brief articles

Self-Assembled Micronanoplexes for Improved Biolistic Delivery of Nucleic Acids

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Abstract: A new method for biolistic delivery of nucleic acids using a combination of cationic micro- and nanoparticles is reported. The new method is simpler to perform than the conventional calcium/spermidine-based formulations and shows 11-fold improved nucleic acid binding capacity and dose-dependent performance both for in vitro and in vivo applications relative to either the conventional preparation or our recently reported direct cationic microparticle method. These features may enable higher throughput gene delivery and genetic immunization programs and open new venues for the biolistic delivery method.

Keywords: Biolistics; gene delivery; nucleic acids; microparticles; nanoparticles; nanoplexes

Introduction

Gene-based therapeutics, such as genetically modified plants, gene therapy and genetic immunization, holds great promise for improving crops and treating diverse diseases, ranging from inherited disorders to cancer. A major obstacle to their effectiveness remains the efficient introduction of bioactive nucleic acids (NA) into live tissue.[1] The two most common vehicles for NA delivery are virus and virus-like-particles (VLPs). Practical applications of viral particle methods are limited by the difficulty of their preparation, very poor storability, and lack of tissue tropism.[2] Among nonviral delivery methods, biolistics (biological ballistics) is unique because it avoids the perils of NA passage through medium (in vitro) or extracellular space (in vivo) by physically propelling it directly across the cellular membranes. Pioneered by J. Sanford and his colleagues over two

decades ago,[3] this method is broadly used for stable and transient transformation of cells,[4] organelles,[5] and organisms.[6] For plastids and a number of cell and tissues, biolistic delivery is the only known route of successful NA administration. Biolistic delivery is also an important genetic immunization route with broad potential for human and animal applications. Clinical trials have shown that this approach elicits both humoral and cellular immune responses, and is one of the most consistently successful methods for delivering gene vaccines.[7]

The biolistic method is based on NA release from NA-coated gold microparticles kinetically propelled inside of the cells with a high-pressure blast. A number of the particle delivery devices, or gene guns, have been described to date,

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and one is commercially available from Bio-Rad Laboratories (Helios gene gun). Surprisingly, for the past two decades most of the efforts to optimize gene gun delivery have been focused on the refinement of the device itself without much attention paid to the microparticles that actually deliver the payload. The conventional biolistics protocol involves coating of spherical gold microparticles (1−2 μm in diameter) with loose calcium−DNA−spermidine precipitate.[4] The coating process is cumbersome, requires a high degree of skill, and is highly sensitive to environmental factors and characteristics of the NA, particularly its size and purity. Namely, the conventional protocol cannot be applied to attach NA molecules smaller than 200 bp, such as siRNA, because spermidine does not efficiently condense these smaller NA fragments. These disadvantages result in inconsistent and suboptimal performance of the biolistically delivered material and limit the scope of applications.

Recently, we reported a new method for preparation of gene gun microparticles in which gold microparticles are coated with positively charged polyethylenimine (PEI) polymer and DNA is directly bound to the surface. The PEI-coated gold microparticles (PEI-AuMP) were constructed by conjugating amine groups of PEI polymer to the carboxyl moiety of tiopronin linker self-assembled at the gold surface via thiol functionality. These new microparticles exhibited a DNA dose-dependent increase in luciferase (Luc) transgene activity.[8]

In a further attempt to increase the efficiency of this intercellular delivery vehicle, we argued that it may be advantageous to enable release of NA from the surface of PEI-AuMP in the form of a complex with nanoparticles (nanoplex) within the cytoplasm, which might not only serve as protection against degradation[10] but possibly assist DNA transfer into the nuclei by other nuclear localization mechanisms. We further hypothesized that some portion of the particles are likely to not directly enter the cytoplasm. These extracellular nanoplexes might dissociate from the microparticles and undergo endocytosis by the surrounding cells, thereby providing another opportunity for transfection. We tested the above concepts with the micronanoplexes (complexes of microparticles with nanoplexes) formed between cationic PEI-AuMP microparticles and anionic nanoplexes formed by complexation of DNA with cationic gold nanoparticles (AuNPs).

