A Bacterial Spore Model of Pulsed Electric Fields on Spore Morphology Change Revealed by Simulation and SEM

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***Abstract*—A two-layered spore model was proposed to analyze morphological change of bacterial spores subjected under pulsed electric fields. The outer layer, i.e. spore coat, was defined by Mooney-Rivlin hyper-elastic material model. The inner layer, i.e. peptidoglycan and spore core, was modeled by applying additional adhesion forces. The effect of pulsed electric fields on surface displacement was simulated in COMSOL Multiphysics and verified by SEM. The electro-mechanical theory, considering spore coat as a capacitor, was used to explain concavity; and the thin viscoelastic film theory, considering membrane bilayer as fluctuating surfaces, was used to explain leakage forming. Mutual interaction of external electric fields, charged spores, adhesion forces and ions movement were all predicted to contribute to concavity and leakage.**

I. Introduction

Pulsed electric fields (PEF) have been used to induce pores or pore-like structures within cell plasma membranes to facilitate trans-membrane exchange of materials such as foreign DNA [1-4]. Compared with wet heat inactivation, PEF, a type of non-thermal inactivation method, can also be used to inactivate fungi and bacteria [5]. Inactivation of vegetative cells and bacterial spores using PEF has been reported [6-10]. Hamilton and Sale studied the killing effect of high-voltage pulsed electric fields (30 kV/cm) on vegetative bacterial cells. Bacterial cell death caused by irreversible loss of the membrane's function as semipermeable barriers was proposed. Inactivation of bacterial cells has been well studied. As bacterial cells, bacterial spores can cause food spoilage and food-borne disease [11]. However, study on inactivation of spores by PEF is still limited. Jin *et al*. applied electric fields at 30 to 40 kV/cm for 2 to 3 ms to *B. subtilis* spores. More than 95% of *B. subtilis* spores were inactivated. Scanning electron microscopy (SEM) revealed that PEF-treated spores had structural changes, i.e. the spores shrank and many wrinkles were formed on their surfaces, similar to those of thermally inactivated spores with wet heat [12]. Inactivation of bacterial spores was related with high voltage; however, less inactivation effect was observed at low voltage. Yonemoto *et al*. treated *B. subtilis* spores with PEF at 5.4 kV/cm. The viability of spores was slightly decreased by about 1% after PEF treatment [13].

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Pore formation is observed after PEF treatment. With an increase in external electric fields, trans-membrane potential increases until a critical voltage of ~1 V is reached across the membrane. Formation of pores increases the permeability and conductivity of the membrane [14]. Reversible breakdown is defined when the pores can reseal due to a self-healing process [15]. If the applied electric fields exist for longer time or exceed the critical electric field more pores with larger sizes are formed, causing irreversible membrane breakdown and mechanical destruction [11].

Two popular models have been developed to describe PEF treatment on membrane bilayers. The electro-mechanical model proposed by Zimmermann describes the electric breakdown of bilayers as a consequence of an electrostatic compression force balanced against the elastic force of the membrane [16]. The thin viscoelastic film model of electric breakdown proposed by Dimitrov considers the membrane as a thin viscoelastic film with fluctuating surfaces. He proposes that electric-field-induced membrane breakdown can be divided into three stages: 1. growth of membrane surface fluctuations; 2. molecular rearrangements leading to membrane discontinuities; 3. expansion of pores resulting in mechanical breakdown of the membrane [17]. The two membrane-bilayer models are well established to explain mechanisms of inactivation and drug delivery by PEF, but study on models for spore inactivation by PEF is still limited.

A bacterial spore model combining electro-mechanical model with thin viscoelastic layer model was proposed in this work. The electro-mechanical model was used to describe PEF-treated spores with concave shape and the thin viscoelastic layer model was used to describe spores with leakage after PEF treatment. This bacterial spore model of pulsed electric fields on spore morphology change was verified by simulation study and SEM.

Bacterial spore is metabolically dormant, and very resistant to a variety of environmental conditions [18]. Spore contains exosporium, outer coat, inner coat, outer membrane, cortex, germ cell wall, inner membrane and core [19]. The spore coat is composed of two layers, outer layer and inner layer, most of which are proteins. The cortex is composed of peptidoglycan with a similar structure to that of vegetative cells. The core contains low levels of water which is an important factor determining the resistance of spores [20].

