Application of Novel VEGF-Loaded PCL-P Based Hybrid Copolymer Scaffold for Bone Regeneration

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Abstract

Background: Polymeric materials are widely known as versatile materials, particularly for biomedical applications. The bioavailability, low toxicity, solubility, and permeability of this material play an essential role in biomedical implants, such as joint replacements, bone plates, ligaments, vascular grafts, intraocular lenses, heart valves, sutures, and dental implants. These materials can be formed as either biodegradable or other naturally bioactive polymers, which are composed of nano-sized particles in the composition of other materials. For example, PCL-P (HEMA-NIPAAm) is a thermosensitive, biodegradable, and biocompatible hydrogel for biomedical applications. In this study, we aimed to create a VEGF-loaded PCL-P (HEMA-NIPAAm) hybrid copolymer and analyze VEGF gene expression in human dental pulp stem cells (hDPSCs) that adhere to the copolymer.

Methods: VEGF (0.1 Â· g/mL) was loaded onto synthesized PCL-P (HEMA-NIPAAm), and hDPSCs were seeded into the scaffolds. To describe the hDPSC changes, VEGF mRNA, VEGF protein, and calcific deposition were examined.

Results: VEGF-loaded PCL-P (HEMA-NIPAAm) increased VEGF mRNA and protein expression in hDPSCs compared to PCL-P (HEMA-NIPAAm) and cells with any scaffold (P<0.05). After 21 days of culture, the color intensity of alizarin red was significantly higher in VEGF-loaded PCL-P (HEMA-NIPAAm) cells than in those with any scaffold (P<0.05) and PCL-P (HEMA-NIPAAm) cells (P<0.05).

Conclusion: Finally, thermosensitive and injectable PCL-P (HEMA-NIPAAm) including VEGF, enhanced the expression of genes associated with osteogenesis and angiogenesis in hDPSCs. Thus, these results imply that the thermo-sensitive PCL-P (HEMA-NIPAAm) scaffold may be a good option for future use as a VEGF carrier for hDPSC implantation.

Introduction

Common causes of bone abnormalities include trauma, fracture non-union, reconstructive surgery, necrosis, and malignancy. Bone tissue regeneration using stem cells is now regarded as a potential therapeutic option for bone repair and rebuilding.1,2 Osteogenesis is closely tied to angiogenesis in bone tissue engineering, in which osteoblast and endothelial cell interactions are critical for bone regeneration. Because the bone is highly vascularized, skeletal integrity requires a tight connection between bone cells and blood vessels.3 As a result, bone rebuilding and regeneration require an adequate blood supply of cells, nutrients, and oxygen.4 Scaffolds, growth factors, and stem cells are necessary factors for a tissue-engineered system.5

Recent progress in the production of advanced biomaterials has provided attractive alternatives to bone grafting. New bioactive materials (second generation) with controlled chemical decomposition, final absorption, and substitution with tissue have been

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developed. Third-generation biomaterials are designed to be osteoconductive and stimulate bone regeneration. However, the development of smart biomaterials with desirable mechanical properties, including adhesion and differentiation of stem cells, remains a challenging issue. Novel biomaterials for the controlled delivery of growth factors and cells can deliver soluble growth factors locally at a controlled rate for long periods for cell growth and differentiation. A biomaterial that can load a sufficient amount of growth factor and release it at a suitable rate during cell growth can be regarded as the second challenge in the design of tissue engineering scaffolds containing growth factors. The minimally invasive delivery of hydrogels through injection has gained significant attention in tissue engineering. The development of injectable and biodegradable hydrogel biomaterials with low toxicity and desirable mechanical properties is the third challenge in preparing bone tissue engineering scaffolds.

A range of biomaterials, including ceramic scaffolds, polymeric matrices, and hydrogel-based materials, have been created and optimized to encourage the creation of bone and blood vessels and to contain various functional components. Numerous porous and water-swollen polymeric networks in hydrogel structures may act as scaffolds for diverse tissues and medicines, and enable homogenous cell seeding during encapsulation. Hydrogel-based materials have many advantages, including easy synthesis and rational design, production of hydrogels with changeable viscoelastic characteristics, biocompatibility, injectability, biodegradability, and the ability to include diverse bioactive compounds or motifs. The PCL-P (HEMA-NIPAAm) hybrid copolymer is a thermosensitive, biodegradable, biocompatible, and hydrophilic hydrogel with superior mechanical characteristics and a distinct porosity structure. PCL-P is composed of poly (N-isopropylacrylamide) (PNIPAAm), poly (ε-caprolactone) (PCL), and poly (2-hydroxyethyl methacrylate) (PHEMA) (HEMA-NIPAAm).

