CHAPTER – III

EXPERIMENTAL DETAILS

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Experimental details

3.1. Materials and Chemicals

Sodium bentonite clay (Na-bent) as host material and Silver nitrate ($AgNO_3$), Titanium isopropoxide [$Ti(OC_3H_7)_4$], Copper sulphate pentahydrate ($CuSO_4.5H_2O$), Zinc nitrate hexahydrate ($Zn(NO_3)_2.6H_2O$, Manganese acetate tetrahydrate [$Mn(CH_3COO)_2.4H_2O$] and Manganese benzoate tetrahydrate ($C_{14}H_{10}MnO_4.4H_2O$), Manganese nitrate tetrahydrate ($Mn(NO_3)_2{\cdot}4H_2O$), are used as precursors as well as guest materials and were purchased from Sigma-Aldrich Chemicals Pvt. Ltd. Sodium hydroxide (NaOH), Sodium borohydride ($NaBH_4$), and Polyvinylpyrrolidone (PVP) $(C_6H_9NO)_n$ were obtained from Merck, India Pvt. Ltd., and these were used as a precipitating, reducing, and stabilizing agents, respectively for the preparation of nanoparticles. N-trimethyl-N-propylammonium bis(trifluoromethane sulfonyl)imide ($C_8H_{16}F_6N_2O_4S_2$) ionic liquid (IL) was used as capping agent for intercalation nanoparticles and it was purchased from Merck (India Pvt. Ltd.). All chemicals were used as received and deionized (DI) water was used throughout the experiment.

The main composition of the pristine sodium bentonite (Na-bent) clay material was 68.7 % SiO_2, 17.2 % Al_2O_3, 3.7 % Na_2O, 2.3 % K_2O, 1.6 % MgO, 0.9 % Fe_2O_3, 0.4 % CaO, 0.13 % TiO_2, and 0.05 % MnO. The ignition loss of the pristine material at 1273 K was found to be 5.0 %.

3.2. Methods

3.2.1. SYNTHESIS OF Mn_3O_4/BENTONITE NANOCOMPOSITES

3.2.1.1. Synthesis of pristine manganese oxide (Mn_3O_4) nanoparticles

In a typical synthesis, pure Mn_3O_4 nanoparticles were obtained using three different manganese precursors by co-precipitation method. Solutions (0.1 M) manganese nitrate ($Mn(NO_3)_2.4H_2O$ for C3) and sodium hydroxide were dissolved in 100 mL DI water, followed by constant stirring for 2 h at room temperature. After stirring, black gel suspension was formed, then kept aside overnight to settle the residue. The residual precipitate was centrifuged, and then washed with DI water for several times to remove unreacted chemicals. The final black product of manganese hydroxide was removed, and then dried in hot air oven at 100 °C for 10 h. The obtained black powder was further calcinated at 600 °C for 5 h in a muffle furnace to form crystalline Mn_3O_4 nanoparticles.

This procedure was repeated with the other precursors 0.1 M manganese acetate ($Mn(CH_3COO)_2.4H_2O$ for C1) and manganese benzoate tetrahydrate ($C_{14}H_{10}MnO_4.4H_2O$ for C2), and 0.1 M sodium hydroxide separately. The final product of Mn_3O_4 nanoparticles was obtained using the above mentioned dried and calcination conditions separately.

3.2.1.2. Synthesis of Mn_3O_4/bentonite nanocomposites

For the synthesized Mn_3O_4/bentonite nanocomposite, the Na-bentonite clay (10 g) and manganese nitrate tetrahydrate (C3 - 4.57 g) were dissolved in 100 ml of DI water. The homogenous solid suspension was obtained with the pH 7. Suspensions were stirred for 3 h at room temperature. The resultant solid suspensions were settled down after 24 h and washed several times with DI water to remove unwanted ions by cationic exchange capacity (CEC) process. The resulting pure solid residue was filtered and dried at 100 °C in an hot air oven for

12 h. The dried sample was calcinated at 600 °C for 5 h and the obtained Mn_3O_4 was grinded by mortar and pestle. Finally the crystalline black powders of Mn_3O_4/bent composite were obtained.

