Original Article

Effect of Quercetin Incorporated Silk Sericin/Gelatin Scaffolds in Wound Healing

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Abstract

Background: Wound healing research aims at developing bioactive material that act as wound dressings for treating chronic wounds that do not heal easily.

Objectives: Herein, we report the fabrication of silk sericin/gelatin scaffold incorporated with quercetin by blending silk sericin extracted from Bombyx mori cocoons with gelatin and quercetin respectively.

Materials and Methods: The scaffolds were analyzed using UV-visible spectroscopy, FTIR-spectroscopy, HR-SEM and energy dispersive spectroscopy (EDAX). The scaffolds were also evaluated for their mechanical property. Hemolytic assay was performed to check the blood compatibility of the scaffolds. The wound healing efficacy of the scaffolds was studied by creating open excision type of wounds in albino rats.

Results: The fabricated scaffolds proved to improve the rate of wound closure in rats treated with them.

Conclusions: Studies showed that the proposed scaffold has better wound healing activity and it can be used as a potential biomaterial for wound dressing.

Key words : sericin, gelatin, quercetin, scaffolds, wounds

Introduction

Skin is one of the largest organs in the human body, which acts as a primary protective barrier from the external environment. It helps in heat regulation and maintenance of body hydration.[1] Skin has very complex structure composing of three different layers such as epidermis at the top, dermis in the middle (that serves as a sheet for the cells) and hypodermis or subcutaneous fat layer at the bottom.[2] The most common skin injuries that are encountered are due to direct contact of skin with the harmful microbes and pathogens or through chemical, mechanical or thermal damages that are caused during day-to-day activity.[3] Wound healing is a highly intricate repairing process encompassed of intracellular and intercellular pathways. To recuperate the loss and integrity of skin tissues, four different succeeding and temporally overlapping stages such as homeostasis (blood clotting), inflammation, proliferation (re-epithelialization) and tissue remodeling will occur in human body.[4] The healing of wounded site will occur

naturally, via the above-mentioned phases of wound healing. But in cases of deep wounds and burns, the healing processes is not satisfactory and thus are prone to risks, infection, inflammation, and scar formation. Additionally, some of the internal factors that could affect the natural healing process and tissue restoration of wounds arise from diseases and life style such as diabetes, existence of foreign bodies, malnourishment, obesity and aging. Thus, chronic wounds fail to heal which could develop to foot ulcers in case of diabetic patients and may lead to amputation or even death.[5,6] This leads to decrease in life quality and causes burden of increasing medical expenses to human population. Millions of people worldwide are affected by chronic wounds and spend billions of dollars each year on wound care.[7,8] Many critical challenges are encountered with use of autograft, allograft and xenograft as skin substitutes, due to limitation of donor morbidity and immune rejections of the substitutes.[9] Even though, plethora of research has been carried out in skin tissue regeneration, there are no promising substitutes which can be used for the regeneration of deep wounds.[10-13] The use of autologous stem cells is effective in skin tissue injuries but harvesting and maintaining the cells is expensive and time consuming.[14] Advanced development in skin tissue engineering aims to regenerate the defective tissues by combining living body cells with highly porous scaffolds, where the scaffold serves as template for cells and guide them for the development of new tissue.[14] An ideal biomaterial is often used in order to fasten the process of wound healing. The biomaterial should have the capability to maintain hemostasis (blood clotting), humid environment on the wound site, inhibit the growth of pathogens or microbes; allow fluid and air-exchange, and stimulate re-epithelialization by releasing bioactive molecules at the wounded site.[15,16] It should have high porosity and good mechanical strength for cell migration, proliferation and adhesion to form a new tissue.

Silk Sericin (SS) is a natural protein obtained from the cocoons of silkworm, Bombyx mori. It is a natural hydrophilic polymer which is extracted by degumming method. Due to its biocompatibility, biodegradability, non-toxicity and ability to enhance mammalian fibroblast cell proliferation, migration and adhesion, sericin is extensively used in the field of biomedicine. Silk sericin consists of 18 amino acids, of which serine and aspartic acid are the major components with strong polar side chain (hydroxyl, carboxyl and amino groups), that facilitate copolymerization, blending and crosslinking with other polymers to improve their biomedical properties.[17,18] Even though, it has potential biomedical properties, silk sericin has not gained a lot of attention in the field of tissue engineering and regenerative medicine as it is a fragile polymer. In this study, gelatin is used as a natural protein polymer to blend with silk sericin to fabricate scaffolds for wound healing application.[19,20] Gelatin is a widely used polymer which is obtained from animal source. It is relatively cost-effective, biocompatibile and easily available. It has superior water solubility and can be used to fabricate thin films. It has been used in the field of research in the form of microspheres, tissue-adhesives and drug carriers for various pharmaceutical and biomedical applications.

