Synergistic Effects of Ultrafast Heating and Gaseous Chlorine on the Neutralization of Bacterial Spores

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ABSTRACT

Improving the neutralization of bacterial spores is of paramount importance for the bioterrorism defeat. In this study, we investigate the synergism between rapid heating ($\sim 10^4$ °C/s to $\sim 10^5$ °C/s) and chlorine gas in the neutralization of *Bacillus thuringiensis* (Bt) spores – a close relative of *B. anthracis* (Ba), which is a known biowarfare agent. Bt spores were heated in a gas chamber with defined concentrations of Cl₂ gas and relative humidity (RH). The critical peak temperature (T_c) of spores, which corresponds to 50% reduction in viability, was decreased from 405 °C when heated at $\sim 10^4$ °C/s in air to 250 °C when heated at the same rate in 100 ppm Cl₂. SEM results show no obvious difference between the morphologies of spores heated in air or in Cl₂ at $\sim 10^4$ °C/s. These results indicate that Cl₂ gas acts in synergy with high temperatures (>300 °C) to neutralize Bt spores. Similarly, the T_c for Bt spores heated at the faster rate of $\sim 10^5$ °C/s was reduced from 230 °C when heated in air to 175 °C when heated in 100 ppm Cl₂. At $\sim 10^5$ °C/s, the treatment of Cl₂ did not alter spore morphology at temperatures below 300 °C. At temperatures above 450 °C with Cl₂, the spore coat detached from the underlying core. The effect of Cl₂ was further examined by changing the RH of Cl₂ gas. The results show that highly humidified Cl₂ (RH = 100%) reduced T_c by 170 °C and 70 °C at $\sim 10^4$ °C/s and $\sim 10^5$ °C/s, respectively, as compared to dry Cl₂ (RH = 0%). Energy dispersive spectrometric (EDS) results demonstrate that Cl on the spore increased with elevated peak temperature, with the majority of the Cl located in the shed spore coat. This study indicates that the major mechanism of spore neutralization by the synergism of Cl₂ and rapid heat is chlorine reacting with the spore surface.

KEYWORDS

Bacterial spores; Neutralization; Ultrafast heating; Chlorine; Hydrogen Chloride

1. Introduction

Bacterial spores have raised serious concerns in the military biodefense [1-3]. Neutralization of spores is a significant challenge since they are much more resistant than their vegetative counterparts to a variety of external stresses such as UV irradiation, extreme pH values, chemicals, and temperature extremes [4-6]. Studies have shown that spore longevity and resilience is correlated to physical features of spores, including a tight proteinaceous spore coat that inhibits chemicals penetration, low water content in the spore core to reduce metabolism, as well as the production of proteins for DNA stabilization (e.g. α/β -type small acid soluble proteins (SASPs)) [6]. One of the primary strategies for spore neutralization is to expose them to autoclaving heat (120-150 °C) for minutes to hours [7-9]; however, this approach is not appropriate for the large-scale neutralization of spores. To further improve the killing efficacy, aerosol based techniques are being developed to rapidly inactivate airborne bacterial spores by rapid heating (>200 °C within a timescale of a second) [10-16]. At this timescale, more than 3-logs reduction in spore population can be achieved when the peak temperature ranges from 200 to 400 °C. An analogous approach of spore inactivation by heat has been tested using heat generated from exothermic reactions of energetic materials such as aluminum-based thermites [17]. This approach is able to produce even higher peak temperatures (> 2200 °C) over a shorter period (~ 0.1 s), and leads to a 7-log reduction of spore viability. Although these methods are capable of neutralize spores, an accurate and quantitative relationship of time-temperature-kill for spores is not available due to the variability in temperature distribution and the resident exposure time of these heating schemes. Nevertheless, a precise time-temperature-kill relationship is needed for predicting and ensuring a successful outcome of large-scale neutralization events. In order to improve the accuracy of measurements of the temperature

history on spores, an alternative approach has been developed by heating spores deposited on conductive surfaces [18] that allows measurement of the transient temperature using the standard electric resistance-temperature relationships [18]. Since the transient temperature of immobilized spores can approximate that on the immobilizing surface [19], the time-temperature-kill relationship for spores can be accurately measured. Results showed that a 6-log reduction in spore counts could be achieved by rapidly heating spores to 600 °C within 50 ms at a heating rate of $\sim 10^4$ °C/s. The neutralization mechanism was likely due to DNA damage as mutants in *sspA* *sspB* are sensitized for killing [6]. Faster heating rates ($\sim 10^5$ °C/s) also improved spore neutralization, which was associated with increased structural destruction of spore coat through increased pressure of vaporization [19].

