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Physiologically clotted fibrin – Preparation and characterization for tissue engineering and drug delivery applications

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ABSTRACT

Fibrin used for biomedical applications is prepared by mixing concentrated solutions of fibrinogen and thrombin in presence of cross-linking agents such as Factor XIII or glutaraldehyde. The main drawbacks associated with this procedure include cost, complexity and time required for fibrin preparation. Hence, present study deals with the characterization of physiologically clotted fibrin (PF) for bone tissue engineering and drug delivery applications. For this the physico-chemical properties of PF were compared with those of the conventionally prepared fibrin (CF). Further MTT and haemolytic assays were performed for both PF and CF to compare their biocompatibility. The amount of alkaline phosphatase produced and calcium secreted by MG-63 cells in the presence of PF and CF were used to relate the osteogenic potency of PF with that of CF. Gallic acid, an anti-cancer drug was loaded within PF and CF and their role in drug delivery was compared.

1. Introduction

Fibrin is a fibrous and viscoelastic protein, commonly used in surgical practices for haemostasis and wound healing [1]. It is a biocompatible and biodegradable polymer and hence serves as a perfect matrix for stem cell differentiation and tissue regeneration [2] and [3]. Human umbilical cord mesenchymal stem cells encapsulated within fibrin-alginate microbeads showed excellent proliferation, osteogenic differentiation and bone mineral formation when compared with the non-encapsulated cells [4]. Keratinocytes loaded within fibrin capsules showed five time increase in the production of transforming growth factor β1 and recombinant human platelet derived growth factor-BB [5]. Apart from tissue engineering applications, fibrin has also been utilized in the form of drug delivery vehicles to attain continuous discharge of drugs and proteins over a specific period of time [6] and [7]. When fibrin was used as a carrier of carboplatin, it lingered cytotoxic on retinoblastoma cells proving its possible use in the field of drug delivery [8].

Fibrin used in the above mentioned applications is prepared using the conventional method of mixing concentrated solutions of fibrinogen with thrombin in the presence of cross-linking agents such as Factor XIII or glutaraldehyde [9] and [10]. The main drawbacks associated with this procedure include cost, complexity and time required for fibrin preparation [11].

In some of the slaughter houses at India, bovine blood is collected to prepare pharmaceutically important products such as haemoglobin and serum [12]. During the process physiologically clotted fibrin (PF) is obtained as a by-product. It has been reported that fibrin obtained naturally (PF) or therapeutically (conventional method), deems to be biocompatible and biodegradable [13]. However the therapeutic applications of PF has not yet been studied extensively; thus the main objective of this study is to investigate the possible use of PF as an alternative for conventionally prepared fibrin (CF) for bone tissue engineering and drug delivery applications. For this purpose, the physical and chemical properties of CF and PF were compared and the similarities between them were assessed. Haemolysis and MTT assay were performed to evaluate the biocompatibility of PF. For bone tissue engineering applications, MG-63 human osteosarcoma cells were treated with PF and the amount of alkaline phosphatase produced and calcium secreted were quantified. Gallic acid was loaded within

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PF and its anti-cancer potency was determined using MCF-7 human breast cancer cells.

2. Materials and methods

2.1. PF and CF

Fresh bovine blood was collected from municipal slaughter house under sterile condition. PF was prepared by churning the blood with a glass rod. It was washed thoroughly with cold distilled water and treated with 0.5 M sodium acetate and 30% hydrogen peroxide [14]. CF was purchased from Hi-Media Laboratories Pvt. Ltd., Mumbai, India. All the chemicals used in this study were of analytical grade.

2.2. Physicochemical characterization of PF and CF

UV–vis spectroscopic measurements were performed using Jasco dual beam UV–vis–NIR spectrophotometer (model V-570). SDS–PAGE analysis was carried out to compare the electrophoretic mobility of PF and CF. For this study, 5 mg per sample was treated with sample buffer containing β–mercaptoethanol, boiled at 100 °C for 5 min and then subjected to electrophoresis. Coomassie brilliant blue R–250 was used to stain the protein bands. JASCO model 815 circular dichroism spectropolarimeter was used to analyse the secondary structure of PF and CF. For circular dichroism, a total volume of 500 μl per sample was prepared in PBS at a concentration of 0.5 mg/ml. To evaluate the functional groups present in PF and CF, FTIR was performed using ABB MB3000 Fourier transform infrared spectrophotometer. The samples were mixed with potassium bromide, made into pellet and then used for the study.

