2022

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Original Publication Citation

Boye, C., Arpag, S., Francis, M., DeClemente, S., West, A., Heller, R., & Bulysheva, A. (2022). Reduction of plasmid vector backbone length enhances reporter gene expression. *Bioelectrochemistry*, 144, 1-8, Article 107981. https://doi.org/10.1016/j.bioelechem.2021.107981

Bioelectrochemistry 144 (2022) 107981

Contents lists available at ScienceDirect

Bioelectrochemistry

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

Reduction of plasmid vector backbone length enhances reporter gene expression

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ARTICLE INFO

Article history:
Received 31 January 2021
Received in revised form 18 September 2021
Accepted 7 October 2021
Available online 29 October 2021

Keywords:
Plasmid DNA
Gene expression
Gene therapy
Gene electrotransfer

ABSTRACT

Gene therapy has a wide range of applications for various types of pathologies. Viral methods of gene delivery provide high levels of gene expression but have various safety concerns. Non-viral methods are largely known to provide lower levels of expression. We aim to address this issue by using plasmid DNA with smaller backbones to increase gene expression levels when delivered using non-viral methods. In this study we compare gene expression levels between two vectors with firefly luciferase encoding gene insert using liposome complexes and gene electrotransfer as delivery methods. A 2-fold reduction in plasmid vector backbone size, disproportionately enhanced gene expression levels more than 10-fold in rat tenocytes *in vitro*, and rat myocardium *in vivo*, while improvements in delivery to the skin were more moderate.

1. Introduction

Gene therapy has a wide variety of applications that have reached the clinical use for some applications [1]. These applications include treating cancer, genetic diseases, cardiovascular disease, neurological disorders, opthalmic and infectious diseases [1]. Current gene therapy clinical trials are largely composed of studies that use viral vectors as a method for gene delivery [1]. While viral vectors as a delivery method are generally regarded as efficient, there are many safety concerns related to using viral vectors, namely insertional mutagenesis [1]. Non-viral gene delivery methods, such as plasmid DNA (pDNA) injection, gene gun, electroporation (also called gene electrotransfer, or GET), and liposome complexes are generally seen as safer, but also less efficient when compared to viral vectors [2]. We aim to improve gene expression when using non-viral delivery methods by using a unique, smaller pDNA backbone that is specifically designed to induce high gene expression.

pDNA consists of two main parts: an expression cassette, which contains the transgene and regulatory sequences, and the bacterial backbone, which is used largely in the production of pDNA, often containing restriction sites and an antibiotic resistance marker [3]. Components of both the expression cassette and the bacterial backbone can affect gene expression [3,4]. Within the expression cassette, regulatory sequences such as the promoter, intron(s), and terminator can affect gene expression [3,4]. The inclusion of the bacterial backbone in pDNA has been seen to negatively affect expression when comparing pDNA to minicircles; however certain components of the bacterial backbone may affect expression more than others [3,4]. The length of the spacer region has been found to be particularly important, as spacer regions over 1000 base pairs (bp) have been found to induce transgene silencing [5]. Lu *et al.* [5] hypothesized that this may be due to the position of the 5′ end of promoter and the 3′ end of terminator regions in relation to gene looping, which has been shown to promote transcription, primarily by reusing RNA polymerase II molecules from the terminator to the promoter region [6]. Hornstein *et al.* later investigated cell entry and found that pDNA cell entry was not related to vector size, and similarly proposed that DNA-protein interaction may instead be the cause of smaller plasmids generally producing enhanced gene expression [7]. Hornstein *et al.* [7] hypothesize that although transcription proteins are more likely to bind to longer DNA, the one-dimensional movement of RNA polymerase is limited by the length of the DNA and may dissociate before reaching the plasmid's expression cassette [8].

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https://doi.org/10.1016/j.bioelechem.2021.107981
1567-5394/

Enhancement of gene expression by using shorter pDNA sequences has been observed both *in vitro* and *in vivo*. Previous studies have shown that decreasing plasmid backbone size enhances transgene expression when using GET both in HeLa cells [7] and in mice [9]. Additionally, minicirles, which do not have a bacterial backbone, typically provide higher expression than conventional pDNA, however scalability and quality control remain problematic [3,10]. These findings served as motivation to investigate potential differences in gene expression between two different commercially available plasmids.

Our *in vivo* experiments use GET as a method of non-viral gene delivery. Generally, the field has explored a wide variety of pulsing conditions to be used in different applications [11]. Work has also been done to optimize pulsing conditions for delivery to specific tissues, including several of our and other researchers' previous work to deliver pDNA to both skin and heart tissues [12–16]. While our pulsing conditions are based primarily on these previous findings, we also test additional parameters within this study. It has been previously established that lower voltages [17] and biphasic pulsing conditions [18,19] can reduce muscle twitching. Additionally, lower voltages can also reduce tissue damage [17]. For these reasons, we also test pulsing conditions that are biphasic, as well as conditions that have lower voltages than what has been previously established to induce transgene expression.