Flavonoids obtained from plants are extensively used in medicine due to their excellent therapeutic properties. They possess anti-inflammation, anti-fungal, anti-bacterial and anti-cancer properties. Numerous studies have shown that flavonoids have proved to substantially improve topical wound healing due to their free radical scavenging ability.[21] Quercetin, (3, 3', 4', 5, 7-pentahydroxy flavone) is a naturally occurring flavonoid which is obtained from various fruits and plants such as berries, grapes, cherries and citrous fruits.[22,23] Since quercetin has both anti-inflammation and anti-oxidant properties, it has been reported to be effective in cutaneous wound healing.[24]

In this study, we fabricated silk sericin/gelatin scaffolds incorporated with quercetin for wound healing application. Physical characterization of the scaffolds was carried out using UV-visible spectroscopy, FTIR, SEM, EDAX and mechanical strength. The wound healing efficacy of the scaffolds were studied by creating own excision wounds in albino rat.

Materials and Methods

Fresh cocoons of Bombyx mori were procured from the local sericulture farm at Bangalore, Karnataka, India. Quercetin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Gelatin Extra pure, Dialysis membrane (12 KDa MWCO) were purchased from Himedia Pvt. Ltd (Mumbai, India). All other chemicals were of analytical grade and used without further purification.

Extraction of silk sericin (S) from Bombyx mori Cocoons

Silk sericin (S) was extracted using high temperature and high-pressure degumming technique with slight modification.[25] Silk cocoons were cut into small pieces, mixed with deionised water and autoclaved at 120°C (1 g of dry silk cocoons/30 ml of water) for 1 h. The resulting solution was centrifuged at 8000 rpm for 15 minutes. Then the sericin solution was subjected to membrane dialysis, lyophilized and stored at - 20°C until used.

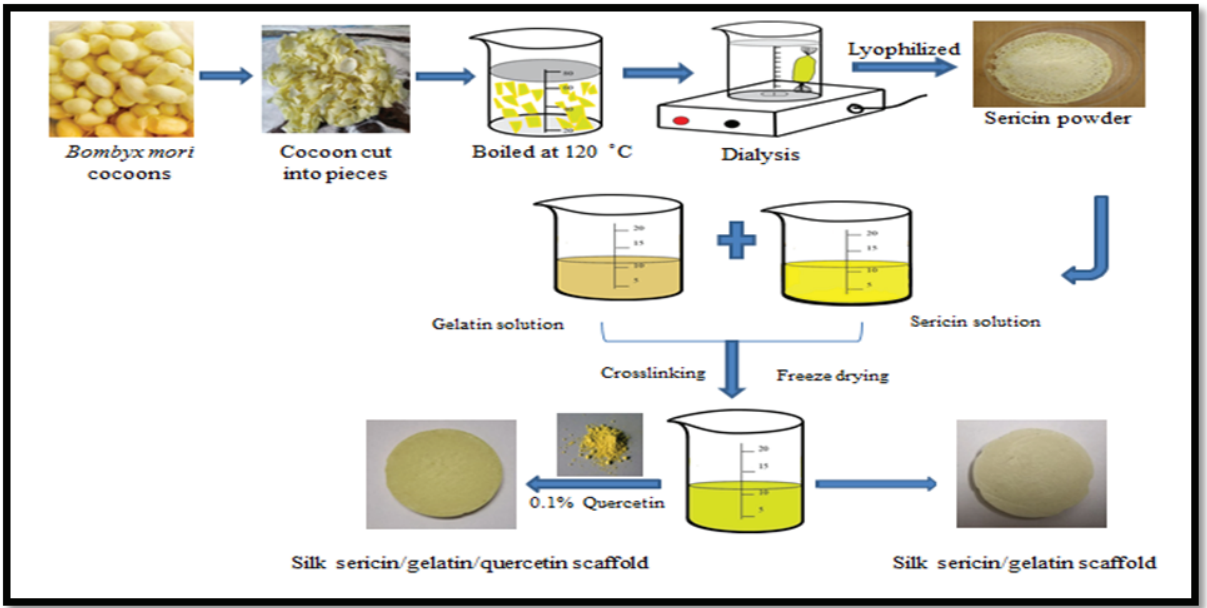

Figure 1: Graphical representation depicting the extraction, purification and fabrication of SS/G and SS/G/Q scaffolds

