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Development and evaluation of a novel beneficent antimicrobial bioscaffold based on animal waste-fish swim bladder (FSB) doped with silver nanoparticles



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ABSTRACT

Treated fish wastes have found many applications in industry and medicine. Besides, nowadays low-cost scaffold with antimicrobial activity which can accelerates the process of wound healing is very demanding. In this study fish swim bladder (FSB), taken from *Rutilus frisii*, which is a disposable waste was doped with silver nanoparticles (AgNPs) and evaluated as antimicrobial wound dressing. The scanning electron microscopy (SEM) micrographs showed the presence of AgNPs on the scaffold. Histological observation confirmed cells and muscle removal from FSB and collagen preservation. There was significant antibacterial activity even in 50 ppm AgNPs concentration against pathogenic bacteria, swelling ratio was rather low, and cytotoxic assay revealed that the AgNPs-FSB scaffold had no toxic effect on human foreskin fibroblast (HFF) cells. Interestingly, despite the porous structure, the AgNPs-FSB scaffold was found to be a suitable barrier to microbial penetration even after 72 h. Further study showed the gradual release of AgNPs during 24 h. In conclusion, biofabricated FSB prepared in this study have appropriate characteristics notably encompassing a high quantity of collagen and broad-spectrum antimicrobial activity. Also, its porous structure made it suitable as a 3-D structure for the growth of cells and adding other antimicrobial nano-sized materials.

1. Introduction

In the last decade, more research has been conducted to find a better way of utilizing the waste product generated from food processing industries. The increasing of industrial byproducts is one of the main reasons for the conversion of these wastes into valuable products. Among the different valuable products from the waste, animal disposable organs could be utilizable wastes, due to their effective applications in biomedical and pharmaceutical industries. One of the most abundant sources is fish organs and its components. Many studies have dealt with the extraction and functional properties of collagen from fish wastes (Jeevithan et al., 2013; Li et al., 2018). These researches have gained enormous interests in the recent past due to the growing need for novel health substances with the least side-effects. Also, fish byproducts are viewed as significant for their noteworthy potentiality as therapeutic compounds.

The swim bladder, gas bladder, fish maw or air bladder is an internal organ that contributes to the ability of a fish to control its floating in water (Finney et al., 2006). This tissue is always considered as garbage in the fishery, but has various applications. For example, biopiezoelectric nanogenerator (BPNG) is fabricated from FSB in which natural collagen of this tissue act as nanofibrils. This nanogenerator is used as a sensor in the biomedical application (Ghosh and Mandal, 2016). FSB is not only biocompatible and biodegradable, but also has a high content of collagen compared to other animal tissues studied so far (Hafidh et al., 2009; Naderi et al., 2013; Shahabipour et al., 2013). Collagen is the main component of the extracellular matrix (ECM) (Pati et al., 2010), this protein gives the skin its tensile strength and plays an

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important role in the process of wound healing. Strikingly, collagen attracts cells, such as fibroblasts and keratinocytes to the wound, which encourages debridement, angiogenesis, and re-epithelialization. In addition, collagen provides a natural scaffold or substrate for new tissue growth (Miranda-Nieves and Chaikof, 2016).

The presence of bacteria delay the wound healing process (Fan et al., 2014). Therefore, preparing scaffolds with suitable antimicrobial activity has been one of the most important concerns in recent research studies. Silver is known as an antimicrobial agent for centuries and has been widely used in the treatment of clinical diseases, including newborn eye prophylaxis, topical burn wounds, and orthopedic infections (Klasen, 2000). Silver is a broad-spectrum antimicrobial agent that inhibits yeast, mold, and bacteria, including methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin-resistant enterococci (VRE), whenever provided at an appropriate concentration (Rai et al., 2014). Silver in nano size has larger effective surface comparing to its bulk and therefore has more antimicrobial activity than its bulk shape (Rai et al., 2009). The bacterial cellulose, a biological structure somehow similar to FSB, impregnated in AgNPs was used as an antimicrobial scaffold (Maneerung et al., 2008). Also, a scaffold made of chitin with AgNPs added antimicrobial activity to the dressing (Madhumathi et al., 2010). In this study, a new bio-scaffold fabricated from the extracellular matrix of fish swim bladder impregnated into colloidal AgNPs to achieve antimicrobial activity for further applications in wound healing and tissue engineering industries.

2. Experiment

2.1. Bioscaffold preparation

After removing the outer layer of FSB and cutting it into smaller pieces, a combination of physical and chemical decellularization methods, including snap freeze-thawing in liquid nitrogen, and treatment with sodium dodecyl sulfate (SDS) were performed to lyse the cell membrane and cellular components for preventing immune response of the body (Crapo et al., 2011). After washing FSB tissue with normal saline, the snap freeze-thawing method was applied using two different protocols. First FSB tissue was soaked in liquid nitrogen for 3 min, and then soaked 5 min in distilled water for rapid thawing. In the second procedure, FSB tissue was soaked 5 min in liquid nitrogen and also 5 min in distilled water. Both experiments were carried out in triplicates (Jackson et al., 1988). In the next step, FSB tissue was washed in phosphate-buffered saline (PBS) at 37 °C and treated with 0.5 and 1% sodium dodecyl sulfate (SDS) for 24 h in a shaker incubator (Crapo et al., 2011). Finally, the FSB scaffold was prepared by soaking the treated tissue in PBS solution followed by 0.3% acetic acid addition.

