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Biodegradable Silver oxide treated hydroxyapatite nanoparticles (AgO@HA) interlaced Poly (ether-imide)/Poly (methyl-methacrylate) membrane for Blood purification: In-vitro study

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1. Experimental methods

1.1. Materials and methods

Commercial Poly (methyl-methacrylate), with acetyl content 39.99 wt. % and polyetherimide procured from Mysore Acetate and Chemicals Company Ltd., India. N-Methyl-2-Pyrrolidone (NMP), Calcium nitrate tetrahydrate (Ca $(NO_3)_2.4H_2O$) and diammonium hydrogen phosphate ($(NH_3)_2$ HPO_4), Silver nitrate, Sodium Lauryl Sulphate (SLS), were purchased from Southern India Scientific Corporation (SRL), Chennai, India. Human monocytic leukemia cells THP-1 were procured from National Centre for Cell Sciences (NCCS), Pune, India. Without further purification, all chemicals were utilized.

The, functional groups are characterized by Fourier transform infrared (FT-IR) spectroscopy (ALPHA-TFT-IR) in the range of 400-4000cm^{-1}. The crystal structure were analyzed by X-ray diffraction studies (XRD) from D4 Endeavor, Bruker AXS Diffractometer (Germany) with Cu Kα (λ=1.54056 in the range of 10-100θ USA). Thermo gravimetric analysis of polymeric material can be analyzed by the, Thermo Gravimetric Analyzer, Netzsch-STA 2500 Regulus. The structure and topography was investigated by scanning electron microscopy (FEI Quanta FEI200) and EDAX (FEI Nova SEM450), nanostructure of the particles were measured by HR-TEM (JOEL, JEM, Fb-2000) operating at 300KeV. The adsorption spectra was calculated through UV-Visible spectroscopy (Analytic Jena specord 200 plus). Hydrophlicity of the material was measured by Kyowa water contact angle meter of DMs-401(Japan).

1.2. Porosity

The membrane porosity P (%) was calculated by dividing the volume of the pores by the total volume of the porous membrane. Eq.1 was used to calculate the porosity of the various membranes. The porosity of the scaffold was evaluated by comparing the sample's measured mass to the mass of a fully dense sample of the same size and measuring the sample's dimensions (i.e., length, width, and thickness). By applying continuous stress to all scaffolds, the thickness was measured with a thickness gauge. Equation was used to calculate the porosity (1).

$$P = \frac{M_{1\_}M_2}{M_1} \times 100(\%) \tag{1}$$

P stands for porosity, Where is the membrane's porosity, M_1 is the wet membrane mass, and M_2 is the dry membrane mass (g), respectively. For comparative purposes, all of the samples used had the same dimensions.

1.3. Mean pore size

The filtration velocity method was used to calculate the mean pore radius rm (mm). rm was calculated using the Guerout-Elford-Ferry equation,

$$r_m = \sqrt{\frac{(2.9 - 1.75\varepsilon) * 8nlq}{\varepsilon * A * \Delta P}} \tag{2}$$

Where Z is the water viscosity (8.9 104 Pa s), l is the membrane thickness (m), Q is the volume of permeate water per unit time (m_3 s1), A is the membrane's effective area (m_2), and DP is the Trans membrane pressure (Pa).

1.4. Tensile Strength

Each membrane type yielded five rectangular samples measuring 30 mm in length and 10 mm in width. Using a thickness gauge, each specimen was measured to be roughly 0.2 0.01 mm thick. During the measurements, a consistent force was applied to all samples. During the preparation, extra caution was used to avoid causing severe harm to the samples. Duct tape was meticulously

applied to each edge of the samples to strengthen their grip on the testing device's iron grips. The uniaxial micro-tensile tests were carried out on a Minimat 2000 tensile instrument with a 200 N load cell (Rheometric Scientific, Shakopee, MN, USA). The span length was fixed to 12 mm for the tests. At a strain rate of 5 mm/min, all experiments were carried out at room temperature until the sample failed. All samples were pre-tensioned before to testing to guarantee that they would extend and receive load at the start of each trial. The scaffolds' apparent mechanical properties (Equations (3) and (4), respectively) can be used to calculate) at any time during the tensile testing.

$$\sigma = \frac{F}{A} \tag{3}$$

$$\varepsilon = \frac{L - L_0}{L_0} \tag{4}$$

Where F denotes the force, A denotes the sample's cross-sectional area, L denotes the displacement, and Lo denotes the span length. Ultimate tensile strength (ε_{max}), young's modulus (E), and strain at break (σ_{max}) are the apparent mechanical properties of the scaffolds max), were measured and examined further.

