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Ultrasound-mediated gene delivery of naked plasmid DNA in skeletal muscles: A case for bolus injections

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ABSTRACT

Localized gene delivery has many potential clinical applications. However, the nucleic acids (e.g. pDNA and siRNA) are incapable of passively crossing the endothelium, cell membranes and other biological barriers which must be crossed to reach their intracellular targets. A possible solution is the use of ultrasound to burst circulating microbubbles inducing transient permeabilization of surrounding tissues which mediates nucleic acid extravasation and cellular uptake. In this study we report on an optimization of the ultrasound gene delivery technique. Naked pDNA (200 μg) encoding luciferase and SonoVue® microbubbles were co-injected intravenously in mice. The hindlimb skeletal muscles were exposed to ultrasound from a non-focused transducer (1 MHz, 1.25 MPa, PRI 30 s) and injection protocols and total amounts as well as ultrasound parameters were systemically varied. Gene expression was quantified relative to a control using a bioluminescence camera system at day 7 after sonication. Bioluminescence ratios in sonicated/control muscles of up to 101× were obtained. In conclusion, we were able to specifically deliver genetic material to the selected skeletal muscles and overall, the use of bolus injections and high microbubble numbers resulted in increased gene expression reflected by stronger bioluminescence signals. Based on our data, bolus injections seem to be required in order to achieve transient highly concentrated levels of nucleic acids and microbubbles at the tissue of interest which upon ultrasound exposure should lead to increased levels of gene delivery. Thus, ultrasound mediated gene delivery is a promising technique for the clinical translation of localized drug delivery.

1. Introduction

Gene therapy has an immense therapeutic potential for treating diseases caused by a genetic disorder as it can effectively compensate for a gene mutation rather than treating the disease on a symptomatic level. The large effort devoted to gene therapy has resulted in more than 1800 clinical trials to date on gene therapy, which have been completed, are ongoing or have been approved [1]. The treatments are mostly utilizing an engineered gene vector that must be delivered into specific cells/tissues either via viral or non-viral routes. Viral vectors that naturally reach a passive high transduction and transgene expression levels are therefore most commonly used in clinical trials (66.8%) [1]. However, safety concerns (e.g. insertional mutagenesis) and their limited capacity to accommodate a larger DNA load have inspired the development and use of nonviral vectors (25.6%) mostly using naked plasmid DNA (pDNA; 18.3%). Although these vectors are safer, naked plasmid-based gene therapy faces an enormous delivery challenge. Plasmid DNA is a large hydrophilic and charged molecule that is not able to extravasate after intravenous injection into the interstitial space, nor does it passively cross cell membranes for eventual nuclear uptake. Furthermore, pDNA can be rapidly degraded *in vivo* by systemic and cellular nucleases. One approach to address these

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shortcomings is the use of delivery vehicles, such as cationic lipids and/or polymers to help protect pDNA in circulation and facilitate the crossing of biological membranes. Although considerable protection is obtained, toxicity and unspecific delivery concerns are not yet addressed [2].

A physical approach such as ultrasound-mediated gene delivery (UMGD), is a potential solution to this problem [3]. This technique employs non-invasive ultrasound (US) waves and intravenously administered microbubbles (MBs) that are currently also used as US contrast agents. These MBs are gas spheres with diameters typically in the range of 1–10 μm that are stabilized with a lipid layer to prevent rapid dissolution in the blood. Upon insonation these circulating MBs are driven to a forced oscillation state led by compression and decompression cycles of their gas core. Depending on the US intensity, the MBs can either simply vibrate and/or collapse. The ensuing mechanical forces, together with the US pressure waves themselves, induce pores in the surrounding endothelium which promotes extravasation of macromolecules that would otherwise remain intravascular. The number of pores and thus the induced overall permeability is expected to increase with the number of MBs [4–6]. Permeability is temporary as the pores close over time leaving a time window for molecules to extravasate that scales inversely with molecule size [7]. Furthermore, oscillating MBs and mechanical forces can induce a transient permeability of cellular membranes, termed sonoporation, which allows cellular uptake of charged molecules like pDNA (Fig. 1). Some authors have used MBs as delivery agents themselves by chemical modifications namely: (multilayer) (antibody targeted) cationic MBs that bind pDNA on the surface; incorporation of pDNA in the MBs; and pDNA carrying nanoparticles linked to MBs [2]. Independently from the route of administration (direct or intravenous injections), several preclinical studies have shown the feasibility of the US–MB technique in a multitude of organs including skeletal muscle [8–15], liver [16,17], heart [18,19], pancreas [20], kidney [21,22], brain [23], tendon [24,25], and tumor [26–29]. In particular, the works of Fujii et al. [19] in improving myocardial perfusion, Sheyn et al. [30] in inducing ectopic bone growth, and Vu et al. [12] in showing improved glucose homeostasis by targeting the skeletal muscle clearly demonstrated the therapeutic potential of UMGD. Thus, it is possible to systemically co-inject MBs and pDNA and reach local transfection only in the areas subjected to US without any further need for transfection and/or binding agents.

