose and PBS (about 1 mL total). The pGL4/MB solution was then infused slowly (~20 μL/s, 45 seconds) to the target lobe, which was exposed simultaneously to US (1.1 MHz frequency, 13.9 Hz PRF, 20 cycles pulse width, 2.7 MPa peak negative pressure) for a total treatment time of 90 seconds. The US system consisted of a combination pulse generator/radio frequency amplifier (RFG1000; JJ&A Instruments, Duvall, WA) that can deliver up to 1000 W of electrical power, driving a 16-mm diameter planar transducer (H158; Sonic Concepts,

Bothell, WA). After treatment, the rats were sutured and allowed to recover from surgery. They were sacrificed 24 hours later; blood samples and liver tissues were collected for luciferase assay (for gene expression) and transaminase assays (alanine- and aspartate-aminotransferase, ALT and AST, respectively, for assessment of liver damage).

2.10 Statistical Analysis

All data are presented as the mean + S.E. The Student's T-Test was used to calculate statistical significance. A p-value less than 0.05 was considered significant.

3. Results

3.1. Microbubble Characterization

In this study, three customized MB formulations: neutral RN16, RN18 and cationic RC5K, were characterized along with the clinically available contrast agent Definity®, which served as a comparison control. The lipid formulations of these MBs are summarized in Table 1. RN16 was made of the same lipids as Definity to emulate its characteristics. RN18 was made of similar phospholipids with longer acyl chain length (C18), and RC5K was made of C18 phospholipids with positively charged DSTAP incorporated in the MB shell. Several lipid ratios were tested for each customized MB formulation, and the molar ratios used for this study were determined based on the formulations that yielded the greatest concentration, stability, and consistency. Thus, slightly different lipid ratios from Definity were used to construct the MBs. We believe that this limitation of small ratio difference is not a major factor that drastically alters MB characteristics. We evaluated the concentrations of MB type (Figure 2A) and their stabilities over time post-activation (Figure 2B). The average concentrations of Definity® ($6.43\pm0.08\times10^9$ MBs/mL), RN16 ($7.20\pm3.9\times10^9$ MBs/mL), RN18 ($5.00\pm0.1\times10^9$ MBs/mL), and RC5K ($2.22\pm0.11\times10^9$ MBs/mL) were within the same order of magnitude as measured on both Coulter Counter and flow cytometry, and all displayed little post-activation fluctuations over time (70–107%). Size profiles of various MBs evaluated by Coulter Counter III showed all MBs have comparable mean, median, and mode values (Fig. 2C). In addition, the mean diameters, MB population peak and distribution of neutral MBs Definity®, RN16, RN18 and RC5K (1.42±0.02 μm, 1.51±0.03 μm, 1.48±0.03 μm and 1.73±0.09 μm, respectively) were also very similar as observed on flow cytometry (Figure 2D). Since RN16 was very similar to Definity®, we focused on comparisons between Definity®, RN18, and RC5K in the subsequent investigations.

3.2. Microbubble Affinity to DNA

There was no statistical difference between the mean zeta potentials of Definity (-4.04±0.32 mV) and RN18 (-1.77±0.38 mV), whereas the surface positive charge of the cationic RC5K was significantly higher (+6.39±0.27 mV) than the neutral MBs. To investigate the MB's binding affinity to DNA, zeta potential measurements were taken for each MB after incubation with DNA at different durations. Relatively minimal changes in surface charges were observed in neutral MBs after incubation with plasmids, (-2.72 ΔmV for Definity and −0.72 ΔmV for RN18); whereas the surface charges of the cationic RC5k decreased steeply as incubation time increased (−46.08 ΔmV), demonstrating a significantly greater binding capacity with negatively charged DNA (Figure 3A). In our quantitative binding assay, the

neutral RN18 similarly showed negligible DNA loading whereas the cationic RC5K could carry approximately 0.06 pg per MB.

To further confirm and quantify their binding capacities, MBs were incubated with Cy5™-labeled plasmid DNA for 1 and 5 minutes and analyzed using flow cytometry. Figure 3B confirms that cationic MBs (RC5K) have greater DNA association compared to neutral MBs (RN18), as indicated by the greater percentages of Cy5™-positive population (68.2% and 35.6%, respectively) and MFI (110 and 54, respectively) after 1 minute incubation. These differences were more pronounced after longer incubation (5 minutes), as the number of Cy5™-positive population and MFI of RC5K increased drastically (Cy5™ positive: 68.2% to 93.4%, MFI: 110 to 273); while those of the neutral RN18 remained relatively unchanged (Cy5™ positive: 35.6% to 51.5%, MFI: 54 to 59). Further analysis showed that the MFI increased as the cationic MB size increased as expected due to an increase in cationic surface area, whereas the MFI of neutral MBs remained the same across all sizes (Figure 3C). On the other hand, the percentage of Cy5™-positive MB population remained the same for all MB size categories in all RN18 and RC5K bound pDNA samples, indicating that DNA associability were dependent on MB shell charge regardless of size (Figure 3D).

3.3. In Vitro US-Mediated Gene Delivery

Next, we evaluated the effectiveness of these MBs in enhancing gene delivery. A reporter luciferase plasmid, pGL4, was used in transfecting 293T cells using US (1 MHz frequency, 20% duty cycle, 100 Hz PRF) at 2 W/cm^2 intensity in the presence of 0.3% (v/v) of MBs for 3 minutes. The results showed that addition of MBs, RN18 and RC5K, dramatically enhanced luciferase activity compared to US-only treatment by 268-fold ($4.0\pm1.7\times10^7$ RLU/mg) and 177-fold ($2.7\pm1.5\times10^7$ RLU/mg), respectively (Figure 4A). The difference of expression levels between MB types was not statistically significant ($p=0.08$). Negative control groups without US exposure including cells transfected with pGL4 only, RN18 and pGL4, or RC5K and pGL4 produced minimal gene expression levels, confirming that both US and MBs are essential for enhancing gene delivery efficiency.

As shown in Figure 4B, when US intensity was increased from 0.5 W/cm^2 to 5 W/cm^2, luciferase expression further increased by up to 4.3-fold, while cell viability remained the same over the same US intensity range. In cells transfected with US and Definity®, luciferase expression plateaud at 1 W/cm^2; while RN18 and RC5K were significantly more efficient in enhancing gene delivery (Figure 4B, $p<0.01$). Transfection experiments using pGFP in parallel were consistent with these findings, as higher percentages of fluorescent cells were observed with increasing power using customized MBs both visually by microscopic analysis (Figure 4C) and quantitatively by flow cytometer evaluation (Figure 4D).

The dosage of MBs used in *in vitro* studies were also investigated. When the concentration of MBs was increased from 0.1% (3 μl) to 5% (150 μl), luciferase expression generally increased. This enhancement was more pronounced with cationic RC5K MBs. Efficiency was normalized to number of MBs, and it was found that RC5K MBs was more efficient than RN18 MBs on a per MB basis by as much as 2.4-fold. However, there was a negative

relationship between MB dosage and cell viability, which dropped dramatically as the volume of either MB types increased (Figure 4E).

