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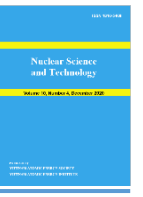

Radiation-crosslinked scaffolds from gelatin/CM-chitin and gelatin/CM-chitosan hydrogels for adipose-derived stem cell culture

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Abstract: Radiation technology has been providing a useful tool to modify polymers involving the radiation crosslinking of hydrogels. In the present work, two kinds of hydrogels composed gelatin and carboxymethyl chitosan (gelatin/CM-chitosan), and carboxymethyl chitin (gelatin/CM-chitin) were prepared by the radiation crosslinking. The resulting hydrogels were freeze-dried, sterilized under gamma-ray at a sterilization dose of 25 kGy, and characterized to be utilized as the scaffolds for culturing adipose-derived stem cells (hADSCs). The physicochemical properties, biodegradability, and cytotoxicity of these scaffolds were also investigated. The results indicated that both CM-chitosan and CM-chitin much improved the swelling capacity, porosity, and pore size of the gelatin-derived scaffolds. The swelling degree of gelatin/CM-chitosan and gelatin/CM-chitin scaffolds was about 7-9 g/g after 6 hrs immerging in PBS, and their porosity was about 70-73% with pore size ranging from 100 to 300 mm. The results also revealed that the compressive modulus of gelatin/CM-chitosan and gelatin/CM-chitin was 45.6 and 66.4 kPa, respectively, which were suitable for soft tissue engineering. Both scaffolds were enzymatically biodegradable by collagenase and non-cytotoxic for hADSCs with an RGR of ~97%. Thus, the resultant scaffolds can be suitably utilized for culturing hADSC in practice to regenerate soft tissues.

I. INTRODUCTION

Tissue engineering is a quickly enlarging interdisciplinary field that attempts to provide solutions for the regeneration of defected or damaged tissues. Designing of biologically active scaffolds with optimal characteristics is one of the most important factors for successful tissue engineering [1]. Recently, hydrogel has been acquired substantial attraction as an immense potential material for engineered tissue scaffolds due to their unique compositional and structural similarities to the natural extracellular matrix (ECM), which is an indispensable component to ensure the survival, communication, proliferation, and differentiation of the cells in tissues [2-4]. In practice, the hydrogel-scaffold materials in tissue engineering ought to meet several design criteria to mimic ECM, which is dependent on the cultured source cells and the targeting tissues. Alternatively, in the soft tissue-regeneration technique from the adult mesenchymal stem cells, a biomaterial scaffold should be owned to the functional specifications as follows: the hydrophilic 3D-

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microporous structure, porosity >70% and pore size ~100 – 300 μm, swelling degree ~6 – 12 g/g, compressive strength ~1 – 80 kPa, admissible biodegradability, non-cytotoxicity, and providing a good platform for cell adhesion, function, differentiation, and transplantation [3, 5-8]. In the last couple of years, the human adipose-derived stem cells (hADSCs), one exciting kind of the adult mesenchymal stem cell sources copiously found in the perivascular area of adipose tissues, have been examined for application in regeneration-tissue therapy due to their abundance, multipotent nature, facile isolation, and non-controversiality [7, 9]. In terms of conventional technology, the polymer-based mesenchymal stem cell culture scaffolds are hydrophilic hydrogels in the form with a 3D microporous structure, which were prepared by chemical crosslinking [3, 8, 9], ionizing radiation followed by freeze-drying treatment [2, 10-12]. Among them, the radiation-crosslinking approach is recognized as a potential way with some advantages such as no chemicals to initiate the reaction and the product sterilized at the same time [1, 13]. The crosslinking reaction in polymer solution occurred through the recombination of free radical macromolecules, which are produced by the indirect and direct effect of radiation interaction [10, 12]. The radiation-crosslinked gelatin is a promising cell culture scaffold due to its chemical resemblances to the ECM [11]. Despite the advantages, poorly mechanical and physicochemical properties, and enzymatic degradation beyond controllability have restricted its applications and levered the production of gelatin-based composite hydrogels [11, 12]. Because of the many inherent useful bio-properties of CM-chitosan and CM-chitin, natural polysaccharide derivatives of modified chitosan/chitin, it has been considered as the leading materials to make hydrogels for biomedical applications. So far, several novel hybrid scaffolds have been developed from a combination of gelatin/CM-chitosan [2, 11, 12] as well as gelatin/CM-chitin [4, 5] for the adult mesenchymal stem cell tissue engineering. However, studies on the effect of CM-chitosan and CM-chitin in mixture with gelatin on the features of hybrid scaffolds are still lacking. This work characterizes the scaffolds derived from gelatin/CM-chitosan and gelatin/CM-chitin hydrogels obtained by radiation-crosslinking in combination with freeze-drying and radiation sterilization for further applications.

