Single-cell analysis reveals microbial spore responses to sodium hypochlorite

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Abstract

Pollution from toxic spores has caused us a lot of problems, because spores are extremely resistant and can survive most disinfectants. Therefore, the detection of spore response to disinfectant is of great significance for the development of effective decontamination strategies. In this work, we investigated the effect of 0.5% sodium hypochlorite on the molecular and morphological properties of single spores of *Bacillus subtilis* using single-cell techniques. Laser tweezers Raman spectroscopy showed that sodium hypochlorite resulted in Ca $^{2+}$-dipicolinic acid release and nucleic acid denaturation. Atomic force microscopy showed that the surface of treated spores changed from rough to smooth, protein shells were degraded at 10 min, and the permeability barrier was destroyed at 15 min. The spore volume decreased gradually over time. Live-cell imaging showed that the germination and growth rates decreased with increasing treatment time. These results provide new insight into the response of spores to sodium hypochlorite.

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Abstract: Pollution from toxic spores has caused us a lot of problems, because spores are extremely resistant and can survive most disinfectants. Therefore, the detection of spore response to disinfectant is of great significance for the development of effective decontamination strategies. In this work, we investigated the effect of 0.5% sodium hypochlorite on the molecular and morphological properties of single spores of *Bacillus subtilis* using single-cell techniques. Laser tweezers Raman spectroscopy showed that sodium hypochlorite resulted in Ca^{2+}-dipicolinic acid release and nucleic acid denaturation. Atomic force microscopy showed that the surface of treated spores changed from rough to smooth, protein shells were degraded at 10 min, and the permeability barrier was destroyed at 15 min. The spore volume decreased gradually over time. Live-cell imaging showed that the germination and growth rates decreased with increasing treatment time. These results provide new insight into the response of spores to sodium hypochlorite.

Key words : atomic force microscopy; *Bacillus subtilis* ; laser tweezers Raman spectroscopy; morphological structure; single-cell analysis

INTRODUCTION

Spores are dormant bodies formed by spore-producing bacteria under nutrient-poor conditions [1]. Spore formation is an extremely complex process involving multiple changes in morphology, structure, chemical composition, and other aspects. During sporulation, trophoblast cells divide asymmetrically to form the prespore and mother cell compartments, where the smaller prespore compartment is used for spore formation and the larger mother cell compartment is used to cultivate the spores and synthesize the external proteolipid layer structure. Until the prespore is fully developed, the mother cell compartment engulfs and fuses with the prespore compartment and forms a multilayered protective layer around the prespore; when the spore is formed, the mother cell ruptures to release the spore into the environment [2]. The spore contains the complete genome of the species and is capable of resuming growth to form a trophoblast under nutritive conditions. In addition, spores have a special multi-layered structure and extremely low water content, giving them the ability to survive for long periods of time, even in extreme environments, making them extremely resilient. For example, spores can survive in low and high temperatures, including space environments, and can be exposed to strong acids and bases, ethanol, etc [3-7]. The resilience of spores is mainly dependent on two types of wall structures, an external protein wall, the spore shell, and a peptidoglycan wall, the spore cortex. The spore shell is resistant to a wide range of chemical and enzymatic treatments and acts as a permeable barrier, limiting the entry of macromolecules into the spore. The cortex, on the other hand, is a special peptidoglycan-rich structure deposited between the inner and outer membranes of the spore, responsible for maintaining the core in a highly dehydrated state, thus contributing to the extreme dormancy and heat resistance [2]. In addition, maintaining the dehydration of the spore core is dependent on the inner membrane, which is relatively permeable and limits the entry and exit of small molecules and chemicals [6]. Degradation of a spore's cortex and damage to the inner membrane can result in the rapid rehydration of the spore core and consequent loss of resistance [8]. Notably, a specific molecule within the spore core, pyridine-2,6-dicarboxylic acid (DPA), chelated 1:1 with Ca^{2+} (CaDPA), plays an important role in spore resistance and stability [9]. Although the exact function of CaDPA in spores is not yet known, a lack of, or low levels of, CaDPA can result in very unstable spores that germinate spontaneously [10].

Currently, chlorination is a popular method for sterilization and sodium hypochlorite (bleach) is widely used by virtue of its cheapness and availability [11]. Many studies have evaluated the effects of sodium hypochlorite on spores in terms of (i) spore viability, (ii) release of budding molecules and (iii) electron microscopic capture of budding structures. These studies have shown that at suitable concentrations and exposure times, sodium hypochlorite is able to kill spores by damaging their shells, germination proteins and DNA [12, 13]. However, there is controversy as to whether CaDPA is released from spores after sodium hypochlorite treatment. It has been shown that sodium hypochlorite destroys the outer shell of spores and damages proteins on the inner membrane, thus disrupting the permeability barrier, and leading to the release of CaDPA from the core and DNA denaturation [14]. However, in another study, spores did not release CaDPA after hypochlorite treatment but released CaDPA at germination and were unable to grow further [15]. The ability of hypochlorite to inactivate spores is not disputed; however, its mechanism of action is not yet fully understood. There are still many unresolved issues, including (i) whether sodium hypochlorite-treated spores release substances, such as DNA, at the time of CaDPA release and (ii) whether the degradation of spore shells by sodium hypochlorite is localized or global. In addition, methods used in previous studies, such as transmission electron microscopy and scanning electron microscopy, require the special treatment of individual organisms, which may cover up some information about treated spores [16]. It should also be noted that the overuse of chemicals to treat spores results in a series of problems, such as strain resistance and contamination of the environment [11, 17].

