Improved electroporation parameters of delivering silver nanoparticles into living C666 cells for surface-enhanced Raman scattering

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Abstract. Electroporation assisted metallic nanoparticle delivery has been shown by our previous work to significantly reduce the time of sample preparation for surface-enhanced Raman spectroscopy (SERS) measurements of biological cells. In this research note, we report our experimental work to optimize the electroporation parameters, including adjustment of the pulse pattern and operation temperature, for fastest delivery of silver nanoparticles into living C666 cells (a human nasopharyngeal carcinoma cell line). The delivery efficiency was evaluated by the integrated intensity of whole cell SERS spectrum. Our work concluded that the silver nanoparticle delivery rate is best under the electroporation condition of using 4 consecutive 350 V (875 V/cm) rectangular electric pulses of 1 ms, 10 ms, 10 ms, and 1 ms durations respectively. Low temperature (0–4°C) is necessary for improving the delivery efficiency of silver nanoparticles.

1. Introduction

With advantages in detection sensitivity, selectivity, and specificity, surface-enhanced Raman scattering (SERS) spectroscopy is an analytical method with rapid expanding applications in chemical and biochemical analysis and detection. One such new application is intracellular SERS measurement, in which the metallic nanoparticles such as colloidal silver or gold are delivered into living cells to serve as the enhancing agent. At present, “endocytosis” is the dominant technique for delivering metal nanoparticles into living cells[1-3]. Besides this technique, biosynthesis of nanoparticles has also been tried in intracellular SERS study[4-5]. However, both the endocytosis technique and the biosynthesis technique are time-consuming and silver or gold nanoparticles have to be incubated with living cells at 37°C for more than several to twenties hours prior to the SERS experiments. For many biomedical applications, such as cancer screening, keeping the cells alive for such a long time and delivering nanoparticles in such a slow fashion bring extra procedures and increase the cost.

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To overcome the disadvantages of the two existing delivery techniques, we have recently developed an electroporation based method that can rapidly deliver silver nanoparticles into living cells within one minute and successfully carried out intracellular SERS measurements[6]. Electroporation involves the application of controlled electrical pulses to induce transient and reversible pores on the cell membrane. For a moment, the cell membrane becomes highly permeable to exogenous substances in the surrounding media[7-8]. Although electroporation is a powerful delivery technique for many molecules[8], silver nanoparticles are not so easy to be delivered into living cells, because, electroporation efficiency is influenced by many factors, such as voltage, pulse width, cell condition, operation temperature, serum concentration in electrical buffer[9-11]. Therefore, the primary objective of this study is to investigate the optimal electroporation parameters for obtaining the greatest delivery efficiency and at the same time ensuring cell viability. This optimization is important for practical applications of electroporation assisted intracellular SERS spectroscopy.

2. Materials and Methods

2.1. Preparation of silver nanoparticles
Colloidal silver was prepared by the aqueous reduction of silver nitrate with hydroxylamine hydrochloride[12]. First 4.5 mL sodium hydroxide (0.1 mol/L) was added to 5 ml hydroxylamine hydrochloride (6 × 10⁻² mol/L) and then the mixtures were added rapidly to 90 mL silver nitrate (1.11 × 10⁻³ mol/L) with vigorous shaking. The silver colloids were kept in dark at room temperature. The absorption maximum of the silver colloid was located at 419 nm, very close to the value from literature of 416 nm[12].

2.2. Cell culture
Human nasopharyngeal carcinoma cells (C666 cell line) were obtained from the Fujian Medical University Union Hospital. C666 cells grow in the complete RPMI 1640 medium (supplemented with 100 IU/mL penicillin/streptomycin and 10% fetal calf serum, hereafter referred to as RPMI 1640) at 37°C and 5% CO₂. Before electroporation, C666 cells were harvested from monolayer cultures, then the cells were washed three times by phosphate buffered saline (PBS) to remove any residual medium. After the cells were centrifuged, the cells were suspended in electroporation buffer with a density of 10⁶ cells/mL, finally 800 μL cell suspension and 200 μL silver colloids were mixed thoroughly and added into an electrode cuvette. The silver colloid concentration used in this experiment was reduced by 10 times compared to our previous work[6] to avoid the excessive accumulation of silver nanoparticles surrounding the cells, which is hard to wash off.