3.4 Effect of DNA-binding on Transfection Efficiency

To assess how MB pre-incubation with plasmid translates in transfection efficiency, 293T cells were transfected with 1×10^7 RN18 or RC5K MBs that were incubated with pGL4 plasmid for 1 or 5 minutes. It was found that without prior incubation, the two MB types facilitated transfection at similar rates. However, Figure 5A shows that RC5K MBs incubated with plasmid for 1 minute produced significantly increased transgene expression levels ($p<0.05$) compared to no incubation, as well as improved expression compared to using neutral RN18 MBs ($p=0.01$). RN18 produced no change in transfection efficiency with pre-incubation with DNA. Similar results were obtained with transfection using pGFP. Flow cytometry analysis indicated that prior DNA incubation with RC5K increased the proportion of GFP-expressing cells by 2.3-fold (Figure 5B). Further, although the size distribution of Definity, RN18 and RC5K are very similar, in order to completely eliminate the possibility that small quantities of large MBs may be responsible for better transfection efficiency, we performed a separate experiment with RN18 and RC5K MBs after filtering out the large size MBs. As shown in Supplemental 2, no difference in enhancing gene transfection efficiency was observed with or without filtration of the MBs larger than 5 μm.

Interestingly, transfection using RC5K with 5 minute incubation did not yield a higher luciferase expression even though binding data suggested that these cationic MBs can load on even greater DNA cargo compared to 1 minute incubation. Cavitation experiments revealed that this may be due to reduced responsiveness to US when MBs are overloaded with DNA. As shown in Figure 5C, after exposure to 2 W/cm^2 US for 10 seconds, RN18 MBs were destroyed with similar extent across all MB sizes regardless of incubation duration, whereas less RC5K MBs were destructed with 5 minutes of prior DNA incubation ($p<0.01$), suggesting a diminished MB response to US stimulation.

When combined with increased US power, the enhancing effect of DNA incubation was augmented further for cationic RC5K but not for neutral RN18 (Figure 6). While transfection using RN18 only improved with increased US intensities, transfection using RC5K not only increased with increased US power (5 W/cm^2), but also further augmented by pre-incubation with DNA (1 minute). The transgene expression reached up to $1.3\pm0.1\times10^8$ RLU/mg, which was 4.7-fold of initial RC5K transfection efficiency at lower US intensity without pre-incubation with DNA. This was the highest attainable expression level with high cell viability (>95%) in our current experimental setting.

In Vivo US-mediated Gene Delivery—To demonstrate the efficiency of these MB types in animal models, US-mediated luciferase transfection experiments were performed on rats. Figure 7 shows the luciferase expression in rat liver using different MBs, 24 hrs after treatment. No significant difference in gene expression was found in rats treated with Definity® ($1.0\pm0.2\times10^5$ RLU/mg), RN18 ($1.3\pm0.2\times10^5$ RLU/mg), and RC5k without incubation ($1.3\pm0.2\times10^5$ RLU/mg). However, luciferase expression in rats treated with RC5k with 1 minute incubation ($2.3\pm0.3\times10^5$ RLU/mg protein) showed a significant

increase compared with RN18 and RC5k without incubation ($p<0.05$), and Definity® ($p=0.016$). Further, there was no apparent difference in liver damage with the associated increase in gene expression, as observed by gross examination during the liver harvest, nor any significant difference in the ALT and AST levels in all rats (Table 2).

4. Discussion

Gene therapy has attracted great interest in recent years for effective treatment of cancer and various genetic diseases. However, potential clinical application has been hampered by concerns of safety, invasiveness, and efficiency. Approaches using viral vectors, while efficient, are limited by immunotoxicity, potential oncogenesis and low targetability [44, 45]. US targeted MB destruction (UTMD)-mediated gene delivery has emerged as a promising non-invasive alternative that can address these issues. Therapeutic application of this system has demonstrated spatial and temporal control [1–5] in addition to being well-tolerated in animal experiments [16–18]. However, it has yet to show sufficient gene delivery efficiency [7], and improvements are required for clinical translation.

To this end, we investigated the development of MB application for US-mediated gene delivery. Given that Definity MBs are already commonly used in clinical diagnostic US and in some therapeutic US research, we aim to improve gene delivery efficiency relative to this standard. Two custom-made MBs were designed to enhance their role as cavitation nuclei that facilitate sonoporation and DNA entry into cells: RN18 has a longer phospholipid acyl chain length of C18 than Definity (C16), which has shown superior stability due to increased shell cohesiveness [31], whereas RC5K has an added cationic surface charge that was hypothesized to further aid gene delivery by associating and protecting anionic genetic cargo by ways of electrostatic interaction [13, 34, 37, 39]. Although both types are similar to Definity in several basic features, including lipid composition, concentration, stability, and size, RC5K has a slightly lower concentration and stability, which is likely due to electrostatic lateral repulsion between cationic lipid head groups at submicrometer radii of curvature [46].

In order to assess the effectiveness of the different MB types in gene transfer, several *in vitro* UTMD transfection experiments were performed. We designed a simple cell culture setup that will address common issues arising from conventional experimental setups. Our setup involves inverting the tissue culture plates and using a silicone stopper to seal the media and the MBs in the well. In this way the MBs floated up and directly interacted with the adherent 293T cells. The silicone stopper also acted as an acoustic absorber, which prevented reflection of US waves and thus, maintaining higher cell viability. It was demonstrated that both US and MBs are required to be simultaneously present for efficient gene delivery. The addition of either neutral RN18 or cationic RC5K drastically enhanced *in vitro* US-mediated gene delivery by 180–267-fold compared to US only control. With a non-inverted traditional set-up, only 1–3 fold increase in gene transfection efficiency can be obtained (Supplemental 3). In addition, both RN18 and RC5K had greater transfection efficiency than that of Definity (150-fold). While it is encouraging to see better performance in custom-made MBs compared to Definity, it is still mechanistically unclear why RN18 consistently outperformed Definity since size, stability, and cavitation rate of both MB were

virtually identical. We speculate that this phenomenon may be due to more efficient sonoporation effects from higher oscillation amplitudes or enhanced resonance, since a longer chain has been reported to have increased cohesiveness during insonification, prolonging the MB activities [32, 46]. More in-depth studies examining MB behaviors are underway to investigate this effect.

Several experimental parameters were tested to maximize the application of new MBs. As the US intensity and MB dosage were increased, we found that transfection efficiency also increased significantly, consistent with results in the literature.[10–13, 15, 16, 47] However, higher intensities and MB dosages lead to more violent cavitations and energy release, which in turn may lead to wider perforations on the cell membranes. Presence of these holes along the membranes can then lead to increased DNA entry; but big holes (>5 μm) and too many perforations may lead to cell death[48]. In our *in vitro* experiments, we observed little cell death even when cells were exposed to relatively higher US powers. However, while higher MB dosages dramatically enhanced gene expression by up to 7-fold, cell viability inversely dropped to as low as 30%, which is consistent with findings of other studies [7, 14, 15, 49]. A balance between more efficient DNA entry and lower cellular damage is required to achieve more effective gene transfer.