To determine the amino acid composition of PF, 50 mg of sample was placed in 15 ml ampoule and treated with 6 N HCl at 110 °C for 24 h under vacuum. The hydrolysed sample was neutralized using 1 N NaOH and diluted with 0.2 M citrate buffer (pH 2.2). 20 μl of the sample was then loaded into the SUPELCOSILTM LC–DABS HPLC column (Agilent Technologies, Santa Clara, California, United States) and the amino acids were analysed [15].

2.3. Cytotoxicity test

MTT (3-(4,5-dimethylazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay was performed to evaluate the cytotoxicity of PF and CF using NIH 3T3 cells (mouse embryonic fibroblasts cell line). MTT is a water soluble yellow coloured substrate which gets reduced into an insoluble purple coloured formazan on reduction by dehydrogenases produced in living cells. For this study, 1×10^4 cells were seeded separately in 96-well plate and incubated overnight under controlled atmosphere. Subsequently the cells were exposed to similar concentrations of PF and CF (25, 50, 75 and 100 μg/ml) for 24 h. Cells without any treatment served as negative control and those treated with Triton X-100 served as positive control. Following incubation, the cells were incubated with 100 μL of MTT solution (0.5 mg/mL) for 3 h at 37 °C. Finally 50 μl of DMSO was added to dissolve the formed formazan and the absorbance was measured at 570 nm [16].

2.4. Haemolysis

Haemolytic assay is deemed to be an easy and dependable method to evaluate the blood compatibility of bio-materials [17]. Human blood samples were collected with the consent from healthy volunteers and centrifuged at 1000 × g for 10 m. The pellet obtained was washed thrice with phosphate buffered saline (PBS) to remove the residual blood plasma. A 5% haematocrit was prepared by suspending the purified red blood cells (RBCs) in PBS. 50 μl of the RBC suspension was treated with 1000 μl of different concentrations of PF and CF (25, 50, 75 and 100 μg/ml) and incubated at 37 °C for 60 min. RBCs treated with PBS and water were used as negative and positive control [18]. After incubation, all the samples were centrifuged and their supernatants were used for determining the optical density at 540 nm.

2.5. Mechanical strength

The tensile strength presents an indication of mechanical strength which is a very important feature for hydrogels used for tissue engineering applications. Tensile strength of PF and CF was determined using Instron 4501 tensile system. The formula used to calculate the tensile strength of the sample is as follows;

$$\text{Tensile strength } (N/mm^2) = \text{Breaking force } (N)/\text{Cross-sectional area of sample } (mm^2)$$

2.6. Rate of degradation

To assess the biological stability of PF and CF, a known weight of each sample was incubated in PBS at 37 °C for 1, 3, 5 and 7 days respectively. After each incubation, both the samples were removed air-dried and weighed. The percentage of weight loss was calculated as follows;

$$\text{Weight loss } (\%) = \text{Initial weight} - \text{Final weight} \times 100$$

2.7. Bone tissue engineering

Various biochemical events such as synthesis of alkaline phosphatase (ALP) and extracellular calcium deposition ensue during new bone formation. Hence these biochemical markers were quantified after treating MG-63 cells with similar concentrations of PF and CF at different time intervals. This was done to compare the osteogenic property of PF with CF.

2.7.1. Cell proliferation assay

MG-63 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin with 5% CO_2. 1×10^5 cells were seeded in 96-well plate and incubated overnight. They were treated with 25, 50 and 100 μg of PF and CF and incubated for 1, 3 and 5 days. After each exposure, the cell culture medium was discarded, 100 μl of 1 mg/ml MTT was added and incubated for 4 h [15]. After incubation, 100 μl of DMSO was added and subjected to UV detection at 570 nm.

2.7.2. ALP Activity

The amount of alkaline phosphatase secreted by PF and CF treated cells was determined by measuring p-nitrophenol present in the cell culture supernatants. Briefly, cells were treated with various