Preparation of Silk Sericin/Gelatin (SS/G) and Silk Sericin/Gelatin/ Quercetin (SS/G/Q) scaffolds

Silk Sericin/Gelatin (SS/G) scaffolds were prepared by dissolving silk sericin (S) in gelatin (G) solution (1:1 ratio). To this EDC and NHS were added and allowed for crosslinking under constant stirring.26 For preparing silk sericin/gelatin/quercetin (SS/G/Q) scaffolds, 0.1% of quercetin (Q) was added to sericin/gelatin solution under constant stirring. The resultant solutions were then poured on to the petri plates and frozen at -20°C and lyophilized (Figure 1).

Characterization

The surface morphology of SS/G and SS/G/Q scaffolds were observed using scanning electron microscopy (HR-SEM, Quanta FEG 200). The UV-vis spectroscopic analysis of the silk sericin, gelatin and quercetin were carried using UV-vis spectrophotometer. FTIR (Fourier Transformed Infrared spectrophotometer) (Bruker, USA) was recorded in the range of 4000-500 cm-1. A universal testing machine (Instron, 3369/ J 7257, USA) equipped with 100 N load cell was used to measure the mechanical properties of the SS/G and SS/G/Q scaffolds.

Hemolytic assay

Hemocompatibility test was determined by soaking scaffolds (1x1cm^2) in 1 ml PBS (pH 7.4). After soaking, the samples were incubated with 50 µl of Human Red Blood Cells (Proposal No.223/IHEC/ 1-19) and incubated at 37°C for 1 h. Then the samples were centrifuged at 3000 rpm for 15 min and the absorbance was measured at 540 nm. The percentage of hemolysis was calculated as previously described.[27]

Hemolysis (%) = [Test sample – Negative control] / [positive control – Negative control] x 100

In vivo wound healing study

In vivo experiment was carried out with the approval from Institute Animal Ethical Committee (IAEC) (ethics approval no: IAEC4/Proposal: 30/A.Lr:12A/ Dt: 28.03.19) of Chettinad Hospital and Research Institute, Chennai India. Male Wistar Albino rats were used for the study. For surgical experiments, the animals were anesthetized with halothane and the dorsal hair was sterilized with spirit and removed using a razor blade. Full-thickness excision wound (2x2 cm^2) were created on dorsal region of rats. After wound creation, the rats were divided into three groups; Group 1: Normal saline (control); Group 2: SS/G scaffold and Group 3: SS/G/Q scaffold. Topical application of the samples was carried out once in every 2 days. The surface of the wounds was photographed and the wound size was measured from the start till the end of the study. The percentage of wound closure was calculated using the following equation.

Wound closure (%) = (Area of actual wound – Area of closure wound) / Area of actual wound x 100

Results and discussion

Characterization

The microstructure morphology of the SS/G and SS/G/Q scaffolds fabricated by freeze drying method was analyzed using HR-SEM (Figure 2). The HR-SEM showed that both SS/G and SS/G/Q

