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FULL PAPER

Loading of VEGF and BMP2 to PNIPAAm-based hydrogel as a scaffold for bone regeneration

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Several techniques have been developed to create 3D biomimetic scaffolds based on hydrogels, and these have recently become popular in tissue engineering. Given that the incorporation of osteogenic reagents such as VEGF and BMP2 improves bone regeneration, in this study, we evaluated a PCL-P (HEMA-NIPAAm) hydrogel consisting of polycaprolactone (PCL) blocks and poly(N-isopropylacrylamide-hydroxyethylmethacrylate) (NIPAAm-HEMA) acrylate blocks for the delivery of two growth factors, VEGF and BMP2, simultaneously. Physical characterization of PCL-P (HEMA-NIPAAm) hydrogel was evaluated by FTIR, SEM, and ^{1}H-NMR. VEGF and BMP2 were loaded on the hydrogel and the release of both growth factors (GFs), cytotoxicity assay, and Alizarin red S staining the human dental pulp stem cells (hDPSCs) were evaluated. The initial release occurred rapidly within the first 24 hours, with 45% and 50% of both growth factors (GFs) being released. By the end of the study period, a total of 80% of BMP2 and 85% of VEGF had been released from the hydrogel scaffold. The cell viability of samples was measured at 91%, 205%, and 470% after 24 h and on the third and fifth day, respectively. In the SEM images, hDPSCs fully adhere, colonize, and proliferate on the hybrid copolymer, which is non-toxic to cells. The produced hydrogel demonstrated no evident cytotoxicity in hDPSCs cell lines and our novel PCL-P-based hydrogel scaffold contained growth factors as a matrix for hDPSCs and cell seeding, which could be a beneficial technique used in 3D constructs with high potential for bone tissue engineering and regenerative medicine.

KEYWORDS

VEGF; BMP-2; copolymer; vascular endothelial growth factor; bone tissue regeneration.

Introduction

Bone defects caused by congenital anomalies, trauma, cancer resection, and degenerative diseases collectively represent some of the most critical global issues [1,2]. The current gold standard for addressing bone defects caused by congenital anomalies, trauma, cancer resection, or degenerative diseases is

to replace damaged bone tissue with allogeneic or autologous bone implants [3]. However, these treatments are ineffective and have several drawbacks for patients, including increased morbidity, pain, and minimal support of the obtained bone as well as an immunogenic response that causes immediate tissue rejection [4]. In recent years, bone and cartilage tissue engineering has emerged as a viable approach. This method aims to enhance the levels of biologically active bone components such as osteogenic factors, which could have a substantial effect on preserving, enhancing, and repairing bone tissue [5,6].

The bone tissue engineering based scaffolds is the most promising technique that can progressively release growth factors (GFs), which are necessary for bone repair [7-9]. Bone morphogenetic protein-2 (BMP-2) is a vital growth factor that plays a crucial role in the bone repair techniques and is increasingly employed in orthotopic and ectopic locations for bone regeneration [10,11]. Fracture healing and skeletal maturation rely heavily on angiogenesis, a process in which vascular endothelial growth factor (VEGF) is widely regarded as a critical molecule. Scaffolds could contain VEGF to promote blood vessel growth [12-14]. The fields of regenerative medicine and bone tissue engineering rely heavily on the development of biocompatible scaffolds [15,16]. These networks create a 3D setting analogous to the tissue-level extracellular matrix (ECM) [17,18]. Compared to 2D cell culture, 3D incubation provides superior signals for cell flattening, adhesion, migration, proliferation, differentiation, and cell-derived extracellular matrix (ECM) formation [19]. Scaffolds for promoting MSC chondrogenic development have been prepared using a wide range of synthetic and natural polymers. Because of the availability of water-swollen and porous polymeric scaffolds, hydrogels have become the focus of attention for developing scaffolds for various conditions and tissues capable of initiating chondrogenesis, maintaining uniform cell seeding, and used in tissue engineering research [20-22].

