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Nanoclay Reinforced Starch-Polycaprolactone Scaffolds for Bone Tissue Engineering

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Abstract

Introduction: Bone tissue engineering is one of the important areas in the field of tissue engineering. Scaffolds should have adequate mechanical properties for proper tissue regeneration and for bearing the weight of the regenerating tissues. Many studies have been done for improving scaffolds mechanical properties.

Objective: this study aimed to make and characterize nonoclay reinforced starch-polycaprolactone scaffolds.

Material and Methods: Scaffolds based on starch/polycaprolactone blend containing montmorillonite nanoclays were prepared by solvent casting-salt leaching technique. The nanoclays were introduced to improve the mechanical properties of the scaffold.

Results: The characteristics of scaffolds were analysis by FTIR, SEM, contact angel, MTT assay and compressive strength tests. FTIR showed some hydrogen bonds between starch and polycaprolactone in scaffolds. In addition, the prepared samples exhibited porosity greater than 70%. The compressive mechanical test showed the range of 3.3 to 5.8 MPa for the compressive elastic modulus of the scaffolds. The contact angle experiments exhibited that incorporation of nanoclays improved the hydrophilicity of SPCL from 136 to 122 degree.

Conclusion: FTIR showed that the nanoclays was successfully incorporated into the starch/polycaprolactone blend based scaffolds. Nanoclays influenced the microstructure of starch/polycaprolactone scaffolds. The MTT assay also indicated that the nanoclays did not a negative effect on the viability of osteoblast cells in scaffolds. The porosity of the scaffolds is appropriate for tissue engineering applications. Therefore, the starch/polycaprolactone -nanoclay scaffolds appear to satisfy some of the essential requirements of scaffolds for bone tissue engineering applications.

Keyword: Scaffold; Tissue Engineering; SPCL Polymer; Bentonite

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1. Introduction

In the United States, approximately a quarter of patients in need of organ transplant die while waiting for a suitable donor [1, 2]. The current demands for transplanting organs and tissues is far outpacing the supply, and manner of projections indicates that this gap will continue to widen [1-3]. Many studies have been done in the field of tissue engineering since the early 1990s when Langer and Vacanti introduced tissue engineering. They define it as an interdisciplinary field that combines the principles of

engineering and life science to develop constructs useful for improving, maintaining, or restoring the function of an organ or tissue [4]. In recent years, tissue engineering has become a potentially interesting way of replacing artificial prosthesis organs. In this new field of science for reconstruction, replacement, or repair of living tissue and organ, cells are seeded in a three-dimensional (3D) scaffold [5-7]. As one can expect, these cells need a backbone that provides the initial structural integrity for proliferation and assembling into a functioning tissue [8]. Thus, the most important characteristics for a scaffold include chemical, biological and mechanical factors [9].

Bone tissue engineering is one of the important areas in the field of tissue engineering. Bone tissue characteristics and its important role in the body, such as providing structural support, protecting internal organs, acting as a reservoir of calcium and phosphate-based minerals and facilitating body movement makes it an interesting tissue for tissue engineering science [10, 11]. Besides the increasing number of knee and hip replacement surgeries recently and current treatment methods limitations, such as autografts, allografts, and different implants result in the increasing importance of bone tissue engineering [12].

Since the 1980s, the blend of starch and polycaprolactone (PCL) has received the most attention. The first company that manufactured a PCL/starch (SPCL) blend under the trademark Mater-Bi® is an Italian company named Novament. SPCL is an interesting blend because of the completely different properties of PCL (a synthetic, hydrophobic, flexible, expensive polymer with a low degradation rate) and starch (a natural, hydrophilic, stiff, abundant polymer with a high degradation rate). With appropriate blending procedure, SPCL can overcome critical limitations of both PCL and starch components, and improve mechanical properties and degradation rate so that SPCL would be suitable for many biomedical applications [13].

Scaffolds should have adequate mechanical properties for proper tissue regeneration and for bearing the weight of the regenerating tissues. Thus, depending on the tissue and the organ function, the mechanical properties of the scaffold should be appropriate. There are different methods for enhancing the mechanical properties of scaffolds such as the addition of micro/nanofibers and particles. Various studies have shown that nanosized fillers are useful not only to get a favorable cell response but also for achieving appropriate mechanical properties [14]. Among the nanofillers, organomodified nanoclays such as montmorillonite (MMT) has been extremely investigated by researches. Some studies showed that the addition of MMT not only effects on mechanical behavior of matrix but also it can influence some biological characteristics such as biodegradability and bioactivity [15]. Traditional view relates the increase in the mechanical properties of nanocomposites to the high aspect ratio of the MMT clay sheets that improve the polymer-filler interactions. However, some experimental and simulation studies showed the development of an altered phase model that attributed the enhancement in the mechanical properties of the nanocomposites [16].

