A Biodegradable Composite Scaffold Prepared Through Freeze-Drying Method for Tissue Engineering Application

Shima Ghanavati Nasab and Abbas Teimouri*

Department of chemistry, Payame Noor University, Iran

Submission: June 29, 2017; Published: July 14, 2017

*Corresponding author: Abbas Teimouri, Department of Chemistry, Payame Noor University, P.O. Box 81395-671, Iran, Tel: +98 31 33521804; Fax: +98 31 33521802; Email: a.teimouri@pnu.ac.ir, a.teimoory@yahoo.com

Abstract

In the present study, a novel scaffold containing chitosan (CTS), Montmorillonite (MMT) and Nano Zirconia (Nano ZrO$_2$) was prepared by the freeze drying method. The CTS/MMT/Nano ZrO$_2$ composite was characterized by SEM, XRD, TGA, BET and FT-IR studies. Cytocompatibility of the CTS/MMT/Nano ZrO$_2$ scaffold was assessed by MTT assay, revealing non-toxicity to the HGF cells. Thus, we suggest CTS/MMT/Nano ZrO$_2$ composite scaffold as a potential candidate for tissue engineering.

Keywords: Chitosan; Biomaterials; Nano composites; Composite materials; Tissue engineering; Montmorillonite; Nano ZrO$_2$

Abbreviations: MMT: Montmorillonite; XRD: X-ray Diffractometer; TG: Thermo Gravimetric; SEM: Scanning Electron Microscope.

Introduction

In recent years, many attempts have been made to replace petrochemical products by biodegradable components. The most challenging part of this approach is to obtain bio-based materials with features equal to those of entirely synthetic products [1]. Biopolymers have privileges such as biodegradability and structural groups similar to natural extra cellular components [2]. Chitosan is a biopolymer derived from deacetylation of chitin and considered as an appropriate functional material for biomedical applications because of such great qualities as biocompatibility, non-anti genicity, biodegradability, antibacterial, blood coagulation and high mechanical strength, thereby making it suitable for tissue engineering [3-8]. To improve mechanical strength, chemical properties, dimensional stability and toughness of chitosan, it can be combined with clays such as Na-montmorillonite [9]. Orbio-inert ceramics like zirconia [10].

Montmorillonite (MMT) is a kind of natural 2:1 type layered clay mineral. With only a low amount of MMT, the mechanical properties and solvent resistance of the composites can be improved largely [11-14]. Zirconia (ZrO$_2$), a highly biocompatible ceramic and a chemically inert inorganic metal oxide with high stability, can increase the properties of chitosan upon the formation of the ZrO$_2$/chitosan composite [15-17]. At tissue level, zirconia has been discovered to be as biocompatible as titanium. Cultured osteoblasts are proliferated and differentiated on zirconia without producing any detrimental reaction [18].

In vivo studies have shown that ZrO$_2$ implants perform great osteointegration and zirconium-related materials, such as zirconia ceramics and coatings, have been used as bone implant materials [19]. In continuation of our recent study on the construction of composite scaffolds [20-22], we concentrated on the preparation and characterization of nano composite scaffolds CTS/MMT/Nano ZrO$_2$ to provide potential prospects of this nano composite for biomedical applications.

Materials and Methods

Chitosan (medium molecular weight) and Glutaraldehyde were purchased from Sigma-Aldrich. Montmorillonite was obtained from Shandong Longfeng Montmorillonite Co., China. Zirconium oxide nano powder was purchased from China Changsha Zhonglong Chemical (Group) Co., Ltd.
Preparation of CTS/MMT/ZrO$_2$ Nanocomposite Scaffold: The Chitosan (CTS), Montmorillonite and Nano Zirconia (ZrO$_2$) composite scaffold was prepared by the freeze-drying method. To summarize, chitosan 2% (w/v) was dissolved in 1% acetic acid solution and then added to MMT/ZrO$_2$ (the amount of MMT was twice that of Nano ZrO$_2$) mixture suspension for 5 h to obtain the nano composites. After 1 h, 25% (v/v) Glutaraldehyde (for cross linking) was added to the mixture in a 1:32 volume ratio and stirred for another 1 h. Finally, the prepared solution was poured into a pre-cooled 24-well plate, frozen overnight at -80°C and then freeze dried in a lyophilizer for 24 h.

Evaluation of CTS/MMT/ZrO$_2$ nano composite scaffold properties: The structural morphology of the samples was evaluated using scanning electron microscope (SEM). The samples were analyzed using Philips X'PERT MPD X-ray diffractometer (XRD) with Cu kα (1.5405 Å). A JASCO FT/IR-680 PLUS spectrometer was used to record IR spectra using KBr pellets. The BET specific surface areas and BJH pore size distribution of the samples were determined by adsorption-desorption of nitrogen at liquid nitrogen temperature by using a Series BELSORP 18. To evaluate the weight loss of the CTS/MMT/ZrO$_2$ composite scaffold during thermo gravimetric (TG) analysis, a test was performed using a DuPont TGA 951 at temperatures ranging from room temperature to 1000 °C in air and at a heating rate of 10 °C/min.

