In-vitro evaluation of PCL-based film for guiding segmental bone defect

Vahid Khodabakhshi
Islamic Azad University

Hamid Soleimanimehr (✉ soleimanimehr@srbiau.ac.ir)
Islamic Azad University

Shahram Etemadi Haghighi
Islamic Azad University

Ali Emam
Islamic Azad University

Research Article

Keywords: polycaprolactone, 3D printing, segmental bone tissue engineering, guide films, large bone defect

Posted Date: October 31st, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2196053/v1

License: ☇ This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

Segmental bone tissue engineering is a highly effective approach for the repair of large bone defects. In this paper, a PCL-based guide film was developed for controlling segmental bone tissue engineering. 3D bioprinting was used to fabricate the PCL/NaCl-based cylindrical films. The effects of the film’s thickness and NaCl concentration on the mechanical properties, degradability, swelling behavior, porosity, and cytotoxicity of the samples were investigated. Response surface methodology was employed to study the mechanical behavior using the central composite design (CCD) results showed that increasing the NaCl concentration up to 10% wt. significantly improved the degradability, swelling, and hydrophilicity of the films. It was also indicated that the maximum stiffness of the guide films under vertical loading was almost 5 times more than the maximum stiffness in the horizontal loading direction, but the samples showed greater compressive strength and elongation under horizontal compressive loading. All the evidence indicated that the mechanical properties of the films were more dependent on the film thickness so the thicker films with an 800µm thickness had better mechanical properties in both vertical and horizontal loading. Cytotoxicity assay also approved the non-toxic effect of the PCL films on the MC3T3 osteoblast cell line. Based on the results, the PCL-based films were a suitable candidate to act as a guide for segmental bone tissue engineering.

1. Introduction

Large bone defects are the most common bone fractures caused by trauma, car accidents, and occupational injury (Daskalakis, Liu et al. 2021). The repair of large bone defects is a major challenge in regenerative medicine. The long recovery period, lack of proper remodeling, and changes in bone length are very common problems in large defect regeneration, which directly affect the patient’s health and quality of life (Alonzo, Primo et al. 2021).

The prevalent methods for treating this type of bone fracture were to use metallic fixation devices or artificial bone cement to prevent any inadvertent movements of broken parts and fix the injured member for a long time (Patel, Singh et al. 2021). The use of fixation devices has shown some major drawbacks, such as infection and immune responses, bone adsorption, and stress shielding, osteoporosis, and the need for a second surgery to remove the implanted devices (e.g., orthopedic screw) after bone healing (Moghaddam, Andani et al. 2016). Bone cement was also an inadequate option due to its low porosity and compact structure, which impelled the bone parts towards death (Mukhopadhaya, Gautam et al. 2021). Segmental bone tissue engineering is a novel approach to the regeneration of large bone defects (Alkindi, Ramalingam et al. 2021). The size of the scaffolds is a key factor that directly affects cell migration and viability. Increasing the size of the scaffold could decrease the cell count in the central areas (Zhang, Rong et al. 2021). Furthermore, in non-vascularized scaffolds, the gas interchanges and delivery of nutrients are very difficult and cause major cell death in the central zones of the scaffolds (Vidal, Kampleitner et al. 2020). In segmental tissue engineering, small size scaffolds are considered as filler blocks that can have pre-cell seeding to increase the bone healing rapidity (Alonzo, Primo et al. 2021). Implantation of the scaffold blocks is a big challenge in segmental bone tissue engineering.
Considering the human tibia bone, scaffolds in a fractured site will require a guided shell to provide support and prevent the scaffolds from dislocating. On the other hand, physically adjoining the blocks demands a plate for fixing the scaffolds by pinning them to the plate. Accordingly, the development of suitable guide films seems necessary for the sustainable development of segmental bone tissue engineering. In the case of controlling segmental bone tissue engineering, the guide film simply serves as a supporting shell to join the segments and hold them together, and any cell growth on the film’s surface is not considered a well characteristic (Entezari, Swain et al. 2020).

Polycaprolactone (PCL) is a biocompatible polymer that has been widely used in biomedical applications. The satisfactory mechanical properties along with the non-toxic effect turned the PCL into the desired polymeric material for biomedical applications (Mohamed and Yusoh 2016). Low hydrophilicity and degradation rate are the two most important weaknesses of PCL, but in controlling segmental bone tissue engineering, these properties are very opportune.

