International Journal of Biomedical Engineering and Technology

ISSN online: 1752-6426 - ISSN print: 1752-6418
https://www.inderscience.com/ijbet

Evaluation of protein/polysaccharide blend biopolymeric material for fabrication of drug eluting wound dressing

Shailendra Singh Shera, Rathindra Mohan Banik

DOI: 10.1504/IJBET.2023.10053529

Article History:

Received:	18 June 2020
Last revised:	14 September 2020
Accepted:	21 September 2020
Published online:	25 January 2023

Evaluation of protein/polysaccharide blend biopolymeric material for fabrication of drug eluting wound dressing

Shailendra Singh Shera

Department of Biotechnology,
Faculty of Engineering and Technology,
Rama University Uttar Pradesh,
Kanpur, India
and
Bioprocess Technology Laboratory,
School of Biochemical Engineering,
Indian Institute of Technology (Banaras Hindu University),
Varanasi, India
Email: shailendra.shera@gmail.com

Rathindra Mohan Banik*

Bioprocess Technology Laboratory,
School of Biochemical Engineering,
Indian Institute of Technology (Banaras Hindu University),
Varanasi, India
Email: rmbanik.bce@iitbhu.ac.in
*Corresponding author

Abstract: Silk fibroin protein and polysaccharide xanthan was mixed in three ratios, i.e., 80:20 (SFX82), 60:40 (SFX64) and 50:50 (SFX55) to fabricate blended dressing and functionalised with antibiotic amoxicillin. The dressings exhibited sustained release of incorporated antibiotics for prolonged period which helped in maintaining therapeutic concentrations of drug for quick wound recovery. The dressings showed biphasic release profile, i.e., burst followed by sustained release. SFX64 showed highest cumulative drug release among all three dressing. Further, SFX64 exhibited smoother surface leading to less bacterial adhesion. Changes in wound size and histological assessments of wound tissues over time confirmed that amoxicillin loaded dressings showed faster healing, higher wound closure rate, regular and thicker formation of epidermis. SFX64 dressing was the best performer with pronounced sustained delivery of antibiotic at therapeutic concentration, smoother surface, and maximum wound recovery of 99.12 ± 0.33%.

Keywords: silk fibroin; xanthan; blends; biphasic; wound healing; wound dressing; sustained drug release; bacterial adhesion; *in vivo* wound healing; histology.

Reference to this paper should be made as follows: Shera, S.S. and Banik, R.M. (2023) 'Evaluation of protein/polysaccharide blend biopolymeric material for fabrication of drug eluting wound dressing', *Int. J. Biomedical Engineering and Technology*, Vol. 41, No. 1, pp.16–34.

Biographical notes: Shailendra Singh Shera has submitted his PhD in Biotechnology at Indian Institute of Technology (Banaras Hindu University), Varanasi and is currently working as an Assistant Professor in Department of Biotechnology, Faculty of Engineering and Technology, Rama University, Uttar Pradesh, Kanpur, India. His research interest includes biomaterials and tissue engineering, nanoparticle synthesis for drug delivery, and 3D bioprinting. He has published more than seven research article in peer reviewed international science citation indexed (SCI) journals and three book chapters published by Elsevier and Springer publications and has presented his research at various prestigious national and international conferences.

Rathindra Mohan Banik is currently a Professor (Higher Academic Grade) at Indian Institute of Technology (Banaras Hindu University), Varanasi with over 30 years of teaching and research experience in fermentation technology, food biotechnology and microbial engineering. His research interest includes media design, optimisation of bioprocess system, food biotechnology, polymeric biomaterials, drug delivery. He has published over 50 research articles in science citation indexed (SCI) journals and five book chapters. He has also attended and presented his research at various national and international scientific conferences.

1 Introduction

A wound is a type of injury in which skin is torn, cut or punctured which damages the skin disrupting normal skin physiology. These wounds, if left untreated quickly catches infection and develops into hard to treat chronic wounds. The application of wound dressing to injured skin plays a critical role in treatment of injury and presents an effective intervention to guide correct sequence of healing events for faster recovery (Vasconcelos et al., 2012). These healing sequences involve inflammation, re-epithelialisation, angiogenesis, granulation, tissue formation, wound contraction, and tissue maturation (Dickinson et al., 2016). Therefore, an ideal wound dressing should possess certain characteristic such as ability to maintain moist milieu, absorb excessive exudates, allow fluids exchange with environment, and protects wound from dust and microbes. In addition, modern wound dressing should not only perform conventional role of wound protection but also be able to simultaneously deliver incorporated drugs at sustained rate to the wound site for faster wound healing (Moraes and Beppu, 2013; Gil et al., 2013).

Wounds can be classified in various categories such as mild, moderate to severe wounds, from small to large wounds, from shallow to deep wounds, from acute to chronic wounds (Shi et al., 2020). Treatments of each of these wound requires specific types of dressing due to dynamic nature and complexities involved in healing. Owing to these reasons many different types of wound dressings such as silver-containing hydrophilic fibre dressing, antibacterial dressing and wet dressing have been fabricated to serve diverse functions of healing. Further, dressings have been fabricated in various materials format for e.g., foams, gauze, transparent films, alginates, composites, hydrocolloids and hydrogel (Lei et al., 2019; Shi et al., 2020). The applications of these dressings are dependent upon types of wounds for e.g., foams can be applied for treatment of partial to full thickness injury having medium to massive exudates, gauge are used only for bandaging while hydrogel and alginates wound dressings are used in

treatment of partial to full thickness wounds, necrotic or desquamate wounds, infected and non-infected wounds with a large amount of exudates. Transparent membrane dressing is effective for superficial wound healing only. Composite dressings are most robust and are suitable for treating Grade II and grade III burn injury, chronic wounds and old granulating wounds (Lei et al., 2019). The existence of many types of dressings with different combinations of biomaterials in the market indicates towards complexity and scarcity of ideal dressings suitable for all types of wounds. Therefore, quest for biomaterial suitable for fabrication of wound dressing is a continuous process.

Biomaterial is an important component of any dressings because it acts as structural supports and medium to hold therapeutic drugs. Though, there are several synthetic materials such as poly(ethylene glycol), poly(N,N-diethyl-acrylamide), Poly vinyl alcohol, poly-ε-caprolactone, polyurethane used for wound dressing but their chemical nature, limited renewability and degradability limits their applications (Murray et al., 2019). Owing to these inherent problems, natural materials, especially biopolymers are being considered for developing suitable biomaterials for dressings. Biopolymeric materials as wound dressings are advantageous because of their similarity to natural extracellular matrix, biocompatibility, biodegradability and their ability to become part of the body after healing (Murray et al., 2019). Further, biopolymeric material can influence various aspect of healing such as skin regeneration, re-epthilialisation naturally without need of incorporating growth factors or other bioactive elements.

