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Novel gamma irradiated agarose-gelatin-hydroxyapatite nanocomposite scaffolds for skin tissue regeneration

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ABSTRACT

Agarose-gelatin-hydroxyapatite composites prepared by freeze-drying technique were gamma irradiated with various doses (25 kGy, 50 kGy and 100 kGy). X-ray Diffraction (XRD) analysis revealed the pure phase of HAp and the intensity of prominent planes of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HAp) were found to decrease on irradiation. Fourier Transform Infrared spectra (FTIR) showed functional groups of HAp and polymer composites, and higher disorder of the polymer matrix on irradiation. In addition, gamma irradiation led to a drastic reduction in the wettability (62%) and the compressive modulus (76%) of the scaffolds. There was significant enhancement (113%) in pore size of the scaffolds at higher fluence (100 kGy). The swelling and the dissolution studies of the gamma irradiated scaffolds showed that it had an appreciable change in the scaffold's mechanical and biological properties viz., compressive modulus, cell proliferation, hemolysis etc. The irradiated biomaterials exhibited enhanced hemocompatibility, antimicrobial activity and cell viability. The above results clearly reveal that the gamma irradiation is a suitable tool to tailor the multifunctional properties of the composites and could be used for various biomedical applications.

1. Introduction

The nano crystal hydroxyapatite (nHAp), has been employed to replace damaged bone/tissues due to its biocompatibility and osteoconductivity properties. Conversely, due to its brittle nature and weak mechanical strength, polymers are incorporated to enhance the mechanical strength and could be used as scaffolds in tissue engineering applications [1–2]. Many studies have been carried out to tailor the properties of the potential scaffolds with the combinations of various biomaterials and polymers in the field of tissue engineering [3–4]. The foremost criteria in developing the scaffolds are to enhance biocompatibility, bioactivity, porosity, and surface area. The enhanced mechanical strength and electrical properties of the scaffolds also played vital role in improving the specific cell growth [5]. The engineering of highly interconnected porous scaffolds provides favorable conditions for cell proliferation and adhesion on the scaffold matrix [6].

Natural polymers like gelatin and agarose are highly biocompatible and bioactive [7–8]. Especially, gelatin has good adhesiveness and hemostatic properties. Agarose was widely used as hydrogels in drug delivery applications and gelatin based scaffolds are employed as wound dressing materials. These properties of agarose and gelatin make them a potential candidate for tissue engineering and could be used as bandages for wound healing treatments [7–9]. Incorporation of HAp ceramics in the polymer matrix could enhance both the mechanical strength and the degradation rates of the scaffolds [10–11]. There are many techniques to fabricate porous scaffolds viz., freeze casting, GELPOR3D [1], solvent casting, freeze drying [12], phase separation, melt molding and solution casting [1,13]. Among them, freeze drying technique is an effective way to reduce the shrinkage of the material and to attain uniform flat surface. The synthesis of ceramic powders by incorporating polymers can permit the bone or any other hard

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tissue to grow in and the cells can be seeded when implanted [14].

Sterilization of biomaterial implants was achieved conventionally by UV irradiation and gamma irradiation [15]. On gamma irradiation, biological properties, molecular weights of the polymers and structural properties of the scaffolds are modified depending on the dosage [16–18]. The most common dosage for sterilizing the biomedical implants and scaffolds was reported as 25 kGy [19]. In the present study, agarose-gelatin-HAp composite scaffolds were synthesized by freeze drying technique and subjected to gamma irradiation with various dosages 25 kGy, 50 kGy and 100 kGy to modify the structural and biological properties of the samples.

2. Materials and method

Agarose (SRL) [molecular weight (1, 20,000), viscosity (1500 mPa s)], gelatin (MERCK) [molecular weight (20,000–25,000), viscosity (25 mPa s)], calcium nitrate tetrahydrate ($Ca(NO_3)_2 \cdot 4H_2O$, Merck), diammonium hydrogen phosphate ($(NH_4)_2HPO_4$, Merck), and ammonia solution (annular grade) were used as the chemicals to synthesize agarose-gelatin-HAp composites.

2.1. Nanocrystalline hydroxyapatite (nHAp) preparation

The nHAp powder was synthesized by a wet-chemical precipitation route, using calcium nitrate tetrahydrate ($Ca(NO_3)_2 \cdot 4H_2O$, Merck), Diammonium hydrogen phosphate ($(NH_4)_2HPO_4$, Merck), and ammonia solution (annular grade). Diammonium hydrogen phosphate of 0.6 M was prepared using triple distilled water. The calcium nitrate solution of 1.0 M was prepared respectively. These mixtures of calcium solution were added drop by drop into 0.6 M of $(NH_4)_2HPO_4$ solution with vigorous stirring, using a magnetic stirrer for 3 h. The pH of the solution was maintained at 10 using the ammonia solution. The slurry of the mixture was washed with deionized water and dried at 80 °C in a hot air oven.