An appropriate guide film for segmental bone tissue engineering, therefore the biocompatibility and desirable mechanical properties should allow the consuming materials (oxygen and nutrients) and excretory material exchange to supply suitable conditions for cell ingrowth and bone healing, but because of the low hydrophilicity of PCL, the diffusion of consuming materials is very difficult and gives rise to necrosis (Entezari, Swain et al. 2020). Fabrication of a porous structure with good mechanical properties is a great idea to increase the interchange between consuming and excretory materials, which is necessary for cell metabolism and regrowth. It was also reported before that the presence of porosity in the PCL matrix can enhance the degradability of PCL (Yedekçi, Tezcaner et al. 2021).

PCL as ink for 3D printing approaches (fused deposition modeling (FDM)) has some great properties such as injectability of fused, suitable solidification rate, and self-setting, but in the case of 3D printing of porous structures with a suspend layer, the PCL ink does not have good bridging capacity to form a suspend layer (Zhang, Wang et al. 2021). In order to solve this problem, the use of a suitable temporary space holder has been reported many times in previous studies (Samuel, Kong et al. 2022). Shim et al. (Shim, Sa et al. 2016) have reported that NaCl can be a good space holder in the PCL matrix due to its good solubility in water and nontoxic effect on the living cells. In the other study, Guarino et al. (Guarino, Causa et al. 2007), showed that the yield stress of PCL scaffolds by addition of NaCl was reduced from 1 to 0.05 MPa for 50 and 91% vol. NaCl. Besides, they have confirmed that the mechanical properties of porous PCL strongly depend on the NaCl ratio.

In this paper, the PCL-based porous films for controlling segmental bone tissue engineering were fabricated by the FDM technique. NaCl particles were added into the PCL matrix from 0 to 10% wt as filler to create porosities. and additively manufactured in the cylindrical shape with varied thicknesses between 600 to 800µm. All the experiments were designed by central composite design (CCD) using design expert software. The effects of NaCl concentration in the PCL matrix and film thickness on degradability, swelling behavior, water contact angle, porosity, and cell viability were investigated. The compressive strength, elongation at break, and compressive modules for all samples were also measured in both
vertical and horizontal loading directions, and the relative responses for X, Z loading directions were statistically modeled by CCD in response surface methodology (RSM) for an accurate prediction of the effects of NaCl %wt. and film thickness on the mechanical properties of the guide films.

2. Experimental Procedures

2.1. Experimental design

NaCl weight percentage and film thickness were considered as two independent factors in the central composite design and investigated at three levels for each factor detailed in Table 1.

<table>
<thead>
<tr>
<th>Independent factors</th>
<th>-1 (low)</th>
<th>0 (middle)</th>
<th>+1 (high)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl weight percentage (B, %)</td>
<td>0</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Films thickness (A, µm)</td>
<td>600</td>
<td>700</td>
<td>800</td>
</tr>
</tbody>
</table>

2.2. Ink preparation

The PCL/NaCl ink for 3D printing was prepared by dissolving 5 gr of polycaprolactone (PCL, 80 KDa, Sigma Aldrich, USA) in 20 ml of trichloromethane (chloroform, 99.99%, Sigma Aldrich, USA) using a magnetic stirrer at 25°C for 3 hours. Following that, the PCL solution was divided into three groups and stored in sterile glass bottles, and different percentages of NaCl powder (with the diameter less than 400 micrometers) were individually added to the PCL solution (according to Table 2) and kept on the magnetic stirrer at room temperature. After 12h, PCL solutions consisting of 0, 5, and 10%wt NaCl were cast into the glass petri dish and kept in the laminar hood for 24h. The dried films were cut and placed into the printer's steel cartridge.

2.3. Samples preparation

To print the samples, a computer-aided design (CAD) file was designed in the shape of a cylinder and exported to the STL readable file for the 3D printer machine. The prepared inks were heated up to 60°C and printed on the glass platform in a cylindrical shape with a different thickness at room temperature. All the properties of the specimens were described in Table 2.
Table 2
Thickness and NaCl %wt. for each sample.