Natural biopolymeric materials have been found effective in wound healing. Both polysaccharide and proteins such as pectins, alginates, chitosan, cellulose, agarose, hyaluronic acid collagens, keratin, and fibroin have been used to fabricate various wound dressings in different format (Mogosanu & Grumezescu 2014). Polysaccharides like Hyaluronic acid incorporated hydrogels have been reported to maintain moist healing environment, impart antibacterial property, promotes angiogenesis and blood clotting at wound sites which accelerated healing process. Similarly, chitosan is reported to accelerate wound contraction and healing due to its antibacterial properties, hydrophilic nature and ability to modulate the functions of inflammatory cells and ability to promote granulation and organisation (Liu et al., 2018; Dai et al., 2011). Dressings made of proteins such as collagen, which is a major protein of extracellular matrix has the ability to influence all four phases of wound healing. It stimulates cellular migration, attracts fibroblasts, and encourages the deposition and organisation of newly formed collagen at wound sites (Fleck and Simman, 2010) which creates a suitable microenvironment around wound site for effective healing. Collagen dressings are suitable for partial and full thickness wounds, burns related wounds with medium to heavy exudates. Silk protein fibroin due to its biocompatibility, biodegradability, high water and oxygen uptake, low immunogenicity, and robust mechanical properties is another attractive choice for fabrication of wound dressings (Farokhi et al., 2018). Silk fibroin based wound dressing has been shown to promote the adhesion of human keratinocytes and fibroblasts as well as enhance the deposition of type I collagen *in vitro* due to its hemostatic properties, low inflammatory potential, and permeability to oxygen and water vapour (Zhang et al., 2017a). Further, silk based wound dressings have shown better wound contraction and skin regeneration *in vivo* compared to the hydrocolloid dressing or porcine dermis/dermal matrix (Gil et al., 2013; Sugihara et al., 2000). It is evident that suitable biomaterial design for wound dressing is essential for successful wound treatment and management.

Several studies have indicated towards the effectiveness of protein/polysaccharides biomaterials for wound healing. A bicomponent biopolymeric material for fabrication of

drug eluting wound dressing is always better than the single component biomaterial because a double component material has higher healing potential than the single component. Roh et al. (2006) in a remarkable study showed the superiority of silk fibroin/alginate (SF/AA) dressing over lone silk fibroin and alginate dressing. Silk fibroin has excellent cell attachment, fibroblast activation and low inflammation whereas alginate has the ability to form gel by interacting with wound exudates thereby maintaining optimal microenvironment near wound area for faster recovery, calcium ion in alginates promotes hemostasis, cell migration and increases proliferation of fibroblast. These individual properties of single original biopolymers were combined in one SF/AA dressings for treatment of full thickness defects in rats. The synergistic effect of SF/AA dressings caused re-epithelialisation at greater rate than the dressing with lone component (Roh et al., 2006). Similarly, Zhang et al. (2017b) showed that silk fibroin/hyaluronic acid scaffold when compared with polyvinyl alcohol hydrogel scaffold promoted collageneous tissue formation, capillaries and micro vessels formation, maturation of granulation tissues *in vivo* during course of healing (Zhang et al., 2017b). Similarly, Luangbudnark et al. (2012) also argued the effectiveness of bicomponent silk fibroin/chitosan blend films. The drug eluting bicomponent dressings are effectively used in treatment of non-healing wounds. Non-healing wounds provides a harsh environment resulting in absence of cells producing the required growth factors and cytokines, or the degradation of those that are present thereby severely affecting the wound healing process (Murray et al., 2019). Therefore, drug eluting can make the microenvironment around non-healing wounds suitable for faster healing. Huang et al. (2018) demonstrated the effectiveness of drug eluting chitosan/hyaluronic acid (SF/HA) bicomponent hydrogel for treatment of non-healing wounds. SF/HA hydrogels were used to achieve sequential delivery of two different compounds, vancomycin and vascular endothelial growth factor (VEGF). This hybrid system was able to inhibit bacteria growth and accelerate vein endothelial cell proliferation *in vitro* (Huang et al., 2018). Similarly, Qian et al. (2020) fabricated silk fibroin/chitosan wound dressing loaded with silver nanoparticles and exosomes for promoting angiogenesis, nerve repair and infected wound healing. This composite system exhibited multifunctional properties such as antimicrobial activity, moisture retention ability, electrolyte balance maintenance and wound healing promotion ability. It is evident that blending together two biomaterial of differing capability results in a hybrid bicomponent material with properties of both the original biomaterials. Therefore, a wound dressing made from bicomponent materials and incorporated with drugs or suitable bioactive agent offers excellent healing potential in less time for treatment of healing as well as non-healing wounds.

The aim of this study was to describe the application of silk fibroin/xanthan (SF/Xa) as biopolymeric biomaterial for fabrication and evaluation of the drug eluting wound dressing. Xanthan being anionic polysaccharide can interact with many cationic species such as ions and proteins, for e.g., neutrophils which help in progression from inflammation stage to next stage during healing and can also influence cell signalling due to presence of saccharide moieties for better healing. However, xanthan has poor mechanical strength which limits its applications as wound dressing. Silk fibroins have highest mechanical strength among natural biopolymers and have long history of use in wound healing applications. These favorable and counteracting properties of xanthan and silk fibroin attracted us to prepare blend with this combination and test it for wound healing applications Blending silk fibroin with xanthan created hybrid bicomponent biopolymeric materials suitable for wound dressings. In this study we evaluated drug

release behavior of incorporated drug, adherence of bacteria, water vapour transmission rate (WVTR) and *in vivo* wound healing behavior.

2 Materials and methods

2.1 Materials

Bombyx mori cocoon was purchased from the local unit of Central Silk Board, Bhadrasi (Varanasi, India). Commercial grade xanthan (MW= 2×10^6 g/mol, with acetyl content 7% and pyruvate content 2.4%), from Sisco Research Laboratory, Mumbai, India. Lithium bromide (LiBr) was purchased from Merck, New Jersey, USA. Sodium Bicarbonate was purchased from Molychem Mumbai, India. Dialysis tube (MWCO 12K-14K), Closure Clip, Phosphate Buffer Saline, methanol were purchased from Himedia, Mumbai, India. Deep Freezer (Thermo Scientific, USA), Lyophilizer (Labtech. Hyderabad, India).

2.2 Fabrication of silk fibroin dressing

The silk fibroin was extracted following a previously described protocol (Shera et al., 2018), A 0.3 % (Weight/Weight) xanthan solution was prepared by dissolving xanthan powder and mixed with 7% silk fibroin solution in following ratios: 80:20 (SFX82), 60:40 (SFX64) and 50:50 (SFX55) to prepare silk fibroin/xanthan (SF/Xa) blend solution. These blend solution were sonicated at cycle of 0.5 with an amplitude of 50% for 30 seconds by using Ultrasonicator (Hielscher, Germany) to achieve a homogeneous mixing of the two components. The solutions were immediately deep frozen at –50˚C to avoid sol-gel transition of silk component in the mixture.

The samples were frozen for six hours followed by lyophilization (Labtech Lyophilizer, Daihan Labtech Co., Korea) of the frozen sample at –50°C for 48 hours at a vacuum of 0–2 Torr to prepare silk fibroin/xanthan porous scaffold. After lyophilisation, the dried porous dressings were immersed in methanol for one hour to induce β-sheet formation and insolubility.

2.3 Incorporation of antibiotics into dressings

The amoxicillin was incorporated into fabricated dressing by immersing dressing in 25 ml of amoxicillin solution at concentration of 5 mg/ml. The mechanism of drug incorporation was physical adsorption and entrapment into the polymeric dressing materials. After defined time period, drug loaded dressings were taken out from loading solution and used for *in vitro* drug release and *in vivo* wound healing studies.

