

Evaluation of Chitosan /Organoclay Composite as Bone Tissue Engineering Scaffold

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ABSTRACT

In this study, a scaffold composed of chitosan (C) and organoclay (OC) was prepared by utilizing freeze-drying method. The composition of resulting composite scaffold was evaluated using FTIR and SEM. The in vivo cytocompatibility of the composite was evaluated in vivo, by seeding MC3T3-E1 cells on composite scaffolds. In-vitro cell viability and proliferation were investigated using the MTT assay, while cell-scaffold constructs were evaluated using scanning electron microscopy. Preliminary in vivo experiments were also performed to evaluate tissue compatibility and ectopic bone-forming potential of C/OC composite scaffolds with MC3T3-E1 cells. The findings suggest that C/OC construct supported the proliferation of MC3T3-E1 cells, presented histobiocompatibility and promoted formation of calcified matrix. Based on the results, we conclude that the characterized C/OC composite system may have potential for use in bone tissue engineering applications.

Keywords:

Bone tissue engineering; Chitosan; Organoclay; Composite scaffold; Organically modified calcium-smectite

INTRODUCTION

The important stage of tissue engineering is choosing the best material to create the most suitable template. Scaffolds should be porous, biocompatible, biodegradable and have sufficient mechanical properties. Furthermore, they also should induce cellular attachment, proliferation and differentiation.

A variety of natural and synthetic polymers have been widely used in tissue engineering studies to create the best tissue scaffold. Natural polymers have been receiving a lot of attention in recent years [1,2]. Among numerous natural polymers, chitosan is considered as a promising material in the synthesis of scaffolds for bone tissue engineering. The characteristic properties which make chitosan useful in tissue engineering applications are its similarity to glycosaminoglycans (GAGs), hydrophilicity, biocompatibility and biodegradability [3,4]. Chitosan can be cast in different forms such as sponges, foams, gels, fibers and so on [5]. Thanks to these properties, it facilitates the attachment of cells and promote bone growth. Despite its advantageous structure, because of the unfavorable mechanical property

and bioactivity of chitosan, it is inconvenient to use it as bone tissue templates as its low mechanical strength cannot match the demand of bone tissue applications in many cases [6,7]. In this regard, incorporation of chitosan with a filler material is an effective way to improve its mechanical properties and biological activity [8]. The combination of polymers and inorganic fillers seem to be a promising approach, because they exhibit remarkable improvement in material properties compared to the free polymers [9].

As a filler, clay minerals are favorable candidates for tissue engineering studies due to their excellent properties such as good biocompatibility, high cation exchange capacity and promise for controlled release [10]. From the economical point of view, they are relatively low cost materials due to their abundance in nature. In order to expand the interlayer distance of clay minerals, they can be modified with organic substances. The obtaining structure is called as 'organo-clay'. The resulting structure can be incorporated with organic materials much easier than that of pure clay minerals.

Article History:

Received: 2017/02/07

Accepted: 2017/04/10

Online: 2017/06/30

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The objectives of this work were to report the fabrication and characterization of the developed porous C-based biocomposite with improved mechanical properties and examine the feasibility of using the obtained material as bone tissue engineered scaffold by conducting cell culture study.

MATERIALS AND METHODS

Chitosan powder ($M_r = \sim 400000$; degree of deacetylation $>85\%$) was purchased from Fluka Chemical Company (Milwaukee, WI). Anilinium modified calcium-smectite (127 meq of CEC/100g) was gifted by Dr. Sibel Uzun. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was supplied from Sigma Aldrich Company except otherwise stated.

2 % chitosan in aqueous acetic acid solution was prepared and organically modified smectite powder was added under continuous mixing and stirring for 12 h [C/OC, 9/1 (w/w)]. Subsequently, the obtained mixture was casted on cell culture dishes and stored in a freezer at -40°C for 12 h. The samples were transferred into a freeze dryer to obtain a porous structure (Alpha 1-4 LD, Christ, Osterode am Harz, Germany) for 24 h and then neutralized by immersion in a 1 M NaOH solution. The samples were rinsed three times in double distilled water and lyophilised again. Finally, the samples were cut into $4 \times 4 \times 4$ mm cubes using surgical blade. The structural characterization of C/OC sponges was carried out by means of IR spectroscopy and SEM.