<table>
<thead>
<tr>
<th>Code</th>
<th>Run</th>
<th>Thickness (µm)(A)</th>
<th>NaCl (%wt.)(B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1</td>
<td>600</td>
<td>0</td>
</tr>
<tr>
<td>D*</td>
<td>2</td>
<td>700</td>
<td>5</td>
</tr>
<tr>
<td>E</td>
<td>3</td>
<td>800</td>
<td>5</td>
</tr>
<tr>
<td>H</td>
<td>4</td>
<td>600</td>
<td>10</td>
</tr>
<tr>
<td>G</td>
<td>5</td>
<td>800</td>
<td>10</td>
</tr>
<tr>
<td>D</td>
<td>6</td>
<td>700</td>
<td>5</td>
</tr>
<tr>
<td>D</td>
<td>7</td>
<td>700</td>
<td>5</td>
</tr>
<tr>
<td>C</td>
<td>8</td>
<td>600</td>
<td>5</td>
</tr>
<tr>
<td>D</td>
<td>9</td>
<td>700</td>
<td>5</td>
</tr>
<tr>
<td>F</td>
<td>10</td>
<td>800</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>11</td>
<td>700</td>
<td>10</td>
</tr>
<tr>
<td>A</td>
<td>12</td>
<td>700</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>13</td>
<td>700</td>
<td>5</td>
</tr>
</tbody>
</table>

* The code “D” was repeated five times by CCD.

After printing the samples, all films were immersed in deionized (DI) water for three days at 37°C to dissolve the NaCl particles and remove the temporary space holder to create porosities. After that, all samples were dried at 25°C in a vacuum dryer until the weight of the samples became steady.

2.4. Mechanical characterization

To evaluate the mechanical properties of samples, the compressive test was utilized according to the ASTM D695 standard test method with a 5 mm/min crosshead speed and 200N loading pressure. The stiffness, compressive strength, and elongation at break for all specimens were measured in the X, Y, and Z-axis. Owing to the symmetrical design of the samples, X and Z directions were assumed to be the same. Figure 2. Presents the loading directions in compressive tests.

2.5. Degradability

In vitro degradation of the guide, films were examined by immersion in phosphate-buffered saline (PBS) for 30 days at 37°C. The degradation of samples was calculated by measuring the weight changes after
3, 7, 14, 21, and 30 days of incubation and the degradation percentages were computed using the following equation:

\[
\text{Degradation (\%) } = \frac{W_0 - W_t}{W_0} \times 100
\]  

(1)

Where \(W_0\) is the weight before immersing in PBS and \(W_t\) is related to the weight of samples after incubation in PBS at 37°C.

### 2.6. Swelling behavior

To inspect the swelling behavior of the films, all samples were incubated in DI water at 37°C for up to 24 hours, and the weight of the samples was measured after 6, 12, and 24 hours of immersion. The swelling behavior was quantified for each group of samples by employing Eqs. (2):

\[
\text{Swelling (\%) } = \frac{(W_t - W_0)}{W_0} \times 100
\]  

(2)

Where \(W_0\) is the weight of dry film before immersion in DI water, and \(W_t\) is related to the weight after immersion of samples for each period of time.

### 2.7. Porosity

The porosity of the films was assessed using the Archimedes method according to the ASTM C830-00 standard porosimetry method. All the samples were first dried in a vacuum desiccator at 25°C and then weighed. They were then immersed in pure ethanol for 24 hours. In the current study, pure ethanol was employed as the penetrated liquid due to its lower surface tension at room temperature and to prevent any degradation or swelling in the samples. The pore volume was calculated by the Eq. (3):

\[
\text{Pores value (ml/gr)} = (W_t - W_0) \times 1.297
\]  

(3)

Where \(W_t\) is for the weight of dried samples, \(W_t\) is the weight after 24 hours in ethanol, and 1.297 is the specific value of absolute ethanol per gr.

### 2.8. Hydrophilicity

The water contact angle was considered as a measure of the hydrophilicity of samples. In order to evaluate the contact angle with water, the films consist of 0, 5, and 10% wt. NaCl was first dehydrated in a vacuum chamber and a 400\(\mu\)l drop of water was placed on the surface (according to ASTM D7334). The water drop contact angles were measured by Image J software.

