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► To cite this version:

Julien Piron, Jean-Michel Escoffre, Kadija Kaddur, Anthony Novell, Ayache Bouakaz. Enhanced gene transfection using ultrasound and Vevo Micromarker[®] microbubbles. 2010 IEEE International Ultrasonics Symposium, Oct 2010, San Diego, United States. <10.1109/ULTSYM.2010.5935896>. <hal-04280337>

HAL Id: hal-04280337

<https://hal.science/hal-04280337v1>

Submitted on 10 Nov 2023

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Enhanced gene transfection using ultrasound and Vevo Micromarker[®] microbubbles

Microbubbles-assisted ultrasound and gene delivery

Julien PIRON, Jean-Michel ESCOFFRE, Kadija KADDUR, Anthony NOVELL, Ayache BOUAKAZ
INSERM U930, CNRS ERL 3106, B1A, CHU Bretonneau, 2 bd Tonnellé, 37044 Tours Cedex 9, France.

Abstract—Contrast agents for ultrasound imaging, composed of tiny gas microbubbles, have become a reality in clinical routine. Recent experimental studies showed that the combination of ultrasound with contrast agent microbubbles increase membrane permeability in a process known as sonoporation. This effect is thought to allow foreign molecules such as therapeutic genes to enter into the cells. The transfection level and efficiency have been shown to depend on the type of microbubbles. In that context, we performed a comparative study using 3 types of microbubbles, i.e. BR14[®], SonoVue[®] and Vevo Micromarker[®] microbubbles. The transfection level and the cell viability of U-87 MG glioblastoma cells were measured. The results showed that the transfection level achieved with Vevo Micromarker[®] microbubbles is higher than BR14[®] and SonoVue[®] microbubbles with a comparable cell viability. The transfection rate obtained with Vevo Micromarker microbubbles reached approximately 70%. These results were correlated with the fact that Vevo Micromarker[®] microbubbles exhibited the lowest attenuation coefficient than BR14[®] and SonoVue[®] microbubbles at the insonation frequency.

Keywords—Sonoporation, Ultrasound, microbubbles, transfection, Vevo Micromarker.

I. INTRODUCTION

The clinical development of gene therapy requires the use of safe and efficient methods that deliver a therapeutic gene to target cells where gene expression can be achieved.

Viral vectors have been preferentially used due to their high gene transfer efficiency and their capacity to induce high-level and long-lasting gene expression in various cells and tissues [1, 2]. The effectiveness of these vectors lies in the infectious properties of viruses that are managed by viral proteins. However, these proteins could induce specific immune responses that would limit the ability to re-administer the viral vector and hinder the efficiency of gene transfer [3, 4]. Moreover, some viral vectors such as retrovirus or lentivirus vectors could evoke insertional mutations during their integration into the host genome [5, 6]. In addition, previous studies have shown that the recombination events could induce the production of replication competent viruses [7].

In contrast, plasmid DNA (pDNA) is just covalent closed circles of double-stranded DNA with no associated proteins. pDNA is simpler, easier to mass-produce and potentially safer than viral vectors [8]. Low immunogenicity and lack of integration of plasmid DNA make it a highly attractive molecule for gene therapy provided that an efficient, safe and targeted delivery can be achieved [8].

Among delivery methods, microbubbles-assisted ultrasound (termed sonoporation) is a promising physical method to transfer therapeutic genes into cells and tissues [9]. This method consists in the rational application of ultrasound waves in presence of gas microbubbles [10-12]. Under the effect of ultrasound waves, the oscillating microbubbles in contact with plasma membrane induce a mechanical constraint on the cell membrane [13, 14]. This membrane stimulation enhances its permeability through likely the creation of membrane transient pores and likely the stimulation of endocytosis mechanisms [15]. The level and the efficiency of gene transfer have been shown to depend on ultrasound parameters and the type of microbubbles. The aim of this study is to evaluate the efficiency of Vevo Micromarker[®] for gene delivery and to compare it with BR14[®] and SonoVue[®] microbubbles (Bracco Research, Geneva, Switzerland).

II. MATERIAL AND METHODS

A. Cell culture and plasmid

Human glioblastoma astrocytoma cells (U-87 MG) were derived from a malignant glioma. Cells were maintained as monolayers in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum at 37°C, in humidified atmosphere in presence of 5% CO₂.

A 4.7 kilo-base pairs plasmid, pmaxFP-Green-C (pEGFP), carrying the gene of the Green Fluorescent Protein under the control of the cytomegalovirus promoter was used for gene transfer assay.

