Antimicrobial Activities and Cellular Responses to Natural Silicate Clays and Derivatives Modified by Cationic Alkylamine Salts

Shan-hui Hsu[1,2], Hsiang-Jung Tseng[2], Huey-Shan Hung[3], Ming-Chien Wang2, Chiung-Hui Hung[2], Pei-Ru Li[1] and Jiang-Jen Lin[1,2,4]

[1].Institute of Polymer Science and Engineering, National Taiwan University, Taipei 10617 Taiwan, Republic of China,

[2].Department of Chemical Engineering, National Chung Hsing University, Taichung, 40227 Taiwan, Republic of China,

[3].Graduate Institute of Basic Medical Science, China Medical University, Taichung, 40402 Taiwan, Republic of China, and

[4].Center of Nanoscience and Nanotechnology, National Chung Hsing University, Taichung, 40227 Taiwan, Republic of China

Corresponding author: Shan-hui Hsu

E-mail: shhsu@dragon.nchu.edu.tw

Corresponding author: Jiang-Jen Lin

E-mail: jianglin@ntu.edu.tw

Abstract

Nanometer-scale silicate platelet (NSP) materials were previously developed by increasing the interlayer space and exfoliation of layered silicate clays such as montmorillonite and synthetic fluorinated mica by the process of polyamine exfoliation. In this study, the antibacterial activity and cytotoxicity of these nanometer-scale silicate clays were evaluated. The derivatives of NSP (NSP-S) which were modified by C18-fatty amine salts via ionic exchange association exhibited the highest antibacterial activity in the aqueous state among all clays. The high antibacterial activity, however, was accompanied by elevated cytotoxicity. The variations of cell surface markers (CD29 and CD44) and type I collagen expression of fibroblasts treated with the clays were measured to clarify the mechanism of the silicate-induced cytotoxicity. The signal transduction pathway involved the downregulation of extracellularsignal-regulated kinase (ERK), which appeared to participate in silicate-induced cytotoxicity. This study helped to understand the antibacterial potential of NSP and the interaction of natural and modified clays with cellular activities.

Key words: montmorillonite, mica, silicate platelet, cytotoxicity , extracellular-signal-regulated kinase (ERK)

1. Introduction

Natural clays such as montmorillonite (MMT) and mica have been used as natural medicine for thousands of years. It is generally believed that the clays possess healing functions for internal detoxifying (e.g. the digestive system and liver), trauma injuries, skin conditions, and so on. The therapeutic functions of clays may be associated with the nanosheet structure. One of the common smectite clays, montmorillonite (MMT), consists of two hydrated aluminum with Si-O tetrahedrons and the sandwiched Al-O$(OH)_2$ octahedron, with a neighboring plate distance at 12 angstrom. The clay materials with multilayer inter-space have been reported to serve as a container for encapsulating organic drugs or DNA and proposed to use for cancer treatment and gene therapy [1-3]. In recent literature, an increasing effort has been directed to the nanosized inorganic materials such as the silicate clays for their properties of bactericidal and interacting with biomolecules [4]. Previously, the MMT and the fluorinated mica (Mica) clays were spatially enlarged by incorporating with the salts of polyether-amines and then embedded with larger biomaterials such as bovine serum albumin (BSA). The process of forming protein-silicate clay hybrids in layered structure was developed [5,6]. Besides the absorption with protein, the aluminum silicates for a few cell lines has been assessed for the cytotoxicity [7,8], but lacking the studies on specific cellular response or signal transduction.

Recently, a new process of exfoliating the layered silicate clays such as montmorillonite has been developed to isolate the nanometer-sized silicate platelets (NSP, *ca.* 100×100×1 nm^3 in dimension) as new forms of silicate materials [9]. The NSP inorganic silicates can be further modified by various surfactants (NSP-S) and consequently their dispersing ability [10]. In this study, we compared their antimicrobial ability and cytotoxicity of these clay materials. Scanning electron

microscopy (SEM) was used to visualize the interaction of these clays with bacterial or cell surfaces. Besides, the expression levels of several surface markers of connective tissue cells such as CD29 and CD44 were examined to elucidate the detailed cellular changes caused by the natural clays and their derivatives. CD29 (integrin β1) is involved in cell attachment, migration, differentiation and survival by mediating the signal stimuli from extracellular matrix to the intracellular cytoskeleton [11]. Fibronectin, collagens and laminins are the ligands that integrin can bind. CD44 is a cell surface glycoprotein which is crucial for cell-cell interactions, cell adhesion and migration [12,13]. The understanding of the fundamental size-compatibility and interaction behavior between the nanosized clays and cell surface is crucial for future developments of new antimicrobial and drug-delivering nanomaterials.

