Materials & Methods

3. Materials and Methods

3.1 Bacterial culture

Bacterial culture of *Pseudomonas aeruginosa* (MTCC NO 2581) was procured from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India.

3.2 Isolation of bacterial nanowires

The culture of *P. aeruginosa* was grown in LB medium from a −80 °C freezer stock. After several days' growth, 100 µl aliquots were taken and used to inoculate replicates of 50 mL flask of LB broth. Isolation of bacterial nanowires was conducted in two different conditions of culture broth: 1. Log phase and 2. Stationary phase. Both log and stationary phase of bacterial cultures were centrifuged at 3,000 rpm for 10 minutes followed by washing with double distilled water for 5 times. To the resulting pellet, 1 mL of distilled water was added. This bacterial suspension was imaged by Atomic Force Microscopy (AFM).

3.3 Separation of bacterial nanowires

Bacterial nanowires were prepared with minor modification of Chandlar and Kulasekharam method (Chandlar and Kulasekharam 1970). Whole bacterial culture grown in LB broth medium was deflagellated for 3 min by two different methods: 1. Sonicator and 2. Magnetic stirrer. Both log and stationary phase bacterial cultures were used in this process. The aggregated cells and flagella were dispersed by vibromixing the culture with 0.4% Nacl and 0.02% sodium dodecyl sulphate (SDS) (1:10) for 3 min. After this, the cells were separated from the flagella using centrifugation 16,000g for 30 min.

The supernatant was then filtered through a Millipore GSWP 0.22 µm membrane and the flagella were resuspended into 0.4 percent of saline from the surface of the membrane by gently rubbing it with a curved glass rod. The resulting flagella suspension was examined under the AFM and a little visible contamination was evident.

3.4 Bacterial nanowires characterization

3.4.1 AFM

AFM images of the isolated and separated bacterial nanowires using various strategies were recorded using Shimadzu SPM 9500-2J scanning probe microscope and the roughness was also measured.

3.4.2 HR-TEM

HR-TEM analysis of the bacterial nanowires of *P. aeruginosa* was carried out in a FEI-200KeV, LaB_6 filament, Tecnai T20 G^2 TEM system. Filtered uranyl acetate (2% solution dissolved in distilled water) was used as the negative-staining reagent. The harvested samples were applied as droplet on a TEM grid, stained with 2% uranyl acetate, and dried in air after removing excess media on the grid using absorbent paper.

3.5 Electrochemical conductivity measurements of bacterial nanowires

3.5.1 CV and LSV studies

Electrochemical experiments were carried out with a CHI 660B electrochemical workstation (CH Instrument Inc. USA) using a conventional one-compartment three-electrode cell. A glassy carbon (GC) electrode was utilized as the working electrode (electrode area: 0.07 cm^2), whereas a silver/silver chloride electrode (Ag/AgCl) was

employed as the reference electrode, and a platinum coil was used as the counter electrode. The GC electrode was double polished using alumina powder (0.05μm) followed by sonication in double distilled water for 3 min and used for the modification of the electrode. Experiments were carried out in a deaerated 0.1 M H_2SO_4.

3.5.2 The bacterial nanowires film

The bacterial nanowire coated modified GC electrode was prepared by mixing a 0.5 mg/mL suspension of nanowire coated with metal oxides with 0.5% of nafion polymer in double distilled water under ultrasonication and a known amount (10 μL) of this suspension was cast on the cleaned GC electrode surface and allowed to dry at room temperature which used for electrochemical measurements at a scan rate of 20 mVs^{-1} in 20±2°C.

3.5.3 EIS

Electrochemical experiments were carried out with a CHI 660B electrochemical workstation (CH Instrument Inc. USA) using a conventional one-compartment three-electrode cell. A Ti substrate electrode was utilized as the working electrode (electrode area: 1 cm^2), where as a silver/silver chloride electrode (Ag/AgCl) was employed as the reference electrode, and a platinum coil was used as the counter electrode. The Ti substrate was cleansed by sonication for 10 min in acetone and in double distilled water for 10 min. Ar was used to purge the solution to achieve an O_2 free condition. All the electrochemical experiments were performed out at room temperature.

EIS responses obtained at Ti substrate electrodes modified with bacterial nanowire and metal oxide coated bacterial nanowire. Redox analyte is 1 mM of $K_3Fe(CN)_6$ in 0.1 M KCl. The electrode was polarized at 0.25 V and the frequency range was 1 Hz to 100 kHz. We have used $[Fe(CN)_6]^{3-/4-}$ couple as the redox probe to study the electrical/conductive behavior of the bacterial nanowire. The Nyquist diagram of the complex impedance represents the imaginary versus the real part of the impedance. The Nyquist plot shows a semicircle at higher frequencies corresponding to the electron-transfer-limited process, and the linear portion at lower frequencies corresponding to the diffusion-limited process. The polarization resistance (R_p) was calculated using 'Z' view software.

3.6 Metal oxide nanoparticles synthesis

Semiconductor metal oxide nanoparticles (CuO, NiO and ZnO) were synthesized using different methods.

3.6.1 Copper nanoparticles (CuO) synthesis

Materials

Copper acetate glacial acetic acid and sodium hydroxide were used in the experiments. All the chemicals used were of analytical reagent grade obtained from Merck (Mumbai, India), and deionized water is used for the preparation of solutions.

