SILVER AND/OR COPPER VERMICULITES AND THEIR ANTIBACTERIAL EFFECT

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ABSTRACT
Vermiculite (V) from Brazil with aqueous solutions AgNO₃ and/or Cu(NO₃)₂ was used for preparation of nanocomposites V/Ag, V/Cu, V/AgCu and V/CuAg using two procedures and their effect on bacteria was compared. Structural changes were evaluated using X-ray diffraction (XRD) analysis, Infrared spectroscopy (IRS) and Scanning electron microscopy (SEM). Elemental chemical composition was determined using X-ray fluorescence (XRF) analysis and the solutions were analyzed by Atomic emission spectroscopy with inductively coupled plasma (AES-ICP).

Antibacterial properties of Ag- and Cu-vermiculites and combined Ag/Cu-vermiculites were tested on two bacteria groups: (1) Gram-positive, G⁺, (Staphylococcus aureus and Enterococcus faecalis) and (2) Gram-negative, G⁻, (Pseudomonas aeruginosa and Klebsiella pneumoniae). The samples under antibacterial testing showed different effective times and minimum inhibitory concentration (MIC) values. The Gram-negative bacteria showed higher sensitivity to the prepared samples than Gram-positive bacteria. The combined Cu/Ag vermiculites stopped the bacterial growth at lower MIC value.

KEYWORDS: vermiculite; silver; copper; structure characterization; antibacterial effect

1. INTRODUCTION
Clay mineral vermiculite belongs to a group of planar hydrous phyllosilicates (layered silicates). Vermiculite structure consists from an octahedral sheet placed between two tetrahedral sheets which together form a 2:1 layer. Between the layers is the interlayer space containing hydrated exchangeable cations as an interlayer material. The negative layer charge of vermiculite 0.6 – 0.9 e⁻ resulting from the substitution of Al³⁺ for the tetrahedral central cation Si⁴⁺ and Al³⁺ and/or Fe³⁺ for octahedral central cation Mg²⁺ is balanced by exchangeable cations present in the interlayer (Shirozu and Bailey, 1966; Bailey, 1980; Martin et al., 1991).

Clay minerals have many applications mainly in ceramics as sorbents, catalysts or fillers in various materials, though as well as a matrix for the preparation of metal nanoparticles. The metal nanoparticles can be anchored on the external surface of the clay mineral silicate layers or in their interlayer space.

The most common procedure employed for the preparation of the metal nanoparticles is the reduction from appropriate salts solutions. For example, silver nanoparticles on montmorillonite substrates were prepared using a silver nitrate solution in water (Magaña et al., 2008; Valášková et al., 2007, 2010). Other authors have described procedures, including an additional step of silver nanoparticles’ preparation with reducing agents such as sodium borohydride or formaldehyde (Patakfalvi et al., 2003; Praus et al., 2010, Miyoshi et al., 2010), ethylene glycol or glycerol (Ayyppan et al., 1996; Valášková et al., 2007), and also by using UV radiation (Huang and Yang, 2008; Darroudi et al., 2009).

Copper bound on the clay minerals matrix as a hydrate complex can be prepared by cation exchange reaction (Morton et al., 2001; Strawn et al., 2004; Furnare et al., 2005). Drelich et al. (2011) prepared copper nanoparticles on the vermiculite matrix from the cation exchanged Cu-form by hydrogen reduction followed by heat treatment.

Silver and copper are known for their antibacterial properties. Most of authors have studied the antibacterial ability of Cu-forms of montmorillonite (Zhou et al., 2004; Hu et al., 2005, Özdemir et al., 2010) and vermiculite (Li et al., 2002). Cu-montmorillonites were studied in connection with their potential utilization in veterinary medicine (Xia et al., 2005; Hu and Xia, 2006; Tong et al., 2005; Yu et al., 2008). The antibacterial influence of Ag/montmorillonites and Ag/vermiculites was less commonly studied (Magaña et al., 2008; Valášková et al., 2010; Özdemir et al., 2010; Malachová et al., 2009). Özdemir et al. (2010) compared the antibacterial effect of Ag-, Cu- and Zn- forms of montmorillonite. The authors have found good antibacterial effect of Cu- and Ag-montmorillonite with...
against bacterial strains Pseudomonas aeruginosa and Staphylococcus aureus.

