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# Novel Biodegradable Antimicrobial Composite Scaffold by Coating Type I Collagen Scaffold with Pepsin-Degraded SIS Extraction

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**Abstract.** The antimicrobial activity of tissue engineering scaffolds is crucial for successful implantations and surgeries by reducing surgical infections. In this study, small intestinal submucosa (SIS) extracts were prepared by *in vitro* pepsin digestion (pSIS) and its antimicrobial active is stronger than those prepared by accelerated hydrolysis (aSIS). Compared with aSIS, the average molecular weight of pSIS seems smaller with more narrow distribution via SDS-PAGE electrophoresis combined with commassie blue staining. Furthermore, pSIS coated porous type I collagen scaffold (pSIS/COL I scaffolds) displayed a strong antibacterial activity and is suitable for cell seeded and growth, indicating that it can be potentially applied to tissue engineering or surgical tissue repairing in the future.

## 1. Introduction

Inappropriate operations of medial materials or instruments were considered to take blames for half of the infection situations in hospitals during surgeries or clinical treatments. Hence, anti-infectious biodegradable materials, which could reduce the suffering of patient and high medical burden as well, are greatly demanded for clinical applications. In order to modify materials with antimicrobial activity, various methods have been established, such as coating with antibiotics, and quaternary ammonium salt, silver ions, as representatives of the cationic surfactant or heavy metal oxides [1-3]. Nano-silver particles were used to enhance the antimicrobial activity of porcine-derived small intestinal submucosa freeze-dried films in repairing contaminated abdominal defects [4]. And vancomycin antibiotics/chitosan-bioactive glass mixture coatings showed good results on antimicrobial activity against Gram-positive *Staphylococcus aureus* bacteria [5]. But such modifications are respectively limited by certain cellular toxicity, narrow antimicrobial spectrum and furthermore, bacterial drug-tolerance and contaminations from other kinds of bacteria [6,7].



As an acellular tissue matrix (ACTM) material, small intestinal submucosa (SIS) is mainly made up of Type I, III, IV collagen, fibronectin, mucopolysaccharide and proteoglycan. These ingredients, under certain concentration or activity, could fulfil their own purposes of regulating tissue repairing. Furthermore, scholars find that SIS contains growth factors such as FGF-2, TGF- $\beta$  and VEGF [8]. As the degradation process of SIS goes by, these growth factors could be released and act on cells or tissues around, improving tissue regeneration. Owing to its outstanding physical and chemical property, biocompatibility, biodegradability and bio-absorbability, SIS has been considerable in the use of tissue engineering and surgical tissue repair [9].

Research indicated that SIS itself does not have antimicrobial property [10], but surprisingly, SIS extract, which was prepared by a high temperature and pressure accelerated hydrolysis process, has shown its antimicrobial activity against Gram-positive and Gram-negative bacteria [11,12]. It is important to identify the nature of those anti-infective agents and what's more, to prepare agents with superior antimicrobial activity, and explore if they could provide new ideas for tissue engineering scaffolds or tissue repair patches fabrication or modification to avoid surgical infections. Hereby, we combine type I collagen scaffolds, which is one of the most widely used model both scientifically and clinically, and bionic from both native tissue extracellular matrix composition and structure aspect, with pSIS and therefore, develop a novel tissue engineering scaffold or surgical tissue repair patch and tested its antimicrobial activity. It suggests that novel pSIS coated type I collagen scaffolds may be applied in clinic treatment and tissue engineering in the future.

## 2. Materials and methods

### 2.1. Materials

Small intestinal submucosa (SIS) was supplied from Beijing Biosis Healing Biological Technology Ltd, China. Glacial acetate acid was purchased from Sinopharm Chemical Reagent Co., Ltd, China. Type I collagen (collagen I, COL I) (genetic type *COL I*) was purchased from BBI Life Sciences Corporation, China. Pepsin was purchased from AOBX, China.