2.2. Histological staining

To prepare the tissue for microscopic examination the following steps were performed: (i) fixation of FSB tissue specimen, before and after decellularization, with Bouin's solution for 24 h to prevent the autolysis and tissue decomposition with bacteria, (ii) dehydration of the fixed specimen with ethylic alcohol to facilitate paraffin penetration, (iii) clarifying in n-butanol, (iv) embedding in paraffin blocks, and (v) sectioning with microtome (Letz, Australia). Finally, the tissue sections were put on the surface of gelatinated lam and was prepared for histological staining (Mohammadie et al., 2017).

The tissue section staining, with picro-fuchsin, was performed before and after the decellularization to investigate the effect of decellularization on collagen and muscle loss. Hematoxylin and Eosin (H&E) staining was used for nucleus detection in the tissue sections (Lillie et al., 1976), while the collagen and elastin were treated by orceinpicroindigocarmine staining (Kiernan, 2001). The stained FSB tissue sections were observed with an optical microscope (Olympus, Germany).

2.3. AgNPs-FSB scaffold

Dispersed colloidal AgNPs with the size of 100-200 nm were prepared by sonicating AgNPs solution in a bath sonicator (GM2070, Bandelin Sonopuls, Germany) for 10 min at 25 °C. Then the treated FSB scaffold was soaked in the colloidal AgNPs in a shaking incubator at 36 °C overnight (Wei et al., 2011). The prepared bioscaffold containing AgNPs was morphologically characterized using SEM. Also to find out the amount of colloidal AgNPs loaded in the prepared FSB scaffold, AgNPs concentration was first determined by UV-visible spectrophotometer S2100 (Unico, Germany) at the absorbance wavelength of 400 nm. Subsequently, one piece of the FSB scaffold was weighed up and immersed in 25 mL of colloidal AgNPs overnight under static condition. Then the scaffold piece was placed in a filter paper for dewatering. The amount of colloidal AgNPs absorbed into the prepared FSB scaffold was determined by subtracting AgNPs concentration of the colloid before and after FSB soaking. Six pieces of FSB were used for estimating the loading capacity of each AgNPs concentration. The loading capacity of AgNPs was calculated as the amount of the absorbed colloidal AgNPs per square centimeter of FSB (mg/cm²) (Wei et al., 2011).

2.4. Swelling studies of FSB scaffold

The dry weight of the scaffolds were noted as Wi. The scaffolds were first immersed in deionized water overnight. Then, the scaffolds were taken, blotted with a filter paper to remove the water adsorbed and the wet weight was recorded as Ws,s. The ratio of swelling was determined using the following formula: swelling ratio = (Ws,s -Wi)/Wi (Zhang et al., 2015).

2.5. In vitro release of AgNPs from FSB scaffold

FSB tissue sections were placed in Erlenmeyer flasks containing 25 ml PBS solution and kept in shaking incubator with a shaking speed of 60 rpm at 37 °C for 24 h. Then, changes in the absorbance of PBS solution were monitored spectrophotometrically at 400 nm wavelength in every 2 h to determine the rate of AgNPs release from the FSB scaffold (Cheng et al., 2015). Experiments were carried out in triplicate and a sample (without AgNPs) was used as a control.

2.6. Antibacterial activity

The antibacterial properties of FSB scaffold containing AgNPs were investigated against Gram-negative bacteria (*Esherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*) and Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*) using disk diffusion, bacterial growth inhibition, test tube dilution methods and microbial barrier property (MBP).

Disc diffusion method was performed in tryptic soy agar (TSA) Petri dish. The FSB tissue section was immersed in AgNPs colloid at various concentrations (30, 50, 70 and 100 ppm). The AgNPs laden FSB scaffold sections were dried using filter paper at room temperature and sterilized by UV light irradiation for 20 min. The bacterial suspension (100 μ l of 10⁴–10⁵ CFU ml⁻¹) was applied on the surface of the TSA agar plate uniformly before placing bioscaffold disks on plates incubated at 37 °C for 24 h. The antibacterial activity of bioscaffold samples was determined by measuring the average diameter of the inhibition zone for each AgNPs concentration and each microbial strain, which based on three replicates (Unnithan et al., 2014).

In bacterial growth inhibition assay, the antibacterial activity of AgNPs-FSB scaffold was determined using culture turbidity as a qualitative measure of cell growth. To examine the bacterial growth rate or the bacterial growth behavior in the presence of AgNPs-FSB scaffolds, a single colony of *E. coli, S. aureus, P. aeruginosa, S. typhimurium,* and *B. subtilis* grown on agar culture medium, was transferred into 100 ml of



Fig. 1. Fish swim bladder (FSB). Before decellularization (A), after decellularization (B), decellularized FSB After impregnation in AgNPs (C).

liquid seed medium. After 12 h of agitated cultivation at 37 °C, 5 ml of cell suspension from the seed culture was introduced into a 250 ml. Erlenmeyer flask containing 100 ml of tryptic soy broth (TSB) and then a piece of AgNPs-FSB scaffold was put in the Erlenmeyer flask. The culture was kept at 37 °C for 24 h under agitation. The samples were drawn every 2 h and analyzed by UV-visible spectrophotometer S2100 (Unico, Germany) at the absorbance wavelength of 600 nm. The experiments also included a positive control (flask containing AgNPs-FSB scaffold and nutrient media, devoid of bacterial inoculum) and negative control (flask containing bacterial inoculum and nutrient media, devoid of AgNPs-FSB scaffold). The negative controls indicated the bacterial growth profile in the absence of scaffold. The absorbance value for positive control was subtracted from the experimental values (flasks containing nutrient media, bacterial inoculum, and AgNPs-FSB scaffold). The percentage of bacterial growth inhibition (GI %) of antimicrobial bioscaffold was evaluated to compare with its positive control using the following equation:

Percentage of growth inhibition (GI%) = $100 - (OD_{630}$ at the presence of antibacterial agent/OD₆₃₀ at the absence of antibacterial agent × 100) (Hosseini et al., 2016).