In the present study, we comprehensively evaluated the response of spores to sodium hypochlorite by using single-cell techniques. We used laser tweezer Raman spectroscopy (LTRS) to detect molecular changes in spores after sodium hypochlorite treatment, evaluated spore morphology before and after treatment using atomic force microscopy (AFM), and observed the germination and growth process of single spores before and after treatment using live-cell dynamic imaging. The results of this study deepen our understanding

of the response of spores to sodium hypochlorite, including internal molecular and morphological structural changes, and provide new insights into the effects of sodium hypochlorite on spores at the single-cell level.

MATERIALS AND METHODS

Strains, media, and spore preparation

Bacillus subtilis CMCC(B)63501 was purchased from the Chinese Medical Bacteria Preservation and Management Centre. Spores were incubated on 30% oligonutrient LB medium agar plates, and the ambient temperature was controlled at about 37°C. After 5 days of incubation, the spores were collected and purified. The purified spores were stored in distilled water at 4°C protected from light, and [?]95% of the spores were observed by phase contrast microscopy.

Measurement of sodium hypochlorite treatment and internal molecules in spores

The sodium hypochlorite solution was purchased from Shanghai Aladdin Biochemical Science and Technology Co. Ltd. (product no. S298682), with a density of 1.2 g/cm^3 and an effective chlorine content of 100–140 g/l. Anhydrous sodium thiosulphate was also purchased from Shanghai Aladdin Biochemical Science and Technology Co. Ltd. (product no. S100824). The spores were incubated in 0.5% sodium hypochlorite solution for 5, 10, 15 and 20 min, followed by addition of 5% sodium thiosulphate solution. Samples were left for 10 min for sufficient reaction, centrifuged (4, 10 min, 10^5 rpm) twice, and the supernatant was discarded. The untreated control was centrifuged in the same manner for consistency. The centrifuged spores were diluted to ˜10^7 spores/ml, stored in distilled water at 4degC, protected from light, and subsequently analyzed for viability and assayed by LTRS.

Individual spores suspended in water were randomly trapped by the laser beam, and Raman spectroscopy was used to assess internal changes in the molecule. The scheme

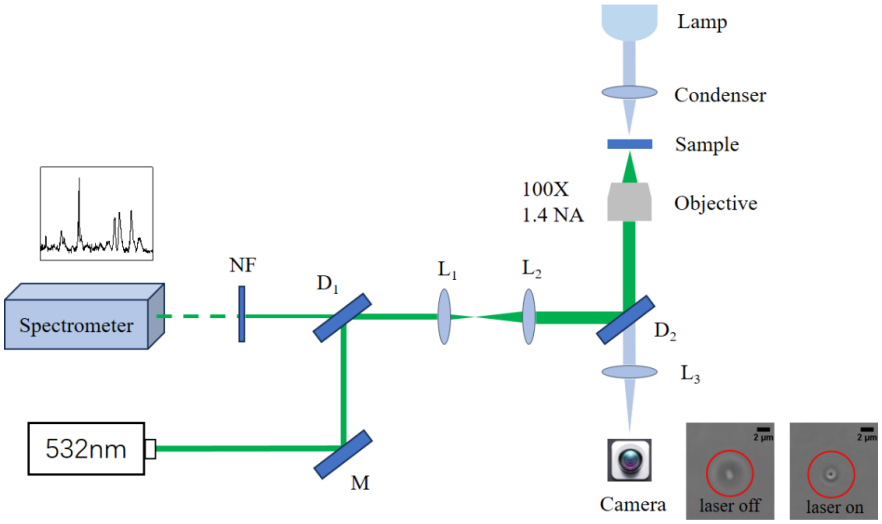


Figure 1 Illustration of the LTRS setup used to acquire Raman spectra of individual spores. The trapping laser is reflected by a planar dichroic mirror and focused through an objective lens to form an optical potential well in the sample cavity. The captured spore Raman scattering excited by the captured laser is returned along the original path and focused into the spectrometer. To illuminate a sample, we used a lamp

and acquired images using a CMOS camera. When the trapping laser is turned on, the trapped spore is imprisoned. When the trapping laser is turned off, the spore can move freely.

of the LTRS system is shown in **Figure 1** . A diode laser of 532 nm wavelength is used for trapping and Raman excitation of the individual spores. The combination of two biconvex lens (L_1 (f_1=100mm) and L_2(f_2=150mm)) is used to expand the beam to overfill the inverted microscope objective (100×, 1.40 NA), which acts as a trapping and Raman excitation and collection objective. The spectrometer (HR Evolution, Horiba Jobin 7 Yvon, Japan) has an 1800 gr/mm grating blazed at 500 nm and a liquid nitrogen-cooled charge coupled device (CCD). The laser power was 2.5 mW and integration time was 30s. The characteristic peaks of CaDPA are located at 660, 825, and 1017 cm^{-1}, the peak at 782 cm^{-1} is attributed to DNA, and the peak at 1004 cm^{-1} to phenylalanine [18, 19]. The content of each type of substance can be determined based on the relative intensities of the characteristic peaks [20].