PNIPAAm-based hydrogels are commonly used as thermosensitive biomaterials due to their lower critical solution temperature (LCST) of 32 °C [23]. Hydrogels made from PNIPAAm have received considerable attention as potential thermosensitive biomaterials. With the addition of hydrophobic or hydrophilic monomers, NIPAM can be copolymerized [20,24]. The LCST of hydrogels may be modified by changing their transition temperatures. Poly(ε-caprolactone) (ε-PCL) is a semicrystalline linear aliphatic polyester with excellent mechanical properties, as well as being biocompatibility, and biodegradability (it may be broken down in a biological setting by hydrolysis of the ester bond) [25]. In this study, the concept of degradable hydrogels and biodegradability of PCL are combined to benefit from the advantages of both polymers.

Acrylates containing hydroxyl functional monomers, such as hydroxyethylmethacrylate (HEMA), can be used for the ring-opening polymerization of cyclic esters [26,27]. The objective of this work was to apply our previously synthesized PCL-P (HEMA-NIPAAm) hydrogel [28,29] for the simultaneous delivery of two growth factors, VEGF and BMP2.

In light of the challenges posed by bone defects and the promising advancements in tissue engineering, this study aims to evaluate the potential of a novel PCL-P(HEMA-NIPAAm) hydrogel scaffold for the simultaneous delivery of growth factors VEGF and BMP2. By characterizing the physical properties of the hydrogel, assessing the release kinetics of the growth factors, and evaluating the biocompatibility using human dental pulp stem cells, our goal is to contribute to the development of effective 3D constructs for bone tissue engineering and regenerative medicine.

Experimental

Materials

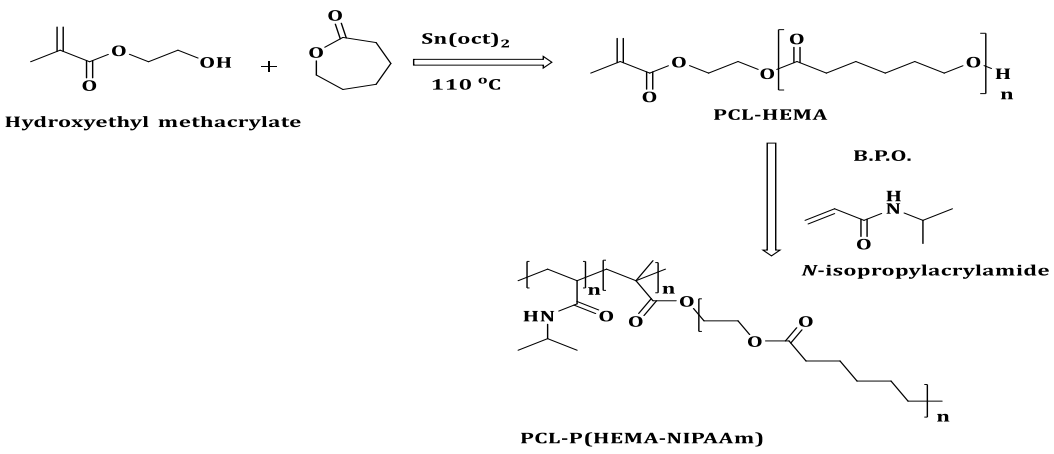
Acros Organics (New Jersey, US), 99% v/v caprolactone monomer and 97% v/v HEMMA were used. HEMA was purified by vacuum distillation. *N*-Isopropyl acrylamide (NIPAAm), refined from *n*-hexane-toluene (90:10 v/v) by recrystallization, was provided by Acros Organics. Merck Chemicals supplied tetrahydrofuran (THF) and toluene as well as the benzoyl peroxide initiator. Tin octanoate ($SnOct_2$), 1,4-dioxane, and dimethyl sulfoxide were provided by Sigma-Aldrich. Gibco provided the phosphate-buffered saline (PBS) and 0.25 percent trypsin/EDTA. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was obtained from Invitrogen. Recombinant human BMP-2 and VEGF were obtained from Sigma-Aldrich. The Tabriz Faculty of Dentistry generously donates stem cells from human dental pulp. All reactants were of analytical quality and utilized as received, and deionized (DI) water was used in all experiments.