2.4 Water vapour transmission rate

The test samples were sealed on mouth of open cup containing anhydrous $CaCl_2$ as a desiccant. The edges of the specimen were thoroughly sealed to prevent inward or outward movement of water vapour through it. The assembly was placed in a desiccators containing saturated solution of NaCl. At an interval of 24 hours, change in weight of permeation cup with specimen was recorded. The water vapour permeation rate was calculated according to equation (1) given by Li et al. (2015):

$$\text{WVTR} = \text{S} \times 24/\text{A}\,(\text{g}/\text{m}^2/\text{day}) \quad (1)$$

where S is the difference between weight of cup at day 0 and day 1 in (g) and A is tested area of sample (m^2).

2.5 Bacterial adherence study

An overnight grown bacterial suspension of *Escherichia coli (E. coli) and Staphylococcus aureus* (*S. aureus) were* centrifuged, pellets resuspended in 0.5% LB medium and diluted to 0.5 Mcfarland standards. Replicate discs of SFX82, SFX64, SFX55, SF and Xa were placed in 6-well flat bottom tissue culture plate and completely immersed with aliquots of the bacterial suspension (4 ml) with a cell density of 1×10^8 CFU ml^{-1}. After the incubation of culture plates containing at 37°C for 48 hrs, the disc were removed from culture plates and non-adherent bacteria were removed by washing with milli Q water for 30 second. The discs were transferred to test tube containing 5 ml of fresh milli Q water, sonicated for 10 min, and vortexed for 30 s to remove adherent bacteria. The number of viable adherent bacteria were stained with trypan blue and counted using hemocytometer under optical microscope.

2.6 In-vitro drug release

The samples were immersed in a solution of drug containing drug at a concentration of 5 mg/ml for three days to adsorb amoxicillin by permeation and physical adsorption. The amounts of amoxicillin loaded per samples were 36.5 ± 1.87 mg. For release study, a 10 mm diameter test specimen loaded with amoxicillin was placed in conical flask containing 50 ml PBS at pH 7.4. The flask was placed in rotary shaker at 37˚C at 50 rpm. At a regular interval, 2 ml aliquot was withdrawn and replenished with 2 ml fresh PBS. The aliquots were measured at wavelength (λ) = 272 nm to determine released concentration of the drug.

2.7 Release kinetics

The drug release kinetics and mechanism of transport can be explained by fitting the experimental drug release data to various drug releases models such as Zero order, First order, Higuchi and Korsmeyer-Peppas models (Shera et al., 2018). The models equations are listed in Table 1. Q denotes the fraction of total drug released up to time t. k_0, k_1, k_H, $k_{1/3}$, and k_{K-P} are the apparent release rate constants of the respective mathematical models.

Table 1 Mathematical model for curve fitting of amoxicillin release data

Drug release model	*Mathematical expression*
Zero-order	$Q = K_o t$
First order model	$\ln(1-Q) = -k_1 t$
Higuchi model	$Q = k_H t^{1/2}$
Korsmeyer-Peppas model	$Q = k_{K\text{-}P} t^n$

2.8 Bacterial inhibition study

The efficacy of released concentration of drug against bacteria was determined by turbidometric based bacterial inhibition analysis. The drug aliquots sample from 8 and 48 hours were used for studying the extent of inhibition of bacterial growth. 100 µl of the released antibiotic amoxicillin was added to each test tube containing 5 ml of *staphylococcus aureus* bacterial suspension and incubated for 24 hours at 37°C. After incubation, all the tested suspension was monitored using UV-Visible spectrophotometer (Shimazdu, Japan) at 600 nm. The percentage of bacterial inhibition was calculated by the following equation (2):

$$\text{Bacterial inhibition } (\%) = \left(I_C - I_S / I_C\right) \times 100 \tag{2}$$

I_C optical density of bacterial suspension at 't' = 0 without drug solution

I_S optical density of bacterial solution with drug.

2.9 In vivo wound healing studies

A total of 48 male albino Wistar rats weighing 200 g were divided into four groups of 12 rats per group for *in vivo* full thickness skin wound healing studies. Full thickness wounds of 6 cm^2 were created on the dorsal surface of rats as per the procedure approved by Central Animal Ethical Committee of the Banaras Hindu University (CAECU-BHU), Varanasi (approval no. CAEC/96 dated 04/08/2017). The European Community guidelines as accepted principles for the use of experimental animals were adhered to. Rats were randomly separated into four groups: group I (undressed, control group), group II (treated with antibiotic loaded SFX82 dressing), group III (treated with antibiotic loaded SFX64 dressings), group IV (treated with antibiotic loaded SFX55 dressing). At pre-defined interval wounds were photographed upto 15 days and dressed again with new dressings. The rate of wound closures was calculated from digital photograph of wounds using Image J (National Institutes of Health, Rockville, MD; http://imagej.net/ImageJ) software by following a method outlined by Sanchez et al.2017 and Shetty et al. (2012). The percentage of wound healing was calculated using following equation (3):

$$\text{Wound healing } (\%) = \left(\frac{\text{Initial wound area} - \text{Wound area on nth day}}{\text{Initial Wound area}}\right) \times 100 \tag{3}$$

2.10 Histological evaluation

The regenerated tissue section along with uninjured skin was excised at the end of 15th day, fixed in 10% formalin solution before embedding in paraffin for further processing. Tissue specimens were bisected vertically (transverse sectioning) into 2 µm thick slice, placed on to glass slide, stained with hematoxylin-eosin stain (HE stain). Histological changes in the tissue sample were viewed through a microscope (Olympus CKX53, Olympus America, Center Valley PA) 40X Plan Achromatic Objectives. digital photographs were taken using inbuilt digital Magnus camera.

2.11 Statistical analysis

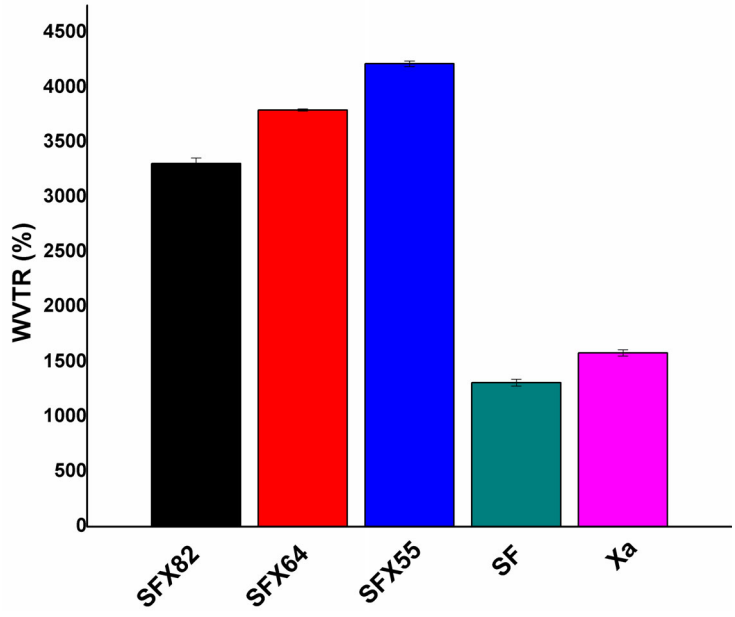
All experiments were carried out in triplicate, and the results were reported as a mean ± standard deviation. Statistical comparison was made using one way ANOVA on computing software OriginPro 2017. A statistically significant difference was defined as $p < 0.05$.

