Paper

Dependence of Antibacterial Activity of ZnO Powders on Their Physico-chemical Properties

Thi Minh Phuong NGUYEN¹, Ken HIROTA^{1*}, Masaki KATO¹, Kazuhiko TSUKAGOSHI¹,
 Hirohisa YAMADA², Atsuki TERABE³ and Hideto MIZUTANI³

¹Department of Molecular Chemistry & Biochemistry, Faculty of Science & Engineering, Doshisha University,
 Tatara-miyako tani 1-3, Kyo-tanabe, Kyoto 610-0321, Japan.

²Department of Chemical Engineering, National Institute of Technology, Nara College, Yata-cho 22, Yamatokoriyama, Nara 639-1080, Japan.

³Sakai Chemical Industry Co., LTD, Ebisujima-cho 5-1, Sakai-ku, Sakai 590-0985, Japan.

Received April 5, 2019; Revised May 17, 2019; Accepted May 28, 2019

ABSTRACT

Recently, bio-safe germicidal ZnO has been attracting much attention because of the interaction with various biomolecules chemically as well as physically. As its antibacterial activity is related to reactive oxygen species (ROS), the ROS have been investigated in terms of luminol chemiluminescence (CL) emitted from the ZnO surface. In this study, the relationship between the disinfect activity of four kinds of ZnO powders and their physico-chemical properties has been studied using XRD (X-Ray Diffraction), SEM (Scanning Electron Microscope), BET (Brunauer–Emmett–Teller), CL, ESR (Electron Spin Resonance), and XPS (X-ray Photoelectron Spectroscopy) and bio-test using *E. coli*. Four ZnO powders tested were as following; No. 1 is newly developed ZnO, prepared via hydrothermal treatment in aqueous Zn(NO₃)₂ solution and then re-oxidized, which reveal a strong antimicrobial activity under dark conditions; No. 2 starting material for No. 1, derived from zinc acetate; No. 3 fine ZnO prepared at low-temperature heating of basic zinc carbonate; No. 4 conventional fine ZnO synthesized from vaporized metal Zn in air. All powders revealed antimicrobial activity in the dark; different amounts and kinds of ROS have been emitted from each ZnO powder, however, it has been cleared that its sustainability depends significantly on the contents of interstitial Zn contained in ZnO particles.

KEY WORDS

zinc oxide, antibacterial activity, reactive oxygen species, physico-chemical properties

1 Introduction

In the present age of advanced technologies, scientist and engineers are still making vast research about recurrence of infectious disease and bacterial contamination in all kinds of materials¹⁾. Nowadays, a number of inorganic anti-bacterial agents have been attracting much attention from food- and medical-related manufacturers due to their high credibility in comparison with organic anti-bacterial agents. Currently, new nanostructured materials with bactericidal properties are being used for preventing microbial growth because of the physico-chemical properties, such as size, structure and surface of materials can enhance the antimicrobial activity²⁾.

Metallic oxide powders, such as TiO₂, especially Anatase-type TiO₂^{3,4)}, and MO^{1,5)}, MO₂^{1,4)} (here, *M*: metal element) have been found to inhibit bacterial growth. On the other hand, zinc oxide ZnO has been reported by the present authors⁶⁻⁸⁾ to reveal sustainable antibacterial activity even under dark conditions by submitting reactive oxygen species (ROS). The generation of ROS

such as hydrogen peroxide (H₂O₂), hydroxyl radicals (OH·) and superoxide (O₂^{·-}) from the surface of ZnO is the main origin of its sterilizing property; and their generation mechanism in the dark has been proposed^{6,7,9)}. Furthermore, the germ's damage caused by bactericidal inorganic substance such as MgO, CaO and ZnO, also has been explained by the changes in sensitivities of root cells⁸⁾.

For the better use of ZnO and in order to assist the expansion of powerful but non-toxic antimicrobial derivatives, there is much need for understanding the action process of ZnO nanoparticles against bacteria.