Phase contrast micrography

To investigate CaDPA release from the core of the spores before and after sodium hypochlorite treatment, the state of spores was observed using phase contrast microscopy. Briefly, 2 μl of the budding suspension was added dropwise to a slide and dried in a vacuum desiccator for 10 min. Approximately 300 μl of sterile water was added to the sample chamber and a cover glass was added to form an airtight space. The sample chamber was fixed on an inverted microscope (Ti2; Nikon, Tokyo, Japan) with a 100× oil lens (Nikon). Images were obtained with a CMOS camera (2048×2048).

Cell viability analysis

Untreated and sodium hypochlorite-treated spore samples were diluted to a suitable concentration of spore suspension, coated on nutrient-rich LB agar medium, and placed in a constant temperature incubator at 37°C for 48 h, after which the colonies were counted and the survival of each group of spore samples was calculated.

AFM imaging of spores

Spore suspensions were added dropwise to Ploy-coated slides, allowed to stand in air, and dried for 30 min. Slides were fixed to sample holders and images were obtained in air. Images were acquired using a Bioscience AFM (NanoWizer4; Bruker, Wissembourg, France) operated in AC mode. The AFM probe used for imaging consisted of a silicon tip on a silicon nitride cantilever beam with a resonance frequency of –320 kHz and an elasticity coefficient of 42 N/m. All images were scanned at 512 pixels per line with a scanning frequency of 0.5–1.0 Hz.

Dynamic live-cell imaging

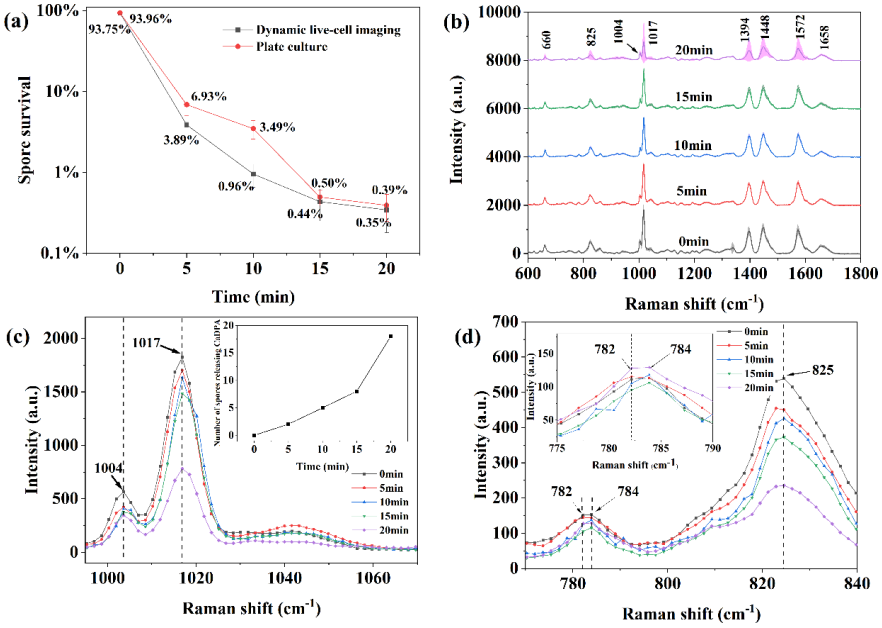
To analyze the sprouting and growth of untreated and sodium hypochlorite-treated spores, dynamic imaging of live cells dynamic imaging (N-storm; Nikon) was used to observe and record growth in real time. A small drop of budding solution was spread on a slide and dried naturally at 25°C for half an hour in order to distribute the spores evenly. A drop of melted 100% enriched LB agar medium was increased by approximately 400 μl to the top of the sample spores to form an agar pad with a thickness of about 3 mm. The agar on one side of the pad was removed to form some small holes for air flow, and was subsequently covered with a coverslip to form an airtight space above the agar. The slides were visualized under an inverted microscope (Ti2; Nikon) and the growth of the spores was recorded with a CCD camera (12bits, 2044 × 2048) at 30 s/frame for 6h at a constant temperature of 37°C.

RESULTS AND DISCUSSION

Survival and Raman spectroscopy analysis of spores after sodium hypochlorite treatment

Sodium hypochlorite (bleach) is an inexpensive and common decontaminant, and a concentration of 0.5% has been reported to kill spores [21]. In addition, sodium hypochlorite degrades organic matter through a variety of reactions such as saponification of fatty acids, neutralization of amino acids, and ammoniation [22]. Therefore, the treatment of spores with sodium hypochlorite can cause damage to lipids, proteins, and DNA.