Materials and Methods

Unless otherwise noted, all chemicals were purchased from Sigma, Inc. (Milwaukee, WI), and used without further purification. Spherical gold microparticles ($d = 1.5$ μm) were from Ferro, Inc. (Cat. No. J5G2000). Transmission electron microscopy (TEM) was done on a Philips CM12S microscope. Scanning electron microscopy was performed on a Leica-Cambridge 360FE microscope. In solution nanosizing and zeta potential measurements were done on a Zetasizer Nano-ZS instrument (Malvern Instruments, U.K.). All spectrophotometric measurements were carried out on a Nano-Drop ND-1000 instrument.

Preparation of PEI-Modified Gold Microparticles (PEI-AuMP). The positively charged microparticles were prepared according to a slightly modified and more efficient procedure than the one previously reported by us.[8] The commercial grade gold microparticles (AuMP, $d = 1.5$ μm, Ferro, Inc.) (20 g) were cleaned in 40 mL of piranha solution (H_2SO_4:H_2O_2 = 3:1; **CAUTION!** *Piranha solution is a corrosive and strongly oxidizing agent*) for 1 h and washed 4× with ddH$_2$O and 2× with ethanol. A solution of 500 mg of 11-mercaptoundecanoic acid (Cat. No. 450561-5G, Sigma-Aldrich, Inc.) in 20 mL of ethanol 200 proof was added, and particles were shaken at 1400 rpm at RT for 2 h. The supernatant was withdrawn after brief centrifugation, and the particles were washed 2× with ddH$_2$O. The modified particles were reacted with 200 mg of 1-ethyl-3-[3[dimethylaminopropyl]carbodiimide hydrochloride (EDC, Cat. No. 22980, Pierce, Inc.) plus 300 mg of *N*-hydroxysuccinimide (NHS, Cat. No. 130672-5G, Sigma-Aldrich, Inc.) for 30 min with vigorous shaking at RT. The excess of reagents was removed by centrifugation/decantation. A solution of 2 g of 50% polyethylenimine (PEI750, MW 750K, Cat. No. P3143, Sigma-Aldrich, Inc.) in 20 mL of ddH$_2$O adjusted to pH 9 was added and reaction allowed to react for 2 h with vigorous shaking (1400 rpm) at RT. The resulting PEI-modified microparticles were washed 3× with ddH$_2$O, dried under vacuum overnight and stored in a dark cool place under nitrogen until further use. The presence of organic layer was confirmed directly by FT-IR and indirectly by DNA binding experiments.[8]

Preparation of Cysteamine Modified Gold Nanoparticles (AuNP). The nanoparticles were prepared according to a literature procedure[9] with slight modifications. Briefly, 400 μL of a freshly made 213 mM solution of cysteamine (Cat. No. 30070, Sigma-Aldrich, Inc.) in argon-purged ultrapure water was added to 40 mL of a solution of 1.42 mM tetrachloroauric(III) acid (HAuCl$_4$, Cat. No. 254169-500MG, Sigma-Aldrich, Inc.) dissolved in argon-purged ultrapure water. The mixture turned yellow and translucent upon addition of cysteamine and was stirred vigorously for 10 min. A freshly prepared solution (10 μL) of 10 mM sodium borohydride (NaBH$_4$) was quickly injected into the reaction mixture with a micropipet. After vigorous stirring for 15−30 min, the wine red solution was stored in the dark at 4 °C under nitrogen blanket for up to two weeks.