Polymeric materials such as PCL are suitable candidates for bone tissue engineering as they exhibit several advantages, such as being bioactive, brittle, and biodegradable polymers. In this case, such materials can exploit ceramic properties, which may be difficult with small elongation of failure (good brittleness), and achieve comparable results in the compressive modulus of the native bone tissue. Hence, the introduction of PCL in biomedical applications, particularly for bone and tissue reconstruction, could be a new dimension in biomedical engineering. This can be proven by the increase in research conducted, which can be found in The Word-Cloud infographic plugins. In this case, the aim of the graphic plugin is to provide a concise visual interpretation of contextual features for better accessibility throughout the intrusion-network mapping analysis of the current review literature.

PNIPAAm is also a thermosensitive hydrogel that is insoluble in water at temperatures below the lower critical solution transition temperature (LCST) of approximately 32°C. Because hydrophilic or hydrophobic monomers affect NIPAM's transition temperature of NIPAM, thermo-responsive PNIPAM-based hydrogels have been used to deliver hydrophilic protein therapeutics. As a semicrystalline polymer made by ring-opening polymerization of a lactone, ε-caprolactone, or polycondensation of a hydroxy carboxylic acid,6-hydroxyhexanoic acid. The strong mechanical characteristics of PCL, its less acidic breakdown, biodegradability, and biocompatibility lead to biomedical applications, such as engineering scaffolds for internal bone fracture and skin and drug administration. Blending PHEMA hydrogels with hydrophobic components increased PHEMAs mechanical strength of PHEMA for the production of amphiphilic materials. Owing to its softness, high water content, biocompatibility, and hydrophilicity, PHEMA has been employed as a scaffold material for drug administration. Most bone regeneration studies have used orthopedically harvested autologous bone marrow mononuclear cells (BM-M), while a limited number of trials have applied dental pulp stem cells expanded by ex vivo culture.

Vascular endothelial growth factor (VEGF) is required for the early phases of angiogenesis, namely, blood vessel development, vascular epithelial, vascular endothelial cell migration, and endothelial cell survival and proliferation. The sustained delivery and bioactivity of VEGF from biodegradable matrices are concentration- and time-dependent; further study is, however, needed. Furthermore, the high cumulative dosage, need for repeated administration, cellular toxicity, short half-life, and high cost can limit the direct injection of VEGF into the wrong location. Human dental pulp stem cells (hDPSCs) were the first stem cells to have osteogenic and mesenchymal capabilities. DPSCs are easier to acquire than BMSCs, and the procedure is less intrusive and uncomfortable. After an initial in vitro growth phase, DPSCs may self-renew and create various cell types, as long as the appropriate conditions are satisfied. DPSCs communicate with perivascular cells and are located in the microvasculature of the tooth pulp microvasculature. Consequently, endothelial cells may influence angiogenesis, leading to the formation of pulp dentin. Both hDPSCs and bone mesenchymal stem cells can be differentiated by VEGF. The regeneration of tooth pulp must create an efficient and appropriate cellular delivery technique. Consequently, this study aimed to create a VEGF-loaded PCL-P (HEMA-NI PAAm) hybrid copolymer and analyze VEGF gene expression in hDPSCs linked to the copolymer. The use of polymer materials, such as PCL, could provide better processing and handling biocompatibility, versatility, adaptability, processability, and degradability for bone scaffold engineering. These polymer materials can exist as natural polymers, natural polymeric-derived composites, and synthetic polymeric materials, which can be used as primary scaffold materials in bone engineering. Finally, the analysis and findings

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obtained from this research work will promote a rigorous analysis of the potential and capability of polymeric materials such as PCL in biomedical applications, especially in bone regeneration.

