International Biodeterioration & Biodegradation 123 (2017) 228–235

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

Top capping of nanosilver-loaded titania nanotubes with norspermidine-incorporated polymer for sustained anti-biofilm effects

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ARTICLE INFO

Article history:
Received 22 June 2017
Received in revised form
7 July 2017
Accepted 7 July 2017
Available online 14 July 2017

Keywords:
Biofilm inhibition
Nanosilver
Norspermidine
TNTs
Coating

ABSTRACT

Detrimental biofilms in water and wastewater treatment systems have become a great concern. Norspermidine is a potent inhibitor for biofilm growth through a non-biocidal mechanism. Nanosilver is a widely used and efficient inorganic biocide. In this study, using a titania nanotube (TNTs) array as the loading carrier of nanosilver and a norspermidine-incorporated polymer poly(lactic-co-glycolic acid) (PLGA) as the top cap of TNTs, a composite anti-biofilm coating (TNTs-Ag-PLGA/norspermidine) with multiple effects was fabricated, and its anti-biofilm efficiency against biofilms by wastewater mixed culture was investigated. Results showed that the addition of a PLGA/norspermidine cap to the top of TNTs greatly retarded silver ion release and extended antimicrobial validity. The TNTs-Ag-PLGA/norspermidine coating showed a biofilm inhibition of 48.42 ± 2.71% after 16 days of leaching, much higher than that without the PLGA/norspermidine cap (4.10 ± 3.32%). Confocal laser scanning microscopy (CLSM) showed that the biofilm attached to the surface of the TNTs-Ag-PLGA/norspermidine showed a much lower exopolysaccharide/exoprotein ratio (0.44) compared to the control (0.94), indicating that norspermidine incorporated into the PLGA layer could act on biofilm bacteria by reducing exopolysaccharides and destroying the EPS matrix. The TNTs-Ag-PLGA/norspermidine coating inhibited biofilm formation both through a killing mechanism by silver and a non-killing mechanism of norspermidine biofilm disassembly. The combination of nanosilver-loaded TNTs and a norspermidine-incorporated PLGA cap provides a novel and effective strategy to mitigate biofilm formation by wastewater mixed cultures and has potential application in water and wastewater treatment systems.

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1. Introduction

Biofilms are surface-associated microorganisms embedded in an extracellular matrix of extracellular polymeric substances (EPS) (Si and Quan, 2017). Adverse biofilms may result in numerous problems for water and wastewater treatment systems, including water pollution, membrane biofouling, pipe blockage and metal erosion (Wu et al., 2015; Lyon et al., 2008). Biofouling and biofilm control remains a challenge for environmental researchers.

One strategy to combat biofilm formation on surfaces is to load anti-microbial agents into surface materials to construct an anti-biofilm coating. A variety of metal nanoparticles have been used as antimicrobial agents, among which silver nanoparticles have been proven to be bactericidal and efficient (Morones et al., 2005; Rai et al., 2009). As the anti-biofilm activity of nanosilver loaded coatings greatly depends on the amount of silver ion (Ag^+) released from nanosilver, these coatings may lose efficiency when Ag^+ is exhausted (Liu et al., 2012). In addition, Ag^+ is less efficient in killing biofilm bacteria than planktonic bacteria. Therefore, the antimicrobial activity of silver nanoparticle-loaded coatings will deteriorate with the colonization of biofilms on surfaces. The resistance and persistence of biofilms requires establishment of composite antimicrobial coatings with multiple chemical and biological agents.

Recently, some small molecules have been found to disperse and disassemble biofilms through disruption of EPS production or

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http://dx.doi.org/10.1016/j.ibiod.2017.07.003
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destruction of the biofilm matrix. Norspermidine is a potent small molecule to disassemble biofilms and inhibit biofilm formed by some pure strains (Ramón-Peréz et al., 2015). Norspermidine coupled to D-tyrosine effectively promoted the dispersal of an old-aged multi-species biofilms (Si et al., 2014). The co-application of norspermidine and Ag^+ in solution increased biofilm removal and disinfection efficiency compared to Ag^+ treatment alone, due to increased penetration of Ag^+ into biofilms (Wu et al., 2016). Norspermidine hosting a poly(acrylicacid) (PAA)/polyethylenimine (PEI) polymer coating was further established and showed good inhibition of pure-strains and multi-species biofilm formation (Si et al., 2015). As norspermidine and nanosilver work on biofilms by different mechanisms, their combination in a composite anti-biofilm coating could provide added benefits, but has not yet been studied.

Titania-based materials have been widely studied as coating materials due to their excellent corrosion resistance and high capability for adhesion to different substrates (Liu et al., 2004). Highly ordered titania nanotubes (TNTs) fabricated on Ti surfaces can serve as a good carrier of antibacterial agents (Aninwene et al., 2008; Popat et al., 2007). Controlled release of antibacterial agents from TNTs is necessary to maintain long-term anti-biofilm efficiency (Song et al., 2009; Xiao et al., 2009). Previous studies showed that polymer capping of TNTs could extend the release of antibiotics (Aw et al., 2011; Gulati et al., 2012).