The Mn_3O_4/bentonite nanocomposite C1 and C2 was prepared using the same procedure as C3 except for the manganese precursor, applying manganese acetate ($Mn(CH_3COO)_2.4H_2O$ - 4.57 g), manganese(II) benzoate tetrahydrate ($C_{14}H_{10}MnO_4.4H_2O$ - 5.41 g), and Na-bentonite (Na-bent, 10 g). The centrifugation, washing, drying, and calcination procedures were the same as for the C3 composite.

3.2.1.3. Preparation of Ag nanoparticles

Silver (Ag) nanoparticles were prepared from $AgNO_3$, $NaBH_4$, and PVP as templating materials. In the typical experiment, 0.025 M $AgNO_3$ solution (A) was dissolved in DI water. Another solution (B) was prepared by dissolving PVP and $NaBH_4$ in DI water and mixed together. The dissolved solution (A) was added into solution (B) drop-by-drop, the aqueous solution that had been chilled in an ice bath. The aqueous solution was mixed and kept at room temperature and continuously vigorous stirred for 30 min. The suspension turned amber yellow color indicating the formation of Ag nanoparticles, confirmed by visual observation. The colloidal amber yellow suspension was centrifuged at 3,800 rpm for 10 min and washed with DI water, then dried in an oven for overnight at 100 °C. Finally, the dried Ag nanoparticles were obtained.

3.2.3. SYNTHESIS OF Ag/TiO_2/BENT NANOCOMPOSITES WITH AND WITHOUT IONIC LIQUID

3.2.3.1. Preparation of TiO_2 nanoparticles

A mixture of 0.1 M of $[Ti(OC_3H_7)_4]$ solution and 0.1 M of NaOH was dissolved in DI water. The solution was stirred vigorously by a magnetic stirrer for 2 h at room temperature and

it was allowed to settle down. The collected thick white suspension was then centrifuged at 4,000 rpm for 15 min. The white solid suspension was washed several times with DI water and then dried in an oven at 100 °C for 7 h. After cooling down to room temperature, the white solid was grinded into a fine powder and it was calcinated at 500 °C for 3 h.

3.2.3.2. Preparation of Ag/TiO_2/bent nanocomposite with IL

The Ag/TiO_2/bent nanocomposite was green synthesized by thermal decomposition method. 3.6 g of Na-bent clay was dispersed with DI water under continuous stirring until a suspension was formed. Then, 0.025 M of $AgNO_3$ was dissolved into the suspension with stirring, followed by the addition of 0.1 M of $[Ti(OC_3H_7)_4]$ solution. The suspension was vigorously stirred at room temperature for 2 h and was centrifuged at 4,500 rpm for 15 min. The final product was washed with DI water. The solid suspension was dried at 100 °C for 9 h and it was calcinated at 500 °C for 3 h. Finally, the calcinated samples were grinded into fine powder. All the samples were stored in a dark place to avoid being pre-activated by daylight.

3.2.3.3. Preparation of Ag/TiO_2/bent nanocomposite without IL

The Ag/TiO_2/bent nanocomposite was prepared using the same procedure as shown in section 3.2.3.2 without addition of IL, applying $AgNO_3$ (0.025 M), $[Ti(OC_3H_7)_4]$ (0.1 M), and pristine Na-bentonite (3.6 g). The centrifugation, washing, drying and calcination procedures were also the same as shown in the section 3.2.3.2.

3.2.4. SYNTHESIS OF Ag/CuO/BENT NANOCOMPOSITES WITH AND WITHOUT IONIC LIQUID

3.2.4.1. Synthesis of CuO nanoparticles

CuO nanoparticles were prepared by dissolving 0.1 M $CuSO_4.5H_2O$ in 100 mL DI water; it was stirred for 10 min and 0.1 M NaOH was added to above solution and the mixture was

stirred for about 3 h at room temperature and the pale blue solution was precipitated instantaneously. Then the precipitate was centrifuged at 4,000 rpm for 10 min and washed thoroughly with DI water. The obtained precipitate was dried in an oven at 100 °C for 12 h. Finally, it was calcinated kept in a muffle furnace at 500 °C for 3 h. After calcination, brownish black powder of CuO nanoparticles was collected.