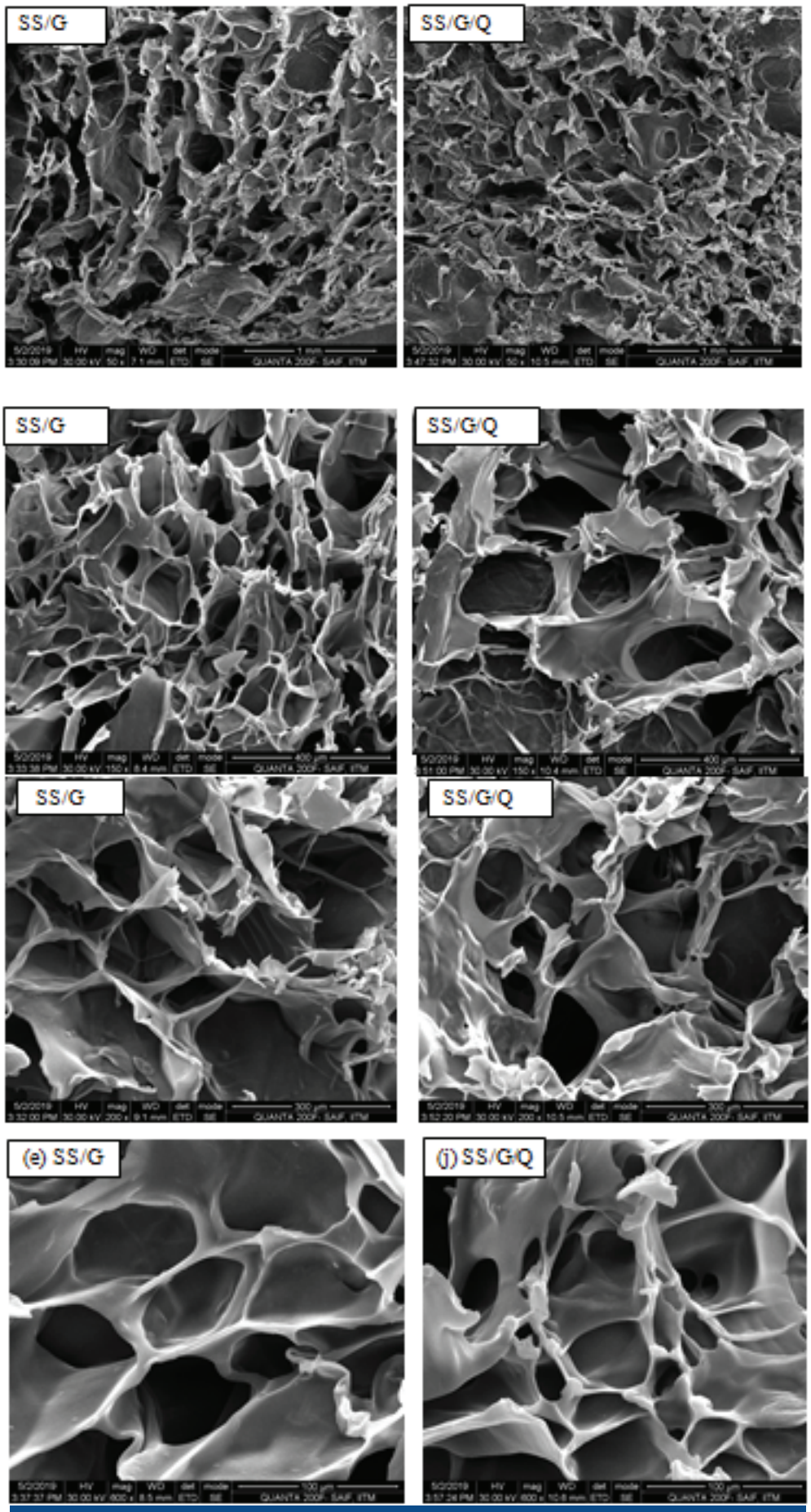

Figure 2: SEM images of silk sericin/gelatin scaffolds (SS/G) and silk sericin/gelatin/ quercetin scaffolds (SS/G/Q) respectively.

