ANTIBACTERIAL EFFECTS OF INTERCALATED MONTMORILLONITE

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Abstract

The evaluation of antibacterial effects of montmorillonite and its four intercalates with cetylpyridinium (MMT-CP), cetyltrimethylammonium (MMT-CTA), silver cation (MMT-Ag^+) and metal silver (MMT-Ag) showed that pure, unmodified MMT had no antibacterial effects. A slight decrease of the absorbance and number of colonies can be explained by the adsorption properties of the material. MMT-CP and MMT-CTA exhibited the biggest antibacterial effect after three hours of action. After a 24-hour incubation, however, both intercalates were inefficient probably because the surface of MMT was covered with a thick layer of bacteria and, therefore, the effective compound could not affect the remaining microorganisms.

After 2-hour and 3-hour incubations, the antibacterial effects of MMT-Ag^+ and MMT-Ag were similar to those of MMT-CP a MMT-CTA but after a 24-hour incubation, a significant difference was observed in the case of MMT-Ag^+. Consequently, the use of intercalated MMT-Ag^+ for disinfection seems to be promising.

Introduction

Phyllosilicates are layered silicates in which tetrahedral silicate layers are linked together in infinite, two-dimensional sheets and condensed with octahedral (metalhydroxyl) layers of AlO_6 or MgO in a ratio of 2:1 or 1:1. The negatively charged layers attract positive cations (e.g., Na^+, K^+, Ca^{2+}, Mg^{2+}) which can hold them together. Their large cation exchangeability enables them to adsorb various cations such as heavy metals and quaternary salts including cationic surfactants. Phyllosilicates modified by cation surfactants exhibit a good ability for retention of hydrophobic toxic compound, such as phenols, polyaromatic hydrocarbons, herbicides etc., from aqueous environments. References to these applications are given in our previous works [1,2].

Recently, a significant sorption and inactivation of *Salmonella enteritidis* and *Escherichia coli* bacteria using montmorillonite (MMT) modified with cationic surfactants have been demonstrated by Herrera *et al.* [3,4]. This idea is quite novel and seems to be promising for disinfection of various types of water. The aim of this work was to prepare phyllosilicate based materials with antibacterial activity and to test them in a laboratory assay. For this purpose, MMT was modified with compounds whose antibacterial effects are well known: cetylpyridinium (MMT-CP), cetyltrimethylammonium (MMT-CTA), silver cation (MMT-Ag^+), and metal silver (MMT-Ag).

Materials and methods

Reagents

The used chemicals were of the analytical-reagent grade: silver nitrate and ethanol (96 %) were purchased from Lachema (Czech Republic), cetyltrimethylammonium (CTA) bromide and cetylpyridinium (CP) chloride from Sigma (USA) and borohydride from Merck (Germany). Pepton and agar were purchased from HiMedia (Czech Republic). Twice-distilled, deionised water used for the preparation of all solutions was obtained with a mixed-bed ion-exchanger (Watek, Czech Republic).

Modification of montmorillonite with CTA and CP

Na^+-rich montmorillonite type Wyoming with an exchanged capacity of 0.9 mequiv/g was used for the adsorption of CTA and CP. MMT intercalation was performed according to a recently published procedure of Praus *et al.* [1]. The cationic surfactants were dissolved in a 50:50 (v/v) mixture of water and ethanol producing concentration of about 1.0 mmol/l. MMT was added and the suspensions were shaken vigorously for 2 hours. Then they were filtered using 1.2 m glass fibre filters (Whatman) and washed seven times with water until the reaction with 1 % $AgNO_3$ solution was negative. MMT-surfactant samples were dried at the laboratory temperature and used for antibacterial tests. The laboratory temperature varied from 20 to 24 °C.

Modification of montmorillonite with Ag^+ and Ag^0

Montmorillonite used for the intercalation with Ag^+ was of Wyoming type with an exchange capacity of about 0.7 mequiv/g (determined by saturation with ammonium). $AgNO_3$ solutions having concentrations of about 15 mmol/l were used for MMT saturation for a period of 2 hours. This step was based on a sorption equilibrium study. The obtained suspensions were filtered through 1.0 m membrane filters. MMT-Ag samples were dried at the laboratory temperature and used for the antibacterial tests. The laboratory temperature varied from 20 to 24 °C.

MMT-Ag materials were prepared by reduction of the Ag^+ intercalates in solutions of 10 mmol/l sodium borohydride [5].

Preparation of standardized bacterial suspensions
Escherichia coli CCM 3988 was obtained from the Czech Collection of Microorganisms (Brno, Czech Republic). Sterile nutrient broth (10 ml) was inoculated with *E. coli* and incubated overnight at 37°C. Bacterial suspensions were diluted with a sterile, 0.15 mol/l saline solution until the absorbance at 625 nm reached a value of 0.35, which corresponded to a concentration of about 2.0 $x10^8$ cfu/ml.

