Available online at www.sciencedirect.com
SCIENCE DIRECT®
Carbohydrate Research 339 (2004) 2859–2870
Carbohydrate RESEARCH

Glycoconjugates for the recognition of *Bacillus* spores

Olga Tarasenko, Sharmin Islam, David Paquiot and Kalle Levon*

The Othmer Department of Chemical and Biological Sciences and Engineering, Polytechnic University, Brooklyn, NY 11201, USA

Received 17 August 2004; accepted 12 October 2004
Available online 11 November 2004

Abstract—Carbohydrates act as ligands in many biological processes, including the folding and secretion of proteins, cell–cell recognition, adhesion, and sporulation in the *Bacillus* genus. Fluorescent-labeled disaccharide glycoconjugates have been applied to evaluate binding to bacterial spores assuming that the spore surface is covered with carbohydrates. This study has shown that specific recognition of bacterial spores is based on interactions between disaccharide glycoconjugates acting as ligands and monosaccharide units expressed on the exterior of bacterial spores. Using fluorophore-assisted carbohydrate electrophoresis (FACE), carbohydrates that are expressed on the exterior of the spores were enumerated. The findings have an impact on how to improve ligand selection, essential for sensor development. In addition, the findings provide new information for inhibition of bacterial spores, and in general, demonstrate how carbohydrates function as recognition signals in nature.
© 2004 Elsevier Ltd. All rights reserved.

Keywords: *B. cereus*; *B. thuringiensis*; *B. pumilus*; *B. subtilis* spores; Glycoconjugate; FACE; Binding studies

1. Introduction

The problems associated with the *Bacillus* genus are as diverse as the environments where they are found. The bacterial spores are the cause of problems such as food spoilage and food-borne illnesses.[1] Spores can withstand a wide range of assaults including heat, UV irradiation, oxidizing agents, desiccation, and physical damage.[2–4] This makes them suitable for incorporation into bioweapons and for concealment in terrorist weapons.[2]

Given the severity of the threat posed by biological weapons, detection of potentially harmful pathogens needs to be rapid and extremely sensitive since the presence of a single pathogen can lead to infection. Furthermore, very selective detection methods are required, as pathogenic bacteria exist alongside non-pathogenic organisms in their environment.

There are several detection systems currently on the market: (i) Anthax Bio-Theat Alert (BTA) test strips (Tetracore, Gaithersburg, MD), (ii) BioWarfare Agent Detection Devices (BADD) (Osborne Scientific, Lakeside, AZ), and (iii) Anthax (spore) SMART II (New Horizons Diagnostics, Columbia, MD); immunomagnetic assay system; PCR-based assays or traditional phenotyping of cultured bacteria.[5–8] These systems are complex, expensive, time consuming, and require a highly qualified technical staff. Other less complex and more portable systems are based on antibody binding to surface antigens.[9] Unfortunately, these simpler systems are flawed. Current antibody-based detectors suffer from a short shelf life, lack of accuracy, and inadequate sensitivity. These deficiencies result in an unacceptably high level of false-positive and false-negative responses, according to federal government trials[10] and independent tests.[11]

The lack of accuracy and sensitivity of the antibody-based and other detectors is compounded by the presence of other spore-forming bacteria of the *Bacillus cereus* group in the environment. Particularly problematic are spores including the human pathogen *B. cereus* and the insect pathogen *Bacillus thuringiensis*. These *Bacillus* species are found to be similar to *Bacillus anthracis* based on genome sequence comparisons.[12] Due to

* Corresponding author. Tel.: +1 718 260 3339; fax: +1 718 260 3125; e-mail: klevon@poly.edu

0008-6215/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2004.10.008

the limitations previously cited, all currently available systems for detecting *B. anthacis* are inadequate for frontline use.[13]

There is an urgent need for a quick and accurate biosensor, especially one specific to *B. anthacis* and capable of distinguishing other species of the *B. cereus* group. It is known that short peptide ligands bind selectively to *B. anthacis* spores.[14] Prof. Turnbough has identified peptides that selectively bind to *B. anthacis* spores and closely related species based on phage technology.[15] Our group focuses on the conjugation of selective ligands such as peptides,[15] lectins, and antibodies with dendrimers, liposomes, and polymers.[16–19] In previous work, we have shown that macromolecular conjugates allow the simultaneous binding of multiple ligands to biological entities, for instance spores.[16–19] This binding is confirmed by fluorescence-based assays, and results clearly show that the affinity of the biomolecules was largely enhanced[16–19] by the polyvalent effect.[20–22] These polyvalent interactions can be collectively stronger than the corresponding monovalent ligands as previously reported.[20–23] Our study focuses on an electrochemical sensor based on potentiometry to identify bacterial spores.[17,24] This approach enables rapid identification of specific ionic interactions between the spore surface and selected ligands.[14–19,24]

Until recently, explanations of cell recognition have mostly focused on proteins while marginalizing carbohydrates. Although carbohydrates are ubiquitously present in both prokaryotic and eukaryotic cells, an appreciation of their diverse biochemistry has merely begun.[25,26] Mammalian and bacterial cellular surfaces are littered with complex carbohydrate structures such as glycoproteins, glycolipids, glycosaminoglycans, and proteoglycans.

In biological systems, glycoconjugates are involved in recognition and signaling processes intrinsic to biochemical functions in cells. They play a central role in cell-to-cell adhesion and in subsequent recognition, receptor activation, and in growth of living organisms.[25–27] Host receptors, which are imperative in microbial recognition, are in fact glycoconjugates.[27] Various antigenic structures identified by host receptors are partly glycoconjugates.[28,29]

The role of carbohydrates in recognition has only been studied in endogenous lectins: proteins forming stable complexes upon binding carbohydrates.[30–32] Carbohydrate groups assist in protein folding or improve their stability.[33,34] It is known that N-glycosylation of an immunoglobulin Fc fragment affects the Fc peptide backbone conformation.[34] Different glycoforms stabilize diverse conformations and can exhibit singular in vivo behaviors.[33] Carbohydrates tend to promote the binding begun by proteins. For example, in case of immunoglobulin binding, protein structures form an initial complex with the antigen.[33,34] However, carbohydrates allow binding into a tighter complex. Given the numerous binding sites on a carbohydrate, structural heterogeneity and conformational multiplicity are anticipated characteristic features of glycoproteins.[34] Complex geometries of immunoglobulin domains and of oligosaccharides mean that even relatively minor changes in the glycan structure may lead to major alterations in protein structure.[33–35]

A model system based on ligands and receptors with corresponding glycoconjugate counterparts was created to better study carbohydrate–carbohydrate interaction characteristics.

The different *Bacillus* species are chemically and morphologically quite distinct.[35] In this study, the spore's specific composition and morphology dictated its role within the model system.

A spore's morphology typically includes the following components: appendages (pilus-like filaments located on the outer spore surface), an exosporium, an outer coat, an inner coat, a cortex, and a core.[1–4,37,38] It is known that *B. cereus* and *B. thuringiensis* spores possess appendages, originating from an exterior basal membrane of the exosporium.[1–4,37,38] Appendages, though present on a variety of *Bacillus* spores, were not observed in *B. subtilis*.[39]

Biochemical compositions of *B. cereus*[35,36] and *B. subtilis*[39–41] spores have been reported. Several key proteins in the exosporium of *B. anthacis* Sterne strain have been identified.[42,43] A collagen-like glycoprotein, BclA,[43] was recently identified as a structural component of the exosporium hair-like nap.[42] Two other unique proteins, BxpA and BxpB, were also identified.[42] In addition, BxpB appears to be glycosylated or associated with glycosylated material.[42]

Exosporium and appendages might be where chemical recognition occurs. Considerable efforts have been directed toward understanding chemical recognition in spores and toward recreating it in vitro. Biomolecules such as polysaccharides, glycoproteins, or lipoproteins embedded in the exosporium or the appendages could play a leading role in the recognition process.

Glycoconjugates are widely used to study carbohydrate-binding sites in histochemical and cytochemical experiments.[26,44–47] The capacity of glycoconjugates to bind carbohydrate epitopes is established for a number of essential cell-surface proteins, for instance clusters of differentiation and adhesion factors.[26,44–48] Furthermore, certain cells are able to selectively bind mono- or oligosaccharides present on glycoconjugate probes.[48] Attempts have been made to develop an effective system to mimic spore recognition.[14–19] Receptor-specific sites of *Bacillus* spp. have not yet been characterized, but identification of specific sites is indispensable to understand recognition events.