As described in the previous research works, silver and copper were sporadically studied on the vermiculite. Preparation of the combined content Ag and Cu on the vermiculite matrix as well as study of their antibacterial effect has not yet been reported. The present work examines the structure and antibacterial effect of Ag/Cu-vermiculites on the bacteria strains: Gram-positive (Staphylococcus aureus and Enterococcus faecalis) and Gram-negative (Pseudomonas aeruginosa and Klebsiella pneumoniae).

2. EXPERIMENTAL

2.1. MATERIALS

Vermiculite from Brazil was used as a starting material. The particle size fraction < 40 μm was obtained by milling in a planetary mill for 20 min and sieving by a sieve with a pore size of less than 40 μm. The half unit cell composition of the 2H-vermiculite sample (Si0.80Al0.20)(Mg2.44Fe0.36, Fe0.02, Ti0.06Al0.04)O10(OH)2 K0.18Ca0.15Na0.08 was calculated from the results of elemental analysis. Silver nitrate, AgNO3 and copper nitrate, Cu(NO3)2 (f y Mach chem., C2) with the purity of 99.99 % were used as sources of silver and copper.

2.2. SAMPLE PREPARATION

Four samples were prepared using two procedures: P1, P2:

P1. Samples V/Ag and V/Cu were prepared from vermiculite (V) and the 0.1 mol.dm-3 aqueous solution AgNO3 and 0.1 mol.dm-3 Cu(NO3)2, respectively, at the rate of 1:10 (grams of vermiculite : millilitre of solution), and the suspension was shaken for 24 hours.

P2. Sample V/AgCu was prepared mixing the sample V/Ag in the 0.1 mol.dm-3 aqueous solution Cu(NO3)2. Similarly, the sample V/CuAg was prepared from the sample V/Cu and the 0.1 mol.dm-3 aqueous solution AgNO3.

All prepared samples were centrifuged and washed with distilled water until nitrate ions were not present, and then dried at 70 °C for 24 h. The samples with silver were protected before the light by aluminium foil during the preparation.

2.3. METHODS

X-ray powder diffraction

The X-ray powder diffraction (XRD) patterns were measured on the X-ray diffractometer from INEL equipped with a 120° curved position-sensitive detector CPSD 120 (reflection mode, Germanochromatized CuKα1 radiation). All patterns were recorded at 30 kV and 20 mA, exposure time 2000 s, with the samples in a flat rotation holder.

Scanning electron microscopy

Morphology and the surfaces of samples were studied using Scanning electron microscopy (SEM) on PHILIPS XL-30 equipped with energy dispersive spectrometer (EDS) for elemental analysis and mapping.

Fourier transform infrared spectroscopy

The mid-infrared spectra were obtained by Fourier transform infrared spectroscopy (FTIR) on a Perkin Elmer 2000. The measurement was carried out in the spectral range 4000-400 cm-1 with a resolution of 4 cm-1 at room temperature using the KBr disc technique.

X-ray fluorescence analysis

The elemental composition of samples and the content of silver and copper were determined by X-ray fluorescence elemental analysis (XRF) on an energy dispersive spectrometer SPECTRO X LAB with Rh X-ray lamp and secondary and polarization targets.

Atomic emission spectroscopy

Atomic emission spectroscopy with inductively coupled plasma (AES-ICP) on a Jobin-Yvon JY-24 spectrometer was used for determination of elements at leaches after modification.

2.4. ANTIBACTERIAL TEST

The antibacterial activity of the prepared nanocomposites was examined using two different groups of experimental bacterial strains. The Gram-negative (G-) strain represented the bacteria Klebsiella pneumoniae (K. pneumoniae, CCM 4415) and Pseudomonas aeruginosa (P. aeruginosa, CCM 1960). The Gram-positive (G+) bacterial strain was studied on the bacteria Staphylococcus aureus (S. aureus, CCM 4395) and Enterococcus faecalis (E. faecalis, CCM 4224).

The broth dilution method (Kneiflová, 1988) was used for determination of the minimum inhibitory concentration of silver and/or copper (MICAg/Cu) in samples as the lowest concentration of silver and/or copper that completely inhibits bacterial growth. The bacterial suspension was prepared by a twenty-hour cultivation in glucose broth. The density of the glucose suspension used for individual bacteria was: S. aureus (1.0 × 109 cfu ml-1), E. faecalis (1.1 × 109 cfu ml-1), K. pneumoniae (1.2 × 109 cfu ml-1), P. aeruginosa (1.1 × 109 cfu ml-1). The silver and copper vermiculite samples were prepared for testing as a 10 % water dispersion, which was further diluted to 3.33 %, 1.11 %, 0.37 %, 0.12 % and 0.041 %. The detailed procedure of antibacterial test is published in Valášková et al. (2010).

