3. Material and Methods

3.1 Chemicals

The phenolic resin-based ACFs were purchased from Nippon Kynol Inc., Japan. Copper nitrate (Cu (NO$_3$)$_2$.3H$_2$O), silver nitrate (AgNO$_3$), Zinc nitrate (Zn (NO$_3$)$_2$), Triton-X 100, ethylene diamine tetra acetate (EDTA), sodium dodecyl sulfate (SDS), and dimethyl sulfoxide (DMSO) were purchased from Merck, Germany. Fetal bovine serum (FBS), streptomycin, penicillin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Himedia, India. RPMI-1640, L-glutamine, Polyvinyl acetate (PVAc), and histopaque-1077 were purchased from Sigma Aldrich, India. Flourescein and the propidium iodide (PI) dye were purchased from Titan Biotech, India and Invitrogen, India, respectively. The reagents used to prepare the Luria Bertani (LB) medium, including tryptone, sodium chloride (NaCl), yeast extract and agar, were purchased from Merck, Germany. Hydrogen, nitrogen, and C$_2$H$_2$ (AAS grade) were purchased from Sigma Gases, India. E. coli K-12, S. aureus RN4220 and Methicillin resistance S. aureus (MRSA) were procured from IMTech, Chandigarh, India. The Cicer arietinum seed was purchased from Indian Institute of Pulses Research (IIPR), Kanpur, India. All solutions were prepared using Milli-Q water (Milli-Q, Integral A-10 system, France). All reagents were used of high purity grade.

3.2 Preparation of ACFs/CNFs

3.2.1 Pretreatment of ACFs

The preparation details for metal-ACF/CNFs are available in previous studies [Bikshapati et al., 2012; Singh et al., 2013]. Briefly, the as-received ACFs were pretreated to remove any undesirable species from their surfaces. A sample of the ACFs (~ 2 g) was treated with 5 ml of 0.03 M nitric acid in 1 L of deionized water at 80 °C for 2 h and then washed several times with deionized water until the pH of the ACF surface became neutral. Next, the sample was vacuum-dried at 120 °C for 6 h to remove any entrapped gases.

3.2.2 Impregnation of ACFs samples

Separate solutions of 0.4 M Cu (NO$_3$)$_2$.3H$_2$O and 0.03 M AgNO$_3$ in deionized water were prepared for impregnating the ACF using the wet incipient method. Fig.
3.1 shows the schematic representation of impregnating set-up. SDS (0.3% w/w) was used as a surfactant to increase the monodispersion of the metal salts in the solution. The impregnated samples were dried at room temperature (~ 30 °C) for 3 h, followed by oven drying at 120 °C for 12 h. The Cu- and Ag-NPs were produced in-situ on the ACFs by calcination at 200 °C and 150 °C for 4 h, followed by H₂ reduction at 330 °C and 250 °C for 2 h, respectively. The prepared ACFs dispersed with metal NPs, Cu-ACFs or Ag-ACFs, were used as the substrate for the growth of the CNFs by chemical vapor deposition (CVD), using acetylene (C₂H₂) as the carbon source. Fig. 3.2, Schematic of catalytic vapor deposition (CVD) set-up. The growth of the CNFs on the ACFs using Cu and Ag occurred by the tip-growth mechanism, as corroborated by scanning electron microscopy (SEM) and discussed later. Fig. 3.3 Growth mechanism of CNFs on the ACFs (M= metals). Some Cu- and Ag-ACF/CNF samples were ultra-sonicated to dislodge the metal NPs from the tips of the nanofibers. The sonication of Cu-ACF/CNF was carried out in 0.05 M HNO₃ for 10 min and that of Ag-ACF/CNF was carried out in 0.03 M HNO₃ for 3 min. Next, the sonicated samples were rinsed in DI water several times to neutralize the excess acid. The sonicated samples are labeled Cu-ACF/CNF-S or Ag-ACF/CNF-S for reference in the subsequent text.

Fig. 3.1 Schematic representation of impregnating unit

Fig. 3.2 Schematic of catalytic vapor deposition (CVD) set-up


Fig. 3.3 Growth mechanism of CNFs on the ACFs (M= metals)
3.3 Preparation of bi-metal ACFs/CNFs

The ACF samples were treated using 5 ml of 0.03 M HNO₃ in 1 l of DI water at 80°C for 2 h to remove undesirable ions from their surfaces. Next, the treated ACFs were washed several times using deionized water until pH ∼7 of the surface. The treated ACFs samples were dried in oven for 12 h at 100 ºC followed by vacuum drying for next 12 h at 200 ºC to remove moisture and entrapped gases.

