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Defensive and simultaneous actions of glycoconjugates during spore decontamination

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Abstract—An estimated $1 billion was lost in decontaminating areas exposed to anthrax in the 2001 attacks. To counter the threat of biological attacks, an effective 'green' decontaminant is vital to minimize the consequences of such attacks. The objective of our research was to study the ability of glycoconjugate ligands to decontaminate *Bacillus cereus* spores on hard surfaces. Polyvalent glycoconjugates (also known as *neoglycoconjugates*) were tested during decontamination of *B. cereus* spores. Resulting colony forming units (CFU) of viable spores were a direct evidence of glycoconjugate decontamination efficacy. Our results indicate a substantial, decreasing CFU count due to *defensive* and *simultaneous* actions of glycoconjugates compared to spores only used as controls. Decontamination of *B. cereus* spores was most efficiently and consistently achieved using Galα1→3GalNAcβ-PAA-flu glycoconjugate under both *defensive* and *simultaneous* conditions. Atomic force microscopy (AFM) allowed us to visualize decontamination at the nanoscale level using glycoconjugates. AFM reveals the size of glycoconjugate agglomerates (clusters) and a noticeably different morphology of glycoconjugate-treated spores during decontamination. Morphological features of untreated spores disappear under a thin layer of glycoconjugate solution. This thin layer is formed due to the defensive action of glycoconjugates. *Simultaneous* action has shown agglomeration of glycoconjugates in solution with *B. cereus* spores in glycoconjugate suspensions. Glycoconjugates might be useful for the development of an environment-friendly decontaminant of bacterial spores.

Keywords: *Bacillus cereus* spores; Polyvalent glycoconjugates (neoglycoconjugates); Decontamination

1. Introduction

Due to the highly fatal nature of anthrax, the ease of production and storage of *Bacillus anthracis* spores and their survival in the environment after a bioattack, this organism has become the primary bacterial agent in biowarfare and bioterrorism.[1] In October 2001, several letters containing *B. anthracis* spores were sent through the US Postal Service. Consequently, five of 23 anthrax cases were fatal.[2,3] As a result, several post offices, mailrooms in government and private office buildings were contaminated with *B. anthracis* spores. These events brought the issues surrounding the deliberate release of biological warfare agents (BWA) into sharp focus and increased concerns that 'weapon grade' biological agents can be obtained or manufactured and disseminated by terrorists or terrorist groups.

B. anthracis has been placed within the *B. cereus* group due to genetic similarities.[4–8] Various *Bacillus* turn into a dormant cell known as spores when exposed to nutrient-poor conditions.[1,9] Spores typically exhibit the following elements: appendages or filaments, an exosporium (a loosely attached exterior membrane), an outer and inner coats, a cortex, and a core.[1,9–11] The biochemical compositions of spores have previously been reported.[12–23] Spores grow vegetatively in presence of amino acids, sugars, at favorable pH and temperature.

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Spores can withstand a wide range of assaults including heat, UV or γ-irradiation, and oxidizing agents[1,24,25] that would destroy their vegetative cell counterparts.[26,27]

There is a high demand of improved decontamination methods of harmful spores.[28,29] Decontamination is defined as the irreversible inactivation of infectious agents.[30] Traditional decontamination methods of BWA involve the use of 'wet' solutions, which include bleaches or Decontamination Solution #2 (DS2) for reactive decontamination.[31] These hazardous chemicals need to meet special guidelines for storage, transport, and disposal during and after usage. Since these chemicals are released into the environment, considerable effort has been focused on identifying 'green' decontaminants.[32] Researchers at the Sandia National Laboratory have created a foam neutralizing BWA.[33] The foam is a cocktail of ordinary neutralizing agents, surfactants, and mild oxidizing substances. A disadvantage with wet methods is that the current decontamination chemicals are corrosive to materials such as metals, plastics, rubber, paint, leather, and skin. Thus, they are not suitable for the use on sensitive equipment.

