**In vitro Studies of Polycaprolactone Nanofibrous Scaffolds Containing Novel Gehlenite Nanoparticles**

**Abstract**

**Background:** Recently, many studies have been done on the physicochemical properties and biocompatibility of polycaprolactone (PCL) scaffolds containing ceramic reinforcing agents in the field of bone tissue engineering. In this study, the physical, mechanical and biological properties of electrospun-fabricated PCL scaffolds containing gehlenite (GLN) nanoparticles (NPs) as a novel bioceramic were investigated. **Methods:** To obtain the appropriate mechanical properties, the solution contains 3%, 5%, 7%, and 10% wt. of GLN NPs were prepared. Fiber morphology was investigated by scanning electron microscopy. In order to evaluate the NPs distribution, Energy Dispersive X-Ray Spectroscopy, X-ray diffraction, and Fourier Transform Infrared Spectroscopy spectroscopy were used. The scaffold hydrophilicity was measured by the water drop contact angle test. The tensile test was used to check the mechanical strength of the scaffold. The proliferation of MG-63 cells was evaluated by the MTT test. Alkaline phosphatase (ALP) activity of MG-63 cells was also examined. **Results:** Average fibers’ diameters and porosity of PCL/GLN7% were obtained 150–500 nm and 80%, respectively. An increase in the scaffold hydrophilicity was observed by the addition of GLN NPs. The strength of PCL/GLN7% was higher than the blank PCL scaffold. Cell proliferation of scaffolds containing GLN was higher than the blank PCL scaffold. A significant increase in the secretion of ALP for GLN-loaded scaffolds was seen. **Discussion:** The results showed that PCL/GLN7% composite scaffold could be a good candidate for bone tissue engineering. **Conclusion:** The overall results indicate that the scaffold (PCL/GLN7%) has suitable mechanical properties, a great cell compatibility for bone tissue regeneration.

**Keywords:** Electrospinning, gehlenite nanoparticles, gelatin, polycaprolactone, tissue engineering

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**Introduction**

Regarding the increase in the average age of the population, bone disorders have become a global concern. This has led to paid particular attention to bone problems and defects.\(^{[1]}\) Despite the self-healing ability of bone, there are some clinical challenges of bone injuries in the case of fractures, joint arthropathies, and dental defects that interfere with the normal bone healing procedure, where autogenous and allogeneic bone grafts cannot solve these problems.\(^{[2‑4]}\) Therefore, the treatment of large bone defects is an issue that remains a significant challenge for orthopedic surgeons.\(^{[2]}\) To reform bone defects, bone grafts have been used regularly.\(^{[1]}\) Besides, tissue engineering techniques and biomaterials-based therapeutics are an alternative way to respond to these issues.\(^{[5‑7]}\)

There are many methods for the fabrication of bone tissue engineering scaffolds;\(^{[8‑10]}\) among them, electrospinning is of particular interest because of extracellular matrix mimicking characteristics (e.g., porosity and mechanical properties), ease of fabrication, high surface to volume ratio, possible surface modification which attracts researchers’ attentions.\(^{[11‑13]}\)

There are many natural and synthetic polymeric biomaterials that are used for biomedical applications.\(^{[14]}\) Polycaprolactone (PCL) is one of the most attractive ones because of desired characteristics including low melting point (59°C–64°C, above body temperature), proper drug loading, biocompatibility, solubility, exceptional mixability properties, and the ability to...

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Gehlenite (GLN) (Ca$_2$Al$_2$SiO$_7$) is a silicate-based mineral compound, and its crystalline structure is classified as a subset of sorosilicates. The values of bending strength, elastic modulus, and fracture toughness of this compound are MPa 142 ± 12.1 MPa, 108 ± 6.8 GPa, and 2.32 ± 0.12 MPa. m 0.5, respectively.$^{[19,20]}$ These values are significantly higher than other bioceramics, such as calcium phosphates, bioactive glasses, and calcium silicates. For example, the bending strength, elastic modulus, and stiffness of the GLN are about three times higher than 45S5 bioactive glass and hydroxyapatite. The biocompatibility of GLN has been studied in some studies.$^{[19,21]}$ Therefore, considering the suitable mechanical and biological properties of GLN, it seems that the use of this compound is very promising for bone tissue engineering applications.