II. MATERIALS AND METHODS

A. Materials

Three polymers including gelatin (CAS 9000-70-8, type A, source from porcine skin with bloom ~300, an average molecular weight ~100.000 Da, and pI 8-9), carboxymethyl-chitosan (CAS 83512-85-0, a deacetylation degree of ~95%, a molecular weight ~60.000 Da, and a substitute degree of 0.87), and carboxymethyl-chitin (CAS 52108-64-2, a deacetylation degree of ~95%, a molecular weight ~110.000 Da, and a substitute degree of 0.62) were purchased from Sigma-Aldrich, Merck, Germany. PBS buffer (pH 7.4) was supplied by Elabscience, USA. The tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was bought from Sigma Aldrich, USA. Dulbecco's Modified Eagle's Medium (DMEM)/F12 cell culture medium was purchased from Gibco Co., USA. The collagenase enzyme, type I (activity 2 U/ml) was a product of Sigma Aldrich, USA. Human adipose-derived stem cells (hADSCs) in the 4^{th} generation were provided by the Faculty of Biology and Biotechnology,

University of Science – Vietnam National University Ho Chi Minh City.

B. Methods

Preparation and sterilization of scaffolds: The solutions with a total polymer concentration 15% (w/v) of gelatin and both composites of gelatin/carboxymethyl-chitosan (G/CM-chitosan) and gelatin/carboxymethyl-chitin (G/CM-chitin) with the weight ratio of 8.5/1.5 were prepared by completely dissolving in distilled water at 50°C for 2 h. The solutions were poured into Petri dishes (diameter 10 cm, thickness 1 cm) and cooled to solidify in pseudo-gel at room temperature. The pseudo-gels were irradiated at a dose of ~35 kGy to obtain hydrogels with crosslinked bonds [10, 11]. The hydrogels were cut into cubic specimens 10×10×10 mm^3 in size and lyophilized (freeze-drying) to create porous scaffolds [5]. Afterward, the porous scaffolds were subjected to γ-irradiation with ~25 kGy for sterilization [1].

Swelling in PBS and mechanical property of scaffolds: Swelling behavior of porous scaffolds in PBS (0.1 M, pH 7.4) at 37°C as a function of immersion-time was evaluated by a weighting method and calculated using Eq. (1) as follows [3, 13]:

$$\text{Swelling degree (g/g)} = Ws/Wd \quad (1)$$

Where Ws and Wd are respectively the weights of swollen scaffolds and dried scaffolds.

The compressive modulus, a parameter representing the mechanical property of scaffolds, was measured using a texture tester (INSTRON 5582, USA) equipped with a 1000 N load cell. To determine the compress modulus, scaffolds were swollen in PBS for 24 h and cut into cylinders (diameter 10 mm, height 10 mm) and applied a constant stain rate of 60 mm/min [5, 11].