1.5. Blood coagulation (APTT & PT)

Healthy human fresh blood (man, 28 years old) was collected using vacuum tubes containing citrate/phosphate/dextrose/adenine-1 mixture solution (CPDA-1) as an anticoagulant (anticoagulant to blood ratio, 1:9). The blood was centrifuged at 1000 rpm for 10 min to obtain platelet-rich plasma (PRP) or at 2800 rpm for 15 min to obtain platelet-poor plasma (PPP). To evaluate the antithrombogenicity of the membranes, the activated partial thromboplastin time (APTT) and prothrombintime (PT) were measured by an automatic blood coagulation analyzer (Sysmex, model-CA104), for whichcitrate-anticoagulated-plasma was used. The APTT and PT were measured as following: the samples (0.5 cm×0.5 cm, 3 pieces) were incubated with 0.2 ml PPP in a transparent plastic tube at 37 ◦C for 30 min, and then the APTT and PT were measured. The test was repeated three times for each sample to get a reliable value. To investigate the effect of calcium ion on the clotting time, CaCl2 solutions with different concentrations were

added intoplasma to measure the APTT and PT. The samples (0.5 cm×0.5 cm, 3pieces) were incubated with 200µl of healthy human bloodplasma (containing 1–5 times calcemia concentration) in a transparentplastic tube at 37 ˚C for 30 min, and then the APTT and PT were measured. The test was repeated three times for each sample to get a reliable value.

1.6. Hemolysis assay

Blood compatibility was evaluated with hemolysis assay. Fresh blood was collected from hepatic vein and stabilized with Heparin. 2 ml of whole blood sample was added to 4ml Dulbecco's phosphate-buffered saline (D-PBS) and then centrifuged at 10000 g for 5 min to isolate red blood cells (RBCs). The RBCs were further washed five times with 10 ml of D-PBS and finally diluted to 20 ml with D-PBS. 0.2 ml of diluted RBC suspension was exposed to (2x2) Membrane suspended in D-PBS to make the final (test group), distilled water (positive group), and D-PBS (negative group). After incubation at room temperature for 4 h and centrifugation for 5 min at 10016g, absorbance of the test samples were measured at 577 nm. The Hemolytic degree was expressed by the hemolytic ratio as the following formula (5)

$$Hemolysis\ ratio = \frac{(Test\ sample\ -\ Negative\ control)}{(Positive\ control - Negative\ control)} \times 100 \quad (5)$$

1.7. Plasma Recalcification Time

Healthy human fresh blood (man, 28 years old) was collected using vacuum tubes containing citrate/phosphate/dextrose/adenine-1 mixture solution (CPDA-1) as an anticoagulant (anticoagulant to blood ratio, 1:9). The blood was centrifuged at 1000 rpm for 10 min to obtain platelet-rich plasma (PRP) or at 2800 rpm for 15 min to obtain platelet-poor plasma (PPP). Different kinds of membranes (3 cm × 3 cm each piece) were immersed in the PBS solution and equilibrated at 370C for 1 h. Then 100 µl PPP was placed on the sample film attached to a watch glass, and incubated statically at 37 ◦C; and then 100 µl of 25 mM $CaCl_2$ aqueous solution was added to the PPP and the plasma solution was monitored for clotting by manually dipping a stainless-steel hook coated with silicone into the solution to detect fibrin threads. Clotting times

were recorded at the first sign of fibrin formation on the hook. The test was repeated three times for each sample to get a reliable value.

1.8. Whole blood clotting time

Blood was collected from a healthy man (28 years old) by venipuncture into sterile plastic disposable taken any syringes. The blood donors had been screened for the study by a medical practitioner to ensure that they had not medications for at least 1week before the blood was collected. The modified membranes (2 cm × 2 cm) were attached to glasses and subjected to the whole blood clotting time test. Starting a stopwatch, 50µl of the blood was immediately placed on the membrane sample. And the whole blood was monitored for clotting by manually dipping a stainless ssteel wire hook coated with silicone into the blood to detect fibrin threads. Clotting times were recorded at the first sign of any fibrin formation on the hook. The experiment was repeated three times for each sample and a mean value was calculated.

1.9. Cell lines and culture medium

Human monocytic leukemia cellsTHP-1 was procured from National Centre for Cell Sciences (NCCS), Pune, India. Cells were cultured in RPMI 1640 supplemented with 20% inactivated Fetal Bovine Serum (FBS), Penicillin (100 IU/ml), Streptomycin (100 mg/ml) and Amphotericin B (5 mg/ml) in a humidified atmosphere of 5% CO_2 at 37˚C until confluent.

1.10. Cell viability

The membranes were tested on THP-1 cell lines and cell viability was assessed using PI staining with the aid of flow cytometry (FACS) (BD FACS calibur) (shaziaanjum *et al*, 2021).THP-1 cells (20,000cells/ml) were seeded and incubated for 18hrswith membranes. At the end of incubation, the cells were centrifuged, pelleted, re suspended in FACS buffer and stained with PI (10µl of 1mg/ml) for 10 min in the dark. Viability of cells was assessed using FACS - FL2 detector and analyzed using BD CELLQUESTPRO software.

1.11. Biodegradability test

The experimental soil pond was used to test biodegradation under real world environments. We buried the specimens 40 cm depth at several locations deeper soil surface in this experimentation, attempting to keep the samples positioned flat. We irrigated the soil from above after burying the samples until it was moist to about 10 cm from the bottom. The grassy seed were then scattered on the soil surface and covered with a thin layer of dirt. To keep the soil moist, we used a sprinkler on a regular basis. For certain period of month, one specimen of each sample was taken out for testing.

For biodegradation report, the specimens were washed thoroughly with distilled water and dried in a vacuum oven until the constant weight was achieved. If the original sample weight is W_0 and the final weight is W_t, so the,

$$\% \quad \text{Weight} \quad \text{loss} \quad = \quad \frac{W_0 - W_t}{W_O} \times 100 \tag{6}$$