



(This article was presented to the 28th National Chemistry Congress and submitted to JOTCSA as a full manuscript)

Preparation and Characterization of Montmorillonite/Polycaprolactone Composite Scaffold Containing Strontium for Bone Tissue Engineering Studies

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Abstract: In this study, composite scaffolds containing strontium-modified MMT and polycaprolactone (SrMMT-PCL) were prepared by using particulate leaching technique. The macrostructure and morphology of composite scaffolds were characterized by X-ray diffraction (XRD), thermogravimetric analysis (TGA), and scanning electron microscopy (SEM). The released amount of Sr²⁺ from scaffolds into cell culture medium was examined by inductive coupled plasma optical emission spectrometer (ICP-OES). The pore size distribution of scaffolds was determined by mercury intrusion porosimetry. The mechanical properties of the scaffolds were also evaluated. The results of XRD confirmed intercalation of PCL into MMT layers. TGA studies concluded that the MMT in PCL promoted the thermal degradation of the matrix. ICP results showed that Sr²⁺ was released from composite scaffolds. The majority of pore volume seems to be occupied by pores around 250-350 µm. SEM observations demonstrated the macroporous structure of the composite scaffolds obtained by using the particulate leaching method. As a result, the gained data suggest that obtained tissue-engineered scaffold has the potential to serve as a suitable template for bone tissue engineering applications.

Keywords: Montmorillonite; polycaprolactone; strontium; bone tissue engineering.

Submitted: July 01, 2016. **Revised:** November 07, 2016. **Accepted:** November 08, 2016.

Cite this: Koç Demir A. Preparation and Characterization of Montmorillonite/Polycaprolactone Composite Scaffold Containing Strontium for Bone Tissue Engineering Studies. JOTCSA. 2016;3(3):669-82.

DOI: To be assigned.

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INTRODUCTION

Every year, thousands of patients around the world suffer from bone fractures and degenerative disease. Although various treatment options are available for repairing bone defects resulting from trauma, tumors, biochemical disorders, and developmental abnormalities, using methodology have some drawbacks including donor site scarcity, inflammation, and the risk of immune rejection. Hence, researchers have begun to focus on bone tissue engineering (BTE) perspectives to find alternative solutions to address common clinical challenges.

The development of suitable three dimensional composite materials play a significant role in tissue engineering studies. An engineered scaffold in bone tissue engineering serves as a temporary artificial extracellular template to support and stimulate bone tissue regeneration via mimicking the native extracellular matrix of the native tissue (1). The scaffold should be 3D and highly porous with an interconnected pore network for cell growth and enable diffusion of nutrients and possess certain mechanical stability, have suitable surface chemistry to allow cell attachment, proliferation and differentiation (2, 3). Natural and synthetic materials, cells and growth factors are three important factors to construct the artificial tissue. Among several choices of synthetic polymers, polycaprolactone (PCL) has been widely used as implantable material in the preparation of scaffolds because of its favorable properties. It is a semicrystalline, biodegradable, hydrophobic polymer belonging to the aliphatic polyester family (4). However, its limited bioactivity and mechanical features do not suit the specific needs of BTE applications. In the recent years, clays receive special interest as reinforcement materials to create a template with improved properties. Among clay minerals, montmorillonite (MMT) offers a wide variety of advantages. MMT is a three-layered smectite group of minerals. Its structure is composed of two silica tetrahedral layers (T) sandwiched a central octahedral sheet of aluminium hydroxide (O). Undergoing to the isomorphic substitution of Al^{3+} with Si^{4+} in the tetrahedral layer and Al^{3+} with Mg^{2+} in the octahedral layer will generate negative charges (5). Excess negative charge in the layers of TOT can be balanced with exchangeable alkaline or earth alkaline cations as K^+ , Na^+ , Ca^{2+} naturally occurring between the layers and also with other positively charged ions like Sr^{2+} , Zn^{2+} . It has wide specific surface area, good cation-exchange capacity, high adsorption capacity and drug carrying capability for polymers (6). Furthermore, it can also serve as a reservoir for different molecules and ions (7).