MC3T3-E1 cells were cultured in the α -Modified Eagle's minimal essential medium (α -MEM), supplemented with 10 % fetal bovine serum (FBS), and 1% penicillin-streptomycin (standart medium; SM) acquired from Sigma Chemical Company. Cells at $\sim 90\%$ confluency were digested using trypsin/EDTA. Cells were suspended in culture medium ($1.0 \times 10^7/\text{mL}$) and the cells were seeded onto each sponge. The loaded-scaffolds were agitated at 37°C for 30 min on a shaker at low speed to allow cells to adhere and then they were transferred in a new culture plate. The culture was maintained in osteogenic medium (SM supplemented 10 nM dexamethasone, 50 mg/mL L-ascorbic acid and 10 mM β -glycerophosphate) at 37°C in 5% CO_2 humidified atmosphere for up to 4 weeks.

Cell morphology on the sponges was inspected by SEM. The samples were washed with PBS and fixed in 2.5% glutaraldehyde solution for 12 h at 4°C . After fixation, the samples were rinsed twice in PBS and dehydrated with increasing concentrations of ethanol in water. The air-dried samples were sputter coated with palladium and analyzed by SEM (400F Field Emission, QUANTA, Hillsboro, OR).

MTT was used to assess cell viability. This assay is ba-

sed on mitochondrial redox reaction. A pale yellow MTT reagent is reduced to a dark blue formazan crystal by mitochondrial dehydrogenase activity of viable cells [11]. Briefly, MTT solution was prepared at 5 mg/mL in PBS and required amount of MTT solution was added to each well containing cell-sponge constructs. After an incubation of 4 h at 37°C and 5% CO_2 to allow MTT formazan formation. The reduced insoluble formazan crystals were observed under an inverted microscope (Nikon TS 100, Tokyo, Japan). After removing the supernatant, the formazan crystals were solubilized in 0.1 N HCl. The absorbance intensity was measured using a microplate reader at 570 nm.

The cell loaded C/OC composite sponges were implanted into the epigastric fasciovascular flap of rats under aseptic conditions to investigate their tissue compatibility and ectopic bone forming capacity. Twelve adult male Wistar rats weighing 200-300 g were used at predetermined time points. All procedures were performed according to the standards of international regulations. The cell loaded-sponges were maintained in a humidified incubator at 37°C with 5% CO_2 overnight before implantation. The samples were implanted into fasciovascular flaps through a 2-3 cm long mid-line incision (Cell free C/OC sponges used as controls) following anaesthetization with Avertin (300 mg/kg; 1.25%). Animals were sacrificed at days 7, 14, and 28 and the implants with surrounding tissue were harvested carefully from the epigastric area together. All the explants were fixed with 2.5% glutaraldehyde for 10 min and then immersed in 0.1 M potassium phosphate buffer (pH 7.4) containing 15% sucrose for 15 min. Finally, the specimens were frozen after embedding in tissue freezing medium (Tissue-Tek, Sakura Finetek, The Netherlands) for 30 minutes and frozen at -80°C . The tissue blocks were cut into $5 \mu\text{m}$ sections using a cryostat (Leica CM 1900, Wetzlar, Germany) and stained with hematoxyline and eosin (H&E) and von Kossa for histological evaluation.

RESULTS AND DISCUSSION

Structural evaluation of composite scaffold

FTIR was used to determine the chemical interactions between C, OC and C/OC composite scaffolds. The spectra of pure chitosan exhibits characteristic peaks at 3424 cm^{-1} (O-H stretch), 2923 cm^{-1} (C-H stretch), 1637 cm^{-1} (amide I) and 1071 cm^{-1} (C-O stretch) (Fig. 1). OC exhibits its characteristic bands at 3626 cm^{-1} (O-H stretch), 2926 and 1637 cm^{-1} (N-H stretch) and 1036 cm^{-1} (Si-O stretch). The spectrums of C/OC includes all the specific absorption peaks of C and OC, indicating that C/OC scaffold was produced successfully.

Fabrication of three-dimensional porous scaffolds with higher porosities having interconnected pore structure is really important, as these properties have an impact

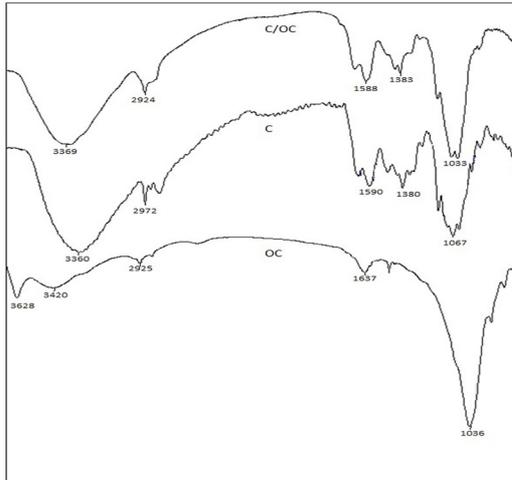


Figure 1. FTIR spectra of C, OC and C/OC composite scaffold.

on cellular behaviour like cell attachment, proliferation and differentiation [12]. The porous structure and surface morphology of the scaffolds were studied using SEM. SEM micrographs indicated that the composite scaffold obtained by freeze drying method had highly heterogeneous porous structure with interconnected architecture. The SEM image also showed that the pore size of composite scaffold was about 50-200 μm which allow cell-cell communication, cell infiltration, growth, and also diffusion of nutrients (Fig. 2) [13].

