APPLICATION NOTE

Pre-clinical *in vivo* imaging

Authors

Jean-Baptiste Gossart
Valérie Kédinger
Géraldine Guérin-Peyrou
Patrick Erbacher
Anne-Laure Bolcato-Bellemin

Polyplus-transfection SA
Illkirch, France

Bioimaging of Gene Delivery with *In Vivo*-jetPEI®

Introduction

The delivery of nucleic acids into cells is essential for basic research as well as medical applications such as gene therapy. Viral vectors are efficient carriers, but their use is limited because of their safety (induction of immune response, virus-associated pathogenicity). These concerns have increased the interest of non-viral methods for *in vivo* gene delivery. Second generation of non-viral vectors offers improved performance and safety, potentially providing an alternative to viral gene delivery.

One of the most promising non-viral vectors is the cationic polymer polyethylenimine (*in vivo*-jetPEI®). It offers high performance in terms of efficiency, reproducibility and robustness. Thanks to its high cationic charge density potential, *in vivo*-jetPEI® can condense DNA to form stable complexes termed polyplexes and promote gene transfer into cells.[1] It is the most widely used technology to deliver gene in animals.[2,3,4]

PerkinElmer imaging systems offer unique opportunities for *in vivo* bioluminescent imaging. This technique allows the non-invasive detection and quantification of plasmid biodistribution in different organs, and can be used to detect luciferase encoding plasmids delivered with *in vivo*-jetPEI®. We took the benefice of this system to determine the essential parameters for efficient gene delivery using *in vivo*-jetPEI® in mice. This application note illustrates the use of the non-invasive whole animal IVIS® imaging system to detect and quantify transgene expression, following *in vivo*-jetPEI® mediated plasmid DNA delivery.

PerkinElmer®
For the Better

Effect of Nitrogen Over Phosphate (N/P) Ratio on Delivery Efficiency

Only positively charged complexes can bind the cell surface via interaction with negatively charged syndecans.[5] The overall charge of the *in vivo*-jetPEI®/DNA complexes is crucial for efficient delivery. It is determined by the DNA to reagent ratio. This ratio which represents the ionic balance within the complexes is classically defined as the N/P ratio, referring to the number of nitrogen residues (N) in the *in vivo*-jetPEI® per phosphate (P) of DNA. *In vivo*-jetPEI®/DNA complexes become positively charged when excess nitrogen residues are present versus phosphate residues of DNA. An N/P > 3 is required.

Examples of complex charge and size at different N/P ratio are showed in Figure 1A. Moderate particle charge is necessary for the colloidal stability of the formulation since strong repulsive forces between the particles prevent aggregation. The charges of complexes obtained with the N/P ratio of 6, 8 and 10 have a good stability for *in vivo* applications whereas the complexes formed at the N/P ratio of 3 are too weak. Higher N/P ratios (6, 8 and 10) give particles of small size, comprised between 43 and 48 nm (Figure 1A) perfectly suitable for *in vivo* application.

The non-invasive IVIS100 system from PerkinElmer was used to follow the luciferase plasmid expression upon systemic delivery with *in vivo*-jetPEI® through retro-orbital injection of the venous sinus. One day post-injection the luciferase signal could only be detected in the lungs and only for N/P ratio > 3 (Figure 1B). The level of luciferase expression in the lung was similar at N/P ratios of 6, 8 and 10 (respectively 6.4×10^4, 5.0×10^4 and 3.9×10^4 photons/s) (Figure 1B). After imaging, different organs, including lungs, were dissected, homogenized and the organ extracts were assayed for luciferase expression. The luciferase signal was measured with a luminometer and expressed as relative light unit (RLU) per mg of protein. As shown in Figure 1C, a good correlation was observed between bioluminescent imaging in whole animal and luciferase assay in lung extract. However, the luciferase assay performed on organ extracts was able to detect much lower levels of luciferase expression that were not detected by whole animal imaging. As shown in Figure 1D, upon organ dissection and homogenization, luciferase expression was determined in spleen, liver, kidney and heart extracts using a luciferase assay. In the spleen, kidney and heart, the N/P ratios of 8 and 10 seem to give higher luciferase expression than the N/P ratio of 6. In the liver, the N/P of 8 is slightly better than the other N/P ratios. Hence the DNA to *in vivo*-jetPEI® ratio and the injection conditions should be adapted to the targeted organ. Collectively, these data show that the IVIS100 imaging system is able to detect 99% of the emitted luciferase signal, while the remaining 1% can be determined with a luciferase assay performed on organ extracts.