It is well known that metallic ions such as strontium, calcium, magnesium, and zinc are necessary for bone growth and development. These elements are involved in the process of bone development by ensuring the activation of bone cells (osteoblasts), while inhibition of bone resorption cells (osteoclasts) via a variety of mechanisms (8). From this point, the release of these ions from materials is very important to support bone regeneration. Due to the increasing evidence for the significant role of strontium in bone formation, the incorporation of strontium

with biomaterials to improve their osteoinductive properties has become a focus of interest (9, 10).

Keeping all of these interesting facts about PCL, MMT, and Sr^{2+} in mind, we aimed to develop Sr^{2+} -containing composite scaffolds by particulate leaching method and investigate the physical properties of Sr-modified MMT-PCL scaffold to determine its feasibility for bone tissue engineering studies.

MATERIALS AND METHODS

Materials

Polycaprolactone was purchased from Aldrich company (Mn: 70,000-90,000). Montmorillonite (MMT) used in this study with a cation exchange capacity (CEC) of 102 meq/100 g was obtained from Ünye Bentonite Company. Other chemicals used were purchased from Sigma.

Preparation of Sr-modified MMT

Sr^{2+} montmorillonites were prepared by a cation exchange method. Briefly, MMT was dispersed in distilled water and mechanically stirred at room temperature for 24 h. Then, strontium chloride solution with various concentrations ranging from 1 to 4 CEC was added dropwise to MMT solution. The mixture was stirred for overnight to allow the ion exchange reactions. The resulting Sr-modified MMT was collected by centrifugation and the pellet was rinsed in distilled water several times until a negative AgNO_3 test was observed. The product was dried at 100°C for 2 days, grinded, and characterized by XRD and XRF to compare the structures obtained by adding Sr^{2+} .

Preparation and characterization of MMT-PCL composite scaffolds

Taking in consideration the XRD and XRF results, Sr-modified MMT was prepared as described above using twice the ratio of Sr^{2+} concentration, for the following experiments. Composite scaffolds were fabricated by particulate leaching method. Briefly, a 10 % PCL in methylene chloride freshly prepared and mixed with the required amount of modified and non-modified MMT. The mixture was homogenized first by mechanical stirring and then by ultrasonic agitation to obtain a homogeneous suspension. The obtained mixture was casted into Teflon molds packed with NaCl crystalline particles (sieved to 200–350 μm). The solvent was evaporated and the resulting MMT-PCL/salt composite was immersed in distilled lukewarm water to dissolve the porogen.

X-ray powder diffractometer (XRD) was employed to analyze the crystalline phases of PCL, MMT, MMT-PCL, and SrMMT-PCL composite structure using a monochromatic Cu-K α radiation (D8 Advance; Bruker, Germany).

Thermal gravimetric analysis (TGA) was performed on the obtained composite material using a thermal analysis system (Shimadzu DTG-60H) under nitrogen atmosphere in the temperature range of 20-800°C.

A mercury intrusion porosimeter (Quantachrome Corporation, Poremaster 60) was used to quantify pore size distribution of the scaffolds. The pressures were applied in the low pressure setting. A contact angle of 140° and a surface tension of mercury of 480 mN/m were determined for the samples.

SEM was employed to confirm the surface/cross-sectional pore morphology of composite scaffolds by using a Gemini 1525 FEGSEM model scanning electron microscope.

The mechanical properties of PCL, MMT-PCL, and SrMMT-PCL were evaluated through compression testing on an Shimadzu AGS-X machine at a constant strain rate (2 mm/min) with a 0.7 N loading cell.

Inductive coupled plasma optical emission spectrometer (ICP-OES) (Perkin Elmer Optima 4300D) was conducted to examine the concentration of the Sr²⁺ ions released from the composite scaffolds into media. Briefly, scaffolds were weighted and soaked in cell culture media (1 mg / 1 mL) in 24-well plates, then samples were transferred into incubator to mimic cell culture condition for up to 7 days. The medium was collected at days 1, 3 and 7. The collected medium was diluted ultrapure distilled water and filtered through a 0.2 µm filter to remove suspended particles before measurement of ions in the supernatant.