In addition to heat, another commonly used disinfection procedure for spores utilizes biocidal chemicals [20]. Commonly used biocides (antibiotics, detergents, alcohol) have little effect on spore viability [21]. In contrast, strong oxidation agents, such as chlorine, iodine, sulfur, silver, and compounds containing these elements, have demonstrated efficacy in spore inactivation [20]. Among these sporicides, Cl_2 is one of the few agents that are gaseous at room temperature. The main advantage of Cl_2 over other aqueous sporicides is that gas provides greater coverage, thus facilitating the neutralization of both airborne and surface-associated spores. Cl_2 can directly chlorinate functional groups on macromolecules in cells to damage proteins, nucleic acids and lipids [22,23]. In addition, Cl_2 can react with water to form hypochlorous acid (HOCl) and hydrogen chloride (HCl). Both compounds can also react with the spore to inactivate them [24][25].

The performance of Cl_2 depends on two characteristic factors: concentration (“C”) and inactivation time (“T”). The US Environmental Protection Agency and the water treatment industry has set the units of Cl_2 concentration in parts per million (ppm) and the inactivation time in minutes [26]. In general, the “CT” product is a constant for spores of a specific *Bacillus* strain required to achieve a defined reduction of viability [27]. Table 1S presents some documented “CT” results for different *Bacillus* spores [24, 26-38]. The “CT” product for a 4-log reduction in spore viability is $\sim 3 \times 10^4$ ppm·min (~ 100 mg·min/l) for most of *Bacillus* spores when the Cl_2 concentration is below 3×10^3 ppm [26-30]. At much higher Cl_2 concentrations (*e.g.* 7×10^6 ppm), this “CT” value is significantly larger than $\sim 3 \times 10^4$ ppm·min, and the minimum exposure time is 5 min [24, 31, 32]. In order to improve the neutralization efficiency at exposure times under a second, which according to the “CT” rule would require concentrations of $\text{Cl}_2 > 10^8$ ppm (close to that of pure Cl_2 liquid). The use of these concentrations of Cl_2 would be impractical as a method to safely neutralize spores [39]. To meet the guidelines set by the U.S. Food and Drug Administration (FDA) for food and drinking water processing (Code of Federal Regulations Title 21 Part 173/178) [40, 41], new approaches are in development to combine heat with 100 - 2000 ppm Cl_2 .

One such potent, more environment-friendly and safer approach is to synergize the neutralization of spores by Cl_2 with heat. Xu et al. [42] studied the inactivation of *Bacillus* spores by Cl_2 (~ 1000 ppm) under high-temperature short-time pasteurization conditions (~ 80 °C, ~ 1 min), and found a viability reduction of 6-logs. Further tests using a higher temperature of 120 °C resulted in inactivation of spores by 6-logs within 16s, whereas it took > 30 mins to achieve viability reduction of 6-logs by employing either Cl_2 gas (1000 ppm) (Table S1) or heat (120 °C)

[7-9]. Based on these results, higher temperatures ($>200\text{ }^{\circ}\text{C}$), and diluted Cl_2 gas ($< 2000\text{ ppm}$) synergistically inactivated bacterial spores.

In this work, we investigated the synergistic effects of ultrafast heating and Cl_2 gas on the inactivation of *Bacillus thuringiensis* (Bt) spores. Bt spores, while closely related to *Bacillus anthracis* (Ba) spores that are considered as a serious bioterrorist weapon, is not pathogenic to humans. Both Bt and Ba spores were reported to have similar sensitivity to biocides [43], so we expect the results in this study can be utilized for the neutralization of Ba spores in the future. For these studies, Bt spores were electrophoretically deposited onto a fine Pt wire [19]. By tuning the heat pulse time (2 ms and 50 ms) and peak temperature (up to $\sim 1200\text{ }^{\circ}\text{C}$) for the Pt wire, the heating rate ($\sim 10^4\text{ }^{\circ}\text{C/s}$ and $\sim 10^5\text{ }^{\circ}\text{C/s}$) and time-resolved temperature for individual spores were measured [19]. Using this thermal approach, we evaluated the effect of 100 ppm (0.3 mg/l) Cl_2 , a concentration below the FDA safety guidelines, in combination with different peak temperatures and heating rates, on the neutralization of spores. Spore viability and morphology were assessed after these treatments by determining the viable number of colony forming units (CFU) and scanning electron microscopy (SEM). To investigate the neutralization mechanism of Cl_2 , spores were also heated in Cl_2 gas with different relative humidities, to see the roles of Cl_2 and its hydrolytic products (hydrogen chloride (HCl) and hypochlorous acid (HOCl)). Energy dispersive X-ray spectroscopy (EDS) was employed to determine the elemental changes of Cl and carbon (C) in spores.