In this study, we compare the gWiz (traditional plasmid) and NTC9385R (Nanoplasmid™) vectors with a reporter gene insert encoding firefly luciferase tagged with the c-myc and DDK tags by analyzing gene expression in rat tenocytes, as well as gene delivery to the skin and ventricular myocardium in a small animal model.

2. Materials and methods

2.1. Plasmids

Plasmids used in this study are gWiz-Luc (Aldevron, Fargo, ND) further tagged with myc and DDK tags (gWiz-Luc-Myc-DDK) and NTC9385R-Luc-Myc-DDK (Nature Technologies Corporation, Lincoln, NE). In this context, luc represents the firefly luciferase reporter gene, and myc and DDK represent the c-myc and DYKDDDDK (or FLAG) tags respectively. These tags are polypeptides that allow for easier detection of cells expressing our transgene in our immunofluorescence analysis. This transgene was inserted into two different vectors: gWiz and NTC9385R. The transgene insert was kept consistent between the two constructs (1.8 kilobases, or kb). The gWiz vector is 5.0 kb and the NTC9385R vector is 1.7 kb. The final overall sizes of the constructs used in this study are 6.8 kb and 3.6 kb respectively. Additionally, the spacer region is 2,678 bp in the gWiz vector and 454 bp in the NTC9385R vector [20]. In addition, gWiz-GFP was used in the *in vitro* transfection, which includes a reporter gene encoding for green fluorescent protein. Plasmid DNA was suspended in sterile saline at 2 mg/mL. Endotoxin levels in both plasmids were <0.1 EU/μg plasmid, confirmed by Aldevron and Nature Technologies. Plasmid DNA maps are shown in Fig. 1.

2.2. Gene electrotransfer in vitro

Primary rat tenocytes were transfected using transfection reagent Trans-IT®-LT1 by Mirus Bio (Madison, WI). Cells were seeded at a density of 5×10^4 cells per well in a 6-well plate and incubated for 24 h. The transfection was then completed according to the manufacturer's protocol, using 7.5 μg TransIT®-LT1 reagent and 1.25 μl respective pDNA per well, keeping the pDNA of equal mass. Three different plasmids were used: gWiz-Luc-Myc-DDK (n = 2), NTC9385R-Luc-Myc-DDK (n = 2), and gWiz-GFP (n = 2). The gWiz-GFP transfection tenocytes were observed under a fluorescent microscope every 24 h to estimate optimal incubation time (typically 24–72 h) for the greatest number of expressing cells. The plate was read and cells were fixed at 72 h.

For the equimolar experiment, B16F10 melanoma cells were transfected using transfection reagent Trans-IT®-LT1 by Mirus Bio (Madison, WI). Cells were seeded at a density of 5×10^4 cells per well in a 6-well plate and incubated for 24 h. To do the equimolar transfection, we calculated the ratio of the molecular weights of the two plasmids as follows:

4.22×10^6g/mol gWiz-Luc-Myc-DDK: 2.21×10^6 g/mol NTC9385R-Luc-Myc-DDK = 1.908 gWiz-Luc-Myc-DDK: NTC9385R-Luc-Myc-DDK

Therefore, 1.25 μl pDNA for the Nanoplasmid™ (n = 3) and 2.39 μl pDNA of traditional plasmid group (n = 3) at 2mg/ml were used per transfection. The plate was read, and cells were fixed at 72 h.

2.3. Animals

Male Sprague Dawley rats initially weighing 250–300 g obtained from Charles River Laboratories (Wilmington, MA) were used for these studies. All animal studies followed an approved Old Dominion University Institutional Animal Care and Use Com-

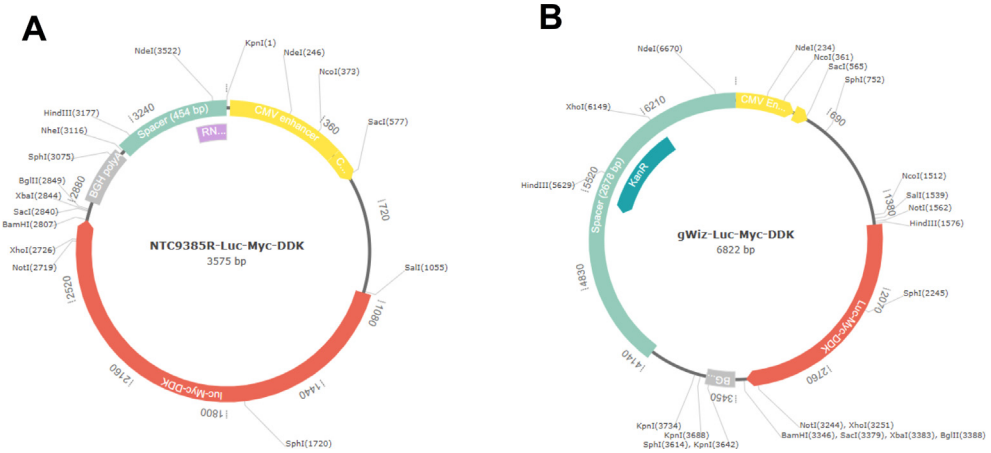

Fig. 1. Plasmid DNA Maps. NTC9385R-Luc-Myc-DDK or Nanoplasmid™ (A) and gWiz-Luc-Myc-DDK or traditional plasmid (B).

mittee protocol, in accordance with the Guide for the Care and Use of Laboratory Animals at an AAALAC accredited facility. Rats were acclimated for 48 h before any procedures were conducted.