2.3. Stimulation parameters and operation conditions for cell electroporation
Based on our previous study[6], we used a constant voltage of 350 volts (corresponding to an field strength of 875 V/cm) for all the experiments. Other parameters including different series of electronic pulses and operation temperature were varied to identify optimum settings for electroporation delivery of silver nanoparticles into living C666 cells. Thus two optimization experiments were performed as outlined below. (1) Influence of different pulse patterns. The electroporation voltage was kept at 350 V (field strength of 875 V/cm), while four different pulse patterns were tested for best silver nanoparticle delivery efficiency: ① 1 ms pulse width pulses for two times, ② three consecutive pulses with pulse width of 1, 10, and 1 ms respectively, ③ four consecutive pulses with pulse width of 1, 10, 10, and 1 ms respectively, ④ five consecutive pulses with pulse width of 1, 10, 10, 10, and 1 ms respectively. For each experiment, the cell/silver nanoparticle mixture was first added into an electrode cuvette and incubated for 10 min at 4°C on ice and then transferred to an electroporation chamber to carry out the electroporation. The electrical buffer used was the complete RPMI 1640 medium. After each electroporation, the cuvette was incubated again for 10 min at 4°C on ice. We also had a cell/silver nanoparticle mixture without electroporation as the control experiment. After the
electroporation procedure, the C666 cells were washed twice with PBS to remove any residual buffer medium. After that, the cells were measured under the Renishaw Micro-Raman spectrometer to obtain the SERS spectrum. In the control experiment, the C666 cells were also washed twice with PBS before the SERS spectral measurements. (2) Influence of operation temperature. For the electroporation experiments with 350 V voltage and four consecutive pulses of pulse width 1, 10, 10, 1 ms and in a complete RPMI 1640 medium buffer, two different operation temperatures were tested: cold (0–4°C on ice) and ambient room temperature (20–25°C).

2.4. SERS measurements
A Renishaw Raman microscope (InVia system) with a ~2 spatial resolution, a 20 mW, 785 nm, semiconductor laser as excitation source, was used for the collection of SERS spectra. The microscope was operated under a 50× objective, which focused the laser beam onto a spot on the sample and the illumination pinhole was adjusted for the laser spot to cover the whole cell. The typical spectral accumulation time in our study was 10 seconds. Raman spectra were scanned over a wavenumber range of 400 – 1800 cm\(^{-1}\) with respect to the excitation frequency.

3. Results and discussion
After electroporation, the cell morphology was observed by 50× microscopy. Figure 1 shows three example images. All the cells treated by pulse pattern ① (1 ms pulse width pulses for two times) and pattern ② (three consecutive pulses with pulse width of 1, 10, and 1 ms) and most of the cells treated by pulse pattern ③ (four consecutive pulses with pulse width of 1, 10, 10, and 1 ms) have normal looking morphology (Figure 1(a)). A significant portion of the cells treated by pattern ④ (five consecutive pulses with pulse width of 1, 10, 10, 10, and 1 ms) are broken (Figure 1(b)); some are fused together (Figure 1(c)). The darker part in Figure 1(b) shows the accumulation silver nanoparticles, probably originated from the silver nanoparticle rushing-in after the cell membrane was irreversibly broken.

We measured the SERS spectra of the control cell and cells at different electroporation treatment experiments. The signal of spectrum measured from the control cell (without electroporation) is very noisy; no reliable Raman peaks are seen. The signal of SERS spectrum measured from individual living C666 cells treated by electric pulse pattern ① (1 ms pulse width pulses for two times) are no reliable Raman peaks; SERS spectrum measured from individual living C666 cells treated by electric pulse pattern ② (three consecutive pulses with pulse width of 1, 10, and 1 ms) are visible, but not good enough S/N. Good quality SERS spectra were obtained from individual living C666 cells treated by electric pulse pattern ③ (four consecutive pulses with pulse width of 1, 10, 10, and 1 ms), containing a wealth of intracellular surface enhanced Raman signals. This is the optimal electroporation parameter for efficient silver nanoparticle delivery into living C666 cells. The quality of example SERS spectrum measured from individual living C666 cells (normal looking morphology) treated by electric pulse pattern ④ (five consecutive pulses with pulse width of 1, 10, 10, 10, and 1 ms) is as good as treated by electric pulse pattern ③. However, a significant portion of the cells treated by pattern ④ are broken, making this pulse pattern unusable. Moreover, SERS spectra from the broken cells are different from each other.

