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Bio-based hyperbranched poly(ester amide)/MWCNT nanocomposites: multimodalities at biointerface

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Materials and method

Preparation of *f*-MWCNT

MWCNT (0.05 wt%) were activated by MW irradiation and covalently functionalized with poly(glycidyl methacrylate) (PGMA) through free radical polymerization using 'fishing process', followed by non-covalent wrapping with PAni nanofiber (1.5 wt%) in a single pot reaction as reported earlier by the same authors.[1] The *f*-MWCNT were washed with ethyl acetate followed by acetone for several times before dispersion in THF (1 g/mL).

Fabrication of castor oil based HBPEA/*f*-MWCNT nanocomposites

Castor oil was transesterified with methanol to produce methyl ester, followed by transamidation reaction with diethanol amine in the presence of sodium methoxide as the base catalyst to obtain fatty amide of the oil.[2] The details of the synthetic protocol of the HBPEA using A_2+B_2+$A'A_2$ approach have been described elsewhere.[3] Briefly, the fatty amide of the oil together with the diacids like maleic anhydride, phthalic anhydride and isophthalic acid served as the A_2 monomers and reacted with diethanol amine, the $B'B_2$ monomer. The condensation reaction between A_2 and $B'B_2$ monomers was carried out at 150 °C for 30 min followed by heating at 185 °C for another 1.5 h and finally at 220-225 °C for 20-25 min with continuous mechanical stirring under the nitrogen atmosphere. The *f*-MWCNT dispersed in THF was added (1, 2.5 and 5 wt%, separately) in the preformed HBPEA at 60-65 °C with vigorous stirring for 30 min, followed by sonication using ultrasonic processor (acoustic power density 460 W/cm^2) operating at a frequency of 24 kHz for a time period for another 15 min. The HBPEA/*f*-MWCNT nanocomposites with varying weight percentages of 1, 2.5 and 5 wt% of *f*-MWCNT were coded as HBPEAM1, HBPEAM2.5 and HBPEAM5 respectively.

Fabrication of porous HBPEA/*f*-MWCNT nanocomposite films

The porous nanocomposite films were fabricated using particulate leaching technique.[4] The HBPEA and nanocomposites were cured by using bisphenol-A based diglycidyl epoxy resin (60:40 weight ratio of HBPEA to epoxy resin) and 50 wt% poly(amido amine) hardener (with respect to epoxy resin).[2] An aqueous sodium chloride solution (maintaining salt particulate:HBPEA=10:1) was added into the above mixture as an porogen additive. The mixture was then cast on glass plates (75 mm × 25 mm × 1.75 mm) under ambient conditions and degassed under vacuum. The cast films were cured at 150 °C for specified period of time. The cured film was immersed in excess amount of hot water wherein the salt particulates leached out to yield the porous HBPEA film. The pore size of the prepared film was calculated from SEM micrograph. The percentage of porosity of the polymer was determined by subtracting the volume of the polymer obtained from its dimensions from the neat volume of the porous scaffold (calculated from its mass and density).

Sample preparation for performance study

The HBPEA and nanocomposites were cured by using bisphenol-A based epoxy resin (60:40 weight ratio of resin to epoxy) and 50 wt% poly(amido amine) hardener (with respect to epoxy resin), followed by casting onto the mild steel plates (150 mm × 50 mm × 1.60 mm) and glass plates (75 mm × 25 mm × 1.75 mm) for mechanical and chemical resistance tests.[2] The casted films were then baked at 150 °C for specified time period. The cured films were then peeled off from the glass plates by immersing the plates in hot water, followed by drying *in vacuo* at 50 °C before testing.

Assessment of antibacterial properties

Colony forming unit (CFU) count. The antibacterial assay of the prepared nanocomposites was assessed with *Bacillus subtilis* MTCC441 (Gram +), *Staphylococcus aureus* MTCC373 (Gram +), *Mycobacterium smegmatis* mc^2 155 (amphicillin resistant, acid fast +) and *Mycobacterium smegmatis* ATCC14468 (acid

fast +), *Escherichia coli* DH5α (Gram -) and *Klepsiella pneumoniae* MTCC618 (Gram -) strains was tested using standard Dynamic Shake Flask Method.[5] The number of individual bacterial colonies growing on the samples inoculated with the nutrient agar and incubated at 37 °C was determined to find out the CFU.[6] All the six test strains were cultured in Mueller Hinton Broth media at 37° C for 18 h and harvested in the midexponential growth phase. The bacteria were then suspended in an isotonic solution (0.85% NaCl) to adjust the McFarland standard[7] of the cells to a turbidity of 0.5 corresponding to approximately 1×10^8 CFU/mL. The prepared nanocomposite films were inoculated with bacterial suspension for 24 h at room temperature and under vigorous shaking condition. The HBPEA film was used as the reference, while the bacterial inoculum without the samples served as the experimental control. The reported results were averaged over a set of three independent experiments. This study provides inkling about the presence of live bacteria.