3 Results

3.1 Water vapour permeability analysis

The permeability of fabricated dressings was measured using water vapour permeability (WVP) test (Figure 1). The SF/Xa blends dressings showed higher permeability than pristine SF and Xa dressings SFX55 showed highest WVP with 4,219 ± 25 g/m^2.Day whereas SFX82 showed lowest permeability with 3,310 g/m^2.Day

Figure 1 WVTR of silk fibroin/xanthan blend scaffold (see online version for colours)



3.2 Bacterial adherence analysis

The numbers of bacteria adhered to the surface of SFX82, SFX64, SFX55 and SF dressings were counted using hemocytometer. Both gram negative *Escherichia. coli* and Gram positive *Staphylococcus aureus* bacteria were used for bacterial adherence study. Table 2 shows the extent of bacterial adhesion to dressing surface within 48 hours. Since Xa dissolved within two days, its attachment results were not reported. SFX64 exhibited lowest bacterial adhesion followed by SF, SFX55, and SFX82 dressings. Among the

individual bacterial strain, *S. aureus* attached to SFX64 in lesser number compared to *E. coli.*

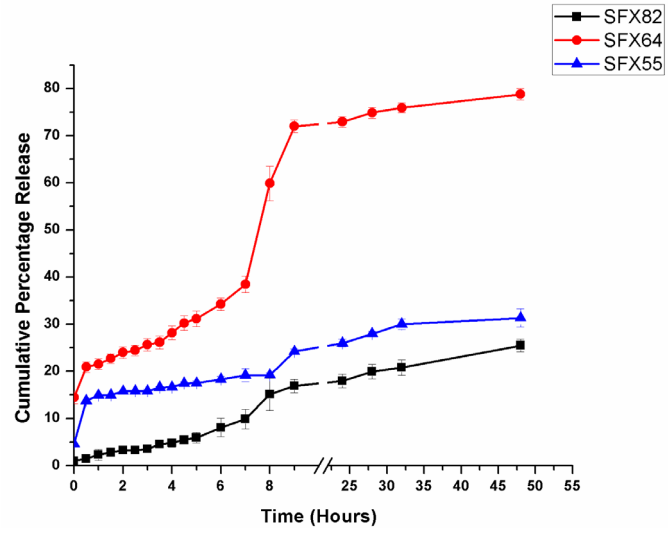
Table 2 Number of bacteria attached to various dressing samples surface during 48 hours

Prepared wound dressing	*E. coli*	*S. aureus*
SFX82	6.41×10^3	3.22×10^3
SFX64	3.81×10^3	2.06×10^3
SFX55	9.19×10^3	5.04×10^3
SF	2.41×10^3	8.43×10^3

3.3 In vitro drug release study

The *in vitro* release behavior of amoxicillin from antibiotic functionalised SFX82, SFX64 and SFX55 dressings were studied (Figure 2). SFX64 and SFX55 showed instantaneous burst release of 14 ± 1.3% and 4.617 ± 0.10%, respectively. SFX82 showed no such burst effect and had an instantaneous release of only 0.99 ± 0.58%. After one hour, all three drugs loaded dressing achieved sustained release profile. The total cumulative release achieved from SFX82, SFX64 and SFX55 in 48 hours were 25 ± 1.34%, 78.7 ± 3.6% and 31.3±1.94%, respectively.

Figure 2 *In vitro* drug release behavior of amoxicillin from SFX82, SFX64 and SFX55 dressings (see online version for colours)



3.4 Bacterial inhibition percentage

The efficacy of released concentration of amoxicillin from amoxicillin loaded dressings was tested using *S. aureus.* The extent of inhibition of growth is shown in Figure 3. The samples collected after 48 hours showed higher reduction in bacterial growth compared to samples collected after 8 hours. Amoxicillin loaded SFX64 dressings showed higher degree of bacterial inhibition compared to other dressings prepared in this study. SFX64

achieved 82.75% of bacterial inhibition after 48 hours which was highest among other amoxicillin loaded dressings. *3.5 In vivo wound healing assessment*

Figure 3 Bacterial inhibition percentage exhibited by different dressing samples as function of released amoxicillin tested using *S. aureus* (see online version for colours)

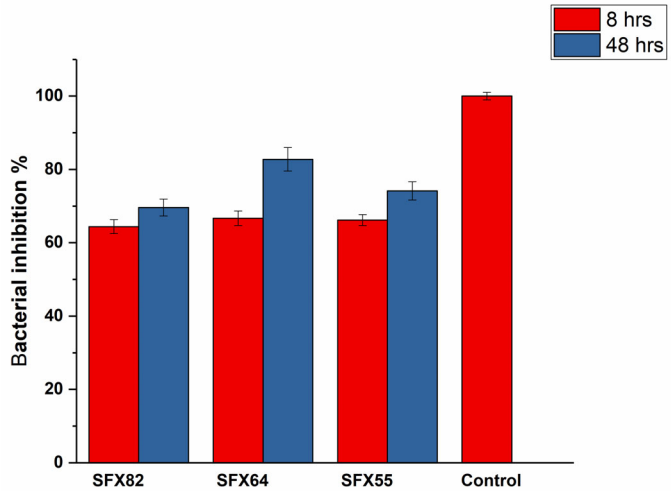
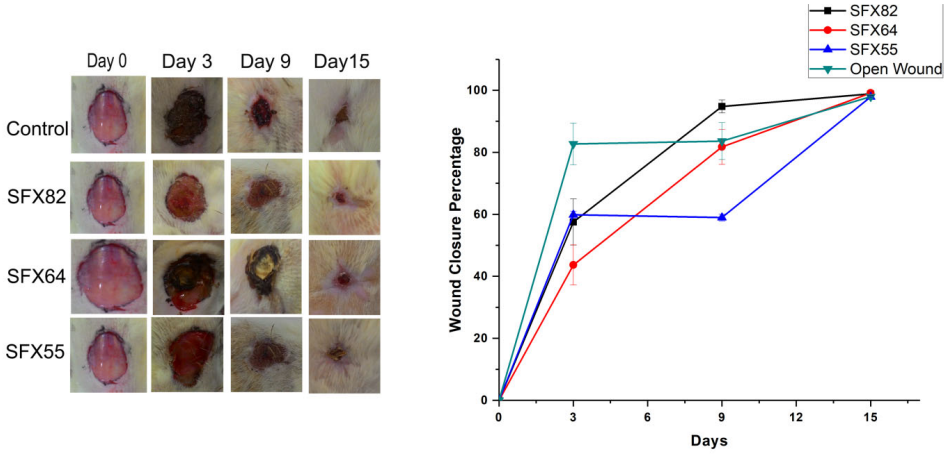


Figure 4 (a) Representative photomicrograph of *in vivo* wound healing evaluation of full thickness wound treated with amoxicillin loaded wound dressings (b) Wound closure percentage (see online version for colours)



(a) (b)

To evaluate the efficacy of antibiotic functionalised wound dressings, full thickness excision wound was created on dorsal surfaces of the Wistar rats and wound closure as function of time was evaluated. The representative photograph of wound closure at respective days is presented in Figure 4(a). The rate of wound closure as a function of time is shown in Figure 4(b). The dressings showed no infections and allergic reactions