The treated ACFs was impregnated using 180 ml aqueous solution of 0.4 M Cu and Zn nitrate salts using wet incipient method. Different volume ratio of metal salts solution were used for preparing bimetallic ACFs. The bimetal ACF samples were classified as (Cu-Zn) ratio as (0-1), (1-0), (1-1), (1-3) and (3-1), which depend upon the relative amounts of Cu and Zn salts used in the impregnating solutions. The nomenclature (1-0) and (0-1) refers to the ACF sample prepared using 0.4 M of both Cu and Zn nitrates, respectively. The nomenclature (1-1) refers to Cu salt and Zn salts in 90 ml each, which is 50% of their respective volume used for impregnation. Similarly, the (1-1) and (3-1) refers to Cu and Zn salts, which are 25%, 75%, and 75%, 25%, volume of the single metal salts, respectively. The SDS (0.3% w/w) was added to the metal nitrates salts solution to enhance loading of metallic salts without agglomeration on the ACF surfaces. The prepared all volume ratio of bimetallic impregnating solution were kept in vials before and after impregnation for determining of metal loading on ACFs samples using AAS analysis.

The impregnated bi-metal ACFs samples were dried overnight at 100 ºC. Next, the bi-metal ACFs samples were calcined at 400 ºC in nitrogen atmosphere under flow rate 200 sccm for 4 h to convert the metal nitrates to their oxides form. The calcined bi-metal ACFs samples were treated at 400 ºC under hydrogen atmospheric condition to converts the oxides forms of metals into metallic states (Cu/Zn). The calcination and reduction temperatures were optimized using temperature program reduction (TPR) analysis. The optimized CVD temperature and time were at 290 ºC for 20 min using optimized flow rate of C₂H₂ during CVD process was upto 30 sccm. Fig. 3.4 describes the schematic representation of various step involved in the synthesis of (Cu-Zn)-ACFs/CNFs and its applications.
3.4 Dispersion of CNFs within the polymeric matrix

The prepared Cu-ACF/CNF was ball milled in CAP to produce the slurry of micron/nano size- (100 nm - 500 nm) fibers. To prepare the dispersion of Cu-ACF/CNF in CAP, approximately 0.1 to 0.5 g of Cu-ACF/CNF was cut into small pieces. The samples were transferred to a nano ball-mill (Retsch, Germany) and milled at 40 Hz-speed for 35 min. The ball milled Cu-ACF/CNF along with different amounts (2.5-30% w/w) of powder CAP were added to 50 ml DI water. The mixture was continuously stirred at 120 ppm for 2 h. The solution containing CAP and dispersed Cu-ACF/CNF were subsequently added to the reactant mixture for esterification, as described in the following section.

3.5 Synthesis of pH sensitive biomaterial

Fig. 3.5 describes the preparation steps for biomaterial. The primary step consisted of the formulation of PVA from PVAc by esterification. A mixture of 124
g-PVAc in 200 cc-volume of methanol was stirred in a beaker to prepare a homogenous solution. Approximately 40 g of the prepared solution was transferred to a 2-1-3 round bottom flask. The temperature of the reaction mixture was kept constant at 60 °C. 25 ml-methanol, 22 ml-methyl acetate and 0.1 ml-deionized water were added to the mixture. The suspension mixture was continuously stirred at 120 rpm until the solution was clear. Next, 10 ml of methanolic solution hydroxide (2.5% NaOH in methanol) was added to the clear solution. Approximately after 15 min, the PVA-gel was formed. At the incipience of the gel formation, a mixture of the ball-milled micron sized web of Cu-ACF/CNF dispersed in CAP was added to the reactant mixture.

![Flow-sheet for the synthesis of PVA-CAP-based polymeric film dispersed with Cu-ACF/CNF](image)

**Fig. 3.5** Flow-sheet for the synthesis of PVA-CAP-based polymeric film dispersed with Cu-ACF/CNF
After approximately 60 min of adding the mixture of ball milled Cu-ACF/CNF and CAP, a black gel of PVA-CAP-Cu-ACF/CNF blend was produced. Next, 100 ml of milli-Q water was added to the reaction mixture. The temperature and stirring speed were held constant at 60 °C and 220 rpm, respectively for the solubilization of the suspension. After approximately 5 h, a homogenous solution dispersed with the blend of PVA-CAP-Cu-ACF/CNF was produced. The pH of the produced solution was maintained constant at ~7.4 during stirring by using NaOH solution. Heater was switched off. When the temperature of the solution decreased to room temperature, black slurry like material was obtained. The stirrer was stopped and the material was removed and casted on a teflon sheet. Next, the material was vacuum dried for overnight at 40 °C before using it for the characterization study and the drug delivery tests. Some samples of PVA-CAP were also prepared without using ACF/CNF. All data are shown for PVA-CAP-Cu-ACF/CNF samples prepared using 0.5 g of Cu-ACF/CNF and 15% (w/w) CAP blended in PVA, unless specified otherwise. Fig. 3.6 schematically describes the release of Cu-ACF/CNF from the encapsulating PVA-CAP polymeric composite.