Nowadays, there are many different scaffold-making techniques. Among them, solvent casting/particulate leaching is an easy and inexpensive method that allows the control of microstructural characteristics such as porosity percent, pore size, and pore interconnection degree [17-22]. In this work, MMT nanoclay was dispersed in the starch-polycaprolactone blend (SPCL), and scaffolds of this nanocomposite were produced by solvent casting/particulate leaching technique.

2. Materials and Methods

2.1. Materials

Polycaprolactone (Mw=80,000 kDa) and biobased starch were purchased from Sigma-Aldrich. Chloroform and Na-MMT nanoclay by the trade name of DK4 was obtained from Merck and Nanoline, respectively. DMEM culture medium, fetal bovine serum (FBS), penicillin, streptomycin, and phosphate buffered saline (PBS) were purchased from Gibco Invitogen. Human osteosarcoma cells (MG 63 cell line) obtained from National Cell Bank of Pasteur Institute.

2.2. Preparation of SPCL blends

For preparing the starch-polycaprolactone blend, starch was plasticized by glycerol and water by the ratio of 5:3:2 for starch, glycerol, and water, respectively. This compound blended completely until a homogenous PS (plasticized starch) was achieved. Then polycaprolactone heated on a hot plate to 200°C for a minute and after that, PS added to it by the ratio of 30-70. The blending process was completed in 5 minutes.

2.3. Preparation of the scaffolds

Solvent casting-particle leaching was used in this work for the scaffold preparation. For dispersing of nanoclays into the polymeric blend, it was stirred in chloroform for 2 hours and was sonicated for 10 minutes. Then, SPCL blend was added to this compound and stirred for 20 minutes. For generating porosity into the scaffold, commercially sugar crystals with a size range of 250-450 µm, as the porogen, added and homogeneously mixed to the polymer solution. The weight ratio of porogen to the solution was 10%. Then, the polymer solution was cast into a cylindrical mold (1 cm diameter, 4 cm height). This kind of mold allows chloroform evaporate slowly, so the pore size and structure of scaffold could be controlled. After drying the scaffolds for four days, they were leached in distilled water for removing the porogen and creating voids into the scaffolds. Finally, scaffolds were dried at ambient temperature, and they were dried in freeze-dryer for four days at -55°C and 100 mTorr for removing residual solvents. As a result, four different scaffolds were prepared by the names of SPCL (0 wt.% MMT), SPCL5 (5 wt.% MMT), SPCL10 (10 wt.% MMT) and SPCL15 (15 wt.% MMT).

2.4. Characterizations

AT-FTIR spectroscopy was executed by using Nicolet 870 FTIR spectrometer. The spectra were collected over the 450–4000 cm^{-1} wavenumber ranges at a resolution of 2 cm^{-1}. This characterization was performed on PCL, Starch and SPCL samples and different scaffolds.

SEM-EDX studies were carried out on dry SPCL, SPCL5, SPCL10, and SPCL15 by using AIS2100 (Seron Technology) scanning electron microscope, in order to study the microstructure of the scaffolds and the dispersion of MMT nanoclays. SEM imaging was also carried out on the scaffolds seeded with human osteosarcoma cells. For this purpose, the cell-seeded scaffold samples, after being incubated for the appropriate time, were washed with PBS. Then, the cells were fixed on the scaffolds by using glutaraldehyde. The scaffolds were dehydrated in ethanol series included: 10%v/v, 30% v/v, 50% v/v, 70% v/v, and 100% v/v and finally, the scaffolds were dried and imaged after gold sputtering.

Contact angle measurements using the sessile drop method by a software program were done to find out changes in hydrophobic characteristics of the blends with increasing MMT nanoclays.

Mechanical tests were done by using a Zwick/Roell Z050 machine. The samples were prepared according to ASTM D575 samples standard: 1 cm in diameter and 2 cm in height.