2. Materials and methods

2.1. Spore attachment on platinum wires

Bt spores were sporulated in Difco Sporulation Medium (DSM) at 30 °C for 48 h. The 250 ml of DSM included 2 g Bacto nutrient broth, 2.5 ml 10% KCl, 0.375 ml 1 M NaOH and 2.5 ml 1.2% MgSO₄·7H₂O. The spore concentration was enumerated by plating to be 8×10⁹ colony-forming units per milliliter (CFU/ml). The purity of spores was found to be more than 99%. Bt spores were electrophoretically immobilized onto a sterilized platinum (Pt) wire with a diameter of 76.8 µm (Omega Engineering, Inc.). The electroplating experiments were conducted in an in-house spore deposition cell [44]. By controlling the biased deposition voltage, pulse frequency and overall charging time (from a 6340 sub-femtoamp remote sourcemeter, Keithley), we are able to prepare a uniform monolayer of Bt spores on the wire after 5 pulses [44]. More information of the spore deposition cell and spore coating scenarios in detail can be found in our previous studies [19, 44].

2.2. Wire heating test in Cl₂ gas filled chamber

The spore coated wire was connected to an in-house built power source, working as a temperature jump probe (Fig. S1). The wire was then inserted into a gas chamber that is connected to a vacuum pump and a Cl₂ gas tank (Fig. S1). Prior to the pulse heating of the wire, the chamber was emptied by vacuum and replenish with the appropriate concentrations of Cl₂ gas. For evaluating the effect of relative humidity (RH) of Cl₂ gas, the chamber is also connected to a water bubbler that can supply water vapor into the chamber. RH was monitored using an attached humidity meter. A defined thermal history for spores on wire during the heating period was measured through the dynamic electric resistance-temperature relationship for Pt (Callendar-Van Dusen equation [18]). The peak temperature (~200 °C to ~700 °C) and the heating rate (~10⁴ °C/s

to $\sim 10^5$ °C/s) can be precisely controlled by tuning the applied voltage and the pulse time (2 ms to 50 ms). The temperature-time profile for spores in the cooled region was calculated according to an energy balance equation which was dominated by the heat conduction. It is estimated that the cooling time scale is between ~ 300 ms and ~ 500 ms [19]. The detailed description of the transient temperature measurement on spores can be found in our previous study [19].

Since Cl_2 will be hydrolyzed to produce HCl and HOCl in the presence of humidity, as a comparison the effect of HCl was also investigated by exposing spores to HCl gas released from a beaker of 36% wt. HCl solution inside the chamber. The solution was extracted from the chamber after ~ 10 mins, when the equilibrium vapor pressure of HCl inside the chamber was achieved (~ 1800 ppm) (calculated based on a known partial vapor pressure of HCl as 0.18 kPa [45]). Then the chamber was vacuumed to $1/18$ atm, followed by air inflation (40% RH) back to 1 atm. The final concentration of HCl inside chamber was kept as ~ 100 ppm (40% RH). The procedure of heating spores in this case is similar to that in the Cl_2 case above.

2.3. Determination of colony forming units

The viable spores after various heat and Cl_2 treatments were enumerated by determining CFUs. Spore-coated wires were completely submerged in 1 ml of Lysogeny Broth (LB) media (10 g tryptone, 5 g yeast extract and 5 g NaCl per liter) and placed on a shaker at the 37 °C for three hours to allow for the germination and detachment of viable spores from the wire surface. After incubation, samples were serially diluted and plated on LB agar plates to count viable CFUs.

2.4. Characterization of morphology of spores

The surface morphology of spores with or without treatment of Cl_2 and rapid heat was investigated by SEM (Hitachi S-4700) using an accelerating voltage of 5 kV. Prior to imaging, the spore-coated wire was attached to stubs and then sputtered with gold/palladium alloy.

2.5. Characterization of hydrocarbon and chlorine contents inside spores

Quantitative EDS X-ray microanalysis using SEM was employed to analyze the elemental contents (carbon, chlorine and calcium) inside spores. Since calcium will not escape from spores by evaporation (boiling point at $\sim 1500^\circ\text{C}$) during the rapid heating in our temperature regimen ($< 700^\circ\text{C}$), the detected calcium content was used as a control to measure the relative Cl and C contents. The tests were conducted in the line scan mode by detecting the elemental intensity along a line drawn across one spore. The ratio of mass fractions of Cl/Ca or C/Ca was calculated by comparing the integrated intensities of Cl/Ca or C/Ca along the line, respectively. For spores with their coats detached, a line was across either the cracked coat or the exposed core, to analyze the distribution of elemental masses in both compartments. Statistical analyses are based on a spore population of ~ 10 .

