Antimicrobial Mechanism and Biosafety Evaluation of PdO Modified WO₃

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Abstract. In this study, PdO loaded WO₃ composite was synthesized by precipitation method, followed by calcination. The as-synthesized composite was characterized and the photocatalytic disinfection of Escherichia coli was tested under visible light-irradiation. The characterization results showed that the PdO nanoparticles decorated on the surface of the WO₃. The antibacterial test demonstrated that the as-synthesized material with 100:1 mole ratio of W:Pd and calcined at 200°C have the highest efficiency. After visible light irradiation of a short time, about 25 min, almost all of the tested E. coli cells were inactivated. Antimicrobial mechanism, revealed by scavengers of reactive oxygen species, demonstrated that the antibacterial activity was primarily due to the oxidation of photo-generated hole. And the oxidation-reduction reaction of superoxide radical and hydroxyl free radicals were assisted for the photo-disinfection of bacteria. Furthermore, the biosafety of the PdO/WO₃ composite was evaluated by analysing the viability of Chinese hamster lung (V79) cells using Cell Counting Kit-8 (CCK-8).

1. Introduction
Every year, millions of people were threatened by water pollution, which contains chemical pollution and microbiological contamination. Water pollution caused by microbiological contamination not only can reduce the crop yields, but also transmit diseases. In order to solve the problem, many water treatments have been carried out, such as: chlorination, ozonation and UV-C lamps. However, such methods present two major shortcomings: the potential generation of carcinogenic and mutagenic by-products even at low concentration, and their high cost of use [1]. Photocatalytic disinfection is considered as an effective method to solve the shortcomings. Firstly, photocatalytic disinfection uses the solar as the energy, which is cost free and environmentally friendly. Secondly, it can purify water and degrade some chemical pollutants in water.

Photocatalytic disinfection activity of TiO₂ semiconductor was firstly reported by Matsunaga et al in 1985 [2]. Since then, photocatalytic disinfection semiconductor has been widely concerned by researchers. TiO₂-based semiconductor is an environmental friendly sterilization materials, which can utilize solar energy as driver force, not only it has broad-spectrum, high efficiency and stability for disinfection, but also degrade cytotoxic and organic contaminant. TiO₂-based semiconductor has been used in many areas, such as: aquaculture industry [3], drinking water [4], food packing [5, 6] etc. Because it can effectively kill the pathogenic bacteria: Bacillus acidophilus[7], Staphylococcus aurous [8], Fusarium graminearum [9]etc.
The photocatalytic semiconductors use the solar irradiation as energy source to drive the chemical reaction. Under the light irradiation, the electrons of ground state were excited, the electron transfer from the value band (VB) to the conduction band (CB), thus forming the electron-holes pairs (e⁻⁻h⁺). The photo-generated holes and electrons trapped on the surface of the semiconductor react with absorbed substance, which can produce the intermediate active groups with the strong redox ability [12,13], which can react with these absorbed substance in the surface of materials. The generated electrons with the strong reductive capacity, which can form more oxidative chemical compounds with the oxygen, such as: superoxide radical (•O₂⁻), hydrogen peroxide (H₂O₂), hydrogen radical (•OH), etc. The generated holes with oxidizing ability, it can react with absorbed OH and H₂O and producing •OH. These free radicals have strong oxidative or reductive activity. The cell wall and cell membrane will be destroyed by the free radicals. Meanwhile, these radicals can enter inner cells, result the bio-macromolecules and cell organs seriously damaged [15, 16]. Finally, the cells die. Therefore, the photocatalysis, that can kill the microorganisms under the light irradiation, is an environmental friendly bactericide.

Tungsten oxide (WO₃) has been used in many fields, because it is cheap, chemical stable and non-toxicity [17, 18]. WO₃, the band gap (Eg) between 2.5eV and 2.8eV, can be excited by visible light (λ=442nm). Compared to the photocatalysis of only using ultraviolet light (UV-light), WO₃ is more safety and efficient. In this work, PdO loaded WO₃ composite was synthesized by precipitation method, followed by calcination. Which has excellent bactericidal performance under the light irradiation. PdO acts as electron trap to improve catalytic efficiency [20], because it can limit the electrons and holes recombination. We demonstrated different scavenges of reactive oxygen species (ROS) in the antimicrobial mechanism. The samples were characterized for microstructures, morphology, using X-ray diffraction (XRD), transmission electron microscope (TEM).X-ray photoelectron spectrocope (XPS), UV-visible spectrophotometer. Further, the photocatalytic antibacterial performance of the samples against *Escherichia coli*-K12 has been carry out under the visible light irradiation and the biosafety of PdO modified WO₃ was further evaluated through Chinese hamster lung (V79) cells.