In the recent findings, the essential process of the antibacterial effect is not coherent^{10,11)}. However, a few studies have shown that the basic cause of the antibacterial function might be originated from the disruption of cell membrane activity^{12,13)}. Another possibility could be the orientation of intercellular ROS, including hydrogen peroxide (H₂O₂) which is a strong oxidizing agent harmful to bacterial cells^{6,13)}. It has also been stated that ZnO can be initiated by UV and visible light to generate vastly ROS such as OH·, H₂O₂, and ·O₂⁻^{13,14)}. The negatively charged OH· and ·O₂⁻

* Corresponding author, E-mail: khirota@mail.doshisha.ac.jp

cannot penetrate into bacterial cells¹⁵⁾.

The purpose of this study is to find the relationship between the physico-chemical properties and the antimicrobial activity of 4 kinds of ZnO powders described as follows; No. 1 the antibacterial ZnO which the authors have developed and reveals sustainable microbial activity in the dark⁶⁾; No. 2 a commercially available ZnO powder (company product's name of "XZ-100F") which is the starting material for No. 1; No. 3 very fine ZnO powder ("FINEX-30") and No. 4 the conventional fine ZnO powder. These powders were prepared in much different methods and conditions. The synthesis ways might strongly affect on their physico-chemical properties. In the present study we have concentrated on both identifications of ROS submitted from the surface of each powder and what kind of ROS has strong influence on the disinfect activity of ZnO powders. From this study the antimicrobial activities of ZnO might be improved furthermore by understanding the nature of inhibitory and lethal effect of ZnO nanoparticles on bacteria.

2 Experimental procedure

2.1 ZnO powder preparation

In this study, 4 kinds of ZnO powders were used as listed in Table 1.

No. 1 sample is "antibacterial ZnO" powder, which was prepared by hydrothermal treatment in $\text{Zn}(\text{NO}_3)_2$ 3M aqueous solution at 443 K for 2.52×10^4 s and then re-oxidized at 773 K for 3.6×10^3 s in air and ball-milling^{7,16)}, using commercially available fine ZnO ("XZ-100F", Sakai Chemical Industry Co., Ltd, Sakai, Osaka, Japan) with BET surface area S_A of $7.83 \text{ m}^2 \cdot \text{g}^{-1}$, particle size P_s of $0.137 \mu\text{m}$ determined using S_A and its theoretical density D_x of $5.61 \text{ Mg} \cdot \text{m}^{-3}$, as the same manner as our previous paper⁷⁾. No. 2 sample is "XZ-100F" ZnO powder, which is a starting material used for No. 1 mentioned above. This sample was prepared from zinc acetate. No. 3 commercially available "FINEX-30" ZnO, (Sakai Chemical Industry Co., Ltd.) was synthesized by heating zinc carbonate at high temperature (673-1073 K) which is known as mechanochemical process¹⁷⁾. No. 4 sample is also conventional fine ZnO powder, which prepared by melting metallic zinc as "French method"¹⁷⁾ at 1173 K, then cooling down to form zinc

oxide as solid powder. All three ZnO No. 2-4 are commercial products.