### 2.2. Preparation of SIS extract

**2.2.1. Preparation of SIS extract by accelerated hydrolysis.** The aSIS was prepared based on the reported method [1]. SIS powders were moistened with double distilled water in a mass/volume ratio of 1g SIS: 9mL ddH<sub>2</sub>O. Then, 0.5N acetate acid was added into the system in a mass/volume ratio of 1g moistened SIS: 2.75mL 0.5N acetate acid. The system was placed in a 121 °C and 0.14MPa environment to process the accelerated hydrolysis for 120min. After a 9000r/min, low temperature centrifugal process, the mixture will be separated in 3 parts, including the upper layer suspension liquid, the middle layer clear solution and precipitation. The clear middle layer solution was neutralized and then refrigerated and freeze-dried, and therefore aSIS is acquired. The aSIS was re-dissolved by ddH<sub>2</sub>O in a mass/volume ratio of 600mg SIS extract: 1mL ddH<sub>2</sub>O, being prepared to proceed further experiments.

The background product sample of accelerated hydrolysis was prepared to prove that there's nothing else with antimicrobial activity except SIS accelerated hydrolysis extract, and its preparation process is exactly the same with the above-mentioned process but without the addition of SIS.

**2.2.2. Preparation of SIS extract by in vitro pepsin digestion.** SIS powders were mixed with ddH<sub>2</sub>O in a mass/volume ratio of 1g SIS: 100mL ddH<sub>2</sub>O. The mixture was boiled for 20min to sabotage the triple helical structure of collagen. After cooling down to room temperature, glacial acetic acid was added to the system to adjust pH to lower than 4. And then pepsin was added to the system according to the mass ratio of 1g SIS: 0.08g pepsin. The system was placed in a 35 °C water bath and stirred until SIS was totally degraded. Then the system was boiled for 10min in order to inactivated the pepsin and concentrate the system as well. After 9000r/min centrifugal process, the mixture will be separated in 3

parts including the upper layer suspension liquid, the middle layer clear solution and precipitation. The middle layer clear solution was refrigerated and freeze-dried, and therefore we pSIS. The pSIS was re-dissolved by ddH<sub>2</sub>O in a mass/volume ratio of 600mg SIS extract: 1mL ddH<sub>2</sub>O, being prepared to proceed further experiments.

The background product sample of *in vitro* pepsin digestion was prepared to prove that there's nothing else with antimicrobial activity except SIS *in vitro* pepsin digestion extract, and its preparation process is exactly the same with the above-mentioned process but without the addition of SIS.

### 2.3. Antimicrobial activity of SIS extract

Staphylococcus aureus (*S. Aureus*), Escherichia coli (*E. Coli*) and Bacillus Subtilis (*B. Subtilis*) were chosen as the experimental bacteria. After resuscitation, the bacteria were cultured in a bacteria Luria-Bertani (LB) liquid medium solution for 10h. The amplified bacterial solution was then diluted to  $10^{-4}$  (bacteria density is approximately  $2.5 \times 10^5$  CFU/mL) and added 200 $\mu$ L into a LB agar medium. The inoculated LB agar medium was then added with 8 tiny glass beads ( $\phi = 5$ mm) and horizontally shake thoroughly in order to spread the bacteria solution evenly.

Filter paper ( $\phi = 10$ mm) was placed on the exact location onto the inoculated LB agar medium, and each piece of filter paper was added with 20 $\mu$ L of the exact according solution. Then, the bacteriostatic annulus was formed and recorded.

### 2.4. Molecular weight and distribution analysis of SIS extracts

The molecular weight and distribution analysis of both SIS extracts was performed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

### 2.5. Preparation of SIS extract and type I collagen composite scaffold

Type I collagen was mixed with 3vt% acetate acid in a mass/volume ratio of 10mg: 1mL and stirred to complete clear solution. A 1500 r/min, 10min centrifugal process was performed to remove bubbles. Then, the collagen solution was added into a 24-well plate for 0.5mL/well. After refrigerated in  $-20^{\circ}\text{C}$  for 1d and freeze-dried, the type I collagen pre-scaffolds were crosslink in our previously reported EDAC crosslinking method [13]. After a freeze-dried process to remove the remain ddH<sub>2</sub>O, the type I collagen scaffolds were acquired.

Moistened with ddH<sub>2</sub>O, type I collagen scaffolds was added with 600mg/ml aSIS or pSIS extracts solutions and kept solid standing for over 6h in order to let the extract solutions disperse thoroughly into the scaffold. Refrigerated in  $-20^{\circ}\text{C}$  and then freeze-dried, SIS extract and type I collagen composite scaffolds (aSIS/COL I scaffolds, pSIS/COL I scaffolds) are acquired.