We hypothesized that cationic MBs, which can bind more DNA and thus localize and concentrate plasmids in the vicinity of cells, can more efficiently facilitate gene delivery without significant compromise in cell viability. Binding experiments using Zetasizer and flow cytometry confirmed enhanced DNA-coupling in cationic RC5K MBs compared to negligible non-specific binding of DNA in neutral RN18 MBs [34]. *In vitro* quantification experiments of DNA loading showed that each cationic MB bound ~0.06 pg of DNA, consistent with the 0.03 and 0.04 pg DNA loading results on other cationic MBs reported previously [34, 39]. By simple incubation, a cationic MB shell can bind and transport genes, among other varieties of nucleotide therapeutics such as RNAi [19–21]. The amount of DNA that can bind to cationic MBs can also be controlled by their sizes. We showed that when sorting MBs by size, larger MBs with greater surface area have dramatically higher MFI when incubated with Cy5-DNA. However, this enhanced DNA loading is limited by the instability of cationic MBs at larger sizes and the difficulty in generating or isolating lipid-based MBs with mono-dispersed sizes. [50]

Our results indicated that transfection with cationic RC5K MBs that were pre-incubated with DNA, significantly enhanced transgene expression ($p<0.05$). This is consistent with the findings of Wang, Panje, and Nomikou et al, and the notion that gene delivery can be augmented with a higher local DNA concentration [11, 12, 51]. However, Tlaxca et al found no difference between cationic and neutral MBs[15]. Differences in findings may be attributed to different experimental setups, particularly the additional step of prior DNA incubation. We found that cationic MBs would only bind and transfect cells significantly better when they were first given time to associate to high concentration of DNA (5660μg/mL), which was not done by other studies. Tlaxca et al carried out low concentration incubation (3.2 μg/mL) for unspecified duration. Interestingly, longer incubation using RC5K that led to even greater DNA-coupling did not yield proportional increase in gene delivery. Follow-up cavitation experiments showed when these MBs had extended

incubation with DNA, they cavitated significantly less than before, while no such change was observed for neutral RN18. These preliminary data suggest that, while cationic MBs can carry a greater DNA payload, this saturated complex formation may affect the MBs' acoustic behaviors and hinder their responsiveness to US. The correlation between ultrasound parameters and DNA payload on the MBs will be investigated in future studies. Since MB destruction requires energy to achieve the desired effect of sonoporation [5, 8], this possibility of diminished cavitation phenomena should be taken into account for optimal application of cationic MBs. The enhanced transfection using DNA-incubated cationic MBs was achieved without compromising cell viability; relative cell viability was sustained above 95% for the entire sample size. This may be because, unlike increased US intensity and MB dosage, increased DNA-loading on MBs supplied no additional mechanical disintegration that can lead to further cell death.

These *in vitro* findings were verified when these MBs were used for gene transfection *in vivo.* Within the complexities of the living system, it is difficult to predict the clinical effectiveness of the present methodology of UTMD-mediated gene delivery. Therefore, exposing the MBs to the interactive physiological environment *in vivo* will assess their potential or identify shortcomings for successful clinical translation and facilitate the development of next-generation therapeutic MBs. As with every model system, the *in vivo* models have limitations and cannot directly lead to conclusions about clinical significance, but consistent findings across multiple different models would be an important step towards clinical relevance.

US parameters used in the rat experiments (PNP, treatment time, PRF, etc.) were based on optimal parameters determined in previous UTMD gene transfer studies [16]. It was shown that 2.7 MPa PNP is sufficient to produce good transfection while minimizing liver damage using Definity MBs. Consistent with *in vitro* findings, transfection with RC5k MBs pre-incubated with DNA resulted in significantly greater transgene expression compared to Definity, RN18, and RC5K without incubation ($p<0.05$). This suggests that the ability of cationic MBs to associate with DNA is persistent even in a dynamic environment. Mild elevation of the transaminase levels post-treatment demonstrates that this enhancement was not associated with significant liver damage. In our previous studies, we observed the peak liver damage at day 1 post-treatment, which gradually recovered to normality within a few days [16].

We find that enhanced DNA-binding of cationic MBs can promote safer and more efficient US-mediated gene delivery by allowing the use of lower DNA dosage, which will make treatments cost-effective and efficient, and by potentially reducing US power requirements, which will minimize cell damage. Customizing MBs for gene therapy clinical applications with therapeutic US can be further improved by incorporating cell-specific ligands in the cationic MB shell for DNA localization at targeted treatment sites.

5. Conclusions

The goal of this study was to present the finding that US-mediated gene delivery can be notably improved by a few simple MB modifications using a setup that we found was both

practical and replicable. It also outlined the steps and assays used to synthesize custom-made MBs improved for therapeutic use. In particular, a series of MBs were synthesized and following initial screening, several types of more stable and consistently generated MBs were further characterized by multiple *in vitro* analysis techniques, cell culture studies and finally animal studies. Therefore, through simple, generalizable modifications of the MB shell using longer lipid chains and adding cationic charge on the surface, we obtained noticeable improvement in US-mediated gene delivery compared to experiments using Definity. Cationic MBs, which bind more DNA, can further facilitate gene transfer without compromising cell viability. Together with the best parameters of US intensity, MB dosage, and DNA incubation, multi-fold enhancement was achieved in gene delivery. These findings demonstrate the potential of MB customizations to generate more efficient US-mediated gene delivery systems, leading closer to clinical application.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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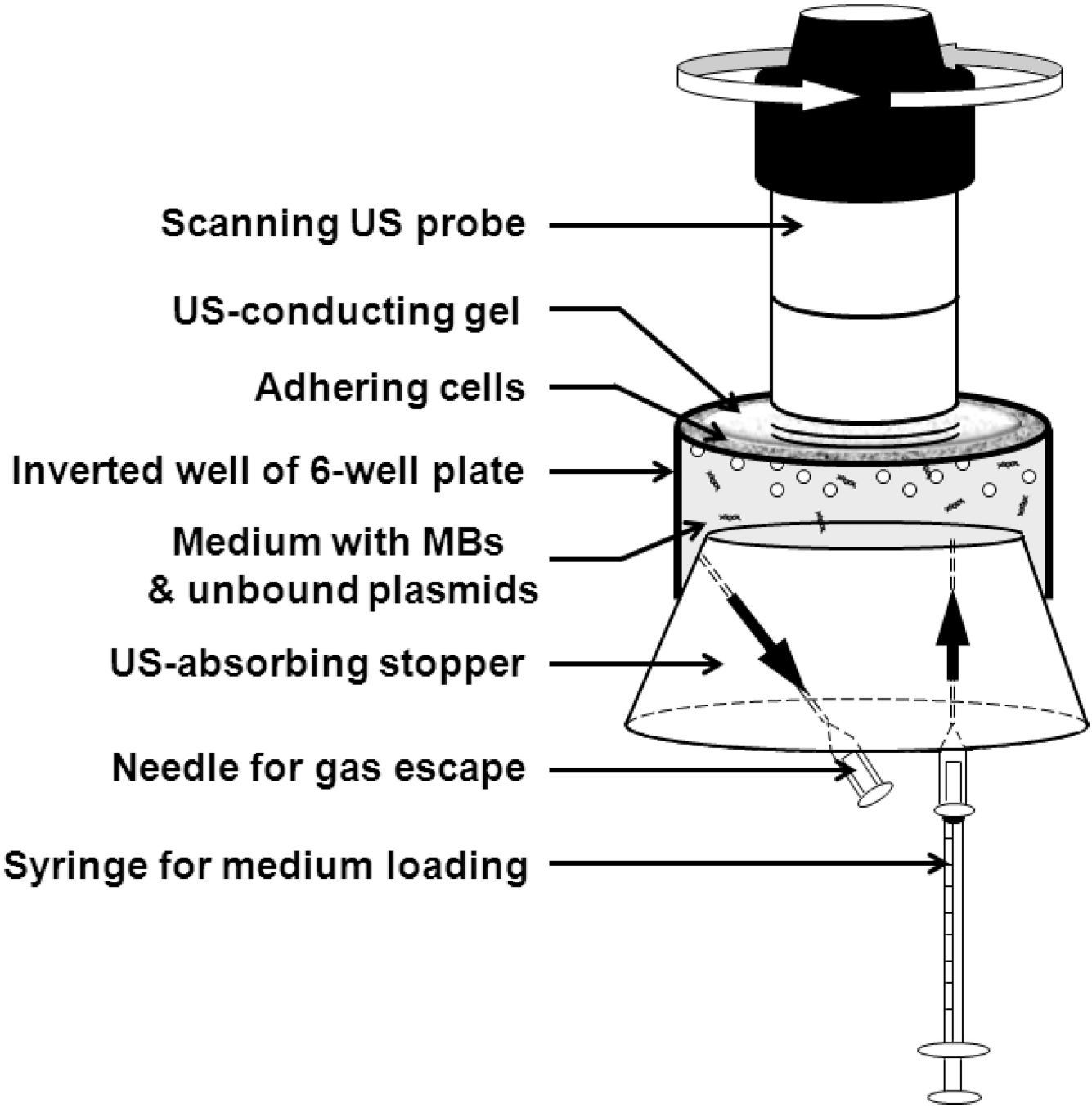