Characterization

Morphological and structural investigations of the product samples were conducted using a 25 kV scanning electron microscope (model EOL, JSM 6700F) (JEOL Ltd., Tokyo, Japan). At room temperature, ^{1}HNMR spectroscopic characterization was conducted using a 400-MHz spectrometer (Ettlingen, Germany) with $CDCl_3$ as the solvent. Functional groups in the materials were identified using KBr pellets and a Tensor 27 FT-IR spectrophotometer (Bruker, Germany) in the 400–4000 cm^{-1} spectral region. Ultraviolet-visible (UV-Vis) spectra were recorded using a UV-Vis spectrophotometer (UV-160 Shimadzu).

Synthesis of PCL-P(NIPAM-co-HEMA) hybrid copolymer

The PCL-P(NIPAM-co-HEMA) hybrid copolymer was synthesized using our previous work [28]. Briefly, HEMA and ε-PCL were reacted in the presence of tin(II) 2-ethylhexanoate ($Sn(Oct)_2$) using toluene as the solvent. To prepare a biodegradable copolymer, PCL-graft-poly(*N*-isoporylacrylamide-co-hydroxyethyl methacrylate) with a lower critical solution temperature (LCST) near the normal body temperature was synthesized (Scheme 1).



SCHEME 1 Synthesis of PCL-P(HEMA-NIPAAm) hybrid copolymer

Loading of growth factors

The PCL-P(HEMA-NIPAAM) scaffold was sterilized overnight using a UV light source. The loading procedure was performed in a laminar flow hood to avoid contamination. Each polymer was placed in 500 mL PBS solution containing either BMP2 or VEGF at a 0.1 g/mL concentration. The suspension was then cooled to 4 °C to permit absorption of the growth ingredients. The mixture was then agitated for three days at 100 revolutions per minute to produce growth factor-loaded scaffolds. The loaded scaffolds were then frozen at 80 °C after centrifugation. The scaffolds were freeze-dried and stored overnight at 4 °C before examination.

The percentages of the encapsulation efficiency (EE) and loading capacity (LC) were computed using the following formulas:

EE (%,W/W)= (Mass of GF in polymer) / (Mass of initially added GF) ×100

LC (%,W/W)= (Mass of GF in polymer) / (Mass of polymer) ×100

Each sample was tested three times, and the mean percentages were recorded.

In vitro release studies

The effects of the growth factor-loaded scaffolds were evaluated in vitro. In a typical experiment, the sample suspension was mixed with 500 µL freshly prepared PBS buffer and incubated at 37 °C. At regular intervals, 100 µL of buffer was withdrawn for analysis and the same amount of new medium was added to maintain a consistent buffer volume. Following the manufacturer's instructions, we used a VEGF and BMP2 Enzyme-linked Immunosorbent Assay (ELISA, Abcam, USA) kit to calculate the overall percentage of growth factor release in the collected supernatant. Each sample was tested three times, and the mean ODs was recorded.

In vitro cytotoxicity studies

To test the cytotoxicity and biocompatibility of the scaffolds, this study employed a specific strain of human dental pulp stem cells (hDPSCs) was used. The PCL-P(HEMA-NIPAAM) scaffold was sterilized with UV light overnight. Briefly speaking, 2 ×104 cells were seeded, trypsinized, and plated in each well of a 96-well plate. The culture medium was withdrawn and replaced with a new growth medium (200 mL) containing the appropriate quantities of the samples as well as blank scaffolds and was then incubated for 24 h, 3 d, and 14 d (in a humidified environment with 5% CO_2 at 37 °C). The medium was then removed, and each well received 5 mg/mL MTT solution before incubating for 4 h. A microplate reader (Elx808, Biotek, USA) was used to measure optical density (OD) at 570 nm. Each sample was tested three times and the mean of the ODs are recorded.