In this study, two methods were used to record spore survival, plate culture and live-cell imaging techniques. The survival of spores after sodium hypochlorite treatment was significantly lower than that of untreated spores and showed a decreasing trend with increasing treatment time (**Figure 2 (a)**). In addition, the maximum difference (5 min) in spore survival obtained using these two methods was ˜3%, indicating that the results obtained using dynamic live-cell imaging were consistent with the actual status of spores before and after treatment.



To observe the effects of sodium hypochlorite on spore properties, LTRS was used to compare the Raman spectra of spores treated with sodium hypochlorite for 0, 5, 10, 15, and 20 min (**Figure 2 (b)**). We found substantial changes in the Raman spectra of treated spores, with the most obvious change in the characteristic peak of CaDPA. Taking 1017 cm^{-1} as an example, its relative intensity decreased dramatically as the treatment timeincreased (**Figure 2(c)**). The percentages of spores releasing CaDPA increased rapidly with an increase in treatment time (**Figure 3**). These two results indicate that sodium hypochlorite treatment caused the spores to release CaDPA and had greater effects on spores over time. It is worth noting that CaDPA is located in the core of spores, and the inner membrane controls the entry and exit of materials from the core. Therefore, the release of CaDPA from spores after sodium hypochlorite treatment is sufficient evidence that sodium hypochlorite disrupts the permeability barrier of the spores, including the inner membrane [14, 23]. The mechanism by which sodium hypochlorite promotes the release of CaDPA from the spores is not fully understood; however, Raman spectroscopy and phase contrast micrography results suggest that CaDPA is released from spores after treatment and that the number of individuals releasing

CaDPA increases with the duration of the treatment are in agreement with the results of a previous report [14]. By comparing the Raman peaks of DNA at 782 cm^{-1}, we detected a slight shift after sodium hypochlorite treatment, although the relative Raman intensity was largely unchanged (**Figure 2(d)**). These results indicated that sodium hypochlorite may damage the DNA of spores, without affecting the DNA content. Among the 30 spectra for single spores measured in each group at 0, 5, 10, 15, and 20min, the spectra of 0, 0, 2, 6, and 15 spores had shifted peaks, respectively, indicating that DNA denaturation occurred and intensified as the treatment time increase [14]. It can be speculated that sodium hypochlorite entered the spores after destroying the permeability barrier, resulting in damage to the DNA but not the loss of DNA. It is

Figure 2 (a) Survival of spores after sodium hypochlorite treatment obtained using plate culture and live cell imaging; (b) average Raman spectra of 30 *Bacillus subtilis* spores after different times of sodium hypochlorite treatment; (c) average Raman spectra in the CaDPA bands and the number of spores releasing CaDPA at different time; and (d) average Raman spectra in the DNA band and the offset at 782 cm^{-1}. a.u., arbitrary units.

worth noting that the 1004 cm^{-1} attributed to phenylalanine decreased after treatment, because sodium hypochlorite reacts with amino acids in a neutralizing manner, damaging spore proteins [24].

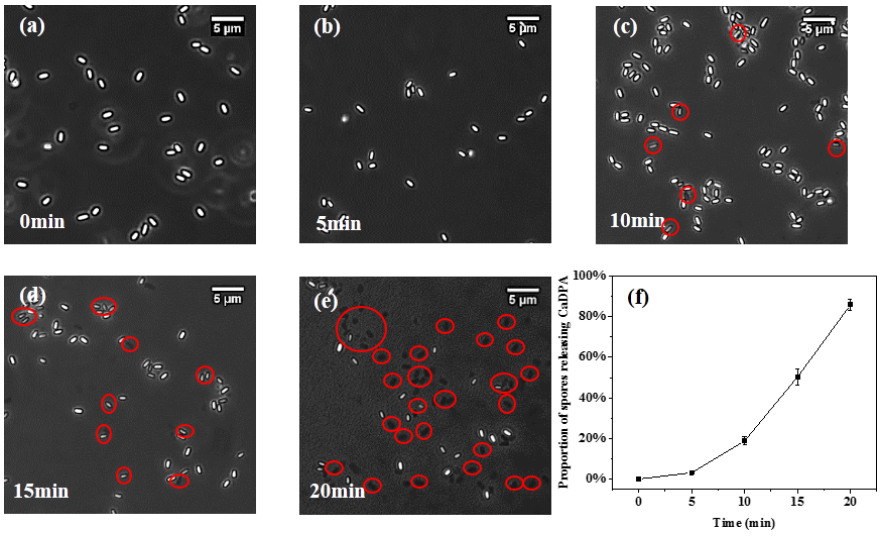
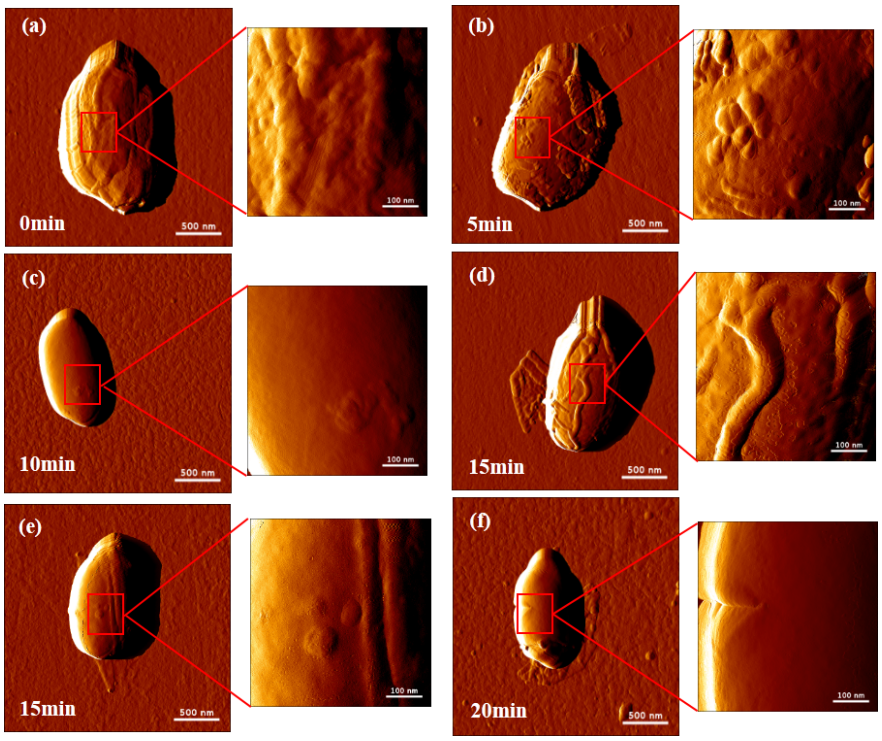