Porosity and pore size determination: The porous scaffolds in the dry state were cut into cubic pieces with the sizes of 10×10×10 mm^3 for measuring porosity. Microscopic structural morphology of the scaffold was observed by the scanning electron microscopy (S-4800, HITACHI, Japan) with an accelerating voltage of 10 kV and a magnification of 1000 for post sputter-coated samples. The pore sizes and morphological shapes of porous scaffolds were also determined using SEM images. The porosity of the scaffolds was measured by the liquid supplanted method [2, 3]. Briefly, the porosity is conventionally equal to the volume of trapped ethanol in the porous scaffolds. The scaffold samples were put into a measuring cylinder containing an exact volume of absolute ethanol (V1). After 24 h of soaking, read the mixture volume of ethanol with immersed scaffolds (V2). Afterward, the scaffold samples were taken out and the residual ethanol volume was determined (V3). The porosity (P, %) was calculated by the Eq. 2 as follows [2]:

$$P\ (\%) = (V1 - V3) \times 100/(V2 - V3) \quad (2)$$

***Biodegradation and cytotoxicity tests*:** Enzymatic degradation of the scaffolds was evaluated through the weight loss after immerging in PBS solution containing colagenase. Briefly, scaffold sample of about 40 – 60 mg (Wo) was incubated in 5 ml enzyme solution, which was prepared by diluting stock collagenase in PBS (0.1 M, pH 7.4) to an activity concentration of 2 U/ml. The biodegradation of scaffolds was carried out in vitro at 37°C for a period of 21 h. At destined time interludes, the remainder of the samples was removed to dry by freeze-drying and weighed (W1). The biodegradability was figured out using Eq. 3 [3, 11].

$$\text{Biodegradability (\%)} = (Wo - W1) \times 100/Wo \quad (3)$$

Cytotoxicity of the scaffolds was appraised by an indirect contact method through extracts and MTT assay according to ISO 10993-5:2009 [11]. The scaffold extraction was prepared by incubating the scaffolds in the DMEM/F12 medium containing 10% FBS and 1% specific antibody with a ratio of 3 cm^2/ml at 37°C for 24 h, then centrifuged to obtain extraction media. The hADSCs with a density of 10^5 cells/ml were incubated into the 96-well culture plate at 37°C, 5% CO_2 for 24 h. After that, the culture medium in the cell-incubated wells was replaced with the extraction media and then incubated for 24 h. The cells cultured with the DMEM/F12 medium set out as the negative control group and latex rubber extraction that was a cytotoxic compound was as a positive control group [9]. The relative growth rate (RGR, %) of the cells in treatments was determined using the MTT assay at 24 h and calculated according to Eq. 4 [4]. The absorbance of MTT-dyed cell suspensions was measured at the wavelength of 570 nm using a spectroscopy equipped microplate reader.

$$\text{RGR (\%)} = As \times 100/Anc \quad (4)$$

Where As and Anc are the optical density of the test sample and the negative control, respectively. The samples with an RGR value higher than 70% are considered non-cytotoxicity or quality [4].

III. RESULTS AND DISCUSSION

In these experiments, the polymeric formulation and the radiation-crosslinking dose were applied based on the findings as reported in the previous paper [10]. And the porous scaffolds from gelatin, gelatin/CM-chitin and gelatin/CM-chitosan hydrogels prepared by radiation-crosslinking in combination with freeze-drying and gamma irradiation sterilization were characterized.

A. *Swelling in PBS and mechanical property of scaffolds:*

The swelling behavior and compressive modulus of scaffolds are the principal parameters. The swelling ability of scaffolds is closely relative to the essential nutrition penetration, the infiltration of culturing cells, and the excretion of secretions [3]. Fig. 1 describes the swelling of the porous scaffolds in the PBS solution. The swelling degree of the gelatin scaffold was the lowest (about 4.8 g/g) in comparison to the G/CM-chitin (about 7.0 g/g) and the G/CM-chitosan (around 9.1 g/g). These differences may be caused by inconformity in crosslinking density and the number of hydrophilic groups in the scaffold, particularly the lower the crosslinking density and the higher the hydrophilic groups, the higher the swelling degree. Yang et al. [11] substantiated that CM-chitosan in a mixture of gelatin/CM-chitosan was able to improve the swelling degree of irradiation-crosslinked gelatin hydrogels based on reducing the crosslinking density and increasing the hydrophilic groups. In addition, Zhao et al. [13] supported that the irradiation-crosslinked extent of CM-chitin was higher than that of CM-chitosan, which could also be the basis for the explanation of the difference in swelling of the G/CM-chitosan and G/CM-chitin (Fig. 1).

