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Enhanced Introduction of Gold Nanoparticles into Vital *Acidothiobacillus ferrooxidans* by Carbon Nanotube-based Microwave Electroporation

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ABSTRACT

A novel method was developed to electroporate gram-negative bacteria (*Acidothiobacillus ferrooxidans*) via multiwall carbon nanotubes (CNTs). CNTs are metallic, ultra strong nanofibers of ~10 nm in diameter and many microns long. When placed in an electric field, the CNTs strongly enhance the electric field at their ends by a factor of 10–100, making them ideal for localized electroporation. Addition of CNTs into a solution containing bacteria and gold nanoparticles (GNPs) and subsequent exposure to microwave radiation facilitates a rapid transport of GNPs across the cell wall, without affecting the cell viability.

In bacteria (both gram-positive and gram-negative), the integrity of the cells is preserved by the cell wall, which protects bacteria from osmotic lysis. In the case of gram-negative bacteria, the cell wall surrounds a double membrane system − the cytoplasmic, or inner membrane, and the outer membrane. The outer membrane is the major permeability barrier in gram-negative bacteria. Thus, while the cell wall constitutes a rigid physical barrier, the cell membrane is a flexible and multifunctional entity. A weakness in the cell wall or a break in the integrity of the cell membrane immediately compromises the essential roles of its structure, leading to cell death. In gram-negative bacteria, many small molecules may pass through the outer membrane, due to the presence of numerous nanopores.[1] These pores are composed of proteins called porins, which act effectively as water-filled, voltage-gated channels across the membrane and which facilitate diffusion of small (<800 Da) polar molecules.[2]

On the other hand, the antibacterial activity of many small, positively charged peptides and proteins is based on pore formation in lipid bilayers.[3] Localized perforation of the cell wall by a new class of biological polymers, cyclic peptide nanotubes, has also been documented.[4] These structures can be embedded in the cell membranes of bacteria and effectively "poke" holes through their membranes. This kind of membrane disruption leads to cell death. Recently, an insertion of vertically aligned carbon nanofibers into hamster ovary cells, for plasmid delivery, shows that the cells subsequently remained viable.[5]

In this study, we use the chemolithoautotroph (mesophilic) *Acidothiobacillus ferrooxidans* (*A. ferrooxidans*), a gram-negative bacterium shown in Figure 1, for electroporation via CNTs. It has optimum growth at pH 1.6 and 30 °C, and was cultivated in ferrous ions according to the procedure described by Touvinen and Kelly.[6] Stock cultures of *A. ferrooxidans* were transferred to a fresh culture medium. The cultures were shaken at 30 °C in darkness, and log phase cells were collected after 10 days of incubation to ensure a high cell concentration.

Multiwall carbon nanotubes shown in Figure 2 were grown by chemical vapor deposition (CVD).[7] CNTs are metallic (high conductivity), chemically and physically robust, exhibit great tensile strength, and do not oxidize or have surface states under ambient conditions. Since CNTs are insoluble in most solvents, sonication and/or surfactant treatment is usually necessary to disperse them. Optically homogeneous stable dispersions of CNTs have been produced using mild bath sonication and subsequent dispersion into water with the aid of the sulfuric/nitric acid treatment, resulting in a

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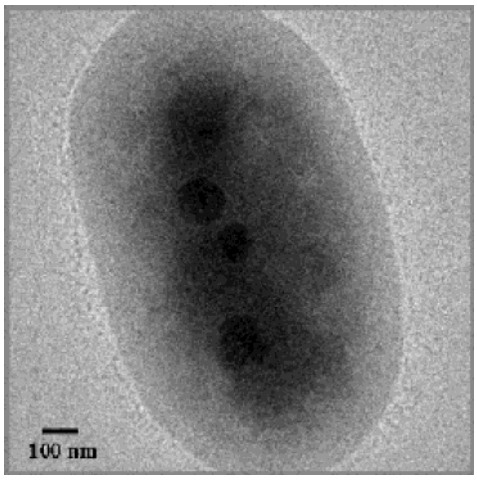

Figure 1. TEM picture showing typical morphology of *Acidothiobacillus ferrooxidans*.