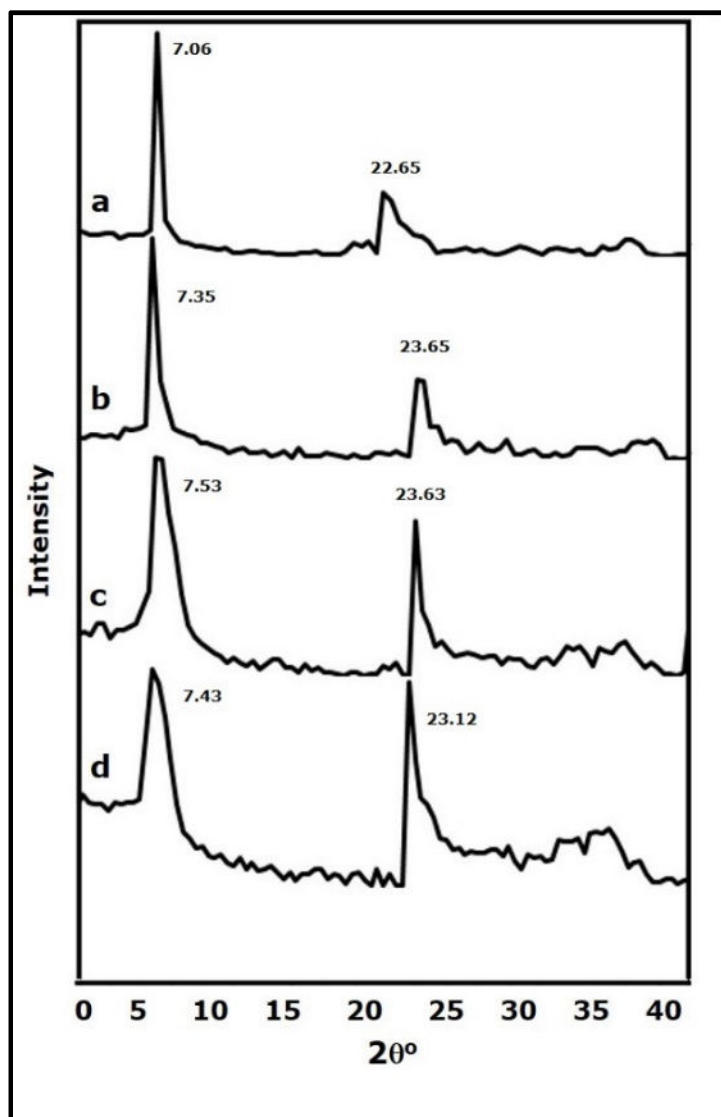
RESULTS AND DISCUSSION

Characterization of Sr-modified MMT

The XRD patterns of MMT and its modified structures with different Sr²⁺ concentration were illustrated in Figure 1. The basal spacing of the MMTs was calculated by using Bragg's equation ($n\lambda = 2d \cdot \sin\theta$). The obtained results showed the basal distance between the layers was not changed with the introduction of the cations, but the intensity of the peaks were increased compared to non-modified MMT (Table 1). XRF was used to estimate the optimum amount of Sr²⁺ incorporated into the MMT. The amount of Sr²⁺ inserted MMT structure was found 0.0139%, 2.709%, 3.6320% and 3.531%, the non-modified MMT (Sr(0x)-MMT), Sr(1x)-MMT, Sr(2x)-MMT and Sr(4x)-MMT, respectively. The Sr content of MMT increased with increasing Sr concentration as expected, but there was no detectable difference between Sr(2x) and Sr(4x). Thus, taking the results of XRD and XRF, twice the ratio of Sr²⁺ was chosen for the modification of MMT.

Table 1. The values of 2θ and the basal spacing of nonmodified and modified MMTs.

	$2\theta_1$	$2\theta_2$	d_1	d_2
Sr(0x)-MMT	7.06	22.65	14.5	4.56
Sr(1x)-MMT	7.35	23.65	13.96	4.36
Sr(2x)-MMT	7.53	23.63	13.96	4.37
Sr(4x)-MMT	7.43	23.12	14.43	4.46

**Figure 1.** XRD pattern of the non-modified MMT (a), Sr(1x)-MMT (b), Sr(2x)-MMT (c), Sr(4x)-MMT (d).

Characterization of MMT-PCL composite scaffolds

The XRD patterns of PCL, MMT, and the composite scaffold were shown in Figure 2. The basal spacing of the samples was calculated by using Bragg's equation ($n\lambda = 2d \sin\theta$). The XRD pattern of the PCL shows a reflection peak at about $2\theta = 25.51^\circ$ and 37.53° . The interlayer distance estimated from Bragg's law was 4.05 and 2.78 nm, respectively. MMT shows two main

diffraction peaks around $2\theta = 7.53^\circ$ and 23.63° . After the incorporation, the peaks of PCL and MMT moves to a lower angle. The increase of interlayer spacing for the composites was indicated the intercalation of PCL into MMT interlayers.