Statistical analysis

GraphPad Prism 8 (GraphPad Software Inc. (San Diego, CA, USA) was used for the statistical analyses. The Shapiro-Wilk normality test was used to test the data distribution. Student's t-test was used to compare the means of the data sets. Statistical significance was set at $P < 0.05$.

Results and discussion

Preparation and characterization of PCL-P(NIPAM-co-HEMA) scaffold

As shown in the FT-IR spectrum of the hybrid PCL-P (Figure 1), the most significant bonds in the PCL-HEMA FTIR spectra were the stretching vibrations of hydroxyl groups (OH) at 3551 cm^{-1}, (CH_2) and (CH_3) at 2924 and 2855 cm^{-1}, and ester carbonyl (C=O) at 1726 cm^{-1}. Absorption maxima for the (C-O-C) stretching vibration absorption bonds of HEMA and (C=C) stretching vibration absorption bonds of HEMA and PCL were observed. In addition, the absorption band at 1452 cm^{-1} is related to the bending vibration of the (CH_2) group. In the FTIR spectrum of PCL-P(HEMA-NIPA), peaks at 1646 cm^{-1} (attributed to NH-CO stretching), 1544 cm^{-1}

(corresponding to N-H bending), and 1383 cm^{-1} (corresponding to C-H stretching vibrations of $-CH(CH_3)_2$) confirmed the presence of NIPAAm groups in the backbone of the copolymer. The stretching vibrations of the carbonyl groups in PHEMA, PCL, and PNIPAAm were attributed to the PCL-P (HEMA NIPAAmprominent) absorption bands at 1726 cm^{-1} and 1618 cm^{-1} in the FTIR spectrum, and the absence of the typical absorption peaks at 1636 cm^{-1} indicated the successful conversion of HEMA to PHEMA via the free radical polymer. The aliphatic -CH stretching vibrations of the (CH_3) and (CH_2) groups are responsible for the absorption maxima at 2855 and 2924 cm^{-1}, respectively.

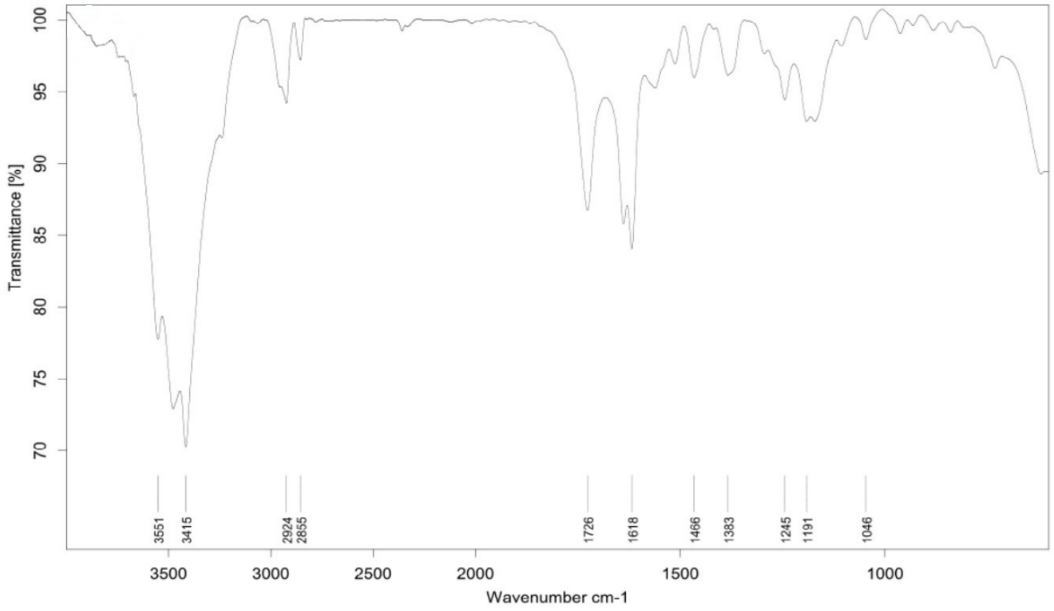


FIGURE 1 Fourier transform infrared spectroscopy (FT-IR) spectra of the synthesized polymer