3. RESULTS AND DISCUSSION

3.1. QUANTITY OF SILVER AND COPPER AT VERMICULITE SAMPLE

The quantity of silver and copper fixed in vermiculite is given in Table 1. Sample V/Ag and V/Cu contained the highest content of Ag and Cu
(0.52 and 0.33 mmol.g⁻¹, respectively). The content of silver in sample V/Ag after the following treatment with Cu(NO₃)₂ solution decreased from 0.52 to 0.15 mmol.g⁻¹ in sample V/AgCu. Additional treatment V/Cu with AgNO₃ solution slightly decreased the content of copper from 0.33 mmol.g⁻¹ to 0.25 mmol.g⁻¹ in sample V/CuAg. The content of silver 0.52 mmol.g⁻¹ in the sample V/Ag was reduced to 0.33 mmol.g⁻¹ in V/CuAg.

These quantity differences resulted from the ways of binding silver and copper cations in vermiculite, and the ability of cations to bind molecules in the interlayer. It was described that (i) the copper on the vermiculite substrate is predominantly positioned in the interlayer where cations are surrounded by water molecules, and also can be adsorbed on hydroxyl edge sites (Clementz et al., 1973; McBride, 1976; Morton et al., 2001) and that (ii) the silver in the interlayer can be present in the form Ag⁺ and also Ag₀ without water molecules and on the surface of vermiculite as the silver nanoparticles (Magaña et al., 2008; Valášková et al., 2007; Tokarský et al., 2010a, 2010b).

The sample V/AgCu contained approximately 70 % lower content of silver than the original sample V/Ag. The sample V/CuAg showed a decrease about 25 % of copper in comparison with the original sample V/Cu. This observation confirmed that the copper was more strongly anchored to vermiculite than silver.

### 3.2. CONTENT OF LEACHED ELEMENTS

The elements released from the vermiculite structure in Table 2 showed the following results: The content of Na⁺ leached from the natural vermiculite was higher in the AgNO₃ solution than in the Cu(NO₃)₂ solutions. This finding confirmed cation exchange of Na⁺ for Ag⁺ and is in agreement with observations reported in literature (Rivera-Garza et al., 2000; de la Rosa Gomez et al., 2008). Similarly, the higher divalent Ca²⁺ exchanged for Cu²⁺ was confirmed by higher content of Ca²⁺ determined after treatment in the Cu(NO₃)₂ solution than in the AgNO₃ solution. It was also observed that the solution Cu(NO₃)₂ contributed to the release not only of divalent cations Ca²⁺ and Mg²⁺ but also Si⁴⁺ from tetrahedra.

### 3.3. X-RAY DIFFRACTION ANALYSIS

The XRD pattern of the original V (Fig. 1) shows the intensive basal diffraction with d(001) = 1.42 nm. The interlayer space value corresponds to the two-layer hydrate phase (Walker, 1956). The other two low-intensive and broad diffractions with d = 1.24 nm and d = 2.32 nm match the one-layer hydrate domains and mix-layered water molecules domains, respectively (Marcos et al., 2009).

Exfoliation of the layered vermiculite structure resulted in a decrease in the intensities of the basal diffractions. If we consider the observed integral intensity of basal reflection (001) with d(001) = 1.42 nm for V as 100 % (relative intensity), we can calculate the percentage decrease in the relative intensity of (001) reflection to 73 % at V/Cu (Fig. 1b) and to 9 % in V/Ag (Fig. 1e). The next broad diffraction with d = 1.24 nm in V shifted to the smaller interlayer space (e.g. to the d = 1.1 nm in V/AgCu) and proved formation of mixtures of less hydrated phases. The metal silver was observed only on the XRD pattern of the sample V/Ag as low intensive diffractions d(111) = 0.236 nm and d(200) = 0.204 nm (Fig. 1e).

### 3.4. INFRARED SPECTROSCOPY

The IR spectra of the samples in Figure 2 showed the shift of the stretching vibration of water O-H groups at 3420 cm⁻¹ in V to 3440 cm⁻¹ in V/Ag and to 3446 cm⁻¹ in V/CuAg.