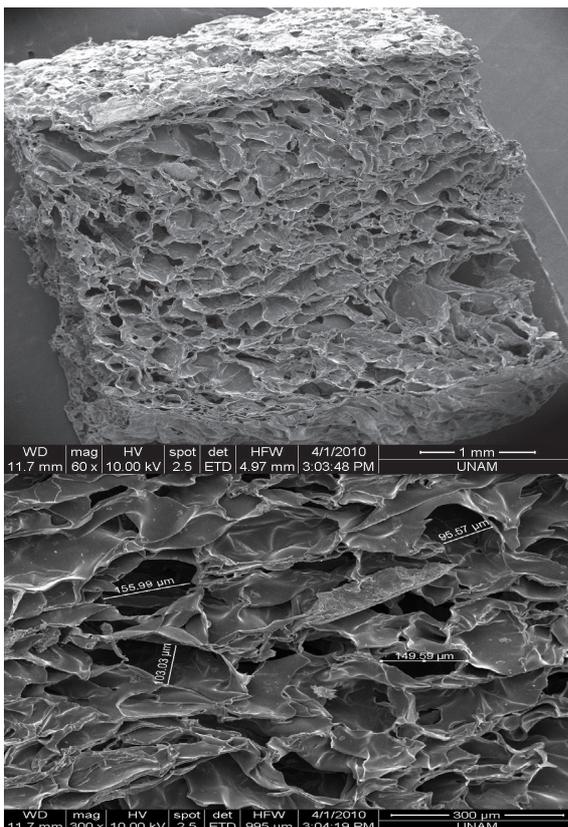


Figure 2. SEM images of acellular composite scaffold.

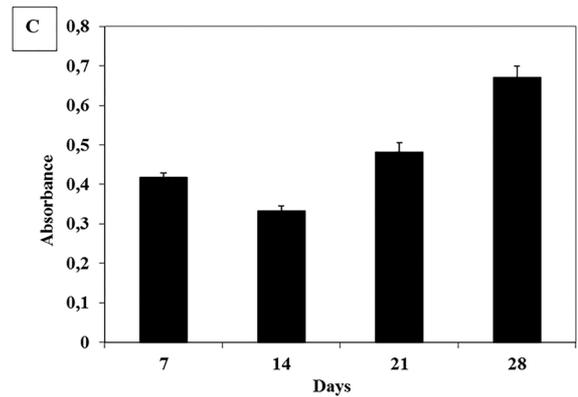
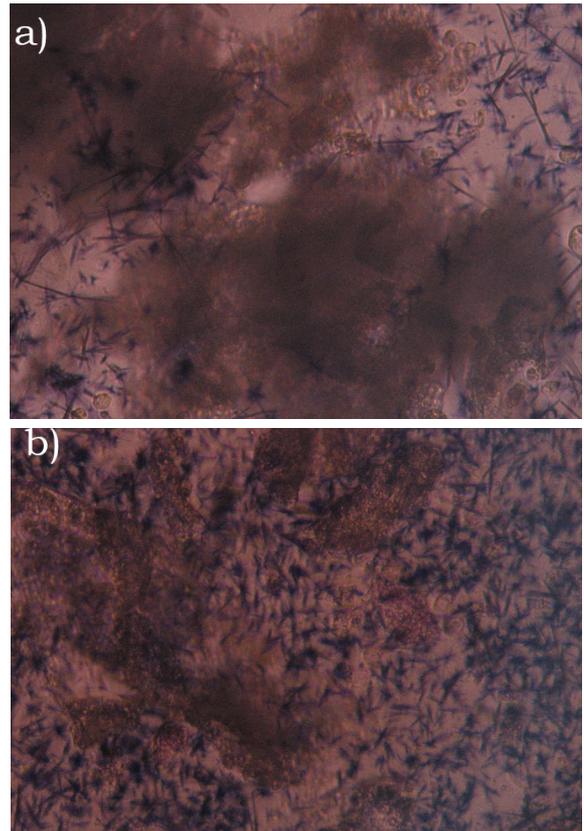


Figure 3. MTT formazan crystals inside sponge pores after 14 (A) and 28 (B) days. Mitochondrial activity of cells cultured on the composite scaffolds (C) (n=3).