Total protein content. The total protein content of the bacteria that were adhered onto the film surface was estimated according to the method described by Bradford.[8] Briefly, 200 µL of (1 g/mL) bovine serum albumin (BSA) standard was mixed with 1.25 mL of Bradford reagent and the absorbance was measured at 595 nm. A standard curve of the absorbance at 595 nm versus protein concentration (mg/mL) with correlation coefficient (R^2=0.9997) was plotted and then the absorbance of the bacteria adhered film was also measured at the same wavelength. The total protein content was estimated using the following linear fit equation:

$$Y=0.2658X \quad (1)$$

where Y is the absorbance and X is the protein concentration.

The long term antibacterial activity was tested by CFU count method for the biodegraded samples.

Nanocomposite-bacteria interaction. UV absorbance at 260 nm was recorded to probe into the mechanistic pathway of antibacterial activity of the nanocomposites.[9] The release of cytoplasmaic constituents, such as DNA and RNA of the microbial cells upon interaction of the same with the nanocomposites can be monitored by their UV absorption at 260 nm. This study is represented as the optical density (OD) ratio of the tested bacterial suspension with nanocomposite to a bacterial suspension without the nanocomposite. The morphological changes in the bacteria upon treatment with MIC value of the nanocomposite was observed using SEM microscopy.

Isolation and culturing of PBMC

The PBMC were separated from the goat blood by sedimentation technique (density gradient centrifugation) using histopaque. The blood was collected in sodium citrate and diluted in 1:1 ratio with phosphate buffer saline (PBS) at pH 7.4. Differential migration of different cells in the blood during centrifugation resulted in the separation of the same into different layers. The blood was then layered with a Pastuer pipette on histopaque in 3:2 ratios in a wide transparent centrifuge tube. The bottom layer contained histopaque-aggregated red blood cells, followed by a diffuse layer containing granulocytes and unbound histopaque with PBMC fraction sandwiched at the interface between the unbound histopaque and uppermost plasma/platelet layer. The interface was collected after centrifugation at 400×g for 15 min and transferred to serum free DMEM (Dulbecco's Modified Eagle Medium). The diluted cell suspension was subjected to multiple washes in 20 mL serum free media at 70×g for 10 min. The final pellet of PBMC was resuspended in 2 mL of serum free medium. The PBMC were cultured for 2 h in RPMI-1640 medium supplemented with 10% fetal calf serum, antibiotic Penicillin-Streotromycin-Neomycin solution and incubated in a humidified 95% O_2 with 5% CO_2 atmosphere at 37 ºC. The non-adherent cells were removed by washing and the remaining adherent cells were cultured in the same media for above mentioned time period.[10]

In vitro biocompatibility assay

Trypan blue exclusion assay. The biocompatibility of the pristine and nanocomposite films was evaluated using mammalian blood derived PBMC. The *in vitro* assessment of cell membrane integrity and proliferation of the PBMC onto the prepared films was determined by trypan blue exclusion assay[11] by the following method. In this test, cells adhered onto the film surface were stained with 0.4% trypan blue solution in PBS (pH 7.4). The visual distinction between unstained viable cells and blue-stained nonviable cells was done using microscope at 20X magnification. The unstained cells as the viable cells were counted by help of a haemocytometer after an incubation period of 3 min. The percentage of viable cells was calculated using the following equation:

$$\%\ \text{of viable cells} = (\text{Number of unstained cells/total number of cells}) \times 100 \quad (2)$$

MTT assay. In the *in vitro* biocompatibility assay, PBMC at a density of 1×10^4 cells/well were seeded onto 96-well microplates at 37 °C under 5% CO_2 in water-saturated atmosphere and allowed to grow for 2 h. The metabolic activity of the viable cells was determined by using colorimetric MTT assay.[12] Film disc of diameter ~4 mm was added into each microplate followed by incubation under the same conditions for 18 h. MTT was added to the seeded wells at different time intervals of 0, 1, 12, 24 and 48 h followed by 4 h incubation under the above conditions. The absorbance of the above cultured media was then recorded spectrophotometrically at 550 nm. The untreated cells were taken as the control and the experiments were performed in triplicates.