In the case of samples V/Cu, V/CuAg and V/AgCu, this band at 3420 cm⁻¹ became wide and the new bands at 3527 cm⁻¹ and 3549 cm⁻¹ appeared, and are probably associated with Cu-for-Mg substitution (Farmer and Russell, 1964). The band 1646 cm⁻¹ belongs to the deformation vibrations of the water O-H groups. The shift of this band was, in sample V/AgCu, to 1635 cm⁻¹, contrary to the V/CuAg, where the new band at 1541 cm⁻¹ indicated different coordinated water forms, probably owing to the type of the interlayer cations (Madejová, 2003). Accordingly, in our samples the wavenumbers shifts depend on the type of predominant cation in the interlayer. The band at 1002 cm⁻¹ belongs to the stretching vibrations of hydroxyl groups shared between Al³⁺ and Mg²⁺ ions (Farmer and Russell, 1964); the band at 812 cm⁻¹ was ascribed to the bending vibrations of hydroxyl groups.

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**Table 1** The content of silver and copper at vermiculite samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>V</th>
<th>V/Ag</th>
<th>V/Cu</th>
<th>V/AgCu</th>
<th>V/CuAg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ag</td>
<td>0</td>
<td>0.52</td>
<td>0.15</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>Cu</td>
<td>0</td>
<td>0</td>
<td>0.33</td>
<td>0.31</td>
<td>0.25</td>
</tr>
</tbody>
</table>

**Table 2** Concentration of elements at solution leached from vermiculite.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Na</th>
<th>K</th>
<th>Mg</th>
<th>Ca</th>
<th>Si</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mmol l⁻¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V/Ag</td>
<td>15.65</td>
<td>1.69</td>
<td>12.76</td>
<td>2.15</td>
<td>0.21</td>
</tr>
<tr>
<td>V/AgCu</td>
<td>2.57</td>
<td>3.94</td>
<td>8.2</td>
<td>1.78</td>
<td>1.64</td>
</tr>
<tr>
<td>V/Cu</td>
<td>12.26</td>
<td>2.89</td>
<td>18.93</td>
<td>13.33</td>
<td>2.39</td>
</tr>
<tr>
<td>V/CuAg</td>
<td>1.13</td>
<td>2.66</td>
<td>9.18</td>
<td>3.58</td>
<td>1.71</td>
</tr>
</tbody>
</table>

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Farmer and Russell, 1964; de la Rosa Gomez et al., 2000; Farberová et al., 2008; Valášková et al., 2007.
Fig. 1 The XRD patterns of basal diffractions (from 2° to 10° 2θ) and Ag diffractions (from 35° to 50° 2θ) of V (a), V/Cu (b), V/CuAg (c), V/AgCu (d) and V/Ag (e).

bending vibrations of tetrahedral Si-O. The position shifted from 685 cm⁻¹ at V to the slightly lower vibrations 683 cm⁻¹ at V/Cu, V/CuAg and V/AgCu involves bending vibrations of Mg₃OH and Mg₂AlOH (Farmer, 1974; Madejová, 2003). The band around 458 cm⁻¹ was assigned to bending vibrations Si-O-M (where M can be Si, Mg, Al or Fe) (Van der Marel and Beutelspacher, 1976).

3.5. SCANNING ELECTRON MICROSCOPY

The images of the samples with the spot elemental analysis are shown in Figures 3 and 4. The characteristic lamellar morphology of vermiculite was, after treatment, partially disturbed. The treatment with Cu(NO₃)₂ solution caused the vermiculite flakes change to the flocy aggregates (Fig. 3c).

3.6. ANTIBACTERIAL ACTIVITY

The samples under antibacterial test showed different effective times and MICₐgₗ₆₃ values (Tables 3 and 4). The sample V/Ag had a positive effect against the G⁻ bacteria and stopped bacterial growth after 24 h (MICₐg = 0.21 mg.l⁻¹). The sample V/Cu also showed the positive effect for all bacteria regarding MIC (MICₐₗ₃ = 0.69 mg.l⁻¹) and short effective time of 5 h to suspend bacterial growth. The sample V/AgCu after 24 h showed the lowest MIC from all of the samples on the G⁺ bacteria P. aeruginosa (MICₐgₗ₃ = 0.06/0.07 mg.l⁻¹). On the other hand the V/CuAg was the most effective in stopping bacterial grow of G⁻ bacteria - S. aureus (MICₐgₗ₃ = 0.13/0.06 mg.l⁻¹) after 24 h.

