DEVELOPMENT OF A NEW PRODUCTION METHOD OF FOAM-LIKE WOUND DRESSINGS FOR SKIN REGENERATION

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Abstract

Chitosan is widely used to prepare films, hydrogels, cryogels, sponges, fibers and other various biomaterials used in the tissue engineering field. It is one of the best processable polysaccharides used in biomedicine. However, its stability is generally lower as compared with others, due to its pH sensitivity and hydrophilic character. Using chitosan in combination with agarose may not only improve chemical and mechanical properties of the resultant material (by the formation of a biocomposite), but also lead to the formation of a gel imitating physical attributes of the extracellular matrix. Moreover, the combination of these two polysaccharides has a promising ability to improve the stability of chitosan and to increase fibroblasts' affinity to agarose. Characteristic advantageous features of these natural polymers raise a wide interest in tissue engineering. The aim of this study was to develop and optimize a new method to produce a highly biocompatible foam-like chitosan/agarose wound dressing for skin healing applications. The production process optimization helped to obtain the absorbent foam-like biomaterial which is non-toxic to skin fibroblasts and does not conduce their adhesion. Employing sodium bicarbonate as the main agent in the foaming reaction not only led to obtaining the foam-like structure but also neutralized the acidic pH, making the material non-toxic and non-irritating to the skin. In conclusion, the new foam-like biomaterial has great potential for biomedical applications as the wound dressing accelerating the healing process of the damaged tissues.

Keywords: *chitosan, agarose, biomaterials, fibroblasts, cytotoxicity*

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Introduction

Skin is the biggest organ in the human body that possesses numerous functions essential for human survival. The primary one is to keep a barrier between the external and internal environment [1]. The wounded epidermis is known to stimulate self-regeneration, but the healing process depends on the type of injury. If the regeneration is not adequate, it may lead to the formation of a chronic wound. Any deep wound resulting in full-thickness skin loss needs grafting for its cure [2]. Frequently, patients with a high percentage of injuries or burns cannot become a donor for self-skin transplantation. Therefore, surgery procedures are often limited by the low accessibility of healthy tissue. Another strategy applied in skin regeneration is the allogeneic skin graft. Nevertheless, non-self-tissues used for allografts may cause immune rejection and infections related to the poor compatibility of tissue. Thus, in the case of extensive injuries or burn wounds, tissue-engineered skin grafts are the best way to overcome the above-mentioned problems [3,4]. The tissue engineering aims to fix, restore, improve or maintain tissue functions that have been lost due to various pathological conditions. The main strategies used can be classified into three primary groups: (1) implantation of the patient cells, (2) implantation of the different scaffolds seeded with patient cells, and (3) supplying tissue-inducing substances [5]. The development of appropriate scaffold design and an effective fabrication method is an important issue in regenerative medicine research and tissue engineering [6]. Among all engineered organs, the skin was the first tissue-engineered product used in patient treatment [7]. Over the last several decades, various natural and synthetic biomaterials for skin regeneration have been developed. A vast majority of them possess basic features of a proper wound dressing i.e. providing an external barrier against microorganisms, controlling the pain, removing exudates and also promoting the skin healing process [8].

Tissue-engineered biomaterials for skin regeneration that are currently available on the world market differ in a few main attributes: the type of biomaterial regarding its function (skin substitute/graft or external dressing), the duration of skin wound coverage (permanent or temporary), and cellular or acellular applications [9]. Interactive (or bioactive) dressings, like hydrogels (e.g. made of alginate, collagen), hydrocolloids, foams, or transparent films (e.g. made of polyurethane) are semi-occlusive or occlusive dressings that are essential for maintaining optimum moisture. They are recommended because of their advantageous influence on the local cellular response [10]. All the mentioned biomaterials may perform various important functions during the skin regeneration process, such as pain alleviation, protection of periwound skin, removal of nonviable tissues and dead spaces [11]. In general, wound dressings made of natural products can improve the wound healing process thanks to their demulcent, emollient, astringent, and antioxidant properties [12]. For instance, fibrinogen, elastin, chitosan, and collagen are natural biocompatible polymers similar to natural molecules present in the human organism [13]. Due to their efficient bioactive properties, natural polymers better interact with the cells facilitating the healing process. Natural polymers which are used in skin regenerative medicine can be divided into three large groups: (1) polysaccharides (e.g. alginate, dextran, chitin, chitosan, cellulose, amylose, glycosaminoglycans), (2) polynucleotides (DNA, RNA), and (3) proteins (e.g. collagen, elastin, gelatine, silk, fibrinogen) [6]. Synthetic polymers used in wound dressing production represent the largest group of biodegradable polymers. They are widely used in tissue engineering as it is possible to adapt their capabilities to the specific applications. Moreover, such biomaterials exhibit a long shelf time and can be produced in large uniform quantities [14]. Biological properties of bioactive dressings can be also improved by impregnation with antimicrobial or debriding agents, e.g. polyhexamethylene biguanide, cadexomer iodine, crystal violet, silver sulfadiazine, etc. [15]. The presence of natural or synthetic polymers and growth factors or other bioactive agents in the modern wound dressings composition makes it possible to predict the changes in the wound environment and to enhance the healing process [16].