In this study, glycoconjugate-polyacrylamide-fluorescein (Glyc-PAA-flu) polymers proved to be the most

suitable synthetic ligands since they can be immobilized on a solid phase.[49] The Glyc-PAA-flu probe binds non-specifically to cellular components.[26,48,49] Both the fluorescent label and the polymer backbone, affect this interaction minimally.[48,49] PAA-flu is hydrophilic and shows low binding affinity and flexibility. In addition, its structure allows shorter ligands to bind. Fluorescent labels work advantageously as efficient markers in different techniques: spectroscopy, fluorescence/confocal microscopy, and microspectroscopy of cells and tissue sections.[49]

The aim of this study is two-fold: (a) to elucidate *Bacillus* spore recognition using disaccharide Glyc-PAA-flu polymers, and (b) to identify interaction receptors expressed on the spore surface. In pursuit of bacterial spore (analyte) recognition, we have developed (i) a glycoconjugate assay using polystyrene microplates, (ii) a novel sensing concept based on a synthetic ligand such as glycoconjugates, and (iii) synthetic ligands for spore-binding studies.

2. Results and discussion

2.1. Binding of glycoconjugates to bacterial spores

Recognition can be easily accomplished using surface-immobilized molecules that interact with other species called targets. As many as 10 glycoconjugates (ligands) were evaluated as tools to distinguish related *Bacillus* spp. (targets) including *B. cereus*, *B. subtilis*, *B. thuringiensis*, and *B. pumilus* (Fig. 1). Figure 1 displays the signal intensity (*y*-axis) for each glycoconjugate–spore binding (*x*-axis). The average signal intensity of each glycoconjugate indicates a strong bonding to a particular spore. Two independent sets of experiments were carried out and repeated twice. The experiment outcomes were nearly identical. It is apparent that (i) glycoconjugates largely bound to *B. cereus* and *B. thuringiensis* are accompanied with a higher OD value than those bound to *B. pumilus* and *B. subtilis* (Fig. 1); (ii) glycoconjugates such as Galβ1-4 Glcβ-PAA-flu and Galα1-3 GalNacα-PAA-flu have a marked affinity for *B. cereus* and *B. thuringiensis*; (iii) all glycoconjugates are bound to *B. subtilis* with extremely low OD values compared with other spores.

The binding data illustrates that (i) despite being the most similar species in spore morphology, with both a developed exosporium and appendages,[37] *B. cereus* and *B. thuringiensis* can nonetheless be differentiated with these synthetic ligands; (ii) glycoconjugates Galβ1-4 Glcβ-PAA-flu and Galα1-3 GalNacα-PAA-flu have a marked affinity for both species of spores. The OD values for glycoconjugates GlcNacβ1-4 GlcNacβ-PAA-flu, and Fucα1-4 GlcNacβ-PAA-flu bound to both *B. thuringiensis* and *B. cereus* spores, were greater in case of *B. thuringiensis* than that of *B. cereus*. The OD value for all glycoconjugates bound to *B. thuringiensis* spores in contrast to those bound to *B. pumilus*, is higher as shown in Figure 1. Particularly, Fucα1-4 GlcNacβ-PAA-flu shows a higher average OD intensity for *B. thuringiensis* spores. The OD value of Galβ1-3 GalNacα-PAA-flu with *B. thuringiensis* spores is high as well.

In case of *B. pumilus*, Galα1-3 GalNacα-PAA-flu showed the highest OD value, followed by Galβ1-4 Glcβ-PAA-flu, and Galβ1-3 GalNacα-PAA-flu. However, these values were significantly lower compared to the values obtained with *B. thuringiensis*. Unlike *B. pumilus*, glycoconjugates bind strongly to *B. thuringiensis*.

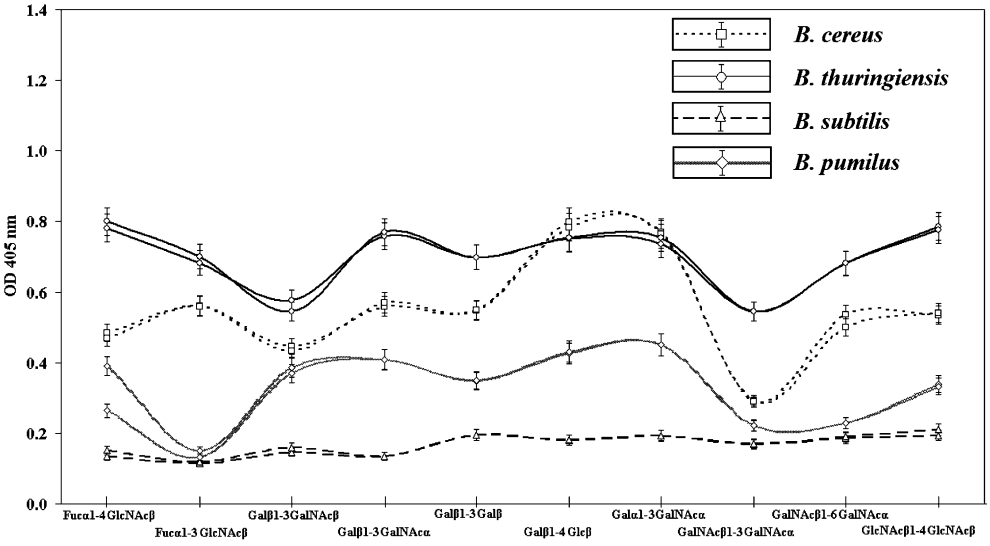

Figure 1. Recognition patterns of bacterial spores with glycoconjugates. The *y*-axis represents OD binding values for each glycoconjugate ligands (*x*-axis) obtained at a wavelength of 405 nm (here and thereafter).

The two types of spores can, therefore, be easily differentiated.

Average OD intensities for *B. subtilis* and *B. pumilus* spores have also been compared. These species possess a different morphology (see Section 2.3). It is known that *B. subtilis* has no more than an exosporium and *B. pumilus* has merely appendages.[31,38,39] In addition, these spores exhibit different neutral monosaccharide expressions on the spore exterior (see Section 2.5). Experimental data illustrates that: (i) both *B. pumilus* and *B. subtilis* reveal a low-affinity binding with the 10 glycoconjugates used in this study. Average OD intensities were below 0.5. (ii) Glycoconjugates such as Galα1-3 GalNacα-PAA-flu, Galβ1-4 Glcβ-PAA-flu, Galβ1-3 GalNacα-PAA-flu, Fucα1-4 GlcNacβ-PAA-flu and GlcNacβ1-4 GlcNacβ-PAA-flu showed the highest average OD intensities for *B. pumilus*. (iii) Glycoconjugates induced noticeably lower average OD intensities with *B. subtilis* than with *B. pumilus*. (iv) Glycoconjugates Galα1-3 GalNacα-PAA-flu, Galβ1-3Galβ-PAA-flu, Galβ1-4 Glcβ-PAA-flu, and GlcNacβ1-4 GlcNacβ-PAA-flu have bound to both spores, although stronger to *B. pumilus* than to *B. subtilis* spores.

Figure 2 represents the dilution effect on recognition abilities of glycoconjugate ligands.

In order to examine concentration effects of ligands during spore recognition, glycoconjugate solutions were prepared according to the manufacturer's protocol. Solutions were then diluted ×10, ×100, ×1000 and used in the spore recognition studies (Fig. 2A–D). In Figure 2, the curves of independent experiments almost entirely overlap.

