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Gold Nanoparticles Enhanced Electroporation for Mammalian Cell Transfection

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Abstract

Electroporation figured prominently as an effective nonviral gene delivery approach for its balance on the transfection efficiency and cell viability, no restrictions of probe or cell type, and operation simplicity. The commercial electroporation systems have been widely adopted in the past two decades while still carry drawbacks associated with the high applied electric voltage, unsatisfied delivery efficiency, and/or low cell viability. By adding highly conductive gold nanoparticles (AuNPs) in electroporation solution, we demonstrated enhanced electroporation performance (i.e. better DNA delivery efficiency and higher cell viability) on mammalian cells from two different aspects: the free, naked AuNPs reduce the resistance of the electroporation solution so that the local pulse strength on cells was enhanced; targeting AuNPs (e.g., Tf-AuNPs) were brought to the cell membrane to work as virtual microelectrodes to porate cells with limited area from many different sites. The enhancement was confirmed with leukemia cells in both a commercial batch electroporation system and a home-made flow-through system using pWizGFP plasmid DNA probes. Such enhancement depends on the size, concentration, and the mixing ratio of free AuNPs/Tf-AuNPs. An equivalent mixture of free AuNPs and Tf-AuNPs exhibited the best enhancement with the transfection efficiency increased 2-3 folds at minimum sacrifice of cell viability. This new delivery concept, the combination of nanoparticles and electroporation technologies, may stimulate various *in vitro* and *in vivo* biomedical applications which rely on the efficient delivery of nucleic acids, anticancer drugs, or other therapeutic materials.

Keywords

Electroporation; Gold Nanoparticles; Gene Delivery; Transfection Enhancement

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1. Introduction

Efficient delivery of nucleic acids often plays important roles in the treatment of various diseases^{1,2}. Its delivery involves the insertion of healthy copies of DNA or RNA probes in specific cells, which relies on either viral infection or nonviral membrane perturbation. Viral vectors offer stable and efficient transduction^{3,4}, but have safety concerns associated with oncogenesis, immunogenicity, and toxicity^{5,6}. Nonviral delivery approaches, including chemical and physical approaches, have been explored as replacements to natural viruses, but yet to reach competitive levels to their viral counterpart⁷⁻²⁹. Among nonviral approaches, gold nanoparticles (AuNPs) have been extensively explored in DNA or RNA delivery for their good biocompatibility and unprecedented combination of therapeutic and imaging capability¹³⁻¹⁸. Through the unique gold-thiol chemistry and/or electrostatic interactions, AuNPs help improve the cellular uptake of molecule probes through similar internalization routes (i.e., endocytosis) as other nanoparticles. The long intracellular delivery barriers generally prevent many nanoparticles from reaching cytosol or nucleus. With the aid of cell penetrating peptides (CPPs), AuNPs-siRNA nanoconjugates were successfully demonstrated to reach cytosol directly to improve the delivery efficiency¹⁹. However, the low release extent of siRNAs from AuNPs still leads to poor transfection efficiency due to the high affinity of AuNPs and therapeutic agents²⁰.

Compared to nanoparticle-mediated delivery routes, physical approaches have been used in the past two decades to directly deliver drugs or gene probes to desired intracellular locations (e.g., cytosol or nucleus) to attain impressive benefits in various biomedical research and clinic trials²¹⁻²⁹. Among them, electroporation figures prominently for their balance of simplicity, transfection effectiveness, less restrictions on probe or cell type, and operation convenience²⁵. In electroporation, short, high-voltage electric pulses are applied to surpass the cell membrane capacitance, making the subjected cells transiently permeable²⁸. The required transmembrane potential (V_m , in V) for reversible breakdown of the cell membrane can be estimated by:

$$\Delta V_m = \frac{3}{2} E_{ext} r \cos \theta \quad (1)$$

where E_{ext} is the electric field strength (in V/cm), r is the radius of cell (in cm), π is the angle between E_{ext} and the membrane surface. From equation (1), the breakdown sites on the cell membrane appear first at those locations which face the two electrodes (i.e., $\pi = 0^\circ$ and 180°).