![Fig. 3.6](image_url) Schematic of Cu-ACF/CNFs release from PVA-CAP-Cu-ACF/CNF. The dissolution of PVA-CAP depends on pH
3.6 Hemolysis assay

The *in-vitro* hemolytic activity of the prepared material was determined using the protocol for the primary toxicity assay test [Fischera et al., 2003] with some modifications. The activity was determined by measuring the lysis of erythrocytes as a consequence of disruption in the cellular membrane caused by the exposure to PVA-CAP-Cu-ACF/CNF biomaterial. Briefly, blood samples were collected from a healthy volunteer and anticoagulated with EDTA for isolating erythrocytes. Blood was diluted with phosphate buffer saline (PBS) in 1:1 volume-ratio to prepare 50% hematocrit. Next, the diluted blood was centrifuged at 2400 rpm for 10 min. The supernatant (plasma) and middle layered (buffy coat) were discarded. The erythrocytes were washed three times with PBS at 2400 rpm for 15 min. Different concentrations (0.1 to 15 mg/ml) of PVA-CAP-Cu-ACF/CNF in the washed erythrocytes suspension was incubated at 37 °C in the shaking water bath for 60 min. PBS reagent and Triton-X-100 (0.2%) were used as negative (0% cell lysis) and positive (100% cell lysis) test controls, respectively. After incubation, the samples were centrifuged at 3000 rpm for 10 min and the supernatant was collected. The lysis of erythrocytes were determined by measuring the optical absorbance (OB) at 540 nm using UV-VIS (Varian Cary 100, Germany). The hemolysis (%) was calculated based on the absorbance. The hemolysis measured less than 10% was considered to be nontoxic. The test was replicated thrice to check the reproducibility. Fig. 3.7 schematically describes of the interaction between the prepared ACFs/CNFs and erythocytic cells.

\[
\text{% Hemolysis} = \left( \frac{\text{Test sample - Negative control}}{\text{Positive control - Negative control}} \right) \times 100
\]

**Fig. 3.7** A schematic representation of the interactions between ACFs/CNFs and blood cells.
3.7 Metal release profile

3.7.1 Metal release profile of bimetal ACFs/CNFs

The metal release profiles of the Cu-and Zn-NPs from the prepared bi-metallic (Cu:Zn) volume ratio of (Cu-Zn)-ACFs and (Cu-Zn)-ACFs/CNFs in Milli Q water were determined using the atomic absorption analysis (AAS) (Varian AA-240, USA). Approximately 40 mg of the (Cu-Zn)-ACFs and (Cu-Zn)-ACFs/CNFs samples were transferred into conical flask containing 20 mL-Milli Q water. The flasks were incubated at room temperature at 100 rpm in orbital shaker. The samples were taken out at different time intervals (0.5, 1, 3, 6, 12, 24, 48, and 72 h). Next, 10 mL-volume of the filtered samples was mixed with 5 mL-Milli Q water for both metals, for the AAS analysis.

3.7.2 Metal release profiles for agricultural application

The release profiles of the Cu-NPs from the Cu-ACFs and Cu-CNFs in water were determined using the atomic absorption analysis (AAS) (Varian AA-240, USA). Approximately 20 mg of the Cu-ACFs and Cu-CNFs samples were transferred into different vials (15 nos for each material) each containing 10 mL-Milli Q water. The vials were kept at room temperature. The materials-dispersed water samples were taken out at different time intervals for 15 days, using syringe filters. Next, 5 mL-volume of the filtered samples was mixed with 5 mL-Milli Q water, to be used for the AAS analysis.