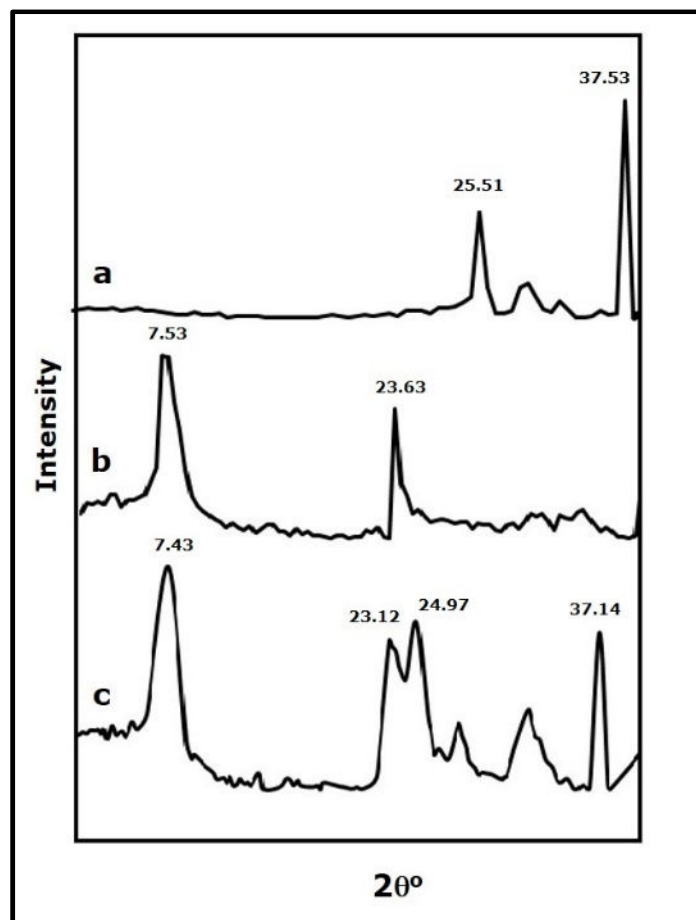


Figure 2. XRD pattern of PCL (a), SrMMT (b) and SrMMT-PCL scaffold (c).

The thermal behavior of MMT, PCL and MMT-PCL were assessed by means of thermogravimetry. The TGA curves of MMT, PCL and MMT-PCL composite scaffold were shown in Figure 3. MMT did not exhibit any significant weight loss in the range of temperature, while PCL exhibited significant weight loss after 600°C . The degradation of PCL in PCL-MMT composite structure was observed over a range of $500\text{--}650^\circ\text{C}$ and the amount of remaining residue was 70 %, implying that MMT promoted the thermal stability of the polymeric matrix.