Figure 3 Phase contrast micrographs of *Bacillus subtilis* spores at different times of sodium hypochlorite treatment. (a) 0 min; (b) 5 min; (c) 10 min; (d) 15 min; (e) 20 min; (f) proportion of CaDPA released at different times. Scale bar of the figure is: 5 μm. Red circles are spores releasing CaDPA.

Effect of sodium hypochlorite on the surface morphology and size of spores



To confirm the effect of sodium hypochlorite on the surface topography and morphology of spores, groups of spores treated with sodium hypochlorite for different times were characterized separately using AFM. Most of the untreated spores had a relatively rough outer surface (**Figure 4 (a)**), with some individuals showing shallow ridges on the surface. After sodium hypochlorite treatment, the external surface of the spore was obviously changed; the surface of the spore individual was uneven and had protrusions consistent with shell protein retention after 5 min. The surface of the spore individual treated for 10 min was extremely smooth, with a few attachments. After treatment for 15 min, obvious ridges appeared on the spore surface, holes of different sizes were detected, and there were many crystal-like attachments around the spore. After 20 min, it became smoother, the individual spores became significantly smaller, and some spores had gaps on the surface (**Figure 4(b)–(e)**). Sodium hypochlorite treatment caused obvious damage to the surface morphology and morphological

Figure 4 AFM images of individual spores treated with sodium hypochlorite for different times. (a) 0 min; (b) 5 min; (c) 10 min; (d) and (e) 15 min; (f) 20 min. (Picture size of individual spores: 2.5 μm, scale bar: 500 nm; picture size of local details of spores: 500 nm, scale bar: 100 nm)

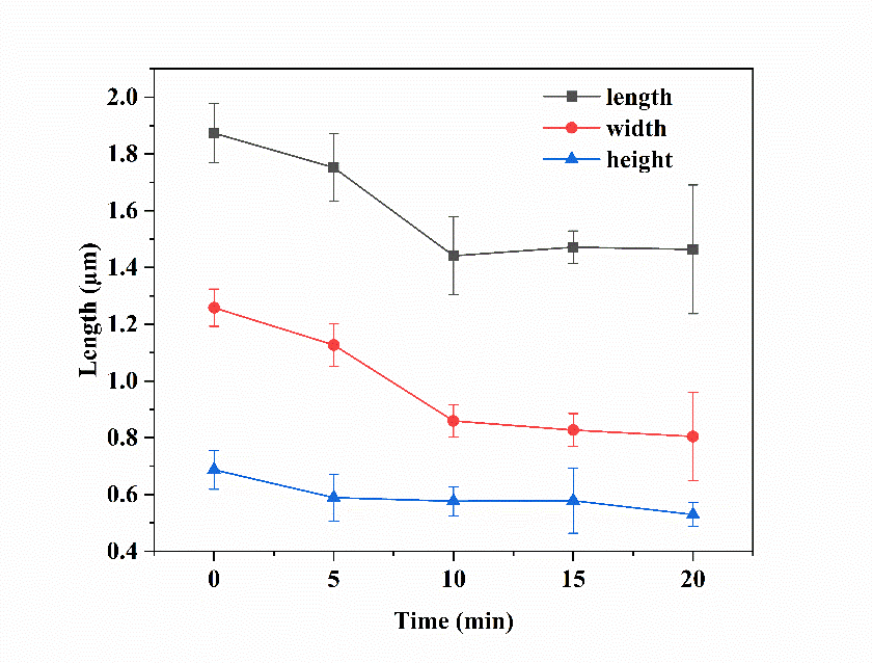


Figure 5 Variation curves of length, width and height dimensions of spores after different times of treatment.