### 2.6. Antimicrobial activity of SIS extract and type I collagen composite scaffold

After resuscitation, amplification and inoculation onto LB agar medium of bacteria in the above-mentioned procedure, SIS extract and type I collagen composite scaffolds were placed on the exact location onto the inoculated LB agar medium, and then the composite scaffolds were moistened by ddH<sub>2</sub>O. Then, the bacteriostatic annulus was formed and recorded.

### 2.7. Cell study

**2.7.1. Culture of MG-63.** The MG-63 cells were cultured with RPMI modified medium supplemented with 10%(v/v) fetal bovine serum and 1%(v/v) antibiotics in plates at a controlled atmosphere (5%CO<sub>2</sub>, 37°C). Culture medium was replaced every two days.

**2.7.2. Cell seeding and cell proliferation investigation.** All the COL I scaffolds and SIS extract/COL I composite scaffolds were sterile by ethylene oxide sterilization. Finally, MG-63 cells were seeded into the scaffold at a density of 7,500 cells/ml. The proliferation of MG-63 was determined by alamar Blue assay.

**2.7.3. Cell morphology investigation.** After 2, 4, 6 and 8 days of culture, the morphology of the seeded cells were stained and observed by fluoresce in diacetate living cell staining assay and an inverted fluorescence microscope.

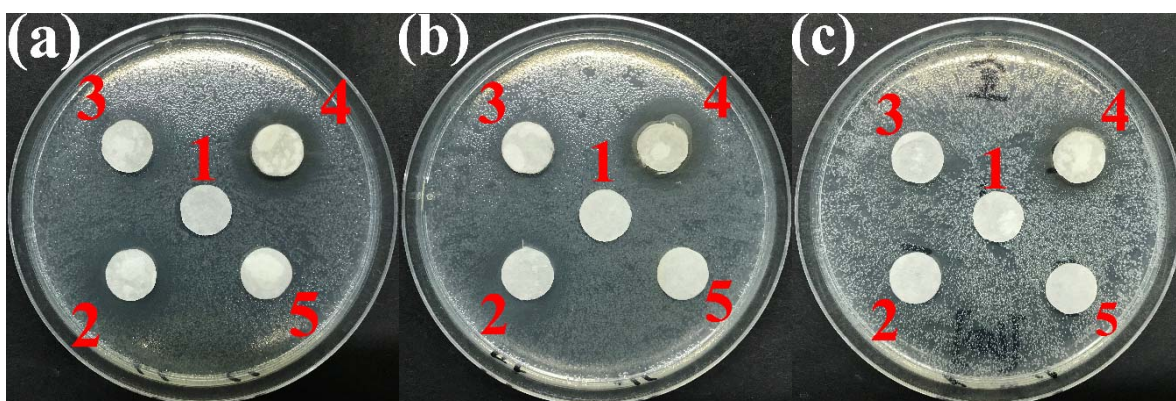
### 2.8. Statistical analysis

All the experiments results were acquired from at least 3 independent experiments at each condition and shown as their average  $\pm$  standard deviations. Statistical analysis was performed using an SSPS 10 statistical package, and values were considered significant at p value  $< 0.05$ .

## 3. Results and discussion

### 3.1. Antimicrobial activity of pSIS

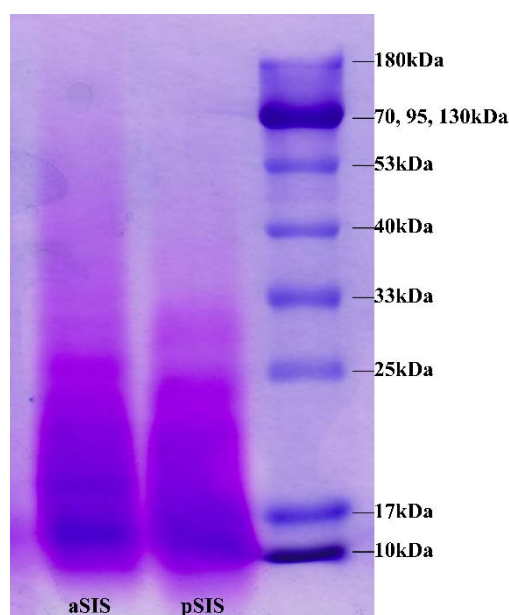
First aim of this research is to identify the antimicrobial activity of pSIS and compare it with aSIS. By employing bacteriostatic annulus assay, the sample with adding pSIS displayed a significant larger bacteriostatic annulus against *E. Coli*, *B. Subtilis* and *S. Aureus* with clear boundaries compared with aSIS did (Figure 1). It seems that *S. Aureus* is not sensitive to pSIS compared with other two bacteria, since the smallest bacteriostatic annulus generated by pSIS against *S. Aureus* was observed at Figure 1. Because of acetate and acetic acid, the reaction buffer of aSIS displayed a slight antimicrobial activity. Hence, it demonstrated that pSIS exhibits a strong antimicrobial activity.



