ACADEMIA ROMÂNĂ
Revue Roumaine de Chimie
http://web.icf.ro/rrch/

Rev. Roum. Chim.,
2018, *63*(7-8), 719-724

Dedicated to Professor Bogdan C. Simionescu on the occasion of his 70th anniversary

ZnO RADIAL MORPHOLOGIES WITH HIGH ANTI-BIOFILM AND ANTIBACTERIAL ACTIVITY OBTAINED BY A POLYMER-ASSISTED HYDROTHERMAL SYNTHESIS

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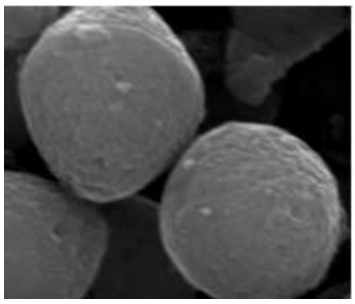
Received November 14, 2017

A one-pot approach for the synthesis of ZnO radial hierarchical architectures was developed. The synergy between methylcellulose, urea and triethanolamine is the key parameter of the synthetic methodology. The synthesized material presents good anti-biofilm and antimicrobial activity.



INTRODUCTION

As a natural evolution of pollution prevention actions, recently, great work has been made in design and synthesis of compounds/materials in a "greener" way that, at the very least, avoids the use and creation of toxic compounds and waste.[1] From the point of view of this eco-friendly aspect of material science, carbohydrates, especially polysaccharides, may be considering a new entry, so far relatively scarce utilized but with very promising results.[2,3] Currently, four forefront research directions have been developed targeting the use of carbohydrates in the synthesis of materials: (*i*) as growth inhibitors and crystal habit modifiers in different wet chemical syntheses;[4-6] (*ii*) as low-temperature fuels in the combustion synthesis of metal oxides;[7-9] (*iii*) as template for different materials (metals, metal oxides, metal/metal oxide composites)[10-14] and (*iii*) as carbon sources for metal–carbon[15.16] or metal/metal oxide–carbon composites.[17-20]

Nanostructure zinc oxide (ZnO) is a versatile and interesting semiconductor material because it possesses a combination of attractive properties: unique electronic, catalytic, optoelectronic, photocatalytic and antibacterial ones, being also bio-safe and biocompatible.[21-25] The shape control of ZnO nanostructures has attracted considerable attention due to two reasons. Firstly, as it is well-known due to the important role (often decisive) of the shape on its physical and chemical properties

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and derived applications.[26,27] Secondly, ZnO possesses probably the richest family of nanostructures among all materials, in both structures and properties terms. Thus, a rational control over the synthetic procedure in order to obtain morphologically diverse and functionally discrete ZnO structure materials represents a must. However, nanometer-scaled materials, such as nanoparticles and nanorods, with high surface- to-volume ratios tend to aggregate, which results in a mitigation of its efficiency in different applicative properties. A convenient and available way to prevent the aggregation is to organize these nanomaterials into hierarchical structures.[28-30]

Among the many synthetic approaches, wet chemistry is an effective route for the production of ZnO nanostructures, and additionally permit the use of diverse capping agents to control the crystal growth and their assembly in diverse nanostructures.[31,32] Several studies mentioned the use of polysaccharide as green additive in wet procedure of metal oxides, mostly dealing with starch and dextran[1,33-36] due to their good coordination abilities and water solubility (lower in starch case). Because of its insolubility in water, cellulose cannot be used *per se* in such synthesis, instead, its soluble derivatives (as cellulose acetate, carboxylmethyl cellulose, methyl and ethyl cellulose, hydroxylethyl cellulose, *etc*.) may be interesting starting materials in controlling metallic materials (oxide or not).

Herein we developed a simple, one-pot hydrothermal synthetic procedure, carried out at low temperature for a short period of time in order to obtain hierarchical radial ZnO materials. As raw materials, besides zinc source (zinc acetate), were utilized methylcellulose as growth inhibitor and crystal habit modifier, triethanolamine and urea as the pH modifiers and chelating agents. The synthesized material proved to be feasible for use as antimicrobial agents.