Current electroporation systems have been reasonably successful while still carrying several major drawbacks which are associated with the high applied electric voltage and/or the lack of uniformity of electric pulses on all treated cells. The low electrical conductivity of the electroporation solution (e.g., for PBS, it is ~ 1.5 S/m) leads to the consumption of a large percentage of the overall applied voltage across the two planar electrodes and the actual voltage allocated on treated cells is much lower than expected, as illustrated in Figure 1a. Because of the physiological conditions requirements, increasing the ion strength (e.g., salt concentration) of the electroporation buffer is not allowed to avoid such additional voltage consumption. To achieve desired probe transfection efficiency, harsh electroporation

conditions (e.g., high-voltage pulses) are therefore necessary to ensure enough permeabilization to the majority of treated cells. This makes electroporation inevitably accompanied with unwanted effects (e.g., strong electrochemical reactions, gas bubble issue, and Joule heating), which are harmful to the survival of treated cells²⁸. Current protocols are often established on the compromise between acceptable transfection efficiency and cell viability. The recent introduction of microtechnology in electroporation research devoted to the reduction of these issues through closely patterning electrode pairs³⁰⁻⁴⁶. But these designs often sacrifice some favorite features of electroporation systems, namely simplicity, low-cost, and operation convenience.

Here we present a simple approach to enhance the transfection performance of electroporation that is compatible to most commercial electroporation instruments as well as the emerging micro/nanoelectroporation systems. In this new approach, free therapeutic probes (e.g., DNA plasmids) are directly introduced into cell cytosol through electroporation while AuNPs are added to locally enhance the electric pulse strength and control the poration area on the cell membrane with minimum operation changes. Because of their high conductivity ($\sim 4.5 \times 10^6$ S/m), the electric voltage consumed by the electroporation buffer is greatly reduced so that most of the applied electric voltage is truly imposed on cells. In addition, as the electric pulses are converged in the vicinity of AuNPs, they work like many virtual microelectrodes when staying around cells with the focused field strength to cause localized poration, as shown in Figure 1b. Different from bulk electroporation with two large breakdown locations at the two poles of cells facing the electrodes, multiple small poration sites are expected to be created on the cell membrane by AuNPs. This could benefit not only the recovery of the cell membrane and the survival of cells, but also the uptake opportunity for the subjected probes from multiple sites. As AuNPs are also randomly dispersed in the electroporation solution, just like cells themselves, they are expected to be present evenly around cells, which might further reduce the polarization variations associated with suspended state of cells in electroporation.

To test our hypothesis, we added AuNPs to the electroporation solution, together with mammalian cells and DNA plasmids. Cells were then executed using both a commercial batch-type electroporator (BTX 830 from Harvard Apparatus) and a home-made semi-continuous flow electroporator (SFE)^{41, 46}. The pulse strength focusing evaluation was done from two aspects: (i) execute cells at the presence of AuNPs under standard electroporation conditions, in which the cell viability should get worse as the electric pulse was focused by AuNPs and cells received higher-than-optimal electric pulses; (ii) perform electroporation under less-effective conditions (i.e., low-voltage, much benign pulses), in which the focusing effect helps gain sufficient electrical strength for better transfection efficiency and/or cell viability. A human chronic leukemia cells (K562 cells), a hard-to-transfect cell line, were used in this investigation so that the localized electroporation with controlled polarization area and locations on the cell membrane could also be conveniently evaluated through ligand-receptor affinity binding. The electroporation enhancement evaluation was focused on the cell viability and the transfection efficiency of a reporter gene (WizGFP). Similar enhancement performance was also observed in NIH 3T3 cells (data not shown), confirming the broad effectiveness of the enhancement roles AuNPs play in electroporation.

2. Materials and Methods

2.1 Materials and reagents

Gold nanoparticles of 5 nm, 10 nm, and 20 nm were obtained from Sigma-Aldrich. DNA plasmids with gWiz™ GFP reporter gene were purchased from Aldevron, Inc. The concentration of 1X AuNPs refers to the stock solution, which has 0.01 wt% of Au (0.1 mg/ml) while the actual particle number varies with the size of AuNPs. Other concentrations of AuNPs were prepared by either concentrating or diluting from the stock solution. All other chemicals were purchased from Sigma-Aldrich and the cell culture reagents were purchased from Life Technologies (Carlsbad, CA) unless specified.

2.2 Cell Culture

K562 lymphoblast cells were obtained from American Type Cell Culture (ATCC, Manassas, VA). K562 cells were cultured in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and L-glutamine. Cells were maintained in T-75 flasks at 37 °C with 5% CO₂ and subcultured using 0.25% (w/v) trypsin with EDTA·4Na.