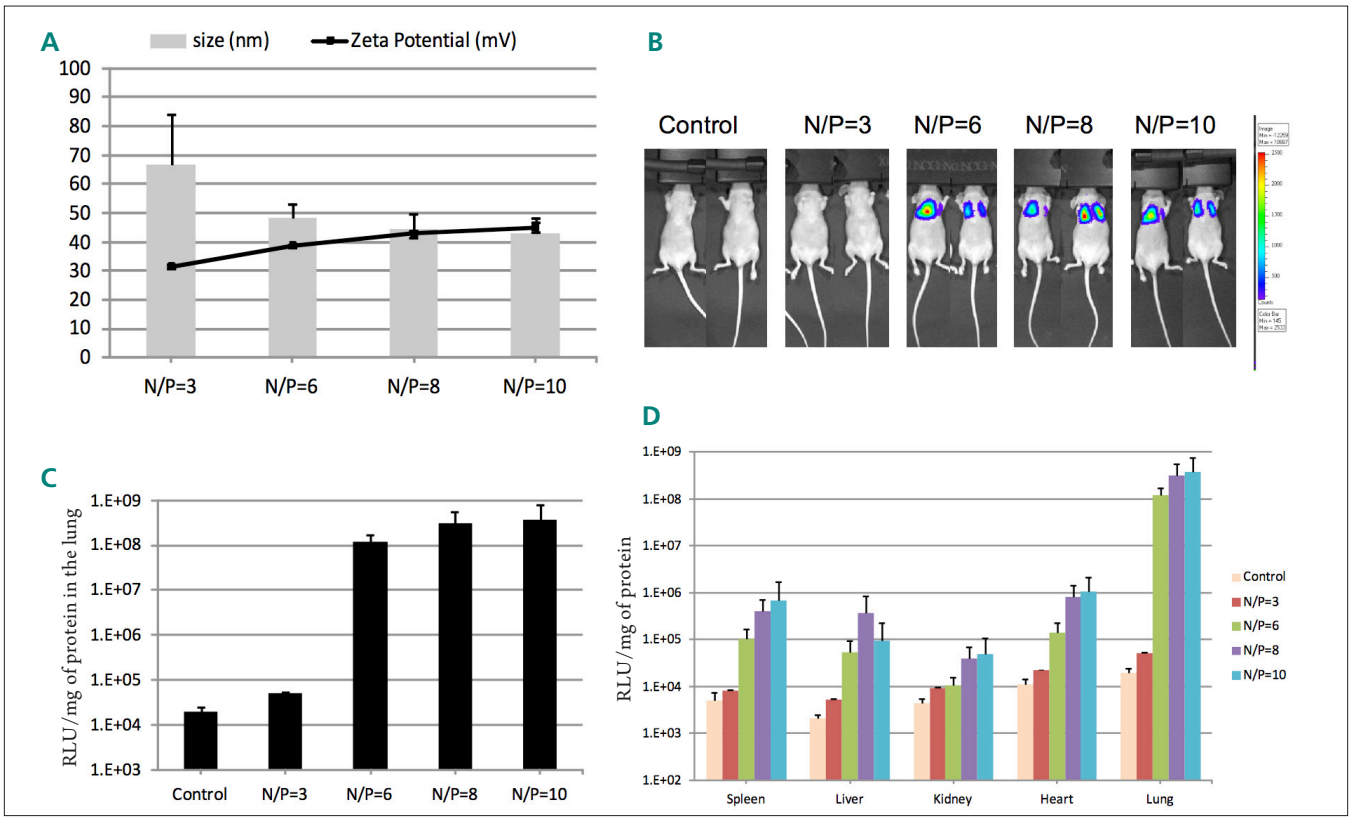


Figure 1. **Effect of nitrogen over phosphate ratio on delivery efficiency.** Forty micrograms of pCMVLuc plasmid were complexed with *in vivo*-jetPEI® at N/P ratio of 0 (control), 3, 6, 8 and 10. Thirty minutes after complexation, particle charge and zeta potential were measured using a zeta sizer (a). Complexes were injected retro-orbitally and mice were imaged with the IVIS100 imaging system 24 h after injection (exposition time 5 s) (b). After imaging, organs were removed and the luciferase level in each organ was analyzed in organ extracts using a luciferase assay and expressed relative to the amount of proteins (c and d), n=6.

Luciferase Expression Time Course in Lungs After Complex Injection

Once bound on the cell surface via interaction with the syndecan, *in vivo*-jetPEI®/DNA complexes are endocytosed into intracellular vesicles.[6] There, *in vivo*-jetPEI® acts as a "proton sponge" in the acidic environment of the lumen of endosomes.[1,7] Multiple protonations of *in vivo*-jetPEI® induce osmotic swelling followed by endosome rupture, leading to plasmid release into the cytoplasm.[8,9] *In vivo*-jetPEI® protects DNA against nucleases present in blood and serum.[10,11,12] Upon intravenous injection (tail vein or retro-orbital), the plasmid is quickly delivered in organs and expressed in cells. We investigated the time course of the luciferase expression in lungs using the PerkinElmer bioimaging system. As shown in Figure 2A, bioimaging of whole animals shows lung luciferase expression as early as 12 h after retro-orbital injection. In lungs, maximum expression is observed between 12 and 24 h