RESULTS AND DISCUSSION

The obtained ZnO exhibits the ZnO-wurtzite structure (hexagonal phase, space group $P6_3mc$) (Fig. 1). The mean crystallite size is 90 ±0.4 nm, and the lattice parameters [a= 0.3241(55) nm and c= 0.5195(2) nm] and tetragonality ratio (c/a=1.36) are almost the same and quite close to the standard ZnO-zincite (a=0.324982(15) nm c=0.520661(15) nm c/a=1.602, JCPDS 36-1451). No impurities have been detected.

The IR investigations of ZnO (Fig. 2) reveal the presence of MC and water, and the formation of ZnO. The characteristic bands of urea and TEA (or their decomposition products) are not identified. MC fingerprints are found in the synthesized material, the FTIR spectrum of ZnO showing resemblance with the polymer one in its characteristic regions.[37-39] In 400–600 cm^{-1} range, the spectrum of ZnO sample reveals the distinct oxide absorption bands.[40]

SEM investigations identified curved morphologies (Fig. 3), spheres and ellipses (Fig. 3b), consisting of assemblies of nanorods radially arranged, from the center to the surface (Fig. 3c). Their surface is smooth and the diameters range between 300-780 nm.

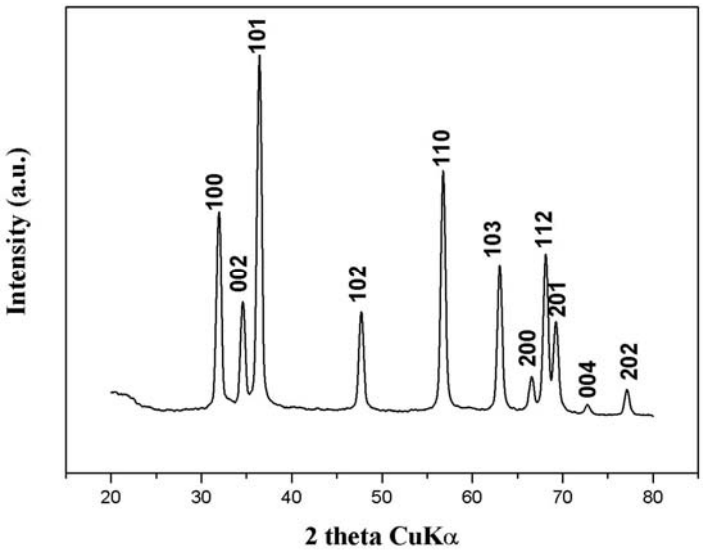

Fig. 1 – XRD pattern of the ZnO sample.

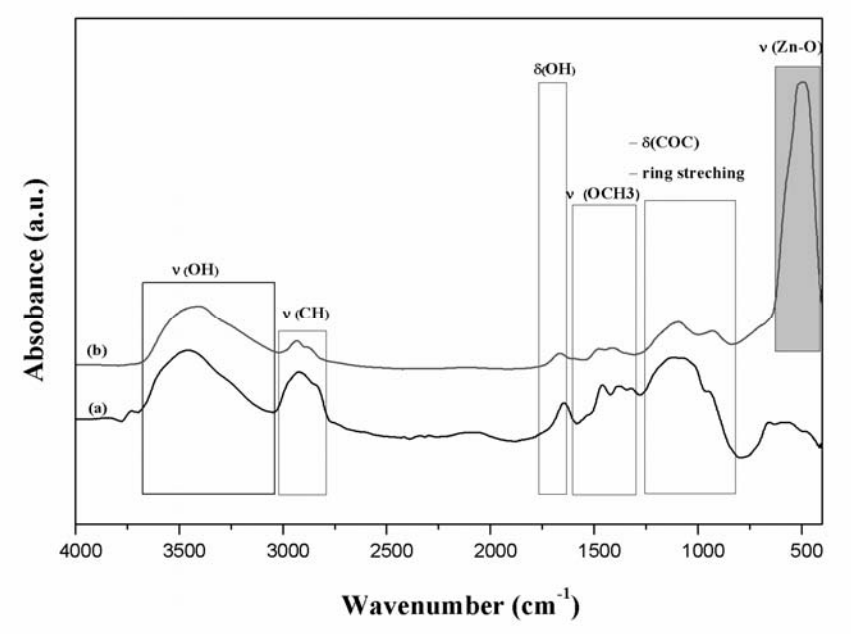

Fig. 2 – FTIR spectra of: (a) methylcellulose raw material; (b) obtained ZnO.