### 2.9. In vitro cell viability
The nontoxic effect of NaCl was verified by in vitro cell culture followed by an MTT assay. The plate-like films of 14 mm in diameter are first prepared from the mixture of PCL/NaCl with 0, 5, and 10% wt NaCl. After removing the NaCl particles from the PCL matrix, the samples were immersed in 70% ethanol for 2 h and washed with sterile PBS twice. Each surface of the samples was exposed to ultraviolet (UV) light for 20 min to completely purify the samples from contamination. The MC3T3 cell line was supplied by the Iran national cell bank at the Pasteur Institute and incubated in eagle medium (DMEM, Gibco, USA) containing 10% fetal bovine serum (FBS, Sigma Aldrich, USA) and 1% penicillin-streptomycin (Sigma Aldrich, USA) to rias up the culture plate conuency up to 80%. The cells were detached from the culture vessel using trypsin (EDTA, 0.05, Gibco, USA) treatment. Samples were paced into the 24 well culture plate and the cells were seeded on the samples at a count of $2 \times 10^3$ cells per ml. The samples were incubated at 37°C in the atmosphere with 95% air, 5% CO2, and 95% humidity. After 48 h, 50mg/ml MTT solution (3-[4,5-dimethylthiazol2-yl]-2,5-diphenyltetrazolium bromide, Sigma Aldrich, USA) was prepared in PBS and added into each well of the culture plate. After 3 hours of incubation of samples with MTT, dimethyl sulfoxide (DMSO, Sigma Aldrich, USA) was used for dissolving the formazan crystals, and the light absorbance was read at 570nm using an ELISA plate reader. The cell viability for each sample was calculated by Eqs. (4):

$$\text{Cell viability} = \frac{\text{sample absorbance}}{\text{control absorbance}} \times 100$$

(4) Cell viability

2.10. Statistical analysis

Based on response surface methodology (RSM), all experimental samples were designed using Design-Expert software (12.0.3 version). Each test was performed on three samples, and the results were presented as the average of three replicates with standard deviations (SD). Analysis of the biological assay results was performed using SPSS v22 software. The one-way analysis of variance (ANOVA) and student t-test was used for the statistical analysis, and at a p-value less than 0.05, the confidence level was considered significant.

3. Result And Discussion

3.1. Mechanical properties

As can be seen in Fig. 3, in vertical pressure loading, the amounts of elongation, compressive strength, and stiffness were significantly higher than the mentioned properties in horizontal loading for all samples. In the case of controlling segmental bone tissue engineering, the guide films should have high compressive strength and stiffness under vertical loading (because of enduring the body’s weight) and low elongation under pressure to prevent any dislocation in the fractured bone segments or implanted scaffolds. However, regarding the Y axis, the most stiffness belonged to the sample A and F (no significant difference) which contained zero percentage of sodium chloride, and sample E was the next sample with a higher stiffness with zero level of sodium chloride. The least stiffness belonged to the samples H and C due to the high content of NaCl. Considering the horizontal analysis, Samples F and E
showed the most stiffness in which their NaCl content was 0% and 5% respectively while their strand diameter was 800 micrometers. The least stiffness belonged to the sample H with a diameter of 600 micrometer and 0% NaCl content. It can be concluded that the presence of porosity reduces the stiffness. Besides, it can be inferred that the diameter of the printed strands play a vital role in stiffness as if in most cases by reducing the diameter, the stiffness was decreased. As shown in Fig. 3b, the mean elongation at break under X, Z axis loading was higher (almost 3 folds) than the elongation that occurred by the Y loading direction. Surprisingly, in all groups by increasing the NaCl content from 0–5%, the elongation was reduced, and then by increasing it from 5–10% the elongation was enhanced.