2.4. Gene electrotransfer to the rat skin

Treatment sites on each flank were randomly assigned to the traditional plasmid, Nanoplasmid™, and injection-only (IO) groups. Each animal had 4 treatment sites per flank (8 treatment sites per animal). After thorough hair clipping of each flank, a conductive electrode gel was placed on the skin to facilitate contact with the grounding plate. Each treatment site then received a 50 μl injection of 2 mg/mL pDNA. A custom-built pulser was used to administer pulses to the skin, capable of biphasic pulses in the 1 microsecond range. A platinum tweezer electrode with a 5 mm diameter (BTX, Holliston, MA) and a 2 mm gap was used to deliver pulses to each treatment site. There were 5 different pulsing conditions were administered, consisting of the conditions shown in Fig. 3. Group 1, delivering 8 pulses at 40 V applied voltage and 150 ms pulse length acted as a positive control, as we had previously observed gene expression in the skin using a similar condition [12]. The other 4 conditions (Fig. 3 A) were biphasic conditions that were hypothesized to lead to similar expression levels, but potentially decrease muscle twitching, which is advantageous in keeping good contact between the tissue and the electrode [18,19]. These conditions were required to keep the same total pulsing time of 1200 ms, as derived from the monophasic Group 1. Group 2 had a pulsing pattern delivering 600,000 pulses at −40 V for 1 μs, a biphasic delay of 1 μs, and a second phase of 40 V for 1 μs followed by a 1 μs pulse gap. Group 3 had a pulsing pattern delivering 30,000 pulses at −40 V for 20 μs, a biphasic delay of 1 μs, and a second phase of 40 V for 20 μs followed by a 1 μs pulse gap. Group 4 had a pulsing pattern delivering 30,000 pulses at 40 V for 1 μs, a biphasic delay of 1 μs, and a second phase of −40 V for 1 μs followed by a 1 μs pulse gap. Group 5 had a pulsing pattern delivering 12,632 pulses at −150 V for 20 μs for the first phase, a biphasic delay of 1 μs, and a second phase consisting of 40 V for 75 μs, followed by a 10 μs pulse gap.

2.5. Heart gene electrotransfer

A left thoracotomy was performed as previously described to expose left ventricular myocardium [13]. Animals were randomly assigned to the traditional plasmid, Nanoplasmid™, and IO groups.

A 1 cm^2 monopolar platinum non-invasive surface electrode was used to administer pulses. A metal grounding plate was placed under the animal on the opposite flank, with electrode gel ensuring contact between skin and grounding plate. The pulser used was custom-built, using a power supply manufactured by Advanced Energy (Fort Collins, CO).

30 μl of 2 mg/ml pDNA was injected in 3 different areas of the left ventricle to minimize damage to the myocardium. For GET groups, the electrode was placed on the surface of the left ventricle near the injection site immediately after injection and pulses were administered. Pulses were delivered during the rise of the R wave in the electrocardiogram to avoid fibrillating the heart. 4 square wave pulses were delivered to each animal and were 100 ms long with 120 V being administered for the traditional plasmid group and 90 V being administered for the Nanoplasmid™ group. The IO group did not receive any pulses. These pulsing parameters originated from previous research on GET to the myocardium, however a different electrode was used in this study and thus the parameters were optimized for this new electrode. Additionally, we hypothesized based on the previous experiments that the Nanoplasmid™ group could achieve relatively high expression levels with lower applied voltage of 90 V, than the 120 V required for high expression in the myocardium with traditional plasmid. Lower voltage is known to reduce potential tissue damage

2.6. Bioluminescence analysis

Tenocytes were imaged at 72 h using the *In Vivo* Imaging System(IVIS (PerkinElmer, Waltham, MA))[1] to quantify gene expression via the bioluminescence levels detected from the luciferase-luciferin reaction. D-luciferin (Gold Bio, St. Louis, MO) was added to fresh media at a concentration of 150 μg/mL as recommended by the manufacturer. Images were taken immediately after adding the luciferin until the peak flux values for each group were observed. A circular region of interest (ROI) was used to get flux values for individual wells. ROI diameter was kept consistent among wells. B16F10 cell bioluminescence was quantified with a luminescence plate reader after D-luciferin addition to the media (Fig. 2A, 2E and Supplemental Fig. 1 Figure Supplementary Fig. 1).

