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A proposed mechanism of action of textile/Al_2O_3–TiO_2 bimetal oxide nanocomposite as an antimicrobial agent

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ABSTRACT

The textile/Al_2O_3–TiO_2 bimetal oxide nanocomposite has been developed as an antimicrobial agent, but its antimicrobial mechanism has still not been clarified. The Al_2O_3–TiO_2 bimetal oxide nanoparticle has been reported as a radical scavenger. This study focuses on investigating the antimicrobial mechanism of the textile/Al_2O_3–TiO_2 bimetal oxide nanocomposite against *Escherichia coli* and their interaction with cell envelope biomolecules. L-α-Phosphatidyl ethanolamine (PE) is used as a model of bacteria to investigate the antimicrobial mechanism of this nanocomposite. The antimicrobial activity of the textile/Al_2O_3–TiO_2 bimetal oxide nanocomposite was investigated by using attenuated total reflectance/Fourier transform infrared (ATR-FTIR) and UV–Vis, while the toxicity of this nanocomposite was also examined through tissue culture test against a fibroblast skin cell. The ATR-FTIR used was able to confirm the destroyed cell envelope of the bacteria. The amounts of reactive oxygen species (ROS) were analyzed by UV–Vis spectroscopy and the toxicity of this nanocomposite was also examined by reacting tissue culture against the fibroblast skin cell. Overall, the antimicrobial mechanism of this textile nanocomposite was first by the attachment of this nanocomposite through the attachment of this nanoparticle to the surface of PE (as the model of bacteria) by hydrogen binding, and then nanoparticles can destroy the cell wall of bacteria through oxidation reaction by the produced ROS. Finally, these nanoparticles scavenged the ROS free radical. Therefore, the attached nanoparticles attached on textile can kill bacteria without any ROS free radical remaining in human body. These results suggest that the antimicrobial mechanism of this nanocomposite is mostly different due to the scavenger ability of this nanocomposite and its lower toxicity.

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KEYWORDS
Antimicrobial mechanism; textile/Al_2O_3–TiO_2 nanocomposite; radical scavenger

Introduction

The reappearance of infectious diseases and the continuous development of antibiotic resistance among a variety of bacteria caused a serious problem to public health worldwide (Adams, Lyon, & Alvarez, 2006). Various new classes of b-lactamases have appeared, due to the broad use of b-lactam antibiotics in the clinical practice over the last several decades. The resistance to third-generation cephalosporin is now observed worldwide in all species of Enterobacteriaceae based on extended spectrum of b lactamases (ESBLs) especially in *Escherichia coli* (Bradford, 2001; Branger et al., 2005). Due to the ability of bacteria to cause resistance to almost all antibiotics, the antimicrobial therapy, morbidity, and mortality related with the bacterial infections remain high (Kolář, Urbánek, & Látal, 2001). Therefore new strategies are needed to develop and investigate next-generation antimicrobial agents or drugs to control the infection caused by bacteria. In this scenario, nano-sized materials have appeared as new antimicrobial agents due to their high surface area to volume ratio and unique physical and chemical properties (Kim, Kim, & Cremer, 2001; Morones et al., 2005). Commonly, the harmful effects of such microorganisms that increase the undesirable effects of textile wound dressing can be prevented by using antimicrobial agents. Previous researchers have reported that metal oxide nanoparticle has toxicity for mammalian cell lines (Brunner et al., 2006), crustaceans (Heinlaan, Ivask, Blinova, Dubourguier, & Kahru, 2008), and bacteria (Adams et al., 2006). The nanosized metal oxides with a greater surface area than their bulk types show higher performance in their applications (Jiang, Mashayekhi, & Xing, 2009). Generally, the cell size of bacteria is in the micrometer range, while the pores of its outer cellular membranes are in the nanometer range. Therefore, the nanoparticles will have a unique ability of crossing the cell membrane when the size of nanoparticles is smaller than bacterial pores.

The antibacterial property of different nanoparticles depends on their size, stability, and concentration added to the growth medium, because this provides greater retention time for interaction between bacteria and nanoparticles. Due to the wide range of biomedical applications of

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nanoparticles, for example in drug delivery, wound healing and imaging, the use of nanosized particles in clinical and experimental settings has increased. The nontoxicity and antimicrobial ability are two important key factors for biomedical applications like wound healing. In this setting, it is broadly accepted that cytotoxicity to animal or human cells depends on some parameters such as mechanism of antimicrobial action (Carré et al., 2014).

