Lethality mechanisms in *Escherichia coli* induced by intense sub-microsecond electrical pulses

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In this letter, the authors present the inactivation kinetics of cells of *Escherichia coli* and its mutants following treatment with high-intensity electrical pulses of 700 and 32 ns durations. Their experimental results suggest that bacterial inactivation by 700 ns pulses is consistent with a mechanism of reversible electroporation, whereas inactivation by 32 ns pulses may occur as a result of damage to intracellular components. They believe that their results represent a first step towards elucidating the mechanism of lethality of submicrosecond pulses of different durations in prokaryotes. © *2006 American Institute of Physics*. [DOI: 10.1063/1.2361271]

Recent technological advances in pulsed power technology have stimulated interest in high-intensity submicrosecond pulses in biological applications, including microbial decontamination of liquid foods.[1,2] Pulse duration and electric field intensity are two key parameters that could potentially be manipulated to achieve a tailored inactivation of cells for specific requirements of intended applications. Pulses of duration greater than 1 μs and of sufficiently high field intensity inactivate bacterial cells through a widely accepted mechanism often referred to as irreversible electroporation (or irreversible breakdown).[3–5] The charging time constant of the cell membrane plays an important role in this process as it represents a temporal threshold that must be overcome to initiate the electroporation process. At the molecular level, the cell membrane is also a primary component that interacts with the applied pulsed electric fields. The cell membrane may be represented in electrical terms as a membrane capacitance in parallel with a membrane resistance. When the applied pulse duration is much longer (i.e., at low frequency) than the charging time constant of the cell membrane, the resistance of the cell membrane insulates the cytoplasm from the external electric field and free charges build up on both sides of the membrane surfaces. This process eventually leads to the formation of pores, and finally to cell lysis due to the irreversible breakdown of the cell membrane.

There is a possibility that the mechanism of cell inactivation might be different if the applied pulse duration is much shorter than the charging time constant of the cell membrane. Much shorter pulses (i.e., at high frequency) might bring about a short-circuiting effect of the membrane capacitance, which would allow the electrical field to penetrate into the cell.[6] If the electric field intensity of such pulses is sufficiently high (100 kV/cm), the resulting effect is the generation of a potential difference across the intracellular structures or organelles without necessarily causing permanent damage to the outer cell membrane. These effects might cause the bacterial cells either to inactivate themselves in a mechanism akin to programed cell death (apoptosis) or to modify their intracellular structures. Experimental verification of this mechanism would be invaluable as it would open the door to a number of important applications ranging from efficient bacterial decontamination to selective destruction of virus-infected bacterial cells. In mammalian cells, submicrosecond pulses have been shown to have effects such as apoptosis of cancer cells.[2,7] However, the dimension and biophysical properties of bacterial cells are significantly different from mammalian cells and it is necessary therefore to examine experimentally such effects in bacterial cells. In this letter, we present results from an experimental investigation to show how the wild-type *Escherichia coli* (*E. coli*) cell and its mutants respond, when they are subjected to the pulses of either 700 or 32 ns duration. This is the first step towards an understanding of the mechanisms of bacterial inactivation by ultrashort and high voltage pulses, which are probably distinct from the irreversible electroporation.

The charging time constant (τ_m) of the *E. coli* cell membrane can be calculated using the spherical model given by Cole.[8]

$$\tau_m = \left[\left\{ \frac{1+2V}{1-V} \right\} \frac{\rho_s}{2} + \rho_c \right] C_m R, \tag{1}$$

where R is the radius of the bacterial cell (in this case *E. coli*), ρ_s is the specific resistivity of the extra cellular medium, ρ_c is the specific resistivity of cytoplasm, V is the volume concentration of the spheres in cell suspension, and C_m is the specific capacitance (capacitance per unit area) of the cell membrane. An approximate value for C_m is given by the equation for a slab dielectric.[6]