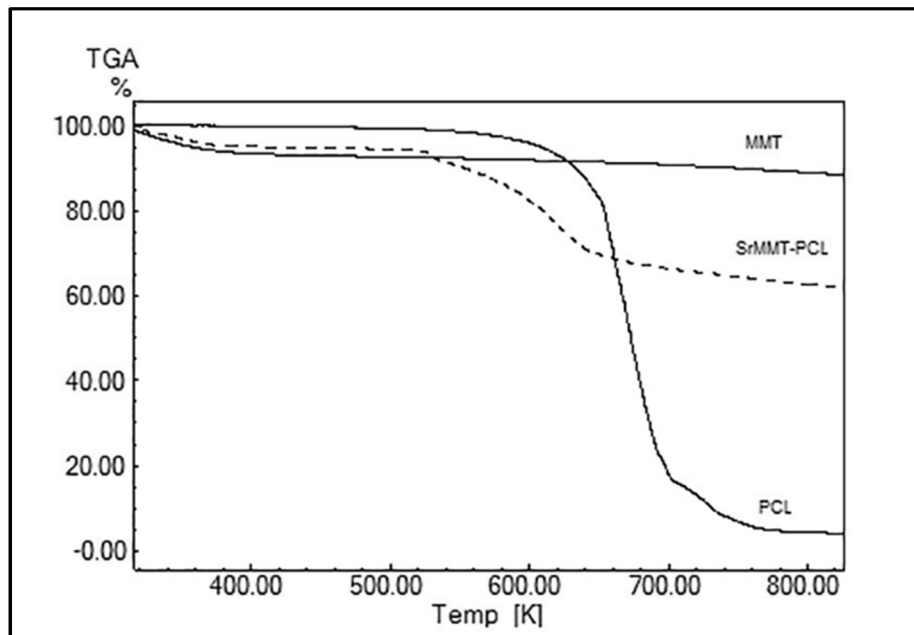


Figure 3. Thermogravimetric curves of MMT, PCL and SrMMT-PCL scaffold.

The pore size and interconnectivity of the engineered biomaterials are really vital factors for creating a suitable scaffold, because these parameters have a strong effect on cellular response such as cell attachment, proliferation, and differentiation (11). The scaffold should be highly porous with an interconnected pore structure for transportation of necessary nutrition factors and oxygen for seeded cell and maintain its mechanical stability between the scaffolds and surrounding tissue (2, 3). The pore size distribution of composite scaffolds was investigated with a mercury intrusion porosimeter. The curves of pore size distribution of PCL and MMT-PCL scaffolds were presented in Figure 4. As shown in the figure, the pore size of composite scaffold was found to vary between 50 and 250 μm . Based on previous study, scaffolds with interconnected pores and pore sizes ranges from 200 μm up to 350 μm have been generally used to support osteoblastic response of mesenchymal stem cells and this range is the convenient range for good interconnection and exchange of nutrients and waste (12).

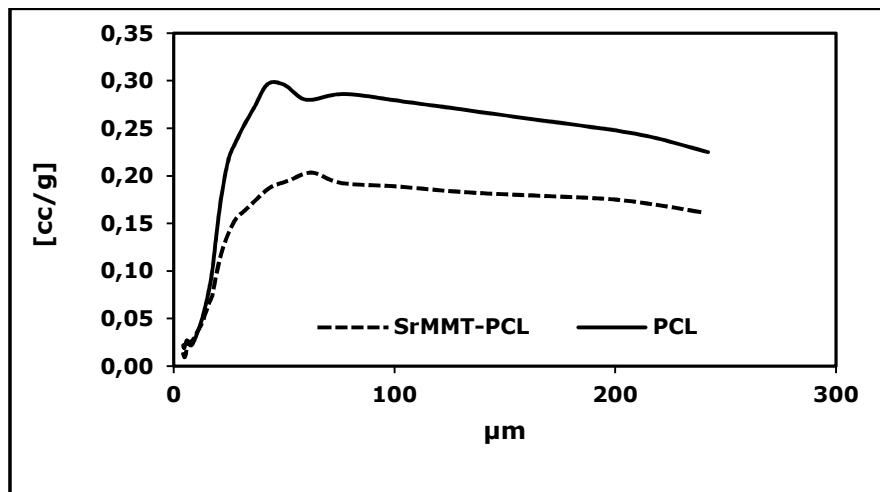


Figure 4. The size distribution of the pores of PCL and SrMMT-PCL scaffold.

The macroporous structure of the MMT-PCL scaffold obtained by using the particulate leaching method was given in Figure 5. Morphological observation by SEM indicated that the composite scaffold had a highly porous morphology with interconnected structure and MMT particles were dispersed homogeneously throughout PCL matrix. MMT particles in PCL matrix will improve cell attachment properties of scaffold via enhancing the surface area. The well-dispersed MMT also helps to improvement of mechanical and thermal properties of the composite (13-15). There was no observable difference in the structures of the non-modified and Sr-modified MMT-PCL composite scaffolds. Associated with pore size distribution data, it is possible to say that, the pore size of scaffold is adequate for viability and function of cells.