The minimum inhibitory concentration (MIC) of AgNPs-FSB at different AgNPs concentrations (30, 50, 100, and 150 μ g ml⁻¹) were determined using the test tube dilution method, in which each tube inoculated with the same amount of testing bacteria at 2.5 × 10 ⁵ CFU/ml concentration. Afterward, test tubes were incubated for 24 h. The concentration in which AgNPs-FSB inhibits 99% of bacterial growth, compared with positive control, was determined based on turbidity.

The microbial barrier property (MBP) of studied bioscaffold was also investigated. First, four 10 ml tubes containing 5 ml nutrient broth were prepared, kept at room temperature and checked for possible contamination for 24, 48 and 72 h. The positive control was an open-up tube without any cover, the negative control was a tube sealed with a sterilized cork, two other tubes sealed with sterilized FSB scaffold and top-covered with sterilized scaffold impregnated in AgNPs. The turbidity of each tube was monitored in time intervals and the ability of bioscaffold to hinder microbial penetration was found as the result of no turbidity after 72 h comparing the positive control tube (Augustine et al., 2015).

2.7. Cytotoxicity of FSB scaffold

The cytotoxicity of the different tested FSB scaffolds and AgNPs on human foreskin fibroblast (HFF) cells was determined by MTT (3-(4, 5-Dimethylthiazol-2-Yl)-2, 5- Diphenyltetrazolium Bromide) assay for 24, 48 and 72 h after the treatment. HFF cells at a density of 3.2×10^3 cells per well were seeded in 96-well plates and incubated at 37 °C under 5% CO₂ for 24 h. Then, the cells were treated with AgNPs (50 ppm) or 5 mm wide ringlets of various tested scaffolds which were placed into the center of each well. The tested FSB scaffolds included six groups: FSB normal tissue denoted as (Tissue), FSB normal tissue impregnated in AgNPs (Tissue + N), decellularized FSB tissue with freeze-thawing (TN₂), FSB tissue section decellularized with freeze-thawing and 0.5% SDS (TN₂₊ SDS), FSB tissue decellularized with 0.5% SDS and freezethawing impregnated in Ag NPs (TN₂₊ SDS + N). The incubated cells with the decellularized FSB tissue with freeze-thawing impregnated in the AgNPs (TN₂₊ N) without SDS supplement and the cells without exposure to FSB tissue were taken as controls. All plates were incubated in a CO₂ incubator at 37 °C for 24, 48 and 72 h. The 20 μ l of MTT reagent (0.5 mg/ml) was added to each plate and the plates were incubated for 3 h in a CO₂ incubator. Then, 150 μ l of DMSO (dimethyl sulfoxide) was added to dissolve the formazan crystals and the absorbance was measured at 570 nm using a microplate ELISA reader (Stat fax 2100, Awareness Technology, Inc., USA). The cell viability was calculated using the following equation:

% Cell viability = $[(A_T-A_B) / (A_C-A_B)] \times 100$

where A_T , A_C , and A_B are the absorbances of the cells incubated with the tested FSB scaffolds or AgNPs, the cells without exposure with FSB tissue (control), and blank respectively, at 570 nm.

Statistical analysis of the data was performed by one way ANOVA and Tukey test in SPSS for comparing the viability of HFF cells in the presence and absence of bioscaffolds. All experiments were accomplished in triplicates. The obtained data are presented in terms of 'mean \pm standard deviation' values. Differences were considered statistically significant at p < 0.05.

3. Results and discussion

3.1. Preparation and characterization of FBS scaffold

After separating the FSB from inside of the fish stomach the outer layer of this organ was removed (Fig. 1A), and then cut into small pieces and decellularized (Fig. 1B and C). Histological study showed that decellularized FSB tissue by 3 min freeze-thawing and soaking in liquid nitrogen in combination with chemical decellularization using 0.05% SDS retained the ECM component without any evidence of cellular and nuclear material (Fig. 2B). Also, our results confirmed the suitable maintenance of ECM collagen component in FSB tissue sections when samples stained with H&E and picro-fuchsine were compared before and after decellularization by 0.5% and 1% of SDS (Fig. 2).

Fig. 2 (G, H and I) showed, after decellularization, staining the paraffin tissue sections with "orcein-picroindigocarmine" made the color of elastin strands light brownish, muscles yellowish and the nucleus disappearance. In our study, the bioscaffold derived from FSB consisted of a large amount of collagen, elastin fibers and muscles. The role of collagen in promoting blood coagulation during the tissue repair process has been reported by Miyata et al., in which the collagen mediates the adhesion and aggregation of platelets leading to thrombus formation (Miyata et al., 1992). Collagen thin layer alone or in combination with other proteins such as glycosaminoglycan, fibrin, and biotin can be used as a based matrix for cultured cells which can be grafted directly onto wound bed to improve the properties of wound



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Fig. 2. FSB staining before and after decellularization with 0.5% and 1% SDS. H&E staining before decellularization (A): decellularization with 0.5% SDS (B); decellularization with 1% SDS (C); FSB tissue staining with picrofuchsin (D); FSB tissue staining with picro-fuchsin with 0.5% FSB (E); FSB staining with picro-fuchsin with 1% SDS (F); FSB tissue staining with picro indigo carmine-orcein, which orcein stain the elastin fibers with dark red color and picroindigocarmin stain the muscles with yellowish brown color (G); FSB tissue staining with orcein-picroindigocarmine after decellularization with 0.5% SDS (H); FSB tissue staining with orcein-picroindigocarmine with 1% SDS (I). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 3. SEM images of AgNPs (A); FSB tissue before decellularization (B); decellularized FSB tissue (C); decellularized FSB tissue after impregnation in AgNPs (D).

dressing (Jeevithan et al., 2013).