**Figure 1.** Antimicrobial activity of pSIS and aSIS tested by bacteriostatic annulus assay. From left to right ((a), (b) and (c)), the bacteria used in this test are *E. Coli*, *B. Subtilis* and *S. Aureus*. Samples are 1. ddH<sub>2</sub>O, 2. aSIS, 3. the reaction buffer of aSIS, 4. pSIS and 5. the reaction buffer pSIS.

### 3.2. Molecular weight and distribution of pSIS

The mechanism of antimicrobial activity could be peptides of certain molecular weight among degradation extracts tangling with cellular membrane of micro-organism by static electricity then killing it through sabotaging the dynamic equilibrium of internal and external ions or substances of membranes, or forming a pliers-like structure, tearing the membranes apart during micro-organism growth or proliferation [14]. By using SDS-PAGE electrophoresis, components of pSIS and aSIS were separated and were stained with commassie blue (Figure 2). The components of aSIS were distributed in a board range of molecular weight, but the ingredients of pSIS were located in a narrow range of molecular weight (Figure 2). The majority ingredients of pSIS locate at the narrow range from 17 kDa to 10 kDa. The distribution of components containing in pSIS is different from that of aSIS, implying that pSIS contains more peptides contribute to antimicrobial activity.

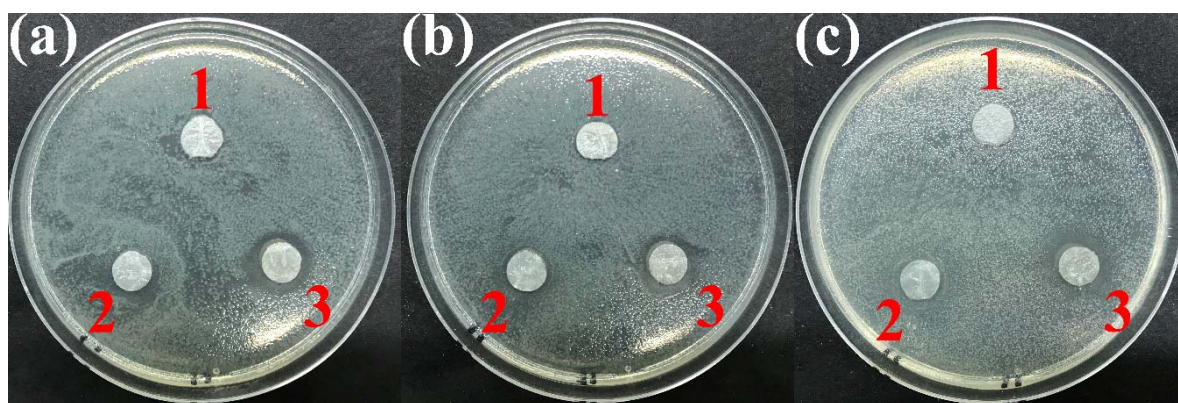


**Figure 2.** SDS-PAGE result. (12wt% SDS-PAGE resolving gel)

(a. SIS accelerated hydrolysis extracts, b. SIS *in vitro* pepsin digestion extracts, c. marker)  
(marker molecular weight ranges from 10kDa – 180kDa with 10 types of purified proteins (10, 17, 25, 33, 40, 53, 70, 95, 130, 180kDa))