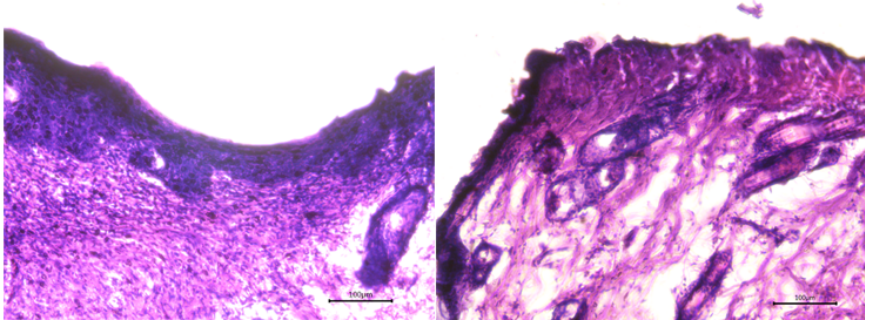
which confirmed its biocompatibility. Taken together with hemocompatibility results it was concluded that the prepared dressings were free from any endotoxins.

Initially, the control group showed higher wound recovery of 82 % ± 6.76. After day 3, wound closure rate in control (untreated, open wound) reduced drastically, achieving only 83.59 ± 5.95%, a change of only 0.91% compared to day 3. However, the wound closure in all three dressed group on day 3 were lower than the control. But from day 9 onwards, dressed group showed higher wound recovery than control with percent change of 37.28 %, 38.09 %, and 0.91% for SFX82, SFX64 and SFX55, respectively. Within the individual dressed group, SFX64 showed highest wound area reduction after day 9 onwards, achieving highest healing of 99.12 ± 0.33% on 15th day than other amoxicillin loaded dressings. The efficacy and superiority of SFX64 were attributed to smoother surface and ability to deliver antibiotic at controlled rate which reduced the attachment and proliferation of bacteria, respectively.

3.5 Histological evaluation

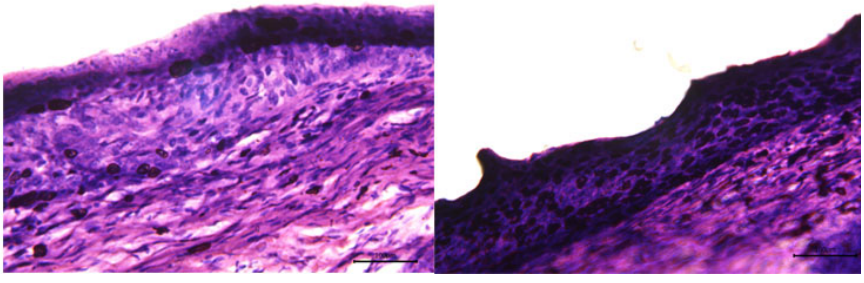
Histological evaluation was carried out on skin section derived from control group, dressed group and unwounded skin, by sacrificing experimental animal on day 15th. As expected, the histological section of unwounded skin did not show any structural modification as compared to the histological section derived from healed wounds [Figure 5(a)]. The control (undressed group) showed irregular formation of epidermis layer and deposition of fibrin at open wound site, indicating partial healing [Figure 5(b)]. Among the amoxicillin loaded dressings, SFX64 showed complete epithelialisation, fibrosis, thicker granulation depth and regular epidermis layer [Figure 5(d)] whereas SFX82 showed progression of epithelialisation and low granulating tissues [Figure 5(c)]. In contrast SFX55 showed partial epithelialisation, discontinuous epidermis formation [Figure 5(e)].

Figure 5 Histological examination of wound closure in rats after 15th day of study, (a) uninjured skin (b) control (open wound without dressing) (c) SFX82 (d) SFX64 (e) SFX55 H&E 40X scale bar = 100 µm (see online version for colours)

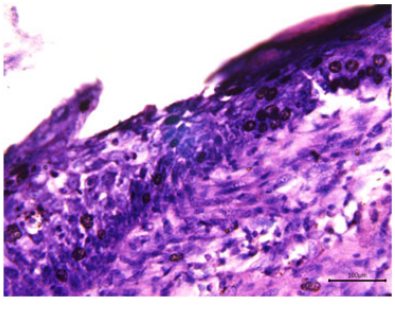


(a) (b)

Figure 5 Histological examination of wound closure in rats after 15th day of study, (a) uninjured skin (b) control (open wound without dressing) (c) SFX82 (d) SFX64 (e) SFX55 H&E 40X scale bar = 100 µm (continued) (see online version for colours)



(c) (d)



(e)

4 Discussions

4.1 Water vapour transmission rate

Control of water loss/evaporation from wound bed is essential because dehydration of wound bed may result in back pressure, and maceration of healthy surrounding tissues (Elsner et al., 2012). In addition, an ideal dressing should be permeable enough to allow gaseous exchange between surrounding environment and wound to prevent excessive build up of wound exudates. Acccording to literature reports, a healthy skin looses moistures in the range 240 g/m^2 to 1,920 g/m^2 × 24 h whereas wounded skin can lose moisture as high as 4,800 g/m^2 × 24 h (Gu et al., 2013). This suggested that WVP of SF/Xa dressing should be within above mentioned specified range. All the SF/Xa dressings had higher WVTR compared to healthy skin throughout indicating the suitability of dressings for wound healing process. This result was similar to observation made by Wibbenmeyer et al. (2012), who suggested that WVP of dressings should be higher than a normal skin to prevent tissue death and infection. However, an intermediate WVTR is desirable for wound healing purpose which led to selection of SFX64 dressing with intermediate permeability as an ideal biopolymeric hybrid biomaterial for wound dressings. SFX64 dressing when compared to WVTR of commercial dressings showed 4.6 fold higher WVTR than commercial Mediafoam® N dressings (WVTR = 811 g/m^2

Day-1) and 1.7 fold reduction than Biofilm® dressings (WVTR 6,595/m^2 Day-1) (Lee et al., 2016; Wu et al., 1995). Therefore, it was concluded that SF/Xa biopolymeric water vapour transmission properties is comparable to ideal wound dressings and this biopolymeric material can be used for wound healing applications.

4.2 Bacterial adherence determination

The main objective of dressing is to protect the wounds from opportunistic microorganisms to control infections. However, dressing also provides an additional surface area for bacterial cell attachment and biofilm formation within wounds which is an undesirable conditon. Further, contamination of dressing necessitates their frequent removal causing discomfort to patients and delaying the healing process. Therefore, an ideal dressing should inhibit adhesion of bacterial population on their surfaces.

Surface topography and surface wettability are two important parameters affecting bacterial adhesion to material surface (Liu et al., 2016). Smoother and hydrophobic surfaces tend to have low surface area for attachment of bacteria to its surface. Table 2 suggests that SFX64 had lowest bacterial attachment for both the gram negative (*E. coli*) and gram positive (*S. aureus*) bacteria among all the SF/Xa scaffold. This suggested that SFX64 had smoother surface and comparatively hydrophobic surfaces. The smoother surface of SFX64 reduced the contact area between the bacterium and solid support leading to reduction in bacterial attachment. This results follows the Wenzel wetting model which states that that lowering of surface roughness will diminish the wettability caused by chemistry of surface (Attension, 2015) and makes the surface hydrophobic. Therefore, it is evident that just by modifying the surface topography and surface wettability, the adhesion of bacteria to dressing surface can be controlled effectively.