structure of spores. Previous studies have shown that DPA within spores exists in the form of CaDPA crystals; accordingly, it is likely that these crystal-like attachments are CaDPA [25]. In addition, sodium hypochlorite degrades the spore shells and cortical peptidoglycan [26]. The overall trend in which the length, width, and height of the spores decreased as the treatment time increased (**Figure5**) is consistent with the degradation of the shells by sodium hypochlorite and core CaDPA leakage.

Our AFM images confirmed that sodium hypochlorite degrades the tissue structure of spores. As the treatment time increased, sodium hypochlorite destroyed and degraded the morphology and structure of the spores from the outside in. At 0–10 min, substantial alterations in surface morphology were detected, and the rough structure of the outer layer gradually disappeared, revealing a relatively smooth structure. However, electron microscopy released that the thicknesses of the outer shell protein layer and cortex of *Bacillus subtilis* were about 200 nm and 70–200 nm, respectively [2, 27]. At 10 min, the length and width of the buds were reduced by ˜400 nm and the height was reduced by ˜100 nm, indicating that at this time, the rough outer shell protein layer of the buds was almost completely degraded and the smooth cortex layer was revealed. Sodium hypochlorite continued to destroy the cortical structure of the spores; at 15 min, many holes were distributed on the surface of the spores, some of the spores swelled locally to form irregular ridges (**Figure 4 (d)**), and some of the spores were obviously cracked (**Figure 4 (e)**). At this time, a large number of spores released CaDPA, indicating that sodium hypochlorite can continue to degrade the cortical structure of the spores, destroying the osmotic pressure barrier of the spores. The enabled outside substances to enter the spores, and the spores swelled to form ridges. When the osmotic barrier was destroyed to a certain extent, the internal substances were released, such as CaDPA. In addition, according to Raman spectra of DNA, as the contact time with sodium hypochlorite increased, more spore individuals showed deviations in characteristic peaks. Approximately 50% of the individuals had deviations by 20min, indicating that sodium hypochlorite is highly likely to enter into the spore and damage DNA. Furthermore, large gaps appeared on the surface of the spores at 20 min, indicating that the degree of damage increased with the time. By comparing the AFM images of spores at different time points, we can see that spore damage cause by sodium hypochlorite occurs from the outside to the inside, and prolonging the treatment time will aggravate damage.

Effects of sodium chlorate on the sprouting and growth of spores

To analyze the effect of sodium hypochlorite on germination and growth, the spores were cultured on 100% enriched LB agar Petri dishes at 37°C, and bright field images were recorded every 30 s for 6 h (**Figure 6 (a)–(e)**). Some of the spores treated with sodium hypochlorite were still able to germinate and grow. However, compared with those of untreated spores, the germination rate of sodium hypochlorite-treated spores was substantially lower (**Figure 2 (a)**), the time of germination was much later (**Figure 7(a)–(b)**), and the growth rate of spores was much slower (**Figure 7 (c)**), indicating that sodium hypochlorite had an extremely strong inhibitory effect on spore germination and growth, and the inhibitory effect was more obvious over longer time periods. The inhibitory effect of sodium hypochlorite on spores' growth can be explained in two ways. (i) Proteins related to sprout growth were damaged. The spore shell has a multilayered structure with a variety of proteins; it serves as a permeability barrier, restricting macromolecules from entering the interior, and is able to sense changes in the external environment, which plays an important role in the process of germination and growth [6, 28, 29]. Sodium hypochlorite degraded the shell of the spores, destroying proteins attached to the shell, making spores unable to receive and transmit the signals for germination quickly. Therefore, there was a lag in the germination and growth of spores and, eventually, failure to germinate. Previous studies have also shown that hypochlorite can cause significant damage to a variety of germination proteins [15]. (ii) It can be explained by DNA damage. Nucleic acids store genetic information for survival and reproduction [6]. Raman spectroscopy and AFM images showed that sodium hypochlorite disrupts the permeability barrier of the spores; while the spores release CaDPA from the core, external sodium hypochlorite enters the interior of the spores and may cause damage to substances, such as DNA and proteins. As a result of DNA damage, genomic instability and the loss of some growth-related functions may arise, leading to abnormal spore growth [30].