Figure 1. In vitro setup for ultrasound (US)-mediated gene transfer studies

Six-well plates containing adherent 293T cells were inverted to allow direct interaction between cells and MBs. An US-absorbing silicone stopper was placed to create a sealed environment, as well as to absorb US waves from a scanning US probe above the plate. Two needles were placed in the stopper for loading of DNA/MB-containing medium and subsequent air escape.

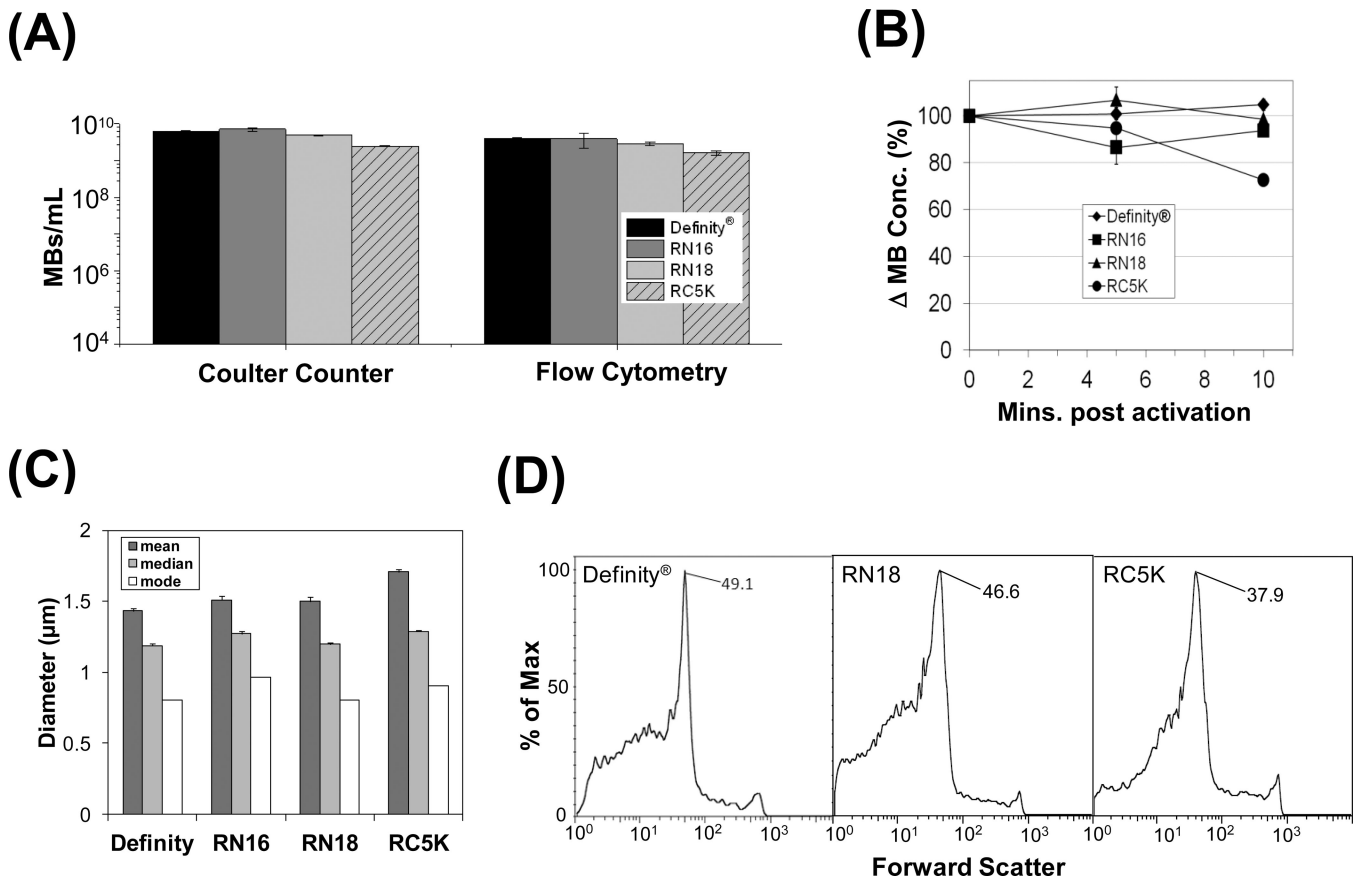


Figure 2. Characterization of MBs using Coulter Counter and Flow Cytometry

(A) MB concentration was measured on Coulter Counter and flow cytometry. All types of MB preparations resulted in similar concentrations: have the same order of magnitude of about 10^9 MB/mL, as measured on both instruments. **(B)** Stability of MB concentrations post-activation. Relative MB concentration was measured on Coulter Counter 0, 5, and 10 minutes post-activation. These were normalized to the initial MB concentration (0 minute). Neutral MBs had similarly sustained MB concentration, while RC5K showed less stability. (C) Size profiles of various MBs evaluated by Coulter Counter III. (D) Size distributions of various MBs. Size histograms obtained from flow cytometry revealed that the peak MB diameters are similar for three MBs. Error bars indicate STD (n=3–5).

