THE INFLUENCE OF ZINC OXIDE CONCENTRATION ON ANTIBACTERIAL ACTIVITY OF THE VERMICULITE NANOCOMPOSITE

ČECH BARABASZOVÁ K.[1], HUNDÁKOVÁ M.[1], PAZDZIORA E.[2]

[1]*Nanotechnology Centre, VSB - Technical University of Ostrava, Czech Republic, EU*
[2]*Institute of Public Health Ostrava, Centre of Clinical Laboratories, Ostrava,*
Czech Republic, EU

Abstract

Antibacterial nanocomposites containing functional zinc oxide were prepared via mechanochemical process. Applying calcination process at 350 and 650 °C for 1.5 hour at prepared nanocomposites having different concentrations of zinc oxide on vermiculite matrix was further characterized. Nanocomposite particles morphology and shape were studied using scanning electron microscopy (SEM). Elemental chemical composition was determined using X-ray fluorescence analysis (XRFS). Structural and phase analysis was performed using X-ray diffraction method (XRD). Morphological and structural differences of vermiculite nanocomposites are depending on grow of zinc oxide in interlayer spaces or on surface of vermiculite particles. The nanocomposite particles size was characterized by the d_{10} and d_{90} diameters, volume-weighted mean diameter (d_{43}) and span value. The surface changes were evaluated using zeta-potential measurements as a function of particle electrophoretic mobility (μ). Antibacterial activity was tested on *Staphylococcus aureus* and *Klebsiella pneumoniae* bacterial strains. The samples under antibacterial testing showed identical minimum inhibitory concentration values (MIC) in different effective times.

Keywords: ZnO nanoparticles, vermiculite particles, zeta potential, antibacterial nanocomposite

1. INTRODUCTION

Antibacterial nanomaterials on the base of clay minerals have attracted significant attention in recent years. The clay nanocomposites are particulate materials with defined particle size and surface. Their layered structures, various morphologies and specific surface properties are investigated in many application areas for a long time [1]. Vermiculite-based nanocomposites are used for their antibacterial, biocompatibility, osteoconductive and nontoxic properties in various medical applications [2]. Their important and desirable properties are additionally supported by their low density, chemical stability and mainly their compositional similarity with the mineral phase of biological (human) tissue. The surface charge is one of the most important characteristic for clay particles, for further application and modification. Zinc oxide nanoparticles are very interesting for antimicrobial utilization. Until recently, these nanomaterials with zinc oxide nanoparticles were used in photocatalysis, solar cells, gas sensors, chemical absorbents; but also in cosmetics or food industry as antibacterial nanomaterials. In recent years, antibacterial properties were intensively studied in association to morphology of the zinc oxide nanoparticles and were checked for antimicrobial activity of: *Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* or *Bacillus Subtilis* [3-8].

For preparation of nanostructured materials and nanocomposite materials especially are often used various physico-chemical methods. One of the solid-state reaction methods is milling process combined with the chemical reaction - mechanochemical process [9]. It is mostly applied to the synthesis of metal oxide nanoparticles such as zinc oxide, cobalt oxide, tin oxide or nickel oxide. This method appears to be a significant process for the production ultrafine nanocomposite materials by grinding solid reactants without or minimal amount of solvent as efficient and economical way. The friction forces accompanying the milling process contribute to the higher agglomeration of particulate materials [10]. The zeta potential (ξ-potential) is very important parameter which can describe the particulate surface property, provide information about total surface changes, and predict the degree of the agglomeration nanocomposite particles and their subsequent utilization [11].

The objective of this work was to prepare the antibacterial nanocomposite material on the base of zinc oxide nanoparticles (in different concentration) on vermiculite substrate via mechanochemical process. Until now, there is one study focused on the preparation (using sol-gel method) and characterization of the photocatalytic behavior of the nanocomposite zinc oxide/vermiculite particles [12]. In our case desirable antibacterial activity was tested on the two bacteria strains: the Gram negative (G-) *Klebsiella pneumoniae* and the Gram positive (G+) *Staphylococcus aureus. K. pneumoniae* resides in the normal flora of the mouth, skin and intestines. *S. aureus* is a constituent of the skin flora that is frequently found in the nose and on the skin. Both mostly associated with nosocomial infections, and may be life threatening in immunodeficient conditions especially in immunocompromised patients and patients hospitalized for a long time.