3.8 In-vitro dissolution profile of the biomaterial

The dissolution profile of the material in aqueous solution was determined at different pH (5.4, 6.4, 7.0, 7.4 and 8.0). Approximately, 0.1 g of PVA-CAP-Cu-ACF/CNF was placed into tubes containing 10 ml of milli-Q water. The tubes were not shaken during dissolution. Samples (200 µl) were taken out from the tubes and replaced with fresh water at different time intervals. The dispersed biomaterials in water were further mixed in 2 ml of dimethyl sulfoxide (DMSO). The absorbance of the mixture was measured at 286 nm using UV-vis spectroscopy. The concentration of released Cu-ACF/CNF or dissolution of polymeric biomaterial was calculated on the basis of the measured absorbance.
3.9 Cytotoxicity assay

3.9.1 Isolation and culture of lymphocytes

Peripheral blood was obtained, with consent, from a healthy volunteer. Blood containing mononuclear cells (PBMCs) consisting of mainly lymphocytes [Sun et al., 2011], was collected in the heparinized vacutainer and diluted to a 1:1 ratio with phosphate-buffered saline (PBS). Lymphocytes were isolated by density centrifugation at 4000 rpm using Histopaque 1077. The lymphocyte interface was removed and washed three times with RPMI-1640 medium using a centrifuge. The cells were incubated if the number of cells was on the order of 1x10^6 cells/ml of the medium. The cells were counted using the trypan blue dye exclusion method [Ghosh et al., 2010] with the aid of a hemocytometer attached to the microscope (Lieca, DM-2000, Germany).

The washed lymphocytes was incubated at 37 °C in a medium consisting of RPMI-1640, 10% (v/v) FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin, under a humidified 5% CO₂ atmosphere. The cells were periodically counted for growth until the cell concentration exceeded 2x10^6 cells/ml of the medium. The incubation time was approximately 24 h. Next, 5-ml volumes of the incubated medium containing the lymphocytes were transferred into 15-ml culture vials for the tests.

3.9.2 Exposure of lymphocytes to ACFs/CNFs

The prepared ACFs and Cu- and Ag-ACF/CNFs (before and after sonication) in small lengths (~ 100 μm) and commercially procured CNT, GAC, silica, alumina, and zeolite, all as powders (~ 500 μm), were tested for cytotoxicity with respect to the harvested lymphocytes. First, the test materials contained in different 15-ml culture vials were sterilized by UV irradiation [Meng et al., 2010]. Next, 5 ml of the incubated cells was poured into each vial. The test was carried out in a CO₂ incubator at 37 °C. Different exposure times (3 h, 12 h, 24 h and 48 h) were used. The lymphocytes exposed to the materials were examined for cell viability and DNA fragmentation. Cells not exposed to the materials served as controls.
3.9.3 MTT Assay

The cell viability was assessed using an MTT assay [Akhtar et al., 2010; Yin et al., 2012]. This assay is based on the ability of viable cells to reduce MTT (yellow) to formazan (blue). As per the method, 0.5 mg/ml of the MTT solution was added to the vials containing cells exposed to the different test materials. The vials were left in the incubator at 37 °C for 4 h. Next, 300 μl of DMSO was added to solubilize the produced formazan salts. Next, the solutions were incubated at the room temperature (30 °C) for 2 h and then transferred to the centrifuge tubes. Centrifugation was carried out at 2000 rpm for 5 min. The test materials settled in the centrifuge were removed, and the absorbance of the supernatant in the tube was measured at 550 nm. The percentage of living cells remaining after exposure to the materials was calculated as follows: viable cells (%) = (Sample absorbance – Blank absorbance)/Control absorbance. The blank sample referred to the media containing the samples without cells.

3.9.4 DNA extraction and fragmentation

DNA was extracted from the lymphocyte-treated samples using the literature method with some modifications [Noaishi et al., 2011]. Briefly, the treated cells were washed three times with PBS using centrifugation. For the lysis of the lymphocytes, 8 ml of the lysis solution (0.5 M sucrose, 1 M Tris-Cl at pH = 7.6, 1 M MgCl₂, 2% sodium azide, 100% Triton X-100) and 14 ml of DI water were mixed thoroughly in the tubes. The lysate was incubated on ice for 2 min and then centrifuged at 3000 rpm for 20 min. Next, 6 ml of the solution containing 1 M Tris-Cl at pH = 8, 0.25 M EDTA at pH = 8, and 10% SDS were added to the suspended lysate produced in pellet form during centrifugation. The solution was mixed vigorously in a vortex mixer. Next, 30 μl of proteinase K was added to the solution. The mixture was incubated in a shaking incubator at 60 °C for 90 min and then on ice for 15 min. After adding 2 ml of NaCl, incubation was continued for 5 min. DNA was precipitated by adding a volume of ethanol twice that of the solution remaining after ice incubation. The isolated DNA from the treated samples was loaded on the 1% agarose gel along with the tris-acetate-EDTA (TAE) buffer and water (1:1 v/v) contained in the electrophoretic unit. The DNA was visualized and photographed using the gel-doc system.
3.10 Antibacterial activity