Hence, alternative decontamination methods are being developed. These include the use of ionizing and nonionizing radiation, thermal and ultrasonic energy, and reactive gases such as those produced by plasmas.[34] These methods do not require mass storage of chemicals, but most do not meet all the standards that form an ideal decontamination method. Ionizing γ-irradiation is effective in decontaminating large areas. This method is usually employed in a heavily shielded fixed facility, but it is not easily implemented in the field and can be detrimental to sensitive equipment. Experiments have shown that dormant spores are highly resistant to UV or γ-rays.[24–26] Thus, the use of nonionizing UV light requires a line-of-sight application and can take a considerable amount of time. The efficiency of thermal decontamination methods is limited by temperature constraints on avoiding damage to equipment and surfaces. Consequently, this method is relatively time-consuming. Ultrasonic removal of surface contaminates is not a 'dry' method either and seems less efficient. An ideal decontaminant should be self-reactive, inexpensive, capable of selectively and effectively destroying biological agents, easily transported, fast working, no mass storage requirement, safe to personnel, and inert to sensitive equipment.

Our group has shown that glycoconjugate polymers (Glyc-PAA-flu) recognize,[23] inhibit,[35,36] and stimulate annihilation of spores during phagocytosis.[37,38] It is apparent that (i) several glycoconjugates predominantly bind to *B. cereus* compared to *Bacillus thuringiensis*, *Bacillus pumilus*, and *Bacillus subtilis*;[23] and (ii) glycoconjugates scarcely bind to *B. subtilis* compared to other spores.[23] Previously obtained data indicate that several glycoconjugates have a marked inhibition affinity for *B. cereus*.[23,35,36] Changes in glycoconjugate concentrations did not potentially hinder binding[23] and inhibition efficacy.[35,36] Even highly diluted glycoconjugate solutions enable binding to and inhibition of *B. cereus*.[23,35,36]

A Glyc-PAA-flu ligand comprises a synthetic 30 kDa multivalent polymer containing 79 polyacrylamide units (PAA), 20 mol % of carbohydrate (Glyc), and 1 mol % fluorescein (flu). Numerous advantages of Glyc-PAA-flu polymers have been reported. It was shown that Glyc-PAA-flu polymers bind nonspecifically to cellular components and can be immobilized on a solid phase.[39–41] It was also shown that the PAA backbone and its flu label minimally affect this interaction.[39–41] PAA-flu is in itself hydrophilic and shows low binding affinity and flexibility.[39–41] The presence of the flu label is advantageous for diverse spectroscopic and microscopic techniques.[41]

In the present study, we evaluated the efficacy of selected glycoconjugates[23,35,36] during decontamination of *B. cereus* spores on ceramic surfaces. *B. cereus* spores were chosen as simulants of *B. anthracis*.

2. Results and discussion

In the present study, we have evaluated the capabilities of water-soluble Glyc-PAA-flu polymers (Fig. 1) in decontamination studies of *B. cereus* spores. As many as six different types of disaccharide Glyc-PAA-flu polymers, namely, Galα1→3GalNAcα-PAA-flu, Galα1→3GalNAcβ-PAA-flu, GalNAcα1→3GalNAcβ-PAA-flu, Galα1→3Galβ-PAA-flu, GlcNAcα1→4GlcNAcβ-PAA-flu, and Fucα1→4GlcNAcβ-PAA-flu were evaluated as decontamination ligands of *B. cereus* spores on ceramic surfaces (Fig. 1).

'*Defensive*' (Fig. 1A) and '*simultaneous*' (Fig. 1B) actions of each glycoconjugate were tested during decontamination studies of bacterial spores. *B. cereus* spores were applied to a hard surface and treated with glycoconjugates during the '*defensive*' action. A mixture of *B. cereus* spores and glycoconjugate was applied to a hard surface during the '*simultaneous*' action. Decontamination results were expressed as mean percent spore survival rate based on colony-forming units (CFU) of remaining glycoconjugate-treated and untreated spores during decontamination (Fig. 1). CFU is indicative of decontamination efficacy of glycoconjugates used. The less CFU formed, the higher the decontamination efficiency, and the more spores were killed. Two independent sets of experiments were carried out and repeated in triplicate.