4.3 In vitro drug release study

Drug eluting wound dressings are used to deliver antibiotics locally at wound site to remove pathogenic bacteria and accelerate the natural healing process. Further, it can be used to prevent colonisation of bacteria and infection at injured site (Gámez-Herrera et al., 2020). Figure 4 suggested that all the SF/Xa dressings were able to release incorporated antibiotic amoxicillin for prolonged period. However, SFX64 and SFXX55 dressings showed biphasic release behaviour consisting of:

1 an instantaneous burst release

2 controlled release.

The burst release observed in case of SFX64 and SFXX55 dressings was attributed to dissolution of most accessible drugs from the surface or larger pores of dressings in the release medium. SFX64 dressing achieved a highest burst release of antibiotic among all dressings. Due to burst release, SFX64 dressing was instantaneously able to release 14 ± 1.3% antibiotics *in vitro*. The instantaneous release of antibiotics in such high concentration from SFX64 dressing can prove to be beneficial for faster relief, sanitising the wound environment to prevent first wave of bacterial colonisation, and provide with a localised targeted effect at wounded area. After one hour, the release rate of antibiotics from SFX64 and SFX55 dressing reduced drastically with observed increment of 6% and 9%, respectively. This reduction in release rate indicated towards the onset of controlled release of antibiotics from SF/Xa dressings. The controlled release of antibiotics from all

three SFX82, SFX64 and SFX55 dressings was attributed to slow dissolution of drugs from interior of dressings due to hindered water penetration. The excessive swelling of xanthan creates such effect by blocking pores of dressings.

It is evident that SFX64 maintained a steady concentration of amoxicillin at controlled rate for prolonged period of 48 hours compared to SFX55 and SFX82 dressings (Figure 4) at sufficiently high concentration and achieved an overall cumulative release of 78.7 ± 3.6%. Still SFX64 dressing had 21.3 % drug left to be released. The controlled release for longer duration is desirable in case of drug eluting wound dressing to maintain sanctity of wound, control bacterial growth and eliminate infections (Karahaliloglu et al., 2017). On the basis of these result it was concluded that dressings made of SF/Xa blends especially, SFX64 was able to release incorporated drug at controlled rate for prolonged period in sufficiently high concentration. These properties of SFX64dressings are beneficial for effective wound treatment at faster rate.

4.4 Release kinetics

The kinetics of amoxicillin release from SF/Xa dressings were elucidated by curve fitting the *in vitro* release data to various drug release model listed in Table 4. The goodness of fit of the experimental data to theoretical models were compared using regression coefficient (R^2) values. On the basis of R^2 values listed in Table 4, it was inferred that, after initial burst release, the release of amoxicillin from dressings proceeded at controlled rate following a Korsemeyer-Peppas model as R^2 values of all SF/Xa dressing showed good fit to this model. Due to biopolymeric nature of fabricated dressing, the better fitting of release data to Korsemeyer-Peppas model was expected because this model best describes the release from polymeric system.

The value of release exponent ‘n’ in Korsemeyer-Peppas model can be used to deduce mechanism of drug release from fabricated SF/Xa dressings. $n \leq 0.45$ shows Fickian diffusion and values between $0.45 < n \leq 0.89$ corresponds to non-Fickian diffusion (Muhsin et al., 2016). The values of release exponent (n) for SFX82, SFX64 and SFX55 dressings were found to be 0.56, 0.44 and 0.46, respectively (Table 4). This suggests that the release of amoxicillin from SFX82 and SFX55 followed non-fickian diffusion mechanism whereas release of amoxicillin from SFX64 was Fickian-diffusion mechanism. The non-Fickian diffusion mechanism was due to combination of swelling controlled and diffusion controlled mechanism. On the other hand, amoxicillin releases from SFX64 followed fickian diffusion due to controlled swelling of SFX64 dressings and hence were able to prolong the release of amoxicillin at sufficiently high concentrations.

4.5 Bacterial inhibition percentage

The bacterial inhibition study was performed to determine the efficacy of released concentration of amoxicillin in controlling the bacterial growth *in vitro.* From Figure 3, it was evident that, the release of amoxicillin from SFX64 among all SF/Xa dressings was sufficiently higher to effect maximum inhibition of growth of gram positive *S. aureus* bacteria. This suggested that, SFX64 dressings if used in wound treatment will be able to control bacterial infection at any given point of time. Further, looking at effective change in inhibition percentage of SFX64 dressings, it was found that effective change during 0 to 8 hours was 66.7 % and during 8 to 48 hours was 16%. Similarly, during the same time

period, the effective inhibition percentage for SFX82 dressings was 64.4 % and 5%, respectively and for SFX55 it was 66.2 % and 8 % only. This suggested that all dressing achieved controlled release after initial burst release, leading to steady reduction in bacterial growth with time, but SFX64 was best among all dressing. Such functionality of drug eluting dressing is desirable because infection is not time bound and can take at any time during course of healing. Since, majority of bacterial population in infected wounds are gram positive, the amoxicillin loaded SF/Xa dressings can be effective in treating wound.

4.6 In vivo wound healing assessment

The antibiotic functionalised SF/Xa wound dressing increased wound healing rate reepithelialisation, dermis proliferation when compared to open wound (Control). The wounds dressed with SFX82, SFX64 and SFX55 showed more or less same healing pattern with significantly faster wound closure rate than the open wound (control group). Within the individual dressed category, SFX64 showed higher wound area reduction throughout; achieving overall wound closure of 99.12 ± 0.33 % till 15th day. During initial healing stage, control group showed higher wound healing than the dressed group [Figure 4(b)]. The higher wound recovery observed at day three in control was possibly due to healing by contraction which is a characteristic of murine model (Dunn et al., 2013). In contrast, the healing in dressed group was due to re-epithelialisation and remodeling of new tissues. The efficacy of antibiotic eluting wound dressing was ascertained by the fact that all the SF/Xa dressing had significantly higher overall wound recovery (98.875 ± 0.62%, 99.12 ± 0.33% and 97.94 ± 0.25% for SFX82, SFX64 and SFX55, respectively) as compared to control (97.87 ± 0.48%) between day 3 and day 15 ($P < 0.05$, Table 3). It was observed that the difference between wound size reduction in control group and that of dressed group was small but were statistically significant. This type of statistical situation arises due to a very large sample size (12 rats per group) and high statistical power (95 %) used in this study. High statistical power detects even small changes in wound area reduction as significant (web references, Rao and Richard, 2012) and observed statistical significance of outcome may be explained on the basis of this criterion.