A key factor in this approach is that higher concentrations of pDNA at the site of sonication translates into higher transfection efficiency [31]. Many publications have used direct injections (e.g. intramuscular) to tackle this challenge but its localized nature relative to injection site and invasiveness is problematic [8]. In this study, we explore a simpler approach to locally achieve higher amounts of pDNA encoding a luciferase reporter gene by co-injecting MBs and naked pDNA intravenously in concentrated bolus volumes. A set of UMGD optimization experiments using bioluminescence imaging to quantify the relative expression of luciferase in sonicated areas was performed to explore the most relevant experimental parameters, such as injection type (infusion vs. bolus vs. multiple-boli), MB concentration, and US parameters (e.g. number of cycles).

2. Materials and methods

2.1. Materials

All reagents and solvents were obtained from Sigma-Aldrich and used without further purification, unless otherwise stated. The plasmid DNA purification kits were obtained from Qiagen (Hilden, Germany).

2.2. Animal studies

All experiments performed in the study were in accordance with the German Law on the Care and Use of Laboratory Animals and approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (LANUV). Female CD-1 mice (25–36 g body weight; Charles River Laboratories, Sulzfeld, Germany) were housed in an enriched environment under standard conditions (23–25 °C, 50–60% humidity, and 12 h light–dark cycles) for at least 1 week before the experiment, with food and water given ad libitum.

2.3. Experimental protocol

Throughout the text the terms 'bolus' and 'infusion' are defined as follows: 'bolus' refers to a rapid manual injection of a solution volume directly into the bloodstream (injection rate approx. 10 μL/s); and 'infusion' is a slow injection (injection rate ≤ 10 μL/min) of a solution volume, controlled by a syringe pump, directly into the bloodstream.

The experimental protocol consisted of five main steps described in Fig. 2.

2.3.1. Animal preparation

The mice were anesthetized with isoflurane and their hindlimbs were shaved and then depilated with commercial hair removal cream (Veet®, Reckitt Benckiser, Slough, UK). A 26G permanent venous catheter (BD Vasculon® Plus, Becton Dickinson GmbH, Heidelberg) was inserted in the tail vein. The mice were then placed in prone position on a heating plate to maintain a 37 °C body temperature, with an acoustic absorber (Aptflex F28, Precision Acoustics, Dorset, UK) underneath to minimize US reflections back into the animal. Degassed US transmission gel (Aquasonics 100, Parker Laboratories) was applied between the absorber and the limbs and between the limbs and the US transducer to create an acoustic path from the transducer to the target region.

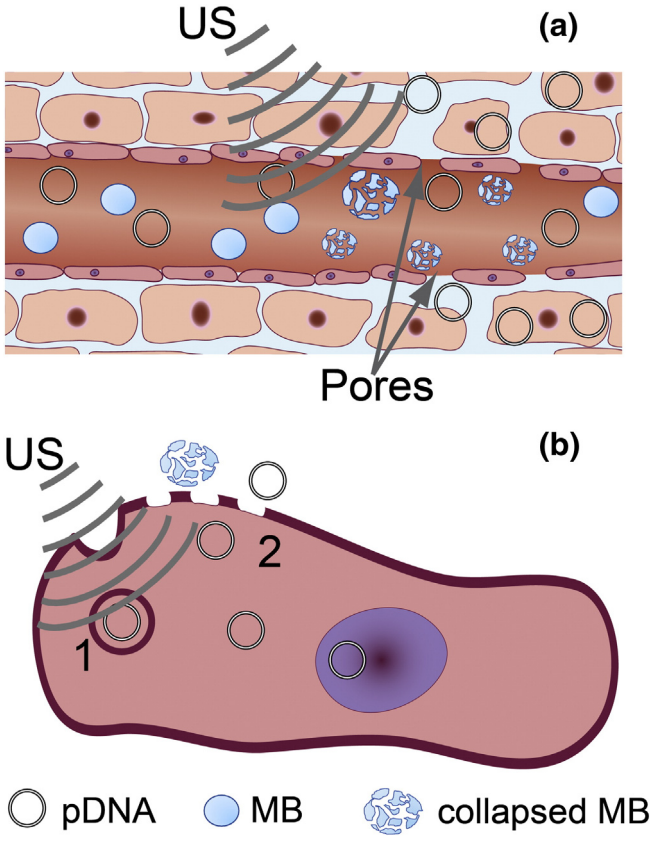

Fig. 1. (a) Ultrasound induced permeabilization of the endothelium and (b) sonoporation of a cell membrane mediating extravasation of pDNA with subsequent intracellular uptake. Two potential mechanisms involved are depicted: (1) endocytosis and (2) temporary pore formation by sonoporation. The symbols are not to scale.

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