Antibacterial activity assay
An amount of 10 mg of MMT or modified MMT (MMT-CP, MMT-CTA, MMT-Ag^+, MMT-Ag^0) was put into test tubes with 20 ml of 1 % nutrient peptone broth. A volume of two ml of the *Escherichia coli* suspension (~ $2.0x10^8$ cfu/ml) was added to the tubes. The tubes were agitated for 2, 3 and 24 hours at 600 rpm and 25°C.The tubes were then centrifuged for 10 minutes at 500 rpm. After removal of clay pellets, absorbances of the supernatants were measured at = 625nm.

Subsequently, the supernatant was 10-fold diluted with a sterile, 0.15 mol/l saline solution to reach a final dilution of 10^{-10}. Volumes of 0.1 ml supernatant aliquots from dilutions of 10^{-6}, 10^{-7} and 10^{-8} were plated on the nutrient agar No.2 for a growth assay. The plates were incubated at 37°C for 24 hours. Then the number of colonies on each plate was counted. Reductions in bacterial counts were determined by comparing the experimental plate counts with the controls. Each growth assay was repeated at least three times using two replica plates for all above-mentioned dilutions of each sample. The values obtained were averaged to give the final data with standard deviations.

Similar experiments were conducted with samples of pure CP, CTA, $AgNO_3$, and colloidal Ag^0.

Results and Discussion

The evaluation of antibacterial effects of MMT and its four intercalates (MMT-CP, MMT-CTA, MMT-Ag^+ and MMT-Ag) showed that pure, unmodified MMT had no antibacterial effects. A slight decrease of the absorbance and number of colonies (Figs. 1 and 2) can be explained by the adsorption properties of the material [6]. Figs 3 and 4 demonstrate adsorption of the *Escherichia coli* bacteria on MMT during 2 and 24 hours. It is obvious that after a 2-hour incubation individual bacteria are well visible but after 24 hours a bacteria coverage is created on MMT. The intercalates MMT-CP and MMT-CTA exhibited the biggest antibacterial effect after three hours of action (Figs. 1 and 2). CP and CTA belong to a group of detergents whose precise mechanism of the effect on bacteria is not known. It is speculated that they cause physical and chemical changes of cellular membranes. It is also known that these compounds easily adsorb to various surfaces and their effective concentration can thus be decreased [7].

By determining the minimal inhibition concentrations (MIC) for CP and CTA and comparing them with the complexes MMT-CP and MMT-CTA, the decrease of the effective concentration of the compounds bound in a complex with montmorillonite was confirmed. The decrease of efficiency was about two-fold (MIC_{CP} = 225mg/l vs. MIC_{MMT-CP} =471mg/l, MIC_{CTA} = 275mg/l vs. $MIC_{MMT-CTA}$ = 535mg/l). After a 3-hour incubation, the effect of MMT-CTA was slightly higher than that of MMT-CP. After a 24-hour incubation, however, both complexes were inefficient (Figs. 1 and 2). This fact can be ex-

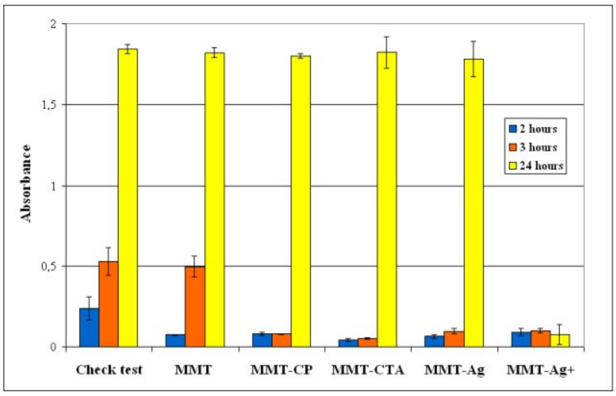

Figure 1. Antibacterial effect of natural and intercalated MMT based on absorbance measurements

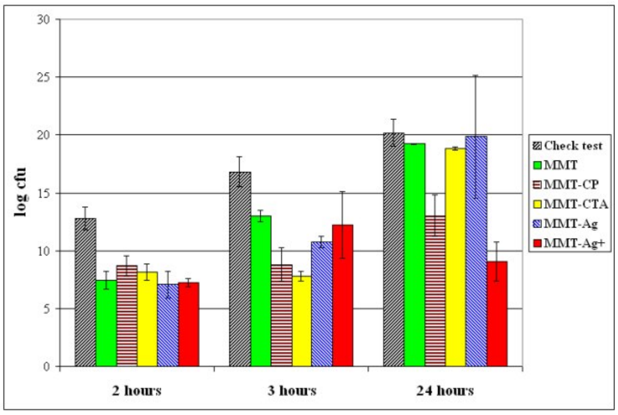

Figure 2. Antibacterial effect of natural and intercalated MMT based on calculation of colonies numbers.