2.3 Transferrin-AuNPs synthesis

Transferrin (Sigma) of 10 mg/mL in 50 mM PBS with 5 mM EDTA was first reacted with Traut's Reagent for 1 hr at room temperature. The thiolated protein was then purified by dialysis (with dialysis cartridge from EMD Millipore, Amicon cartridge, Cat no. UFC805024). AuNPs of 20 nm was then incubated with thiolated transferrin at 4°C overnight. Excess transferrin was removed by repeated centrifugation and washed with PBS. The purified Tf-AuNPs were used immediately or stored at 4°C.

2.4 Electroporation setup and procedure

K562 cells were first centrifuged and re-suspended in fresh OPTI-MEM I (a serum free medium) at a density of 0.5×10^7 cells/ml. Samples were then mixed with AuNPs of various concentrations (0.01-1.0 mg/ml) and sizes (5, 10, and 20 nm) and further added 25 µg DNA. Electroporation with a commercial instrument (ECM 830, Harvard Apparatus) was done in electroporation cuvettes with a 2-mm gap, each containing a 100 µL sample solution. Two pulse conditions were used: 125V, 10 ms (standard electroporation conditions) and 95 V, 10 ms (low-voltage pulse, much benign electroporation conditions) with a single pulse.

SFE microchannel electroporation was done by pumping cells through a serpentine microchannel (length $\times$ width $\times$ depth = 37.5 mm $\times$ 150 µm $\times$ 150 µm) at a low flow rate (e.g., 1.8 ml/hr) and electric pulses (76 pulses with each at 16V, 10 ms) were added through Pt electrodes. Cells were then flushed out with Opti-MEM I medium and collected for further analysis. Detailed SFE operation procedure can be found in our early publication⁴¹.

After electroporation, samples were transferred to 6-well cell culture plates, incubated in fresh medium for another 24 hr, and then harvested for analysis.

2.5 DNA delivery efficiency and cell viability

The transfection efficiency of pWizGFP plasmids was evaluated both qualitatively by visualizing the number of cells with green fluorescence within a representative area selected from the entire culture surface under an inverted fluorescence microscope (Olympus, Japan) and quantitatively by counting cells using a four-color flow cytometry system (FACS Calibur, BD Biosciences, CA) 24 hr post transfection. Briefly, an amount of 1.5×10^6 cells/mL was collected and the percentage of GFP-positive cells was calculated quantitatively via flow cytometer. The unstained samples were run first to adjust the voltage setting and compensation of the flow cytometer. Then the tested samples were processed by CellQuest. At least 10,000 events were collected for each sample.

The cell viability was evaluated by an MTS cell proliferation assay (Promega, Madison, WI). Briefly, the cells in 100 μ L/well of medium were transferred to a 96-well plate and incubated. 20 μ L of CellTiter 96 AQueous One solution (Promega, Madison, WI) was added to each well and the cells were incubated at 37°C for another 4 hr. Absorbance was measured at 492 nm on an automated plate reader (Elx 800, Biotek, VT). Data points were represented as the mean $\pm$ standard deviation (SD) of triplicates, unless otherwise indicated.

2.6 AuNPs imaging and tracking in vitro

The distribution, cellular binding, and uptake of AuNPs in K562 cells were examined by laser scanning confocal microscopy. The mixture of cells and red fluorescence of AuNPs (from Nanopartz, Inc) was washed twice with 1X PBS, followed by fixation with 4% paraformaldehyde for 30 min. Nuclei were stained with 20 μ M of DAPI for 5 min at room temperature. Cells from each sample were then mounted on cover glass slides. Images of phase contrast, red and blue fluorescence channels were taken on a Zeiss 510 META Laser Scanning Confocal microscopy (Carl Zeiss MicroImaging, Inc., NY) and then merged images were produced using the LSM Imaging software.

As AuNPs are well known to quench the fluorescent signal from proximal fluorophores, a sandwich design of AuNPs (from Nanopartz, Inc, having fluorophores separated from the gold surface with polymer spacer) were used to circumvent this problem. Fluorophore labeled AuNPs with CH₃ group terminated surface (FNPs, 10 nm for gold nanoparticle core) were used to represent free, naked AuNPs used in electroporation experiments. To visualize and track Tf-AuNPs, carboxylated AuNPs (FNP-COOH) were used. FNP-COOH nanoparticles (1 mg/mL in PBS) were first incubated with 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (20mg/mL in PBS) for 15 min at room temperature. Transferrin solution (0.4 mg/mL in PBS) was then added and incubated at room temperature for 24 hr to obtain FNPs with transferrin targeting probes (Tf-FNPs). Tf-FNPs particles were then purified by repeated centrifugation and re-suspension in PBS for three times prior to the binding with transferrin receptors (TfR) on K562 cell surface.