Cell viability and proliferation on composite scaffolds

The assessment of viability and proliferation of cells on the material is very important for the determination of the feasibility of materials in tissue engineering applications. There are different methods available to investigate this feature, such as MTT and XTT. In this study, MTT assay was performed to determine the viability of cells on the scaffolds. Firstly, the MTT activity of the seeded cells was assessed qualitatively. Phase-contrast imaging showed that the formazan crystals were formed and clearly visible (Fig. 3 (A, B)). The intensity of formazan

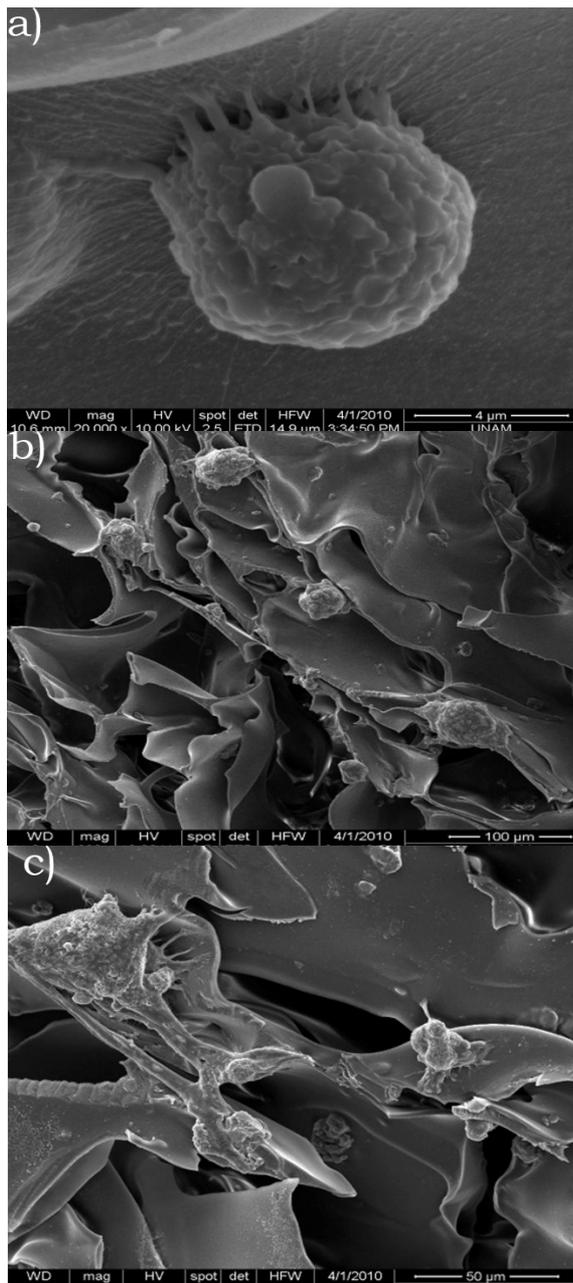


Figure 4. SEM micrographs of cell-loaded C/OC composite scaffolds at days 7 (A, B), and 14 (C).

crystals increased with culturing time. Moreover, the distribution of crystals were homogenous, indicating that the distribution of cells inside the scaffold was uniform. Quantitative data obtained spectrophotometrically revealed that MTT activity of MC3T3-E1 cultured on the scaffolds gradually increased from day 14 to day 28, indicating that cells were viable and proliferated within the scaffolds (Fig. 3C).

SEM was performed to evaluate the attachment and proliferation of cells on the composite scaffolds (Figure 4). SEM observations demonstrated that cells were attached to the scaffold surface in rounded shape on day 7 (Fig.