3. Results and discussion

3.1. Spore viability heated at two ramp rates in air and in Cl_2

The neat effect of fast heating on the viability of Bt spores in air was determined and the relationship between peak temperature and spore viability was plotted in Fig. 1. The viability data fit a sigmoidal model [46] as:

$$S = \frac{1}{1 + \exp(k(T - T_c))} \quad (1)$$

where S is the survival ratio of spores ($\text{CFU}_{\text{final}} / \text{CFU}_{\text{initial}}$), T is the peak temperature, T_c is the critical peak temperature to induce viability reduction by half, and k is the heat resistance parameter. The fitted values of T_c and k are listed in Table 1. Compared to T_c (405 °C) at the temperature rate of $\sim 10^4$ °C/s, T_c at $\sim 10^5$ °C/s decreases to 230 °C. In addition, a reduction of 6-logs was achieved at the peak temperatures of 400 °C and 600 °C at $\sim 10^5$ °C/s and $\sim 10^4$ °C/s, respectively. These neutralization data for Bt spores resemble those for Bs spores in our previous studies [19], indicating that the faster heating rate of $\sim 10^5$ °C/s is able to effectively decrease the peak temperature required for spore neutralization when compared to the heating rate of $\sim 10^4$ °C/s. In addition, the k value of the neutralization curve at the higher heating rate ($\sim 10^5$ °C/s) was larger than that at the lower heating rate ($\sim 10^4$ °C/s), indicating that Bt spores are more sensitive to higher heating rates. These results show a better neutralization effect for Bt spores by higher heating rates and higher peak temperatures.

The effect of Cl_2 gas and rapid heat was evaluated for their ability to synergistically neutralize Bt spores. The resistance of Bt spores to Cl_2 gas at room temperature was first assessed. After 15 minutes of exposure, spore viability was not affected when exposed to 1 ppm (0.003 mg/L) to 100 ppm (0.3 mg/L) of Cl_2 (Fig. S2), whereas spores were completely neutralized by 1000 ppm of Cl_2 . We used 100 ppm of Cl_2 for the rest of this study since this concentration did not neutralize Bt spores in the absence of heat and is within the FDA guidelines (200 – 2000 ppm)

[41, 42]. Heating to 250 °C at $\sim 10^4$ °C/s, and 170 °C at $\sim 10^5$ °C/s (Fig. 1) reduced spore viability. Compared to the viability results by heat treatment alone, the critical peak temperatures T_c decrease by 150 °C and 50 °C at heating rates of $\sim 10^4$ °C/s and $\sim 10^5$ °C/s, respectively (Table 1). In addition, a 6-log viability reduction was achieved at lower temperatures of 300 °C at $\sim 10^5$ °C/s, and 450 °C at $\sim 10^4$ °C/s. These results show a synergistic effect of rapid heat pulses and Cl₂ gas in the neutralization of Bt spores in both heating schemes. It should be noted that the k values for the neutralization curves of spores treated with Cl₂ and without Cl₂ are similar at the same heating rate (> 0.1 for 50 ms pulse and < 0.1 for 2 ms pulse), indicating that the temperature sensitivity of spore neutralization is primarily controlled by the heating rate instead of the addition of Cl₂.

Fig. 1. Survival curves of Bt spores versus peak temperature for 2 ms ($\sim 10^5$ °C/s) and 50 ms ($\sim 10^4$ °C/s) heat pulses, and with or without the presence of Cl₂ (100 ppm). The relative humidity was kept at 40%.

Table 1.
Fitting results for the variables in Eq. 1.

Heating conditions	Cl ₂ (+, -)	RH	k (°C ⁻¹)	T_c (°C)
50 ms heating pulse ($\sim 10^4$ °C/s)	-	40%	0.090	407
50 ms heating pulse ($\sim 10^4$ °C/s)	+	40%	0.065	252
50 ms heating pulse ($\sim 10^4$ °C/s)	+	0%	0.065	352
50 ms heating pulse ($\sim 10^4$ °C/s)	+	100%	0.083	180
2 ms heating pulse ($\sim 10^5$ °C/s)	-	40%	0.109	232
2 ms heating pulse ($\sim 10^5$ °C/s)	+	40%	0.146	176
2 ms heating pulse ($\sim 10^5$ °C/s)	+	0%	0.136	247
2 ms heating pulse ($\sim 10^5$ °C/s)	+	100%	0.131	177

Note: “+” represents the presence of Cl₂ during heating of spores, while “-” represents no Cl₂ during heating of spores.

3.2. Spore viability heated in Cl₂ gas of different RH at two ramp rates

To understand the mechanism of spore inactivation by Cl₂, different humidities were supplemented to the 100 ppm of Cl₂ in the gas chamber used for heat inactivation of Bt spores. The gas chamber for the results described in Section 3.1 has a relative humidity of $\sim 40\%$, similar to that of ambient air. At $\sim 10^4$ °C/s, the critical peak temperature for spores heated in dry Cl₂ (0% RH) is 100 °C higher than that in moderately humidified Cl₂ (40% RH), whereas the critical peak temperature for spores heated in moist Cl₂ (100% RH) is 70 °C lower (Table 1). These results show that the T_c is reduced as the humidity of Cl₂ increases indicating that the synergistic effect of Cl₂ and rapid heat is potentiated by high humidity.