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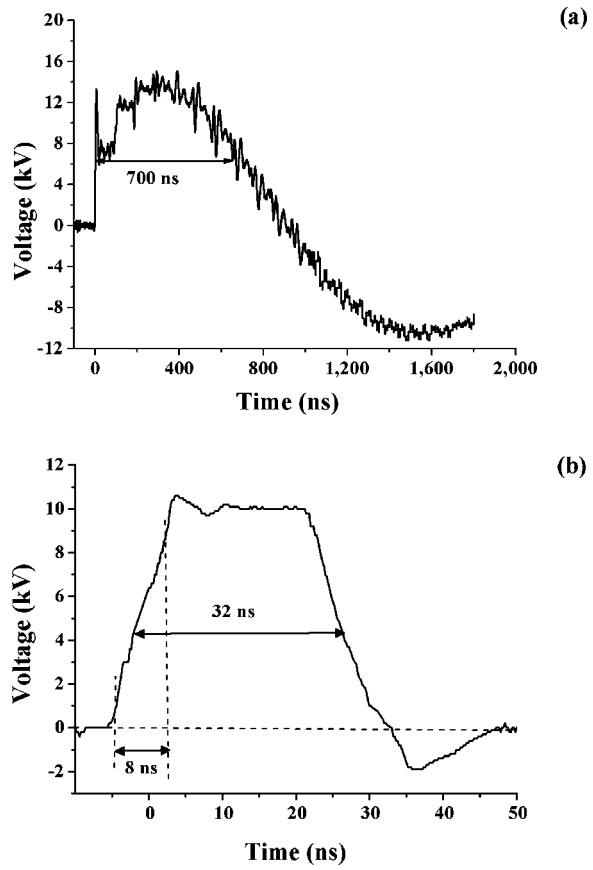

FIG. 1. Temporal profiles and pulse parameters of (a) a 700 ns pulse, (b) a 32 ns pulse.

$$C_m = \frac{\varepsilon_0 \varepsilon_r}{d}, \tag{2}$$

where $d=5$ nm is the uniform thickness of the cell membrane and $\varepsilon_0\varepsilon_r=4.4\times10^{-11}$ A V^{-1} m^{-1} is the permittivity of the membrane forming material.[9] Using these values, C_m was found to be 1 μF/cm^2. The value of V of 0.8 for a face centered cubic orientation is chosen because it is a realistic orientation for nonconductive cells in suspension.[9] The values of R of 0.7 μm (Ref. 10) and ρ_s of 100 Ω cm (Ref. 2) have previously been used for *E. coli*. However, due to the large reported variations in the value of ρ_c between 100 and 500 Ω cm [10–13] both the lower and upper limits were used in calculating τ_m, which was found to lie between 52 and 81 ns. These calculations formed the basis for selecting the durations of the applied pulses, which were set to satisfy the conditions of (a) 700 ns $>\tau_m$ and (b) 32 ns $<\tau_m$.

Details of the pulsed power generators used in our experimental studies are reported elsewhere[14] and the shape and electrical parameters of the applied pulses are shown in Figs. 1(a) and 1(b). The 700 ns pulse has a rise time of 5 ns and an electric field intensity of 30 kV/cm, whereas the 32 ns pulse has a rise time of 8 ns and a field intensity of 100 kV/cm at a repetition rate of 30 pulses/s. The target cells, wild-type *E. coli* K12, and its mutants ΔrecA, ΔsoxS and BL21 (DE3) pLysS were cultured and handled using standard protocols.[14] All data points shown in Figs. 2 and 3 were obtained from three independent experiments and they represent an average and the standard deviation of the three counts.

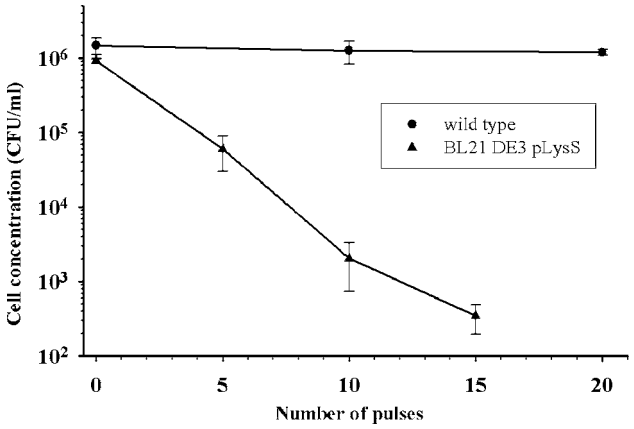

FIG. 2. Inactivation kinetics of *E. coli* K12 and *E. coli* BL21 (DE3) pLysS by 700 ns pulses.