2.7 Simulation on the focusing effect of AuNPs

A commercial finite-element methods (FEM) software, COMSOL (Mathworks, Natick, MA), was used to calculate the electric field around cell at the presence of a single AuNP. We considered an axial symmetric model with one gold nanoparticle ($d = 20$ nm) embedded

in cell membrane (5 nm in thickness). A K562 cell ($D = 15 \mu\text{m}$) was placed at the center of the left side boundary (the symmetrical axis) in the computation domain ($60 \mu\text{m} \times 20 \mu\text{m}$). A polar angle (π) with respect to the electric field direction was defined and the gold nanoparticle was placed at the top of the cell where $\pi = 180^\circ$. An electric field ($E = 475 \text{ V/cm}$) was assigned across the top and bottom of the computation domain and the right side boundary was set as insulated wall. A three-layer cell model, divided as the external medium, the cell membrane, and the cell cytoplasm, was setup here^{47, 48}. The electric field distribution around the nanoparticle and the cell was calculated by solving the Laplace equation using COMSOL:

$$\nabla \cdot (\sigma \nabla V) = 0 \quad (2)$$

where σ is electrical conductivity and V is the electrical potential. The electric field strength was then determined by $E = -\nabla V$. In this three-layer cell model, the electrical conductivity of buffer, cytoplasm, membrane, and gold particle was set as 0.8, 0.2, 5×10^{-7} , and $4 \times 10^7 \text{ S/m}$, respectively.

3. Results and discussions

3.1 AuNPs enhancement on the transfection of mammalian cells

We first did electroporation with K562 cells in both BTX and SFE systems, adopting the pulse conditions which were previously optimized with WizGFP plasmids alone: 125 V (625 V/cm), single 10 ms pulse for the BTX system and 16 V (1067 V/cm), multiple 10 ms pulses for the SFE system. Successful transfection was observed in all four cases: BTX without AuNPs, SFE without AuNPs, BTX with AuNPs, and SFE with AuNPs. Many cells in each case expressed green fluorescence protein (GFP) 24 hr after electroporation (Figure 2a). More quantitative comparison was done by counting the percentage of GFP-positive cells (Figure 2b). Efficiency of pGFP transfection from SFE was generally much better than that from BTX (BTX: $27.5 \pm 1.9\%$, SFE: $51.6 \pm 4.5\%$), which is consistent with our earlier observations^{41, 46}. After adding AuNPs (5 nm at a concentration of 5X or 0.5 mg/ml), the transfection percentage was significantly increased to $50.8 \pm 6.7\%$ for BTX electroporator and to $61.1 \pm 4.8\%$ for SFE microchannel electroporator, respectively.

Some loss on the cell viability was observed (from $78.9 \pm 2.9\%$ to $57.4 \pm 5.1\%$ for BTX electroporator and from $69.9 \pm 4.7\%$ to $52.1 \pm 2.3\%$ for SFE microchannel electroporator), as shown in Figure 2c. This is not surprising considering the focusing effect of AuNPs could shift away the electric pulses from the desired strength. As mentioned earlier, K562 cells in Figure 2 were executed at electroporation conditions optimized without the presence of AuNPs. Considering the high conductivity of AuNPs, their addition greatly reduced the resistance contributed by the buffer solution so that most electric voltage imposed between the two electrodes was allocated to cells. The actual pulse strength on the cell membrane was therefore mitigated to a higher-than-optimal level, resulting in over perturbation to the treated cells. Such harsh conditions increased the percentage of irreversible breakdown of the cell membrane, making the loss of the cell viability inevitable. As this pulse strength focusing effect is generated from the presence of free AuNPs in the electroporation buffer, the cell transfection efficiency can get improved (when reversible breakdown still

dominates) or become worse (when irreversible breakdown becomes the dominant--cells might have probes successfully delivered while not get the subjected transgenes expressed prior to lysis), depending on the concentration and size of the added AuNPs. From data shown in Figure 2c, the field focusing level for 5 nm AuNPs at a concentration of 5X (0.5 mg/ml) belonged to the first case (i.e., reversible breakdown still dominated). The transfection of pWizGFP got improved while accompanied with lower cell viability. Nevertheless, this confirmed the electric field focusing effect of AuNPs to electroporation. Note: the transfection percentage is defined as the number of transfected cell divided by the number of total living cells 24 hr post transfection in each sample and the cell viability is measured as the ratio of the living cells in each sample to that in the negative control samples. These definitions might be different from some others used in literature (divided by the number of cells initial used or cells survived right after transfection) and emphasize the fate of all survived cells, though sometimes show low values on the transfection (or the cell viability) for their large number of the total living cells.