Due to the dilution, the recognition capacity of *B. cereus* with Galβ1-4 Glcβ-PAA-flu (Fig. 2A) has noticeably increased, while it has decreased in case of *B. thuringiensis* (Fig. 2B). The recognition capacity of *B. cereus* using

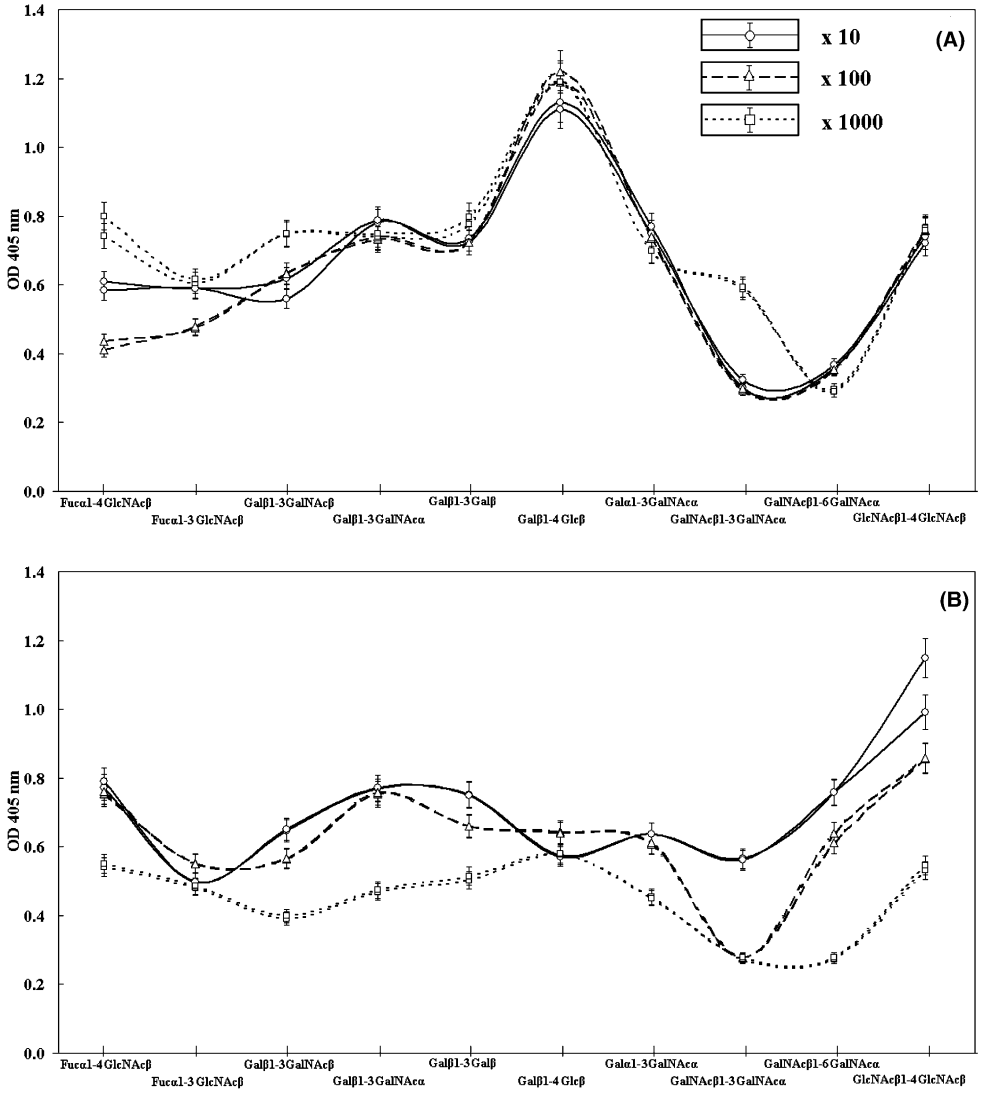

Figure 2. Role of dilution of glycoconjugates on recognition abilities of *B. cereus* (A), *B. thuringiensis* (B), *B. subtilis* (C), and *B. pumilus* (D) pores. Glycoconjugates were used at ×10; ×100; and ×1000 dilutions.

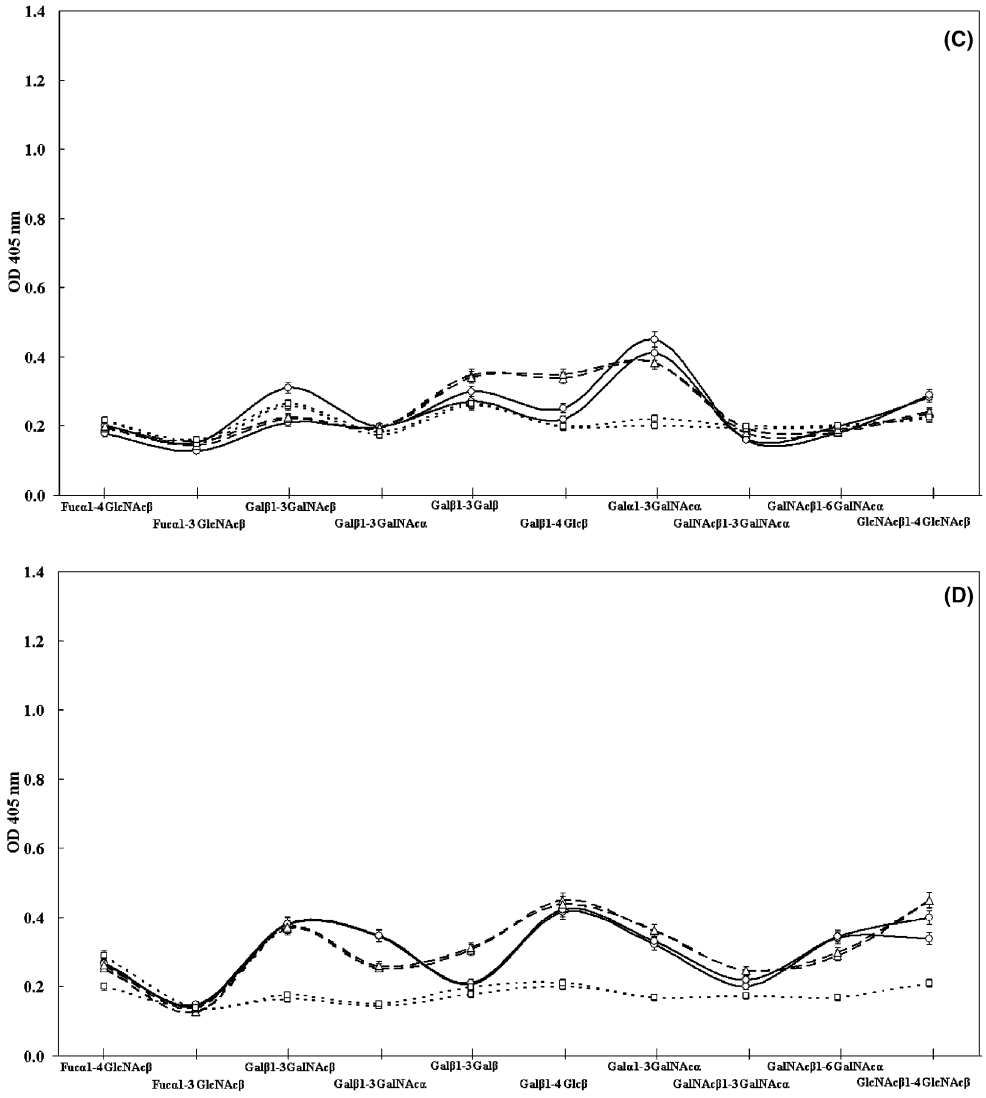

Figure 2 (*continued*)

diluted Galα1-3 GalNacα-PAA-flu, Galβ1-3 Galβ-PAA-flu, and Galβ1-3 GalNAcα-PAA-flu has led to almost identical OD values. Galα1-3 GalNacα-PAA-flu confirmed binding with *B. cereus*, *B. subtilis*, and *B. pumilus*, but OD values were slightly lower with *B. subtilis* (Fig. 2C), and *B. pumilus* (Fig. 2D) spores. Binding capacity of Galα1-3 GalNacα-PAA-flu with *B. thuringiensis* decreased with an increasing dilution (Fig. 2B). The OD intensity for glycoconjugate GlcNacβ1-4 GlcNacβ-PAA-flu increased with respect to *B. thuringiensis*. It, however, decreased for *B. cereus* (Fig. 2A). Solutions of glycoconjugates at 1000-fold dilution made it possible to differentiate *B. cereus* (Fig. 2A) from *B. thuringiensis* (Fig. 2B).