Antibacterial activities of the prepared biomaterials were determined against gram negative, *E. coli* and gram positive, *S. aureus* bacterial strains. *E. coli* (K-12) and *S. aureus* (RN4220) procured from different laboratories in India were stored in -80 °C-Luria Bertani broth (LB broth) medium. The bacterial strains were cultured in LB broth and incubated at 37 °C for 24 h. The antibacterial analysis of PVA-CAP-Cu-ACF/CNF using different amount (2, 4, 8, 12, 16, 24 and 32 mg/ml) was analyzed by plate count method. Approximately 1 ml of pure culture was mixed in 50 ml of sterilized DI water with different amount of the prepared biomaterials. The initial bacterial culture was approximately $10^7$-$10^8$ CFU/mL. Flasks containing test samples in bacterial solution were incubated at 37 °C in a shaking incubator at 100 rpm. 100-μl of samples were taken at different time intervals (3, 12, 24, 48, and 72 h) and spread on LB agar medium, followed by incubation at 37 °C for 24 h. The bacterial suspension without the material served as control. The minimum inhibitory concentration (MIC) and maximum bactericidal concentration (MBC) were also calculated against both gram negative and gram positive bacterial strains to determine the smallest amount of the biomaterial capable of inhibiting and killing the bacterial growth after overnight incubation, respectively [Lara et al., 2010; Singh et al., 2013]. All measurements were performed thrice to ensure the reproducibility.

3.11 Ball milling of ACFs/CNFs for agricultural application

The prepared CNFs/ACFs were ball milled to remove the ACFs from the web and produce the CNFs (~ 200 nm) for the comparison purposes. Some samples of the ACFs and Cu-ACFs were also ball milled to produce the micron size (~ 3 μm) materials for the comparison purposes.

3.12 Seed germination and plant growth

The germination of *Cicer arietinum* seed was carried out using the method described in the study [Wu et al., 2012], with some modifications. Briefly, the uniform size ~6 mm seed were separately disinfected for 10 min, using 70% (v/v) ethanol-water solution. The disinfected seeds were washed several times with water. The seeds were then dried in the oven at 70 °C for 3 h to remove moisture. The sterile petri dishes were used for the germination of the seeds. Two sterile filter papers were kept at the bottom of each dish. The seeds (10 numbers) were placed on the filter
papers. The dishes contained approximately 5 mL of water having different concentrations (10, 50, 100, 300, 400, and 500 μg/mL) of the Cu-CNFs. Next, the seeds were incubated at 25 °C for 3 days. Approximately 2 mL of the Cu-CNFs-dispersed water was added everyday into the petri dishes, as the make-up water to account for the evaporation-loss, and also, for the loss due to the absorption by the seeds and the Cu-CNFs. Some Cu-CNFs settled on the filter paper at the petri dish, which were also required to be replenished. The emergence of radicle from the seed coats was considered to be the criteria for germination. After 5 days, the formation of shoot and root occurred. The seeds were then transferred to the different vials containing approximately 5 mL of the Cu-CNFs-dispersed water. The growth of the plant was monitored for the next 10 days. The tests were repeated for the other nutrient materials (ACFs, Cu-ACFs, and CNFs) for the comparison purposes, as per the identical procedure used for the Cu-CNFs. All experiments were performed three times to check the reproducibility. Different physical, chemical and biochemical parameters were measured during the seed germination and plant growth. The plant grown without using stimulant materials served as a control. Fig. 3.8 shows a schematic representation of seed germination and plant growth using the Cu-CNFs.

3.13 Measurement of germination parameters

The water uptake capacity of the seeds was determined by measuring the increase in the weight of the seeds at 24 h-intervals until the formation of root and shoot occurred. The average germination rate was measured by counting the number of the sprouted seeds in 3 days.
3.14 Measurement of seedling parameters

The shoot and root length of the plants in the vials were measured at 24 h-intervals for 10 days, using a measuring scale. The chlorophyll contents of the plant leaf were determined using the procedure described in the study [Samreen et al., 2013], with some modifications. Briefly, approximately 300 mg-leaves of plant were
cut into small pieces and then ground using a mortar and pestle. The ground leafs were mixed with ~10 mL of 80% acetone and the mixture was left overnight at 4 °C. The incubated leaf samples-dispersed acetone was centrifuged at 3000 rpm for 10 min. The supernatant was collected in a test tube for determining the chlorophyll contents, using the UV-Vis spectrophotometer (Varian Cary 100, Germany). The absorbance was measured at 645 and 663 nm-wavelengths. The chlorophyll consisted of two types and was determined as follows:

\[
\text{Chlorophyll-a (mg/g)} = \frac{(12.7 \times A_{663}) - (2.6 \times A_{645}) \times \text{acetone (mL)}}{\text{(mg) leaf sample}}
\]

\[
\text{Chlorophyll-b (mg/g)} = \frac{(22.9 \times A_{645}) - (4.68 \times A_{663}) \times \text{acetone (mL)}}{\text{(mg) leaf sample}}
\]

\[
\text{Total chlorophyll (mg/g)} = \text{Chlorophyll-a} + \text{Chlorophyll-b}
\]