CuO nanoparticles were synthesized according to the method reported by Zhu *et al*., (2004). A 300 mL aliquot of 0.02 M copper acetate aqueous solution was mixed with 1 mL of glacial acetic acid in a round-bottomed flask equipped with refluxing device. The solution was heated to 100 °C with vigorous stirring; then about 0.8 g of sodium hydroxide solid was rapidly added into the above boiling solution until the

mixture reached pH 6 −7, where a large amount of black precipitatewas simultaneously produced. After being cooled to room temperature, the precipitate was centrifuged, washed once with distilled water and three times with absolute ethanol, respectively, and dried in air at room temperature.

3.6.2 Nickel xide nanoparticles (NiO) synthesis

Materials

Nickel nitrate hexahydrate and Cobalt amide were used in the experiments. All the chemicals used were of analytical reagent grade obtained from Merck (Mumbai, India), and deionized water was used for the preparation of solutions.

Nickel oxide nanoparticles were prepared using Li *et al*., (2007). First, the precursor of the nano NiO was synthesized using a homogeneous precipitation method. According to the molar ratio of nickel nitrate hexahydrate to urea at 1:4, a stoichiometric amount of $Ni(NO_3)_2 \cdot 6H_2O$ (0.08 M) and $CO(NH_2)_2$ (0.32 M) was accurately weighed and dissolved into 80 mL of deionized water, respectively. The two solutions were mixed in a beaker and stirred with a magnetic stirrer at room temperature until a homogeneous solution obtained. Thereafter, the mixture was transferred into a round bottom flask, sealed, and maintained heating at 115°C for 1.5 h in an oil bath. In this process, a kind of light green sediment was formed. After the reaction was completed, the precipitated powders were filtered and washed with deionized water to neutral and colorless. This was to remove the possibly adsorbed ions and chemicals to reduce the potential of agglomeration. After being dried in an oven at 90 °C for 6 h, the precursors were calcined

in a muffle furnace at 400°C for 1h to obtain the products in dark color. The calcined products were then collected for further analyses.

3.6.3 Zinc oxide nanoparticles (ZnO) synthesis

Materials

Zinc sulfate heptahydrate and sodium hydroxide were used in the experiments. All the chemicals used were of analytical reagent grade obtained from Merck (Mumbai, India), and deionized water is used for the preparation of solutions.

ZnO nanoparticles were synthesized using the method reported by Daneshvar *et al*., (2007). To the aqueous solution of zinc sulfate, sodium hydroxide solution was added slowly drop wise in a molar ratio of 1:2 under vigorous stirring, and the stirring was continued for 12 h. The precipitate obtained was filtered and washed thoroughly with deionized water. The precipitate was dried in an oven at 100°C and ground to fine powder using agate mortar.

3.7 Synthesized metal oxide nanoparticles characterization

3.7.1 UV- Visible diffuse reflectance spectra (DRS)

DRS were recorded for metal oxide nanoparticles using the JASCO-Spectra Manager (V-550).

3.7.2 Fourier transform infrared spectroscopy (FT-IR)

The FT-IR spectra of metal oxide nanoparticles were recorded using Shimadzu 8201 PC FT-IR spectroscopy. The spectral region between 4000 and 400 cm^{-1} was scanned and KBr disc method was used for recording the spectra.

3.7.3 X-ray diffraction (XRD)

XRD patterns of metal oxide nanoparticles were recorded by X-ray diffractometer (XPERT-PRO) operating at a voltage of 40 kV and at a current of 30 mA with Cu Kα radiation (λ= 1.54060 Å). The XRD patterns were taken in the 2 θ range of 10º to 80º in a fixed time mode at room temperature.

3.7.4 AFM

AFM images of the metal oxide nanoparticles were carried out by using Shimadzu SPM 9500-2J scanning probe microscope and the roughness was also measured.

3.7.5 SEM

The surface morphology of metal oxide nanoparticles was imaged through scanning electron microscopy (SEM) images. The SEM images were recorded using JEOL (ModelL-6390) scanning electron microscope operated at 10 KV.

3.7.6 HR-TEM

HR-TEM analysis of metal oxide nanoparticles were carried out in a FEI-200KeV, LaB_6 filament, Tecnai T20 G^2 TEM system. Filtered uranyl acetate

(2% solution dissolved in distilled water) was used as the negative-staining reagent. The harvested samples were applied as droplet on a TEM grid, stained with 2% uranyl acetate, and dried in air after removing excess media on the grid using absorbent paper

3.8 Fabrication of metal oxide nanoparticles coated bacterial nanowires film

The bacterial nanowire coated with metal oxide nanoparticles (CuO, NiO and ZnO) were prepared by mixing a 0.5 mg/mL suspension of nanowire coated with metal oxides in double distilled water and a known amount (10 µL) of this suspension was cast on the cleaned glass plate surface and allowed to dry at room temperature which used for atomic force microscopy for metal oxide coated bacterial nanowires surface identification.

3.9 Electrochemical measurements of metal coated bacterial nanowires

3.9.1 CV and LSV studies

The electrochemical experiments were carried out using the same procedure as per 2.5.1 described above. The film prepared for this would be slightly modified as the bacterial nanowire coated with metal oxides (CuO, NiO and ZnO) modified GC electrode was prepared by mixing a 0.5 mg/mL suspension of nanowire coated with metal oxides with 0.5% of nafion polymer in double distilled water under ultrasonication and a known amount (10 µL) of this suspension was cast on the cleaned GC electrode surface and allowed to dry at room temperature which used for electrochemical measurements at a scan rate of 20 mVs^{-1} in 20±2°C.

3.9.2 EIS studies

The EIS was used to study the electrical/conductive behavior of the bacterial nanowire and metal oxides (CuO, NiO and ZnO) coated bacterial nanowires using the same procedure described above in 3.5.3.