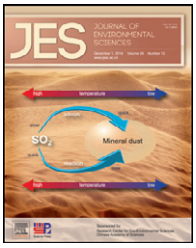
JOURNAL OF ENVIRONMENTAL SCIENCES 26 (2014) 2526–2534

Available online at www.sciencedirect.com

ScienceDirect

www.journals.elsevier.com/journal-of-environmental-sciences



Inhibitory effects of nisin-coated multi-walled carbon nanotube sheet on biofilm formation from *Bacillus anthracis* spores

Xiuli Dong[1], *Eric McCoy*[1], *Mei Zhang*[2], *Liju Yang*[1,*]

1. *Department of Pharmaceutical Sciences, Biomanufacturing Research Institute and Technology Enterprise (BRITE), North Carolina Central University, Durham, NC, USA.* E-mail: xdong1@nccu.edu

2. *Department of Industrial & Manufacturing Engineering, Florida State University, Tallahassee, FL, USA*

ARTICLE INFO

Article history:
Received 6 January 2014
Revised 1 April 2014
Accepted 4 April 2014
Available online 4 November 2014

Keywords:
Multi-walled carbon nanotubes (MWCNTs)
Biofilm
Bacillus anthracis
Nisin

ABSTRACT

Multi-walled carbon nanotube (MWCNT) sheet was fabricated from a drawable MWCNT forest and then deposited on poly(methyl methacrylate) film. The film was further coated with a natural antimicrobial peptide nisin. We studied the effects of nisin coating on the attachment of *Bacillus anthracis* spores, the germination of attached spores, and the subsequent biofilm formation from attached spores. It was found that the strong adsorptivity and the super hydrophobicity of MWCNTs provided an ideal platform for nisin coating. Nisin coating on MWCNT sheets decreased surface hydrophobicity, reduced spore attachment, and reduced the germination of attached spores by 3.5 fold, and further inhibited the subsequent biofilm formation by 94.6% compared to that on uncoated MWCNT sheet. Nisin also changed the morphology of vegetative cells in the formed biofilm. The results of this study demonstrated that the anti-adhesion and antimicrobial effect of nisin in combination with the physical properties of carbon nanotubes had the potential in producing effective anti-biofilm formation surfaces.

Introduction

Biofilms are multicellular structures attached to a solid or liquid surface and embedded in a self-produced matrix of extracellular materials (Costerton et al., 1999; Høiby et al., 2010). They are predominately formed in water/surface interfaces common to nearly all ecosystems. The matrix provides cohesion to the bacterial community and acts as a shield to protect bacteria within the biofilm. As a result, biofilms have significantly higher resistance to desiccation, cleaning procedures, and antimicrobial substances (antibiotics) and even the host immune system than their single cell planktonic counterparts (Donlan, 2000, 2002; Donlan and Costerton, 2002; Leid et al., 2002, 2005). Such resistance makes biofilm a challenge in environment, human health, and industry processes.

Many bacterial species have been identified in the biofilm mode of life in a variety of settings (López et al., 2006). *Bacillus anthracis* is one of such bacteria that have been identified to form biofilms under static and laminar-shear conditions (Lee et al., 2007). *B. anthracis* is the causative pathogen of the life-threatening disease-anthrax in animals and humans. *B. anthracis* spores, the dormant form of cells, are especially problematic because they are highly resistant to extreme temperatures and harsh chemicals. They have been identified as potential bioterrorism agents (National Response Team, 2013) and an actual water threat (U.S. Army Center, 2008) in water and other environment settings, and raise a concern for homeland security and environmental safety. *B. anthracis* spores can survive from 2 to 18 years in pond water and 20 months in seawater or distilled water (Sinclair et al., 2008). Although the major risk of infection by *B. anthracis* spores in water

* *Corresponding author*. E-mail: lyang@nccu.edu (L. Yang).

http://dx.doi.org/10.1016/j.jes.2014.04.010

systems would likely through the ingestion of water contaminated by spores, the contact between a break in skin with spore contaminated water used for bathing, showering, or recreational purposes might also pose a cutaneous risk. The 2001 bioterrorism attack using *B. anthracis* spores as the infectious agent has brought acute public attention and triggered extensive studies on pathogenicity and inactivation of *B. anthracis* spores (Dauphin et al., 2008; Levy et al., 2012). The decontamination of *B. anthracis* spores in drinking water systems and the inhibition of biofilms from *B. anthracis* were among the research areas. For example, Morrow et al. (2008) studied the association and decontamination of *B. anthracis* spores on biofilm-conditioned pipe material surfaces in a simulated drinking water system. Raber and Burklund (2010) studied the decontamination options for *B. anthracis* in contaminated drinking water using a spore surrogate. The use of chlorine dioxide as a disinfectant for decontamination of *Bacillus* spores in drinking-water biofilms using *B. anthracis'* surrogate, *Bacillus globigii* spores, has been reported (Hosni et al., 2011).

Carbon nanotubes (CNTs) have emerged as a novel and promising class of nanomaterials for a wide range of applications (Neupane and Li, 2011; Ajayan et al., 1999; Lam et al., 2006), due to their unique mechanical, electrical, optical, and thermal properties. Most relevant to this study is that CNTs have been identified as good candidates for filtration devices, surface coatings or composite materials for various applications. For example, CNTs have been used to build highly efficient filters for removing bacterial cells and viruses, in the attempt to improve the safety of drinking water (Guan and Yao, 2010; Vecitis et al., 2011). In this respect, although CNTs have demonstrated their strong antimicrobial activity in suspensions, CNT surfaces or CNT-coated surfaces were not suitable to counteract bacterial adhesion and biofilm formation (Pantanella et al., 2011). Our recent study also found that multi-walled CNT (MWCNT)-coated surface exhibited high adsorption capacity to *B. anthracis* spores (Dong et al., 2012). Such high adsorption capacity to bacterial cells or spores raises potential risk for biofilm formation on CNT devices or CNT-coated surfaces, which could be a disadvantage for practical applications of CNTs as filters or surface-coating materials in water systems or other environmental systems.