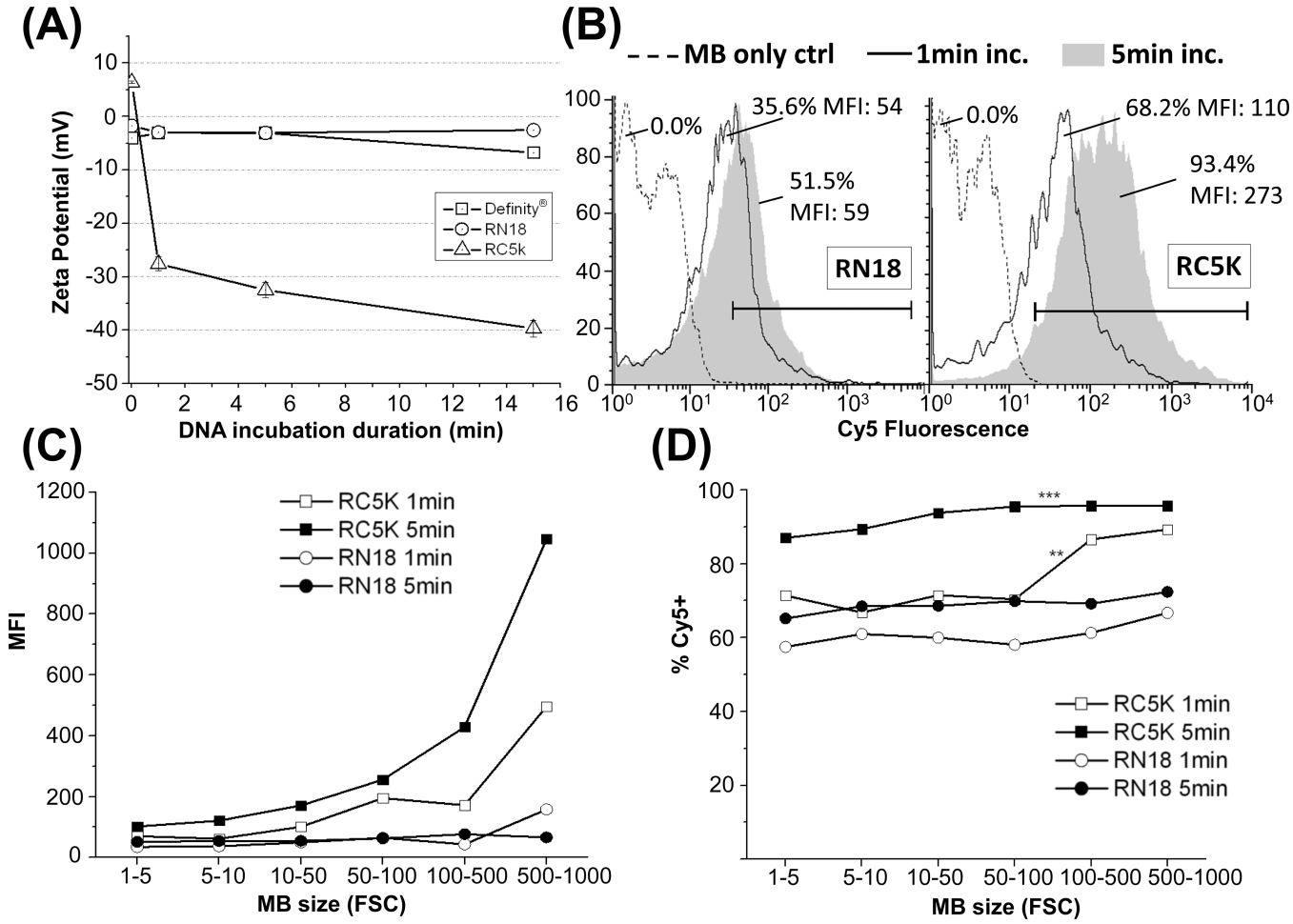
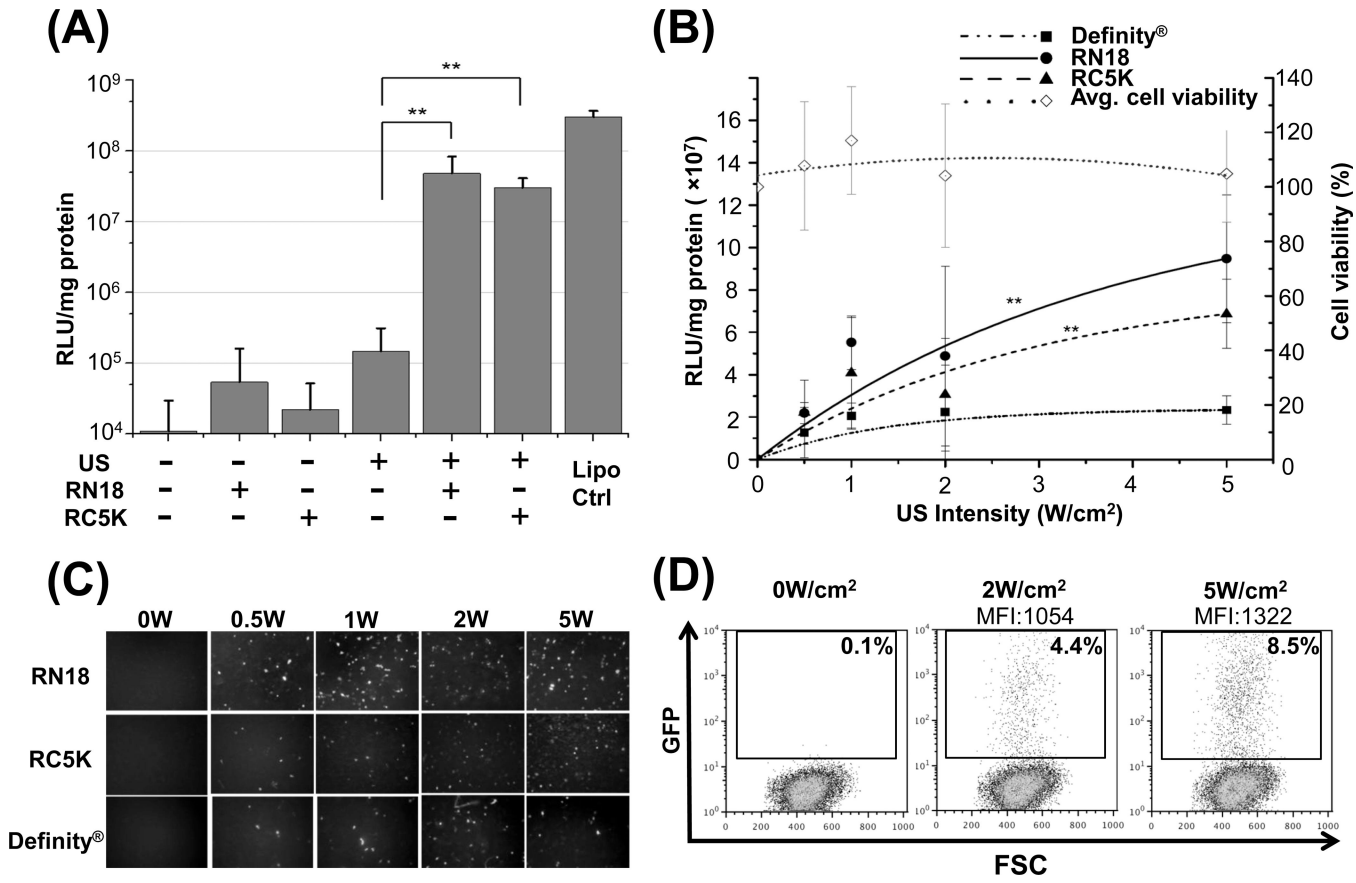