This study aimed to fabricate a PCL/GLN scaffold for use in bone tissue engineering. The morphology of the prepared fibers, mechanical and chemical properties, hydrophilicity, and cellular behavior were also evaluated.

**Materials and Methods**

**Materials**

PCL (Mw = 80,000) provided from Sigma-Aldrich (USA). The solvent 2,2,2-Trifluoroethanol (TFE) is obtained from Roth (Karlsrule, Germany). The synthesizing process for GLN NPs is done according to our previous study.$^{[20]}$

**Methods**

**Scaffolds fabrication method**

The GLN NPs dispersed into TFE solution (24 h) by using ultrasound. Different %wt. of GLN NPs, including 3, 5, 7, and 10% wt. were added to the 10% wt PCL solution to evaluate the effects of varying compositions on the physical and biological properties of scaffolds. The needle gauge, voltage, feeding speed, tip distance for electrospinning step were adjusted, 23G, 22–24 kV, 0.8–1 mL/h, and 18 cm, respectively. Nanofibrous scaffolds are dried in a vacuum oven at room temperature for 7 days.

**Scaffolds characterization**

Structural evaluations of prepared scaffolds, including morphology, fibers’ diameters, and scaffold porosity percentage, were carried out by scanning electron microscope (scanning electron microscopy [SEM], Philips XL30). Image J software was used for the determination of the average diameter of fibers. The diameter of the fibers was calculated as the mean ± standard deviation ($n > 40$).

**Energy dispersive X-ray spectroscopy and X-ray diffraction to analysis of scaffolds**

Energy dispersive X-ray spectroscopy (EDS) is a current method for the evaluation of elements ratio in samples. Therefore, to confirm the presence of GLN NPs in PCL/GLN scaffold, EDS mapping method was used.

The structural and microstructural analyses of all scaffolds including PCL, GLN and PCL/GLN are accomplished through X-ray diffraction (XRD, Philips TW3710, Netherlands).

**Fourier transform infrared spectroscopy analysis**

Chemical analysis was performed using fourier transform infrared spectroscopy (FTIR) (IFS-66 V/S, Bruker, Ettlingen, Germany) in the range of 400–4000 nm$^{-1}$ at room temperature.

**Evaluation of scaffold hydrophilicity**

In order to evaluate the hydrophilicity changes of PCL, PCL/GLN3%, PCL/GLN5%, and PCL/GLN7%, drops of deionized water were placed at three different points on the scaffold. The contact angles were recorded after 2, 5, and 10 s. This test was performed based on ASTM-D7334.

**Tensile strength**

To evaluate the effect of adding different amounts of GLN NPs on scaffolds mechanical property, they were cut 0.3 cm × 5 cm. Then, they were put in tensile testing equipment based on EN ISO, 05/1995 (Zwick Z050, Germany, load cell: 20 N, 1 mm/min, L0 = 2 cm).

**In vitro studies**

**Cell morphology assessment**

One and 7 days after cell culture, the scaffolds were washed three times with phosphate-buffered saline, fixed with glutaraldehyde (3%v/v), and dehydrated with ethanol (50%, 70%, 80%, 905, and 100% v/v). Morphology of MG-63 cells was assessed by SEM images. Cell attachment analysis and confluency levels were evaluated by a fluorescent image analyzer (Olympus, DP72, Japan).