However, previous researcher reported that the cytotoxicity of some metal and metal oxide nanoparticles such as ZnO, TiO_2, and Ag depends to the oxidative stress. This toxicity is dependent on the generation of reactive oxygen species (ROS) free radicals (Azam et al., 2012; Cioffi & Rai, 2012; Haghighi, Mohammadi, Mohammadi, Hosseinkhani, & Shipour, 2013; Iavicoli, Fontana, Leso, & Bergamaschi, 2013; Liu, Zhang, Fang, & Rong, 2014; Parham et al., 2016; Ravishankar & Jamuna Bai, 2011; Roy, Parveen, Koppalkar, & Prasad, 2010; Yun, Kim, Choi, & Lee, 2013). Previous researchers also reported that the toxicity of Al_2O_3 and Al_2O_3–TiO_2 bimetal oxide are not high based on its role as radical scavenger and their ability to block ROS generation (Parham et al., 2016; Sadiq, Chowdhury, Chandrasekaran, & Mukherjee, 2009). The Al_2O_3–TiO_2 bimetal oxide nanoparticles in this nanocomposite can act as radical scavenger because Al_2O_3 in this bimetal oxide nanoparticles has a α structure (Parham et al., 2016). Therefore, the mechanism of action of this bimetal oxide nanoparticles is different with other structure of Al_2O_3 nanoparticles such as γ structure (Ansari et al., 2014). Due to the scarcity of previously published reports on chemical and physical properties of metal and bimetal nanoparticles, there is few information on the antimicrobial or antibacterial properties of Al_2O_3–TiO_2 bimetal oxide nanoparticles and their textile nanocomposite, especially the mechanism of its antimicrobial action.

Besides, to control microbial infections, there is a pressing need to investigate novel strategies and to identify new antimicrobial agents from organic and inorganic materials. Therefore, in this study, the *E. coli* cell membrane biomolecules, specifically the L-α-Phosphatidylethanolamine (PE), are used as models to investigate the cellular interactions of Al_2O_3–TiO_2 bimetal oxide nanoparticles. PE is one of the major components of the outer membrane of Gram-negative (*E. coli*) and Gram-positive bacteria cells, and therefore it has been selected. Due to the absence of outer membrane, Gram-positive bacteria are more receptive to antimicrobial agents than Gram-negative bacteria such as *E. coli*. Therefore, in this study Gram-negative bacteria (*E. coli)* are used because it is more resistant to antimicrobial agents than Gram-positive bacteria. Thus, the overall goal of this study is to better understand Al_2O_3–TiO_2 bimetal oxide nanoparticles bacterial toxicity through nanoparticles-induced biomolecular changes and/or damage. The specific objectives of this study are to investigate the antimicrobial mechanism of textile/Al_2O_3–TiO_2 bimetal oxide nanocomposite by using ATR-FTIR to investigate the Al_2O_3–TiO_2 bimetal oxide nanoparticles-induced alterations/damage in surface molecules or functional groups.

Experimental

Materials

The material used in this study was textile/Al_2O_3–TiO_2 bimetal oxide nanocomposite (Parham et al., 2016). The L-α-Phosphatidyl-ethanolamine (PE) from Gram-negative bacteria *E. coli* (Sigma-Aldrich, St. Louis, MO) used for investigation the antimicrobial mechanism. The cytotoxicity test was carried out with human skin fibroblast (HSF 1184 catalogue no. 90011883, available from ECACC, Salisbury, UK), fetal bovine serum (FBS) (Sigma-Aldrich), phosphate buffered saline solution (PBS) (Sigma-Aldrich), minimum essential media (MEM) (catalogue no. 11095, Invitrogen, Carlsbad, CA), penicillin–streptomycin (PS) (Sigma-Aldrich), Hank's balanced salt solution (HBSS) (Sigma-Aldrich), trypsin/EDTA (Invitrogen), and the ^{TM}Red CMTPX dye (Sigma-Aldrich) were also used.

Methods

Investigation of antimicrobial mechanism

The textile/Al_2O_3–TiO_2 bimetal oxide nanocomposite and 5 mg of L-α-PE (the main phospholipid of bacteria's cell membrane) was added to the culture of *E. coli* at room temperature. Then, the solutions were centrifuged (40,000, 10 min). The toxicity of these solutions on human cell was analyzed with cell tissue or cell culture test. This experiment was repeated by using textile/TiO_2 nanocomposite instead of textile/Al_2O_3–TiO_2 bimetal oxide nanocomposite. This experiment was also repeated by using L-α-PE (the main phospholipid of bacteria's cell membrane), and the damaged structure of L-α-PE was analyzed by ATR-FTIR. The biomolecule samples were made by mixing 5 mg of L-α-PE with textile/Al_2O_3–TiO_2 nanocomposite in 25 mL distilled water or cell culture of *E. coli*, and the samples were shacked for 24 h. Then, a mixture of wet paste was obtained by centrifugation. After air-drying the wet paste on the top of the internal reflection element (IRE), a spectrum was obtained by collecting more than 200 scans with a spectral resolution of 2 cm^{-1} and a scan speed of 0.5 cm/s. This experiment was repeated by using textile/TiO_2 nanocomposite (Kang & Xing, 2007).