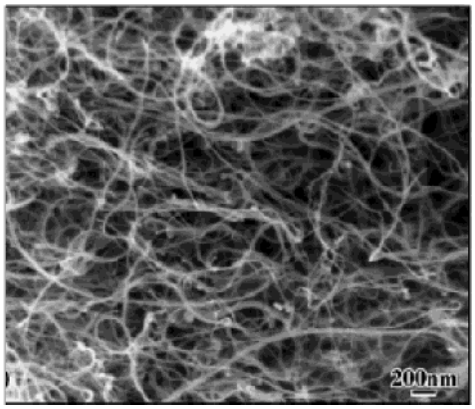

Figure 2. SEM picture of the multiwall CNTs in bulk.

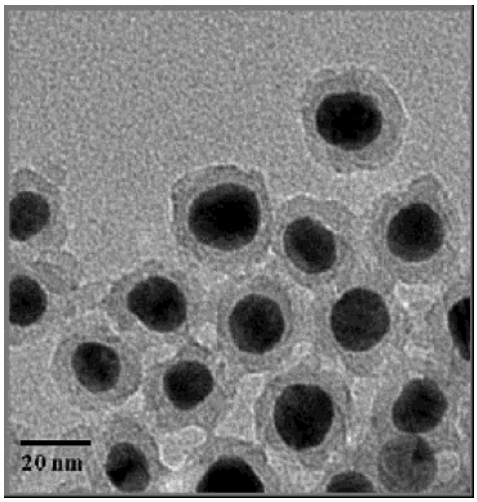

Figure 3. TEM picture of the silica-coated gold nanoparticles.

clear and stable, long-time suspension. The latter was achieved by the chemical generation of mainly carboxylic groups, which contribute to get a negative charge at the ends of the CNTs and in the defects on their side walls.

The silica-coated gold nanoparticles (GNPs), shown in Figure 3, were prepared as described elsewhere[8−9] and concentrated by centrifugation, which also allowed removal of the excess sodium silicate.

Samples for transmission electron microscopy (TEM) analysis were deposited on carbon-coated copper TEM grids. After 2 min, the extra solution was removed using blotting paper, and the grid was allowed to dry prior to measurement. TEM measurements were performed on a Philips model CM12 instrument operated at an accelerating voltage of 120 kV. Exposure to the microwave (MW) field was achieved in a MW cavity, powered by a magnetron source of 1200 W at a frequency of 2.45 GHz. The time of the exposure was controlled.

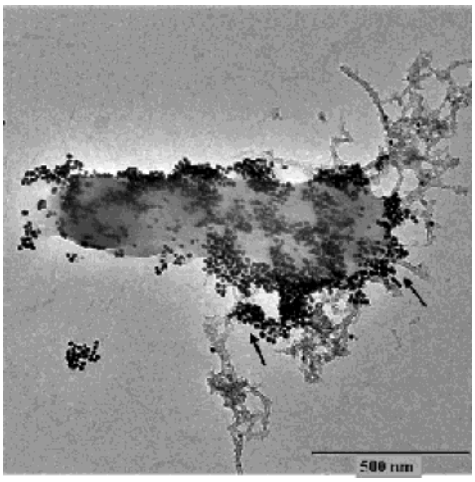

Figure 4. TEM picture of the *Acidothiobacillus ferrooxidans* cell after the exposure to MW (5 s.). Accumulation of CNTs on the cell wall is clearly visible. GNPs also accumulate on the cell wall, but mostly at the CNT−cell contact points (see arrows).