2. Experiment

2.1. Chemicals

The chemicals such as 99% tungsten hexachloride (WCl₆), 98% sodium hydroxide (NaOH), 99% alcohol (C₂H₅OH), 99.99% palladium chloride (PdCl₂), 99.5% sodium oxalate (Na₂C₂O₄), 99.8% potassium bichromate (K₂Cr₂O₇), 99.8% isopropanol (C₃H₈O), 98% nitrogen-oxygen free radical piperidinol (TEMPOL) were obtained from Aladdin industrial corporation (China) and the chemicals were of analytical grade. Tryptone, sodium chloride agar yeast extract and dulbecco's modified eagle medium (DMEM) were purchased by Beijing Solebo Technology Co., Ltd. Fetal bovine serum and Enhanced Cell Counting Kit-8 were brought from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd and Shanghai Biyuntian Biotechnology Co., Ltd, respectively. *E. coli* was purchased from American type culture collection (ATCC) and Chinese hamster lung (V79) cells was supplied by Han Yawei’s laboratory, Zhengzhou University of Light Industry.

2.2. Characterization

The crystal structure was characterized by powder XRD with Cu Kα radiation on a D8 Advance X-ray Diffractometer (Bruker, Germany). TEM images were examined by aJEM-2100 transmission electron microscope (JEOL, Japan).The presence of W, O and Pd in the PdO/WO₃ composite was analyzed by XPS. The XPS measurements were made using an ESCALAB250 X-ray photoelectron spectrometer (Thermo Fisher Scientific Inc., MA) with an AlK anode (1486.6 eV photon energy, 300 W). The optical absorbance was determined from the diffuse reflectance measurements obtained on a U-3900H UV-vis spectrophotometer(Hitachi Limited, Japan) using BaSO₄ as the reference. Steady state photoluminescence (PL) spectra of the materials were obtained on afluorescence spectrometer (Hitachi
F-7000, Japan). To detect the V79 cells viability, CCK-8 was added to the cell cultures and the absorbance was measured by using iMark microplate reader (BIO-RAD, USA) at 450 nm. The visible light was supported by PLS-SXE 300 Xenon lamp (Perfectlight, China).

2.3. Samples preparation
Firstly, 6 mmol WCl$_6$ and 0.12 mmol PdCl$_2$, with a mole ratio of WCl$_6$:PdCl$_2$=200:1, were dissolved in 30 mL ethanol. Secondly, 30 mL alcohol-NaOH solution (the mole ratio XCl:NaOH=1:1) was added dropwise. The mixture was stirred for 30 min at room temperature and transferred into a 100 mL Teflon-lined autoclave and kept at 200°C for 6 h. Under the autogenously pressure and cooled down to room temperature, the obtained samples were centrifuged and washed with distilled water and ethanol respectively and then the samples were dried in a drying oven at 80°C for 12 h. Finally, the samples were calcined in air at 200°C for 5 h before grinding to powder. The samples with different molar ratios were synthesized by the same way.

2.4. Evaluation of photocatalytic antimicrobial
E.coli cells were incubated in Luria-Bertani broth (LB) at 37°C and 150 rpm shaker for 12 h. After that, the bacterial cells were collected by centrifugation at 6000 rpm for 5 min, then washed twice with phosphate buffer solution (PBS, pH=7.0). Finally, the cell density was adjusted to about 2×10$^9$ colony forming units (CFU/mL) by adding PBS liquids.

The photocatalysts (0.1 g) was ultrasonic dissolved in 9.9 mL PBS for 10 min at petri dish. Before exposure to visible light irradiation (440 nm < λ < 700 nm). The suspension was added to the microorganism suspensions. The intensity of light was 10 mW·cm$^{-2}$, as measured by an FZ-A optical radiometer. And 0.1 mL of the solutions were withdrawn per 5 min and then diluted. After that, the cells were cultured at 37°C, 24 h and the cell density was calculated during the culture time.