Figure 3. DNA loading on MBs after incubation

(A) MB surface charge is measured using Zetasizer. Baseline zeta potential for Definity, RN18, and RC5k MBs were −4.04 mV, −1.77 mV, and +6.39 mV, respectively. Neutral MBs Definity and RN18 displayed little change in surface charge after DNA incubation (-2.72 ΔmV, and −0.72 ΔmV, respectively), while the cationic RC5k showed a large decrease in surface charge (-46.08 ΔmV) as the cationic lipid shell binds negatively-charged DNA. **(B)** After incubation with Cy5™ labeled DNA, Cy5™-positive MBs are detected in flow cytometry. RC5k showed increased DNAbinding capacities with longer incubation periods as indicated by higher proportion (93.4% vs. 51.5%) and MFI (273 vs. 59) of Cy5™-positive MBs compared to RN18. (**C)** Post-binding MFI of cationic and neutral MBs was categorized by MB size. This shows that bigger cationic MBs have higher MFIs with longer incubation time; whereas, MFIs of neutral MBs remained the same. **(D)** Post-binding Cy5™ –positive proportions of cationic and neutral MBs as separated by MB size. The proportion of Cy5™ positive population post-DNA incubation was greater in cationic MBs (>85%) but remained the same for neutral MBs (~65%). Error bars indicate STD (n=3). MFI, mean fluorescent intensity.



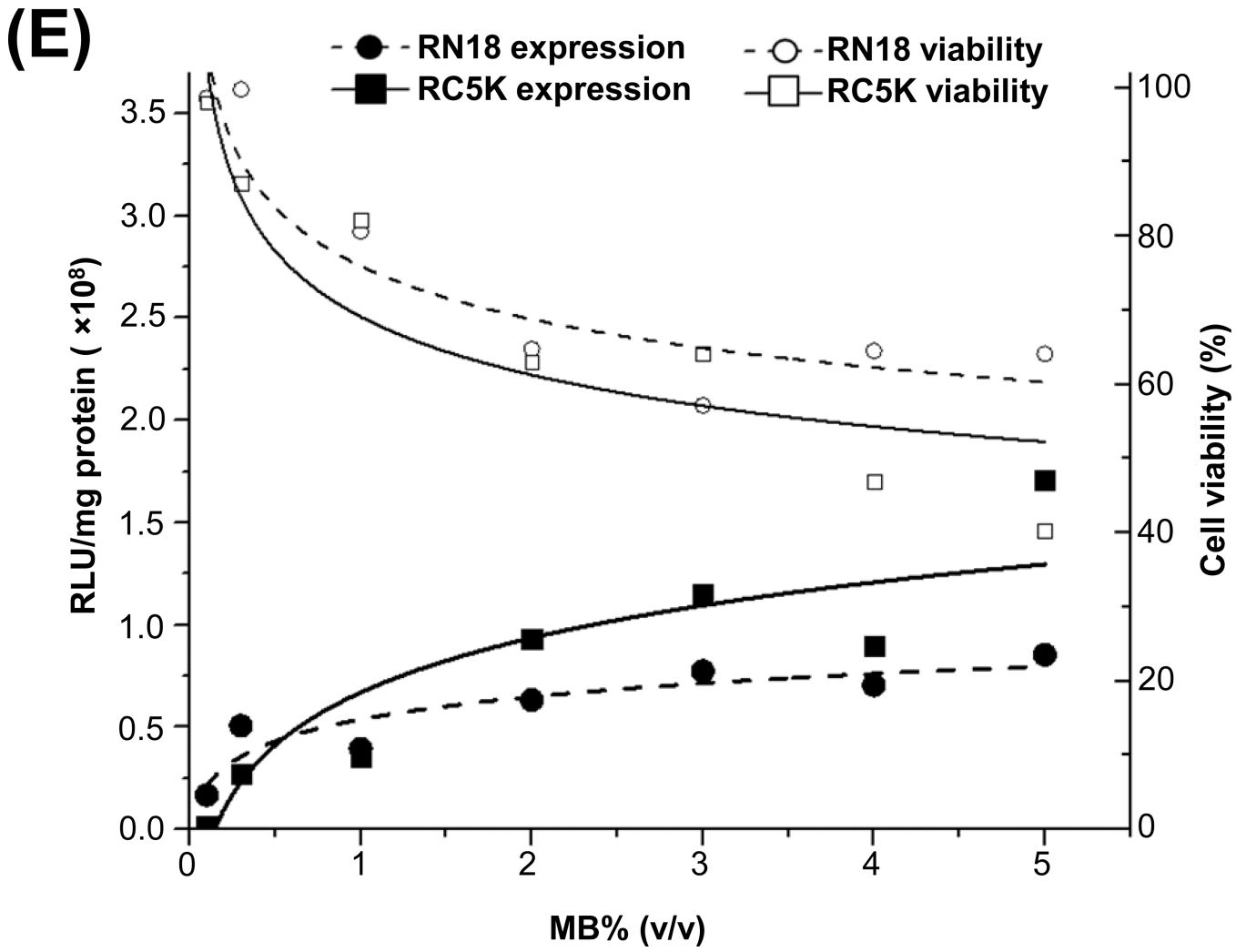


Figure 4. Changing transfection parameters improves in vitro MB/US transfection of 293T cells

(A) The addition of RN18 and RC5k MBs significantly enhanced luciferase expression when 293T cells are exposed to 2 W/cm^2 with or without MBs (1 MHz, 20% duty cycle, 100 Hz PRF) for 3 minutes. Expression is similar to effects of Lipofectamine control. **(B)** As US intensity increased from 0 to 5 W/cm^2, luciferase expression was further enhanced while maintaining cell viability. Results indicate that RN18 and RC5k MBs were more effective in gene transfection than Definity as US intensity increased. **(C)** Representative images from fluorescence microscopy of 293T cells transfected with pGFP and exposed to increasing US intensity in the presence of with different MBs. Higher US intensities and using RN18 and RC5k MBs yielded higher GFP expression, as indicated visually. **(D)** Quantitative measurement by flow cytometry showed a 1.9-fold enhancement in GFP transfection efficiency when cells were transfected at higher US power (5 W/cm^2) compared to lower US power (2 W/cm^2). **(E)** Increasing the MBs dosage up to 5% enhanced luciferase expression; but at the expense of cell viability down to ~40% and ~60% using RC5k and RN18, respectively. Error bars indicate STD (n=2–15). ** p<0.01. RLU, relative light unit. MFI, mean fluorescent intensity.