Our FEM simulation confirmed the enhancement effects of AuNPs to electroporation. The electrical potential distribution is plotted by colorful contours while the electric field lines through buffer, the gold nanoparticle, and the cell are shown by blue lines in Figure 3. Because of the high conductivity, the electric field is clearly focused near the gold nanoparticle. Such localized focusing effect could also help attract charged DNA molecules from the surrounding area towards the focusing spot and enrich them there. As transient pores will form later at the same location, we also compared the total current passing through the pore with and without a gold nanoparticle around. It was found that the current was enhanced 34% (from 1.77 nA to 2.38 nA) when a gold nanoparticle was around (Figure 3c). This suggests that the charged DNA plasmids could transport faster with AuNPs in close proximity and more of them could be delivered into cells before the resealing of the cell membrane.

As the presence of AuNPs greatly affect the actual pulse strength on treated cells, their size and number presented around each cell are critical to the electric pulse strength focusing level and the resulting electroporation performance (the transfection efficiency and cell viability). In the following sections, we evaluated the dependence of the pulse strength focusing effect on the size and concentration of AuNPs.

3.2 The dependence of electroporation enhancement on the concentration of AuNPs

AuNPs of various concentrations (0.1X-10X of the stock solution) were used to evaluate the pulse strength focusing effect using the BTX electroporator. Similar to the aforementioned results, when electroporating cells at their standard pulse conditions (625V/cm, single 10ms pulse), the transgene expression enhancement generally sacrificed some of the cell viability with the increase of the AuNPs concentration (Figure 4a). After adding 5 nm AuNPs at a concentration of 0.1X-1X (1X=0.1mg/mL AuNPs), the cell viability retained at the same level ($71.9\pm5.1\%$ – $68.9\pm2.9\%$) as in electroporation with naked DNA. But that value started dropping gradually when more concentrated AuNPs solutions (2.5X-10X) were used. Such cell viability loss endorsed the field enhancing effect of free AuNPs mentioned earlier. Because the buffer resistance was reduced when adding AuNPs, the local pulse strength on

cells was focused. When starting from the standard pulse conditions, some treated cells were over-perturbed to lethal levels. When increasing the concentration of AuNPs, this pulse focusing effect got continuously enhanced and more cells were over-polarized or died. As the consequence, the cell viability dropped. A threshold concentration of AuNPs existed for this pulse-focusing effect: it did not become obvious until the number of AuNPs reaches a certain level (e.g., 1X, 7.91×10^{13} particles/ml for 5 nm AuNPs). Further enhancing the pulse strength focusing effect led to loss of cell viability, but beneficial to the improvement of the transfection efficiency. For 5 nm AuNPs, the transfection efficiency increased from ~25% (bulk electroporation with DNA only) to the maximum enhancement of ~51% at the concentration of 5X (0.5 mg/mL or $\sim 3.96 \times 10^{14}$ 5 nm AuNPs/ml) when increasing AuNPs concentration and started decaying afterwards due to the significant loss on the cell viability (Figure 4b).

As the electric pulses were generally over-focused when concentrated AuNPs were introduced at standard electroporation protocols, such enhancement might be more beneficial when more benign conditions are used (at these conditions, transfection with naked DNA alone is less effective as the consequence). At these conditions, the cell viability is certainly high and the enhancement is mainly contributed to the improvement on the transfection efficiency of molecular probes. During our investigation, to ensure the local pulse strength was still effective for the majority of cells, we lowered the overall electric voltage (while keeping the pulse duration unchanged) to a minimum field strength that was just enough to transfect a statistically meaningful number of cells with DNA probes alone. For K562 cells, this minimum condition was 475 V/cm (95 V when tested with 2 mm BTX cuvettes) with a $13.6 \pm 1.5\%$ transfection efficiency of naked DNA using the BTX electroporator. As shown in Figure 4c, for all three sizes of AuNPs, similar cell viability ($\pm 10\%$) was achieved within a broad concentration range of AuNPs (0.1X-10X). Different from the enhancement performance at standard conditions, continuous increase on the transfection efficiency was achieved for all cases (Figure 4d). Such improvements were not only significant when compared to that using naked DNA (i.e., ~14%) at the same pulse condition, but also much better than the best performance the BTX electroporator could achieve with naked DNA (i.e., ~25%). This additional gain on the transfection efficiency at benign electroporation conditions further confirmed from another viewpoint the focusing effect of free AuNPs--low-voltage pulses could be focused to high enough levels to provide the needed transmembrane field strength for better transfection efficiency ($40.0 \pm 4.1\%$ for 5 nm, $45.2 \pm 4.0\%$ for 10 nm, $56.1 \pm 3.3\%$ for 20 nm). More important, such delivery enhancement was attained with no or little sacrifice of the cell viability for the application of low-voltage pulses.

