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Bio-studies of scaffolds based on chitosan/tannic acid cross-linked by glyoxal

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ABSTRACT

Scaffolds based on chitosan/tannic acid cross-linked by glyoxal were obtained by the freeze-frying method. Bio-studies were carried out to consider the safety of material used in medical applications. Thereby, the blood and PDLSC cell compatibility studies were carried out.

The results showed that glyoxal is safe cross-linker for chitosan/tannic acid mixtures. Cross-linked scaffolds were nonhemolytic. Moreover, cells cultured on the scaffolds with glyoxal showed higher metabolic activity for 80CTS/20TA composition than others. The current findings allow for the medical application of the proposed materials as wound dressings.

1. Introduction

Scaffolds based on natural polymers have to be modified to improve their stability [1]. The main method is the addition of a cross-linker that reacts with functional groups of polymers. Glyoxal is an aldehyde which may form covalent bonds with amine groups present in chitosan [2]. Chitosan and tannic acid were previously studied by us as a good potential mixture for biomedical purposes [3]. However, the main problem is the low stability of obtained scaffolds. There is a need to search for safe and cheap cross-linker agents. Thereby, we performed novel studies of the chitosan/tannic acid cross-linking process by glyoxal. The essential properties of materials for biomedical application is their biocompatibility with surrounding cells and liquids in our body [4]. The study aimed to determine the biological properties of novel scaffolds based on chitosan and tannic acid cross-linked by glyoxal addition. For this purpose blood and cells studies were carried out.

2. Materials and methods

Chitosan (CTS; DD = 78% and the molecular weight 1.8×10^6), tannic acid (TA; 1701.2 g/mol) and glyoxal (GO; 40 wt% in water; M_w = 58.04 g/mol) are commercial compounds purchased from the Sigma-Aldrich company (Poznan, Poland).

2.1. Samples preparation

Chitosan and tannic acid were dissolved in 0.1 M acetic acid, separately, at a concentration of 2%. Chitosan and tannic acid were mixed in the weight ratios 80/20, 50/50, 20/80. To the mixture, glyoxal was added as a cross-linker in ratios 1 and 5% based on chitosan + tannic acid weight content. Then mixtures were poured into 24-well polystyrene culture plates, frozen, and lyophilized (ALPHA 1–2 LDplus, CHRIST, −20 °C, 100 Pa, 48 h).

2.2. Blood compatibility

200 μl of fresh sheep blood was added to 10 ml of physiological saline solution (0.9%) specimens with the same weight. The positive control (sterile water) and negative control (saline salt) were also prepared. All the tested tubes were incubated for 1 h at 37 °C. After incubation, the suspension was transferred into Eppendorf tubes and centrifuged at 10,000 rpm for 10 min. The supernatant was collected and absorbance was measured by microplate reader Multiscan FC (Thermo Fisher Scientific, Waltham, USA) at 540 nm. Each sample was prepared in triplicate. The hemolysis rate was calculated using the equation:

$$rateofhemolysis[\%] = \frac{[OD]specimen - [OD]negative}{[OD]positive - [OD]negative} * 100\%$$

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2.3. Establishing cell cultures on the experimental scaffolds

2.3.1. Material preparation for cell culture studies

Scaffolds were soaked in 70% EtOH and rinsed with sterile PBS (BioShop) to wash out alcohol residue. Later, the materials were placed at the bottom of the 24-well plates (Nest) and pushed to the bottom with sterile rings made out of medical polypropylene. To complete sterilization, scaffolds were placed under UV light with the laminar flow for 30 min.

2.3.2. Cell culture on the experimental scaffolds

The study was performed on stromal cells harvested from periodontal ligament from a molar tooth of a 31-year-old female, according to the protocol described by Bakkar et al. (2017) [5] (Institutional Review Board protocol nr 1072.6120.253.2017). PDLSCs (periodontal ligament stromal cells) were seeded directly onto scaffolds placed at the bottom of the 24-well plates at a density of $2 \times 10^4/cm^2$ in 100 μl of medium (84% Alpha-MEM Gibco, 15% FBS Gibco, 1% ZellShield Minerva Biolabs) for the cells to attach to the scaffolds. After 1 h, each well was carefully supplemented with the additional 900 μl of the medium. After 2 days, the medium was exchanged. MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega) was carried out on day 6. MTS assay evaluates cell viability, on the basis of their metabolic activity. Cells on scaffolds were rinsed with PBS and then supplemented with 200 μl of phenol-free Alpha-MEM (Gibco) with 10x diluted MTS reagent. The reactions were developed in CO_2 incubator until the visible change of color in comparison to the blank (phenol-free Alpha-MEM with 10x diluted MTS reagent in cell-free well). Next, the products of the reactions were transferred to individual wells in 96-well plates (Falcon) and absorbance was measured at 492 nm using a microplate reader (SpectraMax iD3 Molecular Devices). The intensity of the developed product was directly proportional to the amount of metabolically active cells, according to the technical bulletin of CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega.

2.4. Statistical analysis

Cell cultures and MTS assays were performed in triplicates or quadruplicates on each material. MTS absorbance values (with the subtracted blank value) were averaged (mean value with standard deviation). Results were statistically analyzed with one-way ANOVA and posthoc Tukey with $p < 0.05$ considered significant.

3. Results

3.1. Blood compatibility

The results of blood compatibility measurement are displayed in Table 1. The measurement includes the hemolysis of erythrocytes which are sensitive to the hemolysis due to the shear stress [6]. The rate of erythrocytes hemolysis decreases with an increasing amount of tannic acid – 20% content result in 0.3% hemolysis in comparison to 80% content with hemolysis 0. The addition of glyoxal as cross-linker improves the biocompatibility of scaffolds as the hemolysis rate is equal to 0.