Decontamination data illustrate that (i) glycoconjugates kill spores during decontamination during studied actions (Fig. 1); for example (ii) decontamination ($>IC_{50}$) of *B. cereus* spores was most efficiently and con-

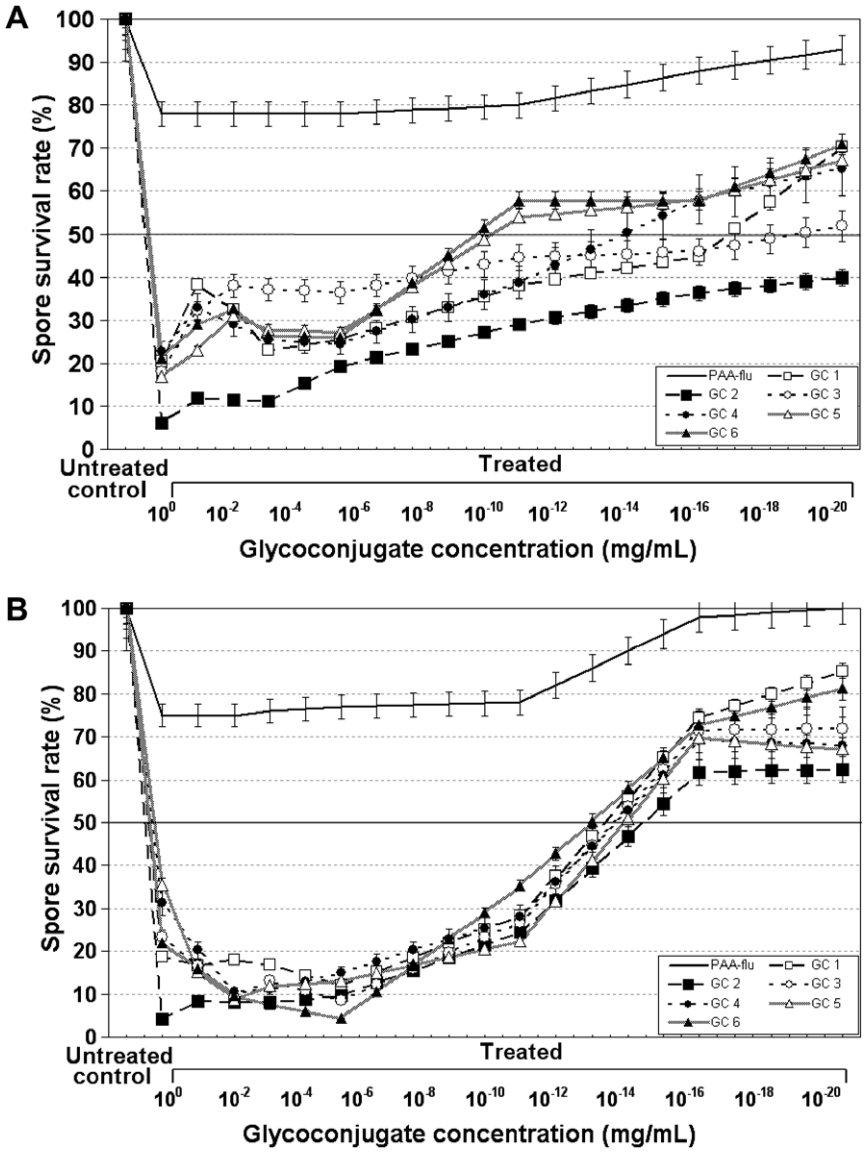

Figure 1. Decontamination of *B. cereus* spores based on *defensive* (A) and *simultaneous* (B) actions of Galα1→3GalNAcα-PAA-flu (GC1), Galα1→3GalNAcβ-PAA-flu (GC2), GalNAcα1→3GalNAcβ-PAA-flu (GC3), Galα1→3Galβ-PAA-flu (GC4), GlcNAcα1→4GlcNAcβ-PAA-flu (GC5), and Fucα1→4GlcNAcβ-PAA-flu (GC6) here and hereafter, $p < 0.01$. PAA-flu and untreated spores were used controls. Half-maximal inhibition values (IC_{50}) of spores during either *defensive* (A) or *simultaneous* (B) actions of glycoconjugates are emphasized by the highlighted line (50%). These results were expressed as mean percent bacterial killing of untreated control and treated spores. These results are representative of triplicate experiments.

sistently achieved using Galα1→3GalNAcβ-PAA-flu glycoconjugate; (iii) the *defensive* (Fig. 1A) action of glycoconjugates resulted in a higher impact than the *simultaneous* action (Fig. 1A); and (iv) glycoconjugate dilutions promote decontamination of spores to a certain degree.

During the *defensive* action (Fig. 1A), Galα1→3GalNAcβ-PAA-flu showed the highest killing rate, followed by GalNAcα1→3GalNAcβ-PAA-flu, Galα1→3Galβ-PAA-flu, and Galα1→3GalNAcα-PAA-flu throughout spore decontamination. Approximately 60–65% of spores were killed throughout the *defensive* action of Galα1→3GalNAcβ-PAA-flu (Fig. 1A). The latter was also the most efficient during the *simultaneous* action (Fig. 1B). The *defensive* action of Galα1→3GalNAcα-PAA-flu and GalNAcα1→3GalNAcβ-PAA-flu yielded the highest spore killing rates compared to rates achieved by other glycoconjugates studied during decontamination. However, the *simultaneous* action of Galα1→3GalNAcα-PAA-flu and GalNAcα1→3GalNAcβ-PAA-flu resulted in a attenuated spore killing rate (Fig. 1B). Fucα1→4GlcNAcβ-PAA-flu has demonstrated lower killing rates compared to other studied glycoconjugates (Fig. 1A and B). Unlike Galα1→3Gal-