The PCL-P(HEMA-NIPAAm) hybrid copolymer created soft and compact scaffolds with a homogeneous morphology, as seen in the scanning electron microscopy (SEM) image (Figure 2), which revealed that the scaffolds were well disseminated and of uniform shape and size. Human dental pulp stem cells (hDPSCs) fully adhere, colonize, and proliferate on the hybrid copolymer, which is non-toxic to cells.

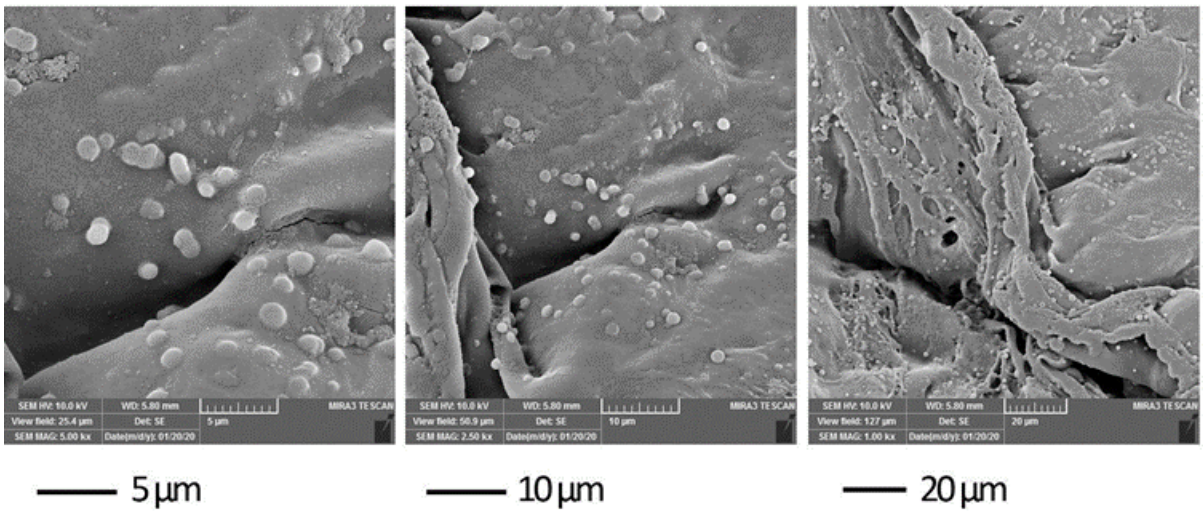


FIGURE 2 Scanning electron microscopy (SEM) images of the PCL-P(HEMA-NIPAAm) hybrid copolymer

Proton nuclear magnetic resonance spectroscopy (¹HNMR)

Figure 3 displays the ¹H-NMR spectra of the PCL-P copolymer. All chemical alterations connected to the PCL segment were observed in the PCL-HEMA spectrum. The signals at 1.283, 4.197, and 6.012 ppm corresponded to the protons in the PNIPAAm region of methyl, ($CH(CH_3)_2$), and ($NH\text{-}CH(CH_3)_2$, respectively. The chemical shifts of the methylene units were 1.505 ppm (g), 1.860 ppm (f), 2.265 ppm (e), and 3.392 ppm (h) (CH_2). At 3.974 ppm, the overlapping resonance in the PHEMA-PCL section corresponded to the methyl ester group (d). The disappearance of the peaks at 4.865 and 5.651 ppm in the spectrum of PCL-P(HEMA-NIPAAm ¹H-NMR) reveals the transformation of the end double bonds into carbon-carbon signal bonds.

