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Preparation and antibacterial efficacy of bamboo charcoal/polyoxometalate biological protective material

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ABSTRACT

Nanocomposites based on Keggin-type polyoxometalate H 2 PV 2 Mo 10 O 40 (POM) and porous bamboo charcoal (BC) were prepared by activation and immobilization processes. The physical properties of the BC/POM composites were examined using FTIR, UV–Vis spectroscopy, 31 P MAS-NMR, SEM and TEM. These techniques indicated that the POM was intact on the surface of the BC matrix after impregnation. The POM particle size was found to be less than 150 nm based on TEM. The antibacterial effects of the BC/POM composites were assessed from the zone of inhibition, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and plate-counting method, and an excellent antibacterial performance was discovered.

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1. Introduction

Polyoxometalates (POMs) are oxygen rich class of inorganic cluster systems exhibiting remarkable chemical and physical properties, which have been applied to various fields such as catalysis, materials and medicines [1–5]. POMs perform significant biological activities, with high efficiency and low toxicity. In particular, the sizes and globular structural motifs of many POMs are similar, and in some cases nearly identical to some water-soluble fullerene derivatives showing fairly good anti-HIV activity [6–8]. So far, various biological effects of the POMs have been reported on antitumor and antivirus activities [9,10]. With regard to the antibacterial activity of the POMs, we found that the polyoxometalates with Keggin, lacunary Keggin, Wells-Dawson, double-Keggin and Keggin-sandwich structures enhanced the antibacterial activity of ß-lactam antibiotics on methicillin-resistant Staphylococcus aureus (MRSA) [11]. Helicobacter pylori [12] and SARS coronavirus (SARS-CoV) [13].

In practice, the applications of POMs as solid phase catalysts have been limited by their low surface areas (1–10 m 2/g) and their low decomposition temperatures. A number of attempts have been made to disperse POMs on inert supports, with the goal of effectively increasing their surface areas and hence the number of accessible active catalytic sites. Several materials such as carbon [14], zirconia [15], titania [16], alumina [17] and porous silica [18,19] have been used as solid supports for the dispersion of POMs. To find a suitable support able to attach itself firmly and to disperse the POMs would be of great practical interest because catalytic activity will be related to the higher surface area and to the thermal stability of the supported heteropolyanion.

Bamboo charcoal has a number of beneficial characteristics, including high electric conductivity and self-lubricity, and can be used as a friction material and an electromagnetic shield material [20]. On the other hand, an increasing number of bioresources have also been utilized in the preparation of adsorbents, and some studies have shown that certain bioresources have high potential for use as adsorbents. Among these, bamboo is recognized as one of the most popular bioresources, and its adsorption characteristics have been the subject of many studies [21,22]. In our research work, we try to combine H 2 PV 2 Mo 10 O 40 with bamboo charcoal, aiming to explore a kind of water-insoluble and biocompatible antimicrobials. In order to explore the antibacterial effects of BC/POM composites, zone of inhibition testing, minimum inhibitory concentrations (MICs), minimum bactericidal concentration (MBC) and the plate-counting method were used in this study to examine the antibacterial activity of the BC/POM composites against gram-negative Pseudomonas aeruginosa (P. aeruginosa), Ciprofloxacin-resistant P. aeruginosa (CRPA), Escherichia coli (E. coli) and E. coli JM109 (pUK18), and gram-positive Staphylococcus aureus (S. aureus), Meticillin-resistant S. aureus (MRSA) and Bacillus subtilis (B. subtilis).
2. Experimental

2.1. Preparation of BC/POM composites

BC powders (particle size <10 μm, Taiwan Paiho) were activated with nitric acid solution under stirring for 1 h. The activated BC powders (5.0 g) were then immersed in 60 ml of ethylacetate under N₂ atmosphere and heating at 60 °C for 3 h. Polyoxometalate (H₄P₂V₂Mo₁₀O₄₀) was purchased from Japanese Inorganic Chemistry Industry Joint-stock Company and was used as received. POM loading of about 1.25, 2.5, 5.0, 10.0 and 15.0 g was dissolved in 60 ml of ethylacetate and added the resulting solutions to the above mixture. Stirring was continued under an inert atmosphere at room temperature for another 3 h. The BC/POM particles were separated and washed with ethanol, then dried in a vacuum at 100 °C for another 3 h. The BC/POM/ring was continued under an inert atmosphere at room temperature.