Animals from the gene delivery to the skin experiment were imaged to measure bioluminescence levels using the IVIS at 24 and 48 h after GET. Animals were anesthetized with 3% isoflurane and given subcutaneous injections of D-luciferin at a concentration of 150 mg luciferin/kg body weight. Animals were imaged approximately every 5 min, alternating flanks. Once peak bioluminescence levels were reached for each treatment site, animals were returned to their cages and observed until they were fully recovered from anesthesia. Circular ROIs were used to examine individual treatment sites. ROI diameter was kept consistent among sites and for all animals.

Animals from the gene delivery to the heart experiment were imaged to measure bioluminescence levels using the IVIS at 24 and 48 h after GET. Animals were anesthetized with 3% isoflurane and given subcutaneous injections of D-luciferin at a concentration of 150 mg luciferin/kg body weight. Animals were imaged approximately every 5 min. Once peak bioluminescence levels were reached for each treatment site, animals were returned to their cages and observed until they were fully recovered from anesthesia. A circular ROI was used to examine the area containing the bioluminescent signal from the heart. ROI diameter was kept consistent among animals.

2.7. Immunofluorescence analysis

Immunofluorescence was used to determine the transfection efficiency of the plasmids. Cells were fixed using a 4% paraformaldehyde (PFA) solution and the gWiz-Luc-Myc-DDK and NTC9385R-Luc-Myc-DDK groups were stained for DDK and counterstained with DAPI. To stain for the DDK protein, a rabbit monoclonal anti-DDK antibody (TA150078, OriGene, Rockville, MD) was used and labeled with an AlexaFluor488 conjugated goat anti-rabbit IgG secondary antibody (A11034, ThermoFisher Scientific, Grand Island, NY). Fluorescent microscopy was used to image all groups by tiling and stitching together 25 images at a 10X magnification (Fig. 2C, D, G, H). DDK-expressing cells were then counted using ImageJ (Bethesda, MD) and reported as a percentage of DDK-positive cells (Fig. 2B, and F).

After 48-hour time point imaging skin samples were harvested and fixed in a 4% PFA solution. Tissue samples were paraffin-embedded and sectioned by IDEXX BioAnalytics (Westbrook, ME). Hematoxylin and eosin staining was also performed by IDEXX BioAnalytics. Unstained slides were deparaffinized using CitriSolv™ (Decon Labs, King of Prussia, PA) and an alcohol gradient. Antigen retrieval was performed using citric acid (pH 6). To stain for the DDK protein, a rabbit polyclonal anti-DDK antibody (TA150078, OriGene, Rockville, MD) was used and labeled with an AlexaFluor488 conjugated goat anti-rabbit IgG secondary antibody (A11034, ThermoFisher Scientific, Grand Island, NY). Negative con-