In the more notable worth, the CM-chitosan and CM-chitin not only ameliorated to the swelling proportion but also improved the mechanical property of the resulted scaffolds from G/CM-chitosan and G/CM-chitin. The compressive modulus or stiffness of scaffolds plays a significant role in the

regulation of stem cell fate as well as adhesion, migration, and differentiation. The crosslinking density of the hydrogels is the main element to affect scaffold stiffness [6]. Fig. 2 shows the compressive modulus of the prepared scaffolds. The attained results indicated that the compressive modulus of the gelatin scaffold (about 167.5 kPa) was effectively diminished to 45.6 kPa and 66.4 kPa for G/CM-chitosan and G/CM-chitin scaffolds, respectively. The stiffness of the G/CM-chitosan and G/CM-chitin scaffolds was believed to be appropriate for the scaffold requirements in the soft tissue engineering (10 – 75 kPa in compressive modulus) [6, 11].

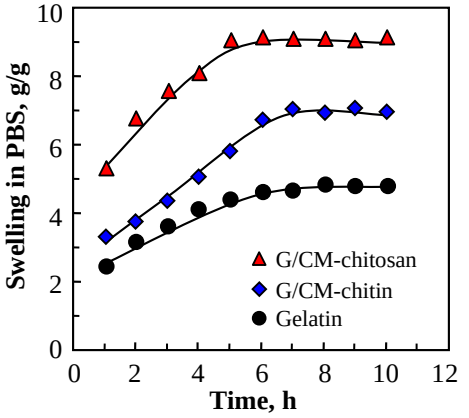

Fig. 1. Swelling degree of scaffolds in phosphate buffer solution as a function time

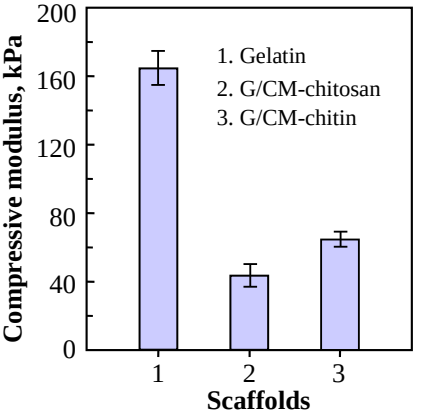

Fig. 2. Compressive modulus of the different scaffolds

B. *Porosity and pore size of scaffolds:*

Table I. The porosity and pore size of the scaffolds.

Scaffolds	Porosity, %	Pore size, μm
Gelatin	$50.0^{b} \pm 3.7$	300 – 450
G/CM-chitosan	$73.3^{a} \pm 4.2$	100 – 250
G/CM-chitin	$70.6^{a} \pm 5.8$	120 – 300

In a column, the means followed by the same letter are not different significantly ($P < 0.05$).

The scaffolds with high porosity and adequate pore size are required to provide a microenvironment for cell-cell and cell-surrounding interactions. The interactions influence an attachment, migration, and penetration of cellular in-growth as well as vascularization in them [8]. The microstructural properties, ascertained from SEM images such as the pore sizes and porosity of the produced scaffolds, were audibly manifested in Table 1 and Fig. 3. The SEM images revealed that the gelatin scaffold possessed a large pore size (300 – 450 μm), thick walls, and almost no interconnecting channels among the pores. Meanwhile, both the scaffolds of the G/CM-chitosan and G/CM-chitin owned a pore size in the range of 100 – 300 μm with thin pore walls and abundance of interconnects. Furthermore, these scaffolds had a significantly higher porosity (around 70 – 73%) than that of the gelatin one

(about 50%). From these results, it was perspicuous that CM-chitin and CM-chitosan in blend with gelatin had a satisfactory effect on the porous microstructural properties of the gelatin scaffold, which could also be mainly due to the change in a crosslinking behavior. More specifically, the porous properties of G/CM-chitosan and G/CM-chitin scaffolds were adequately assumed to culture the hADSCs and generate soft tissue engineering [3, 5, 7].