The protein contents of the plant shoot were determined using the procedure as described in the study [Lopez et al., 2010], with some modifications. Approximately 500 mg of plant shoot were cut into small pieces and then ground using a mortar and pestle. The ground materials were mixed with ~5 mL of the PBS buffer solution in a test tube and incubated at room temperature for 1 h. The incubated material was centrifuged at 3000 rpm for 30 min. The supernatant was collected in a test tube for determining the protein contents. Approximately 1 mL of the supernatant was mixed with ~5 mL of alkaline copper solution. The alkaline solution was prepared by mixing ~5 mL of reagent-A (2% sodium carbonate dissolved in 0.1 N NaOH) mixed with ~1 mL of reagent-B (0.5% CuSO4.5H2O in 1% potassium sodium tartarate). Next, approximately 500 μL of folin reagents were added to the supernatant-mixed alkaline solution and the mixture was incubated in dark at room temperature for 30 min. The protein in the solution was measured at 660 nm-wavelengths, using the UV-Vis spectrophotometer. The entire procedure was repeated for the BSA solution, which served as the standard for the measurements.

3.15 Fluorescence analysis for cytotoxicity

Rhodamine B, a cationic fluorescent probe, was used as the mitochondrial probe. All samples, including ACF, Cu- and Ag-ACF/CNF (before and after sonication) and CNT treated with lymphocyte samples, were washed three times with
PBS using centrifugation. The cells were mixed with 10 μg/ml of rhodamine B dye, and the mixture was incubated at 30 °C for 30 min. Next, the cells were placed on the microscope slide. The cover slip on the slide was fixed using a transparent DPX mount. Images were taken by the fluorescence microscope (Leica, DM-2000, Germany) with a 10X magnification lens using a 555-nm wavelength (red filter) [Chazotte, 2010].

3.16 Cellular disruption of bacteria

The bacterial cellular disruption of the prepared bimetal (Cu-Zn) volume ratio of \((\text{Cu}_{-}\text{Zn})\)-ACFs/CNFs was determined against gram negative \(E.\ coli\), gram positive \(S.\ aureus\) and MRSA. The different bacterial strains in the presence of \((\text{Cu}_{-}\text{Zn})\)-ACFs/CNFs were incubated at fixed dose 2 mg/ml for different time interval (1, 3, and 6 h) at 37°C. After the incubation periods the samples were collected and centrifuged at 6,000 rpm for 3 min. Next, after discarding supernatant and pellet was collected than pellet was washed thrice using PBS, pH ~ 7.3. Next, 100 μl PI dye were mixed in the bacterial cells and incubated in the dark for 30 min at room temperature. Approximately 10 μl bacterial solutions were placed on a glass slide. The image was taken using Leica scanning fluorescence microscope (Leica Microsystems, Buffalo Grove, IL, USA). The bacterial suspension treated with 100 % ethyl alcohol for 15 min served as positive control.

3.17 Bacterial adhesion

The bacteria (\(E.\ coli\)) adhesion on the \((\text{Cu}_{-}\text{Zn})\)-ACFs/CNFs was determined using PI dye. The prepared \((\text{Cu}_{-}\text{Zn})\)-ACFs/CNFs was cut into small pieces and incubated with bacterial solution for 24 h at 37°C. After incubation period 100 μl PI dye were mixed in the samples and incubated in the dark for 30 min. The sample was placed on a glass slide and images were taken using fluorescence microscopy.

3.18 Water uptake capacity and nucleic acid content

The fluorescence images of the plant shoot and root were used to observe their water uptake and nucleic acid content, respectively. Flourescein dye was used as the fluorescent probe for water in the plants. Plant shoot was cuts into thin longitudinal (~3-5 mm) sections, using a stainless steel blade. The sections were immersed in an approximately 100 μg/mL of the dye in PBS and left stained for 30 min. The
photographic images were taken at green channel (filter). PI dye was used as the probe for the observation of nucleic acid in the samples. The plant root was cut into thin transverse sections, using the stainless steel blade. These sections were stained for 10 min in an approximately 10 μg/mL-PI prepared in Milli-Q water. The images were taken at red channel.