PBMC adhesion and proliferation studies. The adhesion, proliferation and morphology of PBMC on the nanocomposite film were evaluated using SEM study. Aliquots containing 1×10^4 cells were seeded onto the nanocomposite films for 48 h and fixed then with 2.5% gluteraldehyde, washed with PBS and finally serial dehydration using graded ethanol solutions.

Reactive oxygen species (ROS) measurement. The toxicity of the nanocomposites was evaluated by the ferric reducing ability of plasma (FRAP) assay[13] using ascorbic acid as the positive control. The specific conditions for this assay is as follows: 300 µL of FRAP reagent (acetate buffer+2,4,6-tripyridyl-s-triazine+$FeCl_3.6H_2O$) was added to 10 µL of nanocomposite followed by dilution with 30 µL of water. The change in the absorbance at 593 nm with and without nanocomposite quantifies the amount of quenched ROS.

Hemocompatibility assay. The hemocompatibility of the prepared films was evaluated through disruption of red blood cell (RBC) using hemolysis assay.[14] The hemolytic activity of the films was tested using goat erythrocytes, which served as the

prototype for mammalian erythrocytic model. Goat blood was collected into heparinized tubes and centrifuged at 1000×g for 20 min at 4 ºC. The erythrocytes were washed with sterile 10 mM PBS (pH 7.4) and centrifuged at 1000×g for 10 min at 4 ºC. Packed erythrocytes were resuspended in PBS to obtain a 5% haematocrit. The catalytic activity of catalase was inhibited by addition of 1 mM sodium azide (NaN_3) and the 5% haematocrit was incubated for 10 min at 37 ºC. Films were incubated with the haematocrit at the concentrations of 0.5, 1.0, 1.5, 2 and 2.5 mg/mL for 60 min at 37 °C. The cells were then centrifuged at 1000×g for 10 min at 4 °C. The hemoglobin concentration was determined by taking the absorbance of the supernatants at 540 nm. The hemolysis caused by 1% (v/v) Triton X-100 and PBS were used as the positive and negative controls respectively.

In vitro biodegradation

The films, cut into small discs weighing about 10 mg each and placed in lipase (~16 U/mg) containing PBS medium (100 mM, pH=7.4). The pH of the medium was maintained throughout the experiment. The pristine and nanocomposite films were incubated in the above medium maintaining the weight ratio of film to degradation medium of 1:3 under a static condition at 37 °C and the results were reported as an average of three replicates. The films were periodically taken out from the medium post incubation 7, 14, 21 and 28 days and rinsed with deionized water to remove adhered salts on the film surface. The tested films were then dried under vacuum for 4 days at 40 °C. The biodegradation of the films were evaluated as per as ASTM F1635-04[15] and degradation percentage was calculated from the residual mass post degradation with time period. Further the intensity of the ester group of the films was assessed from the FTIR spectra, before and after degradation to probe into the changes in the functionalities of the films upon enzymatic erosion.

Results and discussion

Structural characterization of the nanocomposites

FTIR study. The FTIR spectra of HBPEA, *f*-MWCNT, HBPEAM1, HBPEAM2.5 and HBPEAM5 are shown in Fig. S1a. The characteristic bands of HBPEA corresponding to –C=O appeared at 1730 cm^{-1}, amide group at 1632 cm^{-1} and hydrogen bonded -OH stretching at 3422 cm^{-1} were apparent in FTIR spectra.[3] The spectrum of *f*-MWCNT exhibited vibration bands at 915, 1490 and 1576 cm^{-1} ascribed to the presence of epoxide group, benzenoid and quinoid rings of PAni nanofiber adhered onto the nanotube surface.[1] These characteristic bands of *f*-MWCNT shifted to 1462 and 1557 cm^{-1} in the nanocomposites, which is attributed due to π-π interaction of delocalized π-bonds on the *f*-MWCNT (due to sp^2 hybridization) with the π-bonds of the aromatic moieties of the HBPEA.[16] It was observed that the absorbance band of carbonyl group of ester moiety shifted from 1730 cm^{-1} to lower wavenumbers of ~1725 cm^{-1} in the nanocomposites, which corresponds to the interaction between the carbonyl group with the nitrogenous groups of PAni present in the *f*-MWCNT. The shift of amide band from 1632 to 1635-1637 cm^{-1} is attributed to the restriction of the vibrational motion of the carbonyl amide bond of the polymer due to hydrogen bonding with the –NH- group of *f*-MWCNT.[16] The shift in the O-H stretching frequency of the nanocomposites from 3422 to 3407 cm^{-1} as compared to the HBPEA is attributed to the presence of H-bonding between the carbonyl group of HBPEA and O-H group generated by the opening of oxirane ring.