Materials and Methods

Synthesis of PCL-HEMA macromonomer
Hydroxethyl methacrylate was used for the ring-opening polymerization of ε-caprolactone in the presence of tin octoate Sn(Oct)2 catalyst. Specific amounts of ε-caprolactone (50 mmol, approximately 5.707 g), HEMA (10 mmol, 1.3 g), and Sn(Oct)2 (0.05% by weight of the total amount of monomer) were dissolved in anhydrous toluene and transferred to a three-hole balloon equipped with an Ar gas inlet and outlet. After melting the materials, 200-300 μL of Sn(Oct)2 was used as a catalyst to initiate the polymerization. Polymerization was continued at this temperature with gentle stirring and an argon gas flow. To purify the polymer and separate it from unreacted monomers, the synthesized polymer was dissolved in dichloromethane. The polymer solution was then transferred to a mixture containing cold diethyl ether to obtain a precipitate. The product was diluted with 4,1-dioxane and poured into dichloromethane. The polymer solution was dissolved in dichloromethane. The polymer solution was then transferred to a mixture containing cold diethyl ether to obtain a precipitate. The product was purified and dried at 25°C under vacuum.

Preparation of VEGF-loaded PCL-P(HEMA-NIPAAm)
PCL-P (HEMA-NIPAAm) (0.02 g) was sterilized by UV radiation and added to 500 mL of the PBS buffer containing 0.1 g/mL of VEGF, which was chilled to 4°C and agitated for three days to make VEGF-loaded PCL-P (HEMA-NIPAAm). The mixture was then stirred at a shaking speed of 100 rpm for approximately 3 days to prepare the growth factor-loaded scaffolds. The mixture was centrifuged, and the loaded scaffolds were subsequently frozen at -80°C for 4 hours. The scaffolds were freeze-dried overnight and stored at 4°C in a sealed container until further study.

Release of VEGF
For the in vitro release test, 500 μL of fresh PBS was added to the suspension of the prepared samples and incubated at 37°C. At certain time intervals, 100 μL of the sample was taken; to keep the buffer volume constant, the same amount of fresh medium was replaced. The cumulative growth factor release percentage in the collected supernatant was measured using a VEGF enzyme-linked immunosorbent assay (ELISA) kit (Abcam), according to the manufacturer’s instructions.

Dental pulp stem cells isolation and culture
hDPSCs were isolated from the removed third molars of 18–25-year-old human subjects after obtaining informed consent. The excised teeth were cut to reveal the pulp chamber. The pulp tissue was digested at 37°C for 40 minutes in 3 mg/mL collagenase type I (Hyclone, USA) and 0.25% Trypsin/EDTA, seeded in culture flasks containing Dulbecco’s Modified Eagle Medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 1% penicillin-streptomycin (Hyclone, USA), and cultured. hDPSCs were grown in DMEM and changed every 2-3 days. hDPSC passages 3-6 were used in subsequent studies.

Study of proliferation and viability of dental pulp stem cells on the scaffold
The cultured dental pulp cells were first trypsinized and then resuspended in DMEM medium; about 60000-70000 cells were placed on each scaffold. The scaffold was seeded with cells in an incubator at 37°C for 24 hours. The scaffolds were freeze-dried overnight and stored at 25°C under vacuum. For the real-time PCR experiment included 10 l of the YTA 2x SYBR Green qPCR Master Mix kit (Yektajhiz, Tehran, Iran), 1 L of sample DNA, and 500 nM of each primer. In a final volume
of 20 L, 5'-CCAGAAAAAGCAGCAGGGACTGTGT-3' and 5'-GAAGACGCAAGGTGAAGAC-3' were used for VEGF and 5'-GAAGATCATCAGCAATGCCTCC-3' and 5'-TGCCTGTCATCGGAGTGTATCC-3' for 18 sRNA. The 2-CT approach was then used to calculate the fold-change in expression of each sample. Each sample type was tested thrice.

**Western blotting**

Western blotting was used to evaluate VEGF protein expression. After 48 hours of growth on VEGF-loaded PCL-P (HEMA-NIPAAm), hDPSCs were lysed in RIPA buffer (0.1% antiprotease cocktail, 1% SDS, 0.1% EDTA, 0.05 mM Tris buffer, and 150 mM sodium chloride). The protein concentration was determined throughout the investigations using the Bradford technique and kept at -20°C. After boiling for 10 minutes, the protein sample (50 g) was separated by 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide) gel electrophoresis. Following the separation of proteins, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking the membrane with skim milk, it was washed three times with Tris-buffered saline containing 0.1% Tween 20. A mouse monoclonal VEGF antibody was then applied to the membrane and incubated for 16 to 18 hours at 4°C (VEGF C-1: sc-7269, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), followed by incubation for 2 hours at room temperature with horseradish peroxidase (HRP)-conjugated mouse anti-rabbit IgG antibody (IgG-HRP: sc-2357, Santa Cruz Biotechnology Inc., ECL reagents (Thermo Fisher Scientific, USA). X-ray films were used to wash and develop membranes. The mouse monoclonal -actin antibody (-Actin C4: sc-47778, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was then applied as a control for the experiment using the beta-actin protein. The density of the protein bands was determined using ImageJ software. hDPSCs cells cultivated with VEGF were used as the control. However, scaffolds are still lacking. Each sample was tested thrice.