2. Materials and methods

2.1. Clays

MMT, supplied by Nanocor Co., is a smectite silicate clay with a cationic exchange capacity of 120 mequiv/100 g. Mica obtained from CO-OP Chemical Co. (Japan) was a synthetic fluorinated mica of layered silicate clay. The modified clays included NSP (exfoliated MMT), NSP-S (NSP capped with a fatty-amine surfactant) and NMP (exfoliated mica platelet).

2.2. Cell Culture

Human gingival fibroblasts (HGF) and bovine endothelial cells (BEC) were used for cell study. HGF were harvested from healthy gingival tissues obtained during surgical procedures with adequate informed consent [14]. Gingival tissues were cut into small pieces by a surgical knife. BEC were harvested from the bovine carotid artery with the collagenase method [15]. Both cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. When cells grew into confluence, they were passaged at a ratio of 1:3. Cell passages from 3 to 15 were used through this study.

2.3. Bacteria Growth Inhibition Assay

The antibacterial activity was evaluated in two ways, the measurement of the inhibition circle diameter and colony number counts after co-incubation with clays. *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli)* were used to evaluate the antibacterial activity of the clays.

2.3.1 The inhibition circle

The bacterial solution was adjusted to 1×10^7 CFU/ml, and 100 μl was plated evenly on an agar plate. The solutions of clays (1% wt, 100 μl) were absorbed onto a filter paper (10 mm diameter, Toyo Roshi Kaisha, Ltd., Japan) which was then placed on the center of agar plate. After 24 hr, the filter paper was removed and the diameter (D) of clear zone was measured. The size of inhibition ring was represented as (D-10)/2 in mm.

2.3.2 Direct co-incubation with clays

The bacterial solution was first adjusted to 4×10^5 CFU/ml and a portion of 100 μl was added into 20 ml of low nutrient Luria bertani (LB) broth which was diluted to

1/500 with DDW and contained different clays at 10 ppm. The mixed bacterial solutions were incubated in 35°C and shaken at the speed of 110 rpm for 3 hr or 24 hr. At the indicated time point, the bacterial solutions were cooled on ice immediately and diluted serially to proper concentration for colony counting. The aliquot of 100 μl was plated evenly on each agar plate. After 24 hr, the colony numbers were counted to evaluate the antibacterial ability of clays.

2.4. Scanning Electron Microscopy (SEM)

S. aureus suspension was adjusted to 1×10^7 or 1×10^9 CFU/0.9 ml, and 0.9 ml bacterial suspension was transferred to 24 well plates with 1.5 cm round glass coverslip (Matsunami, Japan) in each well. 0.1 ml of NSP at 1000 ppm was then added into each well. In the control well, 0.1 ml double distilled H_2O (DDW) was added. The final concentration of NSP was 100 ppm. The bacterial broth was removed at 0, 0.5, 1, 2, and 3 hr respectively. Bacteria left on the glass were fixed with 2.5% paraformaldehyde at room temperature for a half hour and at 4°C overnight. Then the fixed samples were dehydrated with alcohol followed by field-emission SEM (JSM-7401F, JEOL, Japan) observation. In addition, HGF were seeded on the coverslip glass in a 24-well plate for 24 hr and then the culture media were replaced with fresh media containing 10 ppm of different clays. After 24 hr of incubation, cells were fixed and dehydrated for FE-SEM observation.

2.5. Cytotoxicity Assay

2×10^4 cells were seeded in each well of a 24 well plate. After being treated with 10 ppm various clays, cells were harvested for MTT assay at 24, 48 and 72 hr. Briefly, 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenyltetrazolium bromide (MTT) solution (0.5 mg/ml, 1× phosphate buffered saline (PBS)) was added and incubated for 4 hr at 37°C. The supernatant was removed and the aliquot dimethyl sulfoxide (DMSO) (Tedia, Fairfield, OH, USA) was added into each well to dissolve any resulting formazan crystals for 10 min. The absorbance was measured at 550 nm with an ELISA reader (F-2500, Hitachi, Japan).