In this study, using TNTs as the loading carriers of nanosilver and norspermidine-incorporated polymer as the top cap, a novel composite anti-biofilm coating was established. The biodegradable polymer, poly(lactic-co-glycolic acid) (PLGA) was used not only as the carrier of norspermidine but also as the cap for TNTs to control silver release. PLGA was chosen here because of its good molecular permeability and biodegradability and because it has been widely used as a promising cap or carrier for drug delivery (Jia and Kerr, 2013). The anti-biofilm ability of the established composite coating against wastewater biofilms from a mixed culture was investigated. The study will establish a new strategy to control the corrosion of water purification and wastewater treatment systems, such as water pipes and heat exchange equipment.

2. Materials and methods

2.1. Specimen preparation

Titanium discs (99.8% purity) with a thickness of 0.25 mm were used to fabricate TNTs. TNTs were fabricated through an anodization process using the method described by Mor et al. (2005) and Varghese et al. (2003) with some modifications. Anodization was conducted in an aqueous electrolyte solution of 1.0 vol % hydrofluoric acid at a constant voltage of 40 V for 40 min. Graphite foil served as the cathode. The titanium samples were cleaned using deionized water after anodization and sintered at 500 °C to form TNTs.

After TNTs fabrication, nanosilver was loaded into the TNTs through chemical reduction according to the following procedure. The TNTs samples were first soaked in a 40 g/L $AgNO_3$ solution (pH 7) with stirring for 30 min. Equimolar sodium borohydride was added to the reaction medium as the reducing agent and the pH was adjusted to 10 with NH_4OH. The reduction proceeded for 30 min with stirring. Finally, nanosilver-loaded TNTs (TNTs-Ag) were obtained after rinsing with deionized water and drying under room temperature. The TNTs-Ag plates were covered by containing a PLGA polymer film encapsulating norspermidine according to the following procedure. A mixed solution of PLGA (1% (w/v) in chloroform) and norspermidine was prepared. TNTs-Ag samples were quickly dipped into the mixed PLGA-norspermidine solution, removed and dried in an oven at 70 °C for 10 min. The dip-coating process was repeated 5 times to obtain TNTs-Ag-PLGA/norspermidine composite coatings. TNTs-Ag with only the PLGA cap (TNTs-Ag-PLGA) was also fabricated by dipping the TNTs-Ag samples in a PLGA solution without norspermidine. The surface morphologies of the prepared TNTs before and after polymer coating were observed using a scanning electron microscope (SEM, Hitachi X650, Japan).

2.2. Silver release property of the different Ti coatings

The amounts of Ag^+ released from the TNTs-Ag, TNTs-Ag-PLGA and TNTs-Ag-PLGA/norspermidine samples were measured in phosphate buffered saline (PBS). Each sample was carried out in triplicate tests. The various Ti coating samples were immersed in 5 mL PBS at room temperature without agitation in the dark for one day, removed and immersed in 5 mL fresh PBS. This process was repeated for 16 days. The amount of Ag^+ released into the solution during the leaching experiments was measured using inductively coupled plasma atomic emission spectrometry (ICP-AES, Spectro Arcos Eop, German).

2.3. The anti-biofilm property of the Ti coatings

The anti-biofilm property of the various Ti coating samples (Ti, TNTs-Ag, TNTs-Ag-PLGA and TNTs-Ag-PLGA/norspermidine) was tested against a mixed culture. The source of the mixed culture was activated sludge from a municipal wastewater treatment plant (Beijing, China). The mixed culture mainly consisted of *Comamonas*, *Nakamurella*, *Enterobacteriaceae*, *Clostridium*, *Sphingomonas*, *Pseudomonas*, *Azospira*, *Stenotrophomonas* and *Ferribacterium*, which was characterized using a MiSeq system (Shanghai, China). Nucleotide sequences were deposited in GenBank with the accession numbers from KR815806 to KR815814.

The anti-biofilm experiment was conducted as follows. The prepared Ti coating samples were placed at sterilized Petri dishes containing 30 mL mixed-culture solution suspension and statically incubated at 30 °C for 1 or 6 days to form biofilms. After incubation, biofilms formed on these substrates were observed under a SEM. For SEM analysis, the prepared Ti sample was mounted on the aluminum stub with double-sided adhesive tape and then sputter-coated with gold for 60 s at 20 mA. Once coated, the Ti disc was transferred to a SEM and observed at an accelerating voltage of 20 kV. Moreover, biofilm biomass was measured via crystal violet staining method (Lemos et al., 2014). Briefly, biofilms were washed three times with PBS to remove loosely attached bacteria, and then stained with 500 μL per well of 0.1% crystal violet for 30 min. Finally, crystal violet stained on the biofilms was washed off via immersing in an absolute ethanol solution for 30 min. The biofilm biomass was calculated by measuring the optical density at 600 nm (OD600) using a microplate reader (Tecan Infinite M200, Switzerland). Relative biomass of treated groups was expressed as the percentage of control groups that were assumed to be 100%. In addition, biofilm formation on these Ti coatings after 16-day leaching in PBS was investigated to determine their long-term performance.

2.4. Microscopic observations for the distribution of live/dead cells and EPS in biofilms

To investigate the anti-biofilm properties of the Ti coatings incubated for 1 day, biofilms were stained with fluorescence labeled probes and observed under a confocal laser scanning microscope (CLSM, Carl Zeiss LSM 510, Germany) to view the distribution of total cells (stained by Syto 63), dead cells (stained by

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