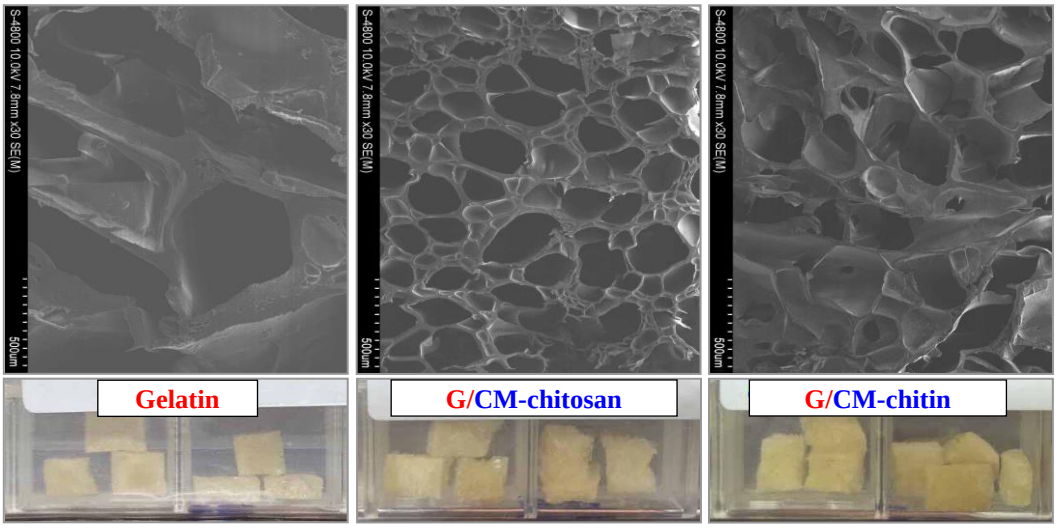

Fig. 3. SEM images and photographs of the scaffolds

C. *Biodegradation and cytotoxicity of the scaffolds*

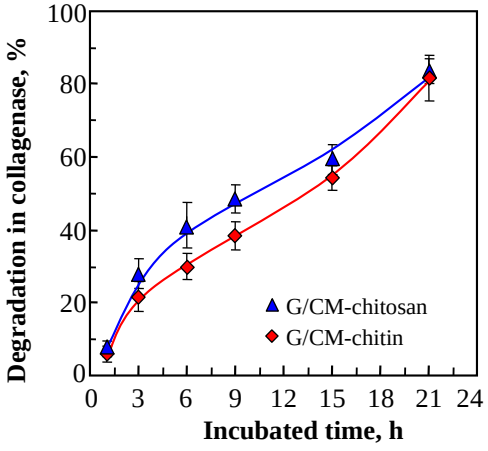

Fig. 4. Enzymatic degradation of the scaffolds in collagenase solution

To be considered and applied in tissue engineering as a scaffold, the biodegradability and non-cytotoxicity are fundamental requirements that need to be satisfied. According to Yang et al [11], the collagenase enzyme is commonly used for evaluation of scaffold biodegradability *in vitro* due to it is able to degrade hydrogels containing proteins. Fig. 4 illustrates the relationship of the biodegradability of the scaffolds with inoculation time. The gained results showed that the scaffolds were to be

almost completed degradation by collagenase enzyme activity. After 21 h incubation, the biodegradability of both the scaffolds was around 81 – 83% evenly. However, the biodegradability of the G/CM-chitosan was slightly faster than that of the G/CM-chitin in the first 15 h. The reason could be explained that the swelling rate and the porosity of the G/CM-chitosan scaffold were moderately higher than that of the G/CM-chitin one, leading to a more favorable invasion of the enzyme [7, 11].

