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Characterisation and cooperative antimicrobial properties of chitosan/nano-ZnO composite nanofibrous membranes

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ABSTRACT

Chitosan was combined with nano-ZnO to increase its antimicrobial activity, using polyvinyl alcohol as a support, and then were electronspun to form composite nanofibres. Through SEM, EDX and XRD observations, chitosan was seen to be able to incorporate nano-ZnO in the composite nanofibres. *Escherichia coli,* expressing recombinant enhanced green fluorescent protein, and *Candida albicans* were used to test the antimicrobial efficacy of the newly synthesised chitosan/nano-ZnO antimicrobial composite. The CdTe quantum dots were used to rapidly detect the residual changes of *C. albicans* and determine the end point of using antimicrobial agents. Minimal minimum inhibitory concentration (MIC), post-antibiotic effect and continuous agent effect of the composite were determined. The MIC of chitosan/nano-ZnO against *C. albicans* was 160 μg/ml, close to the concentration of the treated composite with the lowest fluorescence intensity. The cell damage was observed by SEM, which indicated that nano-ZnO in the nanofibrous membranes played a cooperative role in the antimicrobial process of chitosan.

1. Introduction

Chitosan is a linear polysaccharide composed of polymeric 1→4-linked 2-amino-2-deoxy-β-D-glucose, which is prepared from chitin and commonly found in the shells and exoskeletons of crustaceans and cell wall of fungi (Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003). Chitosan has been examined and used in many applications, such as packaging materials, drug delivery carriers, surgical sutures, bone healing materials, and biological dressings. New research focuses on hybrid films comprising alternating layers of chitosan (Yao, Fang, Tan, Wu, & Yu, 2010). The biodegradable chitosan itself provides bacteriostatic and fungistatic activities (Tomihata & Ikada, 1997; Muzzarelli et al., 1990; Wang, 1992). Due to these reasons, chitosan has been one of the important biomaterials for food and biomedicine industries in recent years.

With growth in world population and the spread of disease, the number of antibiotic resistant microorganisms is rising, along with the occurrence of infections from these microorganisms. Now the overuse, and misuse, of antibiotics have helped fuel the rise of drug-resistant "superbug" infections, like methicillin-resistant *Staphyloccus aureus* (MRSA). Furthermore, Gram-negative *Enterobacteriaceae* with resistance to carbapenem, conferred by New Delhi metallo-β-lactamase 1 (NDM-1), has been a major potential global-health problem (Kumarasamy et al., 2010). Therefore, people are seeking for other alternative compounds for developing resistance conventional antibiotic therapies. Inorganic materials, such as metal and metal oxides, have attracted particular interest for their special antimicrobial properties, as they are not only stable under harsh process conditions but also generally regarded as safe materials to human beings and animals (Fu et al., 2005; Makhluf et al., 2005). The use of nanoparticles of zinc oxide (ZnO) has been considered as a viable solution to stop infectious diseases due to the good antimicrobial properties of these nanoparticles (Stoimenov, Klinger, Marchin, & Klabunde, 2002). The nanoparticles of metal and metal oxides have little or no antibiotic resistance. However, there is only few product of nano-silver for topical dermatologic use (Nel, Xia, Madler, & Li, 2006; Sondi & Salopek-Sondi, 2004), and so far, there have been few papers on the co-utilisation of nanomaterials to strengthen antimicrobial activities.

Rapid and sensitive detection of total bacteria or fungi counts is extremely important in food safety, biotechnology, and medical diagnosis. Due to slow detection speed and complicated procedures, plate count, as a conventional method, could not meet the requirements of fast and efficient microbe detection any more. Recently, Quantum dots (QDs), as novel fluorescent markers, have been applied broadly to biological imaging and analysis. Unlike

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conventional fluorescent dyes, QDs have long-term photostability, high quantum yield, broad absorption spectra and narrow, symmetric emission (Duong & Rhee, 2007). Hence, the sensitivity can be improved, and the optical systems can be simplified if the conventional dyes were replaced by QD as a fluorescent labelling (Kloepfer, Mielke, & Nadeau, 2005). These advantages suggested that QDs bio-conjugates have greater potential in microbe monitoring, such as detection of total bacterial count and pathogens (Kloepfer et al., 2005; Tully, Hearty, Leonard & O'kennedy, 2006). For fungal infection during food processing and storage, *Candida albicans* (*C. albicans*) is an important pathogen. Up to now, no one is able to produce the rapid synchronous monitoring for *C. albicans* during antimicrobial process of nanomaterials.

In this paper, a novel method for fabrication of chitosan/nano-ZnO composite nanofibrous membranes (NFMs) has been proposed, and polyvinyl alcohol (PVA) was used as an appropriate carrier (Bandyopadhyay, Sarkar, & Bhownick, 2005; Huang et al., 2009). The bactericidal effect of the composite was studied using recombinant *Escherichia coli* (*E. coli*), that expressed enhanced green fluorescent protein (EGFP). Meanwhile, a rapid, simple and highly sensitive detection method was developed to evaluate the antimicrobial activity of the resultant nanofibrous membrane against the foodborne pathogen of *C. albicans*.

2. Materials and methods

2.1. Chemicals

Chitosan, with a molecular weight of 20 kDa and deacetylation degree of 90%, was purchased from Zhejiang Tianbao Technology Development Co., Ltd. (China), Nano-ZnO (30 nm) was prepared in our lab (Tongji University, China), and PVA (Polyvinyl alcohol) powder with a polymerisation degree of 1750 ± 50 was purchased from Sinopharm Group Chemical Reagent Co. Ltd. (Shanghai, China). 1-Ethyl-3-(3-dimethyllaminopropyl)-carbodiimide (EDC, Mychems Co, Shanghai, China) was used as an activator. Chloromethyl-2′, 70′-dichlorofluorescin diacetate (DCFH-DA, 95%) was purchased from Sigma–Aldrich. All reagents were of analytical grade and were used without further purification.