Also, the decellularization procedure which we used created more porous scaffold. This facilitates the absorption of extra materials such as metal nanoparticles for bioscaffold development and applications. For example, according to Zhou et al. studies porous hydroxyapatite scaffold could help the improvement of nanosilver doping (Zhou et al., 2015). In a study conducted by Shemsh et al., hematoxylin-eosin (H&E) stain was used for assessing the tissue morphology and the grade of elastin degradation of ligamentum flavum of patients with lumbar spinal canal stenosis (Shemesh et al., 2017). Their results showed the suitability of the staining method for elastin and other tissue component

WD = 11 mm

evaluation as if used for FSB tissue staining procedures.

SEM imaging analysis of FSB before decellularization and also treated FSB tissue with various concentrations of AgNPs showed the presence of polydispersed sphere AgNPs (100–200 nm) on the surface of the FSB scaffold (Fig. 3). Furthermore, energy dispersive spectroscopy (EDS) analysis confirmed the presence of silver element on the surface of the bioscaffold (Fig. 4A and B). AgNPs attached to FSB scaffold through tiny pores and its adsorption have probably been due to electrostatic interactions.

Electrostatic interactions are possibly implicated in the binding between collagen surface with zwitterionic functionality (due to





Fig. 4. EDS analysis of FSB tissue before (A) and after (B) impregnation with AgNPs. (C) AgNPs release profile from FSB scaffold at different times. Error bars are sample standard deviation of triplicate measurements.

presence of zwitterions amino acids) (Beghetto et al., 2013) and nanoparticles. The size, shape and concentration of NPs may affect this interaction (Dos Santos et al., 2014). Rath et al., indicated that nanosilver composite collagen nanofiber had more stability than bare collagen. They concluded that this stability can be related to probable ionic or electrostatic interaction between nanosilver and collagen (Rath et al., 2016). Therefore, electrostatic interaction of collagen-containing FSB with AgNPs can affect the structural stability, allowing the facile and gradual silver ion release.

According to the SEM micrographs, no bacteria were observable in AgNPs doped FSB scaffold samples and the collagen strands seemed as an interconnected network. This is similar to electrospun tubular scaffold fabricated using a blend material such as segmented polyurethane (SPU), styrenated (ST)-gelatin, type I collagen, as well as SPU and polyethylene oxide (PEO) (Hasan et al., 2014). The pores between these networks provide sufficient space for migration, proliferation, and connections between the cells (Fig. 3). There are several research works reported that artificial collagen scaffold are able to provide physical cues which encourage cellular attachment, migration and proliferation (Haugh et al., 2011; Campbell et al., 2017; Türker et al., 2019). Our developed FSB bioscaffold has porous structure almost similar to those already reported (Fig. 3). Therefore, we predict that our FSB scaffold

can provide sufficient space for the migration, proliferation, and also connections between the cells.

To evaluate the potential of AgNPs-FSB scaffold in absorption of exudate of the wound, swelling percentage was calculated. Our results indicated no significant ability in uptaking of wound exudates that might be due to the high density of collagen strands in the FSB tissue. The amount of released silver ions with respect to PBS is quantified by spectrophotometer and the results are shown in Fig. 4C. The PBS turbidity increased during 24 h incubation of AgNPs-FSB scaffold in PBS solution indicating the appropriate release of the AgNPs from bioscaffold leading to higher antimicrobial activity.

Gautam et al. (2014) fabricated a tri-polymer polycaprolactone (PCL)/gelatin/collagen type I composite nanofibrous scaffold by surface modification for skin tissue engineering. Their results, according to FE-SEM (field emission scanning electron microscopy) analysis, highlighted the importance of bioscaffold collagen content in fibroblast cell seeding and proliferation (Gautam et al., 2014).

The AgNPs-FSB scaffold prepared in our studies also contained a large amount of collagen which makes it appropriate for skin tissue engineering. In another study, Karri et al., (2016) prepared a nanohybrid scaffold by incorporating curcumin (CUR) in chitosan nanoparticles (CSNPs) followed by impregnation of the prepared CUR-CSNPs

Fig. 5. Disk diffusion assay. Average inhibition zone diameter (mm) of *Bacillus subtilis* (A), *Escherichia coli* (B), *Pseudomonas aeruginosa* (C), *Salmonella typhimurium* (D), and *Staphylococcus aureus* (E). Tetracycline was used as a control. Error bars are sample standard deviation of triplicate measurements.

into collagen scaffold for tissue regeneration applications. They found that the synergistic combination of CUR (anti-inflammatory and antioxidant), chitosan (sustain drug carrier, wound healing) and collagen (established wound healer as a scaffold) are promising strategies to address various pathological manifestations of diabetic wounds, having better wound healing capability (Karri et al., 2016).

AgNPs -FSB also can create a favorable 3-D bioscaffold for promising wound dressing and tissue engineering applications. 3-D structure scaffolds, mainly defined as device inclusive of polymeric biomaterials or decellularized tissue possessing the superiority to afford structural frame for cell attachment and tissue progress. There have been many attempts to mimic such a suitable 3-D environment for cell growth and proliferation (Goh et al., 2013; Mazza et al., 2015; Venugopal et al., 2008). Similarly, we developed a natural, decellularized scaffold, and the SEM images showed a 3-D interlinked porous structure (Fig. 3). This porous structure provide suitable environment for cell culture. However, further studies are required to approve whether this novel scaffold derived from fish swim bladder could preserve a qualified 3-D environment to promote adheration, multiplication, or differentiation (Yang et al., 2008).