To prepare bacterial suspensions, pellet cells from an early log culture ($7-9 \times 10^6$/mL) were dispersed in a colloidal gold solution containing 0.01% (w/v) hydrophilic CNTs. The resulting coarse predispersion was homogenized using a vortex mixer at low speed. The samples were then filtrated through sterile membranes (pore size 5 μm). After this step, the filtered bacterial suspension was centrifuged at 3000 g for 10 min and the supernatant discarded. Next, we study behavior of cells containing CNTs and GNPs. Cell viability after 5, 15, 20 s MW exposure was determined by transfer of samples (innoculums) into a fresh culture medium. The results of cell viability and cell growth provide insight into the stability of cells in MW, the reversibility of electroporation via CNTs, and the ability of the cells to withstand a high intake of GNPs. After 5 days incubation, only bacteria coming from 5 s MW treatment were viable. The cell growth was slightly reduced compared with untreated control cells. Prolonged exposure of cells to MW irradiation (over 5 s) involved several irreversible events which inevitably led to cell destruction.

When added at random to a bacteria−GNP solution (and after mixing), CNTs and GNPs remained uniformly suspended, without a strong tendency to accumulate on the cells, at least in the time scale of our experiment. However, after a sufficient period of time, GNPs (and CNTs) will eventually accumulate at the cells even before MW is applied.

When exposed to microwaves, however, a large fraction of the CNTs and GNPs accumulated at the cell surface (see Figure 4), and some even penetrated the cell wall (see Figure 5).

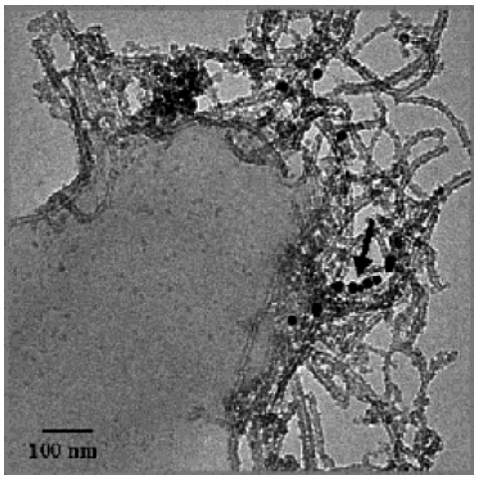

Figure 5. TEM picture of a section of the cell with CNTs attached to the wall, and a stream of GNPs approaching the cell along a CNT (see arrow).

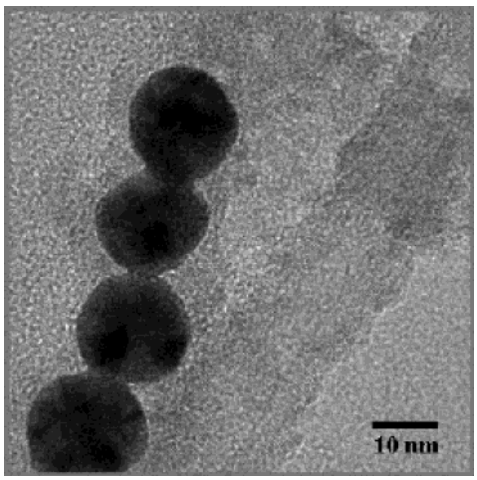

Figure 6. High-resolution TEM picture of a CNT and 4 GNPs aligning themselves at the CNT surface.

GNPs accumulate mostly in the areas of the CNT−cell contact (Figure 4), as well as along the CNT (Figure 5). The reason for this behavior is clear. After the exposure to microwaves, all metallic particles acquire oscillating dipole moments. These charges, in turn, induce effective opposite image charges in the metallic CNTs and other GNPs, consequently leading to the observed attraction of GNPs to CNTs, and to each other. This effect is also clearly visible in Figure 6, which shows a high resolution TEM picture of a CNT (the diagonal feature) and 4 gold nanoparticles attracted to each other, and to the CNT. A similar effect causes attraction of CNTs to the cell surface. In this case, charges are induced in CNTs by the MW field, and these induced charges on the cell surface lead to the tip-first CNT attraction to the cell wall. This is a strong effect, since the electric field of the MW radiation is strongly enhanced at the CNT tips, as described below.

