**INTRODUCTION**

Biolistic DNA delivery is broadly used for stable and transient transformation of cells and organelles (1). The protocol is suitable for most biological targets and is based on mechanical delivery of DNA-coated microprojectiles inside of cells or organelles with a high-pressure blast. Over time, significant advancements have been made in the delivery of DNA-coated projectiles, but their preparation remains virtually unchanged since their first introduction 21 years ago (1). The conventional projectile coating protocol is based on the formation of a highly unstable and fragile DNA/Ca\(^{2+}\)/spermidine complex, which easily shears during preparation, storage, and use. The protocol is highly irreproducible and applicable largely to plasmids and large DNA fragments, as it is virtually useless for DNA fragments, RNA, and polynucleotides smaller than 200 bp (2). Limitations in the current protocol restrain the use of biologic transfection; thus, developing more robust and reliable protocols is highly desirable. Toward this objective, we developed a new type of microprojectile that electrostatically binds DNA, RNA, oligonucleotides, and other negatively charged macromolecules. The new projectiles were made by covering the surface of conventionally used micron-sized gold microparticles with positively charged polyethyleneimine (PEI) using the previously established self-assembled monolayer (SAM) chemistry (3,4). Here we present preparation of such microparticles, their DNA binding properties, and their use for biologic transfection of an animal tissue and cell cultures.

**MATERIALS AND METHODS**

**Nucleic acid reagents**

Mammalian expression vector pCMV\(\beta\)Luc+ (6.6 kb) carrying a codon-optimized firefly luciferase gene (LUC+) from the pGL3-basic vector (Cat. no. E1751; Promega, WI, USA), under control of an enhanced cytomegalovirus (CMV) promoter and flanked by a human growth hormone terminator (5) was prepared using the EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA, USA). The LUC+ linear expression element (LEE, 3.3 kb) was assembled as described in Reference 5 from three independently PCR-amplified pCMV\(\beta\)Luc DNA fragments: CMV\(\beta\) promoter was amplified with 5\'-CGGGCGAGTTACAGCTGAATCTA-3', 5\'-ACUACUACUACUAGCTAAACTATAGTGAGTCAAAATTAGAGTTACAGC-3'; luciferase ORF with 5\'-GUAGUAGUGUAAGAGCCATGGAGAACGGCGACCAGCACCACAAC-3', 5\'-AUGAUAGUAGUAUGAUCTCTAAGATCTACACGGCGATC-3'; and hGH terminator with 5\'-AUCAUCAUAUAUAUAGGGCAUCCCTGTGACCCCTCCC-3', 5\'-GGCTGTGGGACTGGAGAA-3' primers. Silencer firefly luciferase (GL2+GL3) siRNA and irrelevant negative control siRNA were purchased as parts of a firefly luciferase siRNA silencer kit (Ambion, Austin, TX, USA).

**Preparation of conventional DNA loaded gene gun cartridges**

To make a batch of 10 conventional DNA-loaded cartridges for use in the Helios Gene Gun (Bio-Rad Laboratories, Hercules, CA, USA), 10 \(\mu\)g of DNA was mixed with 10 mg of unmodified gold microparticles (Cat. no. J5G2000; Ferro, Cleveland, OH, USA) in 200 \(\mu\)l of deionized water. The mix was agitated by vortexing, during which 200 \(\mu\)l of 2.5 M CaCl\(_2\) and 20 \(\mu\)l of 0.5 M spermidine were quickly added to precipitate DNA. After 10–15 min incubation on ice, the DNA-coated gold particles were spun down by brief centrifugation, liquid was aspirated, and gold was washed in several changes of 100% ethanol. After the final wash, gold particles were resuspended in 550 \(\mu\)l of 100% ethanol supplemented with 0.005% polyvinylpyrrolidone (PVP) and transferred into a piece of Teflon tubing (0.125-in outside diameter, 0.93-in inside diameter) and left on an even surface for gold to settle. After the gold settled, ethanol was gently drained from the tubing and the gold was dried.
by gently flowing dry nitrogen or argon gas through the tubing. Individual gene gun cartridges were made by cutting the tubing into 0.5-in sections. Each section contained 1 mg of gold microparticles coated with 1 μg DNA.

Preparation of PEI-coated gold microparticles (PEI-gold)