When the heating rate for Bt spores increases to $\sim 10^5$ °C/s, T_c increased for Bt spores treated with dry Cl₂ (0% RH) (Fig. 2B), similar to spores heated at $\sim 10^4$ °C/s heating rate (Fig. 2A). In contrast, moderately humidified Cl₂ (40% RH), and moist Cl₂ (100% RH) resulted in similar T_c ,

176 °C and 177 °C, respectively (Table 1). These $\sim 10^5$ °C/s results are distinct from those at $\sim 10^4$ °C/s since there is minimal change of spore neutralization when RH of Cl₂ increases from 40% to 100% (Fig. 2B). Thus, the synergistic effect of Cl₂ in different humidities at $\sim 10^5$ °C/s is reduced as compared to the lower heating rates of $\sim 10^4$ °C/s. The reduction in the effect of humidity can be in part attributed to the reduced change in T_c (~ 60 °C) for spores heated in air and in Cl₂ at $\sim 10^5$ °C/s, which is much smaller than that (~ 160 °C) at $\sim 10^4$ °C/s (Fig. 1 and Table 1). To evaluate the heating-rate-dependent synergistic effects of Cl₂, we assessed the changes to Bt spore morphology and composition in response to Cl₂ and rapid heat.

Fig. 2. Survival curves of Bt spores versus peak temperature for 50 ms ($\sim 10^4$ °C/s) (A) and 2 ms ($\sim 10^5$ °C/s) (B) heat pulses. The blue, red, and black curves fit the survival of spores in response to Cl₂ and heat at 0%, 40%, and 100% relative humidities.

3.3. Spore morphology after treatment of rapid heat and Cl₂ gas

Cl₂ vapor in the chamber surrounds the immobilized spores and is in contact with the surface of the spores. The effect of Cl₂ and heat on Bt spores was assessed by scanning electron

microscopy (SEM). The unheated spores had a general dimension of 1.5 μm (longitudinal) times 1.0 μm (transversal) with wrinkly protrusions along their surfaces (Fig. 3A and Fig. S2B). In the absence of heat, treatment with Cl_2 gas up to 1000 ppm, which was sufficient to completely inactivate Bt spores, did not alter spore morphology (Fig. S2C). The relationship between spore morphology and peak temperature was investigated first for Bt spores exposed to the higher heating rates ($\sim 10^5$ $^\circ\text{C/s}$). The spore coat remains unaffected when heated to a peak temperature of 200 $^\circ\text{C}$ at $\sim 10^5$ $^\circ\text{C/s}$ (Fig. 3B). When heated to 300 $^\circ\text{C}$ at $\sim 10^5$ $^\circ\text{C/s}$, the spore surface started to melt and the surface protrusions were reduced (Fig. 3C). Further increases in the peak temperature to ~ 400 $^\circ\text{C}$ at $\sim 10^5$ $^\circ\text{C/s}$ caused the surfaces of Bt spores to melt (Fig. 3D). At higher peak temperatures of >450 $^\circ\text{C}$, the surface coat was completely melted and only the underlying core remained (Fig. 3E and 3F). When 100 ppm Cl_2 was used, Bt spores were morphological indistinguishable from the spores treated by heat alone when the peak temperature is below ~ 400 $^\circ\text{C}$ (Fig. 3G-3I and Fig. 3A-3C). However, a distinct morphology emerged after ~ 400 $^\circ\text{C}$ when additional surface cracks were formed and the spore coat were detached from the underlying core (Fig. 3J-3K) in contrast to the spore coat melting in the absence of Cl_2 . The disintegration of spore coat was further deteriorated when the humidity of Cl_2 increased to 100% at 400 $^\circ\text{C}$ (Fig. S3), suggesting that the effect of Cl_2 on the destruction of spore coat is associated with RH. Together these results indicate that Cl_2 acts on the surface of the spore to alter the fluidity of the spore coat.

The SEM images of Bt spores at a lower ramp rate of $\sim 10^4$ $^\circ\text{C/s}$ revealed similar morphological changes (Fig. 4A-4D) as that at a higher ramp rate of $\sim 10^5$ $^\circ\text{C/s}$ (Fig. 3A-3D). Bt spores started to melt at the peak temperature of ~ 300 $^\circ\text{C}$, and completely melted at the peak temperature of ~ 400 $^\circ\text{C}$. The addition of Cl_2 did not alter Bt spore morphology (Fig. 4E-4H)

compared to the effect of heat alone (Fig. 4A-4D). These results indicate that the morphological changes were determined by peak temperatures at $\sim 10^4$ °C/s.

10^5 °C/s, no Cl_2 , 40% RH

10^5 °C/s, add Cl_2 , 40% RH

Fig. 3. Spore morphologies when heated to different peak temperatures at $\sim 10^5$ °C/s without (A-F) or with (G-L) the presence of Cl_2 . The relative humidity was kept at 40%. The red arrows and the black arrows in image 4K and 4L designate detached spore coats and exposed spore cores, respectively.

