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Research paper

First evidence of singlet oxygen species mechanism in silicate clay for antimicrobial behavior

Jiun-Chiou Wei, Yi-Ting Wang, Jiang-Jen Lin *

Institute of Polymer Science and Engineering, National Taiwan University, Taipei 10617, Taiwan

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ABSTRACT

Nanoscale silicate platelets (NSPs) from the natural silicate clay minerals were shown to have antimicrobial ability through physical trapping mechanism. The NSP could inhibit the growth of drug-resistant species such as methicillin-resistant *Staphylococcus aureus* and silver ion-resistant *Escherichia coli*. We further found that the NSP generated singlet oxygen species under ultraviolet irradiation. This is the first time to report the ability of generating singlet oxygen species by the platelets of *ca.* 80 × 80 × 1 nm in geometric shape. The ability to emit singlet oxygen species of the NSP clay provides an explanation for "physical trapping" antimicrobial mechanism.

1. Introduction

Recent advances in fabricating the nanostructures of inorganic materials have often led to the discovery of new functions and properties. For example, the nanosize materials, C_{60} derivatives (Lee et al., 2009) and ZnO (Lipovsky et al., 2009), enabled to generate reactive oxygen species and subsequently inactivate bacteria and viruses. Inorganic materials of natural silicate minerals (Ray and Okamoto, 2003) such as montmorillonites, fluorinated micas, hectorite, and saponite, were conventionally considered to be inert to microorganisms (Olphen et al., 1977; Theng, 1974). These clay minerals commonly consist of multilayered stacks of individual silicate layers with large surface areas (*ca.* 720 m^2/g) (Olphen, 1977; Zhao et al., 2003) and ionic surface charges (Shikinaka et al., 2010; Zhang et al., 2009). Conventionally, these clays are used as fillers for polymer nanocomposites (Hsu et al., 2010; Ray and Okamoto, 2003), catalysts (Haydel et al., 2008), and adsorbents (Cunningham et al., 2010). Recent developments have revealed that the hydrated minerals of iron-rich clays could exhibit remarkable antibacterial activity (Bergaya and Lagaly, 2006; Haydel et al., 2008). This bactericidal ability was considered to be derived from compositions such as the Fe^{2+} species that were released from the clay under aqueous buffer pH conditions (Williams et al., 2011).

The use of non-chemical alternatives such as silicate minerals as a substitute for antibiotics in therapies is an important subject because of the serious occurrence of antimicrobial chemical resistance. For example, the mutated species such as methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci have already emerged in common hospital environments and caused the infection of healthy individuals. Recently, we have developed the method of exfoliating or delaminating the multilayered structure of natural clay minerals into nanoscale silicate platelets (NSPs) (Chu et al., 2005; Lin et al., 2006). Examination of these exfoliated nanolayers revealed an unexpected antimicrobial property (Wei et al., 2011). In considering the chemical structure, the exfoliated NSP nanolayers have the same silicate chemical composition as the pristine natural clay, but differences in larger surface area and exposure of surface ionic charges. The nanolayers are comprised of silicates in a thin-layer geometric shape and multiple functionalities of $\equiv SiO^- \ Na^+$ in the estimated amount of 18,000 ions per nanolayer. Previously, the investigation on cytotoxicity and genotoxicity has demonstrated that these layers have a low level of acute toxicity (Li et al., 2010). These unusual characteristics of the NSP nanolayers prompted us to investigate further their mechanism of antimicrobial activity against the drug-resistant bacterial strains. Here, we used a physicochemical method (electron paramagnetic resonance radical trapping) to detect the generation of reactive oxygen species (ROS) and evidence the physical mechanism for antimicrobial properties.

2. Materials and methods

2.1. Materials

Sodium montmorillonite (Mt), a natural smectite aluminosilicate, was obtained from Nanocor Co. (USA). The clay mineral has a generic structure of 2:1 layered silicate to aluminum oxides with 2 tetrahedral sheets sandwiching an edge-shared octahedral sheet, and exchangeable

* Corresponding author. Tel.: +886 2 3366 5312; fax: +886 2 2363 8076.
E-mail address: jianglin@ntu.edu.tw (J.-J. Lin).

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Na^+ counter ions with a CEC of 120 mequiv/100 g. The exfoliated NSPs were prepared by the process involving the polymeric amine-salts for the exfoliation and subsequent purification by biphase extraction to remove organics from water slurry (Chu et al., 2005; Lin et al., 2006). Three surfactants, cationic dodecyltrimethylammonium bromide (DDTMA), nonionic t-octylphenoxypolyethoxyethanol (Triton X-100), and anionic sodium dodecyl sulfate (SDS), were obtained from Aldrich Chemical Co. Two spin probes, 2,2,6,6-tetramethyl-4-piperidone (4-oxo-TEMP) and 5,5-dimethyl-1-pyrroline N-oxide (DMPO), were obtained from Aldrich Chemical Co. Bacterial strains obtained from Super Laboratory Co. (Taiwan) were used as target antibiotic organisms: Gram-negative silver ion-resistant *Escherichia coli* (J53pMG101) and Gram-positive methicillin-resistant *S. aureus*.

2.2. Antimicrobial tests

Cells were cultivated overnight in Luria-Bertani (LB) broth: 100 μL of the suspension was inoculated in fresh medium to restart the cell cycle. A 0.1 mL aliquot of bacterial suspension containing *ca.* 10^8 colony forming units (CFU)/mL was added to 9.9 mL of NSP solution. Bacterial suspensions were then adjusted to 1×10^6 CFU/mL. After agitation, portions of the culture were taken at periods of 0, 3, and 24 h, spread onto agar plates, and subsequently incubated at 37 °C for 24 h. Finally, the number of colonies was counted to determine antibacterial activity (Tortora et al., 2001).