3.3 The dependence of electroporation enhancement on the size of AuNPs

Besides the concentration effect, the size of AuNPs also contributes to the reduction of the buffer resistance and the pulse strength focusing level on cells. Moreover, the size of AuNPs could affect the poration area on the cell membrane if AuNPs are brought close enough. As shown in Figures 1b and 3b, AuNPs converge the electric field on their two poles and when hang around cells. They work as many tiny virtual electrodes with focused pulses pointing towards the cell membrane. This could induce the polarization on the cell membrane within

limited area (i.e., localized poration), which has been found to be beneficial for the electroporation performance^{33, 36, 42}. Therefore, the electroporation enhancement with various sizes of AuNPs reflects a combination of the pulse strength focusing effect and localized electroporation benefits. Three different sizes of AuNPs (5 nm, 10 nm, and 20 nm) were tested here and their effects on the transfection efficiency and the cell viability are included in Figure 4. Similar to AuNPs of 5 nm, AuNPs of 10 nm and 20 nm exhibited similar concentration dependence on the transfection efficiency and the cell viability. As the concentration of AuNPs in Figure 4 were calculated based on the weight percentage of added AuNPs, their buffer resistance reduction effect or the pulse strength focusing level should be similar when the particle concentration is constant. In other words, at the same concentration of AuNPs, the enhancement difference for cases in Figure 4 reflected mainly the contribution of various particle sizes to the localized electroporation benefit.

The enhancement difference on the transfection efficiency among various sizes of AuNPs was marginal at low AuNPs concentrations and became significant only when concentrated AuNPs were used. For 625 V/cm pulses, this started from 1X AuNPs, and for 475V/cm pulses, it was from 2.5X due to their different pulse strength focusing levels and localized electroporation situations. This is reasonable as the pulse strength focusing effect was weak at low AuNPs concentrations. Similarly, localized electroporation was very limited at low AuNPs concentrations, as only a small portion of total free AuNPs could aggregate around cells. In excess of the threshold AuNPs concentration, their contributions on the field focusing effect and localized poration became more pronounced so that the benefit on the transfection efficiency improvement showed up. The larger the size of AuNPs, the better transfection efficiency was achieved. Large AuNPs of a relative lower concentration could also help gain better transfection efficiency than small AuNPs at a higher concentration. For example, electroporation with 2.5X and 5X AuNPs of 20 nm help achieve similar or even better pGFP transfection than 5X and 7.5X AuNPs of 5 nm, respectively (Figures 4b & 4d). Among various particle sizes, the cell viability difference at the same pulse strength focusing level (i.e., the same AuNPs concentration) was marginal in most cases. Therefore, these AuNPs (5 to 20 nm) are appropriate for the electroporation enhancement without extra addition to the cell toxicity.

However, these results also suggested that, with free AuNPs (i.e., AuNPs that are randomly dispersed in the electroporation buffer), the electroporation enhancement was mainly decided by the pulse strength focusing effect or the concentration of AuNPs. Localized poration only became beneficial at high AuNPs concentration when sufficient AuNPs presented around cells during electroporation. But this easily leads to over-perturbation if added AuNPs are all free AuNPs. To further enhance the localized poration effect, sufficient number of AuNPs must be brought close to cells through some pre-concentration approaches.

3.4 Enhancing localized electroporation with transferrin-AuNPs

As many transferrin receptors (TfR) are available on the cell membrane of K562 cells, AuNPs were conveniently brought to cells by grafting transferrin (Tf) molecules on their surface⁴⁹. This was done with the help of the high affinity of sulfhydryl groups to the gold

surface⁵⁰. Specifically, sulfhydryl groups were introduced to transferrin molecules by converting a small proportion of their primary amine groups to sulfhydryl groups with Traut's reagent. The modified transferrin with sulfhydryl groups were then incubated with free AuNPs to form transferrin AuNPs (Tf-AuNPs), as shown in Figure 5a. To evaluate how this transferrin-targeting mechanism affected the localized electroporation, we incubated Tf-AuNPs of 1X with K562 cells for various incubation times and compared their performance on transfection enhancement. As shown in Figure 5b, the best improvement occurred in samples having 4-hr incubation time and the transfection efficiency reached $41.7 \pm 3.2\%$ when compared to that of BTX with naked DNA ($26.4 \pm 1.9\%$) and BTX with free AuNPs ($34.4 \pm 2.9\%$). Such 50% or less enhanced performance resulted from the fact that the gradual depletion of mobile AuNPs in the electroporation buffer because of Tf-AuNPs grafting on the cell membrane. As the consequence, though localized electroporation got improved, the pulse focusing effect from free AuNPs diminished.

