ANTIBACTERIAL ACTIVITY OF CHLORHEXIDINE/NATURAL Mg-VERMICULITE AND
CHLORHEXIDINE/CATION EXCHANGED VERMICULITES

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Abstract

The preparation and characterization of new antibacterial organovermiculites was investigated. The structures and antibacterial effects of the chlorhexidine diacetate (CA)/natural Mg-vermiculite (CA/MgVER) and the chlorhexidine diacetate/cation exchanged vermiculites (CA/ZnVER, CA/CuVER and CA/AgVER) were compared. The vermiculite structures were characterized according to the X-ray diffraction (XRD) patterns. The antibacterial activity of prepared organoclays against Gram-positive bacteria (\textit{Enterococcus faecalis}) and Gram-negative bacteria (\textit{Escherichia coli} and \textit{Pseudomonas aeruginosa}) was evaluated by finding the minimum inhibitory concentration (MIC) that completely inhibited bacterial growth in different time periods.

Keywords: vermiculite, chlorhexidine diacetate, intercalation, antibacterial effect

1. INTRODUCTION

Modifications of clay minerals continue to attract much attention. There are a lot of opportunities to use modified clay minerals in a wide range of industrial and environmental areas as a filler in plastics, paints, fireproof materials and also in clinical treatments, for example as a carriers of drugs [1, 2]. The clay minerals were also used for preparation of antibacterial Ag/compounds [3, 4]. The large surface area and layered structure of clay minerals are very important for their modification. The 2:1 silicate layer of vermiculite consists of the MgO\textsubscript{2} (OH)\textsubscript{4} octahedra sheet bounded by two tetrahedral sheets of silica. An isomorphic substitution of Al\textsuperscript{3+} instead of Si\textsuperscript{4+} in tetrahedra generates net negative charges on the vermiculite layers which are balanced by some interlayer hydrated cations (Na\textsuperscript{+}, K\textsuperscript{+}, Mg\textsuperscript{2+}, Ca\textsuperscript{2+}) in the interlayer space between 2:1 layers [5, 6, 7, 8]. These interlayer cations can be exchanged with antibacterial anorganic and/or organic ions and give rise to resulting antibacterial material [9]. Chlorhexidine acetate is a bisbiguanide antiseptic and disinfectant which is bactericidal or bacteriostatic against a wide range of Gram-positive and Gram-negative bacteria [10]. The structures and antibacterial effects of the chlorhexidine diacetate (CA)/natural Mg-vermiculite (CA/MgVER) and the chlorhexidine diacetate/cation exchanged vermiculites (CA/ZnVER, CA/CuVER and CA/AgVER) were compared.

2. EXPERIMENTAL

Materials

Natural Mg-vermiculite (MgVER) from Letovice (Czech Republic) was ground in a planetary mill for 20 min, then passed through a 0.045 mm sieve and the size fraction under 40 μm was utilized for experiment. The crystallochemical formula (Si\textsubscript{0.13}Al\textsubscript{0.87}) (Mg\textsubscript{2.53}Fe\textsuperscript{3+}_{0.45}Al\textsubscript{0.02}) O\textsubscript{10}(OH)\textsubscript{2} (Mg\textsubscript{0.19}K\textsubscript{0.01}Ca\textsubscript{0.02}) was calculated from the result of the elemental chemical analysis.
The cation exchange capacity (CEC) MgVER was 140 cmol(+) /kg. Further used chemicals were in analytic quality from Sigma Aldrich company: copper dichloride (CuCl₂), zinc chloride (ZnCl₂), silver nitrate (AgNO₃), chlorhexidine diacetate (C₂₂H₃₀Cl₂N₁₀·2C₂H₄O₂) and ethanol as a solvent.

**Modifications**

The MgVER was converted into the ZnVER, CuVER and AgVER using cation exchange procedure from 1.0 M aqueous solutions of their corresponding salts ZnCl₂, CuCl₂ and AgNO₃, respectively. Solutions of chlorhexidine diacetate (CA) in ethanol with the concentration of CA were prepared accordant with the CEC of vermiculite. The solutions of CA were mixed with the MgVER, ZnVER, CuVER and AgVER and then were stirred and heated (6h, 75°C). After centrifugation samples were dried for 24 h at 80°C and labeled as CA/MgVER, CA/ZnVER, CA/CuVER and CA/AgVER.

**Analytical methods**

The XRD patterns were performed on the samples using the X-ray diffractometer INEL equipped with a curved position-sensitive detector CPSD 120 (reflection mode, Ge-monochromatized, CuKα₁ radiation). The measurements were taken in ambient atmosphere (25°C, 43% of humidity) under constant conditions (2000 s, 35 kV, 20 mA). The samples were fixed in a flat rotation holder and measurement was proceeded for 1500 s.