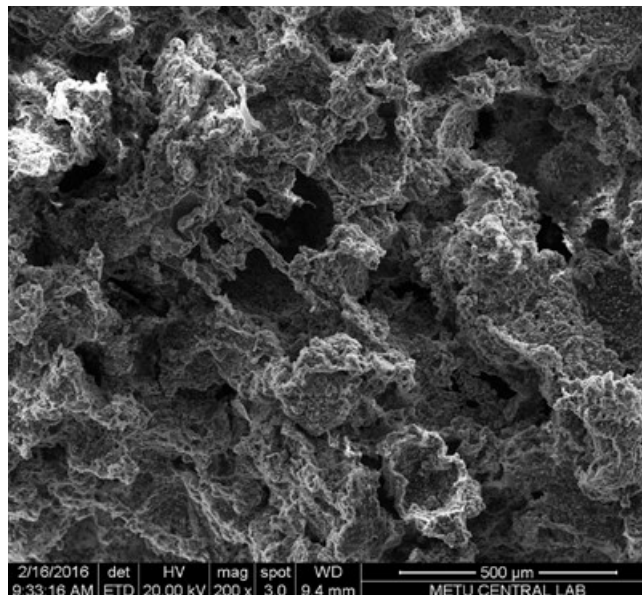


Figure 5. Representative SEM micrograph of MMT-PCL composite scaffold.

Figure 6 presents the stress-strain curves of PCL and SrMMT-PCL scaffolds. Comparison of PCL with SrMMT-PCL shows that the addition of MMT into PCL matrix appeared to lead to significant increase in the mechanical properties of PCL, suggesting that MMT has a positive effect on

improving mechanical properties of polymers due to its small particle size and its intercalation properties.

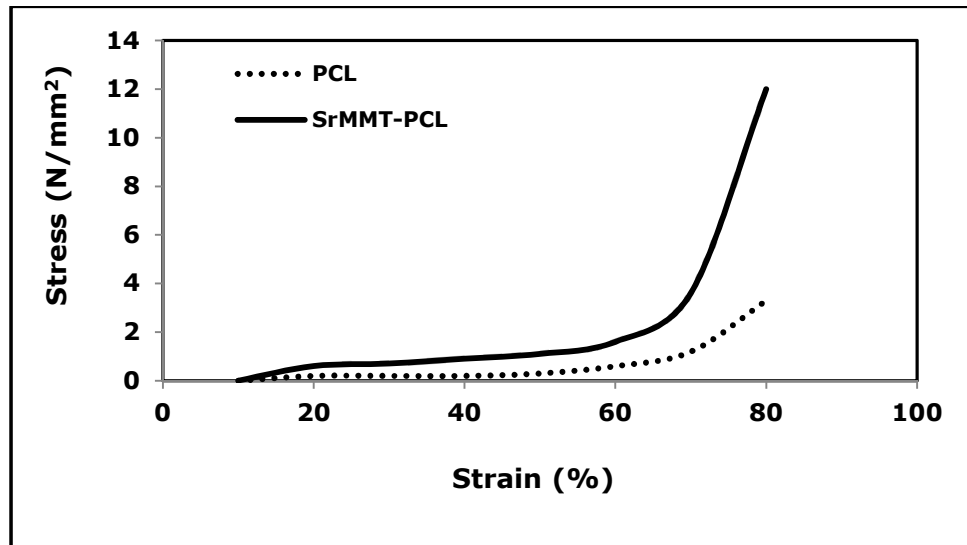


Figure 6. The stress–strain curves of PCL and SrMMT-PCL scaffolds.

Many trace metallic ions present in human body such as strontium, calcium, zinc, magnesium, and boron are known for their effects in bone metabolism (8). Increasing evidence in the literature indicates that the secretion of these therapeutic ions from engineered biomaterials facilitate bone healing process by supplying more appropriate environment to cell proliferation, differentiation and gene expression (16-17). Sr has an opposite effect on bone homeostasis. It increases the pre-osteoblast proliferation and activity, while decreasing the resorbing activity of osteoclasts. In our study, the release of Sr^{2+} from the non-modified and modified composite scaffolds was determined by using ICP-OES. The results showed that the release of Sr^{2+} was increased by the time (Figure 7). There was no Sr^{2+} release from non-modified scaffolds despite their small amount of Sr^{2+} content. Although the concentration of released Sr ions is lower than expected, it will favorably affect the replication of preosteoblast via enhancing the bioactivity of the scaffolds.

