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Cationic microbubbles and antibiotic-free miniplasmid for sustained ultrasound–mediated transgene expression in liver

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ABSTRACT

Despite the increasing number of clinical trials in gene therapy, no ideal methods still allow non-viral gene transfer in deep tissues such as the liver. We were interested in ultrasound (US)-mediated gene delivery to provide long term liver expression. For this purpose, new positively charged microbubbles were designed and complexed with pFAR4, a highly efficient small length miniplasmid DNA devoid of antibiotic resistance sequence. Sonoporation parameters, such as insonation time, acoustic pressure and duration of plasmid injection were controlled under ultrasound imaging guidance. The optimization of these various parameters was performed by bioluminescence optical imaging of luciferase reporter gene expression in the liver. Mice were injected with 50 μg pFAR4-LUC either alone, or complexed with positively charged microbubbles, or co-injected with neutral MicroMarker™ microbubbles, followed by low ultrasound energy application to the liver. Injection of the pFAR4 encoding luciferase alone led to a transient transgene expression that lasted only for two days. The significant luciferase signal obtained with neutral microbubbles decreased over 2 days and reached a plateau with a level around 1 log above the signal obtained with pFAR4 alone. With the newly designed positively charged microbubbles, we obtained a much stronger bioluminescence signal which increased over 2 days. The 12-fold difference ($p < 0.05$) between MicroMarker™ and our positively charged microbubbles was maintained over a period of 6 months. Noteworthy, the positively charged microbubbles led to an improvement of 180-fold ($p < 0.001$) as regard to free pDNA using unfocused ultrasound performed at clinically tolerated ultrasound amplitude. Transient liver damage was observed when using the cationic microbubble-pFAR4 complexes and the optimized sonoporation parameters. Immunohistochemistry analyses were performed to determine the nature of cells transfected. The pFAR4 miniplasmid complexed with cationic microbubbles allowed to transfect mostly hepatocytes compared to its co-injection with MicroMarker™ which transfected more preferentially endothelial cells.

1. Introduction

Due to its fundamental role in metabolism, liver has attracted a number of therapeutic strategies, in particular gene therapy [1]. Various technologies have been applied such as viral or non-viral gene delivery associated or not with physical methods, such as hydrodynamic or ultrasound mediated gene delivery [2]. In terms of physical methods, ultrasound (US) advantageously allows to non-invasively

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reach deep tissues, and therefore shows great interest in gene delivery [3,4].

Various studies have reported the efficacy of ultrasound-mediated microbubbles (MB) gene delivery. The majority of the studies were obtained with therapeutic ultrasound operating at frequency between 1 and 3 MHz and various types of MB, made with gaseous liposomes or polymers [5,6]. In this context, with microbubbles constituted of liposomal nanobubbles, the group of Tanyama has described an efficient IL12 gene expression within tumours [5]. Long lasting gene expression (up to 100 days) was obtained following co-injection of plasmid DNA encoding luciferase gene (pDNA-LUC) and MicroMarker™ MB either in tendons [7] or in the muscle [8]. In the liver, two main studies have been published. The first study used the Optison™ ultrasound contrast agent which was co-injected with pDNA directly inside mice liver followed by ultrasound exposure [9]. The second study used a zwitterionic MB (a mix of DL-α-phosphatidylcholine, dipalmitolyl and DL-α-phosphatidylethanolamine, dipalmitolyl) loaded with pDNA injected into the left ventricle of the heart, followed by an ultrasound application in the liver area [10]. Significant expression of reporter genes such as green fluorescent protein, luciferase, beta-galactosidase, or therapeutic genes such as factor IX were observed in those two studies. The co-injection of Definity® MB with pDNA into a rat liver lobe via a specific portal vein branch promoted as well an increased gene transfection using high intensity ultrasound (1.1 MHz frequency, 20 cycles, 13.9 Hz of Pulse Repetition Frequency – PRF-, 2.7 MPa peak negative acoustic pressure) [11].

It is clear that pDNA complexation with MB could be of interest for systemic delivery as it would allow both MB and pDNA to reach the targeted tissue at the same time. In addition, this complexation could reduce plasmid degradation, and thereby improving gene transfer.

Several strategies have been proposed to anchor nucleic acids at a microbubble surface. Some groups have exploited avidin-biotin linkage to couple nucleic acids complexed with cationic vectors (liposomes or polymers) on microbubble surface [12,13]. Various reports have described the use of cationic liposomes-based microbubbles [10,14–19], formed with gas-filled cationic liposomes made with zwitterionic lipid (DSPC) or cationic lipids such as DSTP or 1,2-stearoyl-3-trimethylammonium-propane (DPTAP). The main challenge is to obtain cationic MB promoting pDNA complexation without any alteration of their acoustic properties.