3.2.4.2. Synthesis of Ag/CuO/bentonite nanocomposite with IL

For the synthesis of Ag/CuO/bent/IL nanocomposite, 3.6 g of pristine Na-bentonite was completely dissolved in DI water and stirred and then 1 ml of IL was added to the suspension at room temperature for 20 min. After 20 min, 0.025 M of $AgNO_3$ and 0.1 M of $CuSO_4.5H_2O$ were slowly added to the above suspension. The sandal color reaction mixture was turned to pale brown color after the addition of silver and copper precursors. The mixture was continuously stirred for 3 h at room temperature. The appearance of the pale brown color suspension was indicated the formation of Ag/CuO/bent/IL nanocomposite. The suspension was centrifuged at 5,000 rpm for 10 min and washed several times with DI water to remove possible remaining ions in the product. The separated solid suspension was dried in an oven at 100 °C for 12 h. The resulting product was dried at 500 °C for 3 h by using a muffle furnace. After 3 h, the final product was allowed to cool down to the room temperature and collected composite was crushed using pestle and mortar. The final resultant pale brownish black Ag/CuO/bent/IL nanocomposite was used for the further studies.

3.2.4.3. Synthesis of Ag/CuO/bent nanocomposite without IL

The Ag/CuO/bent nanocomposite was prepared using the same procedure as shown in section 3.2.4.2 without addition of IL, applying $AgNO_3$ (0.025 M), $CuSO_4.5H_2O$ (0.1 M), and

pristine Na-bentonite (3.6 g). The centrifugation, washing, drying and calcination procedures were also the same as shown in the section 3.2.4.2.

3.2.5. SYNTHESIS OF Ag/ZnO/BENT NANOCOMPOSITES WITH AND WITHOUT IONIC LIQUID

3.2.5.1. Synthesis of ZnO nanoparticles

A mixture of 0.1 M of $Zn(NO_3)_2.6H_2O$ solution and 0.1 M of NaOH was dissolved in DI water. The solution was stirred vigorously by a magnetic stirrer for 4 h at room temperature and it was allowed to settle down. The collected thick white suspension was then centrifuged at 4,000 rpm for 15 min. The white solid suspension was washed several times with DI water and then dried in an oven at 100 °C for 7 h. After cooling down to room temperature, the white solid was grinded into a fine powder and it was calcinated at 500 °C for 3 h.

3.2.5.2. Synthesis of Ag/ZnO/bent nanocomposite with IL

The Ag/ZnO/bent nanocomposite was synthesized by thermal decomposition method. 3.6 g of Na-bentonite clay was dispersed completely with DI water under magnetic stirring and then added 1 mL of IL was injected into the suspension up to 30 min. Then, 0.025 M of $AgNO_3$ was dissolved into the suspension with stirring, followed by the addition of 0.1 M of $Zn(NO_3)_2.6H_2O$ solution. The suspension was vigorously stirred at room temperature for 6 h and was centrifuged at 4,500 rpm for 15 min. The final product was washed with DI water. The solid suspension was dried at 100 °C for 9 h and was calcinated at 500 °C for 3 h. Finally, the calcinated samples were grinded into fine powder. All the samples were stored in a dark place to avoid being pre-activated by daylight.

3.2.5.3. Synthesis of Ag/ZnO/bent nanocomposite without IL

The Ag/ZnO/bent nanocomposite was prepared using the same process as section 3.2.5.2 expects for the IL, applying $AgNO_3$ (0.025 M), $Zn(NO_3)_2.6H_2O$ (0.1 M), and Na-bent (3.6 g). The centrifugation, washing, drying and calcination procedures were the same as for the section 3.2.5.2 nanocomposite.

3.2.6. SYNTHESIS OF Ag/Mn_3O_4/BENT NANOCOMPOSITES WITH AND WITHOUT IONIC LIQUID

3.2.6.1. Synthesis of Ag/Mn_3O_4/bent nanocomposite with IL

Synthesis of Mn_3O_4 nanoparticles were discussed in section 3.2.1.1. For the synthesized Ag/Mn_3O_4/bent/IL nanocomposite, Na-bentonite clay (3.6 g) was dispersed completely with DI water under constant stirring, and then 1 mL of IL was added drop by drop injected into the solution up to 30 min. Then, 0.025 M of $AgNO_3$ was dissolved into the suspension with stirring, followed by the addition of 0.1 M of $Mn(NO_3)_2.6H_2O$ solution. The suspension was continuously stirred at room temperature for 4 h and was centrifuged at 4,500 rpm for 15 min. The resulting blackish brown color suspension was washed with DI water and dried in hot air oven at 100 °C for 10 h. At the end, the dried sample was calcinated at 600 °C for 5 h. Finally, the calcinated samples were grinded into fine powder. All the samples were stored in a dark place to avoid being pre-activated by room light.