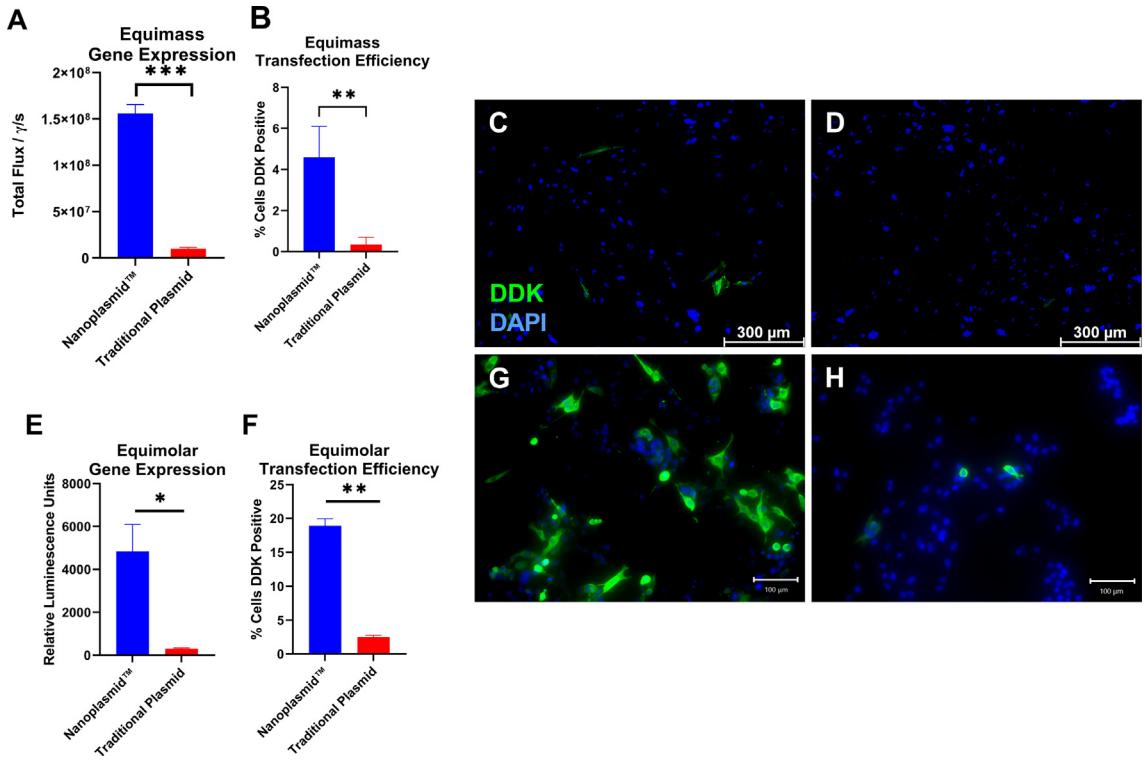

Fig. 2. Reduced vector size significantly increase gene expression *in vitro*. Gene expression levels at 72 h measured via bioluminescence are significantly higher with reduced vector size for equal mass transfection (A). Transfection efficiency is significantly increased with reduced vector size for equal mass transfection (B). Immunofluorescence staining for DDK tag in rat tenocyte transfected with Nanoplasmid™(C) or with traditional plasmid (D) for equal mass transfection. Gene expression levels at 72 h measured via bioluminescence are significantly higher with reduced vector size for equimolar transfection (E). Transfection efficiency is significantly increased with reduced vector size for equimolar transfection (F). Immunofluorescence staining for DDK tag in B16F10 cells transfected with Nanoplasmid™(G) or with traditional plasmid (H) for equal mass transfection. *p < 0.05, **=p < 0.01, ***=p < 0.001.

trol samples did not receive a primary antibody. All samples were counterstained with DAPI. Fluorescent microscopy was used to take representative images of the epidermis and muscle layers.

After 48-hour time point imaging heart tissue was harvested and fixed in a 4% PFA solution. Tissue samples were paraffin-embedded and sectioned by IDEXX BioAnalytics (Westbrook, ME). Hematoxylin and eosin and Masson's trichrome staining were also performed by IDEXX BioAnalytics. Unstained slides were deparaffinized using CitriSolv™ (Decon Labs, King of Prussia, PA) and an alcohol gradient. Antigen retrieval was performed using AR6 buffer (Akoya Biosciences, Marlborough, MA). To stain for the DDK protein, a rabbit monoclonal anti-DDK antibody (TA150078, OriGene, Rockville, MD) was used and labeled with an AlexaFluor488 conjugated goat anti-rabbit IgG secondary antibody (A11034, ThermoFisher Scientific, Grand Island, NY). Negative control samples did not receive a primary antibody. All samples were counterstained with a mouse monoclonal anti-cardiac troponin I antibody (ab10231, Abcam, Cambridge, MA), labeled with an AlexaFluor546 conjugated goat anti-mouse IgG secondary antibody (A11030, ThermoFisher Scientific, Grand Island, NY). Slides were then mounted with Vectashield hardset antifade mounting medium (H-1400, Vector Laboratories, Burlingame, CA). Fluorescent microscopy was used to take representative images of the epidermis and muscle layers. Slides were imaged with a Leica Microsystems (Buffalo Grove, IL) DMi8 microscope.

2.8. Statistical analysis

All quantitative data were evaluated with a one-way analysis of variance, with GraphPad Prism8 software.

3. Results

3.1. Reporter gene delivery in vitro

Use of the Nanoplasmid™ vector delivered in an equal mass as the gWiz vector, significantly increased gene expression and transfection efficiency when measured via bioluminescence and cell count (Fig. 2 A-D). An image of a representative plate is shown in Supplementary Fig. 1.

Equimolar delivery of the Nanoplasmid™ vector, compared to the gWiz vector, significantly increased gene expression and transfection efficiency when measured via bioluminescence and cell count (Fig. 2 E-H).

3.2. Reporter gene delivery to the skin

Multiple pulsing conditions, as described in Section 2.4 are shown in Fig. 3A. Briefly, we included a monophasic group that we have previously observed gene expression in the skin using a similar condition [12]. The other 4 conditions (Fig. 3 A) are biphasic conditions that were hypothesized to lead to similar expression levels, but potentially decrease muscle twitching [18,19].

The monopolar and monophasic control condition (group 1, Fig. 3A) achieved significantly higher expression in both plasmids when compared to their respective injection only (IO) groups when measured via bioluminescence (Fig. 3B). There were no significant differences in expression in the other groups when compared to their respective IO groups, nor between the two plasmids. Further research will be done to improve expression using biphasic conditions.