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Assembly of PEI-AuMP/DNA/AuNP Micronanoplexes. A solution of 8 μg of pLUC DNA in water was mixed with 450 μL of the AuNP solution (60 μg/mL in Au) to give the ratio of Au/DNA = 20:1. The mixture instantly changed color from red to purple indicating formation of DNA−AuNP nanoplexes. A suspension of 8 mg of PEI-AuMP microparticles in 48 μL of pH 6 MES (2-(*N*-morpholino)ethanesulfonic acid) buffer supplemented with 0.5 M NaCl was added in 5 min. The color of the mixture rapidly cleared on gentle vortexing, indicating that the DNA−AuNP nanoplexes entirely settled onto the surface of PEI-AuMP microparticles. The suspension was briefly centrifuged at 4000 rpm to a pellet and the supernatant discarded. The pellet was resuspended in 165 μL of *n*-butanol and cast into 8 bullets in Teflon tubing which were dried under a gentle stream of nitrogen. **CAUTION!** *The n-butanol drying and shooting regimens should be performed in a chemical hood and while wearing an appropriate facial mask, respectively. The n-butanol and the microparticle dust may cause irritation of the respiratory tract if inhaled.*

In Vitro Gene Expression. NIH 3T3 cells were split into the 24-well tissue plate. The cells were grown to near confluence (∼80%). Just before shooting the NIH 3T3 cells, the medium was removed from the wells. The medium was immediately added to the wells after the shooting. As a positive control NIH 3T3 cells were transfected with the pLuc plasmid using FuGENE 6 transfection reagent from Roche Applied Sciences (1 μg of DNA per one well of the 24-well tissue plate). The cells were incubated for 24 h (37 °C, 5% CO_2) and subjected to the luciferase assay.

In Vivo Gene Expression. Two groups of mice ($n = 3$) were shot with pLuc loaded microparticles in the ears. The ears were harvested after 24 h and ground up in a manual homogenizer. The homogenized tissues were treated with 0.5 mL of 1× reporter lysis buffer. The samples were vortexed for 15 min at room temperature and spun at 14,000 rpm for 10 min. The supernatants were assayed by using the luciferase assay.

Luciferase Assay. Assay for luciferase activity was done using Promega's Luciferase Assay System. The media was completely removed from the wells and the cells were washed one time with 1× PBS (pH 7.4). The cells were covered with 1× Luciferase Cell Culture Lysis Reagent (Promega, Inc.) (500 μL of the Luciferase Cell Culture Lysis Reagent per each well of the 24-well tissue plate). Then the cells and all liquid were transferred to a microcentrifuge tube. The tubes were placed on ice. 20 μL of cell lysate were added per one well of the 96-well microplate, and the plate was placed into the luminometer with injector (Clarity Luminescence Microplate Reader, BioTek Instruments, Inc., Winooski, VT). The injector added 200 μL of Luciferase Assay Reagent per well, then the well was read immediately. The light intensity of the reaction was measured for a period of 10 s. 1× Luciferase Cell Culture Lysis Reagent was used for the blank (20 μL per each well of the 96-well microplate) in triplicate. All assays of luciferase activity were done in triplicate as well.
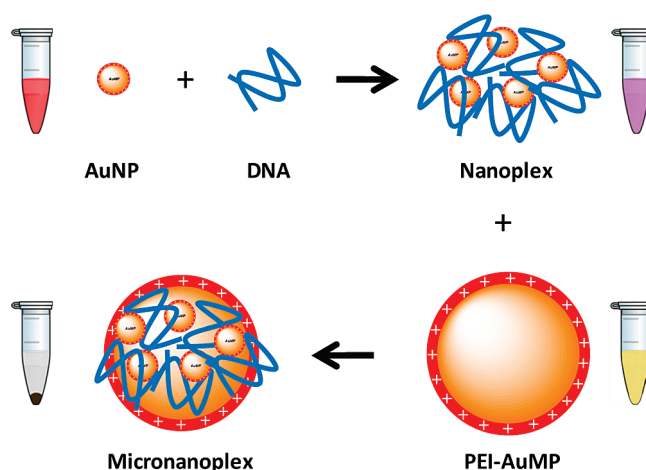

Figure 1. Schematic representation of the micronanoplexes' self-assembly. Cationic gold nanoparticles (AuNP) are complexed with anionic nucleic acids to yield nanoplexes with overall negative charge, which are further complexed with the cationic gold microparticles (AuMP) to form micronanoplexes. The red color of a solution of AuNPs changes to purple on complexation with nucleic acids. Addition of AuMPs leads to rapid precipitation of the nanoplexes to form micronanoplexes.