To retain both the pulse strength focusing and localized electroporation advantages, we added free AuNPs to Tf-AuNPs at various mixing ratios (0%, 25%, 50%, 75%, and 100% Tf-AuNPs). Based on other pioneering work on transferrin targeting, it took about 4 hr incubation to accomplish complete affinity binding of transferrin to the TfRs on cells⁵¹. Therefore, we first incubated K562 cells with Tf-AuNPs for 4 hr and then added the needed quantity of free AuNPs right before electroporation. As shown in Figure 6a, such a combination showed better enhancement on the transfection efficiency under the standard electroporation conditions with only 1X total AuNPs (free AuNPs + Tf-AuNPs) while the actual improvement varied with their mixing ratio: a sustained increase was seen on the transfection efficiency when more Tf-AuNPs were added until reaching a 50%/50% mixture of free-AuNPs and Tf-AuNPs (the transfection efficiency reached $58.2 \pm 1.8\%$), followed by some declines. This provides ~2.5 folds increase on the DNA transfection when compared to electroporation with naked DNA only. Considering the low concentration of AuNPs used here (only 1X), the electroporation enhancement with a combination of free AuNPs and Tf-AuNPs seems more effective than that using free AuNPs or Tf-AuNPs alone. The best enhancement came from an appropriate balance on the pulse strength focusing and localized electroporation advantages AuNPs offered. It is worth to mention that such a transfection efficiency improvement was achieved without sacrificing much of the cell viability (Figure 6a). At more benign conditions (475V/cm), the enhancement was not very obvious, consistent with the free AuNPs enhancement result at low concentrations (0.1X-1X). This insufficient pulse focusing level cannot provide desired transmembrane potential to polarize the majority of cells. When more AuNPs were introduced (e.g., 5X for the total AuNPs concentration), the enhancement on the transfection efficiency became significant for pulses of both 625 V/cm and 475 V/cm and the equivalent mixture of free AuNPs and Tf-AuNPs still offered the best transfection efficiency (Figure 6b). However, because of the over-focusing effect, obvious loss of the cell viability was found for the case with the pulse strength of 625 V/cm, consistent with our early observations.

3.5 AuNP Imaging and tracking in vitro

We tracked the cellular uptake of AuNPs before and after electroporation for both free, naked AuNPs and Tf-AuNPs using confocal microscope and the results were shown in

Figure 7. As free AuNPs were mixed with cells right before electroporation, the short contact time did not provide enough time to allow endocytosis-based uptake of AuNPs and no obvious fluorescently labeled AuNPs (FNPs) were observed (Figure 7a). After electroporation, many FNPs were clearly found in cell cytoplasm (Figure 7b), indicating that AuNPs transported into cells after electroporation. As all samples were fixed shortly after electroporation, we believe the majority of FNPs were taken through the transient openings on the cell membrane, not the endocytosis process. As mentioned in our FEM simulation, AuNPs around transient pores could enhance the cellular uptake of DNA plasmids because the increase of electrical current (see section 3.1). Figures 7c and 7d showed the distributions of Tf-FNPs before and after electroporation (Note: Tf-FNPs alone, not a mixture with free FNPs were used here). The accumulation of Tf-FNPs on the cell membrane was clearly found in samples without electroporation treatment (Figure 7c), confirming the formation of ligand-receptor bonds after incubation. After electroporation, FNPs were also found in the cell cytoplasm (Figure 7d), in consistent with what exhibited in free FNPs electroporation. But unlike in free FNPs electroporation sample, many Tf-FNPs were also found on the cell membrane or regions nearby, suggesting that at least some Tf-AuNPs conjugates and the coupling of Tf-TfR could survive the electroporation process.