ROS analysis. For analyzing the appearance of ROS, biomolecule samples were made by mixing 5 mg of L-α-PE with textile/Al_2O_3–TiO_2 nanocomposite in 25 mL cell culture of *E. coli*. Then, the reaction solutions were centrifuged (40,000 rpm, 10 min). Then the reaction solution was analyzed by liquid UV–Vis after 5 and 14 h, at room temperature. This experiment was repeated by using textile/TiO_2 nanocomposite. Then the reaction solution was analyzed by liquid UV–Vis after 14 h, at room temperature.

Cell culture test. Based on the Freshney protocol, HSF was cultured in MEM with 1% (v/v) PS, 10% (v/v) FBS and 2 mM glutamine (Freshney, 2005). The cell size was about 12 nm. The concentrations of attached cell cultures were retained at 2–9 × 10^5 cells per mL in a humidified incubator

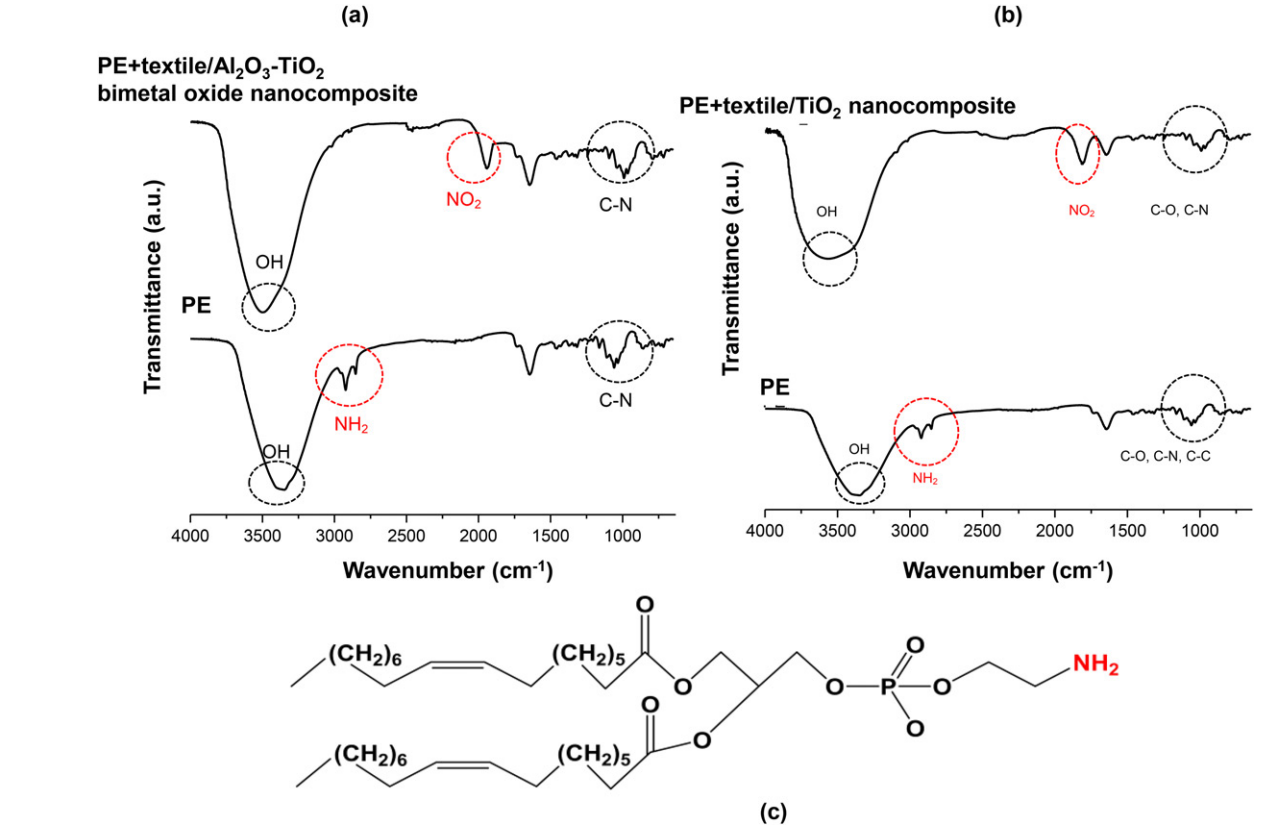

Figure 1. The ATR-FTIR spectra of PE and product reaction between (a) PE and textile/Al_2O_3–TiO_2 bimetal oxide nanocomposite and (b) PE and textile/TiO_2 nanocomposite. (c) The chemical structure of L-a-Phosphatidyl ethanolamine (PE).