**Alizarin Red S staining**

After 21 days of growth on VEGF-loaded PCL-P (HEMA-NIPAAm), the cells were stained with Alizarin Red S to measure the quantity of calcific deposition. Cells cultivated on the VEGF-loaded PCL-P (HEMA-NIPAAm) scaffolds were washed with PBS and fixed for one hour in 70% (v/v) cold ethanol. After washing with water, the scaffolds were dyed with alizarin red S solution (40 mM, pH 4.2) at room temperature for 20 minutes. The scaffolds were rinsed with water for 20 minutes and destained with 10% (w/v) cetylpyridinium chloride in 10 mM sodium phosphate. Scaffolds without hDPSCs served as controls and were stained. The quantities of alizarin red S were determined using an absorbance measurement at 570 nm. Each sample was tested three times and the mean of the ODs was 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 also applied to test the distribution of the data. Furthermore, Student's t test was applied to compare the mean of the data sets. Statistical significance was set at $P < 0.05$.

**Results**

The expression of VEGF in hDPSCs was examined following exposure to VEGF-loaded PCL-P (HEMA-NIPAAm) (factor), PCL-P (HEMA-NIPAAm) (scaffold), or no scaffold (CTR) (Figures 1 and 2). When VEGF gene expression in PCL-P (HEMA-NIPAAm) was compared to that in the control group (6.67%, $P = 0.0793$, $t = 2.341$, $df = 4$), no change was observed. However, VEGF-loaded PCL-P (HEMA-NIPAAm) boosted VEGF expression in hDPSCs compared with PCL-P (HEMA-NIPAAm) (340.62%, $P < 0.001$, $t = 29.86$, $df = 4$) and cells with no scaffold (363.33%, $P < 0.001$, $t = 27.69$, $df = 4$). The degree of VEGF expression in hDPSCs exposed to VEGF-loaded PCL-P (HEMA-NIPAAm) and PCL-P (HEMA-NIPAAm) was examined and compared with hDPSCs not exposed to any scaffolds (Figure 2). VEGF expression was significantly higher in cells exposed to VEGF-loaded PCL-P (HEMA-NIPAAm) than in cells exposed to PCL-P (HEMA-NIPAAm) ($P = 0.0008$, $t = 9.164$, $df = 4$). In addition, VEGF expression was significantly elevated in hDPSCs exposed to VEGF-loaded PCL-P (HEMA-NIPAAm) than in cells not exposed to any scaffold ($P = 0.002$, $t = 13.04$, $df = 4$). Furthermore, the expression of the VEGF protein was enhanced in cells treated with PCL-P (HEMA-NIPAAm); however, this increase was not significant ($P = 0.1210$, $t = 1.964$, $df = 4$).

The differentiation of hDPSCs cultivated on VEGF-loaded PCL-P (HEMA-NIPAAm) was assessed using alizarin red staining (Figure 3). After 21 days of culture, there was a significant increase in the color intensity of alizarin red in VEGF-loaded PCL-P (HEMA-NIPAAm) cells compared to cells with no scaffold ($P = 0.0002$, $t = 8.004$, $df = 6$) and PCL-P (HEMA-NIPAAm) cells ($P = 0.0002$, $t = 7.981$, $df = 6$).

**Discussion**

Osteogenesis and angiogenesis are two significant mechanisms required for bone repair. For a long time, one of the fundamental problems in bone tissue engineering has been discovering a means to retain bone integrity while allowing communication between osteogenesis and angiogenesis. The PCL-P (HEMA-NIPAAm) hybrid copolymer is a thermosensitive, biocompatible, and biodegradable hydrophilic hydrogel that can be used to deliver hydrophilic protein medicines. Direct VEGF injection into the injured tissue is restricted owing to the protein's short half-life, cell toxicity and high cost. Furthermore, the sustained delivery and bioactivity of VEGF depends on the tissue's protein content and time.
of release 8. In this regard, several chemical and natural substances, in combination with VEGF and other growth factors, have been investigated to build scaffolds for the development of faulty cells and growth factor delivery techniques.\textsuperscript{5,32} This study examined the expression of the VEGF gene, which is involved in angiogenesis, in hPDSCs transplanted to a temperature-sensitive injectable scaffold containing VEGF. Cells, scaffolds, and growth factors have been employed in this therapeutic approach to restore injured tissues, particularly the bone. Dental pulp tissue is
widely used in tissue regeneration because of its efficient interaction with biomaterials and its strong differentiation potential.