The maximum compressive strength of samples in the Y axis, was around 13.6 MPa, which is obviously greater than the common bone scaffolds such as porous hydroxyapatite or β-tricalcium phosphate, which means that the guide films can ideally support the scaffold segments (Wen, Xun et al. 2017, Osuchukwu, Salihi et al. 2021). By increasing the NaCl content, the comprehensive strength was reduced and then increased. Based on the results, the stiffness of samples in the Y-axis was in the significant range for the New Zealand white rabbit in vivo model (Schafrum Macedo, Cezaretti Feitosa et al. 2019). The maximum stiffness (114.53 MPa) was observed in the samples with 700µm film thickness and without NaCl (coded A). As can be seen, in the samples with 700µm thickness, by the addition of 5%wt NaCl, the stiffness was reduced from 114.53 to 44.79MPa. By increasing the amount of NaCl in the PCL matrix up to 10%, the stiffness conspicuously decreased and reached 10.42 MPa. By the addition of NaCl into the PCL, after leaching and removing the salt particles, some vacancies appeared in the matrix that made it porous (Haider, Haider et al. 2020). The pores divest the uniformity of the polymeric matrix and caused some defects (pores) in the films that decreased the stiffness. It is also confirmed by Murugan et al. (Murugan and Parcha 2021). In the X, and Z loading directions (X and Z directions were considered the same due to the symmetry of the samples), the maximum elongation at break (71.86%) was observed when the sample G was tested under a 10 MPa pressure load. As can be seen, the compressive strength of samples in the X, Z directions was more than the Y axis loading, which indicates that the PCL guide films provide satisfactory strength during implantation surgery and they are easy to handle for the surgeon (Klemm, Raddatz et al. 2021). The maximum compressive strength (15.73MPa) was for the sample with 5% NaCl and an 800µm film thickness, and the lowest compressive strength was around 1MPa, which was related to the sample C with 5% NaCl and a 600 µm thickness. As presented in Fig. 3c, the mean stiffness of samples in the X, Z directions was almost 14 MPa, which is very ideal for controlling segmental bone tissue engineering (Mauffrey, Barlow et al. 2015). The sample E with 5% wt. NaCl and an 800 m thickness had the highest stiffness in X and Z directions (22.35 MPa), while the sample H with 10% wt. NaCl and 600 µm film thickness had the lowest (9.33 MPa). Like the results for the Y direction, in the X, and Z loading axis, the mechanical properties were intensely dependent on the NaCl weight percentage (at the same thickness), so increasing the amounts of NaCl caused an obvious reduction in the mechanical properties of each group of samples with the same film thickness. The previous study by Kim et al. (Kim, Kim et al. 2017), also clarified the destructive effects of NaCl on the mechanical properties of the polymer matrix. Based on the results, the mechanical properties of all samples in both X, Z, and Y directions were in the good range for implantation and handling during surgery.
3.2. RSM study

The mechanical behavior of samples under X, Z, Y compressing directions was statistically modeled by Central composite design (CCD). Table 3 represents the compressive strength, elongation at break, and stiffness of all samples in different directions. X and Z were considered as same as each other due to the asymmetric geometry of the scaffolds.

By considering the mentioned mechanical properties, as three responses depending on the film thickness (A) and the NaCl concentration (B) and (in X and Z direction), the related responses for stiffness and elongation had the best fit with a simple model and a linear model was determined by CCD-RSM to express the correlation of compressive strength with NaCl concentration and film thickness. The power of responses including stiffness, elongation, and compressive strength were $-3$, $2.2$, $1$ respectively. The related equations for the prediction of mechanical properties in the X, and Z directions are presented in Equations 5, 6 and, 7. Based on Eq. 5, both the NaCl content and strand diameter showed nearly the same impact on the responses (coefficients are the same). According to the Eq. 6, the film thickness plays a vital role in affecting the elongation. Also, in the case of comprehensive strength (Eq. 7), the film thickness is more effective than NaCl content.

\[
StiffnessX (MPa)^{-3} = 0.0005 - 0.0003A + 0.0003B - 0.0002AB - 0.0001A^2 + 0.0001B^2
\]

(5)

\[
ElongationX (\%)^{2.22} = 67.42 + 27.01A + 3.72B - 4.11AB - 26.41A^2 + 4.13B^2
\]

(6)

\[
CompressivestrengthX (MPa) = 8.08 + 5.65A - 0.7983B
\]

(7)

According to the Eq. 8, as can be seen, the coefficient for NaCl Content (B) is greater than the coefficient of A (Thickness), which indicates that the film thickness had a stronger effect on the stiffness of the final structure. Based on the Eq. 9, the NaCl content is more effective in altering the elongation property. In the case of Elongation, the NaCl content depicted its impact two folds of the film thickness. Considering the compressive strength, Eq. 10 shows that the thickness is the main and more effective parameter.

\[
StiffnessY (MPa)^{-0.4} = 0.21 - 0.047A + 0.027B
\]

(8)

\[
ElongationY (\%)^{1.47} = 58.45 - 12.80A + 23.44B - 12.46AB
\]
Table 3 depicts the significance of all models in X, Y, and Z direction. Based on Table 3, the $P$-value of all three responses in each direction was less than 0.05, which confirmed the model's terms are significant and the models are adequate for the prediction of responses. The $F$-values for the stiffness, elongation, and compressive strength were 7.72, 155.34, and 52.13 respectively. The coefficient of determination ($R^2$), adjusted $R^2$, and predicted $R^2$ for the stiffness, elongation, and compressive strength are presented in Table 3. It is evident that there is no significant difference between $R^2$ and $R^2_{adj}$ for all models, which proves that the models are not overfitted. It needs to be highlighted that $R^2$ for all responses were closed to one.

