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Silk based biomaterial in combination with honey and RHEGF for diabetic wound healing

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ABSTRACT

Silk fibroin (SF), a naturally occurring protein polymer, has several unique properties such as biocompatible, slowly biodegradable, minimal inflammatory reaction and endowed with excellent mechanical properties and process ability. The silk blended scaffolds were prepared by using SF as vehicle with dextrin and other healing agent of honey and Epidermal growth factor (rhEGF) is used as drug releasing model. Scanning electronic microscope (SEM) was used to observe the morphology of prepared scaffolds for process versatility and the highly specific surface area. The structure was studied by Fourier transform infrared. The samples, treated at different concentration of honey and EGF, are analysed to investigate the growth inhibition effect of bacterial growth. These data showed the potential application of silk based scaffolds along with honey and rhEGF is intended to provide improved environments for zone of incubation when compared with normal scaffolds without rhEGF. Next the wound pus samples were collected from different diabetic patients and they were tested for antimicrobial test. Silk scaffolds had shown a promising natural product with healing for wound treatment. Processing silk scaffolds with honey and EGF offers a very attractive opportunity for producing a variety of medical products with great potential for bio medical end uses due to its superior biocompatibility of Silk. This prepared silk fibre based protein scaffold would be better in healing of diabetic wound healing as a very widespread model.

Introduction

Silk fibroin (SF), as a naturally occurring structural protein polymer, has recently gained extensive attention in tissue engineering because of their desirable biodegradability, biocompatibility and remarkable mechanical properties[1, 2]. The consumers demand has been growing towards medical textile for human safety and environment. Silk fibroin (SF) has an attractive medical textile application [11]. Due to its permeability to oxygen and water, good cell adhesion and growth characteristics. Silk fibroin scaffolds can be prepared using various methods[4, 8, 9, 12]. Recently silk fibroin is used for blood vessels[13], skin[14], bone [7] and scaffolds. Indeed there were improved ideas in natural medicine for the treatment of different diseases, as a result from animal experiments, these drugs known to have few toxic effects. Of that dextrin in the treatment of the diabetes is particularly interesting it is used in food industry to textile. Theoretically, the dextrin–rhEGF blends have the potential to treat chronic wounds. Especially dextrin – rhEGF combination[6] has been used for a variety of medical therapies such as wound healing and as well as basic scientific research.

In this study the natural extract polysaccharide dextrin and recombinant human epidermal growth factor (rhEGF) were loaded on silk fibroin material. The surface morphology was observed by SEM. Antimicrobial test was conducted to test the effect of microorganism present in diabetic wound

Materials And Methods

Raw Silk was obtained as cocoon from the farmers of Coimbatore district. The recombinant human Epidermal Growth Factor (rhEGF) as REGEN-D™ was purchased from Bharat Biotech International Limited, Hyderabad, India. Honey is purchased from rural area of Masinagudi. Ethanol (absolute GR for analysis) was obtained from Merck (Darmstadt, Germany). Deionized water was used during all experiments.

Preparation of silk scaffold

Silk scaffold was prepared with mixing of dextrin, silk fibroin solution and honey stirring until both dissolved completely. Aqueous silk fibroin was agitated at 50^0 C, meanwhile the dextrin and honey were mixed up with the aqueous silk fibroin solution in the different ratios (Table 1). Then the mixed gel was spread on a watch-glass at a thickness of 4mm, followed by standing and cooling to room temperature to form scaffold. Next, the obtained scaffold was added with rhEGF at controlled temperature.

Bacteriological study

The antibacterial properties of prepared scaffold were tested by using disc diffusion method. The discs with the prepared scaffolds were applied to the surface of an agar plate containing the wound sample to be tested. The line of incubation of antimicrobial agent is shown by the presence of growth inhibition zones measured by using Muller-Hinton (HiMedia) as described by Fingold & Baron[5]. The zones of inhibition appear as clear areas surrounding the disc from which the substances

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with antimicrobial activity diffused. All antibacterial tests were performed by using a gram negative (Escherichia coli) and a gram positive bacteria (Staphylococcus epidermidis). Positive control was chosen as Amoxycillin antibiotic disc. After the 24 h incubation, INT (tetrazolium dye) solution was sprayed over the surface of an agar plate to ensure visible indication of inhibition. INT reacts with the metabolites produced by the microorganisms and the surface with the living microorganisms turn to pink color. The number of colony forming units was counted using digital colony counter (Deep Vision Company, India).