MTT assay utilized for cytotoxicity analyses. In this assay (3-(4, 5 dimethylthiazol-2)-2, 5-diphenyltetrazolium bromide) as a dye reacts with dehydrogenize enzyme and produces a purple colored product known as formazan. The formazan is solubilized, and its intensity is read by using a spectrophotometer. The intensity values indicate the number of live cells. Scaffolds of four different compositions for performing MTT assay were weighted and placed in 24-well plates, then sterilized by 70% pure ethanol for 8 hours and then ultraviolet light was radiated for 2 h. Then, 0.5 ml cell culture medium was added to 25 wells of the plate containing the scaffolds. These samples were incubated at 37°C, 5% CO_2 for a period of 3 days and 7 days. After 3 and 7 days, the cell culture medium was gathered and then 1×10^4 cells were poured into a plate with 96 wells and were incubated for 24 hours at 37°C. Extracted mediums were added to the second plate and incubated for another 24 h. After that, the medium was removed and 100 mL colorless culture medium and 12 mM of MTT solution was added to each well. After 4 h, the solution was removed and dimethyl sulfoxide solution was added for formazan purple crystals formation. Dimethyl sulfoxide concentration was calculated at a wavelength of 545 nm using

ELISA reader and the optical density showed the cell viability rates.

For cell morphology assessments, the cell-seeded scaffolds, which incubated for 24 h, were washed with PBS and then the cells were fixed on these scaffolds using glutaraldehyde. Then, these scaffolds were dehydrated in ethanol series (30% v/v, 40% v/v, 50% v/v, 70% v/v, and 95% v/v) and the dried scaffolds were mounted, gold sputtered, and imaged.

3. Results

3.1. Fourier transform infrared (FTIR)

FTIR-ATR spectra for Starch, PCL and SPCL samples showed in Figure 1. Figures 2 and 3 showed FTIR-ATR spectra for different scaffolds of SPCL, which contain different amounts of nanoclay.

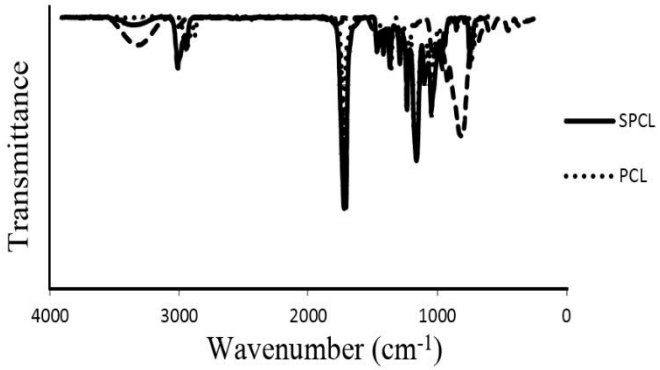

Figure 1. ATR-FTIR spectra for starch, PCL and SPCL

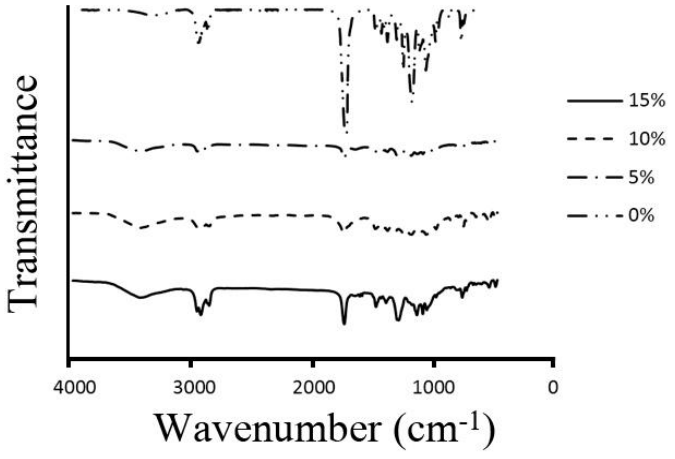

Figure 2. ATR-FTIR spectra for SPCL composites with different weight percentages of MMT nanoclay: 0, 5, 10, and 15