<table>
<thead>
<tr>
<th>Axis</th>
<th>Responses</th>
<th>$R^2$</th>
<th>$R^2_{adj}$</th>
<th>$R^2_{pred}$</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>X,Z</td>
<td>Stiffness</td>
<td>0.98</td>
<td>0.9659</td>
<td>0.8092</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td></td>
<td>Elongation</td>
<td>0.99</td>
<td>0.9847</td>
<td>0.9122</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td></td>
<td>Compressive strength</td>
<td>0.91</td>
<td>0.8950</td>
<td>0.7893</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>Y</td>
<td>Stiffness</td>
<td>0.87</td>
<td>0.84</td>
<td>0.73</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td></td>
<td>Elongation</td>
<td>0.91</td>
<td>0.87</td>
<td>0.78</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td></td>
<td>Compressive strength</td>
<td>0.88</td>
<td>0.86</td>
<td>0.76</td>
<td>$&lt;0.0001$</td>
</tr>
</tbody>
</table>

$R^2$ for all models was very high (> 0.85), which shows that more than 85% of the variations in the related responses can be explained by changes in NaCl concentration and film thickness. Figure 4 shows the predicted versus actual plots for the models. The distribution of terms is not dispersed or S shaped, which indicates that the models were accurate in predicting the responses including stiffness, elongation, and compressive strength.

The contour plots of the models are displayed in Fig. 5 and Fig. 6. As can be seen, the highest stiffness was observed for 800µm thickness and NaCl content of less than 6%wt. Based on the contour plot for the stiffness, by increasing the NaCl content up to 10%, the stiffness decreased and reached 10 MPa for the samples with a thickness of less than 750µm. On the other hand, if the printer machine is adjusted to print films with a 750–800µm thickness, for all amounts of NaCl %wt. (in the range of 0 to10%), a satisfactory stiffness in the range of 15–20 MPa can be attained. According to Fig. 5b, the elongation at break in X,Z direction and Y direction strongly depends on the film thickness and NaCl content respectively. In horizontal direction, the maximum elongation occurred when the samples with the
thickness of 700–800 µm were tested by compressive loading (Fig. 5e, f), while in the vertical direction the most elongation occurred in a high content of NaCl. It was also indicated that the compressive strength had an extreme relationship with the film thickness and significantly enhanced with increasing the sample thickness. This trend was similar in X,Z and Y direction.

3.3. Degradability

Figure 7 demonstrates the degradation curve of samples by immersion in PBS. As can be seen, the degradation of sample G (800µ, 10%wt. NaCl) during 60 days of incubation in PBS was significantly higher than the other samples and was around 21.7%. Furthermore, the weight loss in sample G was more rapid compared to other samples. On the other hand, sample F (800µ, 0%wt. NaCl) had the slowest degradation rate, so at the end of the test period, sample F showed only 2.75% weight loss. In the samples with the same thickness, the degradability was enhanced by increasing the NaCl %wt. In vitro degradation starts by exposing the sample to an aqueous medium (Zada, Kumar et al. 2020). When the samples were immersed in PBS, the surface layer of PCL slowly degraded and the weight was reduced. Since the degradation directly depends on the surface area, more degradability is predictable for the samples with a higher porosity percentage, which is also confirmed by Mandal et al (Mandal, Nandi et al. 2021). As mentioned before, NaCl acts as a temporary space holder to shape the porosity and improve the possibility of exchanging the necessary nutrients for living cells. In the case of degradation, increasing NaCl% wt. from 0 to 10% (in constant thickness) enhances the degradability of the PCL films. The important note about PCL degradability is that the degradation rate of PCL in vivo conditions is much higher than the degradation in the in vitro test (Lam, Hutmacher et al. 2009). Thus, by considering the in vivo environment as the main term of application, all samples were in the good range for degradation in controlling the segmental bone tissue engineering and supplying sufficient time for bone remodeling.

However, based on the defect and its location in the body, the time of bone healing is different. Thereby, it is important to control the time of degradation. But basically, one of the main purposes of this kind of guide is to be removed spontaneously without surgery.