The porosity of the potential wound dressings is a very important feature. It not only provides high material absorption but it is also essential for the cell proliferation, nutrition and tissue vascularization of the skin substitutes seeded with patient cells [17]. The aim of this study was to develop a new production method of the foam-like chitosan/agarose-based wound dressing that can be potentially used in skin tissue regeneration. To obtain a foam-like structure, the freeze-drying method was combined with a gas foaming agent. The reaction of sodium bicarbonate with acetic acid generates carbon dioxide which promotes the formation of a highly porous structure: $CH_3COOH + NaHCO_3 \rightarrow CH_3COONa + H_2O + CO_2\uparrow$. To optimize the production process various concentrations of chitosan, sodium bicarbonate and acetic acid were investigated. The final biomaterials were obtained by lyophilization of the frozen samples. The described method, using sodium bicarbonate as the main agent in the foaming reaction, not only allowed obtaining a foam-like structure but also neutralized the acidic pH, making the material non-toxic and non-irritating to the skin. The obtained dressings were subjected to cytotoxicity tests with human fibroblasts using MTT assay and live-dead staining.

Materials and Methods

Materials

The chitosan powder (Mw 150kDa) of 75-85% deacetylation was purchased from Sigma-Aldrich Chemicals. The agarose powder with the 36°C melting point and ≤10% moisture content, sodium bicarbonate powder (MW 84.01 g/mol), phosphate-buffered saline, Eagle's Minimum Essential Medium (EMEM), penicillin, streptomycin, trypsin-EDTA, sodium dodecyl sulphate, Thiazolyl Blue Tetrazolium Bromide (MTT), Live/Dead Cell Double Staining Kit were supplied by Sigma-Aldrich Chemicals. The foetal bovine serum was obtained from Pan-Biotech, whereas the normal human skin fibroblast cell line (BJ) was obtained from American Type Culture Collections (ATCC).

Preparation of the biomaterials

To optimize the production process of wound dressings, chitosan, solvent (acetic acid) and gas-foaming agent (sodium bicarbonate) were applied at different concentrations. To prepare the foam-like materials, a predetermined amount of chitosan powder (based on the required concentration, see TABLE 1) was added to the acetic acid solution applied at a concentration of 0.5% (v/v) or 1% (v/v) respectively. A beaker with the dissolved chitosan was placed on a hot plate magnetic stirrer and the 2% (w/v) agarose was slowly added to the chitosan solution. The stirrer parameters were matched to the final dissolution of agarose (15 min, 300 rpm, 95°C). Then, the 1% (w/v) or 2% (w/v) sodium bicarbonate was added to the polysaccharide blend and mixed to obtain a foam-forming reaction. Having obtained a homogeneous mass, it was spread on the polystyrene surface. Finally, the produced samples were cooled down at 6°C before freezing at 80°C overnight. All the samples were lyophilized for 20 h in order to receive the foam-like biomaterials. A schematic figure of the preparation of chitosan/agarose-based biomaterials can be seen in FIG. 1. The fabricated B4 biomaterial revealing the most homogenous structure was visualized using the stereoscopic microscope (Olympus SZ61TR).

TABLE 1. Sample design and various components concentrations used during the production process of chitosan/agarose biomaterials.