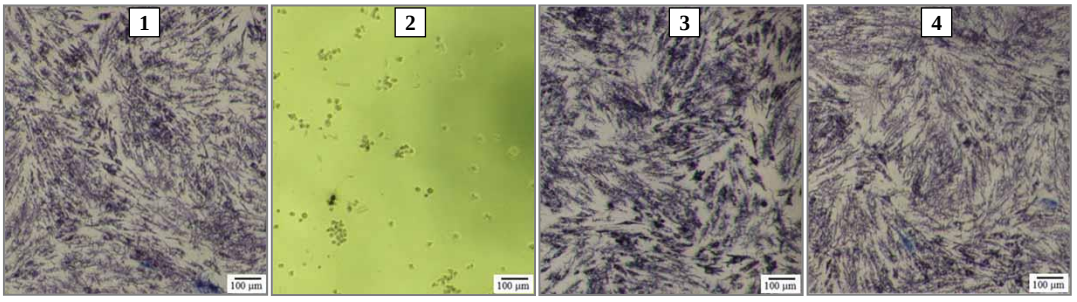

Fig. 5. The confocal micrographs of hADSCs cultured in extraction media and MTT tested: 1) Fresh culture medium (control), 2) Latex extract (control +), 3) G/CM-chitosan scaffold extract, and 4) G/CM-chitin scaffold extract

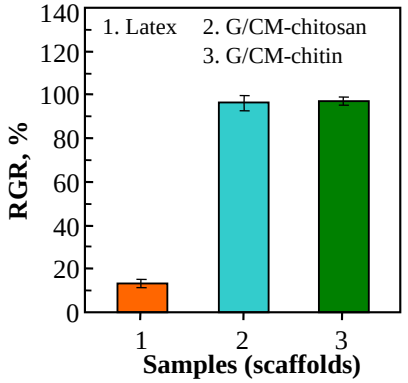

Fig. 6. Relative growth rate of hADSCs in extraction media

Cytotoxicities of G/CM-chitosan and CM-chitin based scaffolds were assessed according to ISO 10993-5:2009 by incubating hADSCs with the extracts of the scaffolds [7, 9]. As a well-known, the MTT assay is a colorimetric test method based on the color intensity of a purple formazan crystal, which depends on the living cell's metabolic activity. The morphology properties and density of cells from the testing extracts after performing MTT assay are presented in Fig. 5. The morphology and cell density for treatments of the G/CM-chitosan and G/CM-chitin were seemingly unchanged from that of the negative control group, while the cells with shrinkage, flaking off, and almost no forming formazan crystals were found in the positive control (latex extract).

From these results, it may be suggested that the G/CM-chitosan and G/CM-chitin scaffold exhibited non-cytotoxicity for the hADSCs. Concerning cell ingrowths, the RGR% values of the hADSCs after an incubation period of 24 h with the different extracts are described in Fig. 6. The RGR values were of 96.2% and 97.1% respectively

for G/CM-chitosan and G/CM-chitin samples, appropriated 7.3-fold higher than that of the positive control group. These values could be assorted to scoring 1 (non-cytotoxicity) and qualified [4, 9]. The superb non-cytotoxicity or cytocompatibility of these scaffolds was assigned to the biocompatibility of the gelatin, CM-chitosan, and CM-chitin [11], as well as a suitable 3 D microenvironment provided by the scaffold materials for the hADSCs [3].

IV. CONCLUSIONS

The mixture scaffolds of gelatin/CM-chitosan and gelatin/CM-chitin were successfully fabricated using radiation-crosslinking combined with freeze-drying. The performance in the same behaviors of ameliorating the inherent properties of the gelatin scaffold by CM-chitosan and CM-chitin was evaluated and compared. Both scaffold materials of gelatin/CM-chitosan and gelatin/CM-chitin possessed the swelling degree, porosity and pore size, mechanical strength, biodegradability, and non-cytotoxicity in suitability for human adipose-derived stem cell culture for soft tissue regeneration. The entire results of this work propound a potential of the scaffolds in the application to hADSC culture. Nevertheless, other prime biological-features, such as proliferation and differentiation of hADSCs on the scaffolds should be further explored.

ACKNOWLEDGEMENTS

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