2.2 Evaluation

X-ray diffraction (XRD, Ultima III, Smartlab, Rigaku, Tokyo, Japan) analysis using $\text{CuK}\alpha$ radiation (wavelength of 0.15418 nm) with a graphite monochromator was utilized for determination of the crystalline phases and lattice parameters. Microstructural observation with field emission-type scanning electron microscopes (FE-SEM, JSM-7001FD, JSM-7800, JEOL Ltd., Tokyo, Japan) was performed on the ZnO powders prepared under various conditions. BET surface areas S_a of powders were measured using a particle characterization analyzer (Tristar II, Micromeritics Japan, Tokyo) at room temperature. Chemiluminescence (CL)^{6,7,16)} of ZnO powders ($100 \mu\text{mol}$) in a 0.25 mL ($2.5 \times 10^{-7} \text{ m}^3$) aqueous luminol solution ($5.0 \mu\text{mol/L}$, $5.0 \times 10^{-9} \text{ mol} \cdot \text{m}^{-3}$) mixed with 3.0 mL ($3.0 \times 10^{-6} \text{ m}^3$) carbonic acid buffer solution ($\text{NaOH}/\text{NaHCO}_3$, $\text{pH} = 10.8$) was observed in the dark condition using a CL detector (CLD-100FC, Tohoku Electronic Industrial Co., Ltd., Sendai, Japan)⁶⁾. After dropping the luminol solution in a $1.2 \times 10^2 \text{ s}$ warming up of the detector, the intensity of CL was integrated between $1.2 \sim 6.0 \times 10^2 \text{ s}$. In the order to determine interstitial Zn contained in crystalline structure of ZnO, *i.e.*, $\text{Zn}_{1+\delta}\text{O}$, the following procedure was performed. (I) The weight of ZnO powder M_{ZnO} after heating at 573 K for $6.0 \times 10^2 \text{ s}$ in air was measured using a precision electric balance (here, this temperature and time was determined in terms of complete elimination of water adsorbed on the surface of ZnO using the DTA/TG). (II) The mass of oxygen content M_o of the same ZnO powder sample was measured using an Oxygen-Nitrogen Analyzer ("EMGA620W", Horiba Scientific, Kyoto, Japan) 5 times. The average value of M_o was determined. (III) From this M_o value, the oxygen mole n_o was calculated. Then the mol of Zn, n_{Zn} , in the same sample was estimated from the following equation: $n_{\text{Zn}} = [M_{\text{ZnO}} - 16.00 \times n_o]/65.41$; here, 16.00 and 65.41 are atomic weights of oxygen and zinc, respectively. The content of interstitial Zn, δ , was determined by an equation of $n_{\text{Zn}}/n_o = 1 + \delta$. As photoluminescence (PL) analysis is ideally suited for compositional analysis of defect evaluation of light-emitting materials¹⁸⁾. PL measurement (Fluorolog[®]-3, 450 W

Table 1 Preparation conditions for 4 kinds of ZnO powders.

Sample	Precursors	Preparation method	Synthesis conditions	Properties
No 1 Antibacterial ZnO	XZ-100F ZnO 3M $\text{Zn}(\text{NO}_3)_2$ aq.	Hydrothermal treatment Ball milling	Reaction temp.: 443 K, $2.52 \times 10^4 \text{ s}$ Drying: overnight, 353 K Calcinaion 773 K, $3.6 \times 10^3 \text{ s}$ in air Ball-milling: 2 mm ^φ YTZ, $3.6 \times 10^3 \text{ s}$	Hexagonal Wurtzite structure $2.69 \text{ m}^2/\text{g}$
No 2 XZ-100F	$\text{Zn}(\text{CH}_3\text{COO})_2$	—	—	Wurtzite $7.83 \text{ m}^2/\text{g}$
No 3 FINEX-30	ZnCO_3	Mechanochemical process	Calcination temp.: 673-1073 K	Wurtzite $27.4 \text{ m}^2/\text{g}$
No 4 Conventional Fine ZnO	Metallic Zinc	French method (indirect process)	Melting temp.: $\sim 1173 \text{ K}$ Cooling ZnO particles	Wurtzite $9.06 \text{ m}^2/\text{g}$

Xe short arc lamp, Xe flush lamp with double monochromator, HORIBA, Kyoto, Japan) was applied to identify interstitial zinc in the crystalline structure of ZnO powders at room temperature. X-ray Photoelectron Spectroscopy XPS or Electron Spectroscopy for Chemical Analysis, ESCA) analysis (ESCA3057, ULVAC-PHI. Inc., Chigasaki, Kanagawa, Japan) was utilized to analyze the surface chemical state of 4 kinds ZnO. A monochromated AlK α X-ray source (1486.6 eV) was used and operated at 150 W in a pressure of 1.333×10^{-12} Pa. All the binding energies were referenced to the Au_{47/2} peak located at 84.2 eV attributed to the surface sputtered-Au. Chemiluminescence (CL) with scavengers such as, 2-5 dimethyl furan (singlet oxygen, $^1\text{O}_2$, Wako Ltd., Tokyo, Japan), nitro blue tetrazolium (superoxide, $\cdot\text{O}_2^-$, Nacalai tesque, INC., Kyoto, Japan), 2-propanol (hydroxyl radical, $\text{OH}\cdot$, Nacalai tesque, INC., Kyoto, Japan), and riboflavin (hydrogen per oxide, H_2O_2 , Wako Ltd.) measurements were utilized at room temperature in the dark to identify the reactive oxygen species (ROS) of ZnO powders⁶⁾. Antibacterial test for various kinds of ZnO powders was evaluated with a colony count method using *E. coli* bacteria on nutrient agar medium in a Na-P buffer solution. The penetrated culture of bacteria was kept at 309 K for 8.64×10^4 s in a laboratory incubator. ZnO powders were added to a small amount of bacteria and mixed with 10 mL Na-P buffer solution in the sunshade.