Results and Discussion

Assembly of Micronanoplex Constructs. Cationic AuNPs have been explored as nonviral gene delivery vehicles to deliver oligonucleotides, plasmids, and siRNA into cells.[11] AuNPs are nontoxic and nonimmunogenic and offer advantages of easy preparation and multiple possibilities for further surface modifications. DNA is first adsorbed onto cationic gold nanoparticles in certain DNA/Au ratios to provide charged nanoparticle−DNA complexes (nanoplexes). By using such formulations, the transfection efficiencies could be increased many-fold compared to the introduction of DNA alone or complexed with other transfection reagents.[9,12,13] Depending on the DNA/Au ratio, nanoplexes can be negatively or positively charged. In particular, we used negatively charged nanoplexes that can be efficiently absorbed by the positively charged PEI-AuMPs to form microparticle−nanoplex complexes (micronanoplexes), as schematically shown in Figure 1.

We prepared cationic cysteamine-modified AuNPs according to a literature procedure and followed the process of their complexation with pLUC plasmid DNA by dynamic light scattering (DLS) and ζ-potential (surface charge) measurements in real time. The titration of cationic AuNPs

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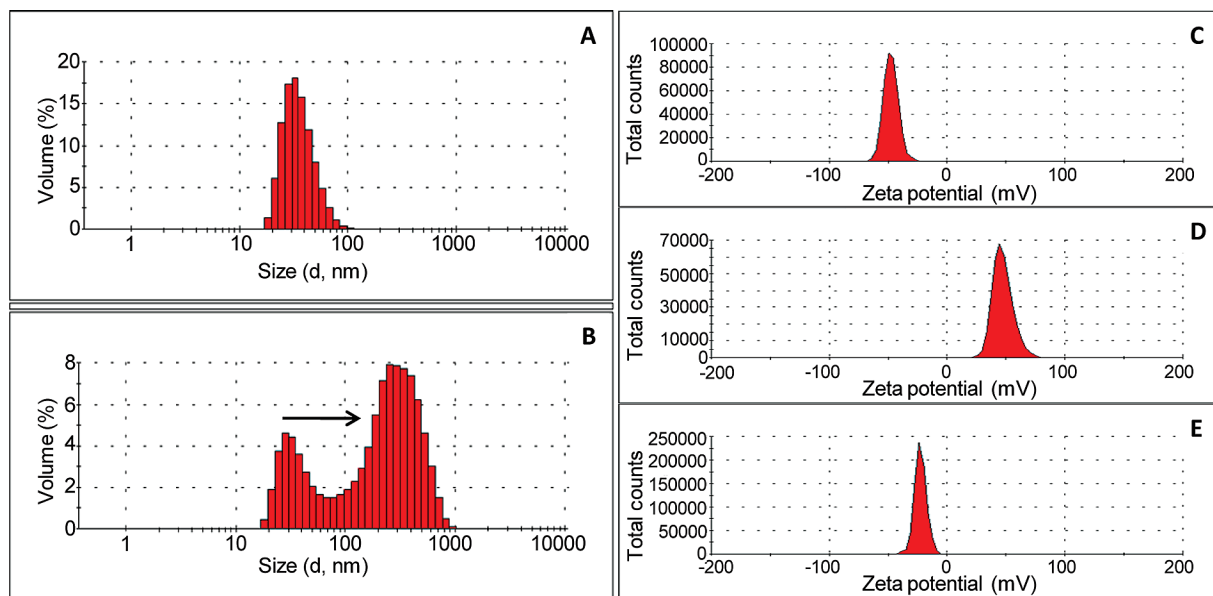
Figure 2. (A) Hydrodynamic diameter of gold nanoparticles determined by DLS. (B) Gold nanoparticles mixed with DNA in a 20:1 Au/DNA ratio. A transient state is shown. Peak corresponding to AuNP nanoparticles disappears entirely in 5 min; zeta potential distributions for (C) pLUC DNA alone; (D) AuNP alone; (E) mixture of DNA and AuNP in a 20:1 Au/DNA ratio.
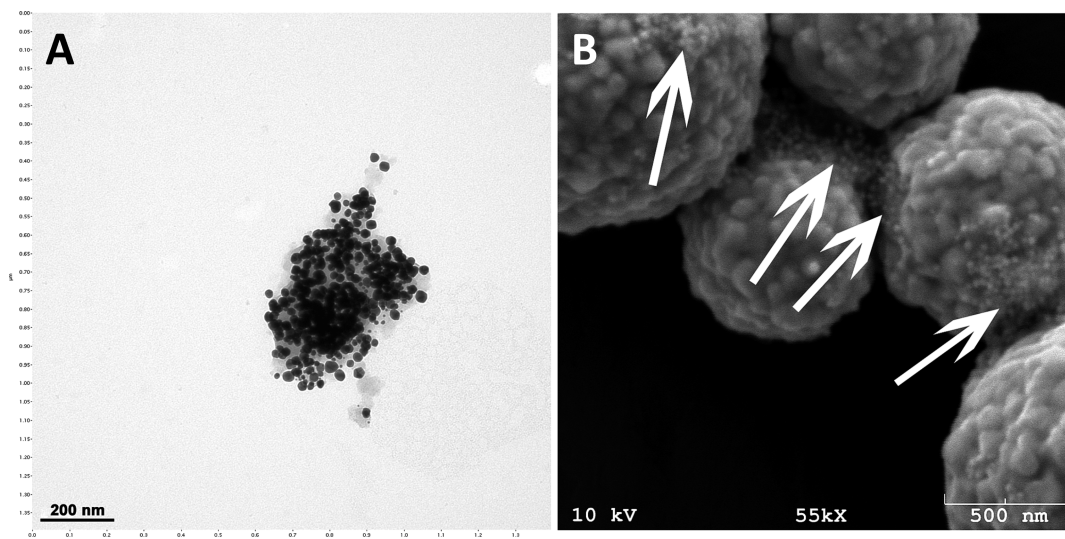