NAcβ-PAA-flu, glycoconjugate Fucα1→4GlcNAcβ-PAA-flu failed to demonstrate strong binding affinities to *B. cereus* under both actions studied.

Spore killing rates of Galα1→3GalNAcα-PAA-flu appear to lay in the 60–65% range during the *defensive action*. The decontamination effect of Galα1→3GalNAcβ-PAA-flu, however, slightly decreased during *simultaneous* decontamination (Fig. 1B). Our previous studies on binding and recognition have shown that GlcNAcα1→4GlcNAcβ-PAA-flu and Galα1→3GalNAcα-PAA-flu show a marked affinity for *B. cereus* spores.[23,35,36] In addition, we have also shown that carbohydrates found on the exterior of spores are recognized by carbohydrate units of disaccharide glycoconjugates engaged in molecular interactions.[23]

In order to examine concentration effects on spore decontamination, glycoconjugate solutions were prepared according to the manufacturer's protocol. Solutions were first diluted 10^{0}–10^{21}-fold, then used in spore decontamination studies (Fig. 1). Due to the dilution effect, the decontamination capacity of glycoconjugates increases noticeably (Fig. 1). *Defensive* decontamination (>IC_{50}) of *B. cereus* spores was efficiently achieved with glycoconjugate solutions diluted up to 10^{20}-fold. In contrast, a $10^{15–16}$-fold dilution allowed *simultaneous* decontamination of *B. cereus* spores. Dilution does not impede the decontamination capacity of glycoconjugates. Galα1→3GalNAcβ-PAA-flu was the most effective glycoconjugate under both the *defensive* and the *simultaneous* actions studied (Fig. 1A and B). Dilution promotes either *defensive* or *simultaneous* actions of Galα1→3GalNAcβ-PAA-flu (Fig. 1A and B) during the decontamination process of *B. cereus* spores.