after systemic injection (respectively 6.9 x 10^5 and 9.1 x 10^5 photons/s) (Figure 2A). Lungs were dissected after imaging, and the lung extracts were assayed for luciferase expression (Figure 2B). Again a good correlation was observed between the two techniques for detection of luciferase expression. The luciferase protein is not very stable and its life time is quite short.[13] Using a different reporter protein, such as eGFP or delivering a plasmid without CpG motives in order to avoid the silencing of gene expression over time[14] can increase transgene expression duration.

Complex Stability and Storage Temperature

We previously showed that *in vivo*-jetPEI® protects DNA from degradation by serum and blood nucleases.[10,11,12] As shown before, a small size of complexes is essential to promote efficient gene delivery *in vivo* (Figure 1). The complexes can be formed and then stored for many hours before their use if they are stable in size and do not form aggregates. We measured *in vivo*-jetPEI®/DNA complex sizes after 30 min complexation time or when stored 24 h at different temperatures (room temperature, 4 °C and 37 °C) by Dynamic Light Scattering. Figure 3A shows that *in vivo*-jetPEI®/DNA complexes are stable for at least 24 h at 4 °C, RT and 37 °C, as no variation of complex size was observed. Luciferase expression in the lungs was determined by bioimaging after storage of the complexes at 4 °C for 24 h and compared with complexes after 30 min complexation time. No significant variation of luciferase expression level was observed after 24 h storage (Figure 3B). Imaging was confirmed by luciferase assay in lung extracts (Figure 3C).