2. MATERIALS AND METHODS

2.1. Materials

Natural vermiculite from Brazil (supplied by Grena Co., Czech Republic) was used as starting material. Vermiculite was milled in planetary ball mill (Retsch PM4) and then sieved through a 0.040 mm sieve (sample was named V). The structural formula of V sample was established on the base of elemental chemical analysis: $(Si_{6.32}Al_{1.58}Ti_{0.1})$ $(Mg_{4.75}Ca_{0.34}Fe_{0.91})O_{20}(OH)_4$ $(Ca_{0.04}K_{0.38})$. Anhydrous sodium carbonate (Na_2CO_3) and sodium chloride (NaCl), were used as catalyst reaction agents; anhydrous zinc chloride ($ZnCl_2$; A) and dihydrate zinc acetate ($Zn(CH_3COO)_2.2H_2O$; O) were used as two different precursors for zinc oxide/vermiculite (ZnO/V) nanocomposites preparation (all supplied from Sigma-Aldrich, Co.).

2.2. Preparation of vermiculite nanocomposite samples

V sample, catalyst (Na_2CO_3, NaCl) and oxides precursors ($ZnCl_2$ or $Zn(ac)_2$) were mixtures in the powder mass ratio 1:1:1:1. These mixtures were ground in planetary ball mill Fritsch Pulverisette 7 equipped with carbide wolfram container for 15 min using 3 wolfram carbide balls (10 mm diameter) in milling process. The milled samples were washed out with distilled water and dried in oven at 75 °C overnight. Heat treatment of the samples was carried out in air atmosphere in a porcelain crucible at 350 or 650 °C for 90 min. The ZnO/V nanocomposite samples were named: VO350 and VO650 prepared from $Zn(ac)_2.2H_2O$ precursor and VA350 and VA650 prepared from $ZnCl_2$.

2.3. Characterization methods

The chemical composition of V and nanocomposite samples was obtained from elemental analysis by the X-ray fluorescence spectroscopy (SPECTRO XEPOS) and atomic absorption spectrometry (UNICAM 989 QZ). Structural and phase analysis was performed using X-ray diffraction (XRD) method using the diffractometer RIGAKU Ultima IV (scintilation detector, CuKα radiation, NiKβ filter, Bragg-Brentano arrangement). Samples in standard holder were measured in ambient atmosphere (40 kV, 40 mA, 2 °/min). The morphology of V and nanocomposite particles was examined using scanning electron microscope (SEM) Philips XL 30. The samples were plated with Au/Pd conducting coats and SEM images were obtained using a secondary electron detector (SE). The particle size (PS) of V and nanocomposite samples was determined by the HORIBA Laser diffraction particle size analyzer (LA-950 instrument) with a two short-wavelength blue and red light source in conjunction with forward and backscatter detection. The parameters used from the PS analysis were: the volume-weighted mean diameter (De Brouckere mean diameter (d_{43})), median diameter (d_{50}) and the span value. The span value represent the width of the particle size distribution (d_{90} - d_{10})/d_{50}), where the d_{10} or d_{90} values represent the 10th or 90th percentile of total volume assuming a spherical shape of particles. Zeta potential (ξ-potential) was measured by a nanoparticle analyzer (HORIBA Nanopartica SZ-100) equipped with a microprocessor unit to directly calculate the ξ-potential. The c was measured at natural pH. Each data point is an average of approximately 8 measurements. All measurements were made at ambient temperature (24.9°C), conductivity (0.19 $mS.cm^{-1}$), suspension viscosity (0.89 mPa.s) and electrode voltage constant (3.4 V).

