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Silk fibroin-Thelebolan matrix: A promising chemopreventive scaffold for soft tissue cancer

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ABSTRACT

Research of improved functional bio-mimetic matrix for regenerative medicine is currently one of the rapidly growing fields in tissue engineering and medical sciences. This study reports a novel bio-polymeric matrix, which is fabricated using silk protein fibroin from *Bombyx mori* silkworm and fungal exopolysaccharide Thelebolan from Antarctic fungus *Thelebolus* sp. IITKGP-BT12 by solvent evaporation and freeze drying method. Natural cross linker genipin is used to imprison the Thelebolan within the fibroin network. Different cross-linked and non-cross-linked fibroin/Thelebolan matrices are fabricated and biophysically characterized. Cross-linked thin films show robustness, good mechanical strength and high temperature stability in comparison to non-cross-linked and pure matrices. The 3D sponge matrices demonstrate good cytocompatibility. Interestingly, sustained release of the Thelebolan from the cross-linked matrices induce apoptosis in colon cancer cell line (HT-29) in time dependent manner while it is nontoxic to the normal fibroblast cells (L929).The findings indicate that the cross-linked fibroin/Thelebolan matrices can be used as potential topical chemopreventive scaffold for preclusion of soft tissue carcinoma.

1. Introduction

The frequency of occurrence of soft tissue cancer is around 21% throughout the world and is expected to increase continuously. Some biomaterial based approaches for the treatment of soft tissue cancer is reported such as oxidized cellulose for breast cancer [1]; tissue engineered conduits for urinary cancer [2]; and epithelium cancers such as bladder, esophagus, trachea, colon, cornea and skin [3]. Many cancer conditions like skin cancer and oral cancer causes extensive normal tissue damage. To treat them, topical chemoprevention should be the new alternative way than systematic medication [4].

Amongst all biopolymers, silk fibroin is widely accepted natural polymer material used in tissue engineering application [5–7]. Silk from silkworms is a good source of silk fibroin. Taking into consideration of its low antigenicity, excellent biocompatibility, biodegradability and tissue specific requirements, *Bombyx mori* silk fibroin and its composites have widely been used for soft tissue engineering (skin, nerve, ligaments, tendons, cardiac, and ocular) [6,8,9].

Thelebolan is a branched *exo*-β-(1 → 3)-glucan, newly reported previously by Mukhopadhyay et al. [10]. It is produced by an Antarctic Ascomycetous fungus *Thelebolus* sp. IITKGP BT-12. Beta-glucans are found in the cell wall of bacteria, fungi, yeasts, mushrooms and plants. The source and diversity of its molecular arrangement leads to variation in its biophysical properties such as, molecular weight, solubility, gelation and viscosity [11]. Beta-(1 → 3)-glucan is recently reported as anticancer agent against skin cancer [12], breast cancer [13], and prostatic hyperplasia [14]. Presently, it is economically advantageous to use Thelebolan for biomedical application, because of its easy processing steps and greater yield (~2 g/L) [10]. Till now, the usefulness of chemopreventive polymer scaffold is less focused in medical and health care field.

In the present work, we hypothesize that being a polymer; fibroin will depict the biomaterial properties. Additionally, Thelebolan will bar the cancer cells from spreading and induce apoptosis to them. For the first time this report insight into the fabrication of an advanced biomaterial matrix composed of silk fibroin

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and Thelebolan. The biophysical properties of the blended matrices with/without cross-linker, genipin are studied. The antitumor properties of the fungal polysaccharide Thelebolan within the fibroin matrix are evaluated *in vitro*. From the findings of this study, it can be evaluated that the fibroin present in the matrices may support the growth of normal tissue; simultaneously, it can release the polysaccharides, which may prevent the carcinogenesis.

2. Materials and methods

2.1. Materials

The materials were purchased as follows: Genipin (Sigma-Aldrich, USA), Protease XIV from *Streptomyces griseus* (3.5 U/mg, Sigma-Aldrich, USA), Cell culture grade chemicals including Dulbecco's modified eagle medium and fetal bovine serum (Gibco, USA), trypsin-EDTA (Himedia, India), penicillin-streptomycin antibiotics (Himedia, India), Alamar blue (Invitrogen, USA), tissue culture plates and flasks (Nunc, Denmark), Live/Dead stain (Live-dead viability/cytotoxicity kit for mammalian cell, Invitrogen, USA), DAPI (Molecular probes, Thermo Fisher Scientific, USA), and other chemicals used were of analytical grade.

2.1.1. Silk cocoon

Bombyx mori silk worm was reared at our own laboratory, IIT Kharagpur West Bengal, India and cocoons were obtained.

2.1.2. Thelebolan

Thelebolan was isolated and purified from submerged mycellial culture of *Thelebolus* sp. IITKGP-BT12 as described previously by Mukhopadhyay et al. [10].