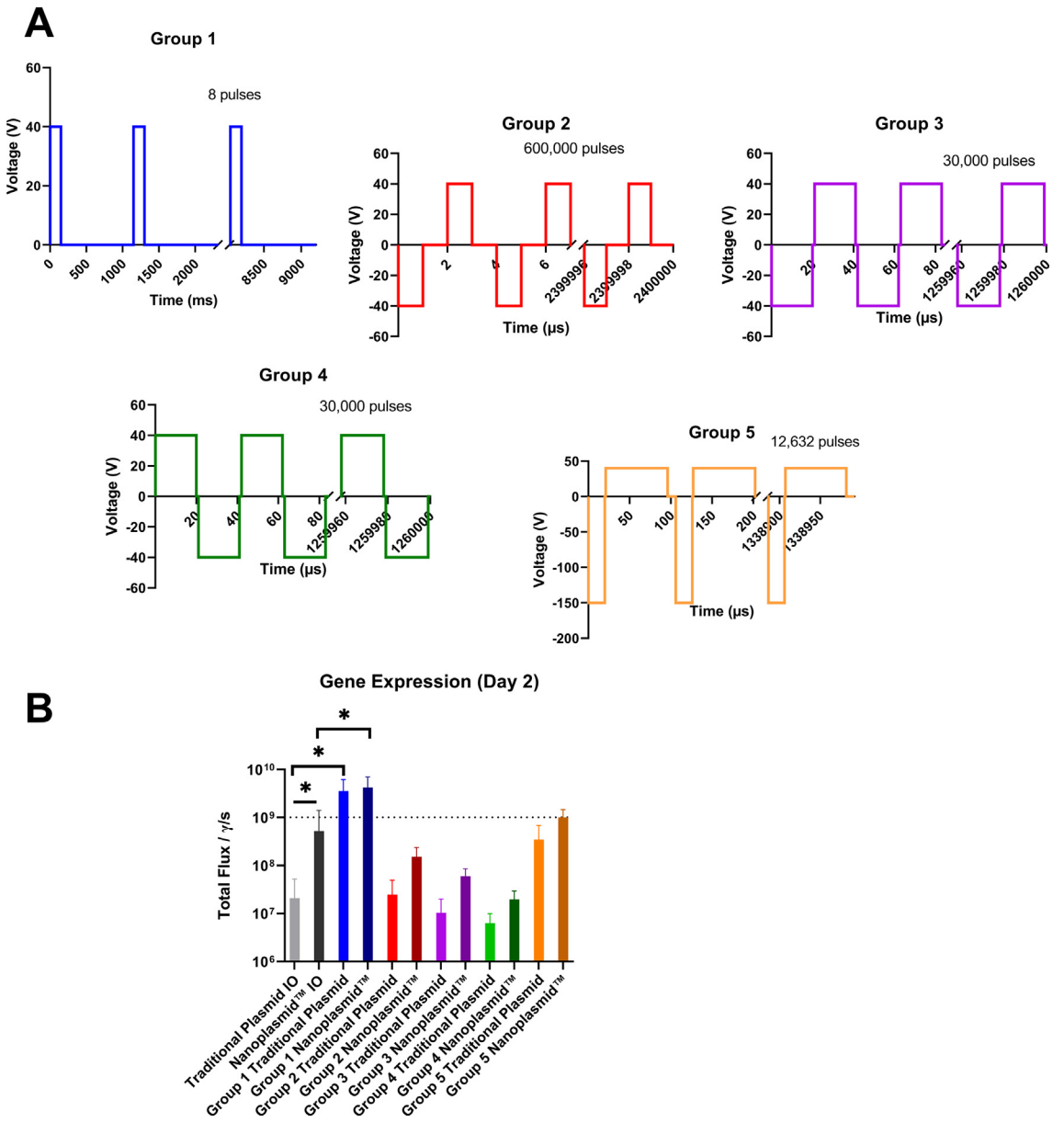

Fig. 3. Gene expression in the skin was significantly enhanced with Nanoplasmid™ vector. Pulsing conditions by group, showing the first 2 pulses and the last pulse for each condition (A). Gene expression after GET to the rat skin as measured by bioluminescence (B). For all conditions n = 4.

Cells expressing the DDK tag were observed in the epidermis (Fig. 4A-B) and muscle layers (Fig. 4C-D) of the skin in both plasmid groups for group 1, the monopolar and monophasic condition. Figure Supplementary Fig. 2 shows corresponding negative control images for epidermis and muscle layers of the skin.

3.3. Reporter gene delivery to the heart

Gene expression levels were significantly increased in the Nanoplasmid™ group at 24 and 48 h after transfection when measured via bioluminescence (Fig. 5 A). Immunofluorescence of both groups shows DDK tag expression throughout the myocardium (Fig. 5 B-C).

4. Discussion

Results suggest that decreasing the plasmid backbone length enhanced gene expression levels and transfection efficiency *in vitro* when transfected using both equal mass and equal moles. Additionally, we found that up to a 1:5 dilution of the Nanoplasmid™ yielded a successful transfection as defined by having greater average gene expression measured by bioluminescence and transfection efficiency when compared to the traditional plasmid group. These results warranted further investigation *in vivo*.

Previous studies show that gene delivery to the skin via GET is typically limited to the epidermis [16,21]. One study required heat to aid GET that resulted in transfected cells within the muscle layer of the skin [12]. With the Nanoplasmid™ and different pulsing parameters, we were able to transfect the muscle layer via GET without the use of additional aids.

Further work will be done to optimize biphasic GET pulsing conditions in skin that result in similar expression levels without damaging tissue, as only group 1 (monophasic) condition achieved optimal gene expression levels. Skin damage was not observed for any of the pulsing groups.