In order to investigate the mechanism of bacterial cell inactivation by 700 and 32 ns pulses, we initially started our experiments with the wild type *E. coli* K12 and the mutant *E. coli* BL21 (DE3) pLysS. The latter contains a plasmid bearing the gene for the enzyme lysozyme that causes the membrane to lyse if significant disturbances are produced at the membrane.[15] If the pulsed electric field treatment affects the cell membrane, it is expected that inactivation of BL21 (DE3) pLysS will be more rapid than the wild type. Inactivation curves of both types of cells subjected to 700 ns pulses are shown in Fig. 2. Wild-type *E. coli* K12 is almost unaffected, but *E. coli* BL21 (DE3) pLysS is rapidly decreased by a 4 $\log_{10}$ reduction after only 15 pulses. As each pulse is 700 ns in width, the total effective pulse-on time of 15 pulses is 10.5 μs. Figure 2 shows a significant difference in the inactivation kinetics of these cells and suggests that it is through an irreversible breakdown of the cell membrane following the pulse treatment. The inactivation curves of *E. coli* K12 and its mutants subjected to 32 ns pulses are shown in Fig. 3. Interestingly, the inactivation kinetics of *E. coli* K12 and BL21 (DE3) pLysS are similar, with up to 2 $\log_{10}$ (99%) reductions being achieved in 300 s. Comparison with Fig. 2 suggests that cell inactivation is not due to the electrical breakdown of the cell membrane but possibly to electric field penetration into the cell membrane and the establishment of an intracellular potential difference before charge buildup can occur at the cell membrane. This suggests that inactivation of cells must be taking place due to

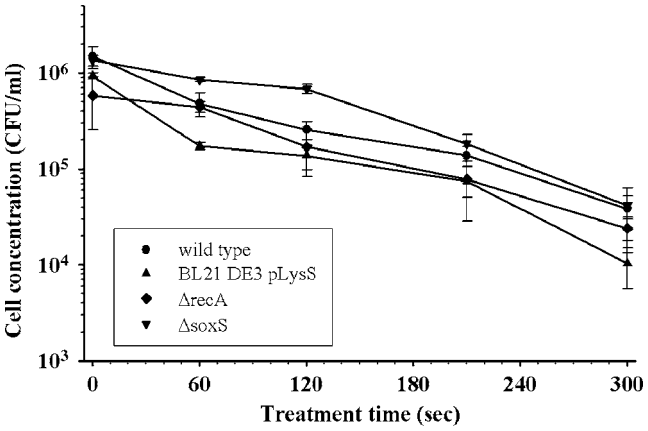

FIG. 3. Inactivation kinetics of *E. coli* K12 and various mutants by 32 ns pulses.

degradation of the intracellular components. Thermal effects were found negligible with the maximum temperature rise less than 10 °C, which is insufficient for *E. coli* inactivation. This is also evident from the very small change in the concentration of viable wild-type *E. coli* K12 cells in Fig. 2.

We carried out further experimental investigations with two additional mutants, *E. coli* K12 ΔrecA and *E. coli* K12 ΔsoxS, to determine what other intracellular targets might be damaged by 32 ns pulses. The former mutant lacks the recA gene that is important in the repair of damaged deoxyribonucleic acid,[16] (DNA), while the latter lacks the gene that is activated to deal with damage caused by oxidative species.[17] Figure 3 shows the inactivation kinetics of the wild-typed *E. coli* K12 cell and its three mutants. It is worth noting that the 32 ns pulses were applied at a repetition rate of 30 pluses/s and so a treatment time consists of 300 s is equivalent to 9000 pulses or an effective pulse-on time of 288 μs. It is clear from Fig. 3 that there is no significant difference in the inactivation kinetics of all three mutants. This suggests that cell inactivation under these conditions is neither due to the oxidative stress nor to DNA damage. However, *E. coli* possesses more than one DNA-repair mechanism and it is therefore not possible to completely rule out the possibility that 32 ns pulses do cause DNA damage. Further studies with a greater number of mutants are therefore needed. The results for *E. coli* ΔsoxS are unsurprising, because the pulse duration and electric field intensity are possibly insufficient to form active species in the interior of the cells. It is worth mentioning that thermal effects were again negligible with temperature rise much less than 10 °C. Hence, the observed similarity in inactivation kinetics of type *E. coli* and its three mutants are indeed indicative of an electrically induced inactivation mechanism associated with degradation of intracellular components.

In summary, we have presented an experimental investigation into the effect of two submicrosecond (700 and 32 ns) pulses on various mutants of *E. coli*. The experimental results suggest that inactivation of bacterial cells by 32 ns pulses is not due to the irreversible breakdown of the cell membrane but instead to a distinct mechanism possibly involving the degradation of intracellular components. Initial experimental results on the effect of 32 ns pulses on potential intracellular targets such as DNA were also obtained. We are in the process of investigating possible synergistic effects in terms of sublethal injury to wild type *E. coli* K12 cells by 32 ns pulses. The significance of sublethally injured cells is very important in food processing operations, as injured cells might not be detectable by normal procedures of estimating food safety.

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