Recognition of *B. thuringiensis* using ×1000 dilute solutions of Fucα1-4 GlcNacβ-PAA-flu, Galβ1-3 GalNacβ-PAA-flu, Galβ1-3 GalNacα-PAA-flu, Galβ1-3 Galβ-PAA-flu; Galα1-3 GalNacα-PAA-flu has decreased. Overall, the presence of Gal in the structure of the glycoconjugates increased binding to *B. cereus* (Fig. 2A) compared to *B thuringiensis* spores.

OD values of Fucα1-4 GlcNacβ-PAA-flu and GlcNAcβ1-4 GlcNAcβ-PAA-flu glycoconjugate interactions with *B. thuringiensis* spores were high with either ×10, or ×100 dilutions. The recognition capacity of Fucα1-3 GlcNacβ-PAA-flu toward *B. thuringiensis* (Fig. 2B) was roughly unwavering, regardless of the dilution. The presence of GlcNacβ within Fucα1-4 GlcNacβ-PAA-flu and GlcNacβ1-4 GlcNacβ-PAA-flu possibly facilitates binding to *B. thuringiensis* spores.

The recognition capacity of *B. subtilis* using Galβ1-3Galβ-PAA-flu, and GlcNacβ1-4 GlcNacβ-PAA-flu has increased with ×10, ×100 dilutions (Fig. 2C). Even when diluted, glycoconjugates were still capable differentiating *B. cereus*, *B. thuringiensis*, and *B. subtilis* spores from each other as shown in Figure 2A–C.

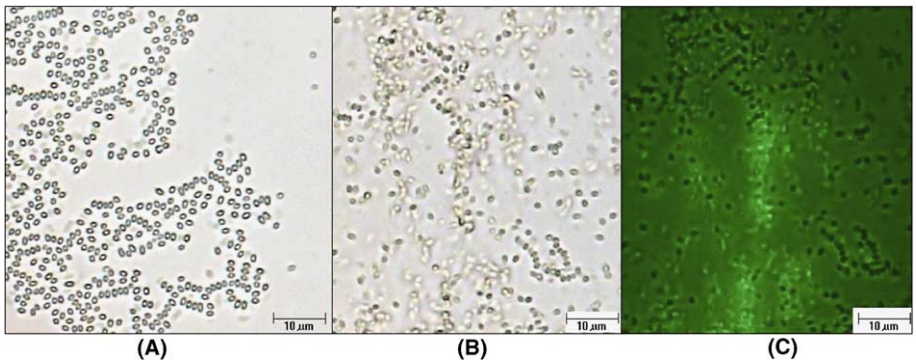

Figure 3. Untreated spores as control (A), glycoconjugate–spore complexes (B) seen under optical microscope. Glycoconjugate–spore interaction is confirmed under fluorescence microscope (C).

A high OD value was observed for GlcNacβ1-4 GlcNacβ-PAA-flu glycoconjugate interactions with *B. thuringiensis* and with *B. pumilus* spores. The latter generally showed lower OD values (Fig. 2D). As a result of the ×10, ×100, ×1000 dilutions (Fig. 2D), OD values of Galβ1-3 GalNacα-PAA-flu, Galβ1-4 Glcβ-PAA-flu increased when treated with *B. pumilus* (Fig. 2D). At ×1000 dilution, the use of Galβ1-3 GalNacβ-PAA-flu, Galβ1-3 GalNacα-PAA-flu, Galβ1-3 Galβ-PAA-flu, Galβ1-4 Gclβ-PAA-flu, and Galα1-3 GalNacα-PAA-flu induces a decrease in OD values. OD values obtained when Fucα1-4 GlcNacβ-PAA-flu and Fucα1-3 GlcNacβ-PAA-flu were used in *B. pumilus* spore interactions were roughly similar. The presence of Gal present in the external layer of *B. cereus* and *B. pumilus* spores [see fluorophore-assisted carbohydrate electrophoresis (FACE) studies, Section 2.5] is involved in the interaction with glycoconjugates containing the very same Gal in their own structure. Unlike in the case of *B. pumilus* spores, diluted glycoconjugates bind strongly to *B. cereus*, thus differentiating both of these spore strains. Differences for appendages found on *B. cereus* and *B. pumilus* spores [see scanning electron microscopy (SEM) studies, Section 2.3] most likely reflect differences in glycoconjugate binding magnitudes (Fig. 2A and D).

The role of diluted glycoconjugates for *B. subtilis* and *B. pumilus* recognition has been compared as well. Dilutions typically decrease binding affinity of glycoconjugates to *B. subtilis* (Fig. 2C) and *B. pumilus* (Fig. 2D). Only Galβ1-3 GalNacβ-PAA-flu, and Galβ1-4 Glcβ-PAA-flu glycoconjugates allowed both spore species to be differentiated (Fig. 2C and D). At ×1000 dilution, the OD values for both species of spores were quite similar, with the exception of glycoconjugate Galβ1-3 GalNacβ-PAA-flu and Galβ1-3Galβ-PAA-flu (Fig. 2C and D).

In summary, glycoconjugates used as synthetic ligands enable recognition and distinction of four different bacterial spores from one another (Fig. 1). Changes in the glycoconjugate concentration did not potentially hinder the recognition (Fig. 2). Even highly diluted glycoconjugate solutions enabled recognition and distinction of individual spores. According to recent research findings, the binding affinity of glycoconjugates to the surface of bacterial spores displaying multiple receptor sites depends both on the antigen, as well as on ligand structures.[20–22,26,45,48] Though both the antigen and ligands may display different bimolecular units in various arrangements,[20–22] glycoconjugates grant us the opportunity to mimic spore recognition.[18,19] FACE made it possible to analyze monosaccharide units of glycoproteins serving as receptors. Previous studies showed that the exterior layer of spores is in fact glycosylated.[42,54,55]

2.2. Optical and fluorescence microscopy studies

Nikon Eclipse E400 Pol, a polarizing optical and fluorescence microscope (Nikon Corp., Tokyo, Japan), was used to visually confirm the time scale for ligand–target complex presence (Fig. 3). Micrographs were taken at constant intervals prior to interaction (Fig. 3A) and during binding studies (Fig. 3B and C), at a magnification of ×400 times (Fig. 3). The ligand–target complex formation is critical in establishing high-affinity binding.[20–22] Figure 3A shows untreated spores as control. The glycoconjugate–spore complexes have appeared under the optical microscope as shown in Figure 3B. Spores have lost their transparency compared to untreated spores. Figure 3B depicts the changes in fluorescence intensities that characterize the interaction with glycoconjugates (Fig. 3C). This method appears to be convenient in revealing the presence of interacted glycoconjugate–spore complexes.

2.3. Scanning electron microscopy (SEM) studies

SEM was carried out for *B. cereus*, *B. subtilis*, and *B. pumilus* (Fig. 4A and B) with the purpose of demonstrating

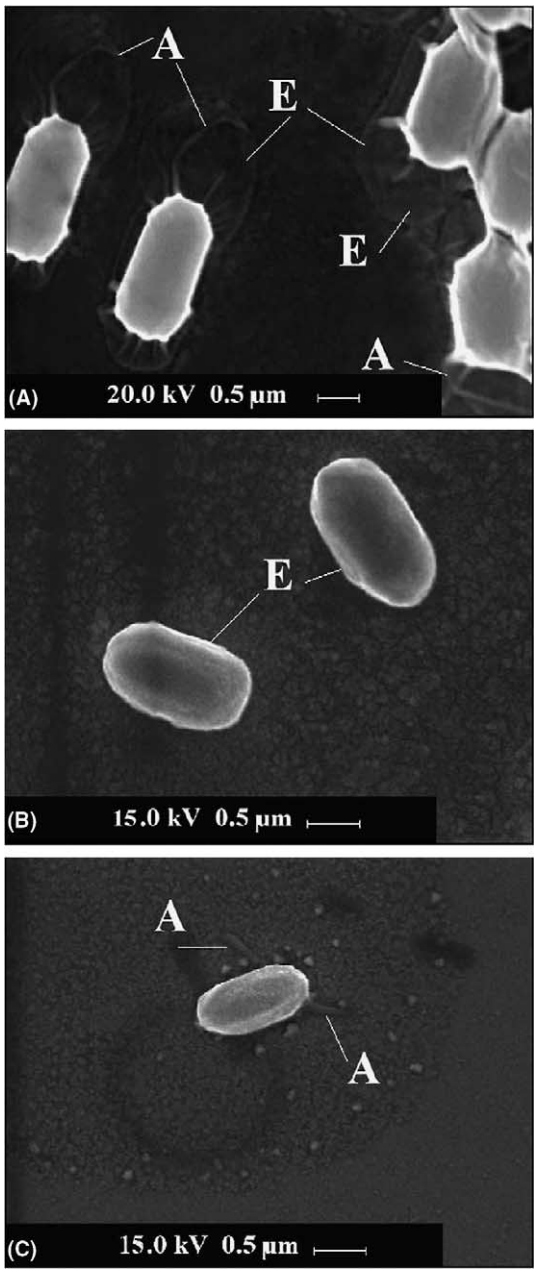

Figure 4. SEM images of *B. cereus* (A), *B. subtilis* (B), and *B. pumilus* (C) spores. *B. thuringiensis* spores are not shown. A stands for appendages, E for exosporium.