According to recent findings, binding affinity of glycoconjugates on the surface of bacterial spores displaying multiple receptor sites depends both on the target and on glycoconjugate ligands.[42,43] Previously reported data have shown that complex carbohydrates are involved in interactions such as adhesion between cells, adhesion of cells to the extracellular matrix, and specific recognition of cells by one another.[44,45]

In summary, glycoconjugates used as synthetic ligands enable decontamination of bacterial spores (Fig. 1). Changes in glycoconjugate concentration did not potentially hinder decontamination (Fig. 1A and B). Even highly diluted glycoconjugate solutions are capable of decontaminating bacterial spores. It is known that carbohydrate–carbohydrate interactions play an important role throughout complimentary binding of glycosphingolipids.[46,47] Though both the target and ligands may display different bimolecular units in various arrangements,[42,43] glycoconjugates grant us the opportunity to bind and recognize,[23] inhibit,[35,36] destroy[37,38] as well as decontaminate bacterial spores (Fig. 1).

In addition, we carried out atomic force microscopy (AFM) observations of the glycoconjugate used (Fig. 2), as well as untreated and glycoconjugate-treated *B. cereus* spores during decontamination (Fig. 3).

AFM helps us to characterize the shape and size of clusters consisting of 1–1000× diluted glycoconjugates