2.4. Antibacterial test

The antibacterial activity of V and ZnO/V nanocomposites were tested on two bacteria strains: the Gram negative (G-) *Klebsiella pneumoniae* (*K. pneumoniae*) and the Gram positive (G+) *Staphylococcus aureus (S. aureus)*. The results were determined using standard microdilution method with 96 hollows which enabled to determine the minimum inhibitory concentration (MIC) that completely inhibits bacterial growth in accordance with their lowest concentration. The first set of hollows on the plate contained 10% (w/v) sample water dispersions. These dispersions were further diluted by a threefold diluting method in glucose stock in such a manner that the second to seventh set of hollows contained sample dispersed in concentrations of 3.33%, 1.11%, 0.37%, 0.12%, 0.04% and 0.01%. The eighth set of hollows contained pure glucose stock as a control test. A volume of 1 µl of glucose suspensions of *K. pneumoniae* CCM 4415 (1.2×10^9 CFU ml^{-1}) and *S. aureus* CCM 3953 (1.1×10^9 CFU ml^{-1}), provided by the Czech collection of microorganisms (CCM), was put into the hollows. Bacterial suspensions were after the elapse of 30, 60, 90, 120, 180, 240 and 300 min, and then during 5 days in 24 h intervals, transferred from each hollow to 100 µl of the fresh glucose stock and bacteria, and incubated in a thermostat at 37 °C for 24 and 48 h. Antibacterial activity was evaluated by turbidity, which is a display of bacterial growth.

3. RESULTS AND DISCUSSION

The V particles of irregular prolonged shape with smooth lamellar morphologies (**Figure 1**) were used as solid substrate for ZnO nanoparticles anchoring. The V particle size was determined in interval values from 9.12 µm (d_{10}) to the 41.83 µm (d_{90}) with mean diameter 22.38 µm (d_{43}) and span value 1.80. The very small V particles were segregated on bigger V particles.

Using two types of Zn-precursors the nanocomposite with different concentration and crystallite size of ZnO nanoparticles were prepared. The ZnO concentration is 21.29 wt% (VO350), 30.67 wt% (VA350), 22.59 wt% (VO650) and 29.62 wt% (VA650).The reaction between the Zn-precursor and catalyst depends on starting condition of reaction. The Zn^{2+} ions from $Zn(ac)_2$ precursor form the coordination compound as ZnO precipitates which is indicating release of CO_2 and H_2O. The OH^- groups decompose to H_2O and oxygen. Five oxygens not released in the heat treated process give to synthesis ZnO. Anhydrous $ZnCl_2$ precursor has only starting role during the preparation process and does not have any important effect on final size of ZnO nanoparticles [13]. The average crystallite size of ZnO obtained from the $ZnCl_2$ precursor was bigger than of those obtained from the $Zn(ac)_2$ precursor.

The lamellar morphology of V matrix is remaining through process (**Figure 1**). It can be observed un-singular shape with a rugged surface and round edged of the V matrix as a result of milling process [10] and the dehydration of V structure as results of heat treatment. The ZnO nanoparticles homogenously decorated the edges of V matrix. In V0350 nanocomposite the ZnO nanoparticles agglomerated or grew together on the V edges.