2.6. Flow Cytometry Analysis

After cultivation with clays for 24, 48 and 72 hours, cells were harvested for the

measurement of surface markers CD29 and CD44. The expression level was quantified by a flow cytometer (BD FACS Caliber, BD Bioscience, USA). Cells were collected and washed with cold PBS then resuspended in 10 μl of PBS. 10 μl of anti-human CD29 antibody conjugated with phycoerythrin (PE) (PharMingen, San Diego, CA, USA) or anti-human CD44 antibody conjugated with PE (Abcam, Cambridge, MA, USA) was added and incubated for 30 min at 4°C in dark. PBS containing 1% BSA was used to eliminate the non-specific binding. The fluorescence intensity was quantified by WinMDI software (Scripps Research Institute, San Diego, USA).

2.7. Reverse transcription polymerase chain reaction (RT-PCR)

Cells were harvested for total RNA extraction after 24 hr of incubation with clays. Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. cDNA was synthesized with RevertAid^{TM} First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany). The cDNA obtained from 1 μg of total RNA was used for PCR by PCR Master Mix Kit (Applied Biosystems, Foster City, CA, USA). The primer sequence used for type I collagen (collagen-I) was: 5'AACCCTGGTGCTGATGGACAG3' (forward) and 5'GGACGACCAGCTTCACCAGG3' (reverse), and for GAPDH was: 5'CCA CCCATGGCAAATTCCATGGCA3' (forward) and 5'TCTAGACGGCAGGTCAGGTCCAC C3' (reverse). The PCR reaction condition was 28 cycles of denaturing at 94°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 45 s.

2.8. Immunoblot Analysis

Proteins were extracted in RIPA buffer (Sigma, St. Louis, MO, USA) and analyzed by SDS-polyacrylamide gel electrophoresis. After being transferred onto a polyvinylidene difluoride (PVDF) membrane, antigens were analyzed with specific primary antibodies. Antibodies against ERK and phospho-ERK were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-human type I collagen antibody was purchased from Chemicon International (Temecula, CA, USA). Antigen-antibody complexes were detected using horseradish peroxide-labeled rabbit anti-mouse IgG and an enhanced chemiluminescence (ECL) detection system (Pierce,

Rockford, IL, USA).

2.9. Statistical analysis

Student's t-test was used for statistical analyses. Statistical significance was indicated by $p<0.05$. Data are presented as mean±SEM. Each experiment was repeated in triplicate.

3. Results

3.1. Growth inhibition effect against S. aureus and E. coli

In order to characterize the antibacterial effect of the clays, the size of growth inhibition circle was measured for two species of bacteria. The inhibitory effect was tested at the concentration of 10, 100, 1000 and 10000 ppm. No clays showed antibacterial effect within the testing protocol until the concentration reached 10000 ppm. The inhibition effect at 10000 ppm (1%) is shown in figure 1. NSP had the greatest growth inhibitory effect among all clays. MMT did not show any inhibitory effect even at 10000 ppm. The antibacterial effect of these clays was ranked in the order of: NSP > NSP-S ≈ Mica ≈ NMP >> MMT.

The antibacterial activity of clays in aqueous state at 10 ppm was proven by direct incubation with *S. aureus*. The result of colony counting after incubation for 3 or 24 hr is shown in figure 2. The antibacterial effect of NSP-S was most dramatic and notably greater than that of original NSP. The ranking of antibacterial activity of these five clays turned to be NSP-S > NSP ≈ NMP≈MMT > Mica.

The interaction of NSP (100 ppm) with *S. aureus* can be visualized by FE-SEM images shown in figure 3. The diameter of *S. aureus* was 0.5~1 μm, and the dimension of the nanosheet structure for NSP was about 100 nm×100 nm×1 nm. The aggregates of NSP attached to and covered the bacteria. As the incubation time increased, some hollow space around the NSP was observed (as the arrow indicated). It seemed that the bacteria were eliminated by incubation with NSP.

3.2. Cytotoxicity to HGF and BEC

Figure 4 shows the viability of cells incubated with 10 ppm of parent clays (MMT or Mica) or the modified clays (NSP, NSP-S or NMP) at 24, 48 and 72 hr. At 24 hr, the viability of HGF in each group was close to control except for the Mica treated group (figure 4A). However, at 48 hr, the viability of HGF treated with NSP-S was significantly lower compared to those treated with the other clays. At 72 hr, HGF treated with Mica or NSP-S showed a remarkable decrease in viability.