3.2. Antibacterial activity of FSB scaffold

The antibacterial potential of the scaffold was evaluated by determining their antimicrobial activity based on disc diffusion and growth inhibition methods against some common bacteria found in wounds such as *B. subtilis, S. aureus, E. coli, P. aeruginosa and S. typhimurium.*

The antibacterial activity measurement was performed by the disc

diffusion method against the bacteria, which was determined as the diameter of the inhibition zone (DIZ) (Fig. 5). Also, changes in the turbidity of the bacterial growth culture were an indication of the antibacterial activity of the bioscaffold (Fig. 6).

According to the results obtained by both methods, significant antibacterial activity of the prepared scaffold, even at low concentration of AgNPs (50 ppm) has been revealed. Furthermore, bioscaffolds with different concentrations of AgNPs (30, 50, 100, and 150 μ g ml⁻¹) were used for test tube dilution method in which the rate of the bacterial growth was determined measuring the optical density (OD). As the concentration of AgNPs increased, the OD of the tested bacteria decreased in a concentration-dependent manner and in concentration range of 50–150 μg ml⁻¹ no bacterial growth was detected (MIC) (Fig. 7A and B). Also, our results showed that S. aureus was the most sensitive bacterial strain to our prepared scaffold. Similar outcomes for antibacterial activity of AgNPs have been reported using the same well diffusion method, in which the DIZ around each well containing AgNPs was an indication for the antibacterial activity of the tested nanoparticles (Logeswari et al., 2015). Furthermore, recently a group of researchers evaluated the long-term antibacterial activity of a wound dressing using radical disc diffusion method against some Gram-positive and Gram-negative bacteria. Their DIZ results confirmed the antibacterial activity of the wound dressing composed of electrospun gelatine mats (Dhand et al., 2017).

In order to evaluate the ability of the scaffold to act as a barrier preventing microbial penetration through wounds, the microbial barrier property (MBP) of the studied bioscaffold was investigated (Augustine et al., 2015). Our results indicated that FSB scaffold having a high density of collagen filaments with distances smaller than 2 μ m

Fig. 6. Growth curve of *Escherichia coli* (A), *Pseudomonas aeruginosa* (B), *Salmonella typhimurium* (C), *Staphylococcus aureus* (D), and *Bacillus subtilis* (E) exposed to the FSB scaffold with different concentration of AgNPs. Error bars are sample standard deviation of triplicate measurements.

(Fig. 2), and multi-fibrils with a pore diameter of about 0.5 μ m can act as an appropriate microbial barrier for the wounds up to three days (Fig. 7C). Moreover, the bactericidal activity of AgNPs which present in scaffold lead to the bacterial cell death in case of any penetration of bacteria (Morones et al., 2005).

3.3. Cytotoxicity of FSB scaffold

An appropriate scaffold should be biocompatible with human cells. To evaluate the biocompatibility of the tested scaffolds with and without AgNPs, MTT assay as a rapid *in vitro* method was performed. The viability of HFF cells in the presence of bioscaffolds with AgNPs has decreased in a time-dependent manner, while the scaffolds without AgNPs showed lower cytotoxicity (Fig. 8, Table 1).

Therefore, the presence of AgNPs in the bioscaffolds seems to increase the cytotoxicity of HFF cell and decreases their viability by time passing. Furthermore, FSB before decellularization caused a noticeable increase in the number of viable cells. Despite of these results, decrease in cell viability could be more preferable in the condition that antimicrobial activity and wound healing functionality is considered. Moreover, using the lower concentration of AgNPs or other ecologically

safe agents could reduce the cytotoxicity (Duraipandy et al., 2015).

The statistical analysis was performed using one-way ANOVA followed by post-hoc Tukey to investigate more differences between all groups in 24, 48 and 72 h. The obtained data revealed no significant difference in the vitality of HFF cells after 24 h (p > 0.05). This result showed that AgNPs have no toxicity in HFF cells after 24 h. Viability percentage calculated after 48 h showed that there is a significant difference in cell viability (p < 0.05) which means that AgNPs are toxic for HFF cells after 48 h. Post-hoc Tukey analysis showed that there is a significant positive difference between the viability percentage of HFF cells treated with AgNPs (N) and the cells treated with FSB tissue (Tissue) and FSB impregnated in AgNPs in comparison with control (HFF) sample after 48 h (p < 0.05). These results revealed that the presence of FSB decreases the cytotoxic effect of AgNPs and encourages cell proliferation. There is no significant difference for other treatments in 48 h time (p > 0.05). The viability percentage measured by MTT assay after 72 h represents a time-dependent pattern as shown, in Fig. 8. The data analysis revealed a significant difference in the viability of HFF cells (p < 0.05).