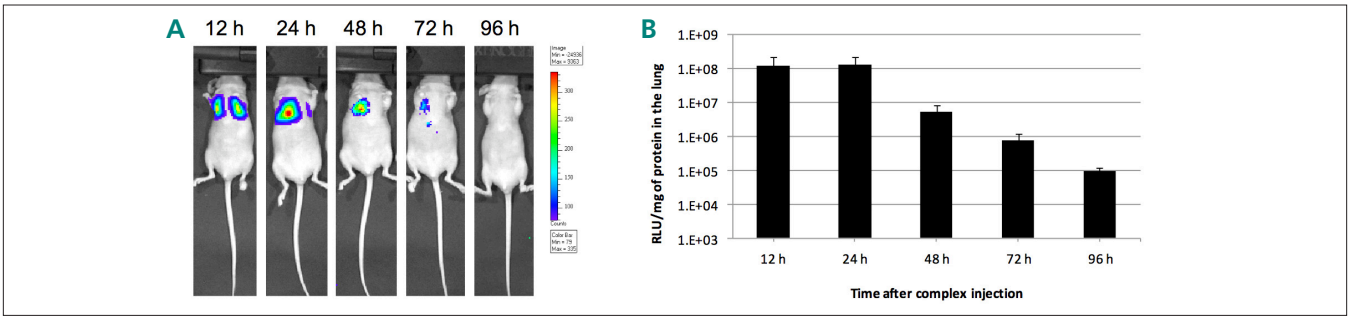
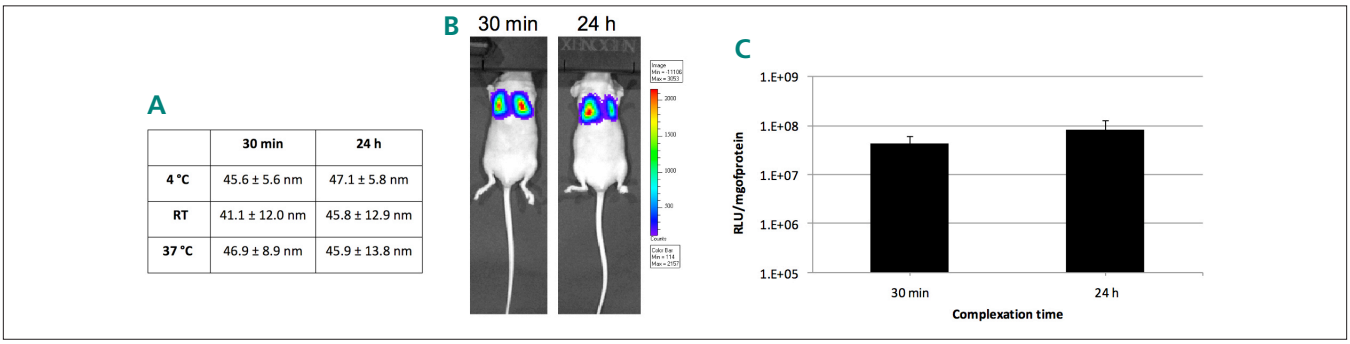


Figure 2. **Luciferase expression time course after *in vivo*-jetPEI®/DNA complex injection.** Forty micrograms of pCMVLuc complexed with *in vivo*-jetPEI® (N/P=8) were injected through the retro-orbital sinus. Mice were imaged 12, 24, 48, 72 and 96 h after complex injection using the IVIS100 imaging system (exposition time 10 s) (a). After injection, lungs were removed and luciferase expression was assayed in lung extracts (Figure 2b, luciferase expression in the lung), n=6.



	30 min	24 h
4 °C	45.6 ± 5.6 nm	47.1 ± 5.8 nm
RT	41.1 ± 12.0 nm	45.8 ± 12.9 nm
37 °C	46.9 ± 8.9 nm	45.9 ± 13.8 nm

Figure 3. **Complex stability at different storage temperatures.** Forty micrograms of pCMVLuc were complexed with *in vivo*-jetPEI® (N/P=8). Complex size was determined after 30 min and 24 h storage at RT, 4 °C and 37 °C by Dynamic Light Scattering using a zeta sizer (a). For *in vivo* imaging, complexes incubated 30 min or stored 24 h at 4 °C were injected through the retro-orbital sinus. The animals were imaged with the IVIS100 imaging system 24 h after injection (exposition time 5 s) (b). After imaging, lungs were removed, luciferase expression in lung extracts was quantified and expressed relative to the amount of proteins (n = 6), Figure 3c.

