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Unique Aggregation of Anthrax (*Bacillus anthracis*) Spores by Sugar-Coated Single-Walled Carbon Nanotubes

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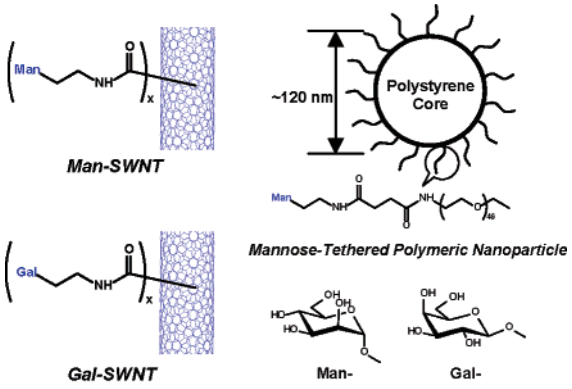
There has been significant interest in the surface structure and characteristics of anthrax (*Bacillus anthracis*) spores as related to their binding by molecular species.[1−6] The investigation of such binding is obviously important to the development of countermeasure technologies for the detection and decontamination of anthrax spores.[1] Various ligands based on natural and synthetic bioactive oligomers and polymers have been explored. For example, Turnbough and co-workers reported that a family of short peptides with a specific sequence could bind selectively to *B. anthracis* spores.[4] Separately, Cole et al. showed that some lectins were able to agglutinate *B. anthracis* spores, for which the binding was attributed to the presence of lectin-specific D-galactose or *N*-acetylglucosamine on the spore surface.[2]

There have been other studies suggesting that various carbohydrates are expressed on the surface of *B. anthracis* spores, including rhamnose, 3-*O*-methyl rhamnose, and galactosamine.[5] The surface carbohydrate residues may be exploited for the binding and agglutination of *B. anthracis* spores through interactions with synthetic multivalent carbohydrate ligands. As reported recently,[7] single-walled carbon nanotube (SWNT) serves as an excellent pseudo one-dimensional scaffold for the ligand display, with the SWNTs tethered with monosaccharide species exhibiting strong cell adhesion to result in efficient agglutination of pathogenic *Escherichia coli*. In the work reported here, we found that functionalized SWNTs displaying a large number of monosaccharide (derivatized mannose or galactose, Scheme 1) molecules bind to *B. anthracis*

Scheme 1



Scheme 1

spores with the mediation of a divalent cation and that the binding is unique to the nanotube-displayed carbohydrates. The resulting significant aggregation of *B. anthracis* spores and the associated substantial reduction in colony forming units (CFU) may potentially find valuable applications in the detection and decontamination efforts.

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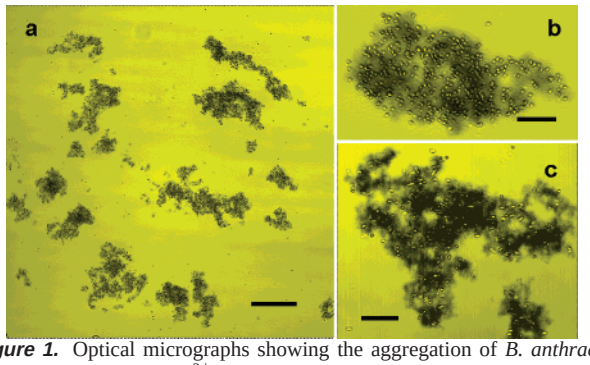

Figure 1. Optical micrographs showing the aggregation of *B. anthracis* spores induced by the Ca^{2+}-mediated binding with Man-SWNT (a and b) and Gal-SWNT (c). Scale bars = 100 μm (a) and 20 μm (b,c).

The sugar-functionalized nanotube samples Man-SWNT and Gal-SWNT were synthesized by attaching 2′-aminoethyl-α-D-mannopyranoside and 2′-aminoethyl-β-D-galactopyranoside, respectively, to purified SWNTs (arc-discharge sample) via the amidation of the nanotube surface-bound carboxylic acid moieties, as reported previously.[7,8] The Man-SWNT and Gal-SWNT samples are readily soluble in water or aqueous buffer, fully compatible with biological species and assays.

B. anthracis spores (Sterne 34F2, a nonvirulent strain, but still requires careful handling in a biosafety level-2 laboratory) were supplied by Colorado Serum Company (Denver, Colorado), and the spore suspension was prepared by following established procedures.[9] It was found that when a sugar-functionalized nanotube solution was added to spore suspension to form an apparently homogeneous mixture, the further addition of divalent cation Ca^{2+} resulted in substantial aggregation of the spores (Figure 1). In a typical experimental procedure, an aqueous suspension of *B. anthracis* spores (20 μL, 10^5 CFU μL^{-1}) was mixed with a Man-SWNT solution (20 μL, mannose equivalent concentration 0.2 mg mL^{-1}) or with distilled water (20 μL) as control. A few minutes later, an aqueous $CaCl_2$ solution (10 μL, 100 mM) was added with gentle mixing. There was immediate formation of aggregates visible to naked eyes in the mixture with Man-SWNT. Nevertheless, the mixtures (sample and control) were rotated for 4 h. There were obviously large aggregates in the sample mixture, while no changes were found in the control (even after rotation for more than 12 h). A small aliquot (10 μL) of the sample was dropped onto a glass slide (covered with cover glass slide) for optical microscopy analyses. As shown in Figure 1, the specimen contained predominantly large aggregates of at least 10 microns in size (compared with a typical spore size on the order of 1 micron in diameter and 2 microns in length).