Table 1

Table 1 - Preparation data of silk fibroin/dextrin/honey scaffold

SAMPLE PROPOTIONS

SDH	SDH – 1	SDH – 2	SDH -3
Silk Solution - 15ml	**Silk Solution - 15ml**	**Silk Solutio- 15ml**	**Silk Solution15ml**
Dextrin - 3gms Honey - 3ml Temperature 80°C Time 45mins	Dextrin - 5gms Honey - 4ml rhEGF – 5µg Temperature 80°C Time 45mins	Dextrin - 5gms Honey - 4ml rhEGF – 10µg Temperature 80°C Time – 45mins	Dextrin - 6gms Honey - 5ml rhEGF – 15µg Temperature 80°C Time – 45mins

SDH – Silk solution + Dextrin + Honey sample, SDH-1 - Silk solution + Dextrin + Honey + rhEGF(5 µg) sample, SDH -2 - Silk solution + Dextrin + Honey + rhEGF(10 µg) sample, SDH -3 - Silk solution + Dextrin + Honey + rhEGF(15 µg) sample.

Water uptake and weight-loss-related tests

The water uptake and degradation behaviour of the silk fibroin scaffolds were assessed after immersion in an isotonic saline solution (ISS; 0.154 M sodium chloride aqueous solution, pH 7.4) for time periods ranging from 1 day to 30 days [Pinho et al., 2009]. All experiments were conducted at 37^{0}C and dynamic condition in controlled condition. After each time period the specimens were removed from the ISS and the weights were determined immediately after adsorption of the excess of surface water using a filter paper. The water uptake was calculated using the following expression:

Water take-up ratio in % = [(Wtf – Wti) / Wti] X 100% (1)

Where Wti is the initial weight of the dry specimen and Wtf is the wet weight of the specimens after being removed from saline solution.

The water stability of regenerated silk fibroin protein based was determined by calculating the weight loss in water. Weight loss was tested by immersing different scaffolds (approx. 20 gm) in PBS at 37^{0}C for 7 days at room temperature. After incubation, samples were dried at 65^{0}C and the remaining mass measured. Mass loss was expressed as percentage of the original mass of each sample. Weight loss was calculated using the formula,

Weight loss in % = [(Wf – Wi) / Wi] X 100% (2)

Where Wi is the initial weight of the dry specimen and Wf is the wet weight of the specimens after being removed from PBS solution. The experiment was repeated thrice and the average value was obtained.

Morphology

Morphologies of the surface and the cross-section of the lyophilized scaffold samples were studied by scanning electron microscope (JEOL, JSEM-6390LV, Japan) with an accelerating voltage of 20 kV after gold coating. SEM observations were performed using the followings magnifications: 1000x and 5000x.

Fourier-transform infrared spectroscopy

The FTIR spectra of sample silk fibroin/honey blend ratios were recorded with Spectra 100 FTIR spectrophotometer (Perkin Elmer India Pvt. Ltd.). The IR spectra in the absorbance mode were obtained in the spectral regions of 400–4000cm−1. Each spectrum of the samples was acquired by accumulation of 32 scans with a resolution of 4 cm−1.

Results

Preparation of silk scaffolds

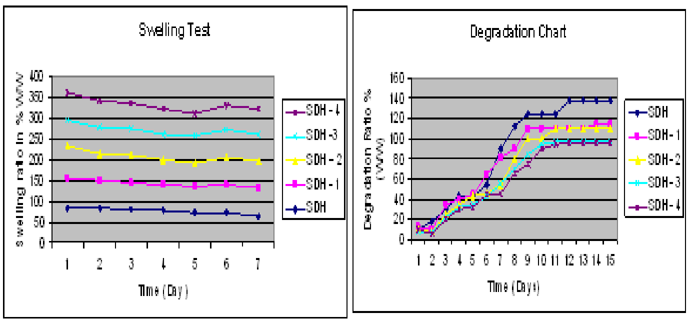
The silk scaffolds were prepared using honey and dextrin with rhEGF at various contents (Table 1), which were uniform and tuft materials. The scaffolds exhibited clay color. All the samples were easily removed from the glass plate and showed as gel surface (Silk scaffold as an example shown in Figure 1).

Figure*: 1 Silk Scaffold*

Water uptake and degradation-related properties

The ability to up take fluids from the surrounding medium plays an important role in tissue engineering. Figure 2 the water uptake ratio of all the scaffolds reached equilibrium after only 7 hours of immersion in aqueous solutions, and can be maintained for up to 15 days. This result shows that the scaffolds possess a good hydration capability and are able to maintain their structural integrity. From Figure 2 also shows that water uptake ratios of the scaffolds increased with increasing dextrin concentration. The differences in water uptake can be attributed to the different porosities of the scaffolds. It was observed that for the scaffolds with higher porosity, the water uptake ratio increased. All the scaffolds after soaking in aqueous solutions for 15 days continue to degrade slowly, one of the main advantages of biodegradable product (Figure 2).