2.3. Reactive oxygen species (ROS) detection method

E. coli, removed from growth media via centrifugation and redispersed in PBS, was incubated with NSP (10, 50, and 100 ppm) for 0.5 h at 37 °C under gentle agitation (150 rpm). After incubation, the bacteria were then centrifuged and redispersed in 2′,7′-dichlorofluorescin diacetate (H_2DCFDA)/PBS buffer for 15 min, and made into the final concentration of H_2DCFDA to be 50 μM. Flow cytometry (FACScan ® BD Co.) was used for further analysis.

2.4. Electron paramagnetic resonance (EPR) measurement

EPR analyses were performed using a Bruker EMX spectrometer under the following conditions: temperature = 298 K, microwave frequency = 9.773 GHz, microwave power = 10.106 mW, modulation amplitude = 1.6 G at 50 kHz, and scan time = 42 s. The inner thickness of quartz cell was 0.25 mm. The light irradiation was generated from a mercury lamp with the main wavelength of 365 nm.

2.4.1. Singlet oxygen detection

Production of singlet oxygen was monitored using 4-oxo-TEMP as a spin-trapping reagent (Lion et al., 1980). Experimental solution with NSP of 500 mg/L in water was prepared by mixing 1 M 4-oxo-TEMP (30 μL), 10 mg/mL NSP (50 μL), and phosphate buffer at pH 7.4 (920 μL). The solution was introduced into a flat quartz cell and subjected to EPR measurement after light irradiation for 0–20 min when 4-oxo-TEMP was oxidized by singlet oxygen to produce 4-oxo-2,2,6,6,-tetramethyl-1-piperidinyloxy radical (4-oxo-TEMPO). The appearance of EPR signals for 4-oxo-TEMPO indicates the formation of $^1O_2$. Rose Bengal was employed as a positive control for the generation of singlet oxygen.

2.4.2. Hydroxyl radical detection

The spin-trapping reagent DMPO was used to trap hydroxyl radical. The slurry solution of the NSP in water at the concentration of 500 mg/L was prepared by mixing 100 μL $CuSO_4$ (10 mM), 100 μL DMPO (100 mM in phosphate buffer pH 7.4), 15 μL NSP (10 mg/mL), and 85 μL phosphate buffer at pH 7.4. The solution was introduced into a flat quartz cell and subjected to EPR measurement after light irradiation for 0–10 min. Hydrogen peroxide was employed for the generation of the hydroxyl radical as a positive control.

2.5. Instruments and analyses

UV–vis spectroscopy measurements were performed with a Hitachi U-4100 spectrophotometer. Interfacial tensions were measured by the Wilhelmy method using a Kruss-K10 digital tension meter equipped with a spherical ring.

3. Results and discussion

3.1. Nanostructures and characterization of silicate layers

A representative smectite clay mineral, Mt, was exfoliated from the multilayered structure into a random form of NSP according to procedures reported previously (Chu et al., 2005; Lin et al., 2006). The two-step process involved exfoliation of layered silicates by a polyamine-salt ionic exchange reaction and subsequent isolation of the silicate layers by toluene/water extraction. In the process, the original clay stacks were totally randomized into individual layers and purified by removing the organic polyamine agent, as reported previously (Chu et al., 2005; Lin et al., 2006). Repeated extractions allowed isolation of the NSP material at >94 wt.% purity based on the organic analysis. It is noteworthy that the exfoliated NSPs have the same composition as the pristine Mt clay mineral, but with totally exposed surface areas and surface-tethered ionic charges of *ca.* 18,000 ions per layer. The silicates exhibited characteristic UV–vis absorption, showing the presence of two peaks at the maximum of 230 and 280 nm (Fig. 1). By comparison, the pristine Mt clay mineral had only a very weak and broad absorbance in the area around 200–300 nm (Banin and Lahav, 1968; Schramm and Kwak, 1982). The newly found UV absorption peaks from the exfoliated silicate nanolayers have not been previously reported. The appearance of the UV absorption peaks could be caused by the transformation of the geometric shape from the layered structure into high-aspect-ratio nanolayers possessing unique dispersing characteristics and self-aggregates in aqueous medium. The geometric shape of the silicate nanolayers and presence of surface charges may contribute to the UV absorption.

3.2. Antimicrobial potency

The randomized silicate nanolayers with ionic charges on surface are believed to have a strong tendency for interacting with polar organic molecules and the microbial cell surface, as previously reported (Wei

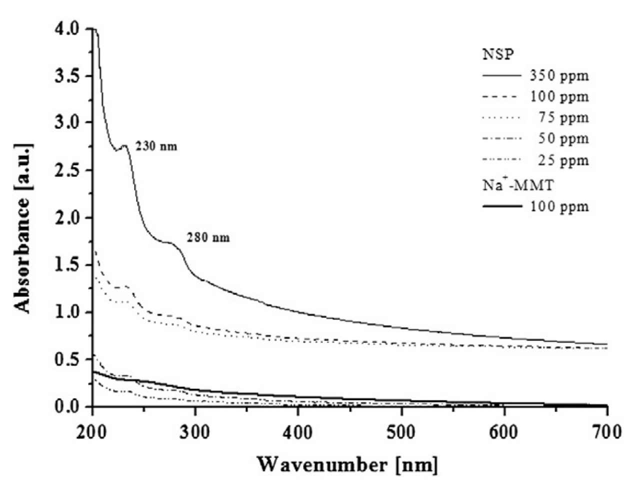

Fig. 1. Absorption spectra of various concentrations of NSP and pristine Mt dispersion in DI-water.