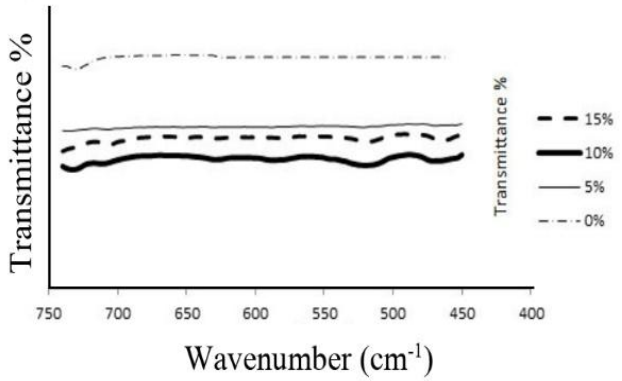

Figure 3. ATR-FTIR spectra for SPCL composites with different weight percentages of MMT nanoclay: 0, 5, 10, and 15 between 450 and 750 cm^{-1}

3.2. SEM-EDX studies

SEM-EDX micrographs of different samples illustrated in Figures 4 and 5. The measurement of the porosity for different samples by using ImageJ software displayed in Figure 6.

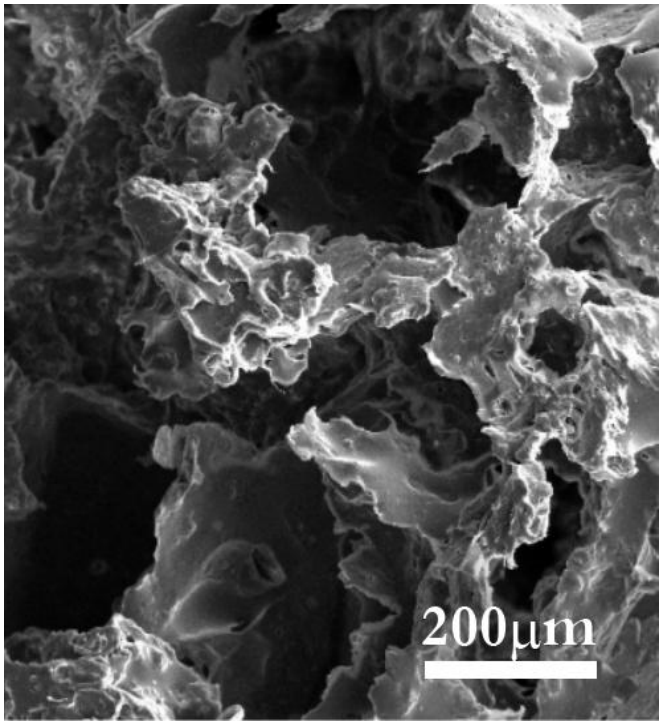

Figure 4. SEM micrograph of SPCL scaffold

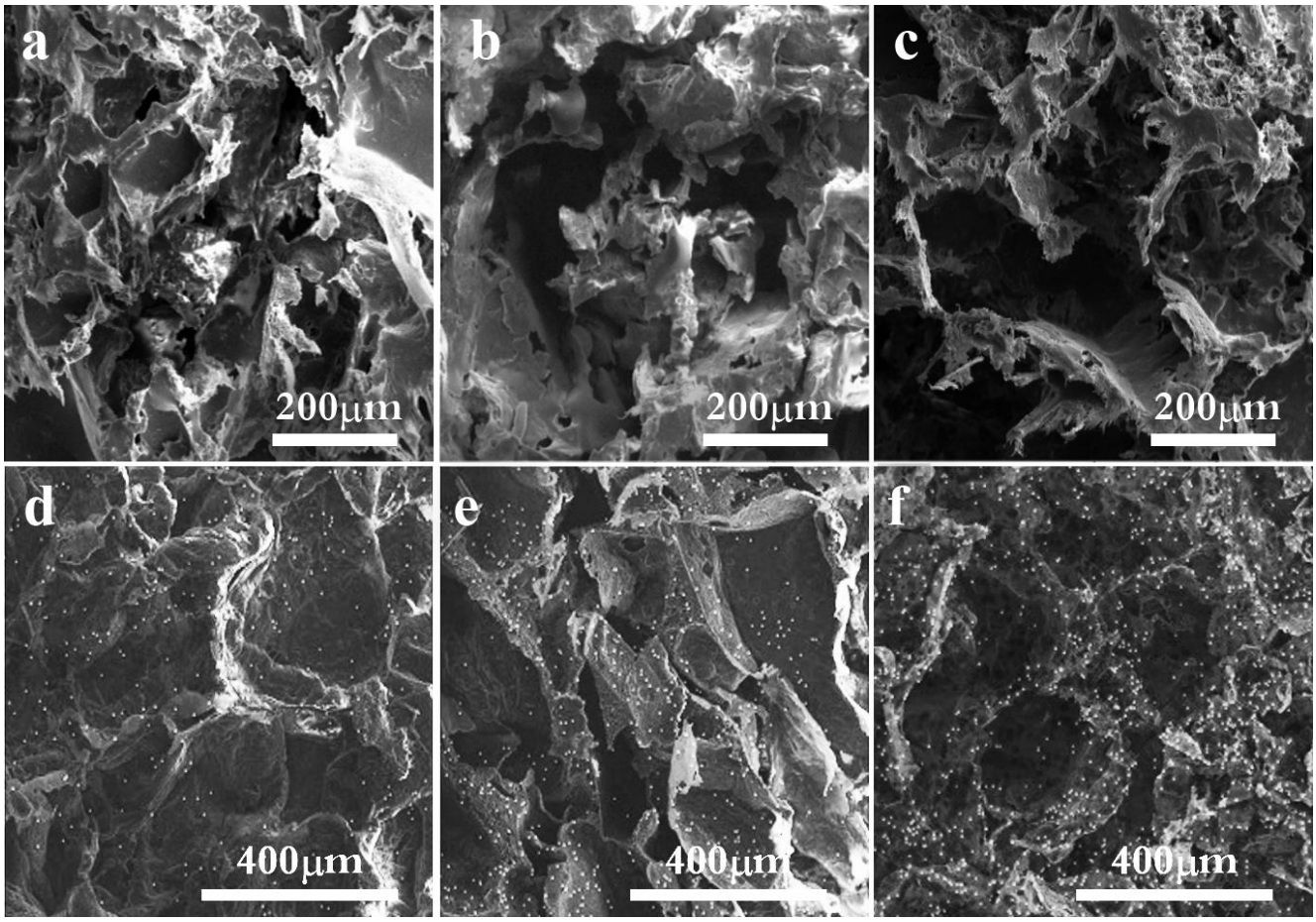

Figure 5. SEM-EDX micrographs for different scaffolds: a, d) SPCL5, b, e) SPCL10 and c, f) SPCL15

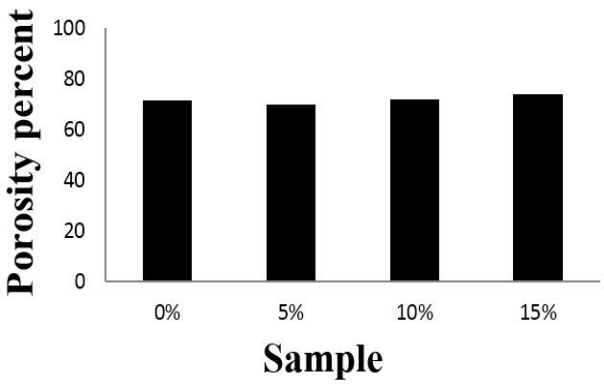

Figure 6. Porosity percent for SPCL with 0-15% nanoclay

3.3. Contact angle test

The results of the water contact angle test illustrated in Table 1.

Table 1. Water contact angle data of different scaffolds

Sample	Name	Contact angle (°)
1	SPCL	136.25
2	SPCL5	131.75
3	SPCL10	129
4	SPCL15	122

3.4. Mechanical properties

Figure 7 and Table 2 showed the compressive stress-strain curves and elastic modulus of different samples, respectively.

3.5. Cell viability test

After characterization, we tested scaffolds for cell viability and cell attachment. The MTT results showed in Figure 8 for 3 and 7 days. These data are significant with a P value < 0.05 in comparison with SPCL as the control sample.

3.6. Cell attachment

The results of cell attachment illustrated in Figure 9. These figures provide the attachment of cells on the scaffolds in two magnifications.