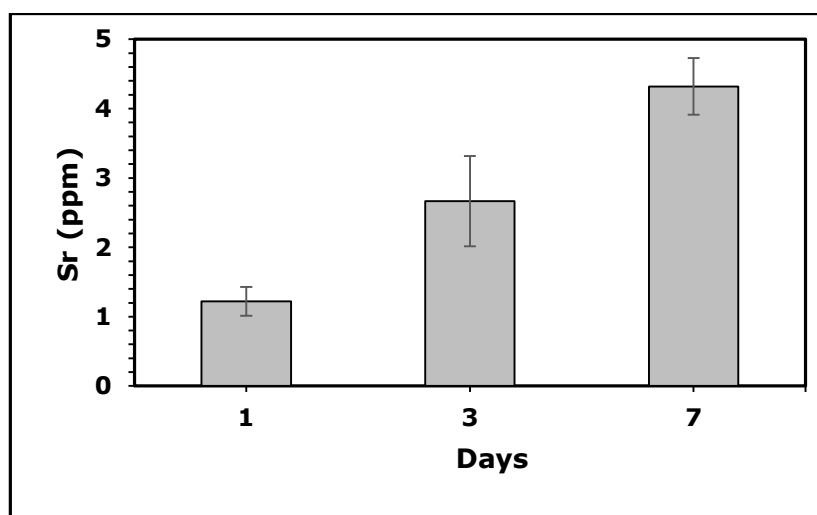


Figure 7. In vitro release of Sr from modified composite scaffolds into cell culture media.

CONCLUSIONS

The scaffold combined with PCL and Sr-modified MMT was successfully developed and characterized in this study. Interest in the use of clay minerals to develop tissue-engineered scaffold to enhance osteogenesis has been growing recently. Among clay minerals, MMT may be considered as an 'ion reserve' due to its properties. Based on this approach, MMT was modified with Sr, which has a role in bone development, afterwards modified MMT was used in fabrication of biocomposite scaffold. The findings of this study suggest that SrMMT-PCL could be a promising biomaterial for bone tissue engineering applications. The release efficiency of MMT might be improved via increasing the concentration of Sr which intercalates into MMT layers. Ongoing studies in our laboratory will perhaps shed light to the biocompatibility of the composite via conducting cell culture experiments.

ACKNOWLEDGMENTS

Financial support has been provided by TÜBİTAK-1002 (214M017). I would like to express my sincere gratitude to Prof. Dr. Y. Murat Elçin for his guidance and for giving me the opportunity to carry out my project at ElcinLab.

REFERENCES

1. Koç A, Emin N, Elçin AE, Elçin YM. In Vitro Osteogenic Differentiation of Rat Mesenchymal Stem Cells on Mineralized PLGA Foams in Microgravity Bioreactor. *Journal of Bioactive and Compatible Polymers*. J Bioact Compat Pol. 2208; 23: 244-261. DOI: 10.1177/0883911508091828.
2. Langer R and Vacanti J. *Tissue Engineering, Science*, 1993; 260: 920-928. DOI: 10.1126/science.8493529.

3. Elçin YM. Stem Cells and Tissue Engineering. Biomaterials. Volume 553 of the series Advances in Experimental Medicine and Biology. 2004: 301-316. ISBN: 978-1-4757-0988-9.
4. Iroh O. Poly(epsilon-caprolactone), edited by J. E. Mark. Oxford Press, Oxford, 1999, pp. 361-362. ISBN: 0195107896.
5. Choudalakis G, Gotsis AD. Permeability of polymer/clay nanocomposites: A review. E Polym J. 2009; 45 :967-984. DOI:10.1016/j.eurpolymj.2009.01.027.
6. Aguzzi C, Cerezo P, Viseras C, Caramella C. Use of clays as drug delivery systems: possibilities and limitations. Appl. Clay Sci. 2007; 36: 22-36. DOI: 10.1016/j.clay.2006.06.015.
7. Ruiz-Hitzky E, Aranda P, Dardera M and Rytwob G. Hybrid materials based on clays for environmental and biomedical applications. J Mater Chem. 2010; 20: 9306-9321. DOI: 10.1039/c0jm00432d.
8. Bose S, Fielding G, Tarafder S, and Bandyopadhyay A. Understanding of dopant-induced osteogenesis and angiogenesis in calcium phosphate ceramics. Trends in Biotechnol. 2013; 31: 594-605. DOI: 10.1016/j.tibtech.2013.06.005.
9. Dahl SG, Allan P, Marie PJ, Mauras Y, Boivin G, Ammann P, et al. Incorporation and distribution of strontium in bone. Bone. 2001; 28(4): 446-453. DOI: 10.1016/S8756-3282(01)00419-7.
10. Qiu K, Zhao XJ, Wana CX, Zhao CS, Chen YW. Effect of strontium ions on the growth of ROS17/2.8 cells on porous calcium polyphosphate scaffolds. Biomaterials. 2006; 27: 1277-1286. DOI: 10.1016/j.biomaterials.2005.08.006.
11. Loh QL and Choong C. Three-dimensional scaffolds for tissue engineering applications: Role of porosity and pore size. Tissue Eng Part B Rev. 2013; 19(6): 485-502. DOI: 10.1089/ten.teb.2012.0437.
12. Koç A, Elçin EE, Elçin YM. Ectopic osteogenic tissue formation by MC3T3-E1 cell-laden chitosan/hydroxyapatite composite scaffold. Artif Cells Nanomed Biotechnol, 2015. DOI: 10.3109/21691401.2015.1036998.
13. Sikdar D, Katti D, Katti K and Mohanty B. Effect of organic modifiers on dynamic and static nanomechanical properties and crystallinity of intercalated clay-polycaprolactam nanocomposites J. Appl. Polym. Sci. 2007;105: 790-802. DOI: 10.1002/app.26284.
14. Yu S, Zhao J, Chen G, Juay YK and Yong MS. The characteristics of polyamide layered-silicate nanocomposites. J Mater Process Tech. 2007; 192-193: 410-414. DOI:10.1016/j.jmatprotec.2007.04.006.