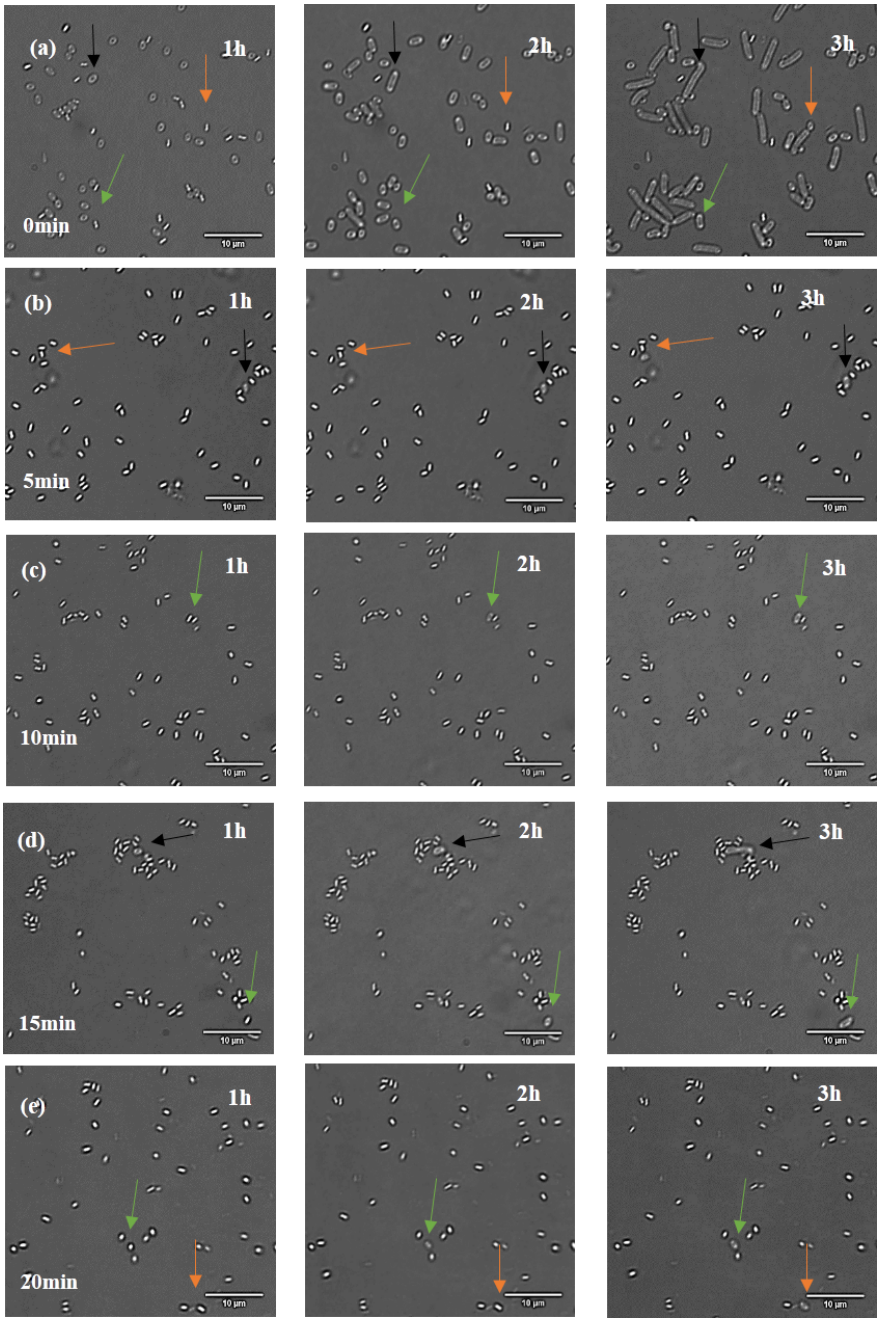


Figure 6 Images of germination and growth of spores after different times of sodium hypochlorite treatment. (a) 0 min; (b) 5 min; (c) 10 min; (d) 15 min; (e) 20 min. Black, green and orange arrows labelled are the sprouted individuals incubated at 37°C for 1h, 2h and 3h respectively. (Scale bar in the figure: 10 μm.)