B. Gene transfer by microbubbles-assisted ultrasound

After washing with Opti-MEM supplemented with 1% FCS, cells were suspended at 500 000 cells per 1.5 mL of this medium. One sample containing 1.5 mL of cells was

introduced into plastic cuvette and maintained at 37°C. Before insonation, microbubbles (i.e. BR14[®], SonoVue[®] and Vevo Micromarker[®]) and pEGFP were added in the sample to a concentration of 5 microbubbles per cell and 5 µg/mL, respectively. Cells were insonated at 1 MHz, with 40% duty cycle during 30s at an applied acoustic pressure of 0.6 MPa. After insonation, cells were cultivated in 24-wells plate and incubated at 37°C, in humidified atmosphere in presence of 5% CO₂. Four hours later, an equal volume of OptiMEM-FCS 10% medium was added to each well.

C. Attenuation measurements

The attenuation coefficient of the three microbubble types were performed before and during ultrasound insonation. Echoes from a perfect reflector were measured using a 2.25 MHz single-element transducer. A wideband excitation pulse (half a sine wave) was transmitted to estimate the contrast agent attenuation in a broad frequency band. The 1 MHz sonoporation transducer was used with insonation parameters (1 MHz, 40% duty cycle, 0.6 MPa) and placed perpendicularly to the first transducer. Attenuation measurements were carried out by positioning a cuvette containing the diluted solution of the microbubbles between the transducer and the perfect reflector.

D. Flow cytometry

Twenty-four hours and forty-eight hours after sonoporation, transfection level (i.e. percentage of eGFP-positives cells) and efficiency (i.e. associated fluorescence intensity) were determined by flow cytometry.

Cell death was determined by propidium iodide staining. This dye was added at 0.5 µg/mL in cell suspension before cytometry measurement.

III. RESULTS

A. Vevo Micromarkers microbubbles

We realized a comparative study of the different microbubbles, i.e. BR14[®], SonoVue[®] and Vevo MicroMarker[®] for gene delivery by ultrasound on U-87 MG cells. The transfection level and the cell death were determined 24h and 48h after insonation.

All the microbubble types induced a successful transfection with pEGFP, using ultrasound at 0.6 MPa, 40% duty cycle for an exposure time of 30 seconds.

Twenty-four hours after insonation, similar transfection level was obtained using SonoVue[®] and BR14[®] microbubbles, i.e. $32 \pm 2\%$ and $33 \pm 2\%$, respectively (Fig. 1). However, the percentage of dead cells was almost twice higher with SonoVue[®] microbubbles compared to BR14[®] microbubbles (i.e. $24 \pm 3\%$ versus $13 \pm 3\%$, respectively) (Fig. 2). The highest transfection level was achieved when ultrasound was combined with Vevo Micromarkers[®] microbubbles. Indeed, at

24h, nearly 45% of the cells were transfected while the percentage of cell death was $16 \pm 2\%$.

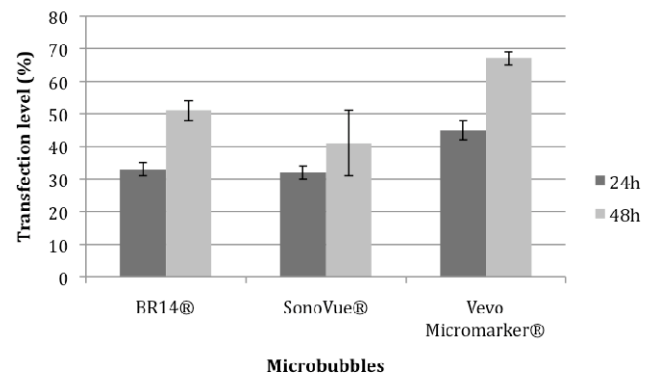


Figure 1. Transfection rate of U-87 MG cells at 24h and 48h after insonation using Sonovue, BR14 and Vevo Micromarker microbubbles (N=5).

All the microbubbles types showed an increased transfection level at 48h whereas the cell death decreased. Indeed, the transfection levels with BR14[®] and SonoVue[®] were $51 \pm 3\%$ and $41 \pm 10\%$, correspondingly. However Vevo Micromarker[®] induced the highest transfection level, i.e. $67 \pm 2\%$ with a weak cell death ($11 \pm 1\%$) (Figs. 1 and 2).