The actual transport of GNP across the cell wall is clearly visible in Figure 7. We confirm this by detailed study of the TEM images, at various focus depths. The interior of the cell is filled with GNPs, and streams of GNPs enter the cell along the CNTs. The intracellular transport occurs in the

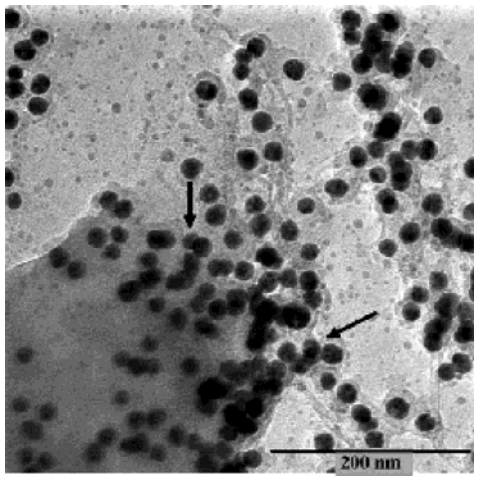

Figure 7. TEM picture of the area of contact of the cell with CNTs, and of massive GNP transfer into the cell. Again, it is clear that GNPs enter the cell by first aligning themselves along a CNT, and then entering the cell in the vicinity of the CNT tip (see arrows).

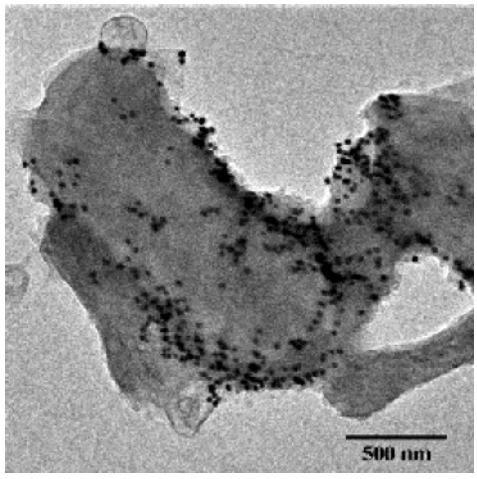

Figure 8. TEM picture of a cell derived from electroporated bacteria. GNPs contained in bacteria move into successive generations of cells.

vicinity of the CNT tips, and we identify it as CNT-induced electroporation. Cells (progeny) derived from the electroporated bacteria still show GNPs in their cytosol (Figure 8).

To determine whether GNPs alone are able to cross the cell wall under MW exposure, we studied gold transport in CNT-free cell solutions. Cells were suspended in a colloidal gold solution and exposed to MW for 5 s. Again, TEM examinations of treated cells indicated that GNPs accumulate only on the outside of the cell membrane in this case (Figure 9).

The activation of CNTs is due essentially to the "lightening rod" effect, and occurs when a metallic rod (e.g., a multiwall CNT) is inserted into a region of a uniform electric field E_0. This causes a strong field enhancement at the CNT tip. A simple estimate of this enhancement for a single, straight CNT is given by

$$E/E_0 = \alpha L/D \qquad (1)$$

where $\alpha \sim 3$ is a constant, E is the field at the tip, L is the

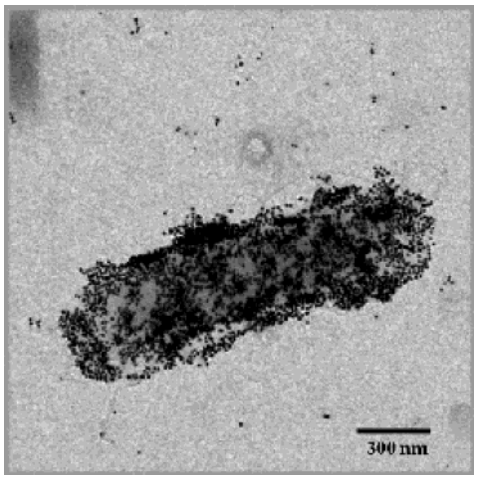

Figure 9. TEM picture of a cell after exposure to MW. Accumulation of GNPs was observed only on the cell surface.

