The Development of a Dental Light Curable PRFe-Loaded Hydrogel as a Potential Scaffold for Pulp-Dentine Complex Regeneration: An *In Vitro* Study

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Abstract

Aim: The study aimed to develop a bicomponent bioactive hydrogel formed *in situ* and enriched with an extract of platelet rich fibrin (PRFe) and to assess its potential for use in pulp-dentine complex tissue engineering via cell homing.

Methodology: A bicomponent hydrogel based on photo-activated naturally derived polymers, methacrylated chitosan (ChitMA) and methacrylated collagen (ColMA), plus PRFe was fabricated. The optimized formulation of PRFe-loaded bicomponent hydrogel was determined by analysing the mechanical strength, swelling ratio, and cell viability simultaneously. The physical, mechanical, rheological, and morphological properties of the optimal hydrogel with and without PRFe were determined. Additionally, MTT, phalloidin/DAPI, and live/dead assays were carried out to compare the viability, cytoskeletal morphology, and migration ability of stem cells from the apical papilla (SCAP) within the developed hydrogels with and without PRFe, respectively. To further investigate the effect of PRFe on the differentiation of encapsulated SCAP, alizarin red S staining, RT-PCR analysis, and immunohistochemical detection were performed. Statistical significance was established at $p < 0.05$.

Results: The optimized formulation of PRFe-loaded bicomponent hydrogel can be rapidly photocrosslinked using available dental light curing units. Compared to bicomponent hydrogels without PRFe, the PRFe-loaded hydrogel exhibited greater viscoelasticity and higher cytocompatibility to SCAP. Moreover, it promoted cell proliferation and migration *in vitro*. It also supported the odontogenic differentiation of SCAP as evidenced by its promotion of biomineralization and upregulating the gene expression for ALP, COL I, DSPP, and DMP1 as well as facilitated angiogenesis by enhancing VEGFA gene expression.

Conclusion: The new PRFe-loaded ChitMA/ColMA hydrogel developed within this study fulfils the criteria of injectability, cytocompatibility, chemoattractivity, and bioactivity to

promote odontogenic differentiation, which are fundamental requirements for scaffolds used in pulp-dentine complex regeneration via cell-homing approaches.

Keywords: Platelet rich fibrin, methacrylated collagen, methacrylated chitosan, injectable hydrogel, visible light crosslinking.

Introduction

Loss of pulp functionality as a consequence of irreversible pulpitis and necrosis disrupts the development of immature teeth and results in short roots with thin walls that are prone to fracture. The tissue engineering platform is a valid treatment modality that has been widely used to temporarily fill root canals and stimulate pulp-dentine complex regeneration. In this methodology, exogenous cells with/without signaling molecules, as well as exogenous scaffolds, have been placed inside the root canal. Early studies on engineering dental pulp-like tissue were performed using polylactic- or polyglycolic acid-based scaffolds loaded with odontogenic stem cells within tooth slices or segments (Cordeiro et al., 2008; Demarco et al., 2010; G. T.-J. Huang et al., 2010; Sakai et al., 2010). Subsequently, cell-laden scaffolds based on other biomaterials were explored, including naturally-derived biomaterials such as fibrin (Galler et al., 2018), collagen (Prescott et al., 2008), gelatine (Khayat et al., 2017), decellularized dental pulp ECM (Hu et al., 2017), hyaluronic acid (Zhu et al., 2018), and chitosan (Yang et al., 2012) or synthetic biomaterials such as self-assembling peptides (Galler et al., 2012; Rosa et al., 2013). However, the problems associated with cell transplantation casts doubt on the clinical practicability of cell-based approaches (Galler & Widbiller, 2017; Eramo et al., 2018; Widbiller et al., 2018). In this context, cell-homing strategies have received much attention recently as a promising alternative to cell-based treatments (Kim et al., 2010; Suzuki et al., 2011; Ruangsawasdi et al., 2014; Li & Wang, 2016; Widbiller et al., 2018). In this approach, the endogenous cells of the host are encouraged to migrate within a custom-made scaffold placed inside the root canal system via the chemotactic effect of endo/exogenous signaling molecules.