morphology of studied spores. SEM has revealed the actual shape of bacterial spores as well as their morphological structure. *B. cereus* spores (Fig. 4A) appeared slightly elongated (Fig. 4A). *B. cereus* spores exhibit a clearly identifiable exosporium as well as appendages. As many as 10–12 appendages were identified in the *B. cereus* spores (Fig. 4A). Only two filamentous appendages of *B. pumilus* have been evidently depicted under the SEM (Fig. 4C).

Our SEM data shows differences in appendage lengths of *B. cereus* and *B. pumilus*. These observed differences may be due to the particular sample preparation techniques applied or different bacterial strains used, although not observed using transmission electron microscopy.[50,51] In case of *B. subtilis*, only an exosporium-like structure was found, whereas no appendages were observed[52] (Fig. 4B) as expected.[39,40,50,51] Conclusive SEM observations indicate that morphological structures of studied spores are noticeably different from one another (Fig. 4). Given the varied structures present in all four spores used in this study, one is convicted that glycoconjugates account for their varied morphology (Fig. 4). Diverse morphology not only refers to the countless different receptors covering the spore surface and their arrangement (Fig. 6), but also to the existence of filamentous appendages on *B. cereus*, *B. thuringiensis* (data not shown) and *B. pumilus* (Fig. 4A and C). Diverse morphology furthermore encompasses the lack of appendages as seen in case of *B. subtilis* (Fig. 4B)[37–39,52] and lack of exosporium as seen in case of *B. pumilus* (Fig. 4C).[37,38,52] These results, at first glance, allude to the role played by structure in interaction avidity.[20–22]

2.4. Atomic force microscopy (AFM) studies

To identify spore surface receptors involved in glycoconjugate–spore interactions, the exterior spore layers were isolated by reducing disulfide bonds with 2-mercaptoethanol as described in the Experimental section and confirmed by AFM (Fig. 5B). Prior to treatment, *B. cereus* spores as they appear in solution are shown as computer-generated 3D AFM renderings (Fig. 5A). Spores were found to be firmly attached to the mica surface and remained sufficiently bound to be scanned with an AFM scanning probe. The spore surface is covered with a loose layer of exosporium that is generally spread out and is eventually stuck to the mica surface (Fig. 5A).

Thin exosporium originating from the outer surface of the spores can be effortlessly identified in 3D AFM images. These appendages are, however, barely visible in 2D AFM images (data not shown). Figures 4B and 4C are very important to this work because they verify that the means to isolate the exterior of the spore, as described in the Experimental section, was successful. AFM pictures that are presented (Fig. 4B and C) consist of two parts: height data (a) of true topography and amplitude image data (b). In the AFM tapping mode, an oscillating cantilever lightly taps a section of the observed sample surface, for instance the spores. As an oscillating cantilever scans the sample surface, amplitude variations (b) are extrapolated and utilized to generate a topographical map of the surface in which height and width (a) of a particular region are embodied. Figure 5B shows a purified mixture of the isolated exterior layer of the spores after treatment as shown in the Experimental (Section 3.2). Due to its smaller size this picture could not be shown in the same 3D format as

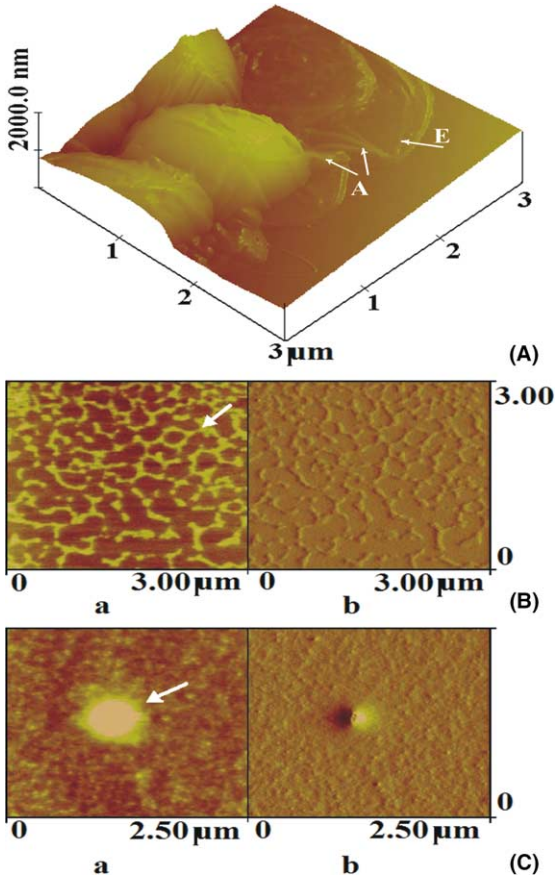

Figure 5. Tapping mode AFM images showing spore prior to (A) and after treatment (B, C). White arrows represent appendages (A), exosporium (E) (Fig. 4A). Mixture of isolated spore's exterior (B) and remaining inner shell (C) of *B. cereus* ATCC 11778 spore: *a*—height and *b*—amplitude.

Figure 4A. Consequently, this purified mixture was used in FACE analysis (Section 2.5) and in the Experimental (Section 3.5). Figure 5C demonstrates spores remaining after treatment.

2.5. Fluorophore-assisted carbohydrate electrophoresis (FACE) studies

Afterwards, the content of the exterior (Fig. 5B) of the *Bacillus* spp. Under study was analyzed using FACE (Fig. 6). FACE allows the examination of monosaccharides from neutral and amino sugar hydrolysis. FACE electrophoresis analysis reveals the presence of several monosaccharides, namely *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), galactose, mannose, and fucose, as well as glucose.

Both, *B. cereus* and *B. thuringiensis* contain neutral and amine profiles. In contrast, the exterior of the spores of *B. subtilis* and *B. pumilus* exclusively exhibit neutral sugar profiles. Galactose has been identified in the exterior of *B. cereus* spores though neutral and amino sugar profiles. A neutral sugar profile of the appendages of *B. thuringiensis* exhibits two monosaccharides: glucose and galactose, whereas the amino sugar profile contained only galactose. Mannose, fucose, and galactose were detected on the exterior of *B. subtilis* spores. *B. pumilus* spores contained galactose and GlcNAc. *B. cereus*[53] and *B. subtilis* typically contain high levels of galactose.[53,54] Glucose is considered the principal neutral carbohydrate found in *B. anthacis*, *B. thuringiensis*, and *B. subtilis* spores.[53,54] FACE has proved to be a powerful tool in identifying monosaccharide units of glycoproteins expressed on exterior layers of spores. Presumably, different units of glycoconjugates are recognized by different receptor units expressed on the exterior layer (Fig. 6) of the spores studied (Fig. 4). We have not yet been able to prove that ligands are actually bound to monosaccharides. Prospective studies, with the assistance of oligosaccharide sequence analysis and bioinformatics will clarify this issue. In addition, previously reported data on protein glycosylation of the external surface of *B. anthacis*,[42] *B. cereus*, and *B subtilis*[53–55] spores confirm