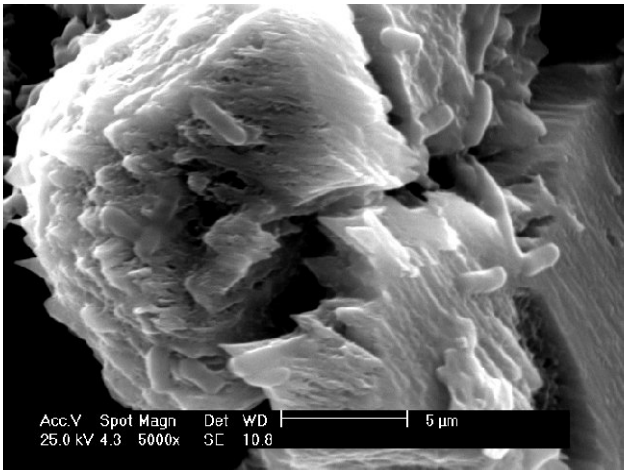

Figure 3. Adsorption of *Escherichia coli* on MMT after a 3-hour incubation.

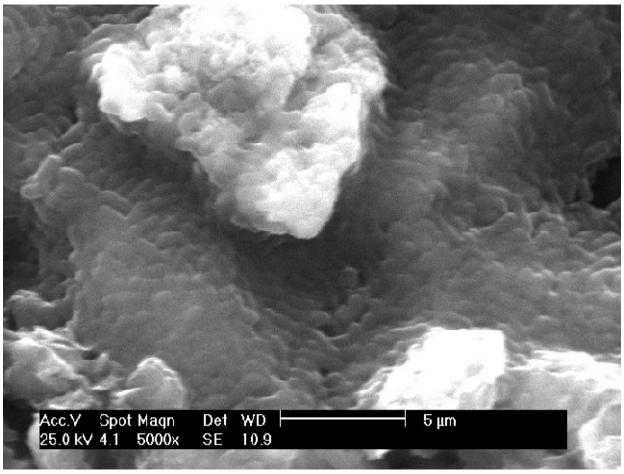

Figure 4. Adsorption of *Escherichia coli* to MMT after a 24-hour incubation.

plained by an observation that, after 24 hours, the surface of MMT was covered with a thick layer of bacteria (Fig. 4) and, therefore, the effective compound could not affect the remaining microorganisms. Besides some other metals, also silver belongs to the compounds with a proven bactericidal effect [6]. Mainly $AgNO_3$ has been applied in human medicine to treat inflammation of the skin and mucosae [6]. Recently, however, the effect of silver in complexes with minerals used for disinfection of water contaminated with coliform bacteria has been investigated [7].

The results of a study of the antibacterial effect of intercalated complexes MMT-Ag^+ and MMT-Ag are shown in Figs. 3 and 4. After 2-hour and 3-hour incubations, the antibacterial effects of the two complexes were similar to those of MMT-CP a MMT-CTA. After a 24-hour incubation, a significant difference was observed in the case of MMT-Ag^+. While the two detergent complexes including MMT-Ag were inefficient, only MMT-Ag^+ was able to retain its antibacterial effect. This finding is in keeping with the results of Rivera-Garza *et al*. [7]. Consequently, the use of intercalated MMT-Ag^+ for disinfection seems to be promising.

Conclusion

1. Our experiments with modified montmorillonite document that intercalates with silver cations exhibit strong antibacterial effects on the bacterium *Escherichia coli*. Other MMT intercalates showed only short-term effects.

2. Consequently, in future, we are going to test the effect of MMT-Ag^+ on *Escherichia coli* and other types of bacteria, such as *Salmonella enterica, Enterococcus faecium*, *Streptococcus faecalis* etc., under various experimental conditions (temperature, salinity, pH, etc.).

3. Using other metals with known biocidal properties (e.g., Ag^+, Cu^{2+}, Zn^{2+}), we intend to prepare phyllosilicate-metal composites and test their application in the area of water disinfection technology. A main advantage of such composites can be their ability of immobilization of disinfecting metals in the rigid phyllosilicate structures that minimizes the risk of their accumulation in human organism when added directly in water as, for example, is the case of the commonly-used $AgNO_3$ and $CuSO_4$.

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References

1. P. Praus, M. Turicová, M, Ritz, S. Študentová, *J. Colloid. Interface Sci*, **304** ,(2006), 29.
2. P. Praus, M. Turicová, *J. Braz. Chem. Soc*., **18**, (2007), 378.
3. P. Herrera, R.C. Burghardt, T.D. Philips, *Veterinary Microbiology*, **74**, (2000), 259.
4. P. Herrera, R. Burghardt, H.J. Huebner, T.D. Phillips, *Food Microbiology*, **21**, (2004), 1.
5. P. Patakfalvi, A. Oszkó, I. Dékány, *Colloids and Surfaces A: Physicochem. Eng. Aspects*, **220**, (2003), 45.
6. H. Lüllmann, K. Mohr, M. Wehling, *Farmakologie a toxikologie*. Praha: Grada Publishing. 2004.
7. M. Rivera-Garza, M.Z. Olguín, I.García-Sosa, D. Alcántara,G.Rodríguez-Fuentes, *Microporous and Mesoporous Materials*, **39**, (2000), 431.