Conclusion

In vivo-jetPEI®-mediated gene delivery is a very useful and easy-to-develop technology. Here we have shown that bioimaging is a perfectly complimentary tool to follow reporter gene expression, and improve gene delivery parameters in mice. The *in vivo* bioluminescence imaging systems developed by PerkinElmer are particularly suitable to follow plasmid expression upon delivery with *in vivo*-jetPEI®. This delivery technology can be adapted to target different organs using various routes of administration including systemic injection or local delivery routes like intra-tumoral, intra-thecal or subcutaneous.[2,10,14,15] Taken together our data showed, that *in vivo*-jetPEI® is a potent reagent for functional and therapeutic studies in animal models. As it is validated for non-viral gene therapy in humans, it is also a good candidate for human therapeutics.

Materials and Methods

Instrumentation

IVIS100 Xenogen system (PerkinElmer).

Reagents

Plasmid: pCMVLuc (Promega)

Transfection reagent: *in vivo*-jetPEI® (Polyplus-transfection)

Buffer: Glucose 10% (Polyplus-transfection)
D-luciferin potassium salt (PerkinElmer)

Table 1. **Complex preparation and injection.**

N/P	Volume of *in vivo*-jetPEI®
3	2.4 µL
6	4.8 µL
8	6.4 µL
10	8 µL

Complexes were prepared in 200 µL of glucose 5% final concentration. 40 µg DNA were diluted in 100 µL (final volume) of glucose 5% and mix by pipetting up and down. The appropriate amount of *in vivo*-jetPEI® (Table 1) was diluted in 100 µL (final volume) of glucose 5% and mix by vortexing. The diluted *in vivo*-jetPEI® solution was added to the diluted nucleic acid solution, mixed by vortexing and left for 30 min at RT before injection. For the complex stability experiments, the complexes were stored at different temperatures at this stage. Complexes were injected through the retro-orbital sinus within 2s.

NMRI Nude female 5-weeks old were obtained from Elevage Janvier and subjected to a week quarantine and acclimation period before use. Animals were maintained under conventional housing conditions (12 h light/12 h night, 22 °C). All animal studies were conducted in accordance to the French Animal Care guidelines and the protocols were approved by the Direction des Services Vétérinaires.

Bioluminescence imaging

For *in vivo* bioluminescence imaging, 150 mg/kg of D-luciferin potassium salt were injected i.p. to the mice. Mice were anesthetized 10 min after injection with isoflurane and imaged with an IVIS100 Xenogen system (PerkinElmer).

Complex charge and Zeta potential determination

Particle size and zeta potential were determined by Dynamic Light Scattering using a ZetaSizer Nano-ZS (Malvern Instrument) with the following specifications: medium viscosity, 1.036 centipoise (cP); refractive index (RI) medium, 1.337; RI particle, 1.47; Dielectric constant, 78.5; temperature, 25 °C.

Luciferase assay in organ extracts

For luciferase analysis in organ extracts, mice were sacrificed; organs were removed and quickly frozen. Organs were then homogenized with an ultra-thurax in appropriate volume (Table 2) of 1x lysis buffer (Promega) and frozen O/N at -80 °C. Lysates were centrifuged at 15,000 g for 5 min and luciferase activity was assessed by using 5 µL of lysate after addition of 100 µL of luciferin solution (Promega). Luciferase activity was normalized per mg of protein by using the BCA assay (Pierce).

Table 2.