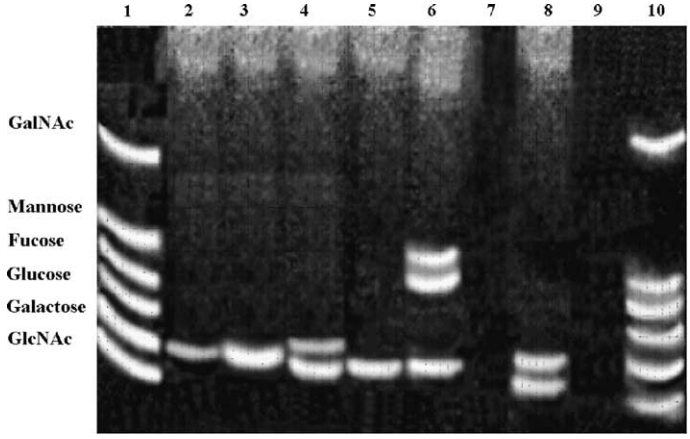

Figure 6. Monosaccharide composition of (2–9) spores exterior and AMAC-labeled monosaccharide standard (1, 10); even numbers represent monosaccharides from neutral sugar hydrolysis; odd numbers stand for amino sugar hydrolysis; lines 2, 3—*B. cereus*; 4, 5—*B. thuringiensis*; 6, 7—*B. subtilis*; 8, 9—*B. pumilus* spore monosaccharide composition.

the involvement of monosaccharides in interactions with glycoconjugate ligands.

Being primarily used as a recognition substrate, fluoresceinated glycoconjugates played a major role in this study. As synthetic ligands, they facilitated the establishment of carbohydrate–carbohydrate interaction relevancy.[26,48] Carbohydrates located on the external surface of bacterial spores serve as a potential multivalent receptor display (Fig. 6). These carbohydrates are recognized by other carbohydrate units of disaccharide glycoconjugates (Figs. 1–3) leading to interactions. The technique presented in this study may be helpful in finding and isolating prospective spore receptor recognition sites. It may also be valuable in discovering the carbohydrate-based inhibitors essential for terminal neutralization of the bacterial spores. Since most pathogens possess unique cell-surface carbohydrates, sequencing of oligosaccharides is a suitable research theme for upcoming carbohydrate-binding sites characterization studies. Specific carbohydrate structures are differentially expressed in each cell type and are believed to be recognized by complementary molecule(s) expressed on the surface of the counterpart interacting cell.[56–59] Previously reported experimental data directly show complex carbohydrates in the recognition processes, including adhesion between cells, adhesion of cells to the extracellular matrix, and specific recognition of cells by one another.[27,28,44–48] It is known that carbohydrate–carbohydrate interactions play an important role for the complimentary binding of glycosphingolipids.[58–60]

The major advantage of our approach is based on glycoconjugate binding capacities to the bacterial spores studied. Furthermore, we applied our method to detect carbohydrate binding and to investigate the selectivity of glycoconjugates in regards to various *Bacillus* spores. Our results demonstrated that disaccharide glycoconjugates were able to recognize and discern *Bacillus* spp. including *B. cereus*, *B. thuringiensis*, *B. pumilus*, and *B. subtilis* spores (Figs. 1 and 2). Furthermore, our findings demonstrated that diluted glycoconjugate solutions do not impede spore recognition (Fig. 2). Moreover, glycoconjugates were observed to interact with monosaccharide epitopes expressed on the exterior layer of spores (Figs. 1, 2, and 6). This observation also suggests that our approach is of value when it comes to discovering unexpected, but biologically relevant, bacterial species. The detection of carbohydrate-binding epitopes is considered practical for this type of application since most pathogens possess unique cell-surface carbohydrates.[53–55] Taken together, the major advantage of our approach lies in the glycoconjugate specificity and selectivity to spore species, reproducibility, and the interaction magnitude (Figs. 1 and 2). Multiple aspects of carbohydrate–carbohydrate recognition lead to applications like the detection of specific bacterial spores. In conclusion, this study provides further evidence that specific recognition of bacterial spores is based on multivalent[20–22] carbohydrate–carbohydrate interactions between disaccharide glycoconjugates acting as ligands (Figs. 1 and 2) and monosaccharide units expressed on the exterior of bacterial spores (Fig. 6).

The findings of the present study provide new a opportunity on how to improve the ligand selection, indispensable for the development of sensors. In addition, the findings provide new information for inhibition of bacterial spores, and in general, demonstrate how carbohydrates function as recognition signals in nature. Prospective research foresees recognition studies of *Bacillus* spp. using different strains of the same bacterial species, glycoconjugates with a much higher molecular weight and an increase in saccharide units coupled to the PAA backbone.

3. Experimental

3.1. Materials

Multivalent disaccharide-PAA-flu glycoconjugates were purchased from GlycoTech, Inc. (Rockville, MD). Tween-20, 30% albumin bovine solution, culture tissue grade water: W3500, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) liquid substrate system, standard proteins were purchased from Sigma–Aldrich (St. Louis, MO). Goat anti-mouse (IgG + IgM)-horseradish peroxidase (HRP) conjugate was purchased from Roche (Indianapolis, IN). Sterile bacterial spore suspensions, namely *B. cereus* ATCC 11778 (10^6 CFU/0.1 mL), *B. thuringiensis* ATCC 29730 (10^6 CFU/0.1 mL), *B. subtilis* ATCC 9372 (10^6 CFU/0.1 mL), and *B. pumilus* ATCC 27142 (10^6 CFU/0.1 mL) were purchased from Raven Biological Laboratories, Inc. (Omaha, Nebraska). The fluorophore-assisted carbohydrate electrophoresis FACE® monosaccharide composition kit was obtained from Glyko Inc., (Novato, CA).

3.2. Isolation of the exterior layer of the bacterial spores

The isolation of the exterior layer of spores was performed as described earlier.[38] Briefly, a 500-μL spore suspension with a concentration of $2 \times 10^6/100\,\mu L$ was mixed with a 2% solution of 2-mercaptoethanol (1 mM carbonate–bicarbonate buffer, pH 10.0), vortexed for 5 min, and incubated at 37 °C for 120 min. After exposing it to the reagent, the mixture was centrifuged at $4000 \times g$ for 20 min. Fractions containing appendages (supernatant) and spore inner constituents (pellets), were washed four times with deionized water. Successful isolation of fractions was confirmed by AFM (Fig. 5) prior to further use.

3.3. Scanning electron microscopy (SEM)

Spore samples (10^4 CFU) for SEM observations were deposited on mica disks and desiccated for 7 days. Samples were then coated with a 10-nm thin film of evaporated gold[61] for 60 s and then examined with a Hitachi S-570 scanning electron microscope (Hitachi Ltd., Tokyo, Japan) at an accelerating voltage of 15–20 kV. Results are shown in Figure 4.

3.4. Atomic force microscopy (AFM)

Solutions of untreated spores (10^4 CFU) and purified exterior layers of spores were immobilized on mica discs (Digital Instruments, Inc., Santa Barbara, CA) using sterile syringes, then dried in air at 20 °C. 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., Santa Barbara, CA) 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. Results are shown in Figure 5.

3.5. Fluorophore-assisted carbohydrate electrophoresis (FACE) of the exterior layer of bacterial spores

FACE was carried out with the FACE® monosaccharide composition kit according to the manufacturer's instructions (Glyko, Inc., Novato, CA). The kit contains reagents and buffers required to hydrolyze oligosaccharides of intact glycoproteins and to analyze neutral and amino monosaccharide constituents. Briefly, glycoproteins (10–20 mg) were isolated from exterior layers of the spore strains. Glycoproteins were then dialyzed against distilled water (50 μL) using 0.5 mL snap-top tubes with cap locks. Neutral monosaccharides were hydrolyzed by dissolving them in 2 M trifluoroacetic acid (TFA) at 100 °C for 5 h, whereas amino sugars were hydrolyzed by dissolving an isolated mixture of the exterior layers in 100 μL of 4 M HCl at 100 °C for 3 h. Following hydrolysis, the mixture was dried under reduced pressure. Dried monosaccharides from the amino-sugar hydrolysis reaction were N-acetylated for a second time by the addition of a re-acetylation reagent M3 (protocol). The dried monosaccharides from both hydrolyses were labeled with a 2-aminoacridone (AMAC) fluorescent tag and incubated overnight at 37 °C. Separation of AMAC-labeled monosaccharides was completed though polyacrylamide gel electrophoresis at a constant current of 30 mA/400–600 V per gel at 5 °C for 75 min. Upon comparison with the monosaccharides, the standard mixture consisted of 100 pmol each of the AMAC-labeled *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), galactose, mannose, fucose, and glucose. They were then loaded into a lane adjacent to the lane of amino sugars and to that of the neutral hydrolysis reaction. The resulting band patterns reflect the monosaccharide composition of the starting material. Band patterns were visualized in a long-wavelength UV light box (ULTRA-LUM, Inc., Claremont, CA) with a peak light output at 370 nm. The results are shown in Figure 6.