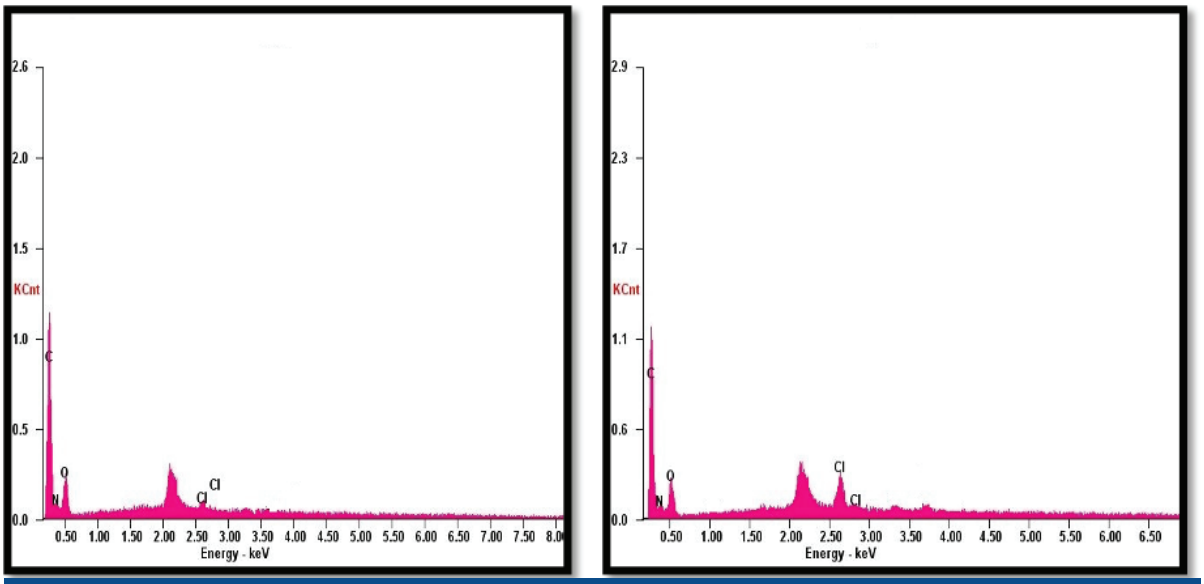

Figure 3: Energy dispersive X-ray spectroscopy of (a) SS/G and (b) SS/G/Q scaffolds

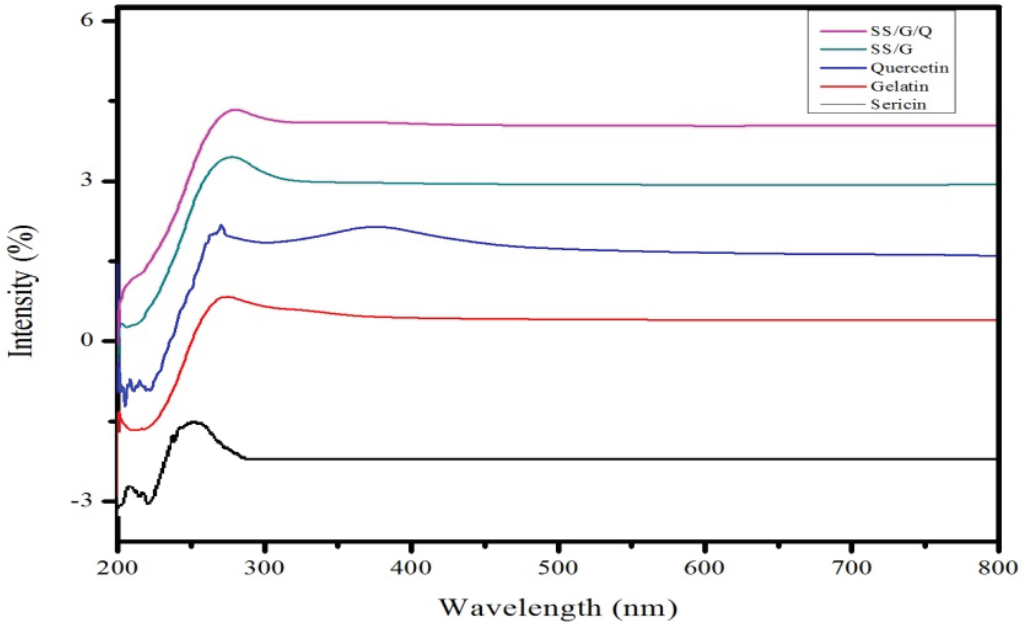

Figure 4: UV-Vis spectroscopic analysis of sericin, gelatin, quercetin, SS/G and SS/G/Q scaffolds respectively

scaffolds has highly interconnected porous structure, which will be better for the cell adhesion and proliferation within the scaffold. To further understand the chemical composition of the biomaterials energy dispersive X-ray analysis was used for analysis of the element. EDAX spectrum for both scaffolds were found to have C, O, and N[28,29] which corresponds to silk sericin, gelatin and quercetin and also some amount of Cl which correspond to crosslinking agents (Figure 3).