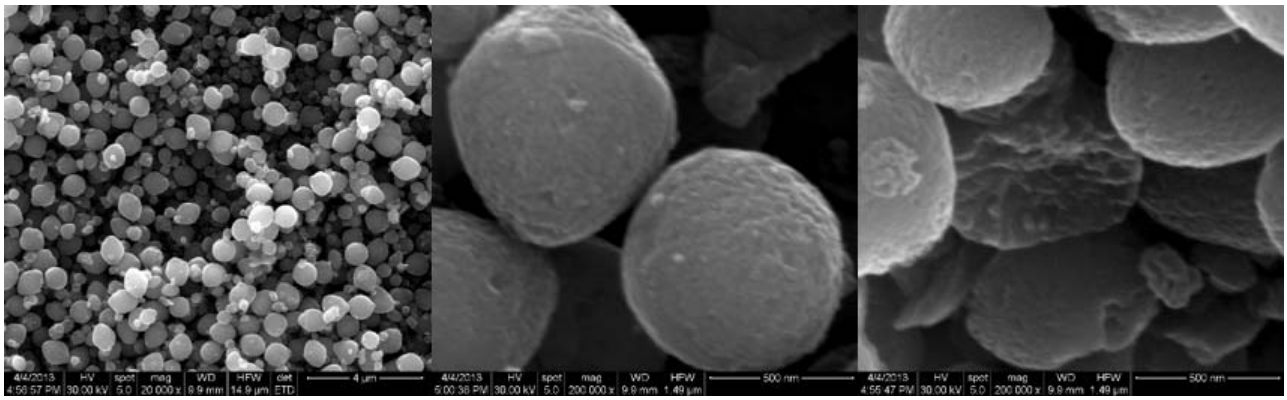

Fig. 3 – SEM micrographs of the ZnO sample.

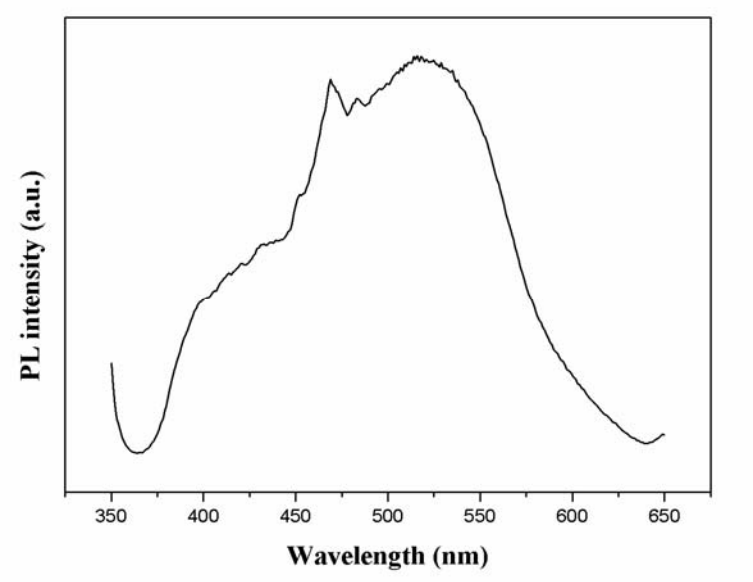

Fig. 4 – Room-temperature photoluminescence spectra of ZnO material.