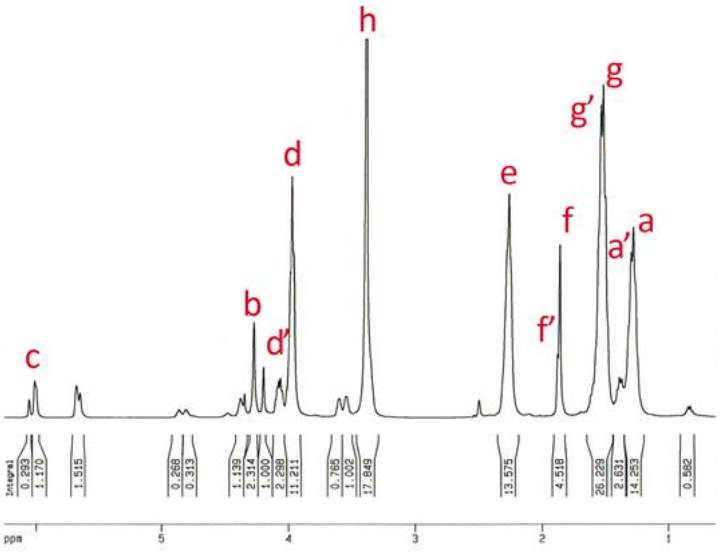


FIGURE 3 Proton nuclear magnetic resonance (1HNMR) spectra of synthesized polymers

Growth factors loading and release studies

A freshly created scaffold was constructed to adapt to the temperature fluctuations. The scaffolds contained growth factors (BMP2 and VEGF). The *in vitro* BMP2 and VEGF release characteristics of the scaffolds were investigated in PBS at 37 °C. The initial release was rapidly over the first 24 h, reaching 45% and 50% of the total loaded BMP2 and VEGF quantities in the scaffold, respectively, according to the release profiles. At 37 °C for approximately 10 days, the total quantities of BMP2 and VEGF released were 80% and 85%, respectively. This biomaterial may provide the optimal quantity of BMP2 and VEGF growth hormones for the development of bone tissue (Figure 4 (A and B)).

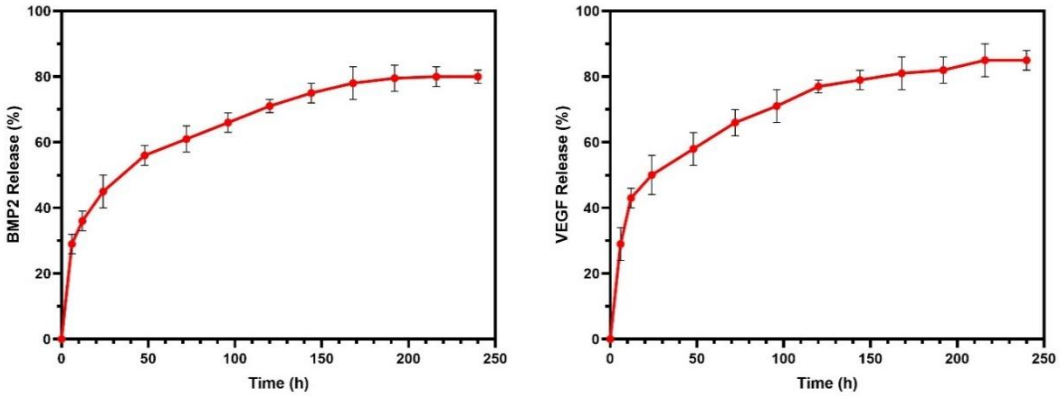


FIGURE 4 Cumulative in vitro release profiles of BMP2 (A) and VEGF (B) after 10 days (data are shown as mean ± standard deviation, n = 3)

In vitro biocompatibility study (MTT assay)