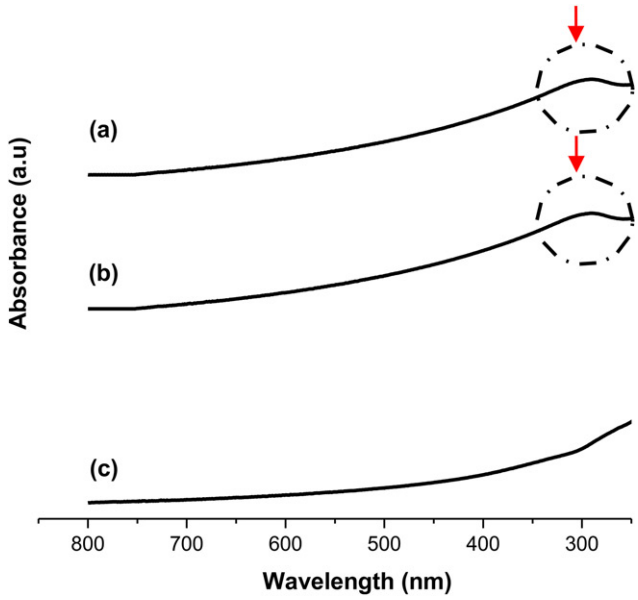

Figure 2. The UV–Vis spectra of solution reaction (a) after 14 h reaction between PE and textile/TiO_2 nanocomposite (b) after 5 h and (c) after 14 h reaction between PE and textile/Al_2O_3–TiO_2 bimetal oxide nanocomposite.

with 5% CO_2 at 37 °C. The cells reach to a confluence stage after 72 h and the cells passages were used; (P11–P15). When the cells are about 80% confluent, the cultures were washed by PBS. Then 0.25% trypsin/EDTA was used to detached the cells. To obtain cells pellets, the cells were centrifuged (at 2100 rpm for 5 min). The cells suspensions were used in 3 mL of MEM with a concentration of 5×10^5 cells per mL. Then ^{TM}Red CMTPX dye was used to stain the cell. The HSF cells in 12-well plates with or without samples were inserted into each well, 24 h before each experiment.

Characterization

The damaged structure of L-α-PE was analyzed by ATR-FTIR (Nicolet IS10). The radical scavenging ability of the samples was measured by using a Shimadzu 1800 UV–visible spectrophotometer in the range of 250–800 nm.

Results and discussion

Antimicrobial mechanism

For achieve the antimicrobial mechanism of textile/Al_2O_3–TiO_2 bimetal oxide nanocomposite, the textile/Al_2O_3–TiO_2 bimetal oxide nanocomposite was added to PE. Figure 1(a) shows the ATR-FTIR spectrum of the reaction product between the textile/Al_2O_3–TiO_2 bimetal oxide nanocomposite and PE and Figure 1(b) shows the ATR-FTIR spectrum of the reaction product between the textile/TiO_2 bimetal oxide nanocomposite and PE. Figure 1(c) shows the chemical structure of L-a-PE. The ROS amount analysis is represented in Figure 2 while Figure 3 demonstrates the toxicity of these nanocomposites which is analyzed by tissue culture test against the fibroblast human skin cell.

The sample became a solution after 5 and 14 h whereby there was reaction between PE as the model with the textile/Al_2O_3–TiO_2 bimetal oxide nanocomposite (Figure 2(b,c)).