Table 1

MIC and MBEC values (μg mL^{-1}) of the tested compound on the Gram-positive and negative reference and clinical strains in planktonic and biofilm form

	Gram-positive strains				**Gram-negative strains**			
	S. aureus ATCC 6538	MRSA	*B. subtilis* ATCC 12488	*B. subtilis* 6683	*P. aeruginosa* ATCC 27853	*P. aeruginosa* 719	*E. coli* O26	*K. pneumoniae* 11
MIC (μg mL^{-1})	31.25	7.81	>500	62.5	62.5	125	0.97	62.5
MBEC (μg mL^{-1})	62.5	15.62	125	125	3.9	31.25	>500	15.62

Room temperature photoluminescence spectrum of the ZnO material presented in Fig. 4 shows only a wide band emission covering the blue–green regions, while the exciton- related near-band edge emission is absent. The mechanism behind visible photoluminescence is still under debate,[41] but we attribute the increased visible emissions to the high density of surface defects (oxygen vacancies, zinc vacancies, oxygen interstitials and zinc interstitials) of the peculiar morphology.[19,42,43] This kind of multi-peaks PL spectrum in the visible region has been rarely reported, being identified for cuboid-shaped ZnO hierarchical structures,[44] micro and nano-sized pencil-like ZnO[45] and ZnO hollow spheres assembled from 1D nanorods[46] or particles.[47] It is worth mentioning, that a high density of surface defects is a prerequisite for antibacterial applications, surface defects being responsible for the increase in lifetime of the charge carriers and hence an increase in the biocide activity.[48]

The tested ZnO material exhibited particularly good antimicrobial activity toward both Gram-positive and negative strains, reference (traceable to ATCC) and clinical ones, including ESKAPE pathogens, in planktonic and adherent, biofilm state. The minimal inhibitory concentration (MIC) and minimum biofilm eradication concentration (MBEC) are shown in Table 1. Several outcomes have to be highlighted. Firstly, as mentioned before except for the activity toward planktonic *B. subtilis* ATCC and *E. coli* O26 biofilm, the biocidal activity can be classified as high (considered for MIC or MBEC values <250 μg/mL). Secondly, the obtained MIC and MBEC values are not always consistent with previous reports showing that biofilm population generally exhibits an enhanced resistance compared to planktonic populations.[49,50] In our study, the MBEC values were often lower than the MIC ones (*B. subtilis*, *P. aeruginosa* both strains, *K. pneumonia*) suggesting that the obtained material could successfully exceed the multiple mechanisms of biofilms tolerance, probably due to a good penetrability through the biofilm extracellular matrix and to their efficiency on persister and non-metabolically active biofilm cells. And finally, very important, the results obtained for the susceptible and resistant *S. aureus* strains represent an example of the inorganic biocides non-differentiating antimicrobial efficiency against both resistant and susceptible microbes,[51] an advantage over the other commonly employed antimicrobials.

The obtained MIC and MBEC values are one of the lowest (and even the lowest) registered for bare ZnO[32,52-55] and doped ZnO materials,[56,57] different ZnO composites[58,59] and even current antibiotics.[60] Further studies that concern the mechanism of antibacterial activity, a screening against a larger spectrum of reference and clinical microbial strains, both susceptible and exhibiting resistance phenotypes of clinical and epidemiological interest, and also a biocompatibility assay of the r material are in progress.

EXPERIMENTAL

Materials

All chemicals (analytical grade) were used as received: methylcellulose (Carl Roth GmbH, Germany, MC), zinc acetate dehydrate (Reactivul, Romania, ZA), urea ($CO(NH_2)_2$, Carl Roth GmbH, Germany, U) and triethanolamine ($N(C_2H_4OH)_3$, Reactivul, Romania, TEA).

Synthesis procedure

The hydrothermal synthesis of the ZnO material was performed using zinc acetate, methylcellulose, urea and triethanolamine in a molar ratio ZA : MC : U : TEA = 1 : 2 : 5 : 4 (for the polysaccharide the repeating unit is considered the molecular mass). In a typical procedure, 2 mmol of MC and 1 mmol of ZA dissolved together in 40 mL distilled water, stirred magnetically for 15 min, after which 5 mmol of U and 4 mmol of TEA was added (pH was ~7-7.5). The solution was placed in a 45 mL Teflon-lined stainless steel autoclave and

heated in an oven at 120 °C for 2 h. The products were filtered off, washed several times with distilled water and finally dried at 70 °C for 10 h.