	Concentrations (%)				
Sample designation	Chitosan (w/v)	Agarose (w/v)	Acetic acid (v/v)	Sodium bicarbonate (w/v)	Observation
B1	3	2	1	2	Chitosan cross-linking and uncontrolled gelation process resulting in inhomogeneous structure
B2	2	2	1	2	Chitosan cross-linking and uncontrolled gelation process resulting in inhomogeneous structure
B3	2	2	0.5	2	Chitosan cross-linking and uncontrolled gelation process resulting in inhomogeneous structure
B4	2	2	1	1	Homogeneous structure

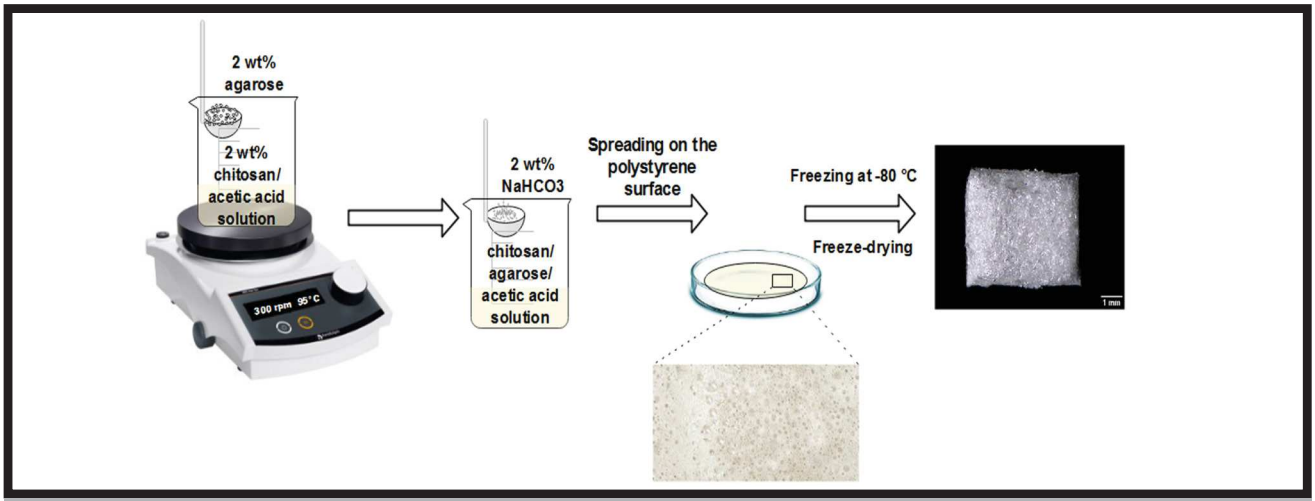

FIG. 1. Schematic representation of the preparation process of chitosan/agarose-based biomaterials.

Cell culture

Normal human skin fibroblasts (BJ cell line) used in this study were bought from ATCC. The line was drawn from skin taken from normal foreskin. The cells were cultured in flasks using Eagle's Minimum Essential Medium supplemented with 10% foetal bovine serum, streptomycin (100 U/ml) and penicillin (100U/ml) to obtain a complete culture medium. The parameters of the culture conditions were as follows: 37°C, 5% CO_2 and 95% of air humidity. The cells were cultured for a period sufficient to approx. 90% confluency and then sub-cultured to obtain a sufficient number of cells. The BJ fibroblasts from the passages 4 -6 were used in the experiments.

Cytotoxicity test using MTT assay

The *in vitro* cytotoxicity test was carried out in accordance with ISO 10993-5 standard using the fluid extracts of the materials. Briefly, the produced biomaterials were weighed and immersed in the EMEM culture medium at the ratio: 100 mg of the sample per 1 ml of the medium. To obtain the extracts the biomaterials were incubated for 24 h at 37°C. The polypropylene extract served as a negative control of cytotoxicity. To assess the cytotoxicity of the prepared biomaterials, the BJ cells were seeded in a 96-multiwell plate in 100 µl of EMEM at the concentration of 2 x 10^5 cells/well. After the 24-hour incubation, the culture medium was replaced with 100 µl of the prepared extracts and the plates were left in an incubator for further 24 hours. Afterwards, the extracts were removed from the wells and 100 µl of 1mg/ml MTT solution was added to each well. The cells were incubated with MTT for 3 h at 37°C and the 0.01% (w/v) sodium dodecyl sulphate solution prepared in 0.01 M HCl was added to lyse the cells and dissolve the insoluble formazan. The absorbance of the obtained solution was measured at 570 nm using the microplate reader (BioTek Synergy H4 Hybrid).