**Cell proliferation assay**

Direct contact cytotoxicity evaluation was done at 1, 3, and 7 days after cell culture based on ISO 10993-5 for PCL and PCL/GLN scaffolds. After the mentioned days, cell culture was removed and substituted by MTT solution (10% v/v...
in Dulbecco’s Modified Eagle Medium, Fisher Scientific, India). After 4 h of incubation time, the solution was replaced by dimethyl sulfoxide and incubated for 1 h. The absorbance was read at 570 nm by a microplate reader (Microplate Reader Model 1680, Bio-Rad, USA).

**Alkaline phosphatase assay**

To quantify alkaline phosphatase (ALP) activity of MG-63 cells after 7, 14, and 21 days, the Alkaline phosphatase assay was done based on the manufacturer’s guidance (Pars Azmoon, Iran). Briefly, the supernatants were obtained, and a p-nitrophenyl phosphate transformed to a p-nitrophenol. The reaction was terminated by adding 100 µl NaOH solution (1 N). The absorbance was read by an ELISA reader (Microplate Reader Model 1680, Bio-Rad, USA) at 405 nm.

**Statistical analysis**

All experiments were repeated at least three times or more. One-Way analysis of variance were used for analyzing the results.

**Results and Discussion**

**Morphology of nanofibers**

SEM micrographs of electrospinning fibers of PCL and PCL/GLN scaffolds with different GLN ratios as well as the fiber diameters are given in Figure 1. The results show that the addition of GLN NPs does not affect the uniformity of fibers in PCL/GLN. There are significant amounts of NPs agglomeration in fibers contain GLN 10%. Therefore, PCL/GLN7% was selected as an optimal sample. The structure of PCL and PCL/GLN7% was uniform. The average diameter and porosity of scaffolds were about 400 nm and 80%, respectively.

The EDS test was used to ensure the presence of NPs in the scaffolds. The results of the EDS analysis [Figure 2] for PCL/GLN7% confirm the presence of calcium, aluminum, and silicon elements, which are attributed to the GLN structure.

**X-ray diffraction analysis**

The XRD patterns of GLN, PCL and PCL/GLN are presented through Figure 3. There are two sharp peaks visible in the XRD pattern of PCL at 21.5° and 23.75° attributing to the crystalline nature of PCL. Besides the two peaks of PCL, there is a peak at 31.46° appeared in the XRD pattern of PCL/GLN7% representing the sharpest peak of GLN NPs (JCPDS 001-0982). Due to the low weight percentages of GLN NPs (7%), the other XRD peaks of GLN are not appeared.

**Fourier transform infrared spectrometry**

The FTIR analysis results of PCL and GLN functional groups are presented in Figure 4. In the FTIR results for PCL different peaks can be seen, including CH, symmetric and asymmetric stretching (2863, 2940 cm⁻¹), C = O stretching (1721 cm⁻¹), C-O and C-C stretching (1292 cm⁻¹), C-O-C asymmetric stretching (1297 cm⁻¹), and C-O-C symmetric stretching (1162 cm⁻¹).[23] In the scaffolds containing GLN were not seen significantly different with blank scaffolds due to overlapping polymer peaks and GLN. Si-O peak (634 cm⁻¹) was observed in PCL/GLN 7%, and it can be because of low amounts of GLN NPs.

**Mechanical analysis**

Figure 5 shows the stress-strain curves of PCL, PCL/GLN3%, PCL/GLN5%, PCL/GLN7%. As the NPs amount increases, the tensile strength of nanofibers increases. On the other hand, the analysis of length increase for scaffolds which contains above 3 wt. % GLN, show that PCL/GLN scaffolds have become more fragile than PCL scaffolds.

**Analysis of surface hydrophilicity**

The hydrophilicity/hydrophobicity properties of scaffolds play an important role in the determination of the initial cell adhesion and migration, mechanical properties, and degradation.[23] Table 1 shows the water droplet contact angle for PCL, PCL/GLN3%, PCL/GLN5%, PCL/GLN7% after 2, 5, and 10 s. Angles >90° indicate hydrophobic property of scaffolds. PCL/GLN7% shows a contact angle of <90°, which confirms that the scaffold is hydrophilic. The contact angle amount is related to different parameters such as the nature of the ingredients, surface properties (e.g., surface roughness).[23] According to GLN NPs hydrophilic nature, by adding GLN NPs to PCL scaffolds, contact angles slightly reduced.