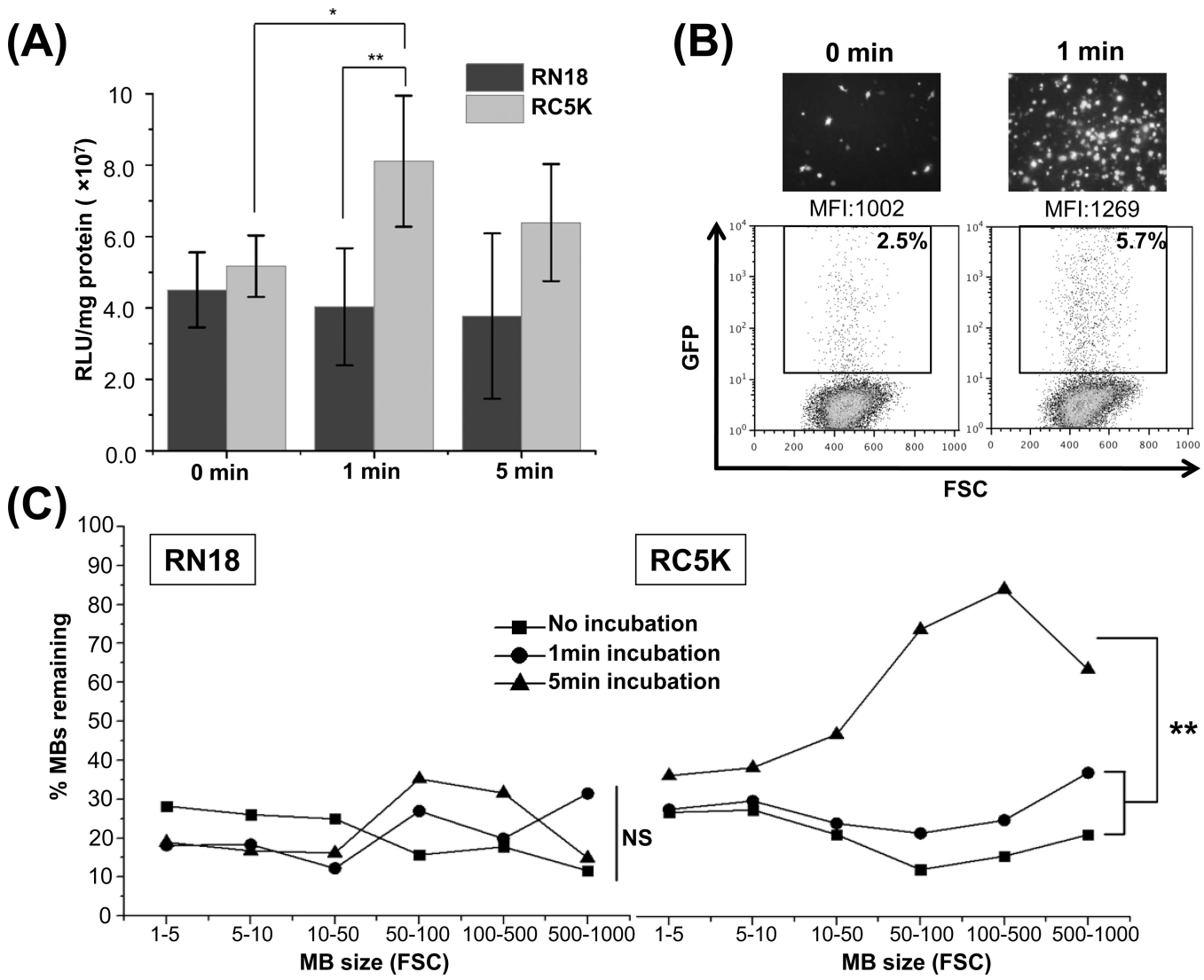


Figure 5. Effect of pre-incubation with DNA on MB application

(A) 293T cells were transfected with pre-incubated RN18 with pGL4, and exposed to US (1 MHz, 2 W/cm^2, 20% duty cycle, 100 Hz PRF). Cells showed no improvement in transgene expression regardless of incubation duration, while those transfected using RC5K showed significant enhancement with 1 minute of DNA incubation compared to cells treated without incubation and cells treated with RN18. **(B)** Flow cytometry analysis of pCMV-GFP-transfected cells using RC5k with prior DNA incubation showed that gene expression was enhanced by 2.3-fold compared to without incubation. **(C)** MB cavitation efficiency was determined after exposure to 10 seconds of 2 W/cm^2 US, separated by MB size. The percentage of MBs remaining after US stimulation stayed the same for RN18 across all MB sizes regardless of DNA incubation duration, while a significant percentage remained for RC5K MBs that were incubated with DNA for 5 minutes, showing a diminished response to US. Error bars indicate STD (n=3–4). * $p<0.05$. ** $p=0.01$.