Cell viability determination by fluorescent technique

The non-toxic biomaterial (B4) selected in the MTT assay was subjected to the cytotoxicity evaluation based on fluorescent staining. The B4 l samples (5 mm x 5mm) were placed in the wells of a 48-multiwell plate and preincubated in the complete culture medium for 24 h. Then, 0.5 ml of the fibroblast suspension with the final concentration of 2 x 10^5 cells/ml was seeded on the biomaterials and incubated for 2 days at 37°C. The BJ cells were grown for 2 days at the surface of the biomaterials and stained with the Live/Dead Cell Double Staining Kit according to the manufacturer instructions, using two reagents: calcein-AM and propidium iodide. Calcein-AM is a fluorescent dye that penetrates through the cellular membrane into cells, and during the test intracellular esterases present in the live cells remove the acetomethoxy group from the calcein, giving green fluorescence. Propidium iodide is used to stain dead cells. It is not membrane-permeable so it cannot penetrate healthy cells. The effect of live-dead reagents on the cell cultures was analyzed for green and red fluorescence using the confocal laser scanning microscope (Olympus Fluoview equipped with FV1000). The dead cells became visible as red ones while the viable fibroblasts - green.

Statistical analysis

The MTT assay was repeated in 3 independent experiments and the obtained data were presented as the mean values ± standard deviation (SD). The differences regarding cell viability of the tested samples were considered statistically significant at $P < 0.05$. All the statistical calculations were performed with the GraphPad Prism 6.0.0 software. The One-way Anova followed by the Tukey's test was used to determine statistical differences by comparing the tested groups.

Results and Discussions

Structural and physicochemical characteristics of the biomaterial (e.g. porosityregarding pore size distribution, pore interconnectivity, pore volume and average pore size, surface chemical composition, wettability) is the key factor that must be considered while developing a new production method. The interconnected porous structure of biomaterials is required for the tissue ingrowth, cell penetration, waste and nutrient transport [18]. The highly porous structure is crucial for the material designed to act as a scaffold for skin cells (fibroblasts or keratinocytes). High porosity makes the biomaterials also more absorbent, which is a highly required property of the dressings to treat exudative wounds.

The foam-like structure of biomaterials was obtained by adding the gas foaming agent and applying the freeze-drying process (FIG. 2). The concentration of sodium bicarbonate (the gas foaming agent) used during the sample fabrication determined the amount of CO_2 gas release, which in turn resulted in the different porosity values of the resultant biomaterials. However, at the stage of adding sodium bicarbonate, the chitosan cross-linking and its gelation forming aggregates was noticed for the B1, B2, and B3 samples. This phenomenon resulted in their inhomogenous structure. It is known that a stable and firm gel is formed by neutralizing acidic chitosan solutions [19]. Thus, it was inferred that the excessive amount of sodium bicarbonate applied during the fabrication process caused the chitosan component gelation, due to the pH decrease. Among all the produced samples, only the B4 material, which was produced using the 1:1 ratio of the solvent and the gas-foaming agent, was characterized by the uniform and homogeneous structure (FIG. 2).