UV-vis spectroscopic analysis of silk sericin (SS), gelatin (G), quercetin (Q), SS/G and SS/G/Q scaffold is shown in Fig. 4. Silk sericin solutions display maximum absorbance at 276 nm, which is similar to previous reports.[30] Gelatin shows maximum absorbance at 272 nm and quercetin at 372.97 nm, SS/G at 278 nm, which indicates that silk sericin and gelatin have merged together at one peak, since both sample are proteins and SS/G/Q at 278 nm and 372.92 nm that indicates the successful incorporation of quercetin.

The FTIR spectral band of the extracted sericin powder is shown in Figure 5. As seen in figure, the sericin powder displayed a characteristic peak at 1074 and 1243 cm^{-1} (C-N stretching vibration) showing the presence of amide II - 1545 cm^{-1} (N-H bending) amide I - 1636 cm^{-1} (C=O stretching vibration) and 2935 cm^{-1} (C-H stretching vibration) and

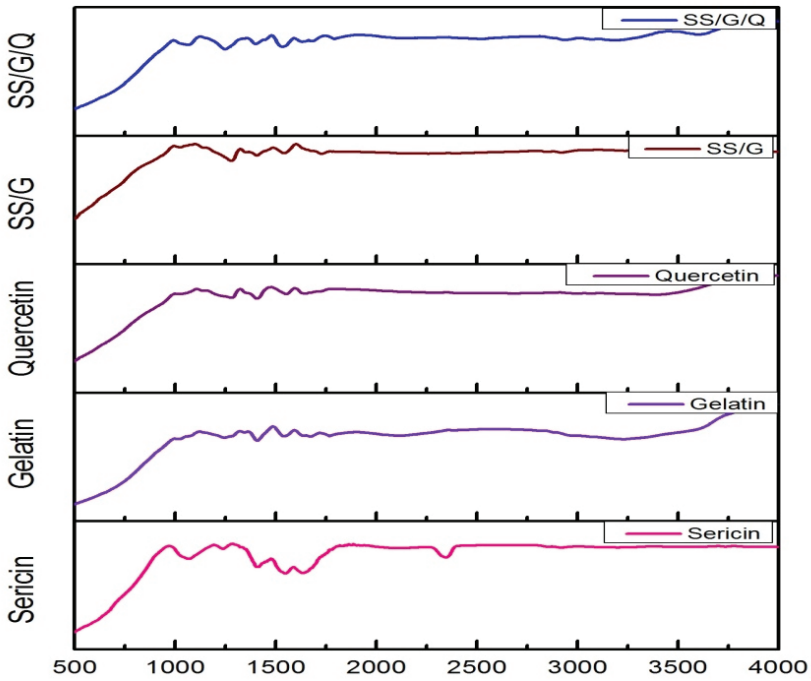

Figure 5: FTIR spectroscopic analysis of silk sericin, gelatin, querectin, SS/G and SS/G/Q scaffolds respectively

3235 cm^{-1} (O-H stretching vibration) which is reported earlier study.[31] Gelatin shows characteristic peaks at 1245 cm^{-1} (C-N stretching, bond in amide III), 1550 cm^{-1} (N-H bending, bond in amide II) and 1650 cm^{-1} (C=O stretching, bond in amide I) respectively.[32] Fig. 5 shows the characteristics peaks at 1648 cm^{-1} (C-C bond stretching vibration) of phenyl ring, found at 1561 cm^{-1}, 1412 cm^{-1}, 1282 cm^{-1}, 1140 cm^{-1} and 1028 cm^{-1}, with the high amount of querectin, attributed to benzene ring bond stretching.[24] The characteristic peaks of SS/G scaffold, depicted similar bands of sericin and gelatin with absorption bands at 1740 cm^{-1} (C=O bond stretching vibration, amide I), 1550 cm^{-1} (N-H bond bending, amide II) and 1285 cm^{-1} (C-N bond stretching vibration, amide III), 2857 cm^{-1} and 2927 cm-1 (O-H bond stretching vibration) respectively. The characteristics peak of SS/G/Q scaffold indicates the presence of new peak at 1073 cm^{-1} and 1247 cm^{-1} corresponding to the stretching vibration as in quercetin. And also the peaks at 1547 cm^{-1}, 1650 cm^{-1} and 1795 cm^{-1} depicted the distinctive peaks of sericin and gelatin respectively.