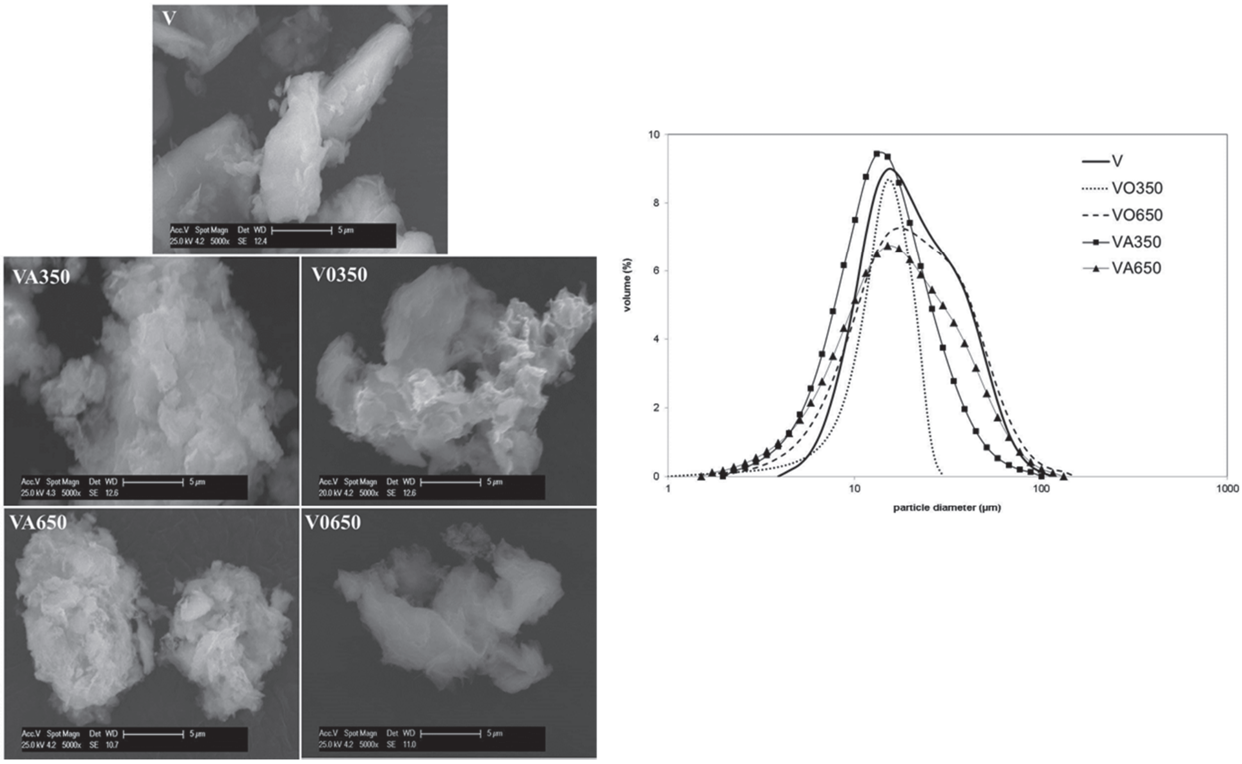

Figure 1 SEM images and particle size distributions (lognormal distributions) of the V matrix and ZnO/V nanocomposite samples

The mechanochemical process contributed to the reduction of the particles size parameters and to the re-arrangement of particle size distributions of all nanocomposite samples (**Figure 1**). The average nanocomposite size was determined in interval values from 7.42 µm (d_{10}) to the 39.05 µm (d_{90}) with mean diameter 18.43 µm (d_{43}) and span value 1.86. VO350 and VA350 samples exhibited very narrow particle size distributions compared to V matrix and VO650 and VA650. The particle of VA650 and V0650 nanocomposites tended to agglomerate. The rate of agglomeration is in agreement with the values of the surface charge, ξ-potential value is -52.3 mV for V matrix and for both nanocomposites reaches -59 mV (VA650) and -56 mV (VO650). The ξ-potential for VA350 is -26.6 mV and -34.5 mV for VO350.

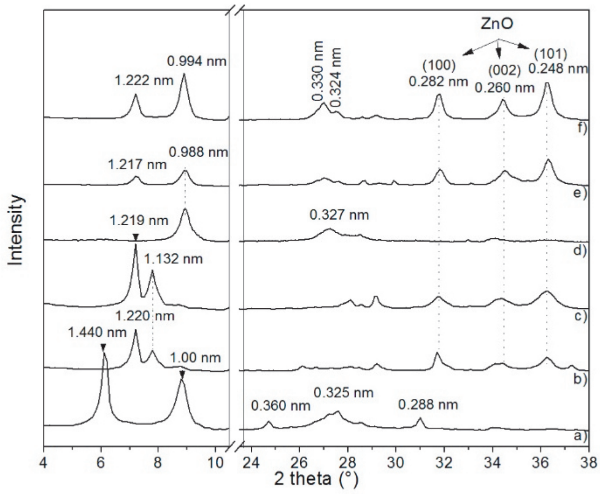

Figure 2 XRD patterns of samples (range 4-11.5° and 23.5-38° 2θ): a) V350, b) VO350, c) VA350, d) V650, e) VO650, f) VA650

XRD pattern of V350 (**Figure 2a**) shows that original V structure partly remained preserved what confirmed the basal reflections with characteristic values d_{002} = 1.440 nm, d_{008} = 0.360 nm and d_{0010} = 0.288 nm. Further reflections with d = 1.00 nm and d = 0.325 nm are present as a result of loss of hydration water from the hydrated cations in the V interlayer [14]. The XRD pattern of V650 (**Figure 2d**) shows dehydrated V structure with d = 0.988 nm and d = 0.327 nm [15,16].