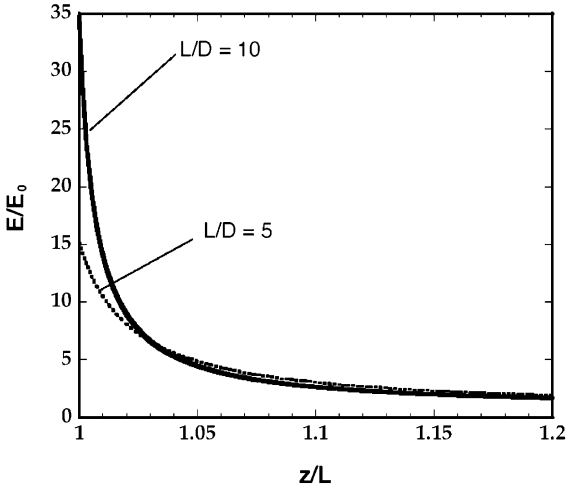

Figure 10. Calculated field enhancement at the tip of a nanotube, simulated as an oblate spheroid with the aspect ratio *L*/*D*.

CNT lengths, and *D* is its diameter. Thus, the larger the aspect ratio (*L*/*D*) of a CNT, the larger the field enhancement at the tip. The same effect is responsible for the lowest observed threshold of < 1 V/μm for the electron field emission from CNT.[10] By modeling a nanotube as a prolate, metallic spheroid of length *L* and width *D*, we can calculate the field enhancement more accurately.[11] Figure 10 shows the electric field along the axis of a CNT calculated for two CNTs with the aspect ratio $L/D = 5$ and 10. The tip of the nanotube is at $z/L = 1$. It is clear, that while the CNT with the aspect ratio 5 enhances the field at the tip by a factor of 15, the tube with the aspect ratio 10 more than doubles that value to 35. Even though these calculations are static, they apply to the microwave fields as well, because the plasma frequencies of both CNT and GNP are much higher (UV range) than the microwave frequencies.

In conclusion, we have demonstrated transmembrane transport of exogenous agents (colloidal GNPs) into cells in the presence of "cell-integrated" multiwall CNTs. The described process constitutes a novel reversible electroporation of gram-negative bacteria. A likely mechanism for pore generation and intracellular transport of GNPs via MW-activated CNTs must primarily consider an induced dipole along the CNTs, whose positive ends make contact with the negatively charged surface of gram-negative *A. ferrooxidans* bacteria. This initial electrostatic contact is enhanced by the MW energy, which in turn determines the onset of CNT-sized pores across the cell membranes. GNPs attached onto the cell surface and having a size comparable to the pore openings take advantage of these transient membrane disruptions and diffuse inside the cells. Further experiments with larger particles ranging in diameter from 50 to about 200 nm will be used to confirm the relationship between the size of nanoparticles and the diameter of pores created by CNTs. We note that the action of the CNTs with *A. ferrooxidans* cells differs significantly from the previously described "carpet-like" behavior of the peptide nanotubes,[4] which do not generate transmembrane pores but rather destabilize the membrane in a manner similar to a detergent action.[12] Furthermore, we observe a very high survivability of the infused cells. This approach also has a great potential for cell discrimination. This can be achieved by properly functionalized CNTs,[13,14] so that only the target cells, to which the functionalized CNT attach, are infused. Thus, this novel technique has great potential for applications in cell biology and medicine.[15]

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