Figure 3. (A) TEM image of a representative nanoplex of ca. 400 nm dimension formed between gold nanoparticles and DNA at 20:1 Au/DNA ratio. (B) SEM images of micronanoplexes formed between the nanoplexes and PEI-AuMP microparticles. The caviar-like nanoplexes attached to the surface of AuMP are shown by arrows. In TEM, large size of the microparticles precluded reliable measurements due to rapid heating of the grid upon electron bombardment that led to partial dissociation of the nanoplexes from the surface of PEI-AuMPs.

($\zeta_{av} = +46$ mV) with DNA ($\zeta_{av} = -47$ mV) was conducted until the reversal of the positive zeta potential to a value of $\zeta_{av} = -21$ mV was observed at the target 20:1 Au/DNA ratio (Figure 2). Figures 2A and 2B show the change in hydrodynamic diameter of the gold nanoparticles and gold nanoparticles complexed with pLUC plasmid. While the AuNPs on average are 36 nm in diameter, the nanoplexes are between 300 and 400 nm. Varying Au/DNA ratio from 1:1 to 80:1 led to almost linear increase in ζ-potential of the nanoplexes from −33 mV to −5 mV. The decreasing surface charge in turn resulted in a sharp increase in the size of nanoplexes at an approximately 60:1 ratio and above, which

is in accordance with diminishing colloidal stability when surface charge approaches zero.[14]

The complexation of the AuNPs with DNA is optically manifested by a distinct blue shift of the surface plasmon band, a phenomenon associated with dispersion to aggregation transition of AuNPs.[15] Figure 3A shows transmission electron microscopy (TEM) image of a typical nanoplex formed in the mixture of AuNPs with DNA. The dimension of the nanoplex agrees with the results of the DLS measurements. When the negatively charged nanoplexes are brought in contact with positively charged gold microparticles, the

electrostatic attraction causes nanoplexes to be absorbed onto the surface of the microparticles creating micronanoplexes shown in Figure 3B,C. The absorption process is conveniently followed by rapid disappearance of the purple nanoplexes upon addition of the suspension of cationic gold microparticles (Figure 1). The color of the suspended microparticles changes from light yellow to dark purple as the complexation proceeds. Treatment of these micronanoplexes with 0.1 M NH_4OH solution releases the DNA and reconstitutes gold nanoparticles to their initial nonaggregated state (red color). A brief sonication is necessary in some cases to accelerate the dissociation process.