A possible strategy to achieve desired properties in CNT-based materials/devices is to build CNT composite materials or chemical modification of CNTs, which can combine the unique properties of CNTs with the useful properties of other composites. This strategy has been successfully used to achieve antimicrobial functions on CNT-based nanocomposite films. For example, the deposited film of nanocomposite MWCNT-epsilon-polylysine exhibited enhanced antimicrobial activities (Zhou and Qi, 2010). Conjugates of MWCNTs and protoporphyrin IX on nitrocellulose filter membranes effectively inhibited 80%–85% of *Staphylococcus aureus* growth upon 1 hr irradiation with visible light (Banerjee et al., 2010). Functionalized MWCNT-ZnO film inactivated 100% of *Escherichia coli* bacteria after 10 min UV–visible light irradiation (Akhavan et al., 2011).

Considering the potential applications of CNT-based filters or CNT-coated surfaces in water systems or other environmental settings, combining CNTs with a non-toxic natural antimicrobial reagent can be a useful approach to prevent the adhesion of bacterial cells or spores and inhibit further biofilm formation on CNT-based devices without any additional treatments or posing any toxicity risk. Nisin is a non-toxic polycyclic peptide with 34 amino acid residues produced by certain strains of food-grade lactic acid bacterium during fermentation. It has been approved and used as a natural, toxicologically safe food preservative (Reunanen and Saris, 2004) in more than 50 countries including the US, the European Union, Brazil, and China. Nisin exhibits a broad spectrum of inhibitory activity against Gram-positive bacteria including their spores (Suganthi et al., 2012) with high efficiency at nanomolar levels (Willey and van der Donk, 2007; Suganthi et al., 2012). It kills Gram-positive bacteria through a multi-step process that destabilizes the phospholipid bilayer of the cell and creates transient pores (Y. Tai et al., 2008). Nisin can be adsorbed on or covalently linked to surface substrates to produce antimicrobial surfaces. Various surfaces, such as silicon (Daeschel et al., 1992), polyethylene film (An et al., 2000), rubber, and stainless steel (Guerra et al., 2005), have been used to adsorb nisin. The adsorbed or covalently linked nisin on surfaces maintains its activity to kill cells that have adhered on those surfaces (Bower et al., 2002; Storia et al., 2013; Qi et al., 2011). Nisin can also be incorporated into polymer-based films and effectively reduced biofilm formation of *Staphylococcus epidermidis* and *Listeria monocytogenes* on the films (Nostro et al., 2010; Kim et al., 2002). In food industry, nisin has been incorporated into packaging materials for antimicrobial purposes (Han, 2000; Kim et al., 2002).

In this study, we developed MWCNT sheets on poly(methyl methacrylate) (PMMA) films, and coated them with nisin. Optimization of nisin coating and characterization of the nisin-coated MWCNT surface were performed. The attachment of *B. anthracis* spores, the germination of attached spores, and biofilm formation from the attached spores on nisin-coated MWCNT surfaces were evaluated. Bare PMMA surface and nisin-coated PMMA surface were used as comparison to evaluate the efficiency of nisin coating and nisin activity on MWCNT surfaces in anti-adhesion and anti-biofilm formation from *B. anthracis* spores.

1. Materials and methods

1.1. *B. anthracis* spore preparation

For safety consideration, we used *B. anthracis* Sterne 34F2 spores in this study. It is an attenuated strain, but still requires careful handling in a biosafety level 2 laboratory. The Sterne strain has been used worldwide by research laboratories involved in research on inactivation of *B. anthracis* and other aspects (Niebuhr and Dickson, 2003; Passalacqua et al., 2007; Rooijakkers et al., 2010). *B. anthracis* Sterne 34F2 was purchased from Colorado Serum Company (Denver, Colorado, USA). *B. anthracis* spores were prepared by following a previously reported procedure (Pantanella et al., 2011). One colony of *B. anthracis* Sterne 34F2 from a Luria–Bertani (LB) agar plate was used to inoculate 15 mL Difco Sporulation Medium (DSM, Difco Laboratories Inc., Detroit, Michigan, USA). The inocula was incubated in the Excella E25 shaker incubator (New Brunswick Scientific, Enfield, Connecticut, USA) at 225 r/min under 37°C until OD_{600} value reached ~0.45, followed by adding 65 mL of pre-warmed (37°C) fresh DSM, and then incubated until >95% of culture was free spores. The culture was heated at 80°C for 20 min to kill the vegetative cells. Then, the culture was centrifuged at 10,000 ×*g* for 10 min, and the pellet was washed with cold deionized (DI)-H_2O. The wash step was repeated eight times to remove cell debris. The purified spores were resuspended in cold DI-H_2O and stored at 4°C for further experimental use. The spores were washed with cold water twice immediately before use in each experiment.

1.2. MWCNT surface preparation

MWCNT sheet on poly(methyl methacrylate) (PMMA) film (defined as PMMA-MWCNT surface) was prepared as previously reported (Zhang et al., 2005). In brief, a freestanding MWCNT forest on a silicon wafer was synthesized by chemical vapor deposition using iron as catalyst. Freestanding MWCNT sheets were fabricated by solid-state draw from the drawable MWCNT