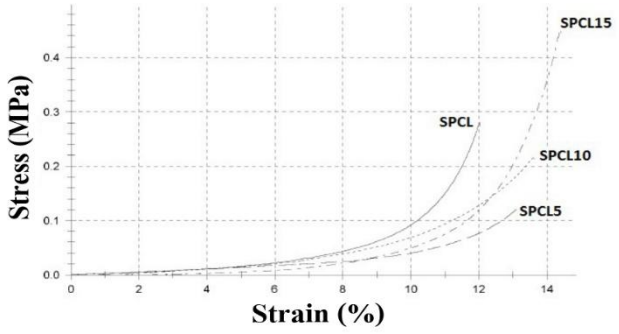

Figure 7. Compressive Stress-strain curves for different scaffolds

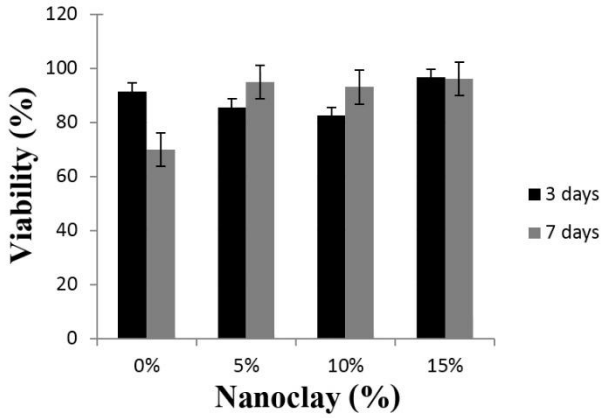

Figure 8. MTT assay results for SPCL with different amounts of nanoclay for 3 and 7 days

Table 2. Compressive elastic modulus of different scaffolds

Sample	Name	Compressive elastic modulus (MPa)
1	SPCL	68
2	SPCL5	66
3	SPCL10	116
4	SPCL15	82

4. Discussion and Conclusion

From FTIR analyses it is obvious in Figure 1 that there is a bending O-H bond between 700 cm^{-1} and 1500 cm^{-1} wavelengths and there are weak hydrogen bonds between tow polymers. In most research works, modifiers were used for PCL and Starch, because of their different characteristics which result in poor adhesion and consequently poor final properties [23]. Figure 2 shows the disappearing of carbonyl bond by adding MMT nanoclay, and Si-O-Si and Al-O-Si bonds respectively in 570 cm^{-1} and 470 cm^{-1} could be seen (Figure 3). These peaks represent for nanoclay and polymeric matrix interactions. Hydrogen bonds and the interaction among starch/glycerol/MMT are proved by the peak associated with –OH stretching located at 3300, 520, and 470 cm^{-1} [24, 25].

In SEM images as can be seen in Figure 4, MMT particles dispersed well in the matrix. Also, because of getting closer the white points in this map by increasing the nanoclay, agglomeration of nanoclay particles may happen. Some studies used mechanical strategies for better dispersion of nanoclays [26, 27], otherwise some studies used different solvents for dispersing nanoclays in the matrix but in this method, the behavior of the solution is affected by nanoclay percent, and as a result, viscosity of the solution increases by increasing nanoclay content [28]. The amount of the porosity (around 70%), which illustrates in Figure 5, is relatively suitable for bone tissue engineering in these scaffold samples. As we mentioned before, incorporation of nanoclay could increase the viscosity of the solution, so that the process of solvent casting and formation of