PEI-gold was prepared from the spherical 1.5-μm diameter gold particles, which were first cleaned of organics with Piranha solution (H₂O₂: H₂SO₄, 3:1), and washed and stored according to recommendations of Asemblon, Inc. (Redmond, WA, USA). First, a tiopronin monolayer was built by incubating 10 g of clean gold microparticles in 20 ml of 25 mg/mL solution of tiopronin [N-(2-mercaptopropionyl)-N′-ethylcarbodiimide hydrochloride; CAS no. 1953-02-2] (Cat. no. M6635; Sigma-Aldrich, St. Louis, MO, USA) in water under continuous agitation overnight at room temperature. The following day gold particles were washed three times to remove excess tiopronin with 0.5 M NaCl, 0.1 M MES pH 6.0. The tiopronin-coated gold was resuspended in 40 ml of the same buffer supplemented with 20 mM EDC [N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride; CAS no. 25952-53-8] (Cat. no. E7750 Sigma-Aldrich) and incubated at room temperature under vigorous shaking to convert tiopronin’s carboxyls into N-sulfosuccinimidyl esters (6). After incubation for 15 min at room temperature the reaction was stopped by twice washing the gold with 0.5 M NaCl, 0.1 M MES pH 6.0. To crosslink PEI to the EDC/sulfo-NHS–activated gold, the gold particles were resuspended in 20 ml of 5% (w/v) solution of PEI (Polyethyleneimine; CAS no. 9002-98-6), MW₅₅₅, 750 K. (Cat. no. P3143; Sigma-Aldrich) in 1× PBS pH 11.5 and incubated for 1 h at room temperature under vigorous shaking. The resulting PEI-gold was washed two times with ionized water and dried. Binding capacity of the PEI-gold is associated with the freshness of the used reagents, especially EDC, which deteriorates quickly and is best if used from a freshly opened vial. Over a period of 2–3 months, PEI-gold preparations kept on the bench lost some DNA binding capacity, likely due to photo-oxidation of the surface thiols (4). To improve its shelf life, PEI-gold should be kept in a dark, oxygen-free environment.

Binding DNA to PEI-gold

To determine binding capacity of PEI-gold, 8 μg of DNA was mixed with 4 mg of PEI-gold in 400 μl of water or other solution for a period specified in the Results and Discussion section. After incubation, the gold was spun down and DNA concentration in the supernatant was determined spectrophotometrically. The quantity of DNA bound by PEI-gold was estimated by calculating the difference between the input DNA amount and the amount measured in the supernatant. For the preparation of gene gun cartridges (unless otherwise specified), DNA and PEI-gold were mixed together at 1 μg/mg, respectively, in 0.5 M NaCl, 0.1 M MES pH 6.0 and incubated with occasional agitation for at least 15 min at room temperature. The gold slurry was then spun down by gentle centrifugation and washed with an equal volume of 100% ethanol. After the final wash, the particles were resuspended in n-butanol at a concentration of 1 mg per 55 μl of gold. The slurry was transferred into the Teflon tubing and treated the same way as described above for the preparation of conventional gene gun cartridges.

Preparation of siRNA loaded gold cartridges

Three milligrams of PEI-gold were mixed with 3 μg of plasmid DNA and 300 pmol of appropriate siRNA(s) (pCMV-IRE-β-gal or pCMV-LUC+) in 200 μl of 0.1 M MES, pH 6.0. The mixtures were incubated at room temperature for 30 min, washed, and loaded into Teflon tubing as described above.

Biolistic delivery and luciferase assay

For cell culture transfection, confluent monolayers of NIH 3T3 cells (National Institutes of Health, Bethesda MD, USA) were grown...
in 24-well tissue culture plates and shot at 100 psi according to the gene gun manufacturer recommendations (Bio-Rad Laboratories). After transfection, cells were incubated for 24 h at 37°C in a CO₂ incubator. Lysates for the luciferase assay were prepared by up-and-down pipetting 200 μl of the cell lysis buffer (Promega), directly added to the well. Cell lysates were transferred into 1.5-ml centrifuge microtubes and clarified by 5 min of centrifugation at 15,000×g. For animal transfection, 2–3 month-old female BALB/c mice (Charles River Laboratories, Raleigh, NC, USA) were shot into the ear pinna at 300 psi. Mice were euthanized 24 h later at which time the ears were collected and ground in 500 μl of the cell lysis buffer using disposable handheld tissue grinders (Fisher Scientific, Pittsburg, PA, USA). The grinded tissue extracts were transferred into 1.5-ml centrifuge microtubes, clarified by 5 min of centrifugation at 15,000×g. Luciferase assays were performed with a commercial assay system (Cat. no. E1500; Promega) according to the manufacturer’s protocol. Luciferase-induced luminescence was measured on a Spectra Max M5 plate reader (Molecular Devices, Sunnyvale, CA, USA).

RESULTS AND DISCUSSION

DNA binding to PEI-gold

To determine whether structural features of a DNA sample influenced binding efficiency, supercoiled plasmid DNA, linear DNA of a variety of lengths, and synthetic oligonucleotides (oligo) were tested. DNA binding characteristics were tested on several independent PEI-gold batches. All of them showed results similar to that presented in Figure 1A. PEI-gold was incubated with slightly excessive (2 μg/mg) amounts of a supercoiled plasmid, linear, or synthetic-oligonucleotide DNA and the gold-bound fraction was determined as described in Materials and Methods. The observed binding kinetics were fastest for the single-stranded synthetic oligonucleotides and slowest for the supercoiled plasmid DNA. At the tested conditions, single-stranded synthetic oligonucleotides saturated PEI-gold after 5 min of incubation; linear double-stranded DNA bound more slowly. Although most of the binding occurred in the first 10–15 min of incubation, a 30-min incubation was required to saturate the same PEI-gold sample with linear double-stranded DNA, and supercoiled plasmid DNA required 2 h. This is in significant contrast to the binding preferences of the traditional Ca²⁺/spermidine protocol, which works best on super-
coiled plasmids and does not work at all on DNA molecules smaller than 200 bp (2).