DNA Binding Capacity. One of the most important advantages of using micronanoplexes is the ability to significantly increase DNA binding capacity. The increased capacity is attributed to the following two factors: (1) the larger surface area of nanoparticles provides more ample scaffolding for attaching more DNA, and (2) the reduction of overall negative charge of the nanoplexes versus that of naked DNA allows an increased electrostatic absorption of nanoplexes per same PEI-AuMP surface charge density. The amount of DNA bound to the gold can be calculated spectrophotometrically as a difference between the input amount of NA and the amount of NA remaining in the supernatant following incubation.[8] In the case of the micronanoplex formulations, this solution measurement is not possible because of the strong residual absorbance of byproduct of gold nanoparticle synthesis.

To determine the DNA binding capacity of the microparticles, we measured the DNA remaining in the supernatant following incubation by gel electrophoresis. One milligram of microparticles was incubated with 1 to 16 μg of DNA complexed with a fixed amount (56 μL) of AuNPs. Once the micronanoplexes were formed and precipitated by centrifugation, the supernatant was assayed on an agarose gel to visualize unbound DNA. Figure 4A shows that when PEI-AuMPs were incubated with up to 11 μg of DNA, no DNA was found in the supernatant. When 12 μg of DNA was added to the microparticles, a small fraction of DNA remained in the supernatant. This indicates that the binding capacity of the PEI-AuMPs is ~11 μg of DNA per milligram, which is significantly higher than either the conventional (1 μg/mg) or directly loaded PEI-AuMPs (3 μg/mg).[8] The binding was stable at hot and cold temperatures and did not dissociate by washing in water, ethanol, or *n*-butanol but, as mentioned above, could be reversed by treatment with dilute ammonia solutions at pH > 9. In this range of pH the amino groups on the surface of PEI-AuMP microparticles become deprotonated and lose their binding affinity to DNA.

In Vitro Gene Expression Efficiency. Conventionally loaded biolistic microparticles show dose-dependent expression of transgenes when loaded with 1 μg or less of DNA, and linear dose responses are lost with any load above 0.5 μg. Loading DNA directly onto PEI-AuMPs represented a significant improvement by providing linear dose-dependent responses for particle loads up to 3 μg.[8] The higher binding capacity of the PEI-indirect microparticles described here suggests that they may provide further improvement in the
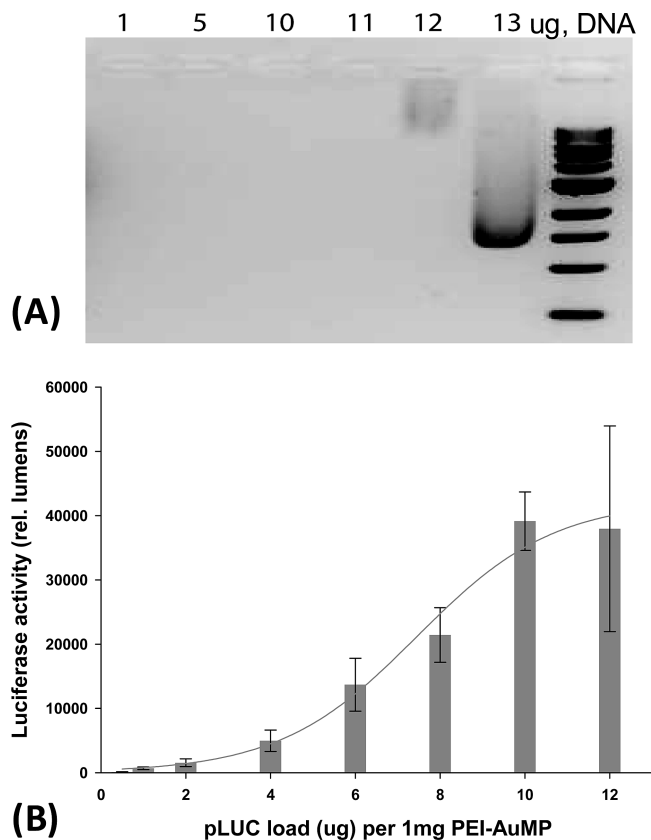