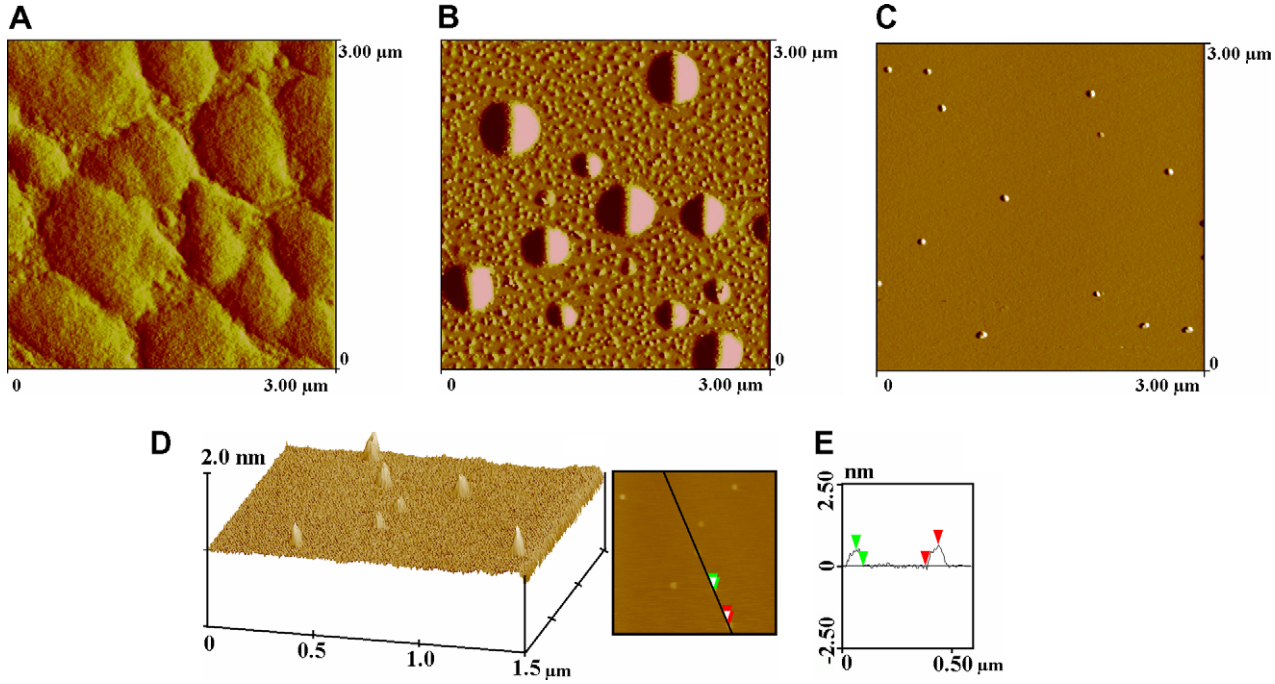

Figure 2. AFM images of 1× (A), 10× (B), 1000× (C) diluted glycoconjugate. 3D rendering (D) and section analysis (E) were carried out 1000× diluted glycoconjugates (C). Amplitude images are shown (A–C).