3.19 Fabrication of electrochemical cell

Fig. 3.9 shows a schematic illustration of the electrochemical cell with the electrode assembly, used as the sensing device in the present study for the detection of ethylene gas from fruit samples. Fig. 3.10-a shows the photograph of the device connected with the Keithley electrochemical probe and the fruit sample container. Fig. 3.10-b shows the photograph of the electrochemical cell. Fig. 3.10-((c-c) and Fig. 3.10-(d-d1) show the photographs of the anode and cathode electrode assemblies, respectively. The anode electrode assembly also served as the gas chamber for the ethylene gas. One of it side was in contact with the electrolyte. The ethylene gas emitted from the fruit samples was stored in the gas chamber through which it gradually came in contact with the prepared electrode, forming the Cu-ethylene complex which was deposited on the cathode, resulting in the increase of the resistance.
Figure 3.9 Schematic representation of the electrochemical cell with the prepared electrode, used as the non-destructive fruit sensing device for the detection of ethylene gas.
Figure 3.10 Photographs of (a) the electrochemical sensing arrangement, (b) electrochemical cell assembly, (c-d) assembled anode-cathode, (c'-d') unassembled electrode holders.

3.20 Synthesis of polymeric electrode

The synthesis of the Cu-CNFe-dispersed PVA-Mannitol-CAP polymeric composite (P-Cu-CNFe) material was carried out, starting with the alcoholysis of polyvinyl acetate (PVAc) to produce PVA. A mixture of 62% (w/w) PVAc in 99% (w/w) methanol was stirred in a beaker to prepare a homogenous suspension. The suspension was transferred into a 2-1-3 round bottom-flask. The temperature of the reaction mixture was kept constant at 60 °C. Approximately 25 ml-methanol, 22 ml-
methyl acetate and 0.1 ml-deionined water were added to the homogeneous suspension of PVAc. The suspension mixture was continuously stirred at 130 rpm until a clear solution was produced. Next, 10 ml of the methanolic solution of sodium hydroxide (2.5% NaOH in methanol) was added to the clear solution. After approximately 15 min, the PVA gel was formed. At the incipience of the gel formation, a mixture of the ball-milled Cu-CNF dispersed in a CAP and mannitol solution was added to the reactant mixture.

The Cu-CNF was separately prepared by CVD, using the commercial phenolic precursor-based ACF as the substrate and the Cu metal NPs as the catalyst, describe earlier in the text. The prepared Cu-ACF/CNF (~0.5 - 2 g) was ball milled for 40 min. The CNFs were detached from the ACFs during the ball-milling. The ball-milled Cu-CNFs (average size = ~ 100 nm) were dispersed in the solution of 30% (w/w)- (powder) CAP and 60% (w/w)-mannitol in a 80 ml-Milli-Q water. The mixture was continuously stirred at 120 rpm for 2 h. A black gel of CAP-Mannitol-Cu-CNF was produced.

After approximately 60 min of adding the mixture of CAP-Mannitol-Cu-CNF into the PVA gel (earlier described), ~50 ml of Milli-Q water was added to the reaction mixture. The temperature and stirring speed were maintained at 70 °C and 200 rpm, respectively for the solubilization of the suspension. The pH of reactant mixture was maintained at ~7.0 during stirring, using NaOH solution. After approximately 6 h, a homogenous solution was produced. Next, the heater was switch off. The temperature of the mixture decreased to room temperature (~30 °C). The stirrer was stopped and the produced black gel was removed and cast on a teflon sheet. Next, the cast material (P-Cu-CNF) was vacuum dried for overnight at 50 °C before using it for the characterization and sensor studies. Some samples of PVA-Cu-CNFs (i.e., without CAP and mannitol) and PVA-CAP-Cu-CNFs (i.e., without mannitol) were also prepared for the comparison purposes.

### 3.21 Electrochemical measurements

The resistance measurement and differential pulse voltammetry (DPV) were carried out using Kiethley probe and Autolab III potentiostat, respectively. A specific design of electrochemical cell with comprise two electrodes system was used for the electrochemical measurements such as DPV and resistance, which comprise of P-Cu-CNF as working electrode (anode) and ACF as cathode and KCl was used as
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electrolyte. All experiments were performed at room temperature (~30 °C) under ambient conditions. All electrochemical measurements were performed in 3M KCL as electrolytes. The resistance measurements were carried out using pulse IV mode at the potential 1 V and current 0.02 amp. The DPV experiments were performed for the determination of ethylene gas entrapped in the P-Cu-CNF with the potential ranges applied -0.4 to 0.4 V.