(A)

(B)

Figure 4. (A) Agarose gel electrophoresis of supernatant over PEI-AuMPs loaded with increasing amounts of pLUC−AuNP nanoplexes. pLUC DNA was mixed with fixed amount of AuNPs and precipitated onto PEI-AuMPs. Supernatant was subjected to electrophoresis in an agarose gel (1% w/v) and stained with ethidium bromide. (B) Dose-dependent luciferase expression levels in NIH 3T3 cells using AuMPs loaded with increasing amounts of pLUC−AuNP nanoplexes.

range of dose-dependent responses. To test this, NIH 3T3 cells were transfected by gene gun using PEI-indirect microparticles loaded with increasing amounts of a luciferase reporter gene plasmid. Cells were harvested 24 h later and assayed for luciferase activity. Figure 4B shows that there was a dose-dependent relationship between luciferase activity and transgene dose up to 12 μg, which was the highest dose tested. Curve fitting of the data shows sigmoid dependence with increasing load, suggesting that saturation of cellular machinery is reached at around 10−12 μg DNA. The intermediate rapid growth in luciferase activity can be explained by increasing size of the nanoplexes, which are more easily released from the surface of the microparticles due to their decreasing surface charge density. These important findings highlight the fact that cellular gene expression machinery is far from being saturated by the currently used protocols.

In Vivo Gene Expression Efficiency. Another set of experiments was performed in mice, using a linear expression construct expressing the same reporter gene. DNAs CMVi promoter, the LUC gene open reading frame (ORF), and the
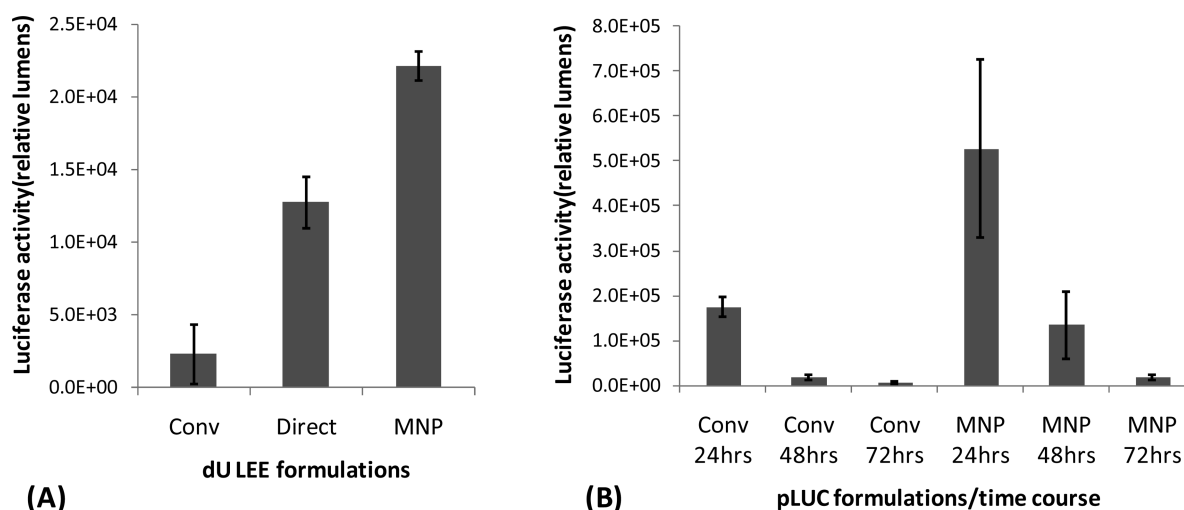
Figure 5. (A) LUC LEE expression measured in mice transfected using conventional calcium/spermidine (conv), directly loaded PEI-AuMP (direct), and micronanoplex (MNP) formulations. (B) Time-dependent course of Luc expression following biolistic delivery of pLUC into mice using conventional calcium/spermidine and micronanoplex formulations.