3 Results and discussions

3.1 Characteristics of ZnO powders

Fig. 1 shows XRD patterns of 4 kinds of ZnO powders; all samples have hexagonal W $\ddot{u}$ rzite structure. There is not so much

Fig. 1 XRD patterns of 4 kinds of ZnO.

significant difference in their lattice parameters among them (Table 2). As No. 1 powder was prepared from No. 2 ZnO by hydrothermal treatment, the lattice parameters of No. 1 are a little larger than those of No. 2. However, *a* and *c* of No. 2 ZnO are a little smaller than the rest, its unit volume is the smallest among them. Crystallite size of No. 3 is the smallest due to its preparation method in which zinc carbonate was calcined at high temperatures, suggesting the finest powder among them. SEM photographs shown in Fig. 2 display the morphology and particle sizes of ZnO powders. ZnO particle's shapes are sphere and some of them are hexagonal column. Besides, the particle size, *P*_s, of 4 samples that evaluated based on BET surface area values as indicated below the photographs are consistent with the SEM observation, predicting that the differences in their morphologies and particle sizes might affect to their antibacterial properties. Fig. 3 shows the integrated chemiluminescence intensity, ΣCL , accumulated for 4.8×10^2 s under dark conditions as the blue bar graphs. Obviously, the CL value of No. 3 FINEX 30 fine powder is much higher than the other ZnO powders. With CL results, No. 3 ZnO powder is expected to reveal strong activity on bacteria. As it is clear that the surface area has much effect on the value of ΣCL , the ΣCL values per unit area, $\Sigma\text{CL}/S_A$, were calculated and shown in red bar graphs in Fig. 3. By comparing these $\Sigma\text{CL}/S_A$ values, there is little difference from No. 2 to No. 4; dare to say, No. 1 reveals a little smaller value.

ZnO is non-stoichiometric chemical compound with the chemical formula $\text{Zn}_{1+\delta}\text{O}$. After various treatments, for example, hydrothermal treatment and following re-oxidation, interstitial zinc ions in the ZnO lattice can be formed. These zinc interstitials are the main source of generation of ROS¹⁹⁾. Fig. 4 displays the values in the formula $\text{Zn}_{1+\delta}\text{O}$ of 4 kind ZnO powders. These values were determined from the oxygen content of ZnO powders as explained in experimental procedure. As δ value reflects the amount of interstitial zinc, this value also affects to the amount of ROS those could be generated from the surface of ZnO powder^{6,7)}. No 1 sample gave the highest δ , on the contrary, No. 3 did the lowest δ , even though high CL. From here it is expected to submit high amount of ROS from the surface of No. 1 ZnO and resulted in the sustainable microbial activity under dark conditions⁶⁾. Fig. 5 shows the photoluminescence spectra (PL) of 4 samples. As all the measured data on Fluorolog3 equipped with a xenon lamp were re-calculated for optical filter transparency correction, some

Table 2 Lattice parameters and crystallite size of ZnO powders.