Characterization

FTIR spectra (KBr pellets, 400–4000 cm^{-1}) were recorded with a FTIR Bruker Tensor V-37 spectrophotometer. X-ray diffraction measurements were carried out at room temperature with a PANalytical X'Pert MPD diffractometer, using Ni-filtered CuKα radiation (λ = 1.5418 Å), with a scan step of 0.02° and a counting time of 20 s per step, for 2θ ranging between 20 – 80°. The average crystallite size (D) of the samples was determined using the Williamson– Hall equation $\beta_{hkl}\cos\theta_{hkl} = k\lambda/D + 4\varepsilon\sin\theta_{hkl}$, where λ is the wavelength of the CuK_α radiation, k a constant equal to 0.9 and β_{hkl} the instrumental corrected broadening measured at the half-maximum intensity of the (hkl) peak at θ_{hkl} Bragg diffraction angle. The HighScore Plus powder diffraction software using the Rietveld method was utilized for the evaluation of the lattice parameters. In order to analyze the oxide particle morphology, scanning electron microscopy (SEM) images using a FEI – Quanta 3D FEG Dual Beam were taken. Photoluminescence (PL) measurements were performed on a JASCO FP 6500 spectrophotometer, using the 350 nm excitation line of xenon light.

Antimicrobial activity assay

The antimicrobial activity of the ZnO compounds was assayed on Gram-negative (*Escherichia coli* O26, *Pseudomonas aeruginosa* ATCC 27853 and 719, *Klebsiella pneumoniae* 11) and Gram-positive (*Staphylococcus aureus* ATCC 6538 and methicillin resistant *S. aureus*, *Bacillus subtilis* ATCC 12488 and 6833) bacterial strains. Microbial suspensions of 1.5 x 108 CFU mL^{-1} (0.5 McFarland density) obtained from 15-18 h bacterial cultures developed on solid media were used. The ZnO compound was suspended in dimethylsulfoxide (DMSO) to prepare a stock solution (1 mg mL^{-1}). The quantitative assay was performed by liquid medium microdilution method in 96 multi-well plates. Twofold serial dilutions of the compounds solutions (ranging between 500 μg and 0.97 μgmL^{-1}) were performed in a 200 μL volume of broth, and each was well seeded with 50 μL microbial inoculum. Culture positive controls (wells containing culture medium seeded with the microbial inoculum) were used. The influence of the DMSO solvent was also quantified in a series of wells containing DMSO, diluted accordingly with the dilution scheme. The plates were incubated for 24 h at 37°C, and the minimal inhibitory concentration (MIC) values were considered as the lowest concentration of the tested compound that inhibited the growth of the microbial overnight cultures, as compared to the positive control, revealed by a decreased value of absorbance at 600 nm (Apollo LB 911 ELISA reader). The microplates used for the MIC assay were emptied, washed three times by phosphate buffered saline. The biofilm formed on the plastic wells wall was fixed for 5 min with cold methanol, colored for 15 min by violet crystal solution and resuspended with a 33% acetic acid solution. Cell density was measured by reading the optical density of the coloured solution at 492 nm. The minimal biofilm eradication concentration (MBEC) values were considered as the lowest concentration of the tested compound that inhibited the development of biofilm on the plate wells.

CONCLUSION

In summary, spherical and ellipsoidal ZnO hierarchical morphologies were obtained by a methylcellulose-assisted one-pot method. The structures consist in assemblies of nanorods radially disposed. The material exhibited particularly good antimicrobial activity toward both Gram-positive and negative strains, reference and clinical ones, in planktonic and adherent, biofilm state, in several cases a higher biotoxicity against biofilm population than toward planktonic one.

Acknowledgments. This work was financially supported by the research project 234 PED-2017 of the Romanian National Authority for Scientific Research (CNCS - UEFISCDI).

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