II. Simulation

This model, simulated in COMSOL, considered a spore as a two-layer sphere (Fig. 1). Spore was assumed to be of a spherical shape with a diameter of 1.5 μm and a thickness of 60 nm [21]. We constructed the spore coat and considered the influence of cortex and core through adding adhesion forces

on the inner surfaces [17]. Based on reported conformational change of PEF-treated spores, i.e. the spores shrank and wrinkles were formed on their surfaces [12], the property of spore coat was assumed to be hyper-elastic. In engineering practice, rubber-like material models are widely used in large deformation nonlinear response simulations [22]. One such model is the well-known Mooney-Rivlin model [22]. The strain energy density function has the following expression:

$$W = C_{1,0}(I_1-3) + C_{0,1}(I_2-3) + D_1(J-1) \quad (1)$$

Where I_1 and I_2 are the first and second invariant of the left isochoric Cauchy-Green deformation tensors; J is the elastic Jacobian; $C_{1,0}$, $C_{0,1}$ are material constants related to the distortional response and D_1 is material constant related to the volumetric response.

The surface charge of a spore depends on many factors, including species, sporulation conditions and buffer conditions [23]. Therefore we assumed the outer surfaces to be positively charged with charge density of -1 $\mu C/m^2$ while the inner surfaces to be negatively charged with charge density of 1 $\mu C/m^2$ respectively. Spore coat and cortex were further assumed to hold together by adhesion forces [21]. The Hook forces were of finite range and the forces would disappear if the deformation exceeded their maximum value, leading to breakdown of linkage between coat and cortex. So we assumed the forces were applied onto the inner surfaces and they increased linearly in a certain range. Two physical conditions, i.e. solid mechanics and electrostatics, were used in this simulation [21].

III. Methods

Spore Preparation. Bacillus atrophaeus (ATCC 9372) spores were sporulated at 37°C , harvested by centrifugation and stored at 4°C. Spores used in this work were free of vegetative or sporulating cells, cell debris, and germinated spores, as observed by phase contrast microscopy [24].

PEF Treatment. A MicroPulser (Bio-Rad, Hercules, CA) was used to generate pulsed electric fields. An exponential decaying PEF waveform was used in this study, with pulse duration of 1ms. PEF treatment was carried out in a plane-plane electrode system using disposable electroporation cuvettes (Bio-Rad, Hercules, CA). The cuvettes had an electrode gap of 2 mm, and they held 400 µl of the spore suspension. Pulses were applied to the disposable electroporation cuvette at 15 kV/cm with pulse interval of 5 s. After 50 pulses were applied, temperature increase of no more than 0.7°C was observed by thermocouple probe (Dickson, Addison, IL).

Samples Preparation for SEM. PEF-treated spores were plated onto 0.2 µm nylon membrane filters frozen in a −85°C deep freezer (llshin Lab Co. Ltd., Kyonggi-Do, South Korea) for 2 hours. After 2 hours of freezing, samples were transferred to a freeze dryer (llshin Lab Co. Ltd., Kyonggi-Do, South Korea) for 24 hours at −35°C and 5 mTorr. Drying completed within 24 hours. The filters were coated with gold from all directions (SC 502 Ion Sputter Coating Machine, Quorum Technologies Co. Ltd., West Sussex, UK). The specimens were examined at 10 kV and photographed with the LEO 1450VP Scanning Electron Microscope (LEO Co. Ltd., Clifton Road, England).

IV. Result

Spore simulation was calculated with PEF at 15 kV/cm, 1 ms for 1 pulse (Fig. 1a) and 50 pulses (Fig. 1b) respectively. Exported animation of morphology and stress change was attached on Appendix. Longer PEF treatment, say 50 pulses, led to more pronounced surface displacement due to accumulated effect of electric forces, which was consistent with experimental results. Also the distribution of surface displacement was uneven due to the mutual influence of electric fields and adhesion forces. Because of interaction of external electric fields with charged spores, the distribution of electric-field lines was uneven. At the center of a spore, the density of electric-field lines was greater than that outside of the spore.

No observable difference of surface roughness was monitored between non-PEF-treated spores and PEF-treated spores at 1 pulse (Fig. 2a, b). However, difference of surface roughness was observed between PEF-treated spores at 1 pulse and 50 pulses (Fig. 2b—d). For spores with longer treatment time, mutual interaction of attraction and rejection forces between charged spores and electrodes partly contributed to serrated features. Longer treatment time, more dosage of attraction and rejection forces applied on charged spores. That could probably be the reason that for shorter treatment time, no observable difference of surface roughness was monitored.