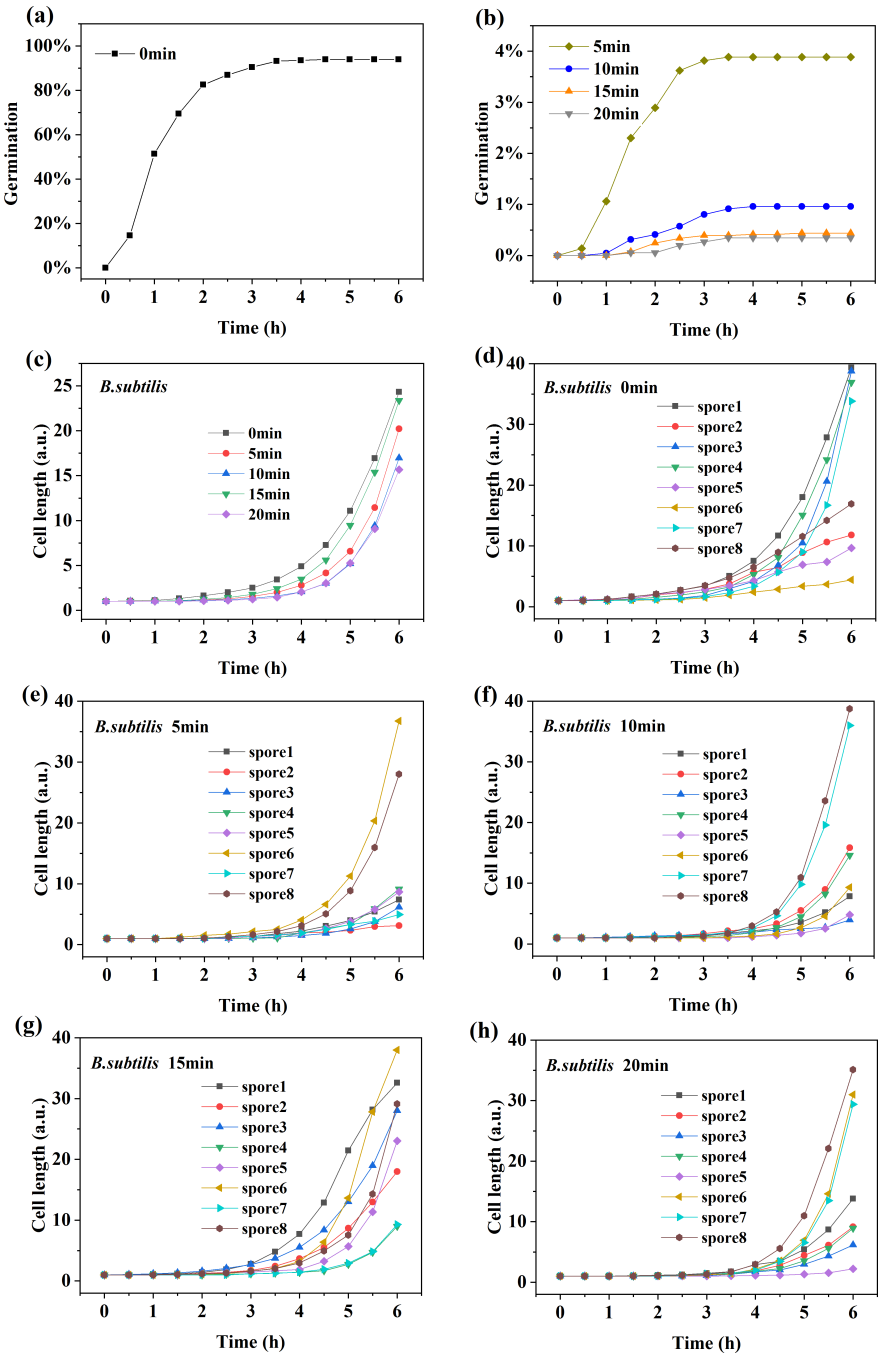


Figure 7 (a) Mean curves of germination rate versus time for untreated spores; (b) mean curves of germination rate versus time for spores after different times of sodium hypochlorite treatment; (c) mean curves of growth length versus time for spores after sodium hypochlorite treatment; (d)–(h) curves of cell length versus time for single spores treated with sodium hypochlorite for 0–20 min.

In addition, we found that surviving spores after sodium hypochlorite treatment were able to germinate and grow to form trophoblasts; however, inactivated spores were unable to germinate despite incubation for up to 3 h (**Figure 6**). Notably, there were differences in the curves of cell length versus time for eight single spores

(treated and untreated groups) (**Figure 7 (d)–(h)**), indicating that there were individual differences in the effect of sodium hypochlorite on spores under the same conditions, reflecting the individual heterogeneity of the spores [31].

CONCLUSIONS

The key contribution of this study was the analysis of the response of spores to sodium hypochlorite at the single-cell level. Using single-cell techniques, our results revealed that sodium hypochlorite inactivates spores by degrading the spore shell, damaging germination proteins, breaking the permeability barrier, and damaging DNA. Upon contact with spores, sodium hypochlorite reacted with the shell protein layer, gradually dissolving the shell until it was completely degraded and the attached budding proteins were destroyed. Subsequently, it damaged the cortex, endosperm, and other structures of the spores, destroying the permeability barrier and causing the spores to release CaDPA. Sodium hypochlorite entered the interior of the spores, causing damage to DNA and other substances and ultimately leading to spore inactivation. In addition, with the prolongation of sodium hypochlorite treatment, the inhibitory effects on germination and growth increased, and damage to the structure of spores was more serious. Long-term sodium hypochlorite treatment will continue to degrade other structures of the spores and even the entire spore. The volume of the spores may decrease dramatically after degradation, making the acquisition of single-cell Raman spectra difficult; therefore, we controlled the treatment time to less than 20 min. The effects of increasing the treatment time on spores need to be investigated in the further.

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Author contributions

LH and HF contributed to conception, design of the study and supervision. WY carried out the experimental design, performed the experiments and the statistical analysis. YY was responsible for writing - reviewing and editing. All authors contributed to the article and approved the submitted version.

Data availability

Data underlying the results presented in this paper are not publicly available at this time but may be obtained from the corresponding author upon reasonable request.

Conflict of interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

Consent for publication

The university is aware about the work has consent for publication. The authors declare consent for publication.

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