2.5. Antimicrobial mechanism
All of the e$^-$, h$^+$ and ROS (O$_2^•$, ·OH) may be assisted for the photo-disinfection of bacteria. In order to identify that the main active substances in the photocatalytic sterilization of PdO/WO$_3$. We used 0.5 mmol/L Na$_2$C$_2$O$_4$, 0.05 mmol/L K$_2$CrO$_4$, 1 mmol/L TEMPOL and 0.5 mmol/L C$_3$H$_8$O to scavenge h$^+$, e$^-$, ·O$_2^•$, ·OH, respectively [21, 22].

2.6. Evaluation of biosafety
To evaluate the biosafety of the composites for the potential application areas. We examined the viability of V79 cells by CCK-8 assay at various concentrations of the composite. Cells were cultured in a 96-well culture plate overnight in an incubator at 37 °C in 5% CO$_2$ for sufficient adherence. Various concentrations (0.1, 0.2, 0.4, and 0.8 mg/mL) of the PdO/WO$_3$ composite in DMEM solution were added to the cell plate as the treated group [23, 24], and the control group was treated with an equal volume of PBS. After 24 h, the cell culture medium was discarded and replaced with fresh medium without PdO/WO$_3$. Then, 10 μL of CCK-8 solution was added to each well of the 96-well plate and incubated at 37 °C for 4 h. The absorbance at 450 nm was then measured using an iMark microplate reader. Those tests were carried out under dark conditions and repeated three times. All the results were analyzed using analysis of variance. Statistical significance was considered at p < 0.05. After the CCK assay, the cells were imaged by an Observer A1 inverted microscope (Zeiss, Germany) [25].

3. Results and discussion

3.1. Phase analysis
Figure 1 shows the X-ray diffraction pattern of the sample, the WO$_3$ cubic phase was formed after calcination. Diffraction peaks at 13.950° (100), 23.196° (002), 28.112° (200) and 36.756° (202) can be indexed to WO$_3$ (JCPDS card No.85-2459). Non-detection of PdO or Pd peaks may be attributed to
its low concentration in the composites. Which was however identified by XPS. Figure 2 shows the SEM images and photographs of the samples PdO/WO₃. As can be seen from Figure 2 (a) that the sample is an irregular shape with the size about 200 nm×500 nm, and the structure of the sample looks like particles. Figure 2 (b) shows the high resolution TEM (HRTEM) image. The d-spacing was determined at 0.317 nm, corresponding to the (200) plane of WO₃ and 0.326 nm corresponding to the (111) plane of PdO. Thus, we determined the PdO distributed in the particles of WO₃.

![Figure 1. XRD spectra of PdO/WO₃.](image)

![Figure 2. SEM images (a)(b) of 1% PdO/WO₃.](image)

### 3.2. X-ray photoelectron spectrum

The sample elemental composition was determined by XPS. And Figure 3 (a) shows the XPS spectrum of 1% PdO/WO₃, the composites contains not only Pd, O and W, but also a small amount of Na element. The presence of Na element may be that the sample was not washed cleanly. The high-
resolution XPS spectra (Figure 3 b) reveals that the bind energies of W4f was located at 36.3 eV, suggesting that W⁶⁺ exist in the PdO/WO₃ composites. The binding energy of 529.5 eV can be ascribed to O1s and the binding energy of 336.9 eV and 342.5 eV can be ascribe Pd3d. So, we confirmed that it is PdO and WO₃.

![Figure 3. XPS spectra of 1% PdO/WO₃.](image)

3.3. UV-Vis diffuse reflectance spectra
Figure 4 shows the UV-Vis diffuse reflectance spectra of WO₃ and as synthesized PdO/WO₃. It is clearly that the bare WO₃ photo-absorption is lower than other samples. With the increasing of PdO
ratio, the photo-absorption of PdO/WO$_3$ composites enhanced. Due to the PdO exist, the samples shows a clear shift of absorbance into the visible light range. Additionally, the PdO/WO$_3$ showed a clear shift of absorbance into the visible light range ($\lambda \geq 400$nm). The sample of 1% PdO/WO$_3$ has the great photo-absorption. This phenomenon may be attributed to the surface plasmon resonance (SPR) effect of PdO. So, the samples could response to the visible light.