Leakage was only observed in PEF-treated spores at 50 pulses in saline (Fig. 2d). In addition, leakage was monitored in a part of surface of a spore and the location of leakage was also diverse. Ions striking, attraction and rejection forces as well as molecular rearrangements were suggested to be partial reasons why leakage occurred. For spores with same treatment time in DI water, leakage was not observed due to lack of ions. Therefore, ions movement affected leakage forming in certain extent, partly because ions movement could strengthen attraction and rejection forces as well as molecular rearrangements. For shorter treatment time, no leakage in appearance was observed because of shorter time for ions movement, resulting in less decrease in coat thickness.

The percentage of spores with concave shape was summarized in Table 1. For different PEF treatment conditions, around 10% of spores were concave. Even though the percentage was almost the same, concavity was different. It was noticed that greater concavity was observed in PEF-treated spores at 50 pulses. For longer treatment time, the interaction of ions striking, attraction and rejection forces as well as molecular rearrangements was enhanced. Also perhaps those spores were more adjacent to electrodes, which indicated those spores were under stronger external electric fields.

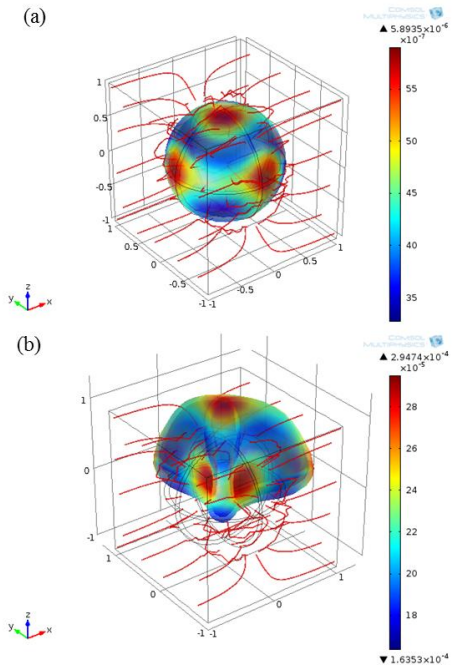

Figure 1. Spore simulation under pulsed electric fields condition at 15 kV/cm, 1 ms. Total surface displacement after pulsed electric fields treatment at 1 pulse (a) and 50 pulses (b) respectively.

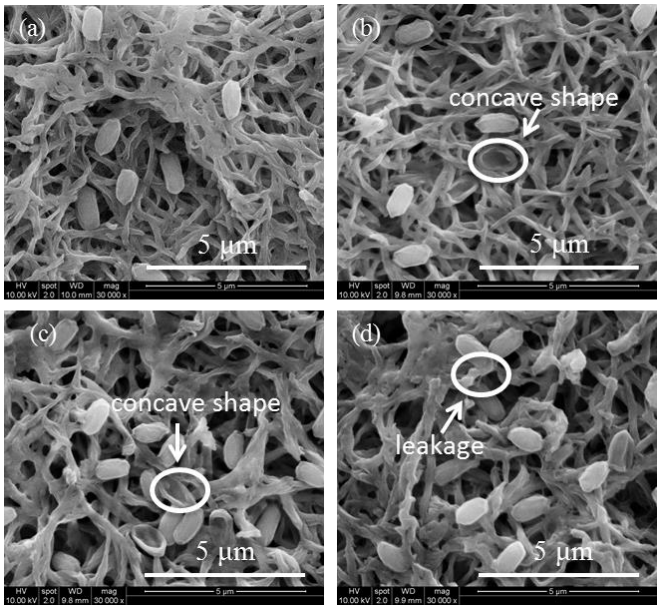

Figure 2. SEM images of *Bacillus atrophaeus* spores treated with PEF at 15 kV/cm, 1 ms. (a) Non-PEF treatment; (b) 1 pulse in saline (0.85%); (c) 50 pulses in DI water; (d) 50 pulses in saline (0.85%).

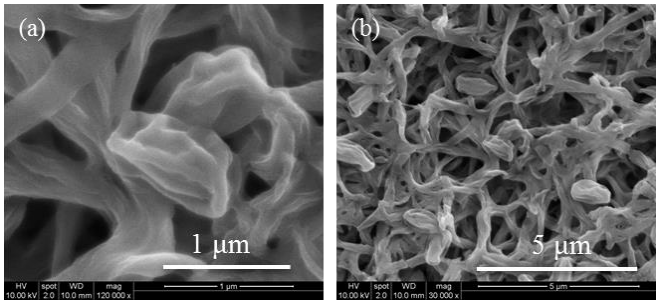

Figure 3. SEM images of *Bacillus atrophaeus* spores autoclaved at 121°C for 30 minutes.