Fig. 4. Spore morphologies when heated to different peak temperatures at $\sim 10^4$ °C/s without (A-D) or with (E-H) the presence of Cl₂. The relative humidity was kept at 40%.

3.4. Changes in C and Cl contents inside Bt spores exposed to rapid heat and Cl₂

To evaluate the effect of Cl₂ on spores, the mass fractions of chlorine (Cl) and carbon (C) within spores treated by different heating schemes were measured using EDS. Calcium was used in these experiments as a standard since the calcium content inside spores will not change within our experimental temperature range (< 700 °C) (Fig. 5). The Cl content of the spores exposed to Cl₂ at room temperature was low indicating that Cl₂ gas does not react readily with the spores at ambient temperatures. The Cl content was increased as the peak temperature rose at $\sim 10^4$ °C/s, with the mass concentration of Cl for spores heated to 400 °C ~ 50 folds higher than that at room temperature. At the higher ramp rate of $\sim 10^5$ °C/s, a similar trend of increased Cl content was observed for spores exposed to higher peak temperature. At each temperature, the Cl content of spores exposed to $\sim 10^5$ °C/s heating rate was higher than spores exposed to the $\sim 10^4$ °C/s heating rate (Fig. 5B). These results demonstrate that both peak temperature and heating rate enhance the

reaction of Cl_2 with the spores. To determine the location on the spore that is reacting with Cl_2 , we took advantage of the SEM observation that the spore coat detaches from the core when heated to 500 °C at $\sim 10^5$ °C/s heating rate in the presence of Cl_2 . EDS of the detached spore coat revealed that there is a Cl/Ca ratio of 0.335 as compared to 0.005 Cl/Ca ratio in the core (Fig 5B). The 70-fold increase in Cl concentration in the spore coat indicates that Cl_2 is reacting primarily with the surface of the spores during the rapid heating event.

Since SEM images reveal that the spores disintegrated at high temperatures, the carbon content within spores was evaluated. Spores heated either in air or in Cl_2 at $\sim 10^4$ °C/s did not lose carbon content (Fig. 5C). Given that the spore coat was not completely melted until 400 °C (Fig. 4), this result shows that the intact spore surface inhibits the release of volatile carbons. When rapidly heated at $\sim 10^5$ °C/s, the C content in spores decreased starting at 300 °C and 400 °C for Bt spores heated in Cl_2 and in air, respectively (Fig. 5D). The decrease in carbon content correlates with the SEM images of revealing damage to the Bt spores heated both in air and in Cl_2 at 400 °C (Fig. 3D and 3J). In addition, at 300 °C the remaining C content for spores heated in 100% RH Cl_2 is 40% less than that for spores heated in 0% RH Cl_2 (Fig. S4), indicating that the carbon release increases at a higher RH. This is consistent with the fact that heating in the presence of Cl_2 gas at a higher RH induces more damage to the spore surface (Fig. S3). The carbon release occurred at peak temperatures that are higher than the corresponding T_c for Bt spores, indicating that release of volatile carbons is not the primary factor for spore neutralization at both temperature rates.

Fig. 5. EDS (energy dispersive spectroscopic) results on the chlorine (A and B) and carbon (C and D) mass contents in spores heated at $\sim 10^4$ °C/s (A and C) and $\sim 10^5$ °C/s (B and D) rates. The right side of the graph (B) shows the relative mass contents of Cl in the coat and core of spores heated to 400 °C at $\sim 10^5$ °C/s. The constant content of Ca inside spores is taken as a control and normalized to 1. Both the relative contents of Cl and C are presented as the mass ratios of Cl/Ca and C/Ca, respectively.

3.5. Spore viability heated at $\sim 10^4$ °C/s in HCl

The viability, morphological and EDS results for spores heated in Cl₂ (Figs. 1-5) indicate that humidity enables to improve the spore neutralization by Cl₂ and rapid heating. The reason for this spore neutralization improvement can be attributed to the generation of new biocidal products from Cl₂ hydrolysis, such as HCl and HOCl. As a control, the spore neutralization in HCl of the same concentration as Cl₂ (100 ppm) was also investigated. At a relative humidity of

40%, Fig. S5 shows that unheated spores in the presence of HCl gas remained their viability. However, spores heated at $\sim 10^4$ °C/s in HCl were killed by $> \sim 4$ logs at the peak temperature of ~ 400 °C, which is similar with that in Cl₂ (~ 4 logs, see Fig. 1). This result indicates that HCl is comparably efficient to synergize rapid heat in neutralizing spores