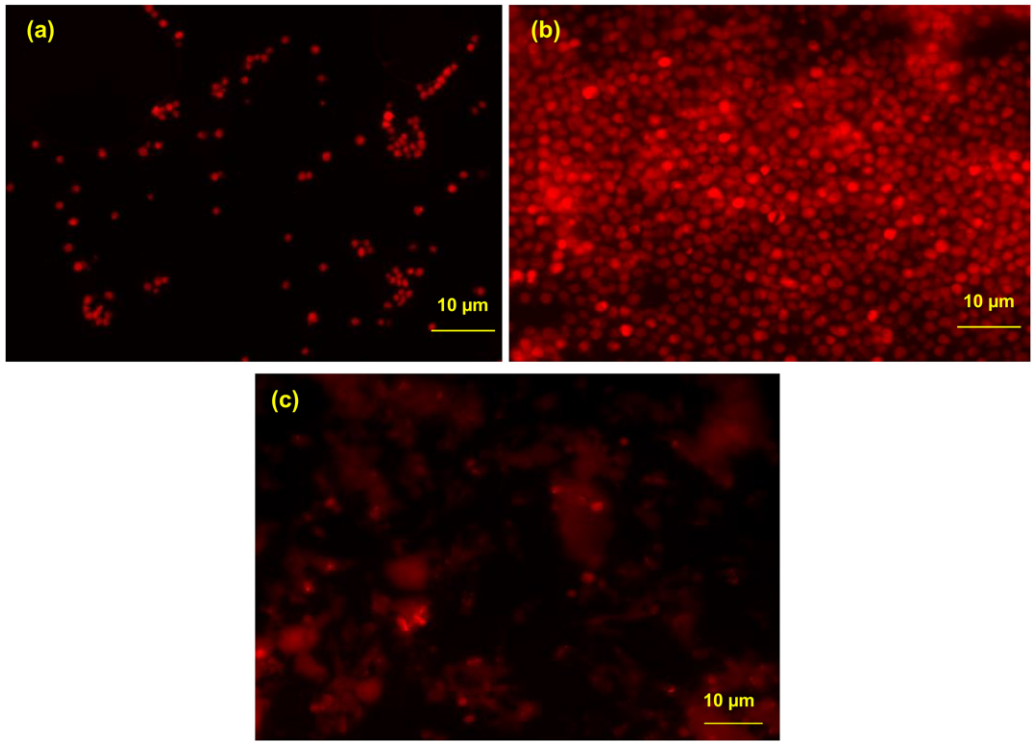

Figure 3. Histology of human skin fibroblasts grown on various cell medium solution of (a) after 5 h (b) after 14 h reaction between PE and textile/Al_2O_3–TiO_2 bimetal oxide nanocomposite (c) after 14 h reaction between PE and textile/TiO_2 nanocomposite.

Therefore, based on this analyzed spectrum (Figure 2(b,c)) shows that after 5 h, a ROS was produced from the oxidation reaction between PE and the textile/Al_2O_3–TiO_2 bimetal oxide nanocomposite. Subsequently, after 5 h, the textile/Al_2O_3–TiO_2 bimetal oxide nanoparticles scavenged ROS free radicals (Figure 2(c)) from the reaction environment. Therefore, finally there were no more ROS free radicals in the solution. Figure 2(a) shows the amount of ROS that remained in the solution after 14 h' reaction between PE (as a model) and the textile/TiO_2 nanocomposite. Therefore, based on this analyzed spectrum, a ROS was produced from the oxidation of PE with the textile/TiO_2 nanocomposite. Thus, finally there were ROS free radicals in the reaction solution.

Compared with the results of other nanocomposites, the textile/Al_2O_3–TiO_2 bimetal oxide bimetal oxide nanocomposite shows the best result. The fibroblast human skin cell can be grown onto standard tissue culture with a reaction solution between PE and the textile/Al_2O_3–TiO_2 bimetal oxide nanocomposite after 5 and 14 h, showing similar morphologies and densities (refer Figure 3). The cell shape was globular and the cell density high. The cell density is higher than in other textile compositions because the radical scavenging ability of this textile nanocomposite is higher than other samples. Therefore, based on current literature, there was no toxicity in the absence of ROS free radicals (Beneš, Ďuračková, & Ferenčik, 1999; Wu & Cederbaum, 2003). Based on Figure 3, this confirmed the result of the UV–Vis spectrum. With that, all results represented that this textile nanocomposite did not show any toxicity for the fibroblast human skin cell. Figure 3(c) highlights the histology of the fibroblast human skin cell grown in standard tissue culture with a reaction solution between PE and the textile/TiO_2 metal oxide nanocomposite (after 14 h' interaction). The cell shape was not globular and the cell wall was completely destroyed in the solution after 14 h of antimicrobial interaction. This phenomenon is related to the antimicrobial mechanism of this nanocomposite. However, the antimicrobial mechanism of the nanoparticles created oxidative stress *via* the generation of ROS, with lipid peroxidation that caused the enhancement of membrane fluidity and disruption of the cell wall (Besinis, De Peralta, & Handy, 2014).