3.4. Swelling

As detailed in Table 4. After the first 6 h of immersion in DI water, only C samples showed the swelling behavior. During the 24h swelling test, samples G, C, and H, showed 6.61, 6.45, and 4.94% swelling, respectively, which were significantly higher than the other samples. In the samples F and I, no swelling behavior during the test period was observed.
Table 4
Swelling behavior during 24h immersion in DI water.

<table>
<thead>
<tr>
<th>Cod</th>
<th>Swelling after 6h (%)</th>
<th>Swelling after 12h (%)</th>
<th>Swelling after 24h (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>D</td>
<td>0.00</td>
<td>0.98</td>
<td>1.96</td>
</tr>
<tr>
<td>E</td>
<td>0.00</td>
<td>0.87</td>
<td>1.74</td>
</tr>
<tr>
<td>H</td>
<td>0.00</td>
<td>1.23</td>
<td>4.94</td>
</tr>
<tr>
<td>G</td>
<td>0.00</td>
<td>2.48</td>
<td>6.61</td>
</tr>
<tr>
<td>D</td>
<td>0.00</td>
<td>0.98</td>
<td>1.96</td>
</tr>
<tr>
<td>D</td>
<td>0.00</td>
<td>0.98</td>
<td>1.96</td>
</tr>
<tr>
<td>C</td>
<td>2.15</td>
<td>5.38</td>
<td>6.45</td>
</tr>
<tr>
<td>D</td>
<td>0.00</td>
<td>0.98</td>
<td>1.96</td>
</tr>
<tr>
<td>F</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>B</td>
<td>0.00</td>
<td>0.00</td>
<td>2.02</td>
</tr>
<tr>
<td>A</td>
<td>0.00</td>
<td>1.04</td>
<td>1.04</td>
</tr>
<tr>
<td>D</td>
<td>0.00</td>
<td>0.98</td>
<td>1.96</td>
</tr>
</tbody>
</table>

It seems that the swelling in samples had a relationship with NaCl %wt. so that the samples without NaCl showed very low swelling or zero (Table 4). In addition to increasing the contact surface by NaCl, the cavities in the PCL matrix (that are formed by NaCl leaching) can trap some water in their porous structure and cause measurement error in the swelling tests. In the case of tissue engineering, the moisture of the devices is a great attribute because most cellular nutrients, such as carbohydrates or amino acids, are soluble in water, and the swelling behavior of the devices for tissue engineering applications can enhance cell feeding and tissue remodeling (Zarrintaj, Bakhshandeh et al. 2017). By considering the swelling behavior as a good property for the guide films, the samples with an 800μm thickness and 10% wt. NaCl (sample G) was the optimum sample for controlling segmental bone tissue engineering (Mauffrey, Barlow et al. 2015, Alkindi, Ramalingam et al. 2021). However, sample C (600μm, 5%wt. NaCl) also showed good swelling behavior but based on the results of mechanical properties, sample G had better characteristics in both swelling and mechanical properties. An appropriate swelling is required to adsorb the output of the cell cycle and remove it from the defected area. In contrast diffusion of nutritions from outside into the defected site is mandatory for cell proliferation and migration. A guide with no swelling will fail for such a characteristic.

4. Porosity
Figure 8 illustrates the results of the porosimetry of the films. As seen, the porosity volume of samples was in the range of 0.005 to 0.02 ml and the highest porosity volume was measured for sample H (600µm, 10%wt. NaCl). In this method for porosimetry of the porous structures, the penetrating liquid (ethanol in this case) diffuses into the pores and increases the weight of the sample after immersion (Zou and Malzbender 2016). Based on Table 9, the porosity values increased by increasing NaCl%wt. In the fabrication of porous structures for biomedical applications, balancing porosity and mechanical properties is a significant challenge (Sabree, Gough et al. 2015). The reduction of mechanical properties by increasing porosity has been reported numerous times in the literature (Wen, Xun et al. 2017, Sadeghzade, Emadi et al. 2020). In the PCL films, the lowest stiffness was for sample H, which had the highest porosity value. Nevertheless, the porosity decreased the mechanical properties of the guide films, but it is necessary to prevent tissue necrosis and infection. By adjusting the number and the size of the created porous area, it is possible to optimize a suitable guide for specific purposes. For instance, a guide for fracture in the leg must own a higher mechanical property than that of the hand.