4. Conclusions

AuNPs were used to enhance *in vitro* delivery of DNA probes for both batch-type and flow-through electroporation systems. Highly conductive free AuNPs were added to the electroporation buffer to reduce the solution resistance so that the pulse strength on cells could be enhanced. Tf-AuNPs were brought to K562 cells through affinity binding with TfR receptors on the cell membrane, serving as many virtual microelectrodes to locally polarize cells from various sites, each affected only limited area. In this way, electroporation was enhanced with better transfection efficiency and the same or higher cell viability. With DNA plasmids carrying a WizGFP reporter gene, we confirmed the pulse strength focusing effect after adding free AuNPs in the electroporation buffer and investigated its dependence on the particle size, concentration, and electroporation conditions. We also evidenced the contributions of localized electroporation with Tf-AuNPs. An equivalent mixture of free AuNPs and Tf-AuNPs was found to provide the best enhancement performance while the optimal concentration of AuNPs was decided by the original pulse conditions. This study offers a new approach to improve the delivery efficiency of nucleic acids or anticancer drugs through the combination of nanoparticles and electroporation technologies. AuNPs were adopted here for their low cost and easy accessibility while other forms of gold nanostructures, such as nanorod, nanoshell, or nanowires, in principle, could be used for similar purposes. As these gold nanomaterials have been widely explored in sensing, imaging, diagnosis, and therapeutic applications, our approach demonstrates a new function of these nanomaterials and/or broadens their potentials for multiple-function applications in drug discovery and clinical practice.

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Figure 1.

Schematic illustration on the mechanism of AuNPs enhancement on electroporation: (a) The pulse enhancement effect through minimizing the electric voltage consumed by the low conductive electroporation buffer during electroporation. By adding highly conductive AuNPs, more percentage of the overall electric voltage across the two electrodes is allocated on cells to have focused pulses when compared to the use of electroporation buffer alone; (b) localized electroporation when AuNPs are brought to the cell membrane through affinity binding with receptors there. The electric field is converged on the conductive AuNPs and these AuNPs could serve as virtual electrodes to polarize only limited area on the cell membrane when stay nearby.

Figure 2.

Gold nanoparticles enhancement on electroporation of K562 cells with a commercial batch electroporation (labeled as “BTX”) system and a home-made semi-continuous microchannel system (labeled as “microchannel”). Panel (a) exhibits fluorescence and phase contrast microscopic images of pGFP plasmid transfection through BTX, BTX with AuNPs, Microchannel, and Microchannel with AuNPs. Panels (b) and (c) are quantitative results of the transfection efficiency (b) and the cell viability (c). The concentration of AuNPs used here is 5X or 0.05 wt% (0.5 mg/mL). n=6 and (***) represents $p < 0.005$.

Figure 3. Simulation of the electric field focusing effect of AuNPs in electroporation. (a) The model and meshes setup for one gold nanoparticle embedded in the membrane of a K562 cell. (b) The calculated electric field lines around the AuNP. (c) The electric field around a transient pore on the cell membrane at the presence of one AuNP around.

Figure 4.

Dependence of the pulse enhancement on the size and concentration of free AuNPs: panels (a-b) are the cell viability (a) and the transfection efficiency (b) with an overall pulse strength of 625 V/cm and panels (c-d) are the results with an overall pulse strength of 475 V/cm (panel c: the cell viability; panel d: the transfection efficiency). The blue and red dash lines refer to the cell viability and the transfection efficiency of electroporation with naked DNA at the optimal conditions (675 V/cm, single pulse of 10 ms), respectively. 1X AuNPs refers to 0.01 wt% or 0.1 mg/mL gold content.

Figure 5. Localized electroporation enhancement with Tf-AuNPs: (a) schematics of grafting Tf-AuNPs as virtual electrodes on the cell membrane, (b) the localized enhancement with Tf-AuNPs alone at various binding stages. The AuNPs used here are 20 nm with 1X concentration (0.1 mg/mL).

Figure 6.

The combined enhancement of the pulse strength focusing and localized electroporation effects using a mixture of free AuNPs and Tf-AuNPs of various mixing ratios with a total AuNPs concentration of 1X (a) and 5X (b) with the pulse strength of 625 V/cm and 475 V/cm. AuNPs of 20nm were used here.

Figure 7.

The confocal microscope images of the cellular uptake of AuNPs before and after electroporation: (a-b) for free FNPs and (c-d) for Tf-FNPs. Images in panels (a) and (c) were taken before electroporation and panels (b) and (d) were after electroporation. FNPs of 10X or 0.1 wt% (1.0 mg/mL) were used in all samples and Tf-FNPs were incubated with cells for 4 hr.