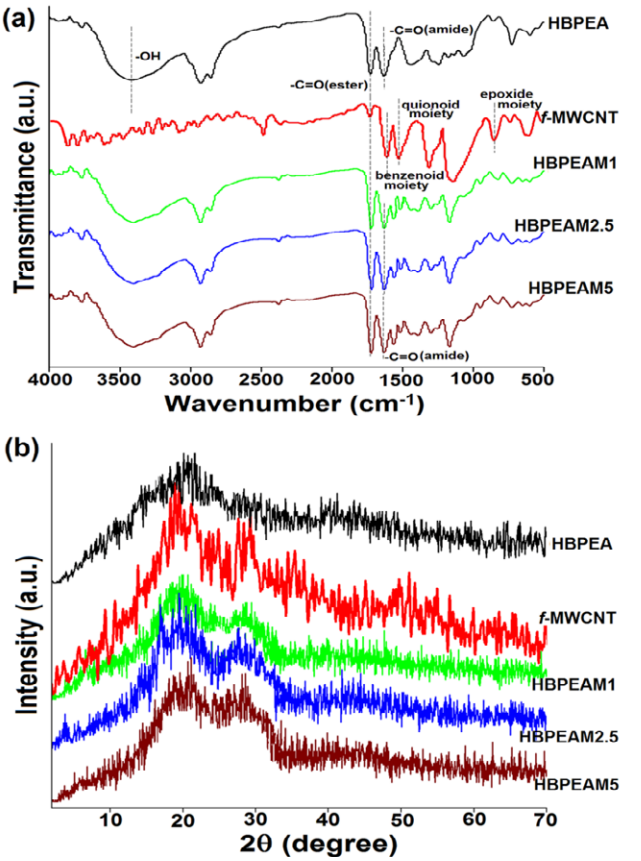

Fig. S1 (a) FTIR spectra and (b) X-ray diffractograms of HBPEA, *f*-MWCNT, HBPEAM1, HBPEAM2.5, and HBPEAM5.

XRD study. The XRD patterns of HBPEA, *f*-MWCNT, HBPEAM1, HBPEAM2.5 and HBPEAM5 thermosets are shown in Fig. S1b. The diffraction peaks of *f*-MWCNT appeared at around 2θ= 20.3° and 25.1°,[1] which are different from that observed peaks for MWCNT and PAni nanofiber. These resulted diffraction peaks are attributed to the π-π interaction between the MWCNT and PAni nanofiber.[1] The XRD of pristine HBPEA exhibited a broad peak attributed to its amorphous structure.[3] The appearance of diffraction peaks at around 19-20° and 25-26° upon nanocomposite formation indicates the occurrence of strong interfacial interaction between the *f*-MWCNT and HBPEA.

The single-line method using a Voigt function is employed to analyze the presence of long range order in the prepared nanocomposites. The employment of the Voigt function was according to the method developed by Kieser et al.[17] The more intense 2θ peak at 20.3° has been deconvoluted computationally to calculate the domain length present in the nanocomposites.[18] The domain length gives an indication about the range of order present in the nanocomposites. According to this method the X-ray profile can be fitted to a Voigt function which is a convolution of a Gaussian and a Lorentz function. The domain length can be calculated as:

$$L=\lambda/\beta cos\theta \quad (3)$$

where β is Cauchy component of integral breadth.

It is evident from the Table 1 that the domain length (range of order) increased from 9.7 to 16.2 Å with increasing *f*-MWCNT content in the nanocomposites. The presence of long range order in the nanocomposites is further supported by the alignment of the *f*-MWCNT in the HBPEA (TEM study).

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