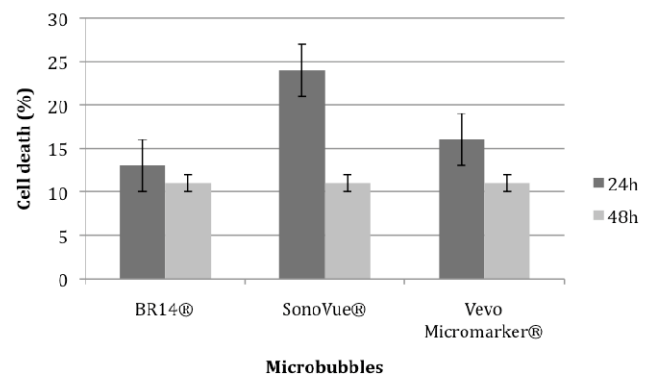


Figure 2. Cell death as induced by ultrasound and microbubbles at 24h and 48h after insonation (N=5).

B. Attenuation measurements

In order to give insight into the differences in transfection levels of the three microbubbles types, attenuation of the three contrast agents were measured before and immediately after sonoporation.

Figure 3 shows the attenuation of the three contrast agents at T0 (solid) and 30 seconds later (dashed). The curves show a negligible change in the attenuation during at least 30 seconds. Moreover, the attenuation is higher for SonoVue[®] microbubbles than the 2 other contrast agents. The attenuation was also measured after applying sonoporation during 30 seconds (dotted). The results show that Vevo Micromarker[®] microbubbles were destroyed almost instantaneously, whereas,

BR14[®] and SonoVue[®] microbubbles were destroyed gradually over time. This finding indicates that the destruction of the microbubbles might be directly correlated to the induced transfection level. Indeed, Vevo Micromarker[®] microbubbles which exhibit the lowest attenuation coefficient (at resonance and at 1 MHz frequency) are also the most effective for gene delivery. However, SonoVue[®] microbubbles which exhibit the highest attenuation are the less effective for gene transfer. This result might suggest that microbubbles destruction plays a role in the transfection efficiency.

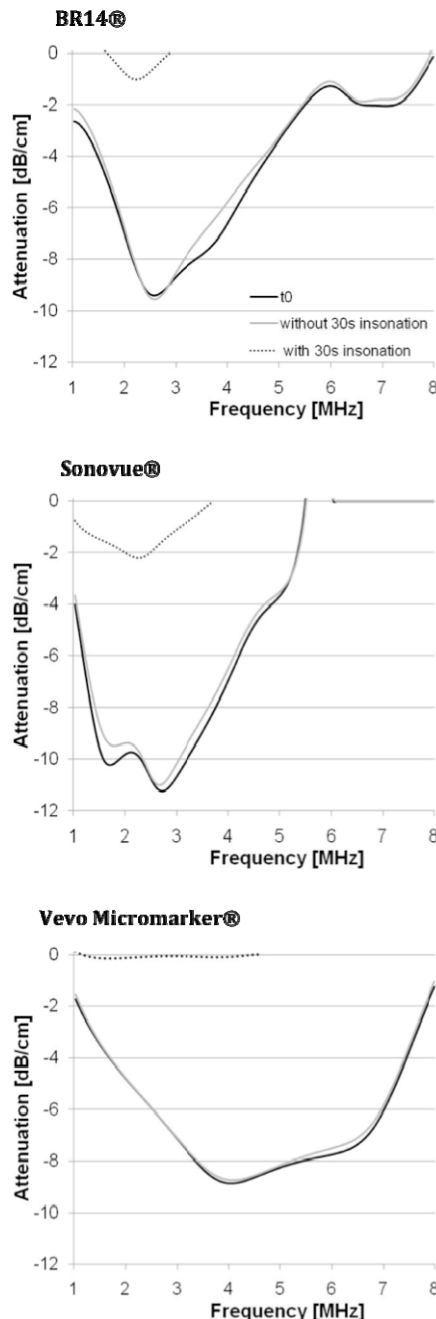


Figure 3. Attenuation of the microbubbles before sonoporation at T0 (solid) and 30 seconds later (dashed). In dotted line is given the attenuation after applying sonoporation during 30 seconds.

IV. CONCLUSIONS

In this present study, we reported the direct evidence that the combination of ultrasound and Vevo Micromarker[®] induce a high level of transfection rate with a low cell death. Moreover, the findings indicate that the attenuation and the destruction of contrast agent might be involved in the sonoporation efficiency.

ACKNOWLEDGMENT

The authors thank Bracco Research for Vevo Micromarker[®], BR14[®] and SonoVue[®] microbubbles' samples generously provided. Project funded in part by the EU Project SONODRUGS (NMP4-LA-2008-213706).

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