**Antibacterial test**

The minimum inhibitory concentration (MIC) of prepared samples was determined by their lowest concentration that completely inhibits bacterial growth. The dilution and cultivation were preceded on the microtitration plate with 96 hollows. The first set of hollows on plate contained 10% (w/v) organomontmorillonites and organovermiculites water dispersion. These dispersions were further diluted by a threefold diluting method in glucose stock in such maner, that second to seventh set of hollows contained sample dispersed in concentration of 3.33%, 1.11%, 0.37%, 0.12%, 0.041% and 0.014%. The eight set of hollows contained pure glucose stock as check test. A volume of 1µl of glucose suspensions of *E. faecalis* CCM 4224 (1.1x10⁹ cfu ml⁻¹), *E. coli* CCM 3954 (1.3x10⁹ cfu ml⁻¹) and *P. aeruginosa* CCM 1960 (1.2x10⁹ cfu ml⁻¹), provided by Czech collection of microorganisms (CCM), was put into hollows. Bacterial suspensions was after the elapse of 30, 60, 90, 120, 180, 240 and 300 min and then during 5 days always in 24 h interval transfered from each hollow to 100 µl of the fresh glucose stock and bacteria were incubated in termostat at 37°C for 24 and 48 h. Antibacterial activity was evaluated by turbidity, which is display of bacterial growth [12.].

3. **RESULTS AND DISCUSSIONS**

**X-ray diffraction analysis**

The XRD patterns (Fig. 1) show the interlayer space values that are given by the (001) basal reflection. The d(001) = 1.43 nm of natural MgVER did not change after substitution of Mg²⁺ for Ag⁺ and Zn²⁺ at AgVER and ZnVER, respectively, and only a small change to the d(001) = 1.27 nm in CuVER was observed. A week intercalation of CA into interlayer space can be assumed at natural MgVER as it can be observed according to new reflection with d = 2.13 nm at CA/MgVER. The XRD patterns demonstrate mounting CA to the surface of the silicate layer rather than CA in the interlayer of vermiculite.
Antibacterial assessment

An antibacterial effect of samples was monitored against Gram-positive *Enterococcus faecalis* bacteria and Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa* bacteria. The effect of MICs was evaluated after 30 min., 60 min., 90 min., 120 min., 180 min., 240 min., 300 min., 1 d., 2 d., 3 d., 4 d., and 5 d. Three groups of antibacterial activities were compared (Table 1). The first group includes MIC values determined after the effect of organovermiculites on the bacteria strains observed for 30 min. The second group includes MICs which were effective after 1 day (24h) and the last group shows MICs after 5 days (5d). We have come to conclusion that CA/MgVER are a less efficient than CA/ZnVER, CA/CuVER and CA/AgVER from comparison of obtained results. The prepared organoclayes of CA/MgVER showed no antibacterial activity in short time period of reacting (30 min). Antibacterial effect was showed after 1 day of reacting and the best result was obtained with *Escherichia coli*. Very resistant bacteria *Pseudomonas aeruginosa* showed no antibacterial effect even after 5 days of reaction. On the other side CA/ZnVER, CA/CuVER, CA/AgVER samples proved good antibacterial activity against all of bacteria in short time. The CA/AgVER had the best antibacterial effect. The CA/ZnVER show the lowest antibacterial action against tested bacteria.
Table 1 MICs (% w/v) of the organovermiculites on the bacteria strains.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Enterococcus faecalis (MIC)</th>
<th>Escherichia coli (MIC)</th>
<th>Pseudomonas aeruginosa (MIC)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 min 24 h 5 d</td>
<td>30 min 24 h 5 d</td>
<td>30 min 24 h 5 d</td>
</tr>
<tr>
<td>CA/MgVER</td>
<td>&gt; 10 0.37 0.37</td>
<td>&gt; 10 0.041 0.041</td>
<td>&gt;&gt;10 &gt;10 &gt;10</td>
</tr>
<tr>
<td>CA/AgVER</td>
<td>&gt; 10 0.37 0.014</td>
<td>3.33 0.014 0.014</td>
<td>3.33 0.12 0.12</td>
</tr>
<tr>
<td>CA/ZnVER</td>
<td>&gt; 10 0.37 0.041</td>
<td>3.33 0.014 0.014</td>
<td>&gt;&gt;10 &gt;10 &gt;10</td>
</tr>
<tr>
<td>CA/CuVER</td>
<td>3.33 0.37 0.014</td>
<td>3.33 0.014 0.014</td>
<td>3.33 1.11 0.12</td>
</tr>
</tbody>
</table>

4. CONCLUSIONS
1. The XRD revealed that natural Mg-vermiculite slightly intercalated CA. Other prepared Ag-, Zn- and Cu-vermiculites did not intercalate CA and the CA has to be outside an interlayer space.
2. The natural CA/MgVER showed lower antibacterial effect against all bacteria than other samples. The CA/AgVER had the best antibacterial effect against tested bacteria.
3. The cation exchange led to the monoionic cation occupation in the interlayer and improving of antibacterial activity of vermiculite. The organics antibacterial materials blocked between the layers appear to be less active than these materials tied on the surface of layers.

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LITERATURE