Sample	Lattice parameters			<i>d</i> (100) (nm)	<i>d</i> (001) (nm)	<i>d</i> (101) (nm)	Crystallite size (nm)
	<i>a</i> (nm)	<i>c</i> (nm)	<i>v</i> (nm ³)				
No 1	0.32490	0.52055	0.04869	0.009606	0.011853	0.007344	60.8
No 2	0.32468	0.52024	0.04860	0.0144575	0.0165865	0.013617	43.3
No 3	0.32484	0.52069	0.04868	0.01929	0.023483	0.020675	23.1
No 4	0.32489	0.52052	0.04868	0.00973	0.011161	0.00759	45

Fig. 2 SEM images of 4 kinds of ZnO powders.

Fig. 3 ΣCL and $\Sigma\text{CL}/S_A$ [S_A : BET surface area (m^2/g)] of 4 kinds of ZnO powders. Fig. 4 δ values for interstitial Zn_i in ZnO powders.

data have obvious intensity gap between the shorter wavelength range and longer wavelength range. These emission spectra that include the intensity gap should not be connected enforcedly using artificial normalization in order to avoid forgery, because these gaps originate from the photobleaching, which is one of well-

Fig. 5 PL spectra of 4 kinds ZnO powders.

known fluorescence feature²⁰). Basing on some previous studies²¹), the peak around 600 nm wavelength corresponds to interstitial zinc and the intensity of PL peak is related to the amount of interstitial zinc. From these spectra, it is easy to notice that the peak at 625 nm of No. 1 sample shows the highest intensity. Therefore, the PL data also support that this sample includes the high amount of interstitial zinc, consisting with high δ value of No. 1 ZnO sample. XPS analysis as shown in Fig. 6 was utilized to understand the surface chemical state of 4 kinds ZnO. Oxygen vacancy V_O peak is clearly observed in No. 1 antibacterial ZnO sample. Moreover, $\text{Zn } 2p_{3/2}$ peak of No. 1 sample shifted to higher binding energy of 0.2 eV. This peak shift would be caused by electron density of Zn^{2+} core level affected by V_O . Therefore, No. 1 antibacterial sample has many active site/surface areas. The surface V_O ratio of No. 3 sample is the almost same intensity as the other non-treated samples. However, the surface area of No. 3 is much higher than

Fig. 6 XPS spectra of Zn $2p^{3/2}$ peak and oxygen 1s peak.

Fig. 7 CL with scavenger for No. 1 ZnO powder.

other samples, so the total active-site number is many more than those of other non-treatment samples. The antibacterial effect of No. 3 might be due mainly to its high surface area.

3.2 ROS from the ZnO surface

The determination of which kind of radical oxygen species ROS generated from the ZnO surface was conducted using various methods. At first, CL-scavenger method was applied; in this method, a specific scavenger that can trap the corresponding ROS. In Fig. 7, the blue and red curves are CL ones before and after scavenger-addition, respectively. For example, in No. 1 as the representative sample, the CL intensity is strongly decreased

after addition of scavenger such as 2-propanol, 2,5-dimethyl furan, riboflavin, and nitro blue tetrazolium; they can trap hydroxyl radical $OH\cdot$, singlet oxygen 1O_2 , hydrogen peroxide H_2O_2 , and superoxide radical $\cdot O_2^-$, respectively. Therefore, it would be concluded that No. 1 sample could submit all 4 kinds of ROS such as $OH\cdot$, 1O_2 , H_2O_2 and $\cdot O_2^-$. Fig. 8 represents that the combination of CL-scavenger curves for No. 2, 3 and 4 ZnO powders. Each powder only submits one kind of ROS; No. 2 1O_2 , No. 3 H_2O_2 and No. 4 $OH\cdot$. To confirm the results obtained from CL-scavenger method, another testing method, which we call, bio-test with scavenger was utilized. The principle of this method is near the

Fig. 8 CL with scavenger for No. 2~4 ZnO powders.