PE is the principal bacterial phospholipid. In Figure 1, the strong absorption at 1740 cm^{-1} was from the C═O of ester carbonyl (Lewis & McElhaney, 2007). For the observed PE, the C═O stretching band was noted to be a summation of at least two major components that are usually near 1738–1742 and 1724–1729 cm^{-1}, reflecting the differences in the degrees of hydration or hydrogen bonding to the ester carbonyl groups (Greenhall, Yarwood, Brown, & Swart, 1998; Lewis & McElhaney, 1998). The textile/Al_2O_3–TiO_2 bimetal oxide nanocomposite slightly shifted C═O from 1740 to 1736 cm^{-1} (refer Figure 1). The shift may be due to the hydrogen bonding of the ester carbonyl group on the nanocomposite surface (Greenhall et al., 1998). The C═O remained in the ester frequency region (Bellamy, 1975; Socrates, 2001), therefore, the ester bond was not broken by the nanoparticles.

The bands at 1636 and 1533 cm^{-1} (refer Figure 1) were due to deformation of RNH_3^+ or RNH_2 (Bellamy, 1975; Socrates, 2001). The two bands around 3000 cm^{-1} belong to NH_2 (the primary amine). These two bands disappeared when PE absorb all the nanocomposites, due perhaps to the interaction of the PE amine moiety with the metal oxide