2.2. Types of strains

Strains of recombinant *E. coli* and EGFP gene plasmid vector used for bioluminescence experiments were donated by the China University of Technology. The recombinant EGFP-expressing *E. coli* (BL 21) was generated by cloning the EGFP gene into an ampicillin-resistant pUC-derived plasmid vector. The microbial strain of *C. albicans* ATCC 10231 was obtained from the University of Tongji culture collection.

2.3. Chitosan/nano-ZnO composite nanofibre characteristic measurements

Nano-ZnO (20 mg) was added to 10 ml solutions containing 4% chitosan and 6% PVA, and then prepared for nanofibres by electrospining. Chitosan NFMs were prepared with 4% chitosan and 6% PVA, and PVA NFMs were electrospun using 6% PVA solution. The morphologies of the electrospun complex fibres were observed by scanning electron microscopy (SEM) (XL-30E, Philips, Japan). EDX elemental composition analyser and X-ray diffraction (XRD, D/max2550VB3+/PC, Rigaku International Corporation) were used to characterise nanofibre structure. X-ray microdiffraction is equipped with a GADDS two-dimensional (2D) detector which captures the data for a fixed range of 2θ (the diffracted angle, 2θ, is defined between the incident beam and the detector angle) and ω (the incident angle, ω, is defined between the X-ray source and the sample), at once. The 2θ ranges of interests for scan were from 10 to 55°.

2.4. Synthesis of CdTe QDs and preparation for CdTe-C. albicans

CdTe QDs were prepared according to the procedure described by Li, Qian, and Ren (2005). Water-soluble CdTe quantum dots coupled with *C. albicans* were prepared through a crosslinking reaction (Xue, Pan, Xie, Wang, & Zhang, 2009). The 300 μl of 1.0 mg/ml CdTe QDs and 100 μl of 0.1 mg/ml EDC were added into 1 ml of 10^7 cfu/ml cells, and then reacted together for 30 min. The treated cells were purified using ultra-filtration membrane (0.22 μm) to wash away broken the cell debris and the excess CdTe QDs. Further, fluorescence intensities of *C. albicans* in water-soluble CdTe QD were obtained using a F-2500 fluorescence spectrophotometer (Hitachi High-Technologies corporation, Tokyo, Japan). Fluorescence images were acquired using fluorescence microscope (BX51, Olympus, Japan) with the excitation wavelength of 400 nm and emission wavelength of 550 nm.

2.5. Antimicrobial efficiency measurements

To determine the minimum inhibitory concentration (MIC) of the composite NFMs, bacteria were cultured in liquid lysogeny broth (LB) medium at 37 °C, and fungi were cultured in fluid sabouraud (SAB) medium at 30 °C. Then each strain's cells were harvested by centrifugation (2000*g*, 10 min), washed and resuspended in PBS. The resuspended cells were inoculated, at numbers of 10^7 cfu/ml, with various concentrations of composite and grown overnight at 37 °C (or 30 °C). The minimum concentration (MIC) of the composite, which gave cultures that did not become turbid and no visible strain was found on LB-agar (or SAB-agar) plates after incubation for 24 h at 37 °C (or for 48 h at 30 °C), was taken to be the MIC. The morphology of the treated *C. albicans* was observed using a scanning electron microscopy (SEM) (XL-30E, Philips, Japan), at an accelerated voltage of 10 or 15 kV. Control experiments were performed with sterile PVA fibrous membrane only.

Post-antibiotic effect (PAE) and continuous agent effect (CAE) were determined according to Ferrara's method (Odenholt-Tornqvist, Löwdin, & Carsb, 1992; Ferrara, Dos, Cimbro, & Grassi, 1996). Each organism was tested at 0.0 × MIC (positive control), 0.5 × MIC, 1.0 × MIC, and 1.5 × MIC of each antimicrobial agent, based on individual MICs. Each assay was performed in 30 ml of culture medium, with an inoculum size of approximately 10^7 cfu/ml. For PAE assay, each isolate was exposed to the respective concentrations for 1 h, centrifuged, washed twice in 10 ml of PBS, and resuspended in 30 ml of fresh medium (initial time = 0) for culture. At different times, a 0.5 ml aliquot was removed from each assay bottle, serial ten-fold dilutions were made in fresh medium, and 0.1 ml aliquots were plated, in triplicate, on agar. For CAE assay, each isolate was exposed to the respective concentrations for 1 h (initial time = 0), and then continuously cultured. At different times, a 0.5 ml aliquot was removed from each assay bottle, serial ten-fold dilutions were made in PBS, and 0.1 ml aliquots were plated in triplicate on agar. All agar plates were incubated at 37 °C for 24 h (or 30 °C for 48 h) and colony counts determined.

The PAE (or CAE) was defined, as the time difference (in hours) for the treated organisms to increase in number by 1 log10(T), minus the same determination for positive control of the same test organism (C). As the physical manipulation of the isolates during the assay probably influences the growth recovery time of the isolates, a PAE (or CAE) value of >1 h was arbitrarily considered a positive result.

To study the bactericidal properties of the composite, EGFP recombinant *E. coli* (BL 21) cells were grown overnight in 300 ml of LB ampicillin medium. The cells were separated by centrifugation,