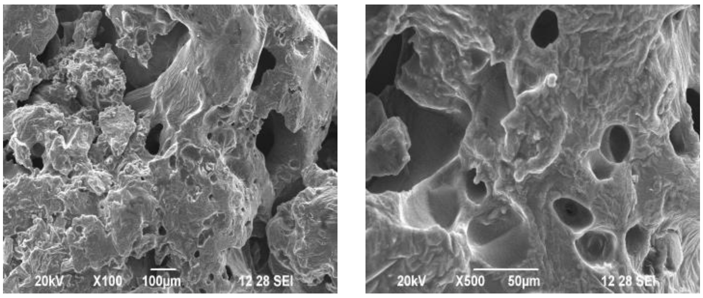
Figure*: 2 Water uptake and degradation*



Morphologies and porosities of the composite scaffolds

In this study, the pores morphology of the prepared silk fibroin scaffolds was investigated using SEM. This observation was conducted to further examine the surface and internal micro architectures of porous SF–Dextrin/honey scaffolds. The microstructure and architecture of the scaffolds are crucial parameters for tissue engineering applications since they can affect the final outcome of the tissue regeneration. Figure 3 shows SEM micrographs of the composite scaffolds with different honey and dextrin along with rhEGF contents. The pure SF scaffold exhibited a macroporous structure with interconnected open pores, and pore sizes varied from 100 to 200 μm (Figure 3). In the three-dimensional (3D) scaffolds, the minimum pore size must exceed 100 μm, otherwise, the tissue can only penetrate the surface of the scaffolds and cannot easily diffuse sufficient nutrients to the inner tissue. After compounding with honey and dextrin, the macroporous structure of the scaffold was still maintained, this suggested that there was no change on the pore structure incurred by adding both (honey and dextrin) into the system.

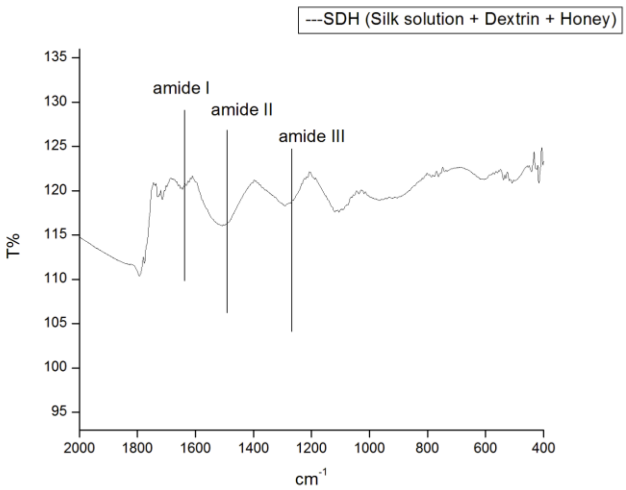
Figure: 3*: SEM image of scaffold prepared with dextrin (3g) and honey (3ml) at* 80°C.



FTIR analysis

In order to confirm the structure change, prepared silk/honey scaffolds with different composition were investigated by FTIR spectra. The FTIR spectrum of SDH (Silk solution + Dextrin + Honey) is shown in Figure 4. Pure silk fibroin had characteristics vibration bands in their FTIR spectra, including 1630–1650 cm-1 for amide I (C=O stretching), 1520–1540 cm-1 for amide II (secondary N\H bending) and 1270–1230 cm-1 for amide III (C-N and N-H functionalities) were assigned as silk I structure[15]. In Figure 4 the characteristics vibration bands of silk particles were obviously shifted to other positions where indicated ß-sheet structure, such as absorption peaks of 1640, 1515 and 1280 cm-1 moved to 1648, 1509 and 1277 cm-1 correspondently. The spectra of dextrin presents bands at 3416 (0-H), 2927(C-H), 1155(C-0), 1016(C-C) cm-1. The characteristics vibration bands of dextrin were obviously moved to 3420, 2935, 1162 and 1020 cm-1. Transform Infrared (FTIR) analysis showed no change in silk fibroin structure in composition with dextrin.

Figure: 4 FT-IR Spectra of Silk blended dextrin and honey scaffold



Antibacterial ability

In this study E. coli and S. aureus were used as the test bacteria to examine the antibacterial properties of prepared silk protein based dextrin and honey scaffolds. It was noted that the numbers of colony of all test bacteria formed on the Silk/dextrin blend membranes were decreased with the increase of rhEGF concentration. Quantitative counting using colony counter found E.Coli - 27 CFU/ml and S.aureus - 33 CFU/ml in SDH -3. SDH -3 sample shows better result compares with other samples of SDH -2 and SDH -1. This prepared silk blend scaffolds demonstrate more effective antimicrobial ability against E. coli than that of S. aureus, as indicated by the lower colony unit. These results indicate that the silk blend scaffolds may be suitable to be used as a wound dressing with antibacterial properties.

Conclusions

In this study, the influence of silk scaffolds along with honey and rhEGF were comprehensively investigated in wound sample by in vitro method. It was found that prepared silk scaffolds could significantly promote the wound healing nature as the result from observation during antimicrobial test. The silk scaffold was definitely shown to be a promising agent with great potential for wound therapy and its healing. Further investigation will more precisely delineate the mechanisms behind the improved antibacterial activity of silk scaffold, and define the types of wounds that can be treated successfully.

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