The delivery efficiency is evaluated quantitatively by the integrated SERS intensity. For each electroporation experiment, SERS measurements were performed on fifteen cells and two spectra were acquired from each cell by repeated measurements. Therefore, in total thirty SERS spectra were obtained during each experiment. While for every spectrum, we calculated the total integrated area under the curve from 400 cm\(^{-1}\) to 1800 cm\(^{-1}\). Then the integrated intensity was divided by the area of the cell to generate a standardized “integrated SERS intensity”, representing the total amount of silver nanoparticles delivered into the cell. With these data processing applied to the four different electroporation experiments and the control experiment, we obtained a comparison of the integrated SERS intensity. For the control experiment and the electroporation experiment with electric pulse
pattern ① (1 ms pulse width pulses for two times), the integrated SERS intensities are minimal, indicating that very little silver nanoparticles were delivered into the cells. Compared with this, the cells treated by the electric pulse pattern ② (three consecutive pulses with pulse width of 1, 10, and 1 ms) achieved considerable integrated SERS intensity. This suggested that considerable amount of silver nanoparticles were delivered into living C666 cells. The cells treated by the electric pulse pattern ③ (four consecutive pulses with pulse width of 1, 10, 10, and 1 ms) showed strong integrated SERS intensity. This result demonstrated that 875 V/cm electric field strength and four pulses of 1, 10, 10, 1 ms width led to a high delivery efficiency of silver nanoparticles into living C666 cells. The integrated SERS intensity of cells treated by the electric pulse pattern ④ (five consecutive pulses with pulse width of 1, 10, 10, 10, and 1 ms) is even higher, however, this is not useful because a significant portion of cells are dead and corresponding Raman signals are very different from the cells that are still alive after the electroporation. In the electric pulse pattern ③ treatment, the silver nanoparticles are effectively delivered into living cells and the cell membrane also recovered its integrity and functions normally, maintained the viability of the cells.

The temperature at the electroporation process might play a role in the efficiency of delivery[13-15]. The electroporation procedure was performed at (0~4°C) or at room temperature (20~25°C) to determine the optimal electroporation temperature with the same electrical parameters (875 V/cm field strength and four pulses of 1, 10, 10, 1 ms pulse width). We also used the integrated SERS intensity to compare the efficiency of silver nanoparticles delivery in these two experiments. Compared with low operation temperature, the integrated SERS intensity of cell electroporation in room temperature decreased dramatically. One explanation for this result is that keeping the cells at low temperature allows the electroporation-induced pores on the membrane to remain open longer and more silver nanoparticles in the medium will get into the cell. Therefore, it is importance to maintain the cells at low temperature condition for the electroporation operation for optimal silver nanoparticle delivery efficiency.

4. Conclusion
In conclusion, we optimized the electroporation parameters for delivering silver nanoparticles into living C666 cells for intracellular SERS spectroscopy. The optimum delivery is achieved under the electroporation condition of using 4 consecutive 350 V (875 V/cm) rectangular electric pulses of 1 ms, 10 ms, 10 ms, and 1 ms durations respectively. Low temperature (0~4°C) is necessary for improving the delivery efficiency of silver nanoparticles. With the optimized electroporation procedure, intracellular SERS analysis can be performed within minutes, offering great promises for biomedical applications such as high-throughput cancer cell screening. However, there are still several questions to be solved and further study on electroporation for intracellular SERS spectroscopy measurements will be given in following research note.

![Figure 1](image_url)

Figure 1. Optical microscopic image of C666 cells after electroporation. (a) normal looking cell; (b) broken cell; (c) cell fusion

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