3.6. Study of glycoconjugate–spore binding

In our system, fluoresceinated glycoconjugates (GlycoTech, Inc., Rockville, MD) were employed as synthetic ligands to recognize and capture *B. cereus*, *B. subtilis*, *B. pumilus*, and *B. thuringiensis* spores (targets). The fluoresceinated glycoconjugate (Glyc-PAA-flu) is comprised of a soluble polyacrylamide (PAA) backbone (~30 kDA) with various carbohydrate side groups (20 mol%) and fluorescein (flu). PAA-flu is known to be an inert carrier due to the lack of charged groups and, therefore, does not show any specific binding to cells.[48,49] The suppleness of the PAA backbone facilitates the conjugates to adjust themselves to targets.[48,49] Glyc-PAA-flu contains 1 mol% flu (2–3 flu residues per polymer chain), sufficient for labeled detection techniques[48,49] such as fluorescence microscopy. Glycoconjugates used: Fucα1-4 GlcNacβ-PAA-flu, Fucα1-3 GlcNacβ-PAA-flu, Galβ1-3 GalNacβ-PAA-flu, Galβ1-3 GalNacα-PAA-flu, Galβ1-3 Galβ-PAA-flu, Galβ1-4 Glcβ-PAA-flu, Galα1-3 GalNacα-PAA-flu, GalNacβ1-3 GalNacα-PAA-flu, GalNacβ1-6 GalNac-PAA-flu, and GlcNacβ1-4 GlcNacβ-PAA-flu, all have the same molecular weight. Two sets of experiments were independently performed twice. Each Glyc-PAA-flu measurement was repeated five times. The binding studies were performed as follows: each microplate well (Nunc, MaxiSorp) was coated with Glyc-PAA-flu (20 μg/mL) and left overnight at 4 °C. Wells were washed three times and subsequently blocked with 100 μL of 3% BSA in PBS at 37 °C for 1–2 h, followed by washing with 50 μL/well with phosphate-buffered saline (PBS) containing 0.1% Tween-20 at 25 °C. Soon after, spores (7.5×10^4 CFU/well) were added and incubated for 1–2 h at 25 °C under continuous shaking. Wells were washed three times with PBS containing 0.1% Tween-20 with the purpose of removing unbound spores. Then, spores bound to glycoconjugate ligands were confirmed under a Nikon Eclipse E400 POL fluorescence microscope at a magnification of ×400 (Fig. 3). The

ligand–target complex formation is critical in establishing binding affinity.[20–22] Subsequently, nonspecific 12.5 μL of anti-mouse (IgG + IgM)-horseradish peroxidase (HRP)-labeled conjugate (1:4000 dilution) (Roche Diagnostics Corp., IN) was introduced to each well and incubated at 37 °C for 1 h. Fluorescence intensity decreased subsequent to spore–ligand bindings. To overcome this shortcoming, a nonspecific goat anti-mouse antibody conjugate was chosen with the purpose of indirectly quantifying retained ligand–target complexes in each well based on OD measurements. The more ligand–target complexes that are available, the more HRP-labeled conjugates will bind to these complexes. HRP-labeled conjugates are, therefore, determined by the amount of available ligand–target complexes. Wells were washed three times with PBS containing 0.1% Tween-20. A substrate solution 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) was then added. The colorimetric reaction was terminated by the addition of 50 μL/well of 1 M H_2SO_4. Spectrophotometry was carried out with a SpectraMax® Plus^{384} microplate reader (Molecular Devices Corp., Sunnyvale, CA) at 405 nm. Data are presented graphically in Figure 1.

3.7. Study of the dilution effect

Solutions of disaccharide glycoconjugates were prepared according to the manufacturer's protocol. The solutions were then diluted ×10, ×100, ×1000 into a PBS/0.2% NaH_3 buffer. The binding studies were carried out using diluted glycoconjugates as described in section above (3.6). Recognition was accomplished using surface-immobilized glycoconjugates as ligands interacting with analytes (spores) in solution (Fig. 2).

3.8. Optical and fluorescence microscopy

Visual confirmation of glycoconjugate–spore complexes was performed during the binding studies. Untreated spore as control and glycoconjugate–spores complexes were visually examined using a Nikon Eclipse E400 POL fluorescence microscope at a magnification of ×400. Digital micrographs of spores and glycoconjugate–spore complexes were acquired in real time (Fig. 3).

Acknowledgements

This research was supported by grant # 0660076225, originating from the Defense Advanced Research Projects Agency (DARPA). We express our deepest gratitude to Joany Jackman, Senior Scientist, Applied Physics Laboratory, Johns Hopkins University, Laurel, MD for valuable discussions during this study. We would also like to thank Prof. Mary Cowman, Othmer Dept. of Chemical and Biological Sciences and Engineering, Polytechnic University, for allowing us to use the AFM. We extend our appreciation to Prof. Said Nourbakhsh, Dept. of Mechanical Engineering for technical assistance during SEM, Prof. Arnost Reiser and Prof. Edward Weil, Othmer Dept. of Chemical and Biological Sciences and Engineering, Polytechnic University, for critical reading of the manuscript.

References

1. Doyle, M. P.; Beuchat, L. P.; Montville, T. J. In *Food Microbiology. Fundamentals and Frontiers*; Doyle, M. P., Beuchat, L. R., Montville, T. J., Eds.; American Society of Microbiology: Washington, DC, 1997.
2. Barnaby, W. In *The Plague Markers: The Secret World of Biological Warfare*; Barnaby, W., Ed.; Vision Paperbacks: London, 1997.
3. Gerhardt, P.; Ribi, E. *J. Bacteriol.* **1964**, *88*, 1774–1789.
4. Setlow, P. *Annu. Rev. Microbiol.* **1995**, *49*, 29–54.
5. King, D.; Luna, V.; Cannons, A.; Cattani, J.; Amuso, Ph. *J. Clin. Microbiol.* **2003**, *41*, 3454–3455.
6. Yu, H.; Stopa, P. J. In *Environmental Immunochemical Methods: Perspectives and Applications*; Van Emon, J. M., Gerlach, C. L., Johnson, J. C., Eds.; ACS: Washington, DC, 1995; pp 297–306.
7. Bell, C. A.; Uhl, J. R.; Hadfield, T. L.; David, J. C.; Meyer, R. F.; Smith, T. F.; Cockerill, F. R., III. *J. Clin. Microbiol.* **2003**, *41*, 524–525.
8. Higgins, J. A.; Cooper, M.; Schoeder-Tucker, L.; Black, S.; Miller, D.; Karns, J. S.; Manthey, E.; Breeze, R.; Perdue, M. L. *Appl. Environ. Microbiol.* **2003**, *69*, 593–599.
9. Hartley, H. A.; Baeumner, A. J. *Anal. Bioanal. Chem.* **2003**, *376*, 319–327.
10. Harris, P. C.; Cloney, L.; Fong, W.; Fulton, R. E. Paper #359. http://www.responsebio.com/pdf/ASM_poster.pdf.
11. www.gsa/gov/mailpolicy.
12. Read, T. D.; Peterson, S. N.; Tourasse, N.; Baillie, L. W.; Paulsen, I. T.; Nelson, K. E.; Tettelin, H.; Fouts, D. E.; Eisen, J. A.; Gill, S. R.; Holtzapple, E. K.; Okstad, O. A.; Helgason, E.; Rilstone, J.; Wu, M.; Kolonay, J. F.; Beanan, M. J.; Dodson, R. J.; Brinkac, L. M.; Gwinn, M.; DeBoy, R. T.; Madpu, R.; Daugherty, S. C.; Durkin, A. S.; Haft, D. H.; Nelson, W. C.; Peterson, J. D.; Pop, M.; Khouri, H. M.; Radune, D.; Benton, J. L.; Mahamoud, Y.; Jiang, L.; Hance, I. R.; Weidman, J. F.; Berry, K. J.; Plaut, R. D.; Wolf, A. M.; Watkins, K. L.; Nierman, W. C.; Hazen, A.; Cline, R.; Redmond, C.; Thwaite, J. E.; White, O.; Salzberg, S. L.; Thomason, B.; Friedlander, A. M.; Koehler, T. M.; Hanna, P. C.; Kolsto, A. B.; Fraser, C. M. *Nature* **2003**, *423*, 81–86.
13. Tengelsen, L.; Hudson, R.; Barnes, S.; Hahn, C. *Emerg. Infect. Dis.* **2002**, *8*, 1093–1095.
14. Williams, D. D.; Benedek, O.; Turnbough, Ch. L., Jr. *Appl. Environ. Microbiol.* **2003**, *69*, 6288–6293.
15. Knurr, J.; Benedek, O.; Heslop, J.; Vinson, R. B.; Boydston, J. A.; McAndrew, J.; Kearney, J. F.; Turnbough, Ch. L., Jr. *Appl. Environ. Microbiol.* **2003**, *69*, 6841–6847.
16. Kazakov, S.; Kaholek, M.; Ji, T.; Turnbough, Ch. L., Jr.; Levon, K. *Chem. Commun. (Cambridge)* **2004**, 430–431.