ZnO nanoparticles preparation by dry milling with V affected the V structure. At samples prepared at 350 °C (**Figure 2b,c**), reflection with d = 1.132 nm (VO350, VA350) correspond to the one water layer hydrate in the

V interlayer [17]. Further reflections with *d* = 1.220 (VO350) nm and *d* = 1.219 nm (VA350) are observed. At samples prepared at 650 °C (**Figure 2e, f**), reflections of dehydrated V structure is present with correspond *d*-values 0.988 nm and 0.330 nm (VO650) and 0.994 nm and 0.330 nm (VA350) [15,16]. Moreover, reflections with *d* = 1.217 nm (VO650) and 1.222 nm (VA650) are observed, similar as in samples at 350°C. These could be ascribed to the ZnO partly intercalated into the V structure [18].

The XRD patterns of nanocomposites show reflections of ZnO (PDF card no. 01-079-2205) on the V surface. The ZnO crystallite size calculated based on (101) reflection according the Scherrer equation [19] is 13.46 nm (VO350), 9.54 nm (VA350), 18.66 nm (VO650) and 23.22 nm (VA650). The calculated values confirm that crystallinity of ZnO increased at samples prepared at higher temperature (650°C).

The antibacterial activity (expressed as the MIC values) was evaluated using two bacterial strains at different period times (**Table 1**). The V matrix did not exhibit antibacterial activity. The MIC values show that all nanocomposite samples were very effective against *S. aureus*, primarily VA350 and VO350 after 120 min and more. Samples VA650 and VO650 were more active after 2 days. The MIC values of VO350 indicated also inhibition effect against *K. pneumoniae* after 1 day and more, while VA350, VO650 and VO650 were more active after 3 days. VA350 had lower antibacterial activity against *K. pneumoniae*. The Gram-positive bacteria *S. aureus* were generally more sensitive to all nanocomposites than Gram negative bacteria *K. pneumoniae*.

Table 1 The antibacterial test. Minimum inhibitory concentration values (MIC; %, w/v).

	Staphylococcus aureus CCM 3953 (MIC)						
Samples	30-90 min	120-300 min	1 day	2 days	3 days	4 days	5 days
V	>10	>10	>10	>10	>10	>10	>10
VA350		3.33	3.33	0.37	0.12	0.12	0.041
VA650		10	10	1.11	1.11	1.11	0.37
VO350		3.33	3.33	0.37	0.37	0.37	0.37
VO650		10	10	1.11	1.11	1.11	1.11

	Klebsiella pneumoniae CCM 4415 (MIC)						
Samples	30-90 min	120-300 min	1 day	2 days	3 days	4 days	5 days
V	>10		>10	>10	>10	>10	>10
VA350			10	10	10	10	10
VA650			10	10	3.33	3.33	3.33
VO350			3.33	3.33	3.33	3.33	3.33
VO650			10	10	3.33	3.33	3.33

4. CONCLUSIONS

Two precursors were used for the zinc oxide/vermiculite nanocomposites preparation by the solid-state reaction method. It was found that the mechanochemical process affects the vermiculite morphology and structure. New nanocomposite samples with different concentration and crystallite size of ZnO nanoparticles have strong antibacterial activity with relatively fast and long-acting. The results of nanocomposites antibacterial activity suggest their potential usage in medicine or in food industry as prophylactic and anti-inflammatory agent.

ACKNOWLEDGEMENTS

This work was supported by the project No. SP2016/49: "The size and concentration dependence of nanostructured materials". Authors thank to S. Kozubová for SEM micrographs.

REFERENCES

[1] ALAA, M. R. Vermiculite as a construction material-A short guide for Civil Engineer. *Construction and Building Materials,* Vol. 125, 2016, pp. 53-62.

[2] BOWEN, L., SHUHUI, Y., JIM, Y. H., SHANGZHAO, S. Antibacterial Vermiculite Nano-Material. *Journal of Minerals & Materials Characterization & Engineering*, Vol. 1, No.1, 2002, pp. 61-68.

[3] ZHANG, L. Investigation into the antibacterial behaviour of suspensions of ZnO nanoparticles (ZnO nanofluids), *Journal of Nanoparticle Research,* Vol. 9, 2007, pp. 479-489.