The cytotoxicity of clays on BEC was also evaluated to obtain the information of variance among different cell types. As shown in figure 3B, BEC treated with 10 ppm of NSP-S had the lowest viability at 24, 48 and 72 hr. BEC treated with 10 ppm of MMT, but not the other clays, had similar viability as the control at 72 hr. Based on

the data in figure 4B, it was deduced that the cytotoxicity of these clays (at 10 ppm) ranked as NSP-S>Mica>NSP ≈NMP>MMT.

Due to the size difference between bacteria and cells, the mechanisms by which the nanosheet structures of clays interacted with bacteria and with cells may have been different. The SEM picture (figure 5) clearly showed that the clays formed aggregates of various sizes on the cell surface. The sheet structure of Mica was larger than the other clays. The aggregates of NMP were not as obvious as that of Mica, but NMP had more obvious sheet structures than MMT derived clays. The particles of MMT, NSP or NSP-S were smaller than that of Mica or NMP. Besides, the extracellular substance of NSP-S treated cells was fewer.

3.3. Effects on expression levels of cell surface markers

Flow cytometry was used to analyze the change in the levels of CD29 and CD44 proteins on HGF. Figure 6A showed that CD29 on cell surface was significantly reduced after incubation with clays, especially Mica and NSP-S, for 72 hr. Although CD29 appeared to be reduced by Mica and NSP-S after 48 hr, there was no statistical significance found between the control group and any clay at 24 and 48 hr. The change in CD44 on the surface of HGF treated with clays was demonstrated in figure 6B. After 48 hr, CD44 was significantly reduced by Mica and NSP-S. The expression profile of CD44 at 72 hr was similar to that at 48 hr for all groups.

3.4. Effects on collagen-I expression level and ERK phosphorylation

The effect of clays on collagen-I expression on HGF cells analyzed by RT-PCR and Western blot analysis is shown in figure 7A and 7B. Collagen-I was significantly reduced in Mica and NSP-S treated groups, both in mRNA and protein levels. In MMT and NSP treated groups, only slight reduction was found. No obvious change was found in the NMP treated group. To clarify if the ERK pathway was involved, the phosphorylated form of ERK protein (p-ERK) was detected by specific antibody. The reduction of p-ERK was particularly obvious in Mica and NSP-S treated groups while in the NMP treated group p-ERK was increased. This finding was consistent with the reduction of collagen-I expression. It is concluded that, Mica and NSP-S showed a certain extent of cytotoxicity and decreased the expression of CD29, CD44 and collagen-I. These effects may be correlated to the reduction of p-ERK.

Table 1 summarizes the antibacterial activity and the cellular response of the modified clays compared to their parent clays. Based on the table, NSP had the highest potential for use in antibacterial applications.

4. Discussion

In this study, two different methods were used to compare the antibacterial activities of the parent clays, MMT and Mica, and their exfoliated silicate platelets, NSP, NSP-S and NMP. In the measurements of inhibition circles, the parent clay, MMT, was shown to have no antibacterial activity toward *S. aureus* and *E. coli*. After exfoliation, NSP had gained the property of antibacterial activity. However, the diffusion kinetics in the filter paper and the extent of dispersion of the clays could influence the size of inhibition circle. If the particles were too large, they might be trapped in the filter paper and more difficult to diffuse or spread to effectively contact with the bacteria. Consequently, the antibacterial behaviors in aqueous state would provide better information for these clays. The direct co-incubation of the bacteria with the clays showed that NSP-S was highly effective for inhibiting the bacteria completely in the test. The ability was significantly higher than that of the other clays examined.

In our previous study [16], NSP were shown to carry significantly more negative charges than MMT. The cell wall of bacteria including the Gram positive and negative bacteria was also negatively charged. After capping with fatty amine which was a cationic surfactant, the surface of NSP was equipped with lipid-like organics and caused a dramatic increase in the antibacterial activity. The interaction of bacteria cell wall and fatty amine on NSP-S would make the bacteria too fragile to sustain the osmotic pressure inside the bacteria then ruptured. The hypothesis of NSP-S interacting with the bacteria cell wall through lipid-like fatty interference was conceptually presented in figure 8. In the study of Herrera et al. [17], cetylpyridinium (CP) was used as the surfactant to increase the antibacterial activity of MMT. The surfactant, CP, increased the antibacterial activity of three CP-exchanged clays to a remarkable level as effective as granulated activated charcoal. The authors suggested that the increased antibacterial activity was resulted from the critical surfactant/clay interactions and was not due to the released surfactant. We believed that, both of the exfoliation to highly surfaced silicates and their capping with fatty amine are crucial to construct the nanoscale materials for the antibacterial ability.