3.2.6.2. Synthesis of Ag/Mn_3O_4/bent nanocomposite without IL

The Ag/Mn_3O_4/bent nanocomposite was prepared using the same process as section 3.2.6.2 extract expects for the IL, applying $AgNO_3$ (0.025 M), $Mn(NO_3)_2.6H_2O$ (0.1 M), and Na-bentonite (3.6 g). The centrifugation, washing, drying and calcination procedures were the same as for the section 3.2.6.2 nanocomposite.

3.3. Characterizations

The crystal structure of the composites was characterized by X-ray diffraction (XRD) analysis using a JEOL IDX 8030 X'Pert Pro instrument with Cu Kα radiation (k = 1.5418 Å) in the range of 10-75° with the step size of 0.02°. The elemental compositions on the surface of the samples were determined by the XPS analysis**.** This spectrum was recorded using Al Kα X-ray source on an AXIS Ultra instrument. The X-ray source of Al Kα target with monochromatic (Al Kα, hα = 1.486 eV) was used with a power of 300 W. Adventitious carbon (internal standard - C1s) was 284 eV. The average particles sizes of the nanomaterials were analyzed by (nano track type ultra serial number: U2475ES).

The morphology and elemental analysis were studied using scanning electron microscope (SEM with EDX) working at a 20 kV accelerating voltage. The surface morphologies and the elements are present in the samples were studied by HR-SEM with EDX from Quanta 250FEG instrument operated at an accelerating voltage of 15 kV. Transmission electron microscope (TEM) (model PHILIPSCM200) was performed at an accelerating voltage of 20-200 kV, with the resolution 2.4 Å. The samples for TEM analysis were prepared by dispersing samples in water by sonicated for 15 min and then drying on a copper grids coated with holey carbon film. The shape and size of the samples were analyzed by FE-TEM with SAED patterns using a JEM-2100F microscope, working at 200 kV accelerating voltage. The HR-TEM was obtained by using a JEOL 4000 Ex instrument operating at 400 kV. The samples for TEM measurements were prepared by smearing a drop of as-prepared products dilute dispersions on gold grids. The specific surface area (m^2 g^{-1}), pore diameter (nm), mesoporosity (cm^3/g) were estimated using the Brunauer-Emmet-Teller (BET) method with the nitrogen (N_2) adsorption isotherm using a Gemini VII 2390 nitrogen adsorption apparatus. The samples were sonicated for 2 h prior to the

for uniform distribution test. FT-IR of the powders was characterized using Perkin Elmer Model Spectrum RX1 and the spectra were recorded in the range of 4,000 to 400 cm^{-1}. The UV-Vis DRS spectra of the powders were recorded using a JASCO UV-Vis 530 spectrophotometer. The bandgap energy (E_g) of the samples was obtained using the following Equation 3.1, where E_g is the band gap (eV) and λ is the wavelength of the absorption edges in the spectrum (nm).

$$E_g = {}^{1240}/_{\lambda eV} \qquad \text{……..} \quad (3.1)$$

The diffuse reflectance data was transformed to the absorption coefficient (α) values according to the Kubelka-Munk Equation 3.2, where R is the absolute reflectance of the sample layer and K and S are the molar absorption and scattering coefficients, respectively.

$$f(R) = \frac{(1-R)^2}{2R} = \frac{K}{S} \qquad \text{……} \quad (3.2)$$

3.4. Antimicrobial assessment

3.4.1. Optical density method

The antibacterial activities of the samples were evaluated by the well diffusion method. Lyophilized bacteria were reconstituted in nutrient broth and cultured overnight at 37 °C. After overnight incubation, cells were pelleted by centrifugation and washed three times with DI water and to remove debris and maintained as a stock solution (1 mL) and stored at -20 °C. The bacteria were grown in a nutrient broth medium at 37 °C for 24 h, and then it was several diluted to get 10^6 (colony-forming unit) CFU/mL. The Muller Hinton agar was poured into petri plates (10 cm in diameter) and allowed to cool. Once the medium was solidified, the above concentration of test organism was swabbed on the agar plate. Once the organism has been absorbed by the agar, three wells (6 millimeters (mm) in diameter) were made and in each well, 100 µL of 1×10^{-4} g mL^{-1} were placed into the well and incubated at 37 °C for 24 h [3]. Potato dextrose agar (PDA) is the most widely used medium for yeast and molds, and can be