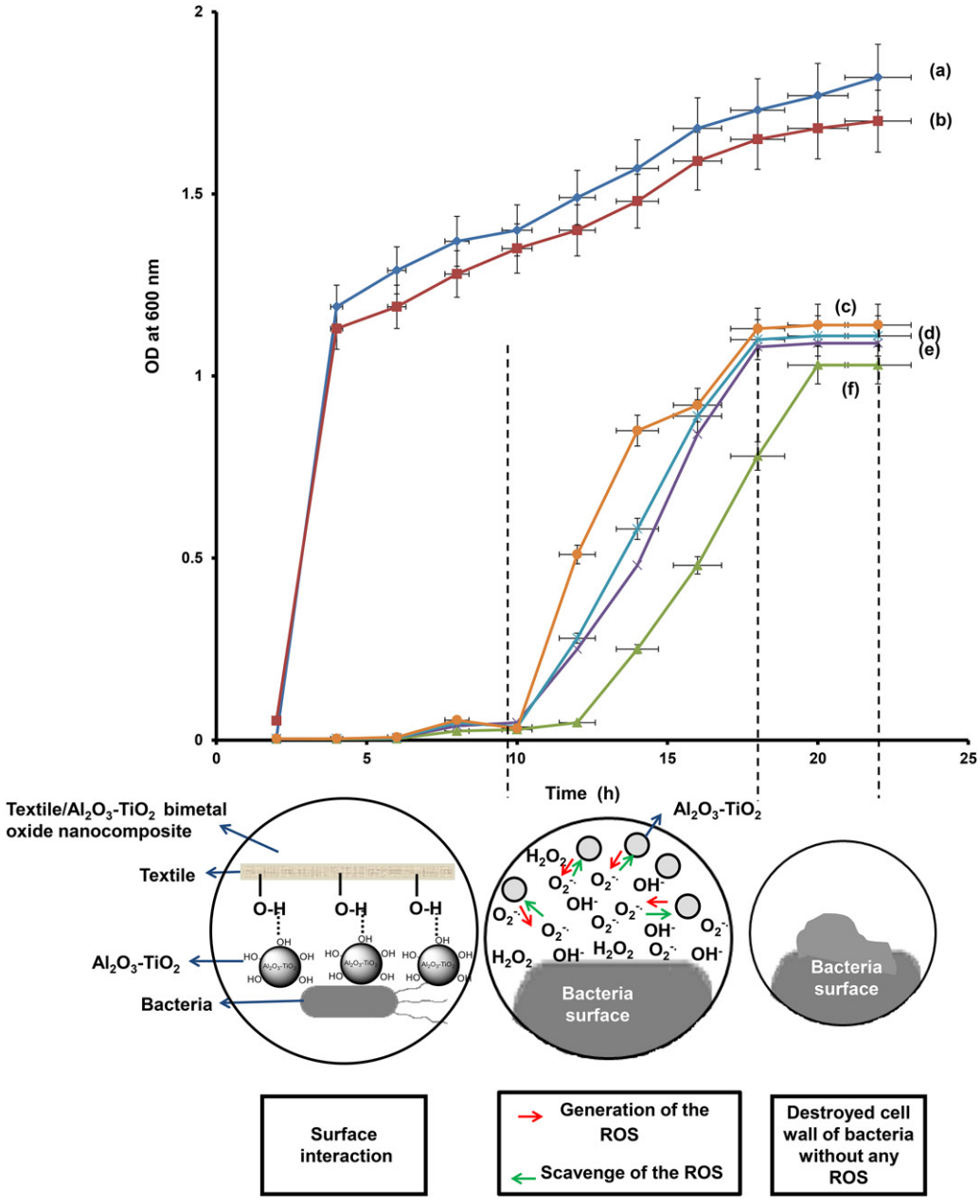

Figure 4. The antimicrobial mechanism of textile/Al_2O_3–TiO_2 bimetal oxide nanocomposite based on Growth curve of *E. coli* on textile/Al_2O_3–TiO_2 bimetal oxide nanocomposite in different concentrations (75, 100, 125, and 150 mmol/L) in liquid medium by optical density (OD) at 600 nm: (a) culture *E. coli*, (b) textile, (c) 75 mmol/L, (d) 100 mmol/L, (e) 125 mmol/L, and (f) 150 mmol/L.

surface. The new peak formed at near 2000 cm^{-1} and belonged to the NO_2 group. Therefore, formation of this peak shows oxidation of PE by this nanoparticle.

Absorption bands between 1200 and 1250 cm^{-1} (refer Figure 1) resulted from the PO_2^- in phosphodiester (Omoike & Chorover, 2004). The frequency is at about 1220 cm^{-1} when the phosphodiester group is fully hydrogen-bonded, and at 1240 cm^{-1} and above when the phosphodiester group is non-hydrogen-bonded (Wong, Papavassiliou, & Rigas, 1991). The peaks of PO_2^- at 1094–1084 cm^{-1} (Kinder, Ziegler, & Wessels, 1997; Lewis & McElhaney, 2007) overlapped with the bands arising from C—N, C—O, C—O—C, and C—O—P stretching modes (Parikh & Chorover, 2006; Socrates, 2001), but had a shoulder peak at 1091 cm^{-1}. Due to the diminishing peak at 1078 cm^{-1} after the PE absorbed the nanocomposite, a sharp (PO_2^-) peak emerged at 1091 cm^{-1} (refer Figure 1).

The absorbance at 1063 cm^{-1} was assigned to the C—O—C (Greenhall et al., 1998; Kiwi & Nadtochenko, 2005), and emerged due to the diminishment of absorbance at 1078 cm^{-1}. The peak at 1078 cm^{-1} was assigned to P═O (Badireddy et al., 2008) and its reduction may be from the phosphodiester breaking or phosphate moiety absorption of the nanocomposites. The broad peak around 3300 resulted from the OH group. When PE was attached to the nanocomposite by the hydrogen band, this band shifted to a lower frequency.

Infrared (IR) spectral changes revealed that the structure of PE could be oxidized by these nanoparticles such as Al_2O_3–TiO_2 bimetal oxide. Nanoparticles produced a ROS

Table 1. The textile/Al_2O_3–TiO_2 bimetal oxide nanocomposite and textile/TiO_2 nanocomposite used as antimicrobial agents together with their antimicrobial mechanisms.

Textile nanocomposite	Proposed mechanism of antimicrobial action	The main factors that influence antimicrobial activity
Textile/TiO_2 nanocomposite	Oxidative stress *via* the generation of ROS; lipid peroxidation that cause to improve membrane fluidity and disruption of the cell wall	Shape, size, and crystal structure
Textile/Al_2O_3–TiO_2 nanocomposite	The ROS generation, lipid peroxidation that cause to improve membrane fluidity and disruption of the cell wall and then scavenge the ROS therefore low toxicity	Crystal structure