The aggregates in the sample mixture were analyzed at a higher resolution by using scanning electron microscopy (SEM). For the preparation of the SEM specimen,[10] the sample mixture was centrifuged at 6800*g* for 5 min. The pellet was washed with distilled water in centrifuging−suspending cycles, and the final pellet was

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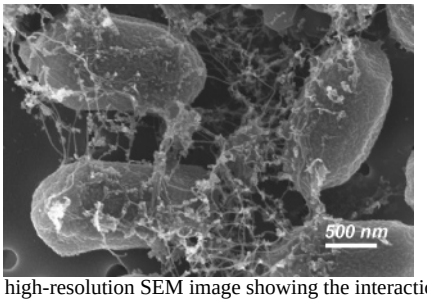

Figure 2. A high-resolution SEM image showing the interactions of Man-SWNT with *B. anthracis* spores.

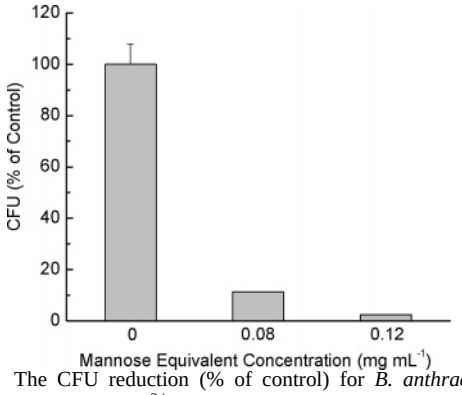

Figure 3. The CFU reduction (% of control) for *B. anthracis* spores aggregated owing to the Ca^{2+}-mediated binding with Man-SWNT.

suspended for the fixing and post-fixing treatment according to established procedures. The final specimen upon critical point drying was mounted on an aluminum stub with double-sided carbon tape and coated with platinum. According to the SEM images (Figure 2), the *B. anthracis* spores in the aggregates are "wrapped" by the functionalized SWNTs.

A similar aggregation effect was observed in the use of Gal-SWNT with Ca^{2+}. The results were qualitatively the same as those obtained with Man-SWNT.

The overall effectiveness in the aggregation of *B. anthracis* spores by Man-SWNT with Ca^{2+} was evaluated in a CFU reduction assay.[11] In the experiment, a suspension of *B. anthracis* with the spore count of 3.8×10^6 (in 40 μL) was mixed with a solution of Man-SWNT (40 μL, two mannose equivalent concentrations used: 0.2 mg mL^{-1} and 0.3 mg mL^{-1}) or with distilled water as control. After 30 min, an aqueous $CaCl_2$ solution (20 μL, 100 mM) was added with gentle mixing, followed by rotation at room temperature for about 12 h. The mixtures (samples and control) were centrifuged at a low speed (25*g*) for 1 min. A portion of each supernatant (50 μL) was used for the assay. Upon serial dilution, the diluted samples (0.1 mL each in triplicate) were spread evenly on solid TSA medium plates for incubation at 37 °C for about 12 h. The plates were counted, and the percentage CFU reduction was calculated in reference to the control. As shown in Figure 3, a reduction up to 97.7% could be achieved under the experimental conditions.

Interestingly and somewhat surprisingly, the binding to aggregate *B. anthracis* spores is unique to the nanotube-displayed monosaccharide molecules, not available to other displaying platforms such as polymeric nanoparticles (Scheme 1, for example). These nanoparticles with a polystyrene core and surface-tethered mannoses or galactoses, while known for their adhesion to various *E. coli* strains to result in substantial cell agglutination,[12] exhibited no interactions with the spores in the presence or absence of Ca^{2+}. It seems that specific arrangements of the carbohydrate ligands are probably required for multivalent interactions with the spore surface, for which the pseudo one-dimensional structure of SWNTs is apparently a viable scaffold.

The observed aggregation and CFU reduction of *B. anthracis* spores are attributed to divalent cation-mediated multivalent carbohydrate−carbohydrate interactions,[13−16] specifically the carbohydrates tethered to SWNTs with those expressed on the spore surface. The role of Ca^{2+} mediation was confirmed by the experiment in which the aggregation of *B. anthracis* spores was reversed with the addition of EDTA, a strong chelating agent of Ca^{2+}. While *B. anthracis* spores were aggregated substantially by Man-SWNT with Ca^{2+} under the specific experimental conditions described above, the further addition of an aqueous solution of EDTA sodium salt (100 μL, 20 mM) with gentle shaking resulted in an immediate and complete redispersion of both the spores and nanotubes (no aggregates at all). The observation is consistent with what is known in the literature on similar carbohydrate−carbohydrate interactions.[14−16]

In summary, the carbon nanotubes represent a unique displaying scaffold for multivalent monosaccharide ligands that bind effectively with *B. anthracis* spores in the presence of a divalent cation. The binding results in substantial aggregation of the spores and corresponding CFU reduction. While an optimization of the binding and the elucidation of related mechanistic details remain to be investigated, the aggregation of spores may be exploited for antibioterrorism applications. For example, aggregated *B. anthracis* spores are considerably less potent for aerosol inhalation,[17] which is considered as the most lethal transmission pathway for anthrax infection.

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