Several investigations on the ability of these cells to develop into osteoblasts for bone regeneration have shown that they have a high potential for bone repair. Furthermore, the ease with which these cells are available suggests that they may be a good alternative for bone marrow stem cells to repair bone injuries. Growth factors induce cell differentiation, and target cells differentiate from those of interest. The release of VEGF from the copolymer changed at 37°C. Li et al also discovered that at 30°C (below LCST), less drug was released owing to alterations in the structure of the micelles, as compared what happened at 40°C (above LCST). When the temperature exceeds the LCST, the PNIPAAm shells are converted into hydrophobic conditions, causing the micelle structure to deform. As a result, VEGF was progressively released from the micelles, and this release behavior persisted for approximately 120 hours until all the loaded growth factors were liberated.

Alizarin red is an anthraquinone derivative that is used to detect calcium in tissue slices and cell cultures in vitro. Alizarin red is widely used to distinguish calcium-containing osteocytes in differential cultures of human and rodent mesenchymal stem cells. In addition, it can be used to assess the differentiation of mesenchymal stem cells into bone cells and osteocytes. According to the findings of the present study, after 21 days of culture, the mineralization indices of VEGF-loaded PCL-P (HEMA-NIPAAm) were significantly higher than those of PCL-P (HEMA-NIPAAm). PCL-P (HEMA-NIPAAm) was shown to have a stimulatory effect, which was greatly amplified in the presence of VEGF.

VEGF can attach to nearby endothelial cells during bone regeneration, thereby increasing neovascularization and osteoblast mineralization and maturation. Furthermore, VEGF may stimulate the production of bone morphogenetic proteins (BMP-2 and BMP-4), which are essential osteogenic growth factors. The relative expression of VEGF and VEGF proteins in PCL-P (HEMA-NIPAAm) did not differ substantially from that in the control group (P>0.05). Nonetheless, there was a statistically significant difference (P>0.05) between VEGF-loaded PCL-P (HEMA-NIPAAm), the copolymer scaffold alone, and the control group. Divband et al also discovered that DPSCs cultivated on VEGF-containing chitosan biguanide-carboxymethylcellulose hydrogels had enhanced mRNA expression levels and increased expression of osteogenesis and angiogenesis-related proteins. Similarly, Chakka et al observed that in the mesenchymal stem cells of bone marrow, VEGF-encoding plasmid DNA (pVEGF) nanoplexes (PLA-PDA-PEI-pVEGF) could stimulate greater VEGF and osteocalcin mRNA expression compared to other scaffolds cultured cells without VEGF. Furthermore, alizarin red staining demonstrated that cells cultivated on PLA-PDA-PEI-pVEGF scaffolds were more mineralized than those cultured on other scaffolds without VEGF. Thus, these findings show that continuous VEGF release from PCL-P (HEMA-NIPAAm) increases the expression of osteogenic genes and proteins in DPSCs. This mechanism converts hDPSCs into bone cells, indicating that VEGF is provided to them.

Conclusion

growing potential to create implants with higher performance as a result of innovations in composite material design and production technologies.

In the present work, an hDPSC-seeded thermosensitive PCL-P(HEMA-NIPAAm) scaffold was fabricated and used for the delivery of VEGF for bone regeneration purposes. The PCL-P (HEMA-NIPAAm) hybrid copolymer was successfully synthesized by copolymerization of PCL, NIPAAm, and HEMA. The levels of angiogenic markers were investigated at the gene and protein levels by real-time PCR and western blotting assays, respectively. The VEGF-loaded PCL-P (HEMA-NIPAAm) scaffold showed significantly higher alizarin red color intensity (P<0.05). The last but not least, VEGF-loaded thermosensitive, injectable PCL-P (HEMA-NIPAAm) increased the expression of genes related to angiogenesis and osteogenesis in hDPSCs.

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