supplemented with acid to inhibit bacterial growth. Fungal strains were incubated at 25 °C for 10 days on potato dextrose broth (PDB) slant. PDB can be made by boiling 4 g potato extract using DI water for 3 min. All fungal isolates were cultured using PDA made from 4 g potato, 20 g dextrose, and 20 g agar, sterilized using an autoclave at 121 °C for 15 min, with final pH maintained at 5.6 ± 0.2 at 25 °C [4]. Heating the acidified medium in acid state will hydrolyze the agar. Once the medium had solidified, the test organism was swabbed onto the plate covered by the solidified medium, and after 10 min (while the organism was absorbed by the agar), samples were dissolved in dimethyl sulfoxide (DMSO) solvent; this suspension was dropped onto the sterile antibiotic disc. A clear zone of inhibition was observed on the next day. Amikacin for bacteria and ketoconazole for fungus and dimethyl sulfoxide (DMSO) were used alone as standard and negative control to compare the antimicrobial behavior. The plates were allowed to stand for 1 h or more for diffusion and then incubated at 37 °C for 24 h. The diameter of inhibitory zones was measured in mm. The inoculated plates were incubated at 25 °C (aerobic atmosphere) for 3 days. The antibacterial activities were performed in triplicate.

3.5. Cytotoxicity Assay

3.5.1. Cell line

The cytotoxicity studies were performed for all synthesized samples by using HEK 293 which was obtained from National Centre for Cell Science (NCCS), Pune, India. The cells were grown in Eagles Minimum Essential Medium containing 10 % fetal bovine serum (FBS). The cells were maintained at 37 °C, 5 % CO_2, 95 % air, and 100 % relative humidity. The cultured cells were passaged weekly and the medium was changed twice a week.

3.5.2 Cell treatment procedure

The adhered cells were detached with trypsin-ethylenediaminetetraacetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium containing 5 % FBS to give a final density of 1×10^5 cells/mL. Then, 100 µL per well of cell suspension were seeded into 96 - well plates at a plating density of 10,000 cells/well and incubated to allow for cell attachment at 37 °C, 5 % CO_2, 95 % air, and 100 % relative humidity. After 24 h of incubation, the cells were treated with serial concentrations of the test samples. They were initially dispersed in phosphate buffered saline (PBS) by sonication and an aliquot of the sample solution was diluted twice to get the desired maximum test concentration with serum-free medium. Additionally, four serial dilutions were made to provide a total of five samples concentrations. Aliquots of 100 µL of the different sample dilutions were added to the appropriate wells that contain 100 µL of the medium, resulting in the required final sample concentrations. Following sample addition, the plates were incubated for an additional 48 h at 37 °C, 5 % CO_2, 95 % air, and 100 % relative humidity. The medium containing without samples were served as control and triplicate were maintained for all concentrations [5].

3.5.3 MTT assay

3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) is a yellow water-soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan is produced directly proportional to the number of viable cells. After 48 h of incubation, 15 µL of MTT (5 mg/mL) in PBS was added to each well and incubated at 37 °C for 4 h. The medium with MTT was then flicked off and the formed formazan crystals were

solubilized in 100 µL of DMSO. Then, the absorbance was measured at 570 nm using microplate reader in Equation 3.3 and Equation 3.4,

The percentage cell growth was then calculated with respect to control as follows,

$$Percentage\ cell\ growth = ([A]\ Test)\ /\ ([A]control)\ \times 100 \quad \ldots\ldots\ldots \quad (3.3)$$

The percentage cell inhibition was determined using the following formula,

$$Percentage\ cell\ inhibition = 100 - Abs\ (sample)/Abs\ (control)\ \times 100 \quad \ldots\ldots\ldots \quad (3.4)$$

Nonlinear regression graph was plotted between percentage cell inhibition and log concentration and IC_{50} were determined using GraphPad Prism software [6].

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