3.4. Disinfection experiments

The photo-antibacterial effect of PdO/WO$_3$ was evaluated by disinfection of $E$.coli under visible light irradiation. Figure 5 showed the cell density of $E$.coli treat with or without photocatalyst. After 25 min illumination treated by visible light, cell density of $E$.coli treated with light only, 1% PdO/WO$_3$ and WO$_3$ was unchanged. Both 0.1% PdO/WO$_3$ and 0.5% PdO/WO$_3$ have an inhibitory effect on bacteria. 0.5% PdO/WO$_3$ can reduce almost three orders of magnitude in 25 min. 1% PdO/WO$_3$ shows an excellent disinfection performance, which can kill almost all of tested $E$.coli in a short time. The best disinfection of the 1% PdO/WO$_3$ samples agrees with its good SPR effect.

![Figure 5](image1.png)

**Figure 5.** Effect of PdO/WO$_3$ on photocatalytic disinfection of $E$.coli.

![Figure 6](image2.png)

**Figure 6.** Photocatalytic inactivation curves in the presence of various scavengers of 1% PdO/WO$_3$. 
3.5. Antimicrobial mechanism analysis
In this section, different scavengers were added to 1% PdO/WO₃ photocatalytic disinfection, such as: oxalate, potassium bichromate (Cr (v)), isopropanol and TEMPO. As can be seen in Figure 6, the disinfection effect have no changed by adding Cr(V) to trapped electron, which indcated the electron has little disinfection efficient. By adding TEMPO to scavenge •O₂⁻, the disinfection effect were decreased about 1 magnitude, that shows •O₂⁻ has a certain role in photodisinfection. However, when the isopropanol and oxalate existence, the disinfection effect were decreased about 3 and 5 magnitude, which means the •OH and h⁺ play the main roles in photodisinfection progress.

![Figure 7. PL spectra of PdO/WO₃ under 369 nm excitation.](image)

3.6. PL spectra
The PL spectra were shown in Figure 7. The PL analysis was used to examine the separation efficiency of the photo-induced electrons and holes. Lower fluorescence emission intensity suggests higher separation efficiency and an enhanced photocatalytic activity. 1% PdO/WO₃ is lower than other samples under 369nm excitation. Moreover, the lower peak intensity of PL implies either slower recombination process with longer decay lifetime or faster migration process with shorter decay lifetime. With the PdO content increasing, the samples’ fluorescence intensity becomes lower and lower, compared to pure WO₃. The 1% Pd/WO₃, with the lowest fluorescence intensity, was indicated higher separation efficiency of the photo-induced electrons and holes, which corroborated the conclusion of photo-disinfection.

3.7. Biosafety evaluation of PdO/WO₃
Hamster lung (V79) cells were used to evaluate the biosafety of PdO/WO₃. Different concentrations of 1% PdO/WO₃ were added to the cell culture fluid for 24h, and the cell viability was determined. There was little cytotoxicity of different concentration of the samples, and the number of living V79 cells were 101.54 ± 4.81%, 108.40 ± 2.28%, 105.68 ± 1.59% and 108.15 ± 2.58% corresponding to 0.1mg/mL, 0.2mg/mL, 0.4mg/mL and 0.8mg/mL respectively. There was no significant difference of cell viability between 0.2mg/mL, 0.4 mg/mL and 0.8mg/mL (P < 0.5). The cell viability between 0.1 mg/mL, 0.2 mg/mL and 0.8 mg/mL were significant. Therefore, the composites have proved no cytotoxicity, which showed the samples is a biosafety materials.
Figure 8. Cytotoxicity of different concentration 1%PdO/WO3 evaluated by V79 cells viability assay for 24h.

4. Conclusions
In summary, 1% PdO/WO3 composites were synthesized via precipitation method, followed by calcination. And PdO was successfully loaded on the surface of WO3, the photocatalytic efficiency has been greatly improved. 1% PdO/WO3 shows excellent photocatalytic disinfection performance. After visible light irradiation for 25 min, all of the E.coli were killed. Furthermore, the scavenger test certified that the •OH and h+ play the main roles in the photodisinfection. The biosafety evaluation shows that the samples have no cytotoxicity to mammalian cell. Thus, the prepared PdO/WO3 composite could be a potential and environmental friendly photodisinfection material.

Acknowledgement
We are grateful for the National Natural Science Foundation of China-Henan Talents Fostering Joint Funds (No. U1504311), and the Key Research Projects of the Science and Technology Department of Henan Province (No. 182102210153) for funding. We thank Professor Yawei Han, Doctor Wenshan Zhou and Miss Panpan Li for their assistance in the cell culture and CCK assay.

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