Table 3 Day wise *P*-value of wound recovery with respect to open wound (control group) ($P<0.05$)

	P-value			*Significance level*
Days	*SFX82*	*SFX64*	*SFX55*	
Day 3	0.6	0.1	0.8	Not significant
Day 9	2.7×10^{-6}	9.05×10^{-5}	5.21×10^{-4}	Significant
Day 15	7.10×10^{-5}	1.0×10^{-8}	4.5×10^{-9}	Significant

Among all the SF/Xa dressing, SFX64 dressing showed better performance with maximum healing due to its ability to maintain sufficiently high concentration of antibiotic at controlled rate for longer duration at wound site and synergistic healing potential extended by silk fibroin and xanthan in the blend dressing. The high antibiotic concentration helps in maintaining infection free environment whereas silk fibroin and xanthan promoted the natural production of growth factors by healthy cells *in vivo* in the

wound site for faster healing. Apart from these, physical design criterion of biopolymeric material based Wound dressing is extremely important to establish and maintain an optimal wound healing environment. For example, a wound dressing should be porous enough to allow exchange of air from wound bed for oxygen exchange. Oxygen is vital for fibroblast proliferation, collagen synthesis, and polymorphonuclear cell functions (Gil et al., 2013). The WVTR studied above indicated that SF/Xa dressings have been designed suitably to allow exchange of air to and fro from wound bed. Therefore, on the basis of these results it was concluded that SFX64 dressing properties was extremely suitable for potential application in wound healing

4.7 Histological assessment of wound

The main purpose of wound dressing is to felicitate reconstruction, revive structural and functional properties of skin. The synergistic effect of SF/Xa dressings provided structural support for cell attachment, migration of fibroblasts, macrophages, endothelial cells, granulocyte cells and collagen responsible for healing. Therefore, HE staining was performed at day 15 to histologically evaluate the effectiveness of drug eluting SF/Xa dressings with open wounds without dressing and uninjured skin. As expected, the uninjured area had intact epidermis and dermis without any white spaces indicative of abundant and densely packed collagen deposition without any damage [Figure 5(a)]. However, in control group lots of white space coud be seen under epithelium which indicated immature granulation, low collagen deposition and loose collagen fibre packing and had visibly incomplete epidermis formation [Figure 5(b)]. SFX82 and SFX55 dressed group showed incomplete healing with irregular epidermis formation and breakage. Further, presence of white spaces in histology image of these two group suggested loose collagen fibre packing and porous structure of healed skin [Figure 5(c) and Figure 5(e)]. SFX64 dressed group in comparison to SFX82 and SFX55 dressed group showed better re-epithelialisation and new full thickness healed skin with intact epidermis and dermis layer. In addition, better granulation, fibrosis and numerous capillaries and sebaceous glands along with densely packed collagen fibre were clearly visible [Figure 5(d)]. SFX64 dressed group showed accelerated healing evident by complete and thicker formation of both epidermis and dermis layer without any discontinuity. However, healing in SFX82 and SFX55 dressed group was still taking place as evident by thinner and irregular epidermis formation, suggesting delayed healing. On comparison of histological image of uninjured skin and SFX64 dressed group, it was clear that the structural integrity of SFX64 dressed group was similar to structural integrity of uninjured skin. The wound recovery observed in case of SFX64 dressed group was comparable to silver nanoparticle loaded silk fibroin/chitosan (SF/CS) wound dressings (Liu et al., 2017). These data suggest that SFX64 dressing had significantly improved the wound healing in rats and can be ideal candidate for wound healing treatment.

5 Conclusions

The silk fibroin/xanthan (SF/Xa) dressings have several unique properties best suited for faster wound healing. SFX64 dressing was best among the rest of blend dressings due to its low bacterial adherence, intermediate WVTR, porous structure, ability to deliver incorporated antibiotics at controlled rate for longer duration and accelerated wound

healing. The use of bicomponent silk fibroin/xanthan blend biomaterial with incorporated antibiotics in dressing had synergistic effect on healing of wounds. This type of system can closely mimic the *in vivo* healing environment which induces secretion of growth factors, induces migration of surrounding healthy cells at wound site for faster recovery. This study showed that a drug eluting dressing made of SF/Xa biomaterial performed dual function of delivering drug to wound site and protecting the wound from dust and cross contamination from opportunistic microbes simultaneously. Therefore, SF/Xa biomaterial is a suitable candidate for fabrication of multifunctional dressings for effective treatment of wounds.

References

Aragón-Sánchez, J., Quintana-Marrero, Y., Aragón-Hernández, C. and Hernández-Herero, M.J. (2017) 'ImageJ: a free, easy, and reliable method to measure leg ulcers using digital pictures', *Int. J. Low Extrem. Wounds*, Vol. 16, No. 4, pp.269–273.

Attension (2015) 'Influence of surface roughness on contact angle and wettability', *Theory Note*, Vol. 7, pp.1–3.

Dai, T., Tanaka, M., Huang, Y.Y. and Hamblin, M.R. (2011) 'Chitosan preparations for wounds and burns: antimicrobial and wound-healing effects', *Expert Rev. Anti Infect. Ther*., Vol. 9, No. 7, pp.857–879.

Dickinson, L.E. and Gerecht, S. (2016) 'Engineered biopolymeric scaffolds for chronic wound healing', *Frontiers in Physiology*, Vol. 7, p.341.

Dunn, L., Prosser, H.C.G., Tan, J.T.M., Vanags, L.Z., Ng, M.K.C. and Bursill, C.A. (2013) 'Murine model of wound healing', *J. Vis. Exp.*, Vol. 75, No. e50265, pp.1–6.

Elsner, J.J, Kraitzer, A., Grinberg, O. and Zilberman, M. (2012) 'Highly porous drug-eluting structures: from wound dressings to stents and scaffolds for tissue regeneration', *Biomatter*, Vol. 2, No. 4, pp.239–270.

Farokhi, M., Mottaghitalab, F., Fatahi, Y., Khademhosseini, A. and Kaplan, D.L. (2018) 'Overview of silk fibroin use in wound dressings', *Trends Biotechnol*., Vol. 36, No. 9, pp.907–922.

Fleck, C.A. and Simman, R. (2010) 'Modern collagen wound dressings: function and purpose', *J. Am. Col. Certif. Wound Spec.*, Vol. 2, No. 3, pp.50–54.

Gámez-Herrera, E., García-Salinas, S., Salido, S., Sancho-Albero, M., Andreu, V., Pérez, M., Lujan, L., Irusta, S., Arreubo, M. and Mendoza, G. (2020) 'Drug-eluting wound dressings having sustained release of antimicrobial compounds', *Eur. J. Pharm. Biopharm*, Vol. 152, pp.327–39.

Gil, E.S., Panilaitis, B., Bellas, E., Kaplan, D.L. (2013) 'Functionalized silk biomaterials for wound healing', *Adv. Healthc. Mater.*, Vol. 2, No. 1, pp.206–217.

Gu, Z., Xie, H., Huang, C., Li, L. and Yu, X. (2013) 'Preparation of chitosan/silk fibroin blending membrane fixed with alginate dialdehyde for wound dressing', *Int. J. Biol. Macromol.*, Vol. 58, pp.121–126.

Huang, J., Ren, J., Chen, G., Li, Z., Liu, Y., Wang, G. and Wu, X. (2018) 'Tunable sequential drug delivery system based on chitosan/hyaluronic acid hydrogels and PLGA microspheres for management of non-healing infected wounds', *Mater. Sci. Eng. C.*, Vol. 89, pp.213–222.

Karahaliloglu, Z., Kilicay, E. and Denkbas, E.B. (2017) 'Antibacterial chitosan/silk sericin 3D porous scaffolds as a wound dressing material', *Artif. Cells, Nanomedicine Biotechnol*., Vol. 45, No. 6, pp.1172–1185.