It is worth restating that there is a need to eliminate wound without the risk of large area infection, necrosis, and deformation of wound

Fig. 7. (A) Determination of minimum inhibitory concentration (MIC) of FSB containing various AgNPs concentrations (30, 50, 100, 150 ppm) for tested bacteria using the test tube dilution method and OD₆₀₀ nm measurement. (B) Bacterial GI% of FSB scaffold with different concentration of AgNPs. FSB scaffold without AgNPs which the inhibitory activity of scaffold equals zero. Error bars are sample standard deviation of triplicate measurements. (C) Microbial barrier property (MBP) of FSB Scaffold after 3 days. Tubes -, +, A and B are respectively indicating for tubes sealed with a sterilized cork, tube without any cover, tube sealed with sterilized FSB scaffold and tube top-covered with AgNPs-FSB scaffold.

borders. To achieve this goal, different types of scaffolds have been developed with different physical properties (Ruszczak and Schwartz, 2000; Vin et al., 2002). Scaffold materials can be either synthetic or naturally occurring, but synthetic materials tend to elicit a foreign material type of response in the host leading to the formation of dense scars and fibrosis. Therefore, biomaterials such as collagen either purified from animal or fish sources and its treatment prior to use have been considered as alternatives to synthetic scaffolds due to its

Table 1

Cell viability percentage of HFF cell line (control) treated with FSB tissue (Tissue), FSB tissue with AgNPs (Tissue + N), FSB tissue decellularized with freeze-thawing (TN2), FSB tissue decellularized with freeze-thawing plus AgNPs (TN2 + N), FSB tissue decellularized with freeze-thawing and 0.5% SDS (TN2 + SDS), FSB tissue decellularized with freeze-thawing and 0.5% SDS plus AgNPs (TN2 + SDS + N) and AgNPs alone (N).

Samples	Cell viability (%)		
	24 h	48 h	72 h
Control (HFF)	84 ± 0.06	85 ± 0.08	90 ± 0.06
Tissue	130 ± 0.062	121 ± 0.05	142 ± 0.08
Tissue + N	86 ± 0.009	119 ± 0.07	113 ± 0.05
TN2	115 ± 0.02	82 ± 0.04	108 ± 0.21
TN2 + SDS + N	121 ± 0.02	95 ± 0.02	62 ± 0.07
TN2 + SDS	139 ± 0.08	94 ± 0.06	105 ± 0.04
TN2 + N	122 ± 0.02	107 ± 0.06	134 ± 0.05
Ν	76 ± 0.02	51 ± 0.02	36 ± 0.03

biocompatibility, nontoxicity to tissues, suitable structural, physical, chemical, biological and immunological properties (Jeevithan et al., 2013). Nevertheless, the marine has been the center of notice in recent decades as a great source for such biomedical materials, especially fish bioactive materials because of their advantages over mammalian analogues (Subhan et al., 2015). In this regard, the exploitation of some disposable organs of fish species has grown in importance, as a way to convert by-products from fish wastes to bioactive materials with antimicrobial, anti-cancer, anti-oxidant, anti-diabetic, antiaging, cholesterol-lowering and anti-atherosclerosis properties (Atef and Ojagh, 2017). However, the properties of such wastes are varied with the

origin of fish species and different parts of fish as well as living environments of fish (Ideia et al., 2019). Thus, more investigations have to be conducted for discovering the application of fish-byproducts properties from all new sources. In this context, fish swim bladder due to the easy availability, eco-friendly and natural-like behavior has been introduced as a suitable dressing for biomedical and pharmaceutical applications. Also, its nanobiotechnological modification explored a new era of the potential application of FSB in tissue engineering which can provide a base for pharmaceutical research in the near future.

4. Conclusions

A novel scaffold with antibacterial activity was successfully synthesized using decellularized fish swim bladder (FSB) and colloidal silver nanoparticles (AgNPs). This scaffold contains a large amount of thin collagen strands that increase migration and proliferation which make it an appropriate artificial scaffold for tissue engineering. Moreover, having prominent characteristics such as encompassing high dense collagen, appropriate antibacterial activity, suitable microbial barrier, good flexibility, biocompatibility and biodegradability with ease of formulation, we propose FSB as an advantageous bioresource for wound dressing.

Declaration of interest statement

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Credit Author Statement

Fadak Mousavi Howaili: Investigation, Methodology, Visualization, Writing - original draft, Writing - review & editing. Mansour Mashreghi: Supervision, Funding acquisition, Data curation, Validation, Writing original draft, Writing - review & editing. Nasser Mahdavi Shahri: Conceptualization, Methodology, Formal analysis, Validation. Writing -Review & Editing. Ahmad Kompany: Validation. Project administration, Writing - review & editing. Razieh Jalal: Methodology, Formal analysis, Validation, Data curation.

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References

- Atef, M., Ojagh, S.M., 2017. Health benefits and food applications of bioactive compounds from fish byproducts: a review. J. Funct. Foods 35, 673–681.
- Augustine, R., Kalarikkal, N., Thomas, S., 2015. An in vitro method for the determination of microbial barrier property (MBP) of porous polymeric membranes for skin substitute and wound dressing applications. Tissue Eng. Regen. Med. 12, 12–19.
- Beghetto, V., Zancanaro, A., Scrivanti, A., Matteoli, U., Pozza, G., 2013. The leather industry: a chemistry insight Part I: an overview of the industrial process. Sci. Ca'Foscari.
- Campbell, J.J., Husmann, A., Hume, R.D., Watson, C.J., Cameron, R.E., 2017. Development of three-dimensional collagen scaffolds with controlled architecture for cell migration studies using breast cancer cell lines. Biomaterials 114, 34–43.
- Cheng, F., Gao, J., Wang, L., Hu, X., 2015. Composite chitosan/poly (ethylene oxide) electrospun nanofibrous mats as novel wound dressing matrixes for the controlled release of drugs. J. Appl. Polym. Sci. 132.
- Crapo, P.M., Gilbert, T.W., Badylak, S.F., 2011. An overview of tissue and whole organ decellularization processes. Biomaterials 32, 3233–3243.
- Dhand, C., Venkatesh, M., Barathi, V.A., Harini, S., Bairagi, S., Leng, E.G.T., Muruganandham, N., Low, K.Z.W., Fazil, M.H.U.T., Loh, X.J., 2017. Bio-inspired crosslinking and matrix-drug interactions for advanced wound dressings with longterm antimicrobial activity. Biomaterials 138, 153–168.
- Dos Santos, C.A., Seckler, M.M., Ingle, A.P., Gupta, I., Galdiero, S., Galdiero, M., Gade, A., Rai, M., 2014. Silver nanoparticles: therapeutical uses, toxicity, and safety issues. J. Pharmacol. Sci. 103, 1931–1944.