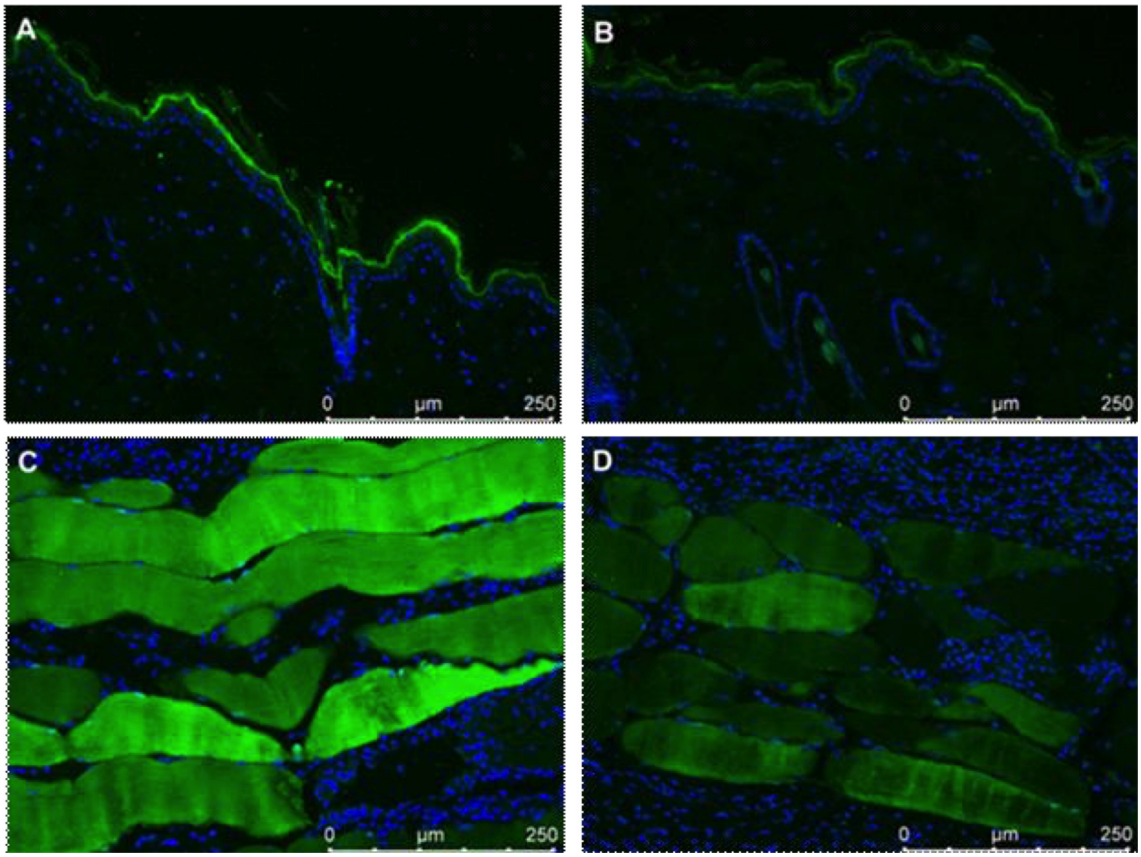

Fig. 4. Reduced vector size has comparable expression distribution, as shown with immunofluorescence staining for the DDK tag for monopolar and monophasic GET to the skin. Abundant expression is observed in the epidermis with Nanoplasmid™ (A), epidermis with traditional plasmid (B), skeletal muscle with Nanoplasmid™ (C), and skeletal muscle with traditional plasmid (D).

By utilizing the Nanoplasmid™ over a traditional plasmid, significantly higher levels of gene expression can be achieved with a lower voltage in GET to the myocardium. Previous literature has established that by lowering voltages used during GET, tissue damage and muscle twitching is reduced [17]. Reducing muscle twitch is ideal when using GET delivery methods to ensure the electrode (s) maintains contact with the tissue during pulsing.

In addition, it is important to note that the differences in gene expression as measured by bioluminescence between the skin and the myocardium are due to difference in cell/tissue types, as well as our ability to detect emitted light. When using the IVIS to measure bioluminescence in the skin, we are able to position the animal so that the flank with the treatment faces the camera detector without any obstructions. Since the heart is an internal organ, there are tissues ventral relative to the heart partially obstruct the signal. Any luminescence emitted from the heart has to travel through the muscle and bone of the thorax, the overlaying muscle and connective tissue, and then the entire thickness of the overlaying skin to reach the camera detector. This leads to an overall lower bioluminescence reading. Therefore, our bioluminescence measurements are intended to compare expression only within the same tissue type.

Nanoplasmids™ are optimized to be produced in large scales when compared to traditional plasmids [20]. Nanoplasmids™ are also antibiotic-free, adhering to European Pharmacopoeia and European Medicines Agency guidelines [20]. Minicircles, a higher-expression alternative to traditional plasmids, may suffer from production and quality issues [3,10], thus creating a need for pDNA vectors that can provide higher expression than traditional vectors without compromising production scalability.