Konop, M., Czuwara, J., Klodzinska, E., Lakowska, A.K., Suljeczak, D., Damps, T., Zielenkiewicz, U., Brzozowska, I., Sureda, A., Kowalkowski, T., Schhwartz, R.A. and Rudnicka, L. (2020) 'Evaluation of keratin biomaterial containing silver nanoparticles as a potential wound dressing in full-thickness skin wound model in diabetic mice', *J. Tissue Eng. Regen. Med.*, Vol. 4, No. 2, pp.334–346.

Lee, S.M., Park, I.K., Kim, Y.S., Kim, H.J., Moon, H. and Mueller, S. (2016) 'Physical, morphological, and wound healing properties of a polyurethane foam-film dressing', *Biomater. Res*., Vol. 20, No. 15, pp.1–11.

Lei, J., Sun, L., Li, P., Zhu, C. and Lin, Z. (2019) 'the wound dressings and their applications in wound healing and management', *Heal Sci. J*., Vol. 13, No. 4, pp.1–8.

Li, X., Qin, J. and Ma, J. (2015) 'Silk fibroin/poly (vinyl alcohol) blend scaffolds for controlled delivery of curcumin', *Regen. Biomater.*, Vol. 2, No. 2, pp.97–105.

Liu, H., Wang, C., Li, C., Qin, Y., Wang, Z., Yang, F., Li, Z. and Wang, J. (2018) 'A functional chitosan-based hydrogel as a wound dressing and drug delivery system in the treatment of wound healing', *RSC Adv*., Vol. 8, No. 14, pp.7533–7549.

Liu, J., Qian, Z., Shi, Q., Yang, S., Wang, Q., Liu, B., Xu, J., Guo, X. and Liu, H. (2017) 'An asymmetric wettable chitosan-silk fibroin blends dressing with fixed silver nanoparticles for infected wound repair: in vitro and in vivo', *RSC Adv*., Vol. 7, pp.43909–43920.

Liu, L., Ercan, B., Sun, L., Ziemer, K.S. and Webster, T.J. (2016) 'Understanding the role of polymer surface nanoscale topography on inhibiting bacteria adhesion and growth', *Biomater. Sci. Eng.*, Vol. 2, No. 1, pp.122–130.

Luangbudnark, W., Viyoch, J., Laupattarakasem, W., Surakunprapha, P. and Laupattarakasem, P. (2012) 'Properties and biocompatibility of chitosan and silk fibroin blend films for application in skin tissue engineering', *Sci. World J.*, Vol. 2012, No. 697201, pp.1–10.

Mogoşanu, G.D. and Grumezescu, A.M. (2014) 'Natural and synthetic polymers for wounds and burns dressing', *Int. J. Pharm.*, Vol. 463, No. 2, pp.127–136.

Moraes, M.A. and Beppu, M.M. (2013) 'Bioblends membranes of sodium alginate and silk fibroin fibers for biomedical applications', *J. Appl. Polym. Sci.*, Vol. 130, No. 5, pp.3451–3457.

Muhsin, M.D., George, G., Beagley, K., Ferro, V., Wang, H. and Islam, N. (2016) 'Effects of chemical conjugation of L-leucine to chitosan on dis-persibility and controlled release of drug from a nanoparticulate dry powder inhaler formulation', *Mol. Pharm.*, Vol. 13, No. 5, pp.1455–1466.

Murray, R.Z., West, Z.E., Cowin, A.J. and Farrugia, B.L. (2019) 'Development and use of biomaterials as wound healing therapies', *Burn Trauma*, Vol. 7, p.2.

Qian, Z., Bai, Y., Zhou, J., Li, L., Na, J., Fan, Y., Guo, X. and Li, H. (2020) 'A moisturizing chitosan-silk fibroin dressing with silver nanoparticles-adsorbed exosomes for repairing infected wounds', *J. Mater. Chem. B.*, Vol. 8, No. 32, pp.7197–7212.

Rao, P.S.S.S. and Ricchard, J. (2012) *Introduction to Biostatistics and Research Methods*, 5th ed., Prentice Hall, India.

Roh, D.H., Kang, S.Y., Kim, J.Y., Kwon, B.Y., Kweon, H.Y., Lee, K.G, Park, Y.H., Baek, R.M., Heo, C.Y., Choe, J. and Lee, J.H. (2016) 'Wound healing effect of silk fibroin/alginate-blended sponge in full thickness skin defect of rat', *J. Mater. Sci. Mater. Med.*, Vol. 17, No. 6, pp.547–552.

Shera, S.S., Sahu, S. and Banik, R.M. (2018) 'Preparation of drug eluting natural blends scaffold using response surface methodology and artificial neural network approach', *Tissue Eng. Regen. Med.*, Vol. 15, No. 2, pp.131–143.

Shetty, R., Sreekar, H., Lamba, S. and Gupta, A.K. (2012) 'A novel and accurate technique of photographic wound measurement', *Indian J. Plast. Surg.*, Vol. 45, No. 2, pp.425–429.

Shi, C., Wang, C., Liu, H., Li, Q., Li, R., Zhang, Y., Liu, Y., Shao, Y. and Wang, J. (2020) 'Selection of appropriate wound dressing for various wounds', *Front Bioeng. Biotechnol*., Vol. 8, p.182.

Sugihara, A., Sugiura, K., Morita, H., Ninagawa, T., Tubouchi, K., Tobe, R., Izumiya, M., Horio, T., Abraham, N.G. and Ikehara, S. (2000) 'Promotive effects of a silk film on epidermal recovery from full-thickness skin wounds', *Proceedings of the Society for Experimental Biology and Medicine*, Vol. 225, No. 1, pp.58–64.

Vasconcelos, A., Gomes, A.C. and Cavaco-Paulo, A. (2012) 'Novel silk fibroin/elastin wound dressings', *Acta Biomaterialia*, Vol. 8, No. 8, pp.3049–3060.

Wibbenmeyer, L., Williams, I., Liao, J., Heard, J., Kealey, G.P. and Miller, R. (2012) 'A pilot study of the use of biocide-impregnated gauze as an adjunct to wound care in a burn population', *J. Burn Care Res.*, Vol. 33, No. 3, pp.358–363.

Wu, P., Fisher, A.C., Foo, P.P., Queen, D. and Gaylor, J.D.S. (1995) 'In vitro assessment of water vapour transmission of synthetic wound dressings', *Biomaterials*, Vol. 16, No. 3, pp.171–175.

Zhang, Q., Chen, S., You, R., Tariq, Z., Huang, J., Li, M. and Yan S. (2017a) 'Silk fibroin/hyaluronic acid porous scaffold for dermal wound healing', *Fibers Polym*., Vol. 18, pp.1056–1063.

Zhang, W., Chen, L., Chen, J., Wang, L., Gui, X., Ran, J., Xu, G., Zhao, H., Zeng, M., Ji, J., Qian, L., Zhou, J, Ouyang, H.W. and Zou, X. (2017b) 'Silk fibroin biomaterial shows safe and effective wound healing in animal models and a randomized controlled clinical trial', *H. Adv. Healthcare Mater*., Vol. 6, p.1700121.

Websites

https://statisticsbyjim.com/hypothesis-testing/sample-size-power-analysis/ (accessed 30 August 2020).

https://web.csulb.edu/~msaintg/ppa696/696stsig.htm (accessed 30 August 2020).

https://www.statisticsdonewrong.com/significant-differences.html (accessed 30 August 2020).