### 3.3. Antimicrobial activity of pSIS coated type I collagen scaffolds

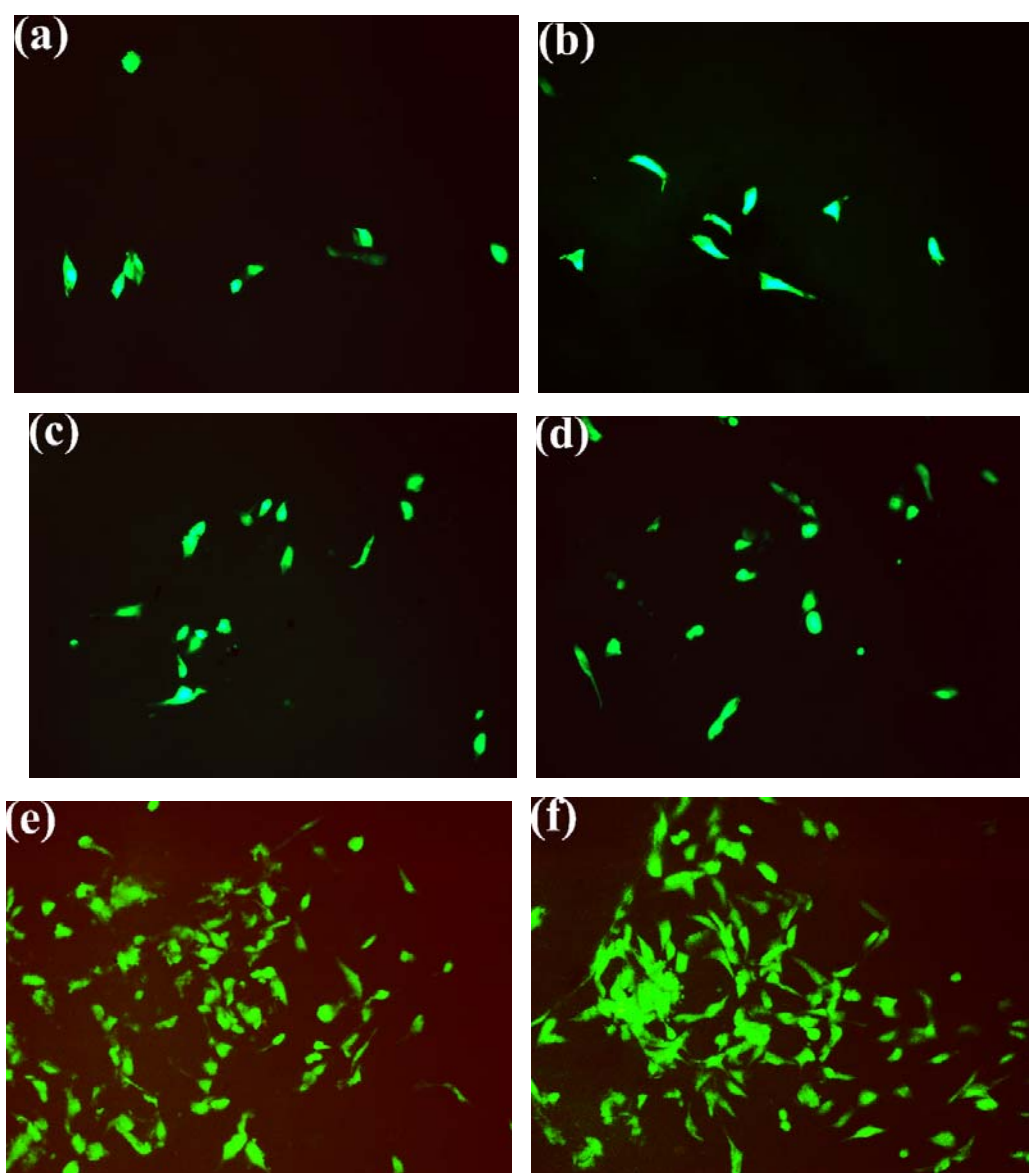
To test whether pSIS can be used as an antimicrobial coating layer of biodegradable tissue engineering scaffolds, pSIS coated COL I scaffolds have been prepared with the method described as 2.5. To examine the antimicrobial ability of these novel composite scaffolds, the same bacteriostatic annulus assay was used as well. It is not surprising that COL I scaffolds showed no antimicrobial activity, and pSIS coated COL I scaffolds exhibited a strong antimicrobial activity (Figure 3) compared with aSIS coated COL I scaffolds. It demonstrated that pSIS coated COL I scaffolds are able to resist the growth of bacteria, implying that this novel scaffold may be use in surgery to reduce clinic infection.



**Figure3.** Results of SIS extract and type I collagen composite scaffolds bacteriostatic annulus assay (from left to right ((a), (b) and (c)), the bacteria are *E. Coli*, *B. Subtilis* and *S. Aureus*) (Samples number 1 to 3 are respectively 1. Antimicrobial negative control (COL I scaffolds), 2. Antimicrobial positive control (aSIS/COL I scaffolds) and 3. Antimicrobial narrative (pSIS/COL I scaffolds).)