**Viability of MG-63 cells**

The MTT test was performed to evaluate the proliferation of MG-63 cells on PCL and PCL/GLN7% on days 1, 3, and 7 after culture [Figure 6]. There were no significant differences between samples on 1 and 3 days ($P \geq 0.05$). Less hydrophilic property of PCL can be a reason for less cell survival in comparison to GLN-loaded scaffolds. On day 7, the cell proliferation of PCL/GLN7% is higher than PCL scaffold ($P < 0.05$). This suggests that GLN NPs stimulate cell proliferation rates after 7 days. These results may prove the role of GLN NPs in cell proliferation and adhesion.[19]

**Cell adhesion and cell morphology on the scaffolds**

To investigate cell adhesion behavior of scaffolds, SEM, and fluorescent images were collected 1 and 7 days after culture [Figure 7]. Based on SEM images on 1 day, the cells attached well. Both scaffolds showed acceptable cell
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compatibility, adhesion, and expansion. However, PCL/GLN7% scaffold had far more cells than PCL scaffold. On day 7, both SEM and fluorescent images showed higher cell density in PCL/GLN7% scaffold. The presence of GLN NPs increased cell proliferation and adhesion on PCL/GLN7% scaffold compared to PCL scaffold. These results were compatible with the results of the MTT test.

**ALP analysis alkaline**

The evaluation of ALP activity is one of the most common osteogenesis assessments and is used to measure bone cell...
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Differentiation.\textsuperscript{[24,25]} ALP activity of MG-63 cells cultured on composite scaffolds were evaluated at 7, 14, and 21 days after culture [Figure 8]. On day 7, no significant differences were seen between scaffolds (\( P \geq 0.05 \)). On the 14 and 21 days, significant ALP activity was observed in PCL/GLN7\% compared to PCL scaffold and control sample (\( P < 0.05 \)), which can be due to the presence of GLN. Silicate ceramics release Ca and Si ions around the environment.\textsuperscript{[19]} In one study, the release of Si ions from silicate ceramics has been introduced as one of the most effective factors in osteogenic properties.\textsuperscript{[26]} In another one, the release of Si ion in the surrounding environment increased cell proliferation, protein synthesis, and ALP activity of osteoblasts.\textsuperscript{[27]}

**Conclusions**

In this study, composite GLN-loaded scaffolds were prepared using the electrospinning method, and their properties were also investigated. According to results, PCL/GLN7\% scaffold showed a porous and homogeneous microstructure, with the appropriate porosity, cell adhesion, and proliferation. The scaffold containing 7wt.\% GLN was chosen as the optimal sample because of a large amount of agglomerated GLN in GLN10\%.

The higher tensile strength of PCL/GLN composite scaffold showed that the presence of these NPs in the polymer substrate strengthened the resulting composite and improved its mechanical strength. The hydrophilicity of scaffolding increased after the addition of gehlenite and GLN, which is suitable for osteoblasts adhesion and proliferation.

The results of the cell proliferation test showed that both scaffolds with and without GLN had no significant toxicity. MG-63 cells also showed better adhesion and proliferation on PCL/GLN nanofibers than PCL nanofibers. The amount of ALP secretion in PCL/GLN
Figure 8: ALP activity analysis of scaffolds on days 7, 14, and 21 after cell culture

nanocomposite scaffolds was significantly higher than PCL scaffolds, and this could indicate better activity of osteoblast cells on this scaffold. The results show that the PCL/GLN7% can be a good candidate for bone tissue engineering.

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Conflicts of interest
There are no conflicts of interest.

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