Within the range of available scaffolds, *in situ* forming hydrogels, which completely fill the irregular space of the root canal system, better meet the criteria required for pulp-dentine complex tissue engineering via cell homing. Their tuneable composition and physical

properties make hydrogels promising candidates for soft- and hard-tissue engineering applications. In addition, precise control over their degradation rate as well as their microarchitecture mean that hydrogels offer substantial potential for the controlled delivery of a variety of signaling molecules (Shirzaei Sani et al., 2019). In the cell homing approach, signaling molecules play a key role, since they can promote chemotaxis to mobilize host endogenous stem cells and guide their differentiation inside the root canal toward the formation of pulp-like tissue (Galler & Widbiller, 2017). Pulp-like tissue has been reported to be regenerated in an ectopic mouse model using a collagen gel containing bFGF, VEGF, PDGF, NGF, and BMP7 (Kim et al., 2010).

Platelet-rich fibrin (PRF) is an endogenous source of signaling molecules widely used in regenerative dentistry (Miron et al., 2017; Metlerska et al., 2019). The presence of growth factors, such as TGF-β1, VEGF, PDGF, FGF, *etc.*, in PRF (He et al., 2016; Qiao et al., 2017) makes it an appropriate candidate for guiding odontogenic differentiation during pulp-dentine complex tissue engineering. It has been reported that the extract of PRF (PRFe) promoted the proliferation of dental pulp cells *in vitro* (He et al., 2016). Additionally, PRFe inhibited inflammation and enhanced odontogenic differentiation of dental pulp cells treated under inflammatory conditions (Kim et al., 2017).

In the present study, an *in situ* forming photocrosslinkable hydrogel, which takes advantage of the availability of light curing units in all dental clinics, was developed based on methacrylated collagen (ColMA) and methacrylated chitosan (ChitMA). To improve bioactivity, PRFe, as an endogenous source of key cytokines and growth factors, was incorporated in the developed hydrogels, and the effect of PRFe incorporation on the physical, mechanical, rheological, and morphological properties of the hydrogel was investigated *in vitro*. Also, the cytocompatibility of engineered hydrogels with and without PRFe was evaluated via three-dimensional (3D) encapsulation and two-dimensional (2D) surface seeding of SCAP. Lastly, the ability of the

PRFe-loaded hydrogel to support odontogenic differentiation of encapsulated SCAP was investigated *in vitro*.

Materials and methods

The manuscript of this laboratory study has been written according to Preferred Reporting Items for Laboratory studies in Endodontology (PRILE) 2021 guidelines (Nagendrababu et al., 2021). The PRILE 2021 flowchart (Figure S1) summarizes the key steps.

Materials

Medium molecular weight chitosan (deacetylation degree in the range of 75–85%), methacrylic anhydride, eosin Y (EY), triethanolamine (TEA), 1-vinyl-2-pyrrolidinone (NVP), 2,4,6-trinitrobenzenesulfonic acid (TNBS), lysozyme from chicken egg white, phosphate buffered saline (PBS), acridine orange, propidium iodide, paraformaldehyde, 4′,6-diamidino-2-phenylindole (DAPI), tetramethylrhodamine B isothiocyanate (TRITC)-labelled phalloidin (P1951), dexamethasone, l-ascorbic acid 2-phosphate, and β-glycerophosphate were purchased from Sigma-Aldrich (GmbH, Sternheim, Germany). High glucose Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin (Penstrep), l-glutamine, non-essential amino acids (NEAA), trypsin-EDTA (0.05%), collagenase type I from *Clostridium histolyticum*, and 3-(4,5-dimethylthiazol-2-yl) 2,5diphenyltetrazolium bromide (MTT) were purchased from Gibco® Life Technologies (Carlsbad, CA, USA). All the other materials were purchased from Merck