that creates free radicals by oxidation reaction. These nanoparticles could change the chemical structure of PE that could bind to these nanoparticles through hydrogen binding. These nanoparticles when induced could cause structural changes in phospholipids. It may lead to the loss of amphiphilic properties. It may also bring about the destruction of the membrane and leaking of the cell. When the nanoparticles can penetrate the membrane, and accumulate inside the bacterial cell, it causes pit formation, perforation, disorganization, and finally, this disturbed its proper function. Biomolecular changes on the cell surface revealed by the ATR-FTIR spectra provide a better understanding of the cytotoxicity of these nanoparticles. Based on Figure 4, the antimicrobial mechanism of the textile/Al_2O_3–TiO_2 bimetal oxide nanocomposite goes through several different steps. The attachment of this nanocomposite through the attachment of Al_2O_3–TiO_2 bimetal oxide nanoparticles to the surface of PE (as the model of bacteria) is by hydrogen binding. Then, these nanoparticles oxidize the cell wall of the bacteria, by producing a ROS. These nanoparticles induced structural changes in the phospholipids which may lead to the loss of amphiphilic properties, destruction of the membrane and leaking from the cell. Therefore, the penetration and accumulation of nanoparticles inside the bacterial cell cause pit formation, perforation, and disorganization, and thus drastically disturb its proper function. For the textile/Al_2O_3–TiO_2 bimetal oxide nanocomposite, ROS free radicals are scavenged by the special structure of Al_2O_3–TiO_2 bimetal oxide. Since Al_2O_3–TiO_2 bimetal oxide are radical scavengers, this can directly inhibit the production of ROS, which can rapidly induce the ROS defense system to be activated before the program of cell degradation is complete. Figure 4 shows the antimicrobial mechanism of the textile/Al_2O_3–TiO_2 bimetal oxide nanocomposite for all steps based on growth curve of *E. coli* on textile/Al_2O_3–TiO_2 bimetal oxide nanocomposite in different concentrations (75, 100, 125, and 150 mmol/L) in liquid medium. Figure 4 demonstrates the growth cells of bacteria. There is the relation between growth cells of bacteria and number of cells of bacteria, when the growth cells of bacteria are high, the number of bacteria's cells is also high and when the growth cells of bacteria are low, the number of bacteria's cells is also low.

Table 1 shows the textile/Al_2O_3–TiO_2 bimetal oxide nanocomposite and textile/TiO_2 nanocomposite used as antimicrobial agents together with their antimicrobial mechanisms. The antimicrobial property of TiO_2 nanoparticles is related to its size, shape, and crystal structure (Haghighi et al., 2013). A particularly important proposed mechanism for TiO_2 nanoparticles is the oxidative stress *via* the generation of ROS. ROS will then cause damage to the site-specific DNA and structure of the cell wall ROS is very toxic and it can be caused to cancer (Fu, Xia, Hwang, Ray, & Yu, 2014). Therefore, based on Table 1, the antimicrobial mechanism of this nanocomposite is mostly different due to scavenger ability of this nanocomposite and lower toxicity.

Therefore, there are no ROS free radicals in the human body for the textile/Al_2O_3–TiO_2 bimetal oxide nanocomposite. In order to confirm this result, the reaction solution was tested by the fibroblast human skin cell. The result is represented in Figure 3, where it can be noted that the antimicrobial mechanism of the textile/Al_2O_3–TiO_2 bimetal oxide nanocomposite was first on the surface interaction between the bacteria and nanoparticles, and then the nanoparticles destroyed the cell wall of the bacteria through the oxidation reaction produced by ROS. Finally, these nanoparticles scavenged the ROS free radicals. Therefore, these nanoparticles attached on the textile can destroy the bacteria without any ROS free radicals remaining in the human body. Conclusively, it can be used as a potential for wound dressing to reduce wounds' infection.

Conclusion

Overall, the textile/Al_2O_3–TiO_2 bimetal oxide's antimicrobial mechanism has been successfully investigated by using L-α-PE of bacteria's cell membrane as a model compound. ATR-FTIR studies have suggested the interaction of PE with this nanocomposite. The intracellular Al_2O_3–TiO_2 bimetal oxide nanoparticles might have interacted with cellular biomolecules and caused adverse effects eventually triggering the bacteria cell death. IR spectral changes revealed that the PE could bind to these nanoparticles through hydrogen binding. This nanoparticle-induced structural changes in phospholipids may lead to the loss of amphiphilic properties, destruction of the membrane and cell leaking. Therefore, the antimicrobial mechanism of this textile nanocomposite was first by the attachment of this nanocomposite through the attachment of Al_2O_3–TiO_2 bimetal oxide nanoparticles to the surface of PE (as the model of bacteria) by hydrogen binding. Then the nanoparticles destroy the cell wall of bacteria through oxidation reaction by producing ROS. Finally, these nanoparticles scavenged the ROS free radical. Therefore, these attached nanoparticles on textile can kill bacteria without any remaining ROS free radical in human body. The results of using PE as a bacteria cell wall model represented the antimicrobial mechanism of this nanocomposite. These results suggest that the antimicrobial

mechanism of this nanocomposite is mostly different due to scavenger ability of this nanocomposite and its lower toxicity. The outstanding features of the results indicate that this textile nanocomposite can be used as a potential wound dressing to improve infectious wounds and to improve wound-healing process.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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