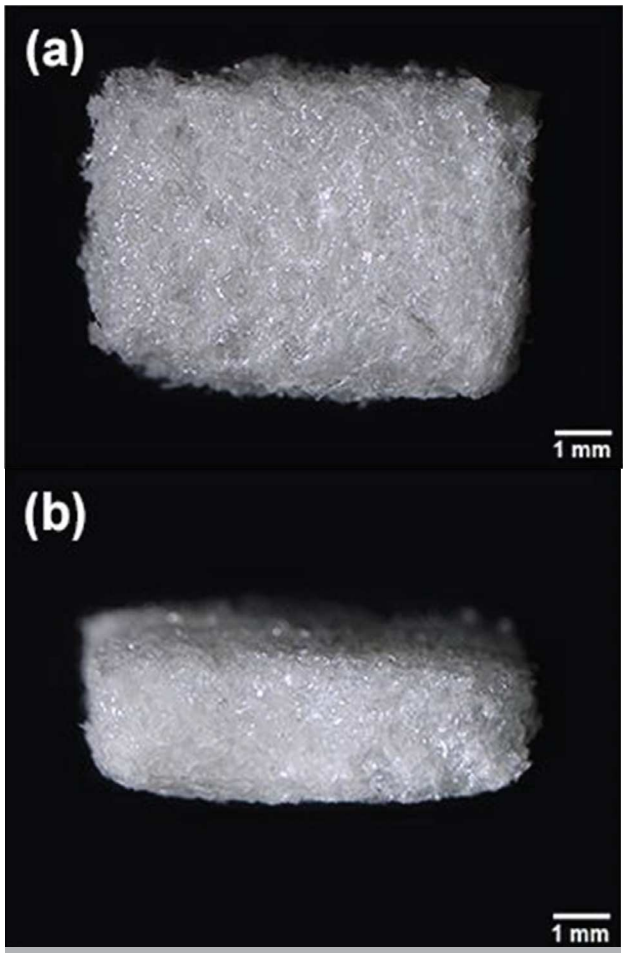

FIG. 2. The B4 foam-like biomaterial: (a) the top surface; (b) the cross-section.

The production method described in this work is unique because the individual components (chitosan, agarose), the gas foaming agent (sodium bicarbonate) and the solvent (acetic acid) were applied at concentrations that enabled the neutralization of the acidic chitosan solution. It was also possible to maintain the homogeneous structure without any traces of the chitosan precipitation or gelation. According to the available literature, other biomaterials based on agarose and chitosan were produced using two main approaches: the pH neutralization after the production process [20], or the production without the neutralization, which probably made the biomaterials toxic to the cells [21].

In this study, it was hypothesized that employing the foaming reaction and lyophilization may increase the specific surface area and the porosity of the resultant materials. The described method also enhances the material absorption capacity which is necessary to remove exudates from the wound. Nevertheless, future studies (e.g. the porosity liquid retention ability tests) need to be performed to confirm this assumption.

The conducted MTT assay showed that among all the tested biomaterials produced applying different concentrations of the components, only the B4 material was non-toxic to human skin fibroblasts (FIG. 3). Namely, although the chemical composition of all samples was the same, only the B4 biomaterial did not reduce cell viability. The B1-B3 biomaterials were characterized by the cell viability reduced to 8-11% as compared to the non-toxic polypropylene control. The viability of the fibroblast cell line exposed to the extract of the B4 material exceeded 92%. Cytotoxicity of the B1-3 samples may be explained by the excess of sodium bicarbonate in their structure which caused a pH significant increase the culture medium, exerting a lethal effect. According to the ISO standard, the biomaterial shall be considered non-toxic to the cells if the relative cell viability is over 70%, as compared to the control sample [22].

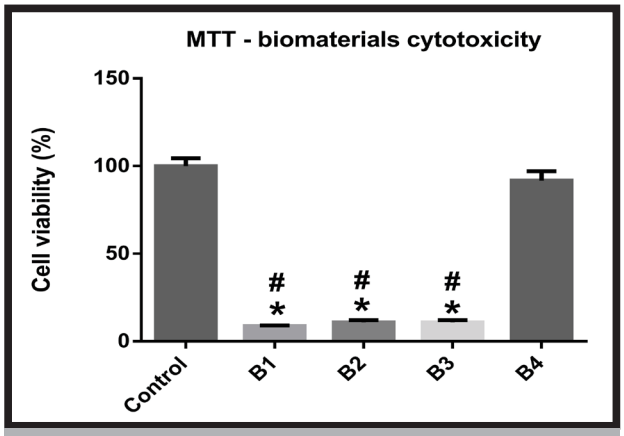

FIG. 3. Comparison of human fibroblasts viability after exposure to the extracts of the produced biomaterials based on the MTT assay; *statistically significant differences compared to the control (cells exposed to polypropylene extract), #statistically significant differences compared to the B4 material, $P < 0.05$, One-way Anova followed by Tukey's test.