3.2. Establishing cell cultures on the experimental scaffolds

Considering cell studies, the addition of glyoxal to the scaffolds with the base of 80CTS/20TA increased PDLSCs viability, for both 1 and 5% cross-linker addition. Cell metabolic activity is higher for cross-linked scaffolds, but not statistically significant, with the addition of 1% glyoxal. The addition of 5% glyoxal shows statistical differences in comparison to control. The addition of neither 1% nor 5% changes also the viability of PDLSCs on 50CTS/50TA and 20CTS/80TA materials. For scaffolds based on 50CTS/50TA 1% glyoxal addition slightly enhanced cell viability, however, 5% cross-linker addition decreased cell response (Fig. 1).

Table 1
The rate of hemolysis for the scaffolds based on chitosan and tannic acid with glyoxal.

Specimen	Hemolysis rate [%]
80CTS/20TA	0.30 ± 0.17
80CTS/20TA + 1%GO	0*
80CTS/20TA + 5%GO	0*
50CTS/50TA	0.18 ± 0.07
50CTS/50TA + 1%GO	0*
50CTS/50TA + 5%GO	0*
20CTS/80TA	0*
20CTS/80TA + 1%GO	0*
20CTS/80TA + 5%GO	0*

* measured values for material were lower than for control.

4. Discussion

Aldehydes are excellent cross-linkers for chitosan-based materials as their aldehyde groups react with amine and covalent bonds are formed. The risk of using aldehydes for biomaterials is that their unreacted monomers may be harmful. Thereby, it is important to consider the appropriate rate of chitosan:cross-linker to obtain safe and nontoxic material. Two methods used by us to determine the biocompatibility of produced scaffolds were studies in contact with blood and cells.

According to the ASTM F756-00 standard materials with a hemolytic index between 0 and 2% are classified as nonhemolytic [7]. All tested scaffolds showed hemolysis lower than 2% in the blood-material contact. The addition of a cross-linker does not influence the erythrocytes degradation rate. Concerning the study of the cells we may assume that the influence of glyoxal on cell viability depends on the scaffold composition. Interesting is that the viability of the cells significantly increase for cross-linked scaffolds

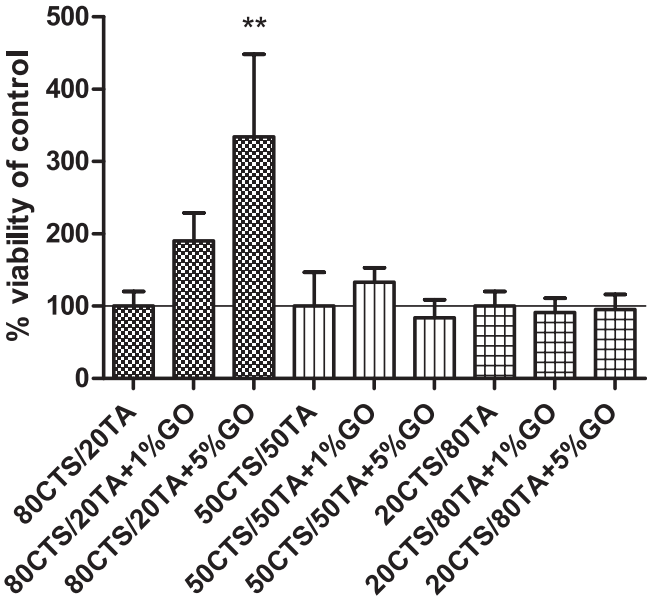

Fig. 1. Metabolic activity of PDLSCs cultured on scaffolds made out of different proportions (80/20, 50/50 or 20/80) of chitosan and tannic acid, with the addition of either 1 or 5% of glyoxal. Materials without the addition of glyoxal are considered as 100% viability in each group. * - results statistically significant.

in the composition of 80CTS/20TA compared to uncross-linked as well as to other composition.

Our studies of biological properties are new as they concern the novel composition of the scaffold. El-Feky et al. [8] proposed glyoxal as 5% addition to produce chitosan-based material for wound dressing. Glyoxal was determined as an effective cross-linker which improves the properties of obtained materials. Glyoxal was also proposed as a cross-linker for the obtainment of food packaging materials where the mechanical properties were improved [9]. It was also effective to modify the properties of soy protein-based materials dedicated to wound dressing [10]. Glyoxal was also used as a cross-linker for gelatin-based films [11]. Based on the published results we it may be assumed that glyoxal is safe and nontoxic cross-linker, however, only if it is covalently bound to the polymeric chain.

5. Conclusion

In this study, glyoxal was studied as a potential cross-linker for chitosan/tannic acid scaffolds. The presence of glyoxal improves blood biocompatibility (hemolysis equal to 0). Based on the cell viability studies we may determine that the most suitable scaffolds are one obtained from 80CTS/20TA + 5%GO. Such scaffolds are safe as they are nonhemolytic and promote cell proliferation, thereby, they may find application in tissue regeneration purposes as regeneration of soft tissues. Materials may find commercial application as the preparation method of such scaffolds is fast and easy to control.

Availability of data and material

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

Author contribution

BKS conceptualization of the study, supervision, writing - original draft and review; OM investigation of cell viability studies; MMS investigation of hemolysis; AMO methodology of cell viability study.

CRediT authorship contribution statement

B. Kaczmarek-Szczepańska: Conceptualization, Supervision, Writing - original draft. **O. Miłek:** Investigation. **M. Michalska-Sionkowska:** Investigation. **A.M. Osyczka:** Methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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