To evaluate DNA size-dependent bias of PEI-gold binding we incubated a PEI-gold sample with two times the excessive amount of a 100-bp dsDNA ladder (New England BioLabs, Ipswich, MA, USA) and analyzed the composition of the PEI-gold bound and unbound fractions by agarose gel electrophoresis. Both samples were identical to the original mixture with respect to the relative intensity of all 12 components of the ladder, ranging from 100 bp to 1.5 kb (data not shown). We concluded that DNA binding to PEI-gold is size-independent and occurs en masse.

DNA binding was only modestly sensitive to either pH or salt (Na⁺) concentration, with acidic, high-salt solutions being favored (Figure 1, B and C). Binding was not disrupted by vortexing or sonication in water, ethanol, or n-butanol.

**Biological activity of PEI-gold delivered DNA**

Although PEI-based nanocarriers are broadly used for DNA delivery it was unclear if DNA would release from the PEI-coated gold particles (7–11). To evaluate intracellular release and biological activity of the PEI-gold–bound DNA, we conducted several experiments using a luciferase gene as a reporter. Tissue cultured or mouse ear skin cells were biolistically transfected with 1 μg of pCMViLUC+ or LUC+ LEE DNA delivered on 1 mg of either PEI-modified or conventional gold microparticles and assayed for luciferase activity 24 h later. All tested samples transfected with DNA delivered on the PEI-gold showed 20–30% higher Luc expression than those delivered on the conventional gold (Figure 2A). This may be due to the higher stability of a polycationic polymer association relative to the naked DNA (10). Indeed, >10% of the peak Luc expression was detected 3 d after biolistic DNA delivery in the tissue transfected with PEI-gold, whereas the same samples transfected with the use of the conventional gold expressed <3% in its peak activity (data not shown).

**Dose dependent activity of PEI-gold delivered genes**

A significant drawback to the conventional protocol is the limitation of DNA amount deliverable on a single microparticle. The protocol is limited to 1 μg DNA per 1 mg of particles. Greater amounts of DNA can be loaded, but due to fragility of the DNA/Ca²⁺/Spermidine complex, most of it sheds from the particles during their preparation and handling.
and as a result does not lead to higher transgene expression. To compare DNA behavior on the surface of PEI-modified microparticles, we made two identical sets of bombardment cartridges with increasing amounts of pCMViLUC+ DNA. One set was prepared with the described protocol here using PEI-gold and the other set by the traditional Ca^2+/ spermidine precipitation on the conventional gold microparticles. Samples were shot into mouse ears, which were harvested 24 h later and assayed for luciferase activity. Results of this study are shown in Figure 2B. DNA delivered on conventional microparticles stimulated dose-dependent Luc expression only when ≤1 μg DNA was loaded per 1 mg of particles. DNA loads heavier than 2 μg and loaded onto conventional microparticles were often associated with particle aggregation and did not stimulate higher gene expression. By contrast, the same DNA amounts loaded onto the PEI-gold did not cause any notable aggregation and also stimulated dose-dependent Luc expression at all tested conditions. Other data suggest that such a trend extends at least to 10 μg/mg dose with linear increase of Luc expression (unpublished data).

Biolistic transfection with siRNA

To explore the oligonucleotide binding capacity of PEI-gold, we tested its potential use for biolistic delivery of short interfering RNAs (siRNAs). Three sets of PEI-gold based biolistic cartridges were made: one set was loaded only with pCMViLUC+, another with pCMViLUC+ supplemented with an equimolar mixture of GL2 and GL3 LUC+ specific siRNAs, and third with pCMViLUC+ supplemented with an siRNA irrelevant to the Luc gene. The samples were shot into confluent monolayers of NIH 3T3 cells. Twenty-four hours later cells were harvested and assayed for luciferase activity. Results of this experiment are summarized in Figure 3C. Cells transfected with pCMViLUC+ alone expressed regular luciferase activity for 1 μg DNA dose. Cells transfected with the mixture of pCMViLUC+ and LUC+ irrelevant siRNA expressed similar levels of luciferase activity, whereas the cells transfected with the mixture of pCMViLUC+ and LUC+ specific siRNAs showed activity reduced by 90%. The observed sequence-specific 10-fold reduction is consistent with the reduction levels affected by gene-specific siRNAs delivered by liposomal transfection methods (12).

Here we report a new method for preparing microprojectile bullets for gene gun transfection which is more robust than the originally developed Ca^2+/spermidine-based protocol. Use of PEI-gold offers a number of advantages over the standard method: (i) the nucleic acid loading protocol is simple and can be conducted in a broad range of pH and salt concentrations; (ii) nucleic acid binding occurs independent of its size or conformation; (iii) significantly greater amounts of biologically active nucleic acids can be delivered; and (iv) the electrostatic basis of the interaction between PEI-gold and nucleic acids provides an opportunity for delivering pre-formulated DNA or DNA mixed with components to control stability, microparticle release, gene expression, nuclear transfer or other desirable activities.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

REFERENCES