Bioactive and cell adhesion study in vitro: The composite scaffolds were soaked in SBF according to the procedure described in Kokubo et al. [23]. Fibroblast cells were cultivated in culture flasks for 2 weeks, and cell suspension 2×105 cells/cm$^2$ was seeded in scaffolds and cultured in 24-well plates. Cell-scaffolds were cultured in a humidified incubator. After 72 h, the cell-scaffolds were taken out and observed by SEM.

Results and Discussion

X-ray diffraction studies: As can be seen in (Figure 1) pure MMT typically showed a diffraction peak at 6.94°, while for CTS, the maximum was observed at 19°. In the XRD of ZrO$_2$, the sharp lines at 30.4, 51.0 and 60.2° were related to its tetragonal phase. Also, the XRD studies of CTS/MMT/ZrO$_2$ nano composite scaffold Figure 1a showed the characteristic peaks of chitosan (2θ=19° and 22.5°), MMT (2θ=28°) and ZrO$_2$ (2θ=51° and 60.2°), there by suggesting the presence of them in the scaffold.

FT-IR analysis: Figure 2 shows the FTIR spectra of pure CTS, pure MMT, pure ZrO$_2$ and the prepared CTS/MMT/ZrO$_2$ nano composite scaffolds. The IR spectra of pure MMT represented the typical band at 3363 cm$^{-1}$, which was related to the stretching vibrations of the N-H and OH bonds. Also, the band at 1653 cm$^{-1}$ was related to the C=O bond of the acetyl group, while the band at 1070 cm$^{-1}$ corresponded to C-O-C bond [24]. The FTIR spectra of ZrO$_2$ indicated a significant band at 542 cm$^{-1}$ was assigned to the vibration of the Zr-O bond. From Figure 2d, it could be understood that the bands in CTS (O-H and N-H stretching), which could be seen to overlap with the bands of MMT (-OH was stretching of H$_2$O) at 3424 cm$^{-1}$, in addition to the band around 542 cm$^{-1}$, which was related to ZrO$_2$, could be observed in the IR spectra of the nano composite.

SEM analysis: The external morphology of CTS/MMT/ZrO$_2$ scaffold was investigated by SEM. The results gave a view on the porous structure of the scaffold and the distribution of pores. In this study, SEM was used to indicate any morphological changes in CTS/MMT/ZrO$_2$ scaffold surface after synthesis. (Figure 3) indicates the SEM images of CTS

- MMT
- ZrO$_2$
- CTS/MMT/ZrO$_2$ scaffold
- Chitosan.

Due to its extensive surface, could act as a bed/support for MMT and ZrO$_2$. It could be seen from (Figure 3d) that MMT...
and ZrO$_2$ were scattered on the surface of CTS. The SEM image showed that the formation of scaffold was favoured, confirming both the FT-IR and XRD analysis results.

**BET Analysis:** The nitrogen adsorption/desorption isotherm and the pore size distribution (inset) is shown in (Figure 4). The surface area was calculated by applying the BET equation to the isotherm [25] and the pore size distribution was estimated by BJH method [26]. The BET surface area, pore volume and average pore diameter were 12.180 m$^2$/g, 0.014 cm$^3$ g$^{-1}$ and 0.994 nm, respectively. The sample was also mesoporous with N$_2$ adsorption-desorption isotherms of type IV and a H3 hysteresis loop according to IUPAC classification. The Type H3 loop, which does not display any limiting adsorption at high p/p$_0$, is typically seen with the aggregates of plate-like particles giving rise to slit-shaped pores [27].

**Thermal degradation:** Thermal degradation of the scaffold was carried out using TGA (Figure 5). It can be seen from (Figure 5a) and (Figure 5b), the pure MMT and CTS/MMT began to decompose at around 450°C and 200°C, respectively. They were decomposed completely by the time the temperature reached 650°C. The thermal degradation of CTS/MMT/Nano ZrO$_2$ scaffold began to decompose at about 270°C and decomposed fully at 900°C. The results demonstrated that thermal stability for the CTS/MMT/Nano ZrO$_2$ was better than CTS, MMT and CTS/MMT.

The *in vitro* bio-mineralization studies noted that the inclusion of zirconia enhanced the bio mineralization and bioactivity of the CTS/MMT/ZrO$_2$ scaffold (Figure 6). Fibroblasts were cultured on the scaffold for 3 days. The results showed an increase in the cell activity in culture media containing CTS/MMT/ZrO$_2$ scaffold during incubation, thereby indicating no cytotoxic effect on cell culture media.
In vitro evaluation of cytotoxicity: Cyto compatibility of the CTS/MMT/Nano ZrO₂ composite scaffolds was assessed using the MTT assay. The results propose that there are no significant toxic leachates in the CTS/MMT/Nano ZrO₂ scaffolds after incubation of the cells with the extract containing the leachates obtained after 24 and 72 h of incubation in the medium (Figure 7). No significant increase in cell growth was seen in the control, CTS, CTS/MMT and CTS/ Nano ZrO₂ groups after culturing for 72 h due to the space deficiency in the multi-well culture dishes, but the cells related to the composite groups were not like this.