2.2. Characterization

FTIR and UV–vis spectra of the samples were recorded on a Tensor 27 (Bruker) and an UV–3000 spectrophotometer. ³¹P MAS-NMR spectra were recorded on a Bruker DEX 400WB solid state NMR spectrometer at a resonance frequency of 400 MHz. The morphology of the composites was observed using a scanning electron microscope (SEM, Hitachi S-800) and a transmission electron microscope (TEM, Philips CM-200) equipped with an energy-dispersive X-ray (EDX, Hitachi S-300) microanalysis system. The Bruauer–Emmett–Teller (BET) specific surface areas (SBET) of the BC/POM composites were determined by a NOVA 1000e automatic physical absorber using highly purified nitrogen gas at 77 K.

2.3. Test of antibacterial properties

P. aeruginosa (ATCC 27853), CRPA, E. coli (ATCC 25922), E. coli JM109, S. aureus (ATCC 25923), MRSA and B. subtilis were obtained from the Food Industry Research and Development Institute, Taiwan, and were used as the reference strains in antibacterial testing. The antibacterial spectrum of BC/POM composites was evaluated by zone of inhibition test. A standard inoculum of the test organism with 10⁷ colony-forming units (CFU)/ml was swabbed onto the surface of a Muller–Hinton agar plate, and then discs of filter paper impregnated with antibacterial agents (6 mg/ml) were placed on the agar. The plates were incubated overnight at 37 °C, and the clear zones around the disc were measured.

The antibacterial effects of the composites were evaluated by the determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using the broth dilution method. Tubes containing 3 ml MH broth with 10-fold dilutions of the BC/POM composites ranging from 4 mg/l to 256 mg/l were inoculated with 10⁵ CFU/ml of bacteria. The inoculated tubes were then incubated at 37 °C for 18 h. After incubation, tubes were examined without shaking for visible turbidity; the MIC was determined as the lowest dilution of composites that produced no visible turbidity [15]. The test was performed three times for each strain, and results in agreement on two or more occasions were adopted as the MIC of the strain. After the MIC has been determined, 0.1 ml of inoculum from each of the tubes broth without visible turbidity was subcultured on nutrient agar plate and incubated at 37 °C for 24 h. The number of grown colonies on this subculture after incubation period was counted and compared to the number of CFU/ml in the original inoculum. The lowest concentration of BC/POM composites that allowed less than 0.1% of the original inoculum to survive, was said to be the MBC [23].

To further investigate the antibacterial effects of the composites, the plate-counting method was used [24]. Approximately 10⁷ CFU of S. aureus were cultured on MH agar plates supplemented with BC/POM composites. BC/POM-free MH plates cultured under the same conditions were used as a control. The plates were incubated at 37 °C for 18 h and the numbers of colonies were counted. The test process was described as follows: 60 mg of the BC/POM composites were added into 3 ml of MH broth containing 10⁷ CFU/ml bacteria. The mixture was aerobically incubated at 37 °C under vibration for 24 h. The above suspension (30 μl) was cultured on an agar plate and incubated at 37 °C for 18 h.

3. Results and discussion

3.1. Structure characterization

Fig. 1 shows the FTIR spectra of pure POM and BC/POM samples from 400 to 1400 cm⁻¹. The band at about 1062 cm⁻¹ of POM is attributed to the v₁(P–O₅) vibrations, while the bands at about 962, 865 and 780 cm⁻¹ are generally assigned to stretching vibrations due to (M–O₁), (M–O₅–M) and (M–O₁–M) species (M = Mo or V) [25]. It has been reported that when POM is bound to a support, a shift in the band position due to the Keggin complex is observed, resulting from a change in the chemical environment and the number and types of counterions [26]. As shown in Fig. 1, BC/POM samples, the absorption bands assigned to the M–O₁–M and M–Oₛ–M stretching at 865 and 780 cm⁻¹, respectively, are shifted to 873 and 800 cm⁻¹. These results indicate that there were interactions between the BC particles and the POM. Fig. 2 shows the UV–vis spectra of the POM and BC/POM composites. The UV–vis spectra of POM and BC/POM composites dissolved in solution show prominent bands at 217 and 309 nm, its intensity distinctly increased with increasing POM content. These bands are generally assigned to charge transfer from the bridging oxygen to the metal in polyoxometalates [27].