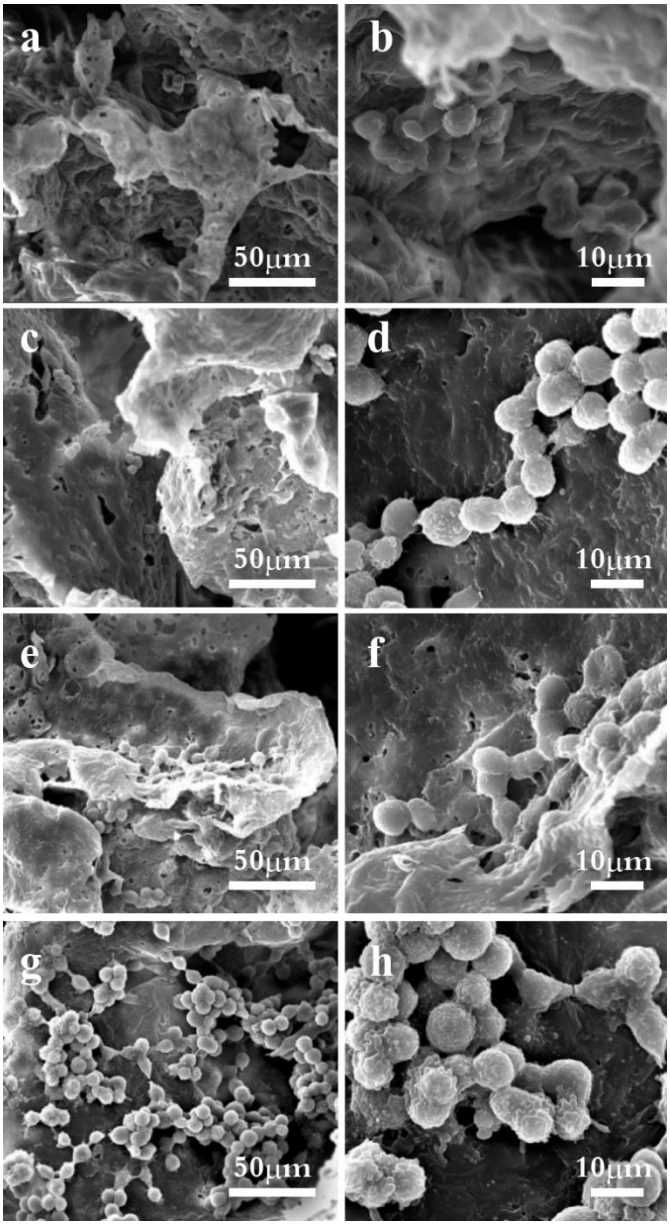

Figure 9. The morphology of cells on SPCL samples: a, b) SPCL, c, d) SPCL5, e, f) SPCL10 and g, h) SPCL15 in different magnifications

hemogenous porosities in the scaffolds could be difficult.

For hydrophilicity analysis, it is obvious from Table 1 that the contact angle reduced from 136.25 to 122 by increasing up to 15% nanoclay. This indicates some increase in hydrophilicity of samples. Montmorillonite nanoclay has been proved to increase the hydrophilicity of matrixes [29].

The mechanical evaluation reveals that the compressive modulus increases by increasing the amount of nanoclay (Figure 6 and Table 2). In Figure 6 the maximum stress is shown for different samples, and also displacement for samples in maximum stress is shown; as we can see the displacement for SPCL15 is 5.5 mm which comparing with 8 mm for SPCL showed that increasing nanoclay content results in compressive stress increase. These results are in agreement with other research works which showed improving mechanical properties of the different matrixes by adding montmorillonite nanoclay [14, 30, 31].

For MTT analysis as we can see, the results for 7 days were even better than 3 days, interestingly. It shows nanoclay improved the viability of cells. These results showed MMT nanoclay makes a favorable environment for osteoblast cells. For analyzing the cell attachment as we can see in Figure 8, there are obvious differences between samples and by increasing in MMT nanoclay percent cell attachment and morphology is improved. The number of cells on SPCL15 is much more than those on SPCL, and it shows MMT nanoclay increases the matrix hydrophilicity and also is compatible with osteoblast cells. As shown in Figure 8 in SPCL15 sample cells tend to have relatively flat morphology after 24 hours and started to attach to the scaffold surface these results are in accordance with other research works which used montmorillonite and concluded that this mineral is really compatible with cells and cells can proliferate and differentiate in the presence of it [14, 32-34].

The scaffolds of starch-polycaprolactone reinforced by nanoclay were prepared by solvent casting- salt leaching method. By this method, we could reach the porosity percent above 70%, and we could control the size of the pores by controlling the size of the porogen. Nanoclay added to the matrix by dispersing it at chloroform for two hours, and the results showed good dispersion. Biocompatibility tests and MTT assay showed that MG63 cells viability has improved with increasing nanoclay percent and the cells have an acceptable attachment to the scaffolds. Mechanical and contact angle experiments showed that by increasing in MMT nanoclay content the compressive

modulus and strength of samples increases and contact angle get decreases, so samples get hydrophilic. The elastic modulus for the SPCL sample is 68 MPa, and by increasing the nanoclay, it increased to 116 MPa. The higher value for compressive elastic moduli is 116 MPa for SPCL10, so this sample is better than SPCL15. It is because of the different effects of MMT nanoclay. By increasing in nanoclay percent, the viscosity of the solution increases and uniformity of structure decreases. SPCL contact angle is 136.25°, and by increasing nanoclay, it decreases to 122°.

Conflicts of interest

The authors declare that they have no conflict of interests.

Acknowledgments

None declared.

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