human growth hormone terminator were individually generated by PCR and assembled into an expression cassette by the early published procedure.[16] This DNA was loaded onto the AuMP by using (1) the conventional calcium/spermidine protocol, (2) directly onto PEI-AuMP particles, and (3) indirectly using the micronanoplex formulation. These three formulations were delivered with the gene gun into mouse ear pinnas (1 shot per ear). Luc activity in the ear tissue was measured 24 h later. Reporter gene activity measured in tissue shot with the micronanoplexes was remarkably 11-fold higher than that of tissue shot with conventional AuMPs and nearly 2-fold higher than that of ears shot with the previously reported directly loaded PEI-AuMP (Figure 5A).[8] These results highlight the utility of charged microparticles for the delivery of smaller DNA fragments, such as linear expression elements (LEEs)[16] or siRNA,[8] where the conventional calcium/spermidine protocol fails to efficiently precipitate such fragments onto the surface of uncharged microparticles.

We also followed the durability of LUC gene expression following conventional and micronanoplex particle delivery to mice ear pinnas. Transgenes delivered with the micronanoplexes showed higher and more prolonged expression levels than those delivered by the conventional protocol (Figure 5B).

Mechanism of Enhanced Gene Expression. Finally, we evaluated the ability of the nanoplexes to produce secondary transfection by nonclassical nuclear localization. We applied conventional calcium/spermidine/pLUC precipitates on uncharged AuMPs, AuNP/pLUC nanoplexes, AuNP/pLUC/

PEI-AuMP micronanoplexes used in the biolistic delivery experiments, and control Lipofectamine pLUC lipoplexes directly to dendritic cell cultures and saw no Luc expression in all cases (data not shown). This is not surprising since transformation of dendritic cells by using nonviral methods in general has proven to be difficult. In a control experiment with NIH 3T3 cells, only Lipofectamine transfection produced moderate Luc activity. Based on these results, we conclude that biolistic delivery using micronanoplexes does not provide benefits of secondary transfection; instead the higher and more prolonged reporter gene activity are attributed to better nucleic acid protection against degradation using micronanoplex formulations than in the case of conventional calcium/spermidine/NA formulations.

At this point, the mechanism of intracellular DNA release from the micronanoplexes remains unclear and is in need of further elucidation. We can only speculate that the release of DNA−AuNP nanoplexes from the surface of PEI-AuMPs occurs followed by simultaneous or delayed free DNA release by a mechanism similar to the mechanism of DNA release from other nano-, poly-, and lipoplexes in the traditional chemical transfections or from histone−DNA complexes in nucleosomes.

Conclusions

In summary, we developed a new set of formulations for biolistic delivery of nucleic acids using cationic gold microparticles. The advantages offered by these formulations may open new possibilities for various applications of gene delivery such as genetic modifications of plants or immunization of animals. In addition to gold nanoparticles, the cationic surface of the microparticles can be used for the attachment of other anionic NA complexes, including polyplexes and lipoplexes currently used in chemical transfections. Layer-by-layer (LbL) alternating charge assembly using anionic, e.g. DNA, hyalouronate or alginate, and cationic, e.g. PEI, chitosan and polylysine, polyelectrolytes

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may enable new gene delivery applications where slow or controlled release is of critical importance. Furthermore, the outer layer polymers can be covalently modified to include specific membrane targeting agents in order to enhance the possible postbiolistic delivery and uptake of NA complexes. Finally, the technique described here offers unique opportunities for biolistic delivery of other charged species including proteins and polysaccharides.

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