15. Kojima Y, Usuki A, Kawasumi M, Okada A, Kurauchi T, and Kamigaito O. Synthesis of nylon 6–clay hybrid by montmorillonite intercalated with e-caprolactam. *J Polym Sci A*. 1993; 31: 983–986. DOI: 10.1002/pola.1993.080310418.
16. Beattie JH, Avenell A. Trace element nutrition and bone metabolism. *Nutr Res Rev* 1992; 5(01):167-188. DOI: 10.1079/NRR19920013.
17. Nielsen F. New essential trace elements for the life sciences. *Biol Trace Elem Res*. 1990; 26-27(1):599-611. DOI:10.1007/BF02992716.

Türkçe Öz ve Anahtar Kelimeler**Kemik Dokusu Mühendisliği Çalışmaları için Stronsiyum İçeren Montmorillonit/Polikaprolakton Kompozit İskelesinin Hazırlanması ve Karakterizasyonu**

Aysel Koç Demir

Öz: Bu çalışmada, stronsiyum ile modifiye edilen MMT ve polikaprolakton içeren kompozit iskeleler partikül uzaklaştırma yöntemi kullanılarak hazırlandı (SrMMT-PCL). Kompozit iskelelerin makroyapı ve morfolojileri X-ışını kırınımı (XRD), termogravimetrik analiz (TGA) ve taramalı elektron mikroskobu (SEM) ile karakterize edildi. İskele yapılarından hücre kültürü ortamına salınan Sr^{2+} miktarı indüktif eşleşmiş plazma optik emisyon spektrometresi (ICP-OES) ile belirlendi. İskele yapılarının gözenek boyut dağılımı civa porozimetresi ile incelendi. İskele yapılarının mekanik özellikleri de değerlendirildi. XRD sonuçları PCL'nin MMT tabakaları arasına girdiğini gösterdi. TGA çalışmaları, PCL içindeki MMT'nin, matrisin ısı bozunma sıcaklığını yükselttiğini gösterdi. ICP sonuçları, Sr^{2+} iyonunun kompozit iskele yapılarından salındığını gösterdi. Gözenek hacminin büyük kısmının 250-350 μm civarındaki gözeneklerden oluştuğu tespit edildi. SEM görüntüleri partikül uzaklaştırma yöntemi ile elde edilen kompozit iskelelerin makrogözenekli yapıda olduğunu gösterdi. Sonuç olarak, elde edilen veriler, hazırlanan doku mühendisliği iskelesinin kemik doku mühendisliği uygulamaları için uygun bir malzeme olma potansiyeline sahip olduğunu gösterdi.

Anahtar kelimeler: Montmorillonit; polikaprolakton; stronsiyum; kemik doku mühendisliği.

Sunulma: 01 Temmuz 2016. **Düzeltilme:** 07 Kasım 2016. **Kabul:** 08 Kasım 2016.