TABLE I. PERCENTAGE OF SPORES WITH CONCAVE SHAPE

Concavity	Treatment Conditions			
	1 pulse in DI water	*1 pulse in saline*	*50 pulses in DI water*	*50 pulses in saline*
Percentage (%)	7.7	11.8	12.3	10.4

V. DISCUSSION

This work analyzed morphological changes of spores under pulsed electric fields. Spore was assumed to be spherical and simulated in COMSOL based on Mooney-Rivlin model. This simulation results implied that longer PEF treatment time, larger surface morphological change due to accumulated effect of electric forces. The theoretical analysis and simulation were based on some assumptions such as the property of spore coat was hyper-elastic. Therefore SEM measurement was performed to verify spore model.

It is noted that difference of surface morphology change between PEF-treated spores and wet-heat associated with high pressure inactivated spores is observed. Spores shrank and many wrinkles were formed after autoclaving at 121°C for 30 minutes (Fig. 3). Wet heat does not inactivate spores by DNA damage or oxidative damage [25]. Instead, spores inactivated by wet heat are associated with protein denaturation and enzyme inactivation [25]. Mechanisms of inactivation by PEF treatment and autoclave are different, but protein denaturation and enzyme inactivation may contribute to mechanism of inactivation by PEF treatment.

PEF-treated spores with concave shape were observed in all PEF treatment conditions. The electro-mechanical model proposed by Zimmermann [16] can describe this phenomenon. This model considers membrane bilayer as a capacitor. Under external electric fields, the attraction of opposite charges induced on the inner and outer surfaces of the membrane bilayer, compression pressure occurs resulting in a decrease in membrane thickness. For membrane bilayer, if the critical electric-field strength is exceeded, the membrane is permeabilized by pore formation. However, for bacterial spores, the spore coat is more rigid (elastic modulus of *B. subtilis* spore coat is approximately 13.6 GPa [21]) than membrane bilayer which means pores are hard to form on the spore coat. Instead, attraction forces of opposite sides of spore coat across the peptidoglycan layer rises resulting in concave shape as revealed by SEM. Spores with concave shape were observed under all PEF treatment conditions and the percentage at different treatment conditions was all around 10% which suggests spores with concave shape are little related with treatment time and conditions. It is hypothesized that those spores are adjacent to electrodes, i.e. under stronger electric fields.

Compared with PEF-treated spores at 50 pulses in DI water and in saline respectively, leakage is only clearly observed in saline. The thin viscoelastic film model proposed by Dimitrov [17] can describe this phenomenon. This model considers the membrane bilayer being always fluctuating.

Breakdown of membrane bilayer occurs after growth of fluctuation, molecular rearrangements and expansion of pores. Since spore coat is more rigid than a membrane bilayer, it is hard for spore coat to fluctuate like a membrane bilayer. However, charged molecules or proteins on spore coat have the tendency to rearrange under the external electric fields. Thus forces to pull spore coat apart will be generated under this tendency of rearrangements. In addition, considering the different PEF treatment conditions in DI water and in saline, only the difference is conductivity. Under the external electric fields, sodium ions and chloride ions are attracted by cathode and anode respectively. As a result, a large number of ions move fast to hit spore coat. In addition, the trans-membrane potential rises under the external electric fields, which means the attraction forces between the inner and outer surfaces of spore coat increases. As described above, breakdown happens in weak areas under the three factors of interaction, i.e. tendency of forces to pull spore coat apart, ions striking and rise of trans-membrane potential.

The bacterial spore model can successfully explain the PEF-treated spores with concave shape and leakage. However, the location of leakage and pore size cannot be determined by SEM. For future study, SEM will be used to study the location of leakage as well as pore forming and evolution.

VI. Conclusion

A bacterial spore model, combining electro-mechanical model and thin viscoelastic film model, was proposed to explain morphology change of spores induced by PEF treatment. This model can interpret why concave shape is formed and leakage occurs. Future study is needed to explore the effect of PEF treatment parameters on leakage occurring and particular locations of pores and pore sizes at different PEF treatment conditions.

Acknowledgment

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Appendix

Animation of surface morphology and stress change of a single spore:

https://www.youtube.com/watch?v=QlKuF_Oond0&feature=youtu.be

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