Reducing plasmid backbone length significantly improved transgene expression levels in rat tenocytes and rat myocardium. This enhanced transgene expression was also observed in different gene delivery methods: a commercially available transfection reagent and GET. Improved transgene expression levels can aid in gene therapies in a variety of contexts. Particularly, non-viral gene transfer methods are often considered safe but have lower transgene expression levels when compared to viral gene transfer methods [2]. Utilizing plasmids with smaller backbone lengths may allow non-viral gene transfer methods to reach therapeutic transgene expression levels, providing a safe and effect method of gene transfer.

5. Conclusion

Reducing plasmid DNA backbone length significantly increased transgene expression levels *in vitro* in rat tenocytes and *in vivo* in rat myocardium. This effect was observed in both GET and a commercially available transfection reagent as gene delivery methods. The Nanoplasmid™ was also able to transfect multiple layers of the skin, including the muscle, which previously has only been done with additional aids [12]. Improving transgene expression levels has a variety of applications in the area of gene therapy.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

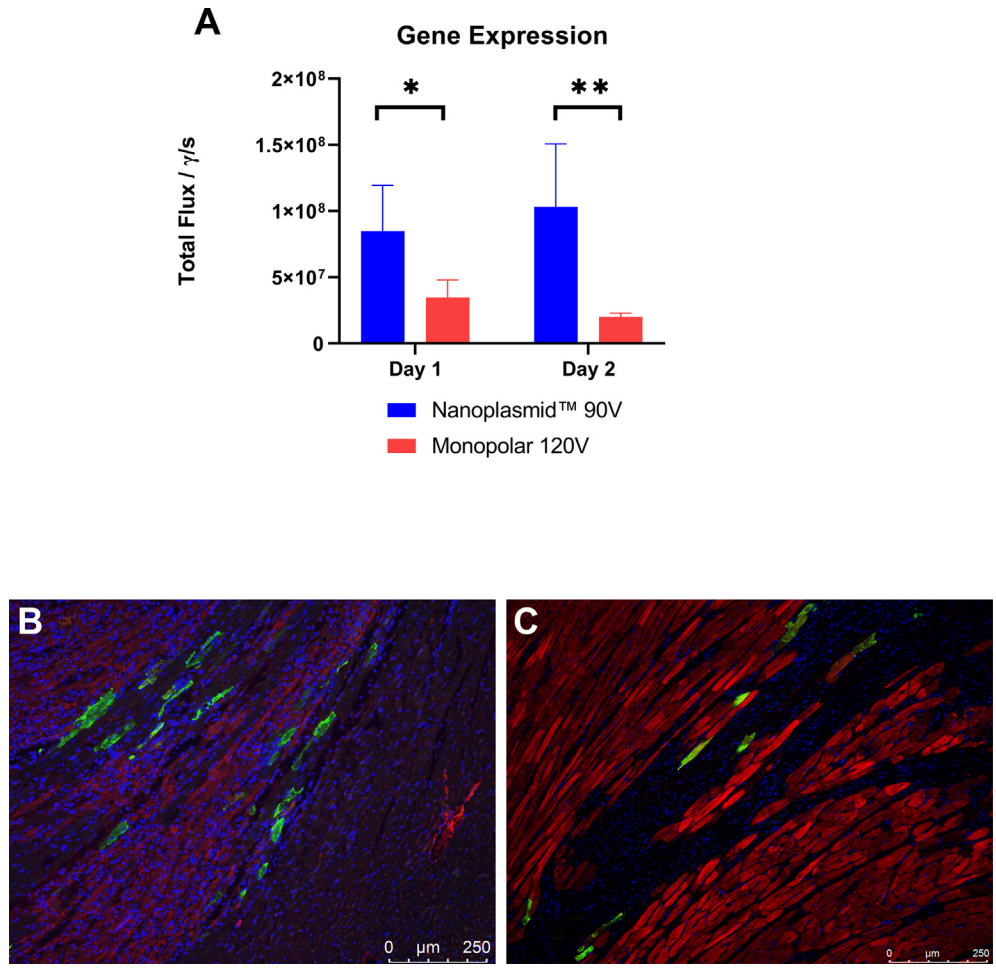

Fig. 5. Reduced vector size significantly enhanced gene delivery in rat myocardium. Luciferase expression was significantly increased(A). Nanoplasmid™ n = 2, traditional plasmid n = 3. *= $p < 0.01$, **= $p < 0.001$. Immunofluorescence staining for the DKK tag (green) and cardiac troponin (red) in heart with reduced vector size (B) and traditional vector (C).

Acknowledgements

The authors would like to thank Dr. Shu Xiao and Dr. Mark Jaroszeski for custom-built pulse generators utilized for biphasic pulsing to the skin and ECG synchronized pulsing to the heart respectively.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioelechem.2021.107981.

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