Duraipandy, N., Lakra, R., Srivatsan, K.V., Ramamoorthy, U., Korrapati, P.S., Kiran, M.S.,

2015. Plumbagin caged silver nanoparticle stabilized collagen scaffold for wound dressing. J. Mater. Chem. B 3, 1415–1425.

- Fan, Z., Liu, B., Wang, J., Zhang, S., Lin, Q., Gong, P., Ma, L., Yang, S., 2014. A novel wound dressing based on Ag/graphene polymer hydrogel: effectively kill bacteria and accelerate wound healing. Adv. Funct. Mater. 24, 3933–3943.
- Finney, J.L., Robertson, G.N., McGee, C.A.S., Smith, F.M., Croll, R.P., 2006. Structure and autonomic innervation of the swim bladder in the zebrafish (Danio rerio). J. Comp. Neurol. 495, 587–606. https://doi.org/10.1002/cne.20948.
- Gautam, S., Chou, C.-F., Dinda, A.K., Potdar, P.D., Mishra, N.C., 2014. Surface modification of nanofibrous polycaprolactone/gelatin composite scaffold by collagen type I grafting for skin tissue engineering. Mater. Sci. Eng. C 34, 402–409.
- Ghosh, S.K., Mandal, D., 2016. Efficient natural piezoelectric nanogenerator: electricity generation from fish swim bladder. Nanomater. Energy 28, 356–365.
- Goh, S.-K., Bertera, S., Olsen, P., Candiello, J.E., Halfter, W., Uechi, G., Balasubramani, M., Johnson, S.A., Sicari, B.M., Kollar, E., 2013. Perfusion-decellularized pancreas as a natural 3D scaffold for pancreatic tissue and whole organ engineering. Biomaterials 34, 6760–6772.
- Hafidh, R.R., Abdulamir, A.S., Baker, F.A., 2009. Comparative histological and histochemical inter-species investigation of mammalian submandibular salivary glands. Era Mod. Diag. Pathol. 384.
- Hasan, A., Memic, A., Annabi, N., Hossain, M., Paul, A., Dokmeci, M.R., Dehghani, F., Khademhosseini, A., 2014. Electrospun scaffolds for tissue engineering of vascular grafts. Acta Biomater. 10, 11–25.
- Haugh, M.G., Murphy, C.M., McKiernan, R.C., Altenbuchner, C., O'Brien, F.J., 2011. Crosslinking and mechanical properties significantly influence cell attachment, proliferation, and migration within collagen glycosaminoglycan scaffolds. Tissue Eng. 17, 1201–1208.
- Hosseini, M., Mashreghi, M., Eshghi, H., 2016. Biosynthesis and antibacterial activity of gold nanoparticles coated with reductase enzymes. Micro & Nano Lett. 11, 484–489.
- Ideia, P., Pinto, J., Ferreira, R., Figueiredo, L., Spínola, V., Castilho, P.C., 2019. Fish processing industry residues: a review of valuable products extraction and characterization methods. Waste and Biomass Valorization 1–24.
- Jackson, D.W., Grood, E.S., Wilcox, P., Butler, D.L., Simon, T.M., Holden, J.P., 1988. The effects of processing techniques on the mechanical properties of bone-anterior cruciate ligament-bone allografts: an experimental study in goats. Am. J. Sports Med. 16, 101–105.
- Jeevithan, E., Qingbo, Z., Bao, B., Wu, W., 2013. Biomedical and pharmaceutical application of fish collagen and gelatin: a review. J. Nutr. Therapeut. 2, 218–227.
- Karri, V.V.S.R., Kupusamy, G., Talluri, S.V., Mannemala, S.S., Kollipara, R., Wadhwani, A.D., Mulukutla, S., Raju, K.R.S., Malayandi, R., 2016. Curcumin loaded chitosan nanoparticles impregnated into collagen-alginate scaffolds for diabetic wound healing. Int. J. Biol. Macromol. 93, 1519–1529.
- Kiernan, J.A., 2001. Histological and Histochemical Methods. Theory Pract, Arnold Ed. . Klasen, H.J., 2000. A historical review of the use of silver in the treatment of burns. II.
- Renewed interest for silver. Burns 26, 131–138.
 Li, J., Wang, M., Qiao, Y., Tian, Y., Liu, J., Qin, S., Wu, W., 2018. Extraction and characterization of type I collagen from skin of tilapia (Oreochromis niloticus) and its potential application in biomedical scaffold material for tissue engineering. Process Biochem. 74, 156–163.
- Lillie, R.D., Pizzolato, P., Donaldson, P.T., 1976. Nuclear stains with soluble metachrome metal mordant dye lakes. Histochemistry 49, 23–35.
- Logeswari, P., Silambarasan, S., Abraham, J., 2015. Synthesis of silver nanoparticles using plants extract and analysis of their antimicrobial property. J. Saudi Chem. Soc. 19, 311–317.
- Madhumathi, K., Kumar, P.T.S., Abhilash, S., Sreeja, V., Tamura, H., Manzoor, K., Nair, S.V., Jayakumar, R., 2010. Development of novel chitin/nanosilver composite scaffolds for wound dressing applications. J. Mater. Sci. Mater. Med. 21, 807–813.
- Maneerung, T., Tokura, S., Rujiravanit, R., 2008. Impregnation of silver nanoparticles into bacterial cellulose for antimicrobial wound dressing. Carbohydr. Polym. 72, 43–51.
- Mazza, G., Rombouts, K., Hall, A.R., Urbani, L., Luong, T.V., Al-Akkad, W., Longato, L., Brown, D., Maghsoudlou, P., Dhillon, A.P., 2015. Decellularized human liver as a natural 3D-scaffold for liver bioengineering and transplantation. Sci. Rep. 5, 13079.
- Miranda-Nieves, D., Chaikof, E.L., 2016. Collagen and elastin biomaterials for the fabrication of engineered living tissues. ACS Biomater. Sci. Eng. 3, 694–711.
- Miyata, T., Taira, T., Noishiki, Y., 1992. Collagen engineering for biomaterial use. Clin. Mater. 9, 139–148.
- Mohammadie, Z.M., Parivar, K., Shahri, N.M., Fereidoni, M., Hayati-Roodbari, N., 2017. Decellularized bovine articular cartilage matrix reinforced by carboxylated-SWCNT for tissue engineering application. Braz. Arch. Biol. Technol. 60.
- Morones, J.R., Elechiguerra, J.L., Camacho, A., Holt, K., Kouri, J.B., Ramírez, J.T., Yacaman, M.J., 2005. The bactericidal effect of silver nanoparticles. Nanotechnology 16, 2346.
- Naderi, S., Zadeh, J.K., Shahri, N.M., Abady, K.N.S., Cheravi, M., Baharara, J., Rad, S.A.B., Bahrami, A.R., 2013. Three-dimensional Scaffold from decellularized human Gingiva for cell cultures: glycoconjugates and cell behavior. Cell J 15, 166.
- Pati, F., Adhikari, B., Dhara, S., 2010. Isolation and characterization of fish scale collagen of higher thermal stability. Bioresour. Technol. 101, 3737–3742. https://doi.org/10. 1016/j.biortech.2009.12.133.
- Rai, M., Kon, K., Ingle, A., Duran, N., Galdiero, S., Galdiero, M., 2014. Broad-spectrum bioactivities of silver nanoparticles: the emerging trends and future prospects. Appl. Microbiol. Biotechnol. 98, 1951–1961.
- Rai, M., Yadav, A., Gade, A., 2009. Silver nanoparticles as a new generation of antimicrobials. Biotechnol. Adv. 27, 76–83.
- Rath, G., Hussain, T., Chauhan, G., Garg, T., Goyal, A.K., 2016. Collagen nanofiber containing silver nanoparticles for improved wound-healing applications. J. Drug