3.22 Surface Characterization

3.22.1 Field electron scanning electron (FE-SEM) microscopy and EDX

The field electron scanning electron microscopy (FE-SEM) has been used to investigate the surface morphology of the materials. The FE-SEM is considered to be the simplest microscopic technique because of its simple image interpretation by scanning of sample surface by high energy beam of electrons which is emitted by electron gun. The focused beam hits the sample surface producing secondary electrons which is attracted and collected by detector and converted into signals. These signals further amplified, analyzed and translated into images of the sample. Therefore, for FE-SEM analysis surface of the sample must be conducting. The sputtering machine is used to make sample conductive using gold sputtering ionized gas molecules of gold tended to melt on sample for approximately 60 sec. The gold sputter coating prevents charging of the samples, which would otherwise occur because of the accumulation of static electric fields. The gold coating also increases the amount of secondary electrons that can be detected from the surface of specimen in the imaging of FE-SEM and therefore increases the signal to noise ratio. Moreover, the analysis must be performed in a vacuum to prevent electron scattering by air molecules.

In the present study, the field emission scanning electron microscope (FE-SEM) (Carl Zeiss NTS GmbH, Oberkochen (Germany) Model: SUPRA 40VP) was used to understand the surface morphology of prepared polymeric nanomaterials and MW-CNFs. The FE-SEM images of the prepared polymeric nanofibres mat and Fe-MWCNfs were taken at different magnification with the accelerating voltage of 10 kV. Prior to the SEM imaging, the sample was mounted on carbon tape adhere with copper stubs and gold sputtering for better conductivity.
Additionally, energy-dispersive X-ray spectroscopy (EDX) elemental spectra of a few spots on the samples were taken for determining the elemental compositions.

3.22.2 BET Surface area and total pore volume analysis

The technique was named BET after its inventor S. Brunauer, P. H. Emmet and E. Teller (BET). It is the first method developed to measure the specific surface area of porous solids. The BET method is based on the adsorption of gas on a surface. The amount of gas adsorbed at a given pressure allows for the determination of surface area.

The surface area and the porosity of samples were determined by the Quantachrome Autosorb-1 BET analyzer. Approximately 0.1 g of each sample was loaded into a capillary glass tube and degassed of the samples at 25°C for 6 h under nitrogen atmosphere. The nitrogen adsorption and desorption were carried out at 77K.

3.22.3 Atomic force microscopy (AFM)

The surface morphology of prepared nano-materials was studied through atomic force microscopy (AFM). The average surface roughness (Ra) of the surfaces could be calculated from the roughness profile determined from the AFM images. The surface roughness of the prepared biomaterials, PVA-CAP encapsulating agent and the PVA-CAP-Cu-ACF/CNF polymer-carbon-metal composite, was measured using AFM.

3.22.4 Fourier Transform Infrared spectroscopy

Fourier transform infra-red spectroscopy (FT-IR) was performed to analyze the surface functional organic groups on the metal doped carbon beads and carbon nanofibers. FTIR analysis were carried out (Tensor 27, Bruker, Germany) over the wave-number range of 400-4000 cm⁻¹ using an attenuated total reflectance (ATR) with a germanium (Ge) crystal. The sample chamber of the instrument was purged with N₂ gas to reduce the effect of atmospheric carbon dioxide and moisture throughout the analysis. The background spectrum was recorded before the experimental measurements were performed. The resolution was set to 4 cm⁻¹ and a total of 100 scans were collected for each sample. The introduction of new functional groups in the functionalized samples was also determined using these techniques.
3.22.5 Thermal investigations

The thermal gravimetric analysis (TGA) mainly used for the determination of thermal stability of the materials that exhibit loss of mass due to their decomposition, oxidation, or moisture present in the samples. The TGA analysis describes the thermal degradation of the blended polymer that can provides the interaction of the compatibility of the polymer, hence mechanical properties of the obtained electrospun fibers.

In the present study, the thermal stability of the materials was determined using TGA analyser (Toledo-Mettler, USA). Approximately 10 mg of sample was taken and temperature ranges between 20 to 700°C at 10 °C/min heating rate in nitrogen atmosphere. All the graphs were plotted for temperature against the weight loss of sample.

3.22.6 Zeta-potential analysis

The surface charge on the prepared materials was measured by zeta potential. The ζ potential of the materials was determined using a Zeta-Nanosizer ZS instrument (Malvern, UK). The ζ potential analysis reveals the distribution of charge on the surface of solid or colloidal particle. To determine the solute-solute interaction in aqueous system it is necessary to know about the surface charge developed after combination of two components and its interaction with the adsorbent functionality.

3.22.7 Particle Size

Particle size distribution is an index to signifying the percentage of the particle size of material. The PSD of a material can be important in understanding its physical and chemical properties. It affects the strength and load-bearing properties of rocks and soils.

3.22.8 Statistical Analysis

All statistical analysis was performed using software, Origin pro 8.5, USA.