An ideal wound dressing material must possess good mechanical strength and should not get damaged during handling. For this purpose, tensile strength [MPa] was investigated to evaluate the influence of quercetin on the mechanical properties of scaffolds (Table 1). Tensile testing serves as an indication of strength and elasticity of scaffolds.

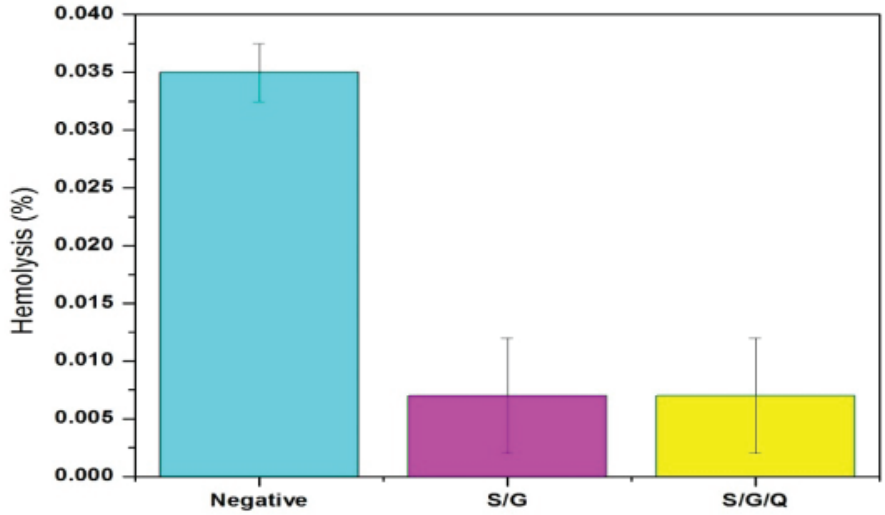

Figure 6: : Hemolysis percentage of SS/G and SS/G/Q scaffolds

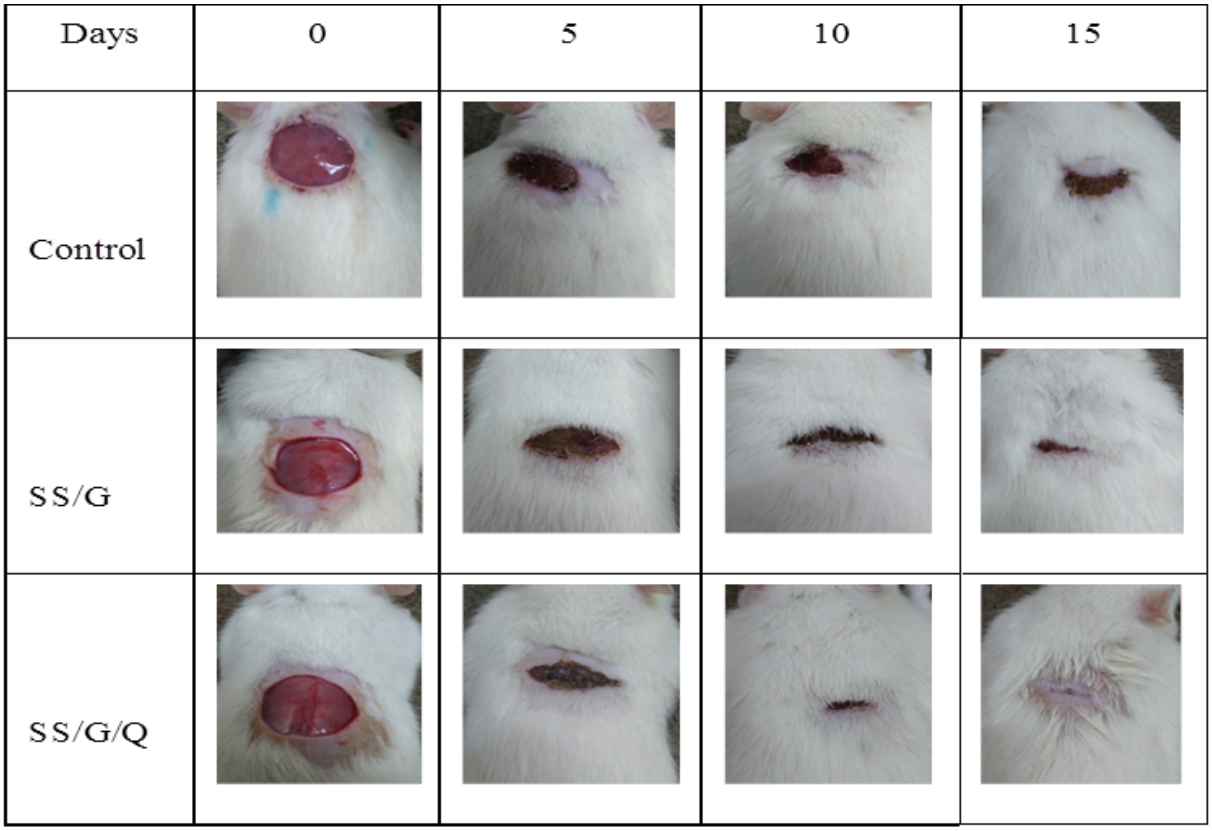

Figure 7a: Photographs of wound healing after treatment with control, SS/G and SS/G/Q scaffold on days 5, 10 and 15 respectively

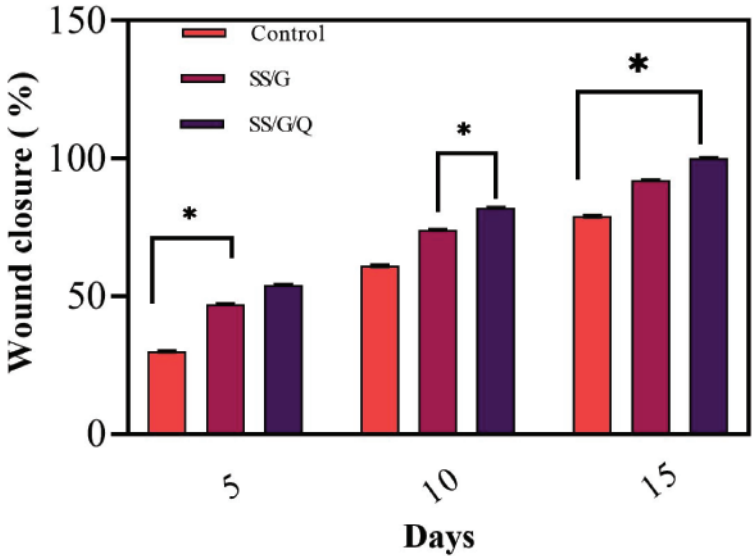

Figure 7b: Closure rate of wounds treated with control, SS/G and SS/G/Q scaffold on days 5, 10 and 15 respectively

SS/G and SS/G/Q scaffolds exhibited maximum tensile strength of 0.045 MPa and 0.035 Mpa, property presented due to the interaction between —NH2 and —C-O-H group of silk sericin and gelatin.

Hemolytic Assay

The blood compatibility of both the scaffolds was studied to determine the behaviour of the scaffold when it comes in contact with blood. Both the scaffolds displayed less percentage of hemolysis which is less than 1%, hence it is considered to be hemocomapatible (Figure 6).

In Vivo Studies:

From Figure 7 (a and b) it is apparent that SS/G/Q treated wounds exhibited faster rate of wound closure than control and SS/G/Q treated groups.

Conclusion

Two scaffolds such as SS/G and SS/G/Q were successfully prepared by lyophilization method. The presence of silk sericin, gelatin and quercetin in SS/G and SS/G/Q scaffolds were confirmed using UV-vis spectroscopy, FTIR and EDAX. The porous scaffolds mean diameter was measured by using HR-SEM was found in the range of about 53 µm for SS/G scaffold, whereas for SS/G/Q scaffold was in range of 18 µm respectively. Hemolytic studies show the hemocompatibility of both the scaffolds. The fabricated SS/G/Q scaffold shows a potential source when treating for skin wound healing in albino rats. Considering a large availability and flexibility of SS in bulk from waste material and the bioactive properties of sericin could be provide a various approach in tissue engineering application at a reasonable products.The result shows that the SS/G and SS/G/Q scaffolds have highly porous interconnected structures. They have excellent water absorption properties with good hemocompatibility. In Vivo studies showed that SS/G/Q scaffolds have better wound healing activity when compared with SS/Q scaffold. Hence SS/G/Q it could be used as a potential biomaterial for wound dressing applications.

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Conflicts of interests

No potential conflicts of interest were disclosed.

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