The difference of activity of bacteria G⁺ and G⁻ are caused by structural variations of their cell walls. While the G⁺ bacteria have an outer membrane layer of cells formed by lipopolysacharides and phospholipids, and only a thin layer of peptidoglycan, the G⁻ bacteria cell is composed of many layers of peptidoglycan and lipoteichoic acid. The G⁺ bacteria cell is simpler and more resistant, probably thanks to a rough layer of peptidoglycan.

Distinct action of Ag and Cu on bacteria was ascribed to several mechanisms (Abad et al., 1994; Tong et al., 2005). Both elements can be attached by the negatively charged bacterial cell and disturb the cell walls and permeability and react with sulphur- and phosphorus- groups of enzymes, proteins and DNA as well. These metals can also cause oxidative stress by generation of hydroxide radicals (Samuni et al., 1981; Thurman and Gerba, 1989). The increased negative effect of combined Ag, Cu - vermiculites on bacteria could be ascribed to their synergic effect on bacteria (Lin et al., 1996).
4. CONCLUSIONS
In this study the antibacterial vermiculites with Ag and/or Cu were prepared and characterized. Vermiculite layered structure after treatment with AgNO_3 and/or Cu(NO_3)_2 became the mixtures of differently hydrated layers domains.

The sample V/Ag showed the highest content of Ag. Samples V/Cu and V/AgCu contained the highest amount of Cu.

The natural vermiculite had no effect on bacteria growth. The Gram negative *P. aeruginosa* and *K. pneumoniae* were more sensitive to sample V/Cu than to the V/Ag, and the antibacterial effect was observed after 5 h and 24 h, respectively. Combined Cu/Ag vermiculites stopped the bacterial growth at lower MIC values. The Gram negative *P. aeruginosa* growth was inhibited after 24 h at the lowest concentration of the sample V/AgCu when the MIC of the Ag and Cu was 0.06 mg.l^{-1} and 0.07 mg.l^{-1}, respectively. The Gram positive bacteria *S. aureus* was the most sensitive to the sample V/CuAg and inhibited growth after 24h at the lowest MIC of Ag and Cu of 0.13 mg.l^{-1} and 0.06 mg.l^{-1}. Gram positive *E. faecalis* stopped growth after 48 h action of sample V/Cu (MIC of Cu 0.69 mg.l^{-1}).

ACKNOWLEDGEMENTS
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The authors thank to J. Seidlerová for ICP-AES analysis, M. Heliová and S. Kozubová for SEM images, S. Holešová for IR spectra and D. Casten for linguistic correction of the text.

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Fig. 3  SEM images of V (a), V/Ag (b) and V/Cu (c) and their spectrum of spot elemental analysis.

Table 3  The results of antibacterial test for the Gram-positive bacteria.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>S. aureus (G⁺)</th>
<th>E. faecalis (G⁺)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dilution (%)</td>
<td>MIC₅₀ (mg. l⁻¹)</td>
</tr>
<tr>
<td>Sample</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V/Ag</td>
<td>1.11</td>
<td>0.62/0.00</td>
</tr>
<tr>
<td>V/Cu</td>
<td>3.33</td>
<td>0.00/0.69</td>
</tr>
<tr>
<td>V/CuAg</td>
<td>0.37</td>
<td>0.13/0.06</td>
</tr>
<tr>
<td>V/AgCu</td>
<td>3.33</td>
<td>0.55/0.66</td>
</tr>
</tbody>
</table>
**Fig. 4** SEM images of V/AgCu (a) and V/CuAg (b) and their spectrum of spot elemental analysis.

**Table 4** The results of antibacterial test for the Gram-negative bacteria.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>P. aeruginosa (G)</th>
<th>K. pneumoniae (G)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>Dilution (%)</td>
<td>MIC$_{Ag/Cu}$ (mg. l$^{-1}$)</td>
</tr>
<tr>
<td>V</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>V/Ag</td>
<td>0.37</td>
<td>0.21/0.00</td>
</tr>
<tr>
<td>V/Cu</td>
<td>3.33</td>
<td>0.00/0.69</td>
</tr>
<tr>
<td>V/CuAg</td>
<td>1.11</td>
<td>0.39/0.17</td>
</tr>
<tr>
<td>V/AgCu</td>
<td>0.37</td>
<td>0.06/0.07</td>
</tr>
</tbody>
</table>

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