2.2. Agarose-gelatin-composite (AGH)

Agarose (0.5 wt%) and Gelatin (7.5 wt%) were mixed together in triple distilled water and heated to 100 °C. The nHAp powder (147 nm) (1 wt%) was added to the copolymer mixture and stirred for 5 h at 100 °C to get a homogenous mixture. The mixture was poured into the petridish. After gelation, the samples were frozen at −20 °C for 12 h. The pre-frozen samples were further freeze dried at −110 °C for 24 h using the SCAN VAC freeze dryer. The scaffolds were cut into 1 cm × 1 cm sheets having the thickness of 0.5 cm for further processing and evaluation.

2.3. Gamma irradiation

The composite scaffolds were irradiated by 1.25 MeV ^{60}Co gamma source using 1200 Gamma Chamber (Inter University Accelerator Center, New Delhi, India) at various doses (25 kGy, 50 kGy and 100 kGy). Hereafter, the composites of agarose–gelatin–HAp and gamma irradiated at 25 kGy, 50 kGy and 100 kGy doses were denoted as AGH, AGH-25, AGH-50 and AGH-100 respectively.

2.4. Characterization

The composite scaffolds were analyzed with PANalytical X'Pert Powder XRD System CuK_α radiation (0.154 nm) with step size 0.02° in a continuous scan mode ranging from 10° to 80°. The functional groups of the pristine and gamma irradiated samples were studied by Fourier Transform Infrared Spectrometer (FTIR-6300) in ATR mode in the range 4000 cm^{-1} to 400 cm^{-1}. The surface morphology of these AGH both pristine and gamma irradiated were examined using scanning electron microscopy (F E I Quanta FEG 200—High Resolution Scanning Electron Microscope). The contact angle measurements were carried out using KRUSS DSA-10 contact angle measuring system. The compressive modulus of the scaffolds were measured using Texture Analyser (CT3 10K, Brookfield, USA0) using the TA10 probe. The speed was 0.5 mm/min and 5 cycles of compression were carried out to ensure the repeatability.

2.5. Swelling test

The stability of the scaffolds can be studied using swelling study, which was carried out using phosphate buffer saline (PBS) pH 7.4. The prepared PBS contains a final concentration of 137 mMNaCl, 10 mM PO_4^{3-} and 2.7 mMKCl. The composite scaffolds of 100 mg were weighed (W_0) and the samples soaked in a container containing 20 mL of PBS was incubated at 37 ± 0.1 °C. The swelling was determined as,

$$\text{Swelling}(\%) = \frac{(W_t - W_0)}{W_0} \times 100$$

Where, W_t–the weight of the scaffolds after immersion time t and W_0–the weight of the dried scaffolds. The experiments were carried out in triplicates.

2.6. Dissolution test

Dissolution is directly proportional to on-set-of-action (immediate relief) and material turn over in biological system with standard condition. The dissolution of the scaffolds was also carried out using PBS solution. The samples were weighed 100 mg (W_0) and immersed in PBS for 24 h at 37 °C incubator. After the incubation time, the samples were dried and weighed (W_1). The experiments were carried out in triplicates.

$$\text{Dissolution Ratio}(\%) = \frac{W_1}{W_0} \times 100$$

2.7. Hemolysis

Hemolysis is the test used to find the toxic reaction of prepared scaffolds towards the blood components, which was performed by adding human blood with Acid Citrate Dextrose (ACD). Anhydrous citric acid of 0.55 g, trisodium citrate dehydrates of 1.67 g and dextrose of 1.84 g were mixed in 100 mL of triple distilled water for the preparation of the ACD solution and stored at 4 °C. The ACD solution of 1 mL was gently mixed with 9 mL of freshly drawn human blood to prevent the blood cell lysis and stored at 4 °C. The scaffolds of 100 mg were sterilized under UV light for 1 h and then it was transferred to the centrifuge tubes containing 1 mL of saline solution. The tubes were kept in incubator at 37 °C for a day. The saline from the centrifuge tubes was removed after the incubation period. The prepared ACD blood of 0.25 mL was added to the centrifuge tubes and incubated at 37 °C for 20 min. Then 2 mL of saline was added to the tubes containing scaffold and blood samples, and incubated for another 1 h. The tubes were spun at 750 rpm for 5 min with the help of SIGMA 3–30 KS ultra-speed centrifuge. The separated serum from the centrifuge tubes was removed carefully and then stored in separate tubes. The positive control of the experiment was the ACD human blood with 2 mL distilled water and the negative control was the ACD human blood with the serum. The optical density (OD) values were obtained at 545 nm. The percentage of hemolysis was calculated using the