Organ	**Volume of Lysis Buffer (mL)**
Spleen	1
Liver	2
Kidney	1
Heart	1
Lung	2

References

1. Boussif, O., Lezoualc'h, F., Zanta, M.A., Mergny, M.D., Scherman, D., Demeneix, B., and Behr, J.P. 1995. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc Natl Acad Sci USA* 92:7297-7301.

2. Demeneix, B., Behr, J., Boussif, O., Zanta, M.A., Abdallah, B., and Remy, J. 1998. Gene transfer with lipospermines and polyethylenimines. *Adv Drug Deliv Rev* 30:85-95.

3. Wightman, L., Kircheis, R., Rossler, V., Carotta, S., Ruzicka, R., Kursa, M., and Wagner, E. 2001. Different behavior of branched and linear polyethylenimine for gene delivery in vitro and in vivo. *J Gene Med* 3:362-372.

4. Wiseman, J.W., Goddard, C.A., McLelland, D., and Colledge, W.H. 2003. A comparison of linear and branched polyethylenimine (PEI) with DCChol/DOPE liposomes for gene delivery to epithelial cells in vitro and in vivo. *Gene Ther* 10:1654-1662.

5. Labat-Moleur, F., Steffan, A.M., Brisson, C., Perron, H., Feugeas, O., Furstenberger, P., Oberling, F., Brambilla, E., and Behr, J.P. 1996. An electron microscopy study into the mechanism of gene transfer with lipopolyamines. *Gene Ther* 3:1010-1017.

6. Kopatz, I., Remy, J.S., and Behr, J.P. 2004. A model for non-viral gene delivery: through syndecan adhesion molecules and powered by actin. *J Gene Med* 6:769-776.

7. Behr, J.P. 1996. [Gene transfer with amino lipids and amino polymers]. *C R Seances Soc Biol Fil* 190:33-38.

8. Kichler, A., Leborgne, C., Coeytaux, E., and Danos, O. 2001. Polyethylenimine-mediated gene delivery: a mechanistic study. *J Gene Med* 3:135-144.

9. Akinc, A., Thomas, M., Klibanov, A.M., and Langer, R. 2005. Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. *J Gene Med* 7:657-663.

10. Lechardeur, D., Sohn, K.J., Haardt, M., Joshi, P.B., Monck, M., Graham, R.W., Beatty, B., Squire, J., O'Brodovich, H., and Lukacs, G.L. 1999. Metabolic instability of plasmid DNA in the cytosol: a potential barrier to gene transfer. *Gene Ther* 6:482-497.

11. Pollard, H., Remy, J.S., Loussouarn, G., Demolombe, S., Behr, J.P., and Escande, D. 1998. Polyethylenimine but not cationic lipids promotes transgene delivery to the nucleus in mammalian cells. *J Biol Chem* 273:7507-7511.

12. Pollard, H., Toumaniantz, G., Amos, J.L., Avet-Loiseau, H., Guihard, G., Behr, J.P., and Escande, D. 2001. Ca2+-sensitive cytosolic nucleases prevent efficient delivery to the nucleus of injected plasmids. *J Gene Med* 3:153-164.

13. Alvarado, M.C., Zsigmond, L.M., Kovacs, I., Cseplo, A., Koncz, C., and Szabados, L.M. 2004. Gene trapping with firefly luciferase in Arabidopsis. Tagging of stress-responsive genes. *Plant Physiol* 134:18-27.

14. de Wolf, H.K., Johansson, N., Thong, A.T., Snel, C.J., Mastrobattista, E., Hennink, W.E., and Storm, G. 2008. Plasmid CpG depletion improves degree and duration of tumor gene expression after intravenous administration of polyplexes. *Pharm Res* 25:1654-1662.

PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com

PerkinElmer®

For a complete listing of our global offices, visit www.perkinelmer.com/ContactUs

Copyright ©2013, PerkinElmer, Inc. All rights reserved. PerkinElmer® is a registered trademark of PerkinElmer, Inc. All other trademarks are the property of their respective owners.

010877_01