17. Ji, T.; Kaholek, M.; Kazakov, S.; Tarasenko, O. M.; Sharma, N. K.; Zhou, Y.; Yu, B.; Levon, K. 226th ACS National Meeting, New York, NY, 7–11 September 2003, Polymer Preprint 44 (2) 483–484.
18. Tarasenko, O.; Alusta, P.; Kudasheva, D.; Paquiot, D.; Cowman, M.; Levon, K. 224th ACS National Meeting, Boston, MA, 18–22 August 2002; COLL-0110.
19. Levon, K.; Tarasenko, O.; Yu, B. U.S. Patent Application Serial No.: 10/719,688, filed: November 21st, 2003; PCT Application: PCT/US03/37401 filed: November 21st, 2003.
20. Bertozzi, C. R.; Kiessling, L. L. *Science* **2001**, *291*, 2357–2364.
21. Kiessling, L. L.; Pohl, N. L. *Chem. Biol.* **1996**, *3*, 71–77.
22. Kiessling, L. L.; Gestwicki, J. E.; Strong, L. E. *Curr. Opin. Chem. Biol.* **2000**, *6*, 696–703.
23. Roy, R. *Curr. Opin. Struct. Biol.* **1996**, *6*, 692–702.
24. Zhou, Y.; Yu, B.; Levon, K. *Chem. Mater.* **2003**, *15*, 2774–2779.
25. Varki, A. *Glycobiology* **1993**, *3*, 97–130.
26. Bovin, N. V. In *Glycosciences: Status and Perspectives*; Gabius, H. J., Gabius, S., Eds.; Chapman & Hall: London, 1997; pp 277–289.
27. Feizi, T. *Immunol. Rev.* **2000**, *173*, 79–88.
28. Karlsson, K. A.; Angstrom, J.; Bergstrom, J.; Lanne, B. *Apmis Suppl.* **1992**, *27*, 71–83.
29. Wang, D.; Kabat, E. A. In *Structure of Antigens*; Van Regenmortal, M. H. V., Ed.; CRC: Boca Raton, 1996; pp 247–276.
30. Frazier, W.; Glaser, L. *Annu. Rev. Biochem.* **1979**, *48*, 491–523.
31. Barondes, S. H. *Annu. Rev. Biochem.* **1981**, *50*, 207–231.
32. Sharon, N. *Biol. Cell* **1984**, *51*, 239–246.
33. Mimura, Y.; Sondermann, P.; Ghirlando, R.; Lund, J.; Young, S. P.; Goodall, M.; Jefferis, R. *J. Biol. Chem.* **2001**, *276*, 45539–45547.
34. Harris, L. J.; Skaletsky, E.; McPherson, A. *J. Mol. Biol.* **1998**, *275*, 861–872.
35. Marz, L. L.; Beaman, T. C.; Gerhard, P. *J. Bacteriol.* **1970**, *101*, 196–201.
36. Scherer, P. S.; Somerville, H. J. *Eur. J. Biochem.* **1977**, *72*, 479–490.
37. Hachizuka, Y.; Kojima, K.; Sato, T. *J. Bacteriol.* **1966**, *91*, 2382–2384.
38. Kozuka, S.; Tochikubo, K. *Microbiol. Immunol.* **1985**, *29*, 21–37.
39. Driks, A. *Microbiol. Mol., Biol. Rev.* **1999**, *63*, 1–20.
40. Warth, A. D.; Ohey, D. F.; Murrell, W. G. *J. Cell Biol.* **1963**, *16*, 578–592.
41. Kornberg, A.; Spudish, J. A.; Nelson, D. L.; Deutscher, M. *Annu. Rev. Biochem.* **1968**, *37*, 51–78.
42. Steichen, C.; Chen, P.; Kearney, J. F.; Turnbough, C. L., Jr. *J. Bacteriol.* **2003**, *185*, 1903–1910.
43. Sylvestre, P.; Couture-Tosi, E.; Mock, M. *J. Bacteriol.* **2003**, *185*, 1555–1563.
44. Hiragi, Y. *J. Gen. Microbiol.* **1972**, *72*, 87–99.
45. Bovin, N. V.; Shiyan, S. D.; Mikhalchik, E. V. *Glycoconjugate J.* **1995**, *12*, 427.
46. Rye, P. D.; Bovin, N. V. *Glycobiology* **1997**, *7*, 179–182.
47. Houseman, B. T.; Mrksich, M. *Top. Curr. Chem.* **2002**, *218*, 1–44.
48. Bovin, N. V. *Glycoconjugate J.* **1998**, *15*, 431–446.
49. Galanina, O.; Feofanov, A.; Tuzikov, A. B.; Papoport, E.; Crocker, P. R.; Grichine, A.; Egret-Charlier, M.; Vigny, P.; Le Pendu, J.; Bovin, N. V. *Spectrochim. Acta, Part A* **2001**, *57*, 2285–2296.
50. Hachisuka, Y.; Kozuka, S. *Microbiol. Immunol.* **1981**, *25*, 1201–1207.
51. Hachisuka, Y.; Kozuka, S.; Tsujikawa, M. *Microbiol. Immunol.* **1984**, *28*, 619–624.
52. Tarasenko, O.; Kudasheva, D.; Nourbakhsh, S.; Cowman, M. Levon, K. 2004, unpublished data.
53. Fox, A.; Black, G. E.; Fox, K.; Rostovtseva, S. *J. Clin. Microbiol.* **1993**, *31*, 887–894.
54. Tarasenko, O.; Islam, Sh.; Levon, K. 226th ACS National Meeting, New York, NY, 7–11 September 2003, Division of Environmental Chemistry, Targeting Chemical and Biological Warfare Agents, Preprints, Vol. 43(2), pp 1–6.
55. Tarasenko, O.; Islam, Sh.; Levon, K. 228th ACS National Meeting, Philadelphia, 24–26 August 2004; AGFD-189.
56. Janeway, C. A.; Travers, P.; Walport, M.; Capra, J. D. *Immunobiology. The Immune System in Health and Disease*, 4th ed.; Current Biology Publications: London, 1999.
57. Hakomori, S. *Annu. Rev. Biochem.* **1981**, *50*, 733–764.
58. Kojima, N.; Hakomori, S. *J. Biol. Chem.* **1989**, *264*, 20159–20162.
59. Kojima, N.; Hakomori, S. *J. Biol. Chem.* **1991**, *266*, 17552–17558.
60. Stewart, R. J.; Boggs, J. M. *Biochemistry* **1993**, *32*, 10666–10674.
61. Bergeret, G.; Gallezot, P. In *Handbook of heterogeneous catalysis*. Vol. 2, Ertl, G., Knözinger, H., Weitkamp, J. Eds.; Wiley-VCH: Weinheim, Germany, 1997.