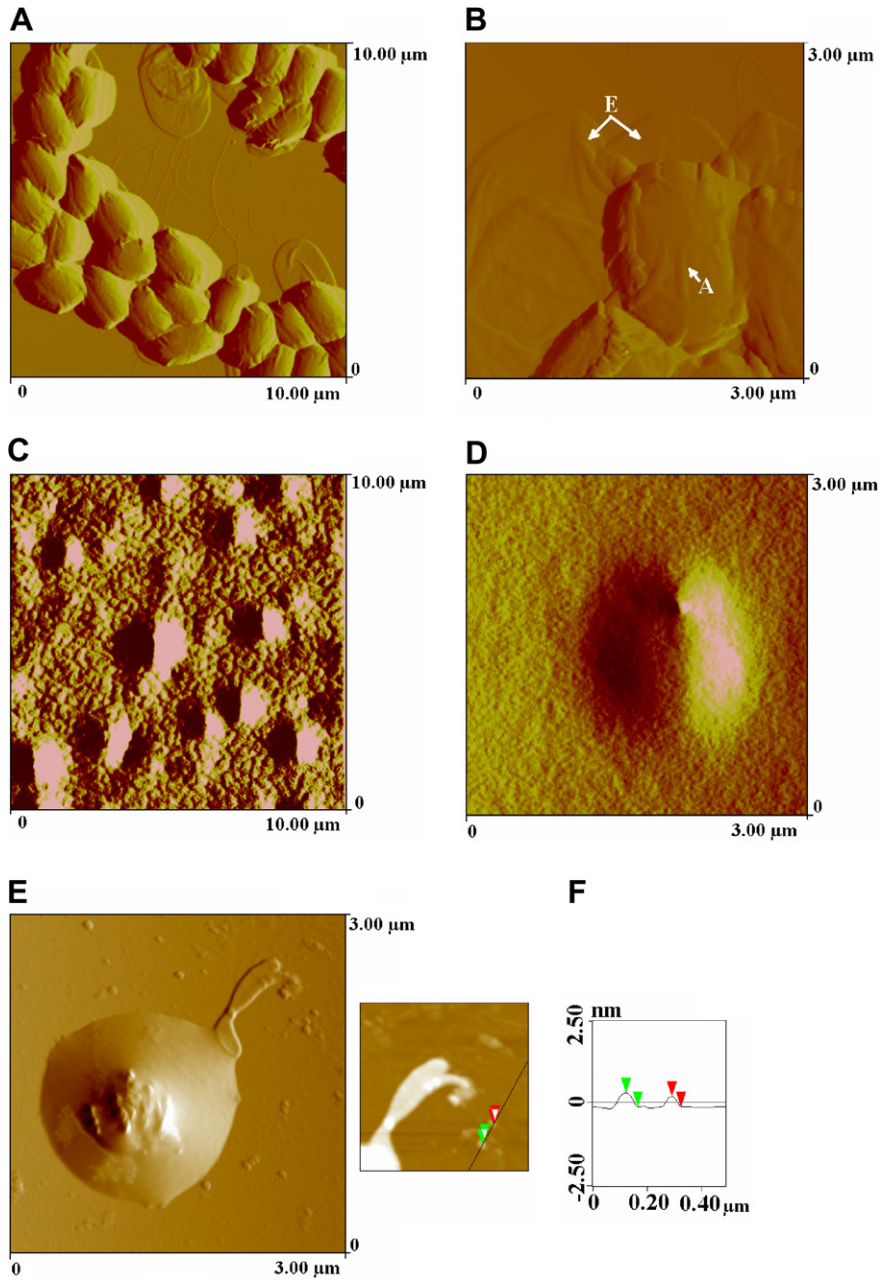

Figure 3. AFM images of untreated (A, B) and glycoconjugate-treated (C–E) *B. cereus* spores at both low (A, C) and high resolutions (B, D, E). Defensive (D) and simultaneous (E) actions of used glycoconjugate, and section analyses (F) are shown. White arrow *A* stands for appendages, *E* for exosporium. Amplitude images are shown (A–F).

at a nanoscale level (Fig. 2). Diluted glycoconjugates (1×) form clusters sizing 300 × 800 nm (Fig. 2A). A dilution of 10× allowed the formation of spheres ranging from 100 to 500 nm (Fig. 2B). Surprisingly, 1000× diluted glycoconjugates formed uniform clusters as seen in 2D (Fig. 2C) and 3D rendered AFM micrographs (Fig. 2D). Section analysis of 1000× diluted glycoconjugates revealed clusters of a size 0.5–0.6 nm vertically and 34–58 nm horizontally (Fig. 2E).

In addition, AFM visually demonstrated changes in spore morphology during glycoconjugate treatment (Fig. 3A and B). AFM permitted visual confirmation of defensive and simultaneous actions of glycoconjugates (Fig. 3C and D) at a nanoscale level. AFM micrographs were taken prior to (Fig. 3A and B) and after decontamination (Fig. 3C and D).

Untreated spores were found to be firmly attached to the mica surface used as base and remained sufficiently bound to be scanned with an AFM scanning probe (Fig. 3A and B). AFM has revealed the actual shape of *B. cereus* spores as well as morphological traits (Fig. 3A and B). Spores appeared slightly elongated

(Fig. 2A and B). The spore surface is covered with a loose layer of exosporium that is generally spread out and eventually attaches itself to the mica surface (Fig. 2A and B). *B. cereus* spores exhibit a clearly identifiable exosporium as well as appendages (Fig. 2A and B). AFM observations of glycoconjugate-treated spores indicate that the resulting morphology of the spores is noticeably different (Fig. 3C–E) from that of untreated ones (Fig. 3A and B). Tiny morphological features such as exosporium and appendages appear to disappear under a thin layer of glycoconjugate solution.

This thin layer is formed due to the defensive action of glycoconjugates (Fig. 3C and D). Simultaneous action has shown agglomeration of glycoconjugates in solution with *B. cereus* spores in glycoconjugate suspensions (Fig. 3E and F). Section analysis reveals agglomeration as well as the size of glycoconjugate clusters (Fig. 3F) to be similar as observed under section analysis of the 1000× diluted glycoconjugate (Fig. 2E).

In summary, glycoconjugates used as synthetic ligands enable decontamination of *B. cereus* spores. Changes in glycoconjugate concentrations did not potentially hinder decontamination (Fig. 1). In addition, AFM allowed visualizing details of untreated and glycoconjugate-treated spores during decontamination, as well as glycoconjugates clusters used at a nanoscale level (Fig. 2).

The technique presented in this study may be helpful in identifying glycoconjugate-based inhibitors among other conjugates essential for the decontamination of bacterial spores. Prospective research foresees decontamination studies of *Bacillus* species involving different strains, on different surfaces, time of decontamination, usage of glycoconjugates of higher molecular weight, and comparing the role of anomeric linkages on decontamination abilities of glycoconjugates. In addition, only highly selective and specific glycoconjugate structures will be used for the synthesis of multifunctional glycoconjugates. The existing glycoconjugate library (database) will be expanded in order to identify highly selective and specific ligands among other inhibitory conjugates for decontamination of *B. anthracis* spores.