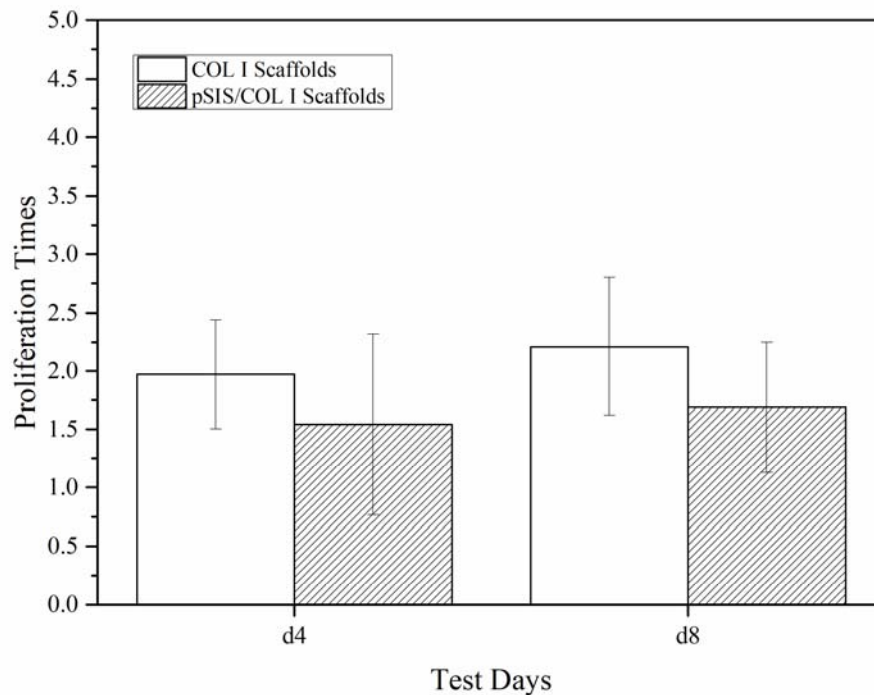
### 3.4. Biocompatibility of pSIS coated COL I scaffolds

It has been suggested that peptides with certain molecular weight is able to interfering the membrane of bacteria and killing them. Hence, the biocompatibility of this pSIS coated COL I scaffolds was tested to confirm that human cells with larger size immune to the influence of small peptides. To investigate the biocompatibility of pSIS coated COL I scaffolds, MG-63 cells were cultured on these scaffolds and COL I scaffolds respectively for 8 days. The proliferation of MG-63 was monitored by using fluorescein diacetate (FDA) staining, which displays living cells only (Figure 4). The morphology of MG-63 cells on both COL I scaffolds and pSIS coated COL I scaffolds exhibited typical polygon shape with filopodia and gradually spreading within scaffolds, indicating that the biocompatibility of pSIS coated COL I scaffolds is suitable for cell growing.



**Figure 4.** FDA staining images of MG-63 inside COL I scaffolds ((a), (c) and (e)) and pSIS/COL I scaffolds((b), (d) and (f)). (a) and (b) were incubated for 2d, (c) and (d) were incubated for 4d, (e) and (f) were incubated for 8d.

To further confirm the results obtained by FDA staining, the harmless alamar Blue assay was choose to detect the cellular proliferation of MG-63 cells cultured on pSIS coated COL I scaffolds and COL I scaffolds respectively (Figure 5). The proliferation of MG-63 cells cultured on pSIS coated COL I scaffolds is closed to that of MG-63 cells cultured on COL I scaffolds. It demonstrated that the biocompatibility of pSIS coated COL I scaffolds and COL I scaffolds are similar.



**Figure 5.** Results of alamarBlue assay. The proliferation rate of MG-63 on 2 type of scaffolds following 4 and 8 days of culture (culture day 2 as cardinal data).

#### 4. Conclusion

The strong antimicrobial ability of pSIS has been confirmed by using the bacteriostatic annulus assay. By applying pSIS as the coating layer, pSIS coated COL I scaffolds were prepared successfully and displayed strong antimicrobial activity without reducing the biocompatibility of COL I scaffolds, suggesting its potential application in tissue engineering and surgery.

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