2.2. Fabrication and post processing of silk fibroin/Thelebolan matrices

Silk protein fibroin (**SF**) was isolated from *B. mori* silkworm cocoons following the standard extraction procedure [15]. Thelebolan (**T**) solution (2% w/v) was prepared by dissolving in deionized ultrapure water. **SF** and **T** solution each of 2% were used for blending. The SF/T blend with 3:1 ratio was prepared volumetrically and kept under constant stirring for 30 min at room temperature. The protein was cross-linked for the same blend ratio using genipin (0.1% w/w) under constant stirring in dark condition for 6 h at 37° C. Finally, silk fibroin (**SF**), Thelebolan (**T**), fibroin-Thelebolan blend (**SF/T**) and genipin cross-linked fibroin-Thelebolan blend (**Cross-linked SF/T**) were used to fabricate the scaffolds and the films. The 3D scaffolds were prepared by pre-freezing the solutions at −20° C for 12 h followed by lyophilization for 48 h. In addition, 2D films were prepared by solvent evaporation at room temperature.

2.3. Scanning electron microscopy (SEM)

The structural morphology of the freeze dried 3D scaffold was observed using a scanning electron microscope (JEOL-JSM 5800, USA). The dehydrated and vacuum dried materials were secured on aluminium stabs by conductive carbon adhesive tape; further they were coated with gold using a sputter coater. Pore sizes of the fabricated sponges were measured by analyzing the micrographs using Image J software (NIH, USA).

2.4. Atomic force microscopy (AFM)

Surface analysis and topographic imaging of air dried 2D films were performed in intermittent-contact mode using scanning probe microscope (Multiview 1000^{TM}, Agilent Technologies, USA) where tips mounted on silicon cantilevers with spring constant of 40 N/m. Measurements were carried out at room temperature with a scan area of 40 μm × 40 μm.The surface roughness of the matrices was analyzed with the help of Pico Image Basic Software, USA.

2.5. Porosity

The porosity of the fabricated 3D scaffold was measured by liquid displacement method [16]. Briefly, the scaffolds were immersed in a known volume (V_1) of hexane in a measuring cylinder for 5 min. The total volume of hexane and hexane-impregnated scaffold were recorded as V_2. The hexane-impregnated scaffolds were then removed from the cylinder and the remaining hexane volume was recorded as V_3. The porosity of the scaffold (ε) was calculated in terms of percentage by the following equation:

$$\varepsilon(\%) = (V_1 - V_3) * 100/(V_2 - V_3) \quad (1)$$

2.6. Fourier transform infrared (FT-IR) spectroscopy

FT-IR was performed using a Nicolet 170SX FT-IR spectrometer (Spectrum One, Perkin-Elmer Corp., USA) to analyze the chemical structure of the 2D films. Attenuated total reflectance mode was used to record the absorption IR spectra over a wave number range of 4000–400 cm^{-1} under ambient conditions. Because of the color development of the crosslinked blend solutions during incubation (due to crosslinking of the protein with genipin) the reflectance mode was chosen for data collection from all the fabricated films.

2.7. Mechanical property

Tensile strength of the 2D film matrices was measured using a universal testing machine (UTM Instron Electroplus E1000, UK). An extension rate of 1 mm/min was used while the UTM was fitted with a 1 kN load cell. For the tensile test, the samples (0.2 mm thick) were prepared according to ASTM D 638-5 (dumbbell shapes, 50 mm × 10 mm). The distance between the clamps was kept 2.8 cm for all the samples. The thickness of the films was measured using a digital slide caliper with 0.02 mm sensitivity. The testing was carried out at 25 °C and 50% humidity to determine the tensile strength and elongation at break. The experiment was repeated twice for each sample.

2.8. Differential scanning calorimetry (DSC)

Thermal property of the fabricated 3D scaffold was deduced using differential scanning calorimetry (DSC NETZSCH DSC 200PC, Germany). Approximately 10 mg of each scaffold was transferred to a DSC aluminium pan. A scanning rate of 10 °C/min was employed along with nitrogen flow of 50 ml/min to get the scan over 40–350 °C. The linear baseline to integrate peaks was determined. The decomposition temperature (T_m) and enthalpy of fusion (ΔH) were calculated from the DSC thermograms of all the samples.

2.9. Determination of swelling ratio

Swelling ratio of the fabricated 3D scaffold was determined to know about the swelling property of the matrices. In brief, completely dried scaffolds were allowed to swell in PBS (1X, pH 7.4) at room temperature for 0–24 h. The swelling ratio was calculated as follows:

$$\text{Swellingratio} = (W_s - W_d)/W_d \quad (2)$$

Where, W_s – the weight of swollen scaffold and W_d – the weight of the completely dried scaffold (dried in oven at 70° C for 24 h).The swelling ratio was calculated for all the time frame (0, 1, 2, 3, 4, 5, 6, 12, 24 h) of each sample in triplicate. This experiment was not