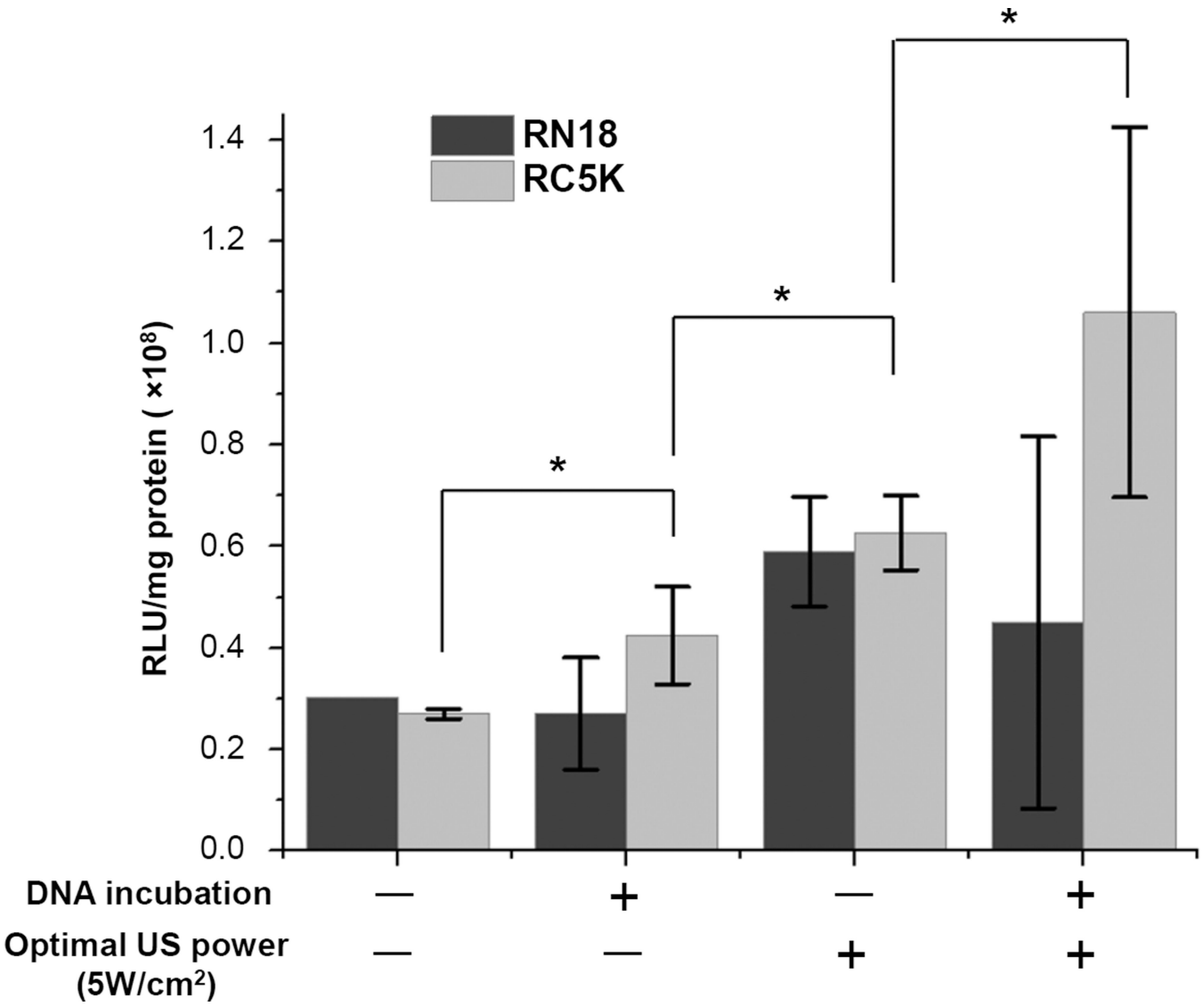


Figure 6. Additive effect of DNA incubation and optimal US

A significant step-wise enhancement was observed for transfection with RC5K when pre-incubation with DNA, optimal US intensity (5 W/cm^2, as opposed to 2 W/ cm^2) were applied alone. When both parameters were applied together, transfection efficiency showed additive enhancement for RC5K, reaching an average of 1.3×10^8 RLU/mg, which was 4.7-fold of initial RC5K efficiency. This effect was not seen in neutral RN18, which only showed improved efficiency with optimal US, consistent with the finding that pre-treatment DNA incubation with neutral MBs does not enhance MB-DNA binding and thus has no additional effective for gene delivery. Error bars indicate STD (n=2–5). * $p<0.05$.

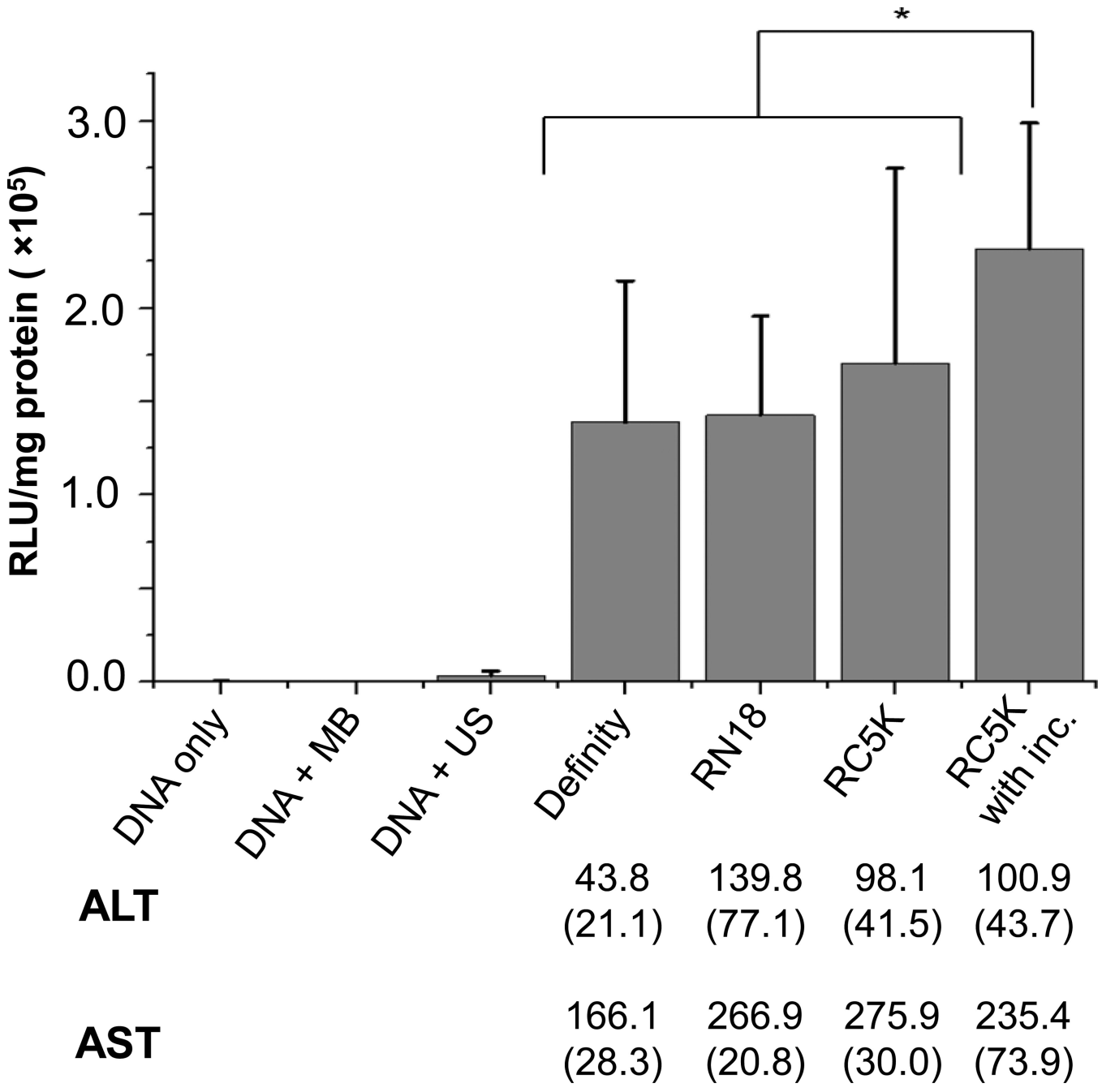


Figure 7. Luciferase gene expression following US/MB-mediated gene delivery into rat liver

A solution of pGL4 plasmid, MBs, and glucose was injected slowly (45 seconds) into a portal vein branch using a 24-ga catheter, with simultaneous exposure to US (1.1 MHz, 20 cycle pulses, 13.9 Hz PRF, and 2.7 MPa PNP) for 90 seconds. Blood samples and treated lobes were harvested after 24 hours. Average luciferase expression for each group is shown, and error bars indicate STD (n=4–6). * $p<0.05$.

Table 1

Molar Percent of Lipids in Each Microbubble Formulation

Microbubble	Surface	Lipid shell (mol %)							Total lipid/vial (mg)
		DPPC / DSPC		DPPA / DSPA		MPEG5000 −DPPE / −DSPE		DSTAP	
Definity®	Neutral	82	-	10	-	8	-	-	1.3
RN16	Neutral	78	-	10	-	12	-	-	1.3
RN18	Neutral	-	82	-	10	-	8	-	1.2
RC5k	Cationic	-	74	-	-	-	5	21	1.3

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