4. Discussion

4.1. Neutralization of Bt vs Bs spores at two heating rates without Cl₂

Our results (Fig. 1 and Table 1) show that the neutralization of Bt spores by rapid heat treatment alone is determined by the peak temperature and heating rate. The critical peak temperatures for Bt spores heated at $\sim 10^4$ °C/s and at $\sim 10^5$ °C/s are 407 °C and 232 °C, respectively (Table 1). Previously, we have also studied the neutralization of Bs spores under similar heating schemes [19]. The critical peak temperatures for Bs spores heated at these two ramp rates are 20 - 30 °C lower, indicating that Bs spores are more sensitive to rapid heat than Bt spores. SEM observations of Bs and Bt spores heated at $\sim 10^4$ °C/s show that spore coats melt at 410 °C [19] and 425 °C, respectively. When heated at $\sim 10^5$ °C/s, the coats of Bs spores started to generate visible fissures at a peak temperature of 300 °C [19]. In contrast, at the same heating rate and temperature, there are only very small pin holes observed on the surface of Bt spores (Fig. 3C). Together, these results indicate that the spore coat of Bt is less temperature sensitive, which corresponds to the fact that Bt spores possess a higher T_c to rapid heat.

The previously reported neutralization mechanisms for Bs spores depend on the heating rate [19]. At $\sim 10^4$ °C/s, inactivation is likely due to the thermal damage to DNA since mutants

lacking small acid-soluble proteins that protect the DNA within spore showed enhanced sensitivity to heat. At $\sim 10^5$ °C/s, the spore coat was compromised by elevated internal vapor pressurization from rapid heating [19]. Since neutralization of Bt and Bs was increased at the elevated $\sim 10^5$ °C/s heating rate, Bt spores likely undergo the same neutralization mechanism as Bs spores. This is supported by the similarity of SEM morphological changes in response to heating at $\sim 10^4$ °C/s. Furthermore, heating of Bt at $\sim 10^5$ °C/s to 300 °C caused the emergence of holes on the surface of the spores indicating that the Bt spores experienced a similar pressurization event as the Bs spores (Fig. 3C). Although the size of the holes on the surface of Bt spores are reduced in size, heating of Bt spores at $\sim 10^5$ °C/s to $> 400^\circ\text{C}$ in the presence of Cl_2 led to the entire spore coat detaching from the underlying core. This phenomenon also supports the idea that internal pressure lead to rupture of the fixed spore coat. Results presented here and in earlier studies demonstrate that Bs and Bt spores undergoing rapid heat treatments can be neutralized through two different mechanisms depending on the rate of heating.

4.2. The interaction of Cl_2 and spores

Cl_2 synergizes with rapid heat pulses to inactivate spores at both the $\sim 10^4$ °C/s and $\sim 10^5$ °C/s heating rates (Fig. 1). Our results thus raise two central questions: 1) which form of Cl_2 is enhancing the synergistic killing of Bt spores? 2) where is this form of Cl_2 acting on the spore? The mechanism by which Cl_2 synergizes with heat pulses is not completely understood. Cl_2 gas, in addition to being a potent oxidizer, can form chemical bonds with numerous organic and non-organic molecules [23]. Our study has revealed several important findings regarding the form of Cl_2 that is enhancing synergistic killing with heat. First, increased water vapor concentrations in

the chamber enhanced the potency of Cl_2 (Fig. 2). Cl_2 can readily react with H_2O to form HCl and HOCl [23]. Since Cl_2 synergizes with heat pulses minimally in the absence of humidity (Fig. 2), the compounds that actively enhance neutralization are likely HCl or HOCl rather than Cl_2 gas. We have estimated that it only requires seconds to reach the reaction equilibrium for $\text{Cl}_2/\text{H}_2\text{O}$ (see the detailed calculation in the supplemental information), which is much shorter than our experimental time scale (minutes). Additional evidence for the higher neutralization activity of HCl/HOCl than Cl_2 is that when the peak temperature increases from $100\text{ }^\circ\text{C}$ to $300\text{ }^\circ\text{C}$, the molar ratio of Cl_2/HCl decreases from ~ 4 to ~ 0.13 due to a biased reaction equilibrium from $\text{Cl}_2 + \text{H}_2\text{O}$ towards $\text{HCl} + \text{HOCl}$ at high temperatures (according to CEA (NASA Chemical Equilibrium with Applications) calculations [47] given that the initial concentration of Cl_2 is $4.2 \times 10^{-3}\text{ mol/m}^3$ with a RH of 40 %). Correspondingly, the spore viability reduced significantly as the temperature increased suggesting that HCl/HOCl is more effective in spore neutralization.