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Target. 24, 520-529.

Ruszczak, Z., Schwartz, R.A., 2000. Modern aspects of wound healing: an update. Dermatol. Surg. 26, 219–229.

- Shahabipour, F., Mahdavi-Shahri, N., Matin, M.M., Tavassoli, A., Zebarjad, S.M., 2013. Scaffolds derived from cancellous bovine bone support mesenchymal stem cells' maintenance and growth. In Vitro Cell. Dev. Biol. Anim. 49, 440–448. https://doi. org/10.1007/s11626-013-9591-7.
- Shemesh, S., Sidon, E., Kaisler, E., Sheinis, D., Velkes, S., Ohana, N., Benayahu, D., 2017. Diabetes mellitus is associated with increased elastin fiber loss in ligamentum flavum of patients with lumbar spinal canal stenosis: results of a pilot histological study. Eur. Spine J. 1–9.
- Subhan, F., Ikram, M., Shehzad, A., Ghafoor, A., 2015. Marine collagen: an emerging player in biomedical applications. J. Food Sci. Technol. 52, 4703–4707.
- Türker, E., Yildiz, Ü.H., Yildiz, A.A., 2019. Biomimetic hybrid scaffold consisting of coelectrospun collagen and PLLCL for 3D cell culture. Int. J. Biol. Macromol. 139, 1054–1062.
- Unnithan, A.R., Gnanasekaran, G., Sathishkumar, Y., Lee, Y.S., Kim, C.S., 2014. Electrospun antibacterial polyurethane-cellulose acetate-zein composite mats for

wound dressing. Carbohydr. Polym. 102, 884-892.

- Venugopal, J., Low, S., Choon, A.T., Ramakrishna, S., 2008. Interaction of cells and nanofiber scaffolds in tissue engineering. J. Biomed. Mater. Res. B Appl. Biomater. 84, 34–48.
- Vin, F., Teot, L., Meaume, S., 2002. The healing properties of Promogram in venous leg ulcers. J. Wound Care 11, 335–341.
- Wei, B., Yang, G., Hong, F., 2011. Preparation and evaluation of a kind of bacterial cellulose dry films with antibacterial properties. Carbohydr. Polym. 84, 533–538.
- Yang, Q., Peng, J., Guo, Q., Huang, J., Zhang, L., Yao, J., Yang, F., Wang, S., Xu, W., Wang, A., 2008. A cartilage ECM-derived 3-D porous acellular matrix scaffold for in vivo cartilage tissue engineering with PKH26-labeled chondrogenic bone marrowderived mesenchymal stem cells. Biomaterials 29, 2378–2387.
- Zhang, D., Zhou, W., Wei, B., Wang, X., Tang, R., Nie, J., Wang, J., 2015. Carboxylmodified poly (vinyl alcohol)-crosslinked chitosan hydrogel films for potential wound dressing. Carbohydr. Polym. 125, 189–199.
- Zhou, K., Dong, C., Zhang, X., Shi, L., Chen, Z., Xu, Y., Cai, H., 2015. Preparation and characterization of nanosilver-doped porous hydroxyapatite scaffolds. Ceram. Int. 41, 1671–1676.