Fig. 9 Bio-test using scavengers under dark conditions.

same as CL-scavenger method, in other words, some specific scavengers (catalase [H_2O_2], superoxide dismutase SOD [$\cdot\text{O}_2^-$], 10% DMSO [$\text{OH}\cdot$]) which can catch the corresponding ROS to ZnO powders: (1), (2), (3) and (4) correspond to No. 1, No. 2, No. 3 and No. 4, respectively, in Fig. 9, were adopted. At first, 10% dimethyl sulfoxide DMSO which is a scavenger for $\text{OH}\cdot$, was used for the first testing condition. As shown in Fig. 9, after 8.64×10^4 s testing on *E. coli* under dark conditions, all samples proved that they could kill all bacteria. These results are not consistent with the CL-scavenger results, so that the number of bacteria was

increased from 10^5 to 10^7 /mL and also the amount of ZnO powder was decreased from 1.0×10^{-4} to 1.0×10^{-5} kg, the results are shown in Fig. 9. From this, it is easily noticed that both No. 1 and 2 ZnO powders could submit ROS. From the results of CL and bio-test with scavengers, Table 3 summarizes what kinds of ROS are emitted from each sample (○: submission of ROS was confirmed by the above-mentioned measurement, ×: no submission, - : submission process itself was not performed); No 1, as mentioned, submitted all ROS. No. 2 XZ-100F generates hydrogen peroxide (H_2O_2) and superoxide radical ($\cdot\text{O}_2^-$). No. 3 two kinds of ROS *i.e.*,

Table 3 Summary of ROS submitted from 4 kinds of ZnO powders.

Sample	OH·		H ₂ O ₂		¹ O ₂		·O ₂ ⁻	
	CL	Bio-test (DMSO)	CL	Bio-test (catalase)	CL	Bio-test	CL	Bio-test (SOD)
No. 1 Antibacterial ZnO	○	○	○	○	○	—	○	○
No. 2 XZ-100F	×	—	×	○	×	—	○	○
No. 3 FINEX 30	×	—	○	○	×	—	×	○
No. 4 Fine zinc oxide	○	—	×	○	×	—	×	○

○: submission of ROS was confirmed, ×: no submission, —: submission process was not performed.

Table 4 MRSA bio-test results.

Germ (Bacteria)	Object (samples)	Concentration	Number of active germs (/mL)	
			before	after 8.64 × 10 ⁴ s
MRSA	antibacterial ZnO	1 mass%	—	<10
	XZ-100F	1 mass%	—	<10
	FINEX-30	1 mass%	—	<10
	Fine zinc oxide powder	1 mass%	—	<10
	Reference		1.9 × 10 ⁵	1.1 × 10 ⁵
Reference: a physiological salt				
Culture condition: 298 K in the shading				
<10: not detected				

H₂O₂ and ·O₂⁻, and No. 4 three kinds of hydroxyl radical (OH·), H₂O₂ and ·O₂⁻, respectively.

3.3 MRSA (Methicilin-resitant Staphylococcus aureus) test

Finally, MRSA which is very famous as resistant bacteria and harmful to patients in the hospitals, was bio-tested in Kobe Center of Japan Textile Products Quality and Technology Center; all 4 kinds ZnO powers revealed antibacterial activity on MRSA even under dark conditions, Table 4 shows the results of MRSA bio-test after 8.64 × 10⁴ s at 309 K, all ZnO powders killed almost 100% the amount of bacteria.

4 Conclusions

Although all ZnO powders revealed strong antibacterial activity under dark conditions, we found a new relationship between the antibacterial properties and physico-chemical properties from the results of the δ in nonstoichiometric compound Zn_{1+δ}O and photoluminescence (PL) spectra. As the δ indicates the content of Zn_i in the ZnO lattice, and these Zn_i have strong effect with submission of ROS, and high PL peak at 625 nm also reflects the content of Zn_i, therefore No. 1 antibacterial ZnO powder can produce much ROS continuously. From the application viewpoints, FINEX-30 or XZ-100F can be applicable for one and short time usage.

Acknowledgment

The authors express their appreciation to Mr. Yuichi Kitagawa of J HORIBA TECHNO SERVICE CO., LTD, Kyoto, Japan, for

his technical assistance and advice in the PL measurement of ZnO. The authors thank Ms. M. Toda and Ms. J. Morita of the Doshisha University Research Center for Interfacial Phenomena, for FE-SEM observations of the samples.

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