The target of Cl_2 inactivation on the spore is also an area of interest since this can reveal vulnerabilities of *Bacillus* spores that can be exploited in biodefense. The EDS data on Cl support the idea that the heat treatment with Cl_2 leads to an accumulation of Cl on the detached spore coat (Fig. 5) indicating the chlorine is interacting primarily with the surface of the spore during the heating pulse. As being said, the two major biocidal agents that interact with spore coat are HCl and HOCl , which have unique chemical properties. HCl is a strong acid that can act to denature proteins on the spore coat [48-50]. In contrast, HOCl can react with organic molecules leading to chlorine covalently attached to the molecules on and in the spores [23]. Therefore, the Cl detected in the spore coat by EDS is either deposited as Cl^- anion from deprotonated HCl or covalently attached through chemical reactions of HOCl to cellular organic molecules. The effect of HCl on *Bt* spores was tested by heating spores at $\sim 10^4\text{ }^\circ\text{C/s}$ in the

presence of only HCl gas. Results show that HCl also synergized with heat to neutralize spore similarly to the heat treatment with Cl₂ with 40% RH (Fig. 1 and Fig. S5). These results suggest that HCl produced by the reaction between Cl₂ and water vapor is one of the active biocidal agent at these heating rates and peak temperatures. Since HOCl is known to form covalent bonds with organic molecules, we believe that both HCl and HOCl promote synergistic killing with rapid heat pulses. Future studies of the synergistic effect of HOCl with rapid heat pulse would provide direct evidence to support the active compound that inactivates spores. Furthermore, future identification of the spore coat protein(s) that are covalently modified by chlorine will reveal the bacterial target(s) that confers sensitivity.

4.3. Synergistic neutralization mechanisms for Bt spores by rapid heat and Cl₂

There are two potential mechanisms for the observed synergistic effect in spore neutralization between rapid heat and Cl₂: 1). A chemical mechanism in which heat activates Cl₂ to become a more potent biocide or 2). A biological mechanism in which Cl₂ and heat damage different targets in the spores to enhance inactivation. Although these two potential mechanisms are not mutually exclusive, the synergistic effect of heat and Cl₂ is at least in part due to the heat pulse overcoming the activation energy of reactions between chlorine and the spore. The EDS data shows that exposure to Cl₂ alone does not increase Cl content in the spore. Only when heated does the Cl content of the spore increase, supporting the idea that heat enhances the reactivity of Cl₂. In addition to the increase in Cl₂ reactivity, the biological targets of heat and Cl₂ act on different parts of the spore. Heat pulses at $\sim 10^4$ °C/s damage DNA [19], whereas Cl₂ targets the spore coat. The combination of coat damage and DNA damage can act synergistically neutralize

spores. Support for this model of inactivation is that heat pulses at $\sim 10^5$ °C/s, which inactivates spores primarily through physical damage of the spore coat [19], synergizes minimally with Cl₂. Thus damaging different targets within the spores, rather than the same target, enhances synergistic neutralization.

Another feature of the synergism of Cl₂ with heat pulses is that this effect occurs at peak temperatures and heating rate far below what was required to fix and detach the spore coat (Fig 5). An open question is whether the effect observed at the higher peak temperature ($>500^\circ\text{C}$) and high heating rate ($\sim 10^5$ °C/s) applies to the lower peak temperature and heating rates, which nonetheless synergize with Cl₂ to neutralize Bt spores. Despite the lack of visual changes of the spores as detected by SEM at peak temperatures of under 300 °C, the Cl content on the spore increases as detected by EDS. One interpretation of these results is that heat pulses between 200-300 °C activate Cl₂ to interact with spores. Even this lower level of chlorine modification of the spore surface can negatively impact the function of the spore coat and increase spore inactivation.

5. Conclusion

Surface immobilized Bt spores subjected to the synergistic effects of ultrafast heating and biocidal chlorine gas were characterized by several observations including changes in viability, morphology, and composition. At the heating rates of $\sim 10^4$ °C/s, the critical peak temperatures for spore neutralization were reduced from 407 °C in air to 252 °C when exposed to 100 ppm Cl₂. At the higher heating rates of $\sim 10^5$ °C/s, the critical peak temperatures for spore neutralization are decreased from 232 °C in air to 176 °C when exposed to 100 ppm Cl₂. Cl₂ synergizes with rapid heat pulse to enhance spore neutralization. Additional experiments revealed that the synergistic effect of Cl₂ and heat was increased in high humidity, whereas the synergistic effect decreased in low humidity. Cl₂ can react with water to generate HCl and HOCl, which in turn react with the spore. Despite enhancing neutralization, Cl₂ and heat pulses under 300 °C did not alter the

morphology of the spores. However, treatment of spores at peak temperatures $>450\text{ }^{\circ}\text{C}$ at $\sim 10^5\text{ }^{\circ}\text{C/s}$ caused the spore coat to detach completely from the endospore. EDS results showed that Cl is present in heated spores and the majority of the chlorine is present in detached spore coat indicating that chlorine is acting on the surface of the spores. Our results suggest that the spore surface damage caused by Cl_2 , via HOCl and/or HCl , is the major mechanism in enhanced spore neutralization by rapid heat.

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