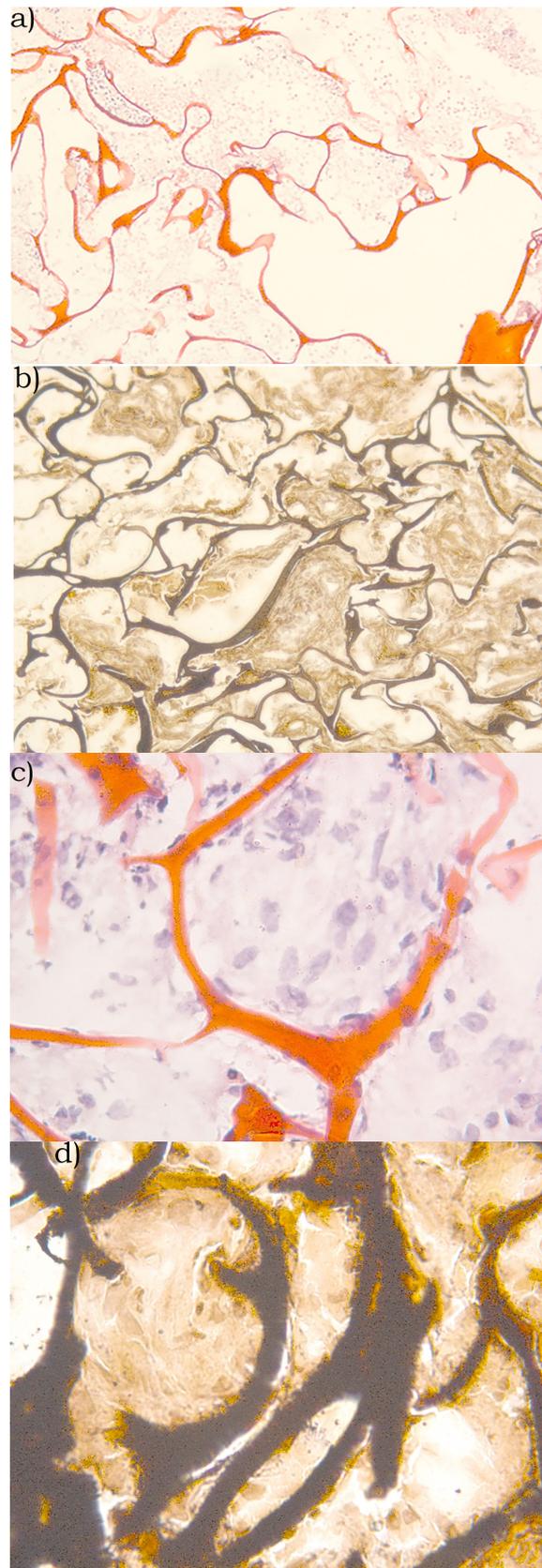


Figure 5. H&E staining of cell-seeded C/OC scaffolds at days 14 (A) and 28 (C); von Kossa staining of cell-seeded C/OC scaffolds at days 14 (B) and 28 (D).

4A). This image also confirmed that cells migrated and proliferated on the composite scaffold with time, and also the cell-pseudopodia have connected with the matrices, indicating that the developed composite scaffold shows no toxic effect to the cells and it is a convenient template as bone tissue engineering scaffold. SEM micrographs also revealed that the composite scaffold preserved the porous structure during the experimental periods.

In vivo findings

In vivo experiment was performed to determine the effect of the cell-loaded scaffolds on the formation of ectopic bone-like tissue. Although acute inflammatory reaction was observed at the first week post-implantation, cell-loaded scaffold did not cause a significant reaction, indicating that the material was biocompatible. The H&E staining was performed to document the formation of new tissue. Histological observations confirmed that cells attached and proliferated on the scaffold and bone-like tissue was formed by the time.

The cell-loaded scaffold was filled with fibrous connective tissues (Fig. 5 A, C). Von Kossa or Alizarin Red S are generally used to detect bone tissue development [13]. In this study, in vivo differentiation and calcification of MC3T3-E1 cells were evaluated by von Kossa staining. Von Kossa staining was performed on the 14-day and 28-day specimens. The obtained light microscopy images show the presence of calcium produced by the cells. Von Kossa was visible starting from the second week of implantation and gradually increased up to 4 weeks. The gained data indicates that the scaffold are able to provide an osteogenic environment to MC3T3-E1 cells and hence it can be used as bone tissue engineering scaffold.

CONCLUSION

Various composite materials have been widely investigated to create an ideal hard tissue template. In this study, the C/OC composite scaffold was successfully produced and characterized. The findings of this study suggest that obtained scaffold provides a suitable environment to MC3T3-E1 cells. Owing to the biocompatibility and sufficient porosity of the developed organic-inorganic composite system which supports cell attachment and proliferation, we conclude that it can be a promising template for bone engineering applications.

ACKNOWLEDGMENTS

I wish to express my deepest and sincere gratitude to Professors Y. Murat Elçin and A. Eser Elçin for their guidance, and for giving me the opportunity to completion of this study at ElcinLab. I also thank Dr. Sibel Uzun for providing me the organoclay.

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