Since the B4 biomaterial revealed both the optimal foam-like structure (FIG. 2) and non-toxicity against skin fibroblasts, it underwent the cytotoxicity test based on the live-dead fluorescent assay. The confocal microscope observation confirmed its non-toxicity against BJ cells (FIG. 4a and b). A monolayer of viable (green fluorescence) and well flattened cells with normal fibroblastic morphology were visible around the biomaterial (FIG. 4c and d) and no flattened fibroblasts were noticed on the top surface (FIG. 4a). The human fibroblasts seeded on the biomaterial were viable and showed green fluorescence. Still they were round and not attached to the sample surface, which may indicate that the B4 biomaterial did not support the attachment of adherent cells, despite its lack of toxicity. In addition, no dead cells stained with red were observed, confirming the high biocompatibility of the B4 sample.

The tested biomaterial can be potentially used as an external wound dressing providing the barrier function between the wound and the external environment. Moreover, due to its foam-like structure, it reveals the high absorption ability, which will remove the wound exudates [23]. Since the B4 biomaterial was proved to be non-toxic and unfavourable to the fibroblast adhesion, it may act as a promising temporary dressing to cover the wound. What is more, after the treatment it can be removed without causing trauma to the wound bed.

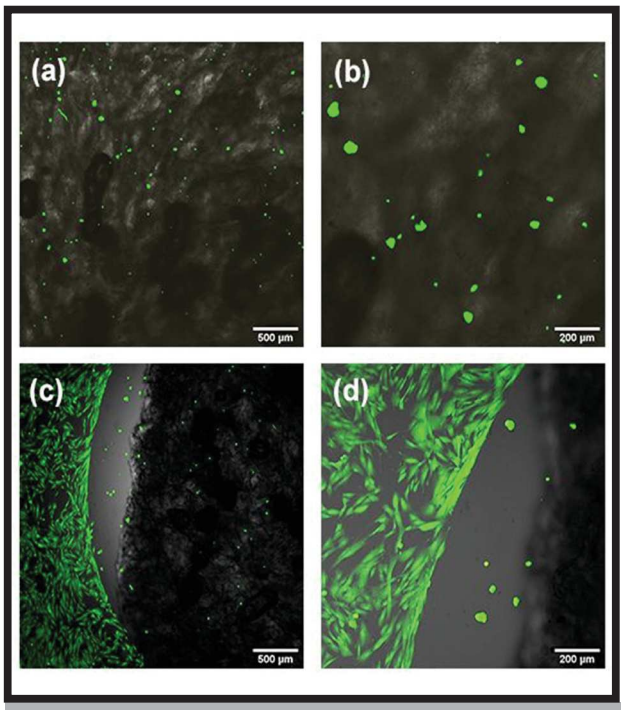

FIG. 4. Qualitative cytotoxicity evaluation of the B4 biomaterial: (a) and (b) live-dead staining of BJ cells grown directly on the biomaterial; (c) and (d) human fibroblasts cultured on the polystyrene next to the B4 biomaterial (merged images with green fluorescence – viable cells, red fluorescence – dead cells, Nomarski contrast highlights biomaterial structure).

Conclusions

The presented study demonstrated that adding sodium bicarbonate to the chitosan/agarose mixture prepared in acetic acid allows obtaining the foam-like structure of the resultant biomaterial. It was proved that the material is non-toxic when sodium bicarbonate and acidic acid are applied at the 1:1 ratio. The biomaterial with the most promising biomedical potential was produced using 2% w/v chitosan, 2% w/v agarose, 1% v/v CH_3COOH and 1% $NaHCO_3$. The described here production method, involving the chitosan and agarose dissolution in acetic acid and the use of the foaming agent, may be potentially applicable for fabricating foam-like skin wound dressings. The biomaterial developed via this method is non-toxic and at the same time its surface does not support cell adhesion. Therefore, the biggest advantage of this biomaterial will be the painless removal of wound dressings after the successful healing process. Nevertheless, to fully confirm the biomedical potential of the developed material, the further detailed analysis regarding its mechanical, antibacterial, and biodegradable properties is recommended.

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