In addition to the improvement of physical gene delivery techniques, a particular attention was paid on plasmid design in the present study, which was identified to be of crucial importance. Indeed, in liver, non-viral gene vectors mostly display much more transient gene expression than that observed in other organs such as muscle, partly due to heterochromatin formation [20,21]. Removal of all or part of bacterial sequences from gene vectors (e.g. in minicircles or in pFAR4 miniplasmids, respectively) allowed to alleviate this silencing effect [22,23]. Alternatively, the use of molecular tools (DNA transposons or integrases) that mediate transgene integration into host genome led, in mice, to high and prolonged therapeutic levels of Factor IX that is deficient in hemophilia B patients. DNA transposon was also used to obtain gene insertion and long-term expression in combination with ultrasound using a low frequency unfocused transducer [11]. The use of unfocused transducer is of importance because it allows to treat a larger area, as compared to focused ultrasound, and is thus more appropriate for a large organ such as the liver.

Taking into account all these data, we ought to design positively charged MB to increase plasmid amount reaching the liver. Based on our former screening of positively charged lipids [24], we chose a highly charged bicatenar lipid with a branched polyspermine head [25]. We designed MB composed of a unique cationic lipid, providing a simple and reliable vector [26] able to interact with high efficiency with DNA. The other originality of our study comes from the pDNA used. We chose a miniplasmid devoid of antibiotic resistance sequence, called pFAR4 [27]. The absence of this sequence leads to a small size plasmid which was shown to be highly efficient in transfecting cells *in vitro* [27] and *in vivo* [23]. Finally, the parameters of low-energy unfocused ultrasound were also optimized using image-guided delivery. In this article, we report that the combination of plasmid molecular optimization and US non-invasive physical trigger led to a stable gene expression in the liver over 6 months, with a mild and transient apparent toxicity.

2. Material and methods

2.1. Plasmid preparation

The pFAR4 derivative, pFAR4-CMV LUC BGH (pFAR4-LUC), which is a plasmid devoid of antibiotic resistance gene, was propagated using a dedicated bacterial producing strain [27]. The plasmid was purified using Nucleobond Endofree plasmid preparation kits (Macherey Nagel, Hoerdt, France). Endotoxin levels were validated using the LAL (Limulus Amoebocyte Lysate) procedure (Lonza, Verviers, Belgium).

pTG11, full name pTG11033 with 9514 bp was obtained from Transgene S. A. (Strasbourg, France). It encodes the luciferase gene under the cytomegalovirus promoter. pTG11 was propagated from *Escherichia coli* DH5α cultures. Supercoiled plasmid was isolated from bacteria with the standard alkaline lysis method, and purification was carried out with the Qiagen Mega Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions.

2.2. Ultrasound exposure system for in vitro gene transfer

The ultrasound system used in this study was previously described [28]. US were generated from a 0.5-in diameter, IBMF-014 transducer with a centre frequency of 1 MHz (Sofranel, Sartrouville, France). The transducer was placed in a custom made polystyrene tank filled with degassed pure water. A signal consisting of 1.0 MHz centre frequency, 40% duty cycle and a pulse repetition frequency of 10 kHz was generated by a 33220A arbitrary function generator (Agilent technologies, Les Ulis, France) and amplified by a RF power amplifier (ADECE, Artannes sur Indre, France) was used as the input for the transducer. Peak negative pressure of 150 kPa was used corresponding to an MI of 0.15. Ultrasound stimulation time was set to 60 s. The transducer was calibrated in a Perspex container using an HGL-200 PVDF bullet type hydrophone (Onda, Sunnyvale, CA) placed at 3 cm, the natural focal distance of the transducer. The transducer was positioned in front of the sonoporation cuvette (Sarstedt AG & Co, Nümbrecht, Germany). The attenuation of the cuvette walls was measured separately and found to be negligible (< 10%).

2.3. Microbubble formulations

The cationic lipid, DMAPAP, synthesized as previously described [25], was diluted in 5% sucrose and placed in 2 mL vials containing 1 mL of lipid suspension. The vials were capped and air was displaced by perfluorobutane gas (Matrix Scientific, Columbia, SC, USA) at atmospheric pressure. MB were formed by vigorous mechanical agitation using a Vialmix system (DuPont Pharmaceuticals Company, Billerica, MA, USA) for 45 s. PEGylated MBs were obtained post agitation by addition of 20% of home-made N-hydroxysuccinimide polyethylene glycol (PEG) – 2000 at pH 7.5. All samples were left for 10 min before use and were used within 1 h following agitation. Prior to pDNA interaction, approximatively 200 μL of MB were collected into 1 mL syringe with a 27 g needle. The syringe was kept in vertical position with the plunger up for 2 min in order to separate MB by buoyancy. The largest MB formed a cake against the syringe plunger and the rest of the suspension containing monodisperse microbubbles were collected by pushing the plunger. Then pFAR4-LUC (50 μg) was added dropwise to interact with MB prior to intravenous (IV) injection.

Cationic MB formulation with DOTAP lipid was also prepared as