3. Experimental

3.1. Materials

B. cereus spores ATCC 11778 (3.5×10^6 CFU/0.1 mL) were purchased from Raven Biological Laboratories, Inc. (Omaha, NE). Fluoresceinated disaccharide glycoconjugate polymers were obtained from GlycoTech, Inc. (Rockville, MD). Tryptic soy agar (TSA), scrapers, water W3500 tissue culture grade, sterile tubes (0.5 mL), Petri ultra-dishes, and pipette tips were purchased from Fisher Scientific Co. (Houston, TX). Ceramic tiles 1 × 1 cm were obtained from Plaid (Norcross, GA).

3.2. Decontamination studies

All processes involving spore handling were carried out under a Biological Safety Cabinet Class IIA/B3 (Forma Scientific, Inc., Marietta, OH). Each ceramic tile was autoclaved using a Consolidated SSR series autoclave (Boston, MA) in order to prevent cross contamination. Untreated spores (0.5 μL) were used as control (2.4×10^6 CFU/0.1 mL).

Glycoconjugates (0.5 mg) were hydrated in 0.5 mL of 0.3 M phosphate buffer (0.1 M NaH_2PO_4, 0.2 M Na_2HPO_4) (1 mg/mL), then serially diluted (10^{-1}–10^{-21} mg/mL) prior to their use in decontamination studies.

For the '*defensive*' (Fig. 1A) action, samples were prepared by inoculating 0.5 μL (2.4×10^6 CFU/0.1 mL) of *B. cereus* spores (target) onto each autoclaved ceramic tile. After overnight incubations at 22 °C, ceramic tile surfaces were treated with 5.0 μL of serially diluted glycoconjugates (1–10^5×). After incubation, ceramic tiles hosting spores were placed into sterile Petri dishes. Soon after, each ceramic tile was extensively washed in 1.0 mL tissue culture water under continuous shaking and scraping for 20 min at 22 °C with the purpose of removing spores off the ceramic tiles. Mixtures were concentrated by centrifugation using an accuSpin Micro centrifuge R (Walham, MA) for 10 min at 5000 rpm. The resulting pellets were serially diluted (10^{-1}–10^{-6}), and 10 μL of each dilution was plated onto trypticase soy agar petri dishes. Resulting CFU were counted after an overnight incubation at 37 °C.

For the '*simultaneous*' condition, mixtures consisting of glycoconjugates (5.0 μL) and spores (0.5 μL) were incubated within 1 h at 22 °C and then placed directly on a ceramic surface. Ceramic tiles were incubated overnight at room temperature and handled as described above.

3.3. Atomic force microscopy (AFM)

Solutions of untreated spores, glycoconjugate-treated spore samples (10^4 CFU) were deposited on mica disks using sterile syringes, then dried in ambient air at room temperature (20 °C) for 14 h. Prepared samples were later mounted on an AFM sample holder for imaging. All AFM observations were carried out at 20 °C, using a Nano Scope® IIIa controller as well as a MultiMode™ microscope (Digital Instruments, Inc.) operating in the tapping mode (amplitude) together with an E-scanner. A 125-μm silicon Nanoprobe (Digital Instruments, Inc.) was also employed. The calculated spring constant was 0.3 N/m. The resonance frequency remained in the range of 240–280 kHz, and the scan rate was of 1 μm/s. Flattening and high-pass filtering of the image data were performed in order to remove the substrate slope

from images as well as high-frequency noise strikes, otherwise more pronounced in the high-resolution tapping mode imaging.

3.4. Statistics

Results were considered statistically significant at p-values <0.01 using ANOVA based on SAS v. 9.0.

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