On the other hand, the negatively charged NSP showed only a low antibacterial activity compared to NPS-S. The mechanism of NSP antimicrobial behavior is likely to be different from that of Cu(II)-exchanged MMT as reported [18,19]. From the pictures of SEM, a direct contact may be essential for the bacteria deterioration.

Nevertheless, the exact mechanism is needed to be further clarified.

Comparing the two parent clays, Mica did not show a significant result for inhibiting the growth of *S. aureus* in aqueous state but MMT actually lowered the colony number count 33% after co-incubation for 3 hr. The exfoliated NSP did decrease the colony number more than 42% than MMT, while NMP decreased 61% more than Mica after 24 hr incubation. The increased surface area may be the major factor contributed to the inhibitory effect boosted by exfoliation. The difference in the platelet size of Mica (300-1000 nm) and MMT (80-100 nm) affects the spacing enlargement and surface exposure [20, 21] and consequently the antibacterial activity.

It is necessary to consider the safety issue if these clays are used as the bactericidal reagent. Cell viability assays showed that Mica was more toxic to HGF and BEC than MMT. The growth inhibition effect of NSP-S was not restricted only to bacteria but also to eukaryotic cells in a same trend. Certainly, the eukaryotic cell membrane was also negatively charged and sensitive to the positively charged amine group on NSP-S as well as bacteria cell wall. To investigate the signal transduction pathway related to the cytotoxicity from the clays, cells from human source (i.e. HGF) were selected.

CD29, the integrin β1 subunit, and also known as fibronectin receptor, is highly associated with cell adhesion and migration [11]. Integrins are an important family of surface proteins that can receive the growth signals from extracellular matrix. The decrease in the expression level of CD29 in Mica and NSP-S treated cells could adversely affect the cell attachment and/or interfere with the growth factor binding and eventually may result in the cell death. CD44 is another transmembrane protein closely associated with cell-cell and cell-substrate interaction. It plays a crucial role in regulating cell migration and differentiation [12]. The interplay of CD44 with its ligands has been reported to modulate adhesiveness, motility, matrix degradation, proliferation and cell survival [13]. Therefore, the decrease of CD44 in Mica and NSP-S treated cells could reduce the binding of CD44 with its ligand which might initiate a subsequent cascade to turn the cell fate to death.

Type I collagen is one of the extracellular matrix components. The gene expression was found to be remarkably decreased in Mica and NSP-S treated cells. The reduction of type I collagen expression could result in cell detachment. Many factors were reported to regulate the gene expression of type I collagen (reviewed in

ref. 22). Especially, it was found that in Mica and NSP-S treated groups the phosphorylated ERK was significantly reduced. This finding did not agree with an earlier report [23] which concluded that the activation of ERK MAPK pathway may downregulate type I collagen gene expression. Signaling pathways may interact with each other and form a network to determine the cell fate. In this study, the expression level of type I collagen and p-ERK were both downregulated when cytotoxicity occurred. Certainly, there are still other signaling pathways related to cell survival such as PI3K/Akt or PKC pathways that can be further investigated. The complicated interaction of these pathways may contribute to the silicate-induced cytotoxicity.

Although detailed mechanism of understanding the interaction of the clays with bacteria or cells required an in-depth study, the antibacterial effect has been realized. The cytotoxicity was accounted to the downregulation of cell surface receptors responsible for cell-substrate interaction, and ERK pathway.

5. Conclusion

The silicate platelet materials, especially surface modified with a cationic fatty amine surfactant, were found to have an antibacterial activity. The effect is attributed to the silicate nanosize and high surface area. The results of cytotoxic studies indicated that cytotoxicity was accompanied by the reduced expression of type I collagen and phosphorylated ERK. With the lipid-like fatty modification on the platelet surface, the nanometer-sized silicate clays are proven to be viable for using as antimicrobial agents.

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