[4] TAM, K.H., DJURIŠIĆ, A.B.,□ CHAN, C.M.N., XI, Y.Y., TSE, C.W., LEUNG, Y.H., CHAN, W.K., LEUNG, F.C.C., AU, D.W.T. Antibacterial activity of ZnO nanorods prepared by a hydrothermal method. *Thin Solid Films*, Vol. 516, 2008, pp. 6167-6174.

[5] YAMAMOTO, O. Influence of particle size on the antibacterial activity of zinc oxide. *International Journal of Inorganic Materials*, Vol. 3, 2001, pp. 643-646.

[6] JIN, T., SUN, D., SU, J.Y., ZHANG, H., SUE, H.J. Antimicrobial efficacy of zinc oxide quantum dots against *Listeria monocytogenes, Salmonella enteritidis*, and *Escherichia coli* O157:H7. J. *Food Sci*., Vol. 74, 2009, pp. 46-52.

[7] LIU, Y., HE, L., MUSTAPHA, A., LI, H., HU, Z.Q., LIN, M. Antibacterial activities of zinc oxide nanoparticles against *Escherichia coli* O157:H7. *J. Appl. Microbiol.* Vol. 107, 2009, pp. 1193-1201.

[8] MERUVU, H., VANGALAPATI, M., CHAITANYA CHIPPADA, S., RAO BAMMIDI, S. Synthesis and characterization of zinc oxide nanoparticles and its antimicrobial activity *Against Bacillus subtilis* and *Escherichа Coli*. *Rasayan J. Chem*., Vol.4, No.1, 2011, pp. 217-222.

[9] AVVAKUMOV, G.V., SENNA, M., KOSOVA, N. V. Soft *Mechanochemical Synthesis A. Basis for New Chemical Technologies.* Edition Number 1, *Springer US*, 2000, 208 p.

[10] ČECH BARABASZOVÁ, K., VALÁŠKOVÁ, M. Characterization of vermiculite particles after different milling techniques. *Powder Technology,* Vol. 239, 2013, pp. 277-283.

[11] ČECH BARABASZOVÁ, K., KALIVODA, P. The methods of Zeta-potential measurement as surface property of defined particles. Journal of nanocomposites and nanoceramics, Vol. 4, No. 1, 2013, pp. 7-13.

[12] JIN, L., DAI, B. Preparation and Properties of Zno/Vermiculite Composite Particles. *Advanced Materials Research*, Vol. 455-456, 2012, pp. 265-270.

[13] MUSIC, S., SARIC, A., POPOVIC, S. Dependence of the microstructural properties of ZnO particles on their synthesis. *Journal of Alloys and Compounds*, Vol. 448, 2008, 277-283.

[14] PÉREZ-MAQUEDA, L.A., BALEK, V., POYATO, J., PÉREZ-RODRIQUEZ, J.L., ŠUBRT, J., BOUNTSEWA, M., BECKMAN, N., MÁLEK, Z. Study of natural and ion exchanged vermiculite by emanation thermal analysis, TG, DTA and XRD, *Journal of Thermal Analysis and Calorimetry*, Vol. 71, 2003, pp. 715-726.

[15] KAWANO, M., TOMITA, K. Dehydration and rehydration of saponite and vermiculite, *Clays Clay Miner.*, Vol. 39, 1991, pp. 174-183.

[16] REICHENBACH, H., BEYER, J. Dehydration and rehydration of vermiculites: I. Phlogopitic Mg-vermiculite, *Clay Minerals*, Vol. 29, 1994, pp. 327-340.

[17] MARCOS, C., ARANGO, Y.C., RODRIGUEZ, I. X-ray diffraction studies of the thermal behaviour of commercial vermiculites, *Appl. Clay Sci.,* Vol. 42, 2009, pp. 368-378.

[18] ABDUL LATIF, M.H., Preparation and characterization for a new ZnO-montmorillonite hybrid from Iraqi (Treifawi) clay minerals, *Chemistry and Materials Research*, Vol. 7, 2015, pp.18-23.

[19] CULLITY, B.D. Elements of X-ray Diffraction, *Addison-Wesley, Reading*, 1978, Massachusetts, USA.