³¹P MAS-NMR is a useful tool in detecting the local environments of POM, since the chemical shift of the phosphorous atom depends not only on its local environment within the metal cluster but also on factors such as associated water molecules, metal ions and solid supports [28]. Fig. 3 shows the ³¹P MAS-NMR spectra of unsupported bulk POM and BC/POM composites. All the samples displayed more than one phosphorus resonance, which indicates that the structure is complex. Bulk POM exhibited a sharp and in-
The main peak indicates the presence of P in a highly uniform environment in a hydrated structure of POM. The small shoulders at $-2.3$ and $-4.2$ ppm are due to the presence of some P atoms in environments with different degrees of hydration [19]. After supporting POM on BC, the $^{31}$P NMR signals broadened considerably and shifted to high field ($\delta = -3.9$ and $-7.9$ ppm). The up-field shift of
the resonance signal suggests some destruction of the heteropolyanion. Therefore, the broadness of the 31P NMR resonances of supported samples can be attributed to a significant distortion of the heteropolyanion symmetry and the long-range order created by water molecules in the hydrated state is lost, due to a chemical interaction between the POM anion and mesoporous BC support [19].

SEM and TEM images were used to evaluate the surface morphology and size distribution of the POM deposited on the BC surface. As shown in Fig. 4, ultra-fine and disaggregated POM particles were homogeneously distributed on the surface of the BC. Pure BC has a porous surface (Fig. 4A). The POM particles are granular in nature and seem to be nanosized, typically in the range of <100 nm (Fig. 4D). EDX analysis confirmed the existence of POM in the BC matrix and qualitatively revealed the POM nanoparticles content (Fig. 4E). Table 1 gives the POM content, \( S_{BET} \) and pore diameter of each BC/POM composite, and shows that POM content increased with increasing initial concentration of POM solution, pore diameter of the BC/POM composites was about 3.40 nm, and \( S_{BET} \) of the BC/POM composites decreased with increasing POM content, due to the direct blockage of pores by POM particles. Particle size and \( S_{BET} \) can be controlled by changing the POM content. Too small a value of \( S_{BET} \) will lead to weak adsorption capability, rendering the BC/POM composite useless in the purification process. Thus, as the antibacterial activity of BC/POM composites is significantly influenced by the loading amount of POM on the BC particles, further studies should be performed to estimate the amount of POM required to suppress bacterial growth and ensure strong adsorption capability.

### 3.2. Antibacterial effects

The antibacterial efficacy of the BC/POM composites against bacteria was tested based on zone of inhibition tests, MIC, MBC and the plate-counting method. Fig. 5 and Table 2 detail the relative retention of activity (zone of inhibition) of BC and the BC/POM composites against bacteria. After 24 h of incubation, the zones of inhibition of the BC/POM composites against bacteria ranged from 9.4 to 27.0 mm, whereas BC did not show any zone of inhibition. The BC/POM composites exhibited significant efficacy against bacteria, especially against \( S. aureus \), MRSA and \( E. coli \) JM109, and the efficacy increased with increasing POM content. The results in Tables 3 and 4 show that the composites have good efficacy against these bacteria. The MIC and MBC values of the BC/POM composites against bacteria were 4–128 \( \mu \text{g/ml} \) and 16–256 \( \mu \text{g/ml} \), respectively. The BC/POM composites have a strong antibacterial activity regardless of gram class, including Ciprofloxacin-resistant \( P. aeruginosa \) (CRPA), \( E. coli \) JM109 and Methicillin-resistant \( S. aureus \) (MRSA), which are much more difficult to kill. Thus, it is likely that the highly negative charge of POM will stimulate the cell to result in the easiness of the bacterial morphological change from bacillary form to coccid form, reflecting the bacterial death [12]. In addition, the antibacterial efficacy of the BC/POM composites was better than that of zeolite/Ag (MIC:

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**Table 1**

| Sample      | C (wt%) | O (wt%) | P (wt%) | Mo (wt%) | V (wt%) | \( S_{BET} \) (m²/g) | Pore diameter (nm) |
|-------------|---------|---------|---------|----------|---------|----------------------|---------------------|
| BC          | 86.24   | 11.11   | 0       | 0        | 0       | 284                  | 3.42                |
| BC/POM-0.25 | 79.51   | 13.96   | 0       | 0        | 0       | 245                  | 3.42                |
| BC/POM-0.5  | 79.30   | 14.06   | 0       | 0        | 0       | 211                  | 3.40                |
| BC/POM-1    | 76.25   | 15.40   | 0       | 0        | 0       | 196                  | 3.40                |
| BC/POM-2    | 68.41   | 15.53   | 0.55    | 14.31    | 1.20    | 164                  | 3.39                |
| BC/POM-3    | 61.96   | 16.52   | 0.66    | 18.73    | 2.14    | 128                  | 3.34                |

**Table 2**

| Bacteria       | BC | BC/POM-0.25 | BC/POM-0.5 | BC/POM-1 | BC/POM-2 | BC/POM-3 |
|----------------|----|-------------|------------|----------|----------|----------|
| S. aureus      | 11.4 | 12.1 | 14.9 | 16.9 | 21.0 |
| B. subtilis    | 12.3 | 12.4 | 14.4 | 16.4 | 20.9 |
| P. aeruginosa  | 9.4  | 10.3 | 13.0 | 14.4 | 19.3 |
| E. coli        | 12.6 | 13.9 | 15.4 | 16.0 | 18.6 |
| MRSA           | 12.0 | 14.2 | 18.1 | 21.6 | 27.0 |
| CRPA           | 10.6 | 10.7 | 14.4 | 16.4 | 21.4 |
| E. coli JM109  | 12.3 | 14.0 | 15.7 | 17.7 | 22.9 |
Table 3
The MIC values of BC/POM composites on bacteria

| Bacteria   | Minimum inhibitory concentrations (µg/ml) |
|------------|------------------------------------------|
|            | BC | BC/POM-0.25 | BC/POM-0.5 | BC/POM-1 | BC/POM-2 | BC/POM-3 |
| S. aureus  | 0  | 128         | 64         | 16       | 4        | 4        |
| B. subtilis| 0  | 128         | 64         | 16       | 4        | 4        |
| P. aeruginosa| 0| 128         | 64         | 16       | 4        | 4        |
| E. coli    | 0  | 128         | 64         | 16       | 4        | 4        |
| MRSA       | 0  | 128         | 64         | 16       | 4        | 4        |
| CRPA       | 0  | 128         | 64         | 16       | 4        | 4        |
| E. coli JM109| 0| 128         | 64         | 16       | 4        | 4        |

Table 4
The MBC values of BC/POM composites on bacteria

| Bacteria   | Minimum bactericidal concentrations (µg/ml) |
|------------|------------------------------------------|
|            | BC | BC/POM-0.25 | BC/POM-0.5 | BC/POM-1 | BC/POM-2 | BC/POM-3 |
| S. aureus  | 0  | 256         | 128        | 64       | 16       | 16       |
| B. subtilis| 0  | 256         | 128        | 64       | 16       | 16       |
| P. aeruginosa| 0| 256         | 128        | 64       | 16       | 16       |
| E. coli    | 0  | 256         | 128        | 64       | 16       | 16       |
| MRSA       | 0  | 256         | 128        | 64       | 16       | 16       |
| CRPA       | 0  | 256         | 128        | 64       | 16       | 16       |
| E. coli JM109| 0| 256         | 128        | 64       | 16       | 16       |

Fig. 6. Number of S. aureus colonies as a function of the BC/POM composites (20 mg/ml) put into 10^7 CFU of bacterial colonies. The inserted photograph of MH plates incubated under the condition in (A) BC/POM-0.25, (B) BC/POM-0.5, (C) BC/POM-1, (D) BC/POM-2 and (E) BC/POM-3.

Fig. 7. Number of S. aureus colonies as a function of the inoculation time of BC/POM composites. The inserted photograph of MH plates incubated under the condition in Fig. 7: (A) 0.2 h, (B) 4 h, (C) 8 h, (D) 16 h, (E) 24 h, (F) 36 h, (G) 48 h and (H) 60 h.

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