As depicted in Figure 5, the MTT cell assay was conducted on the human dental pulp stem cell (hDPSCs) line to determine the biocompatibility of these growth factors loaded on scaffolds as a growth factor delivery system. After 24 h and on the third and fifth days, the cell viability of samples was measured at 91%, 205%, and 470%, respectively, and even at high concentrations, there was no discernible cytotoxicity of blank samples in Human Dental Pulp Stem Cells (hDPSCs) lines. Consistent with Valipour *et al.* [30] and Xue *et al.* [31], which demonstrated a lack of cell toxicity in the stem cells plated on the PCL-P(HEMA-NIPAAm) S-2 or PCL hydrogel and higher viability in the presence of the hydrogel. In addition, they suggested that the presence of PCL in the scaffold enhance the adhesion, growth and proliferation of the cells, as well as, the 3D and porous structure of the scaffold could effect on cell survival. Our results also indicate that the porous and three-dimensional structure of scaffolds affects cell adhesion, proliferation, and growth. These results indicate that the scaffold is biocompatible and can be used as a biomaterial in medicine.

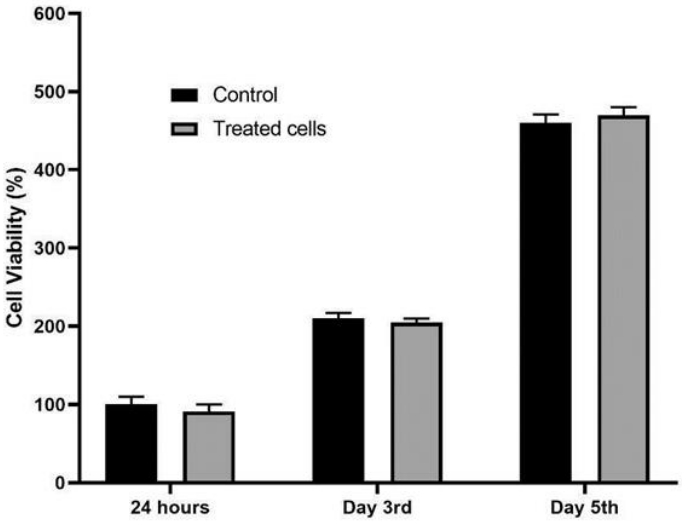


FIGURE 5 Biocompatibility of the developed polymeric scaffolds towards the hDPSCs cell line for 24 h, the 3rd day and the 5th day. Each sample was tested three times, and Student's t-test was used to compare the mean OD each day

Conclusion

By copolymerizing PCL, NIPAAm (a thermoresponsive monomer), and HEMA, we successfully created a novel PCL-P(HEMA-NIPAAM) hybrid copolymer for the delivery of growth factors (VEGF and BMPs) to their crucial roles in launching various stages of bone development, including the recruitment of mesenchymal stem cells (MSCs) and their differentiation into osteoblasts. VEGF and BMPs effectively loaded growth factors. The produced hydrogel demonstrated no evident cytotoxicity in HFFF2 cell lines, according to the MTT assay. Our novel PCL-P-based hydrogel scaffold contained growth factors as a matrix for human dental pulp stem cells (hDPSCs) and cell seeding, which could be a beneficial technique used in 3D constructs with high potential for bone tissue engineering and regenerative medicine.

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Authors' Contributions

Conceptualization was done by Soodabeh Davaran and Saeid Shabestari Khiabani; methodology was done by Marziyeh Aghazadeh; formal analysis was done by Peyman Keyhanvar; data curation was done by Pourya Gholizadeh; writing original draft preparation was done by Saeid Shabestari Khiabani; writing, review, and editing were done by Tayebeh Zivari-Ghader, Farahnaz Reyhanifar , Zahra Shabestari Khiabani, and Soodabeh Davaran; project administration was done by Soodabeh Davaran; funding acquisition was done by Soodabeh Davaran, Saeid Shabestari Khiabani, Marziyeh Aghazadeh, and Peyman Keyhanvar.

Conflict of Interest

The authors declare no conflict of interest

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