From the results it can be concluded that a homogeneous incorporation of MMT and Nano ZrO₂ into CTS scaffold led to higher cell viability compared to that of the CTS-only scaffold or the CTS/MMT and CTS/ Nano ZrO₂ scaffold blended. Generally, the scaffolds prepared in this work were seen to possess favorable cell-compatible characteristics and can be considered as suitable materials for tissue engineering applications.

Cell attachment studies: SEM imaging was used to study the attachment and morphology of the cells on the scaffolds. (Figure 8) shows the SEM images of the cells after incubation for 24 h on the scaffolds and as shown in the Figure the cells attached and spread within the pore walls offered by the scaffolds. Cell attachment studies showed that the CTS/MMT/Nano ZrO₂ composite scaffold significantly increased the cell attachment which could largely attribute to the increase in its surface area, further supporting the suitability of these composite scaffolds for use in tissue engineering.
9. M Darder, M Colilla, E Ruiz-Hitzky (2003) Biopolymer-clay nanocomposites based on chitosan intercalated in montmorillonite. Chem Mater 15(20): 153774-3780.

10. S Pattnaik, S Nethala, A Tripathi, S Saravanan, A Moorthy, et al. (2011) Chitosan scaffolds containing silicon dioxide and zirconia nano particles for bone tissue engineering. Int J Biol Macromol 49(5): 1167-1172.

11. Y Kojima, A Usuki, M Kawasaki, A Okada, Y Fukushima, et al. (1993) Mechanical properties of Nylon 6-clay hybrid. J Mater 8(5): 1185-1189.

12. FH Lin, YH Lee, CH Jien (2002) A study of purified montmorillonite intercalated with 5-fluorouracil as drug carrier. Biomater 23: 1981-1987.

13. Y Kojima, YH Lee, A Usuki, M Kawasaki, O Kamigaito, et al. (1993) Synthesis of nylon-6 clay hybrid. J Mater 8(5): 1179-1184.

14. M Kawasaki, N Hasegawa, A Okada (1997) Preparation and mechanical properties of polypropylene-clay hybrids. Macromolecules 30(20): 6333-6338.

15. L Kljajevic, B Matovic, A Radosavljevic-Mihajlovic (2011) Preparation of ZrO$_2$ and ZrO$_2$/SiC powders by carbo thermal reduction of ZrSiO$_4$. J Alloys Compd 509(5): 2203-2215.

16. HL Liu, XF Sun, CQ Yin, C Hu (2008) Removal of phosphate by mesoporous ZrO$_2$. J Hazard Mater 151(23): 616-622.

17. H Jiang, P Chen, Sh Luo, X Tu, et al. (2013) of novel nanocomposite Fe$_3$O$_4$/ZrO$_2$/chitosan and its application for removal of nitrate and phosphate. Appl Surf Sci 284: 942-949.

18. L Wang, RM Shelton, PR Cooper, M Lawson, JT Trifftit, et al. (2003) Evaluation of sodium alginate for bone marrow cell tissue engineering. Biomaterials 24(20): 3475-3481.

19. NVR Majeti (2000) A review of chitin and chitosan applications. React Funct Polym 46: 1-27.

20. A Teimouri, L Ghorbani, A Najafi, R Emadi (2014) Fabrication and characterization of silk/forsterite composites for tissue engineering applications. Ceram Int 40: 6405-6411.

21. A Teimouri, R Ebrahimi, R Emadi, R Hashemi Ben, A Najafi (2015) Nano-composite of silk fibroin-chitosan/Nano ZrO$_2$ for tissue engineering applications: Fabrication and morphology. Int J Biol Macromol 76: 292-302.

22. A Teimouri, R Ebrahimi, A Najafi, R Emadi (2015) Fabrication and characterization of silk fibroin/chitosan/Nano γ-alumina composite scaffolds for tissue engineering applications. RSC Adv 5: 27558-27570.

23. T Kakubo, H Takadama (2015) How useful is SBF in predicting in vivo bone bioactivity?. Biomater 27: 2907-2915.

24. S Brunauer, PH Emmett, E Teller (1938) Adsorption of Gases in Multi molecular Layers. J Am Chem Soc 60: 309-319.

25. SJ Gregg, KSW Sing (1982) Adsorption, Surface Area and Porosity. In: editors (Eds.) Academic Press (2nd edn) New York, USA.

26. KSW Sing, DH Everett, RA W Haul, L Moscou, RA Pierott (1985) Reporting physiosorption data for gas/solid systems with special reference to the determination of surface area and porosity. Pure Appl Chem 57(4): 603-619.