4.1. Water contact angle

As displayed in Fig. 9. The contact angles for PCL films with 0, 5, and 10%wt, NaCl were 87.2, 84.5, and 82.3° respectively. The greatest hydrophilicity was determined for PCL films containing 10% wt. NaCl. This improvement in the hydrophilicity of PCL did not occur due to a change in the surface functional groups but was due to the surface morphology and roughness that were also approved by Kajau et al. (Kajau, Motsa et al. 2021). Based on the results, the addition of NaCl to the PCL can significantly enhance the hydrophilicity of pure PCL films. It can also be claimed that by increasing the hydrophilic behavior of the PCL guide, it is possible to adjust its degradation period. Higher surface area is obtained by creating porosities. However, increasing the hydrophilicity will let the products of cells cycle to diffuse and move across the guide.

4.2. Cell viability

Cell viability for all three samples was more than 85% which is proof of the biocompatibility of the PCL films (Sadeghzade, Emadi et al. 2020) (Fig. 10). Based on the results from the MTT assay, the cell viability improved by increasing the NaCl %wt. that the rationale reason can be creating more porosity. However, increasing the NaCl content changed the cell viability slightly which might be caused by residual NaCl in the polymeric structure that was also reported by Olmo et al. (Olmo, Franco et al. 2021). Anyway, the MTT results approved a good range of biocompatibility for biomedical applications. By the way, previous studies reported the good biocompatibility of PCL polymer. It can be concluded that the presence of a PCL guide will not leave a negative effect on the cell cycle and also bone healing.

5. Conclusion

Fabrication of the porous PCL films with regard to controlling segmental bone tissue engineering has been reported in this research work. The effects of film thickness and NaCl weight percentage on different
characteristics of the films were investigated. The results showed that the mechanical properties were more affected by film thickness, so that increasing the film thickness, enhanced the mechanical properties in both horizontal and vertical loading directions. It was also discovered that the degradability, swelling, and hydrophilicity of the samples were highly related to NaCl %wt. and could be improved by adding NaCl (5 and 10%wt) to the PCL matrix. The results extracted from the CCD-RSM statistical models showed that by adjusting the film thickness on 700–800µm it is possible to reach their maximum compressive strength and elongation in horizontal (X, Z) loading, but the stiffness has little dependence on the salt percentage and reaches its maximum value by adjusting the salt percentage to less than 6% in 800 micron films. In the vertical loading direction, mean stiffness was almost 4 times greater than mean stiffness in horizontal loading direction, and had a good capacity to bear body weight during bone healing. The use of 3D printed guide films for controlling segmental bone tissue engineering, in addition of reducing the need for metallic pins and plates, can be a customized fixation device that can be fabricated individually for patients by rapid prototyping methods and can be a promising approach in bone tissue engineering.

**Declarations**

**Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Author contribution**

Hamid Soleimanimehr: Supervision; Validation; Conceptualization; Shahram Etemadihaghighi: Data curation; Formal analysis; Methodology; review & editing. Vahid Khodabakhshi: Project administration; Resources; Software; Ali Emam: Visualization; Roles/Writing - original draft; Writing.

**Acknowledgment**

This study received no financial support.

**Data Availability**

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

**References**


Table 9
Table 9 is not available with this version.

Figures
creating new PCL granolas containing NaCl particles to be used in 3D printing.

**Figure 1**

Vertical and horizontal loading directions in compressive tests

**Figure 2**

Vertical and horizontal loading directions in compressive tests
Figure 3

a) stiffness, b) elongation at break, and c) compressive strength of samples in both Y and X, Z loading directions.
Figure 4

Predicted vs actual plots for different responses including a) stiffness, b) elongation, and c) compressive strength (X,Z direction), d) stiffness, e) elongation, and f) compressive strength (Y direction).
Figure 5

Contour and 3D plots for stiffness (a, b), elongation (c, d) and, compressive strength (e, f) (X,Z direction). (The color blue low level and red high level of response).
Figure 6

Contour and 3D plots for stiffness (a, b), elongation (c, d) and, compressive strength (e, f) (Y direction). (The color blue low level and red high level of response).
Figure 7

Degradation curves of all samples after 60 days of incubation in PBS.
Figure 8

The results of the porosimetry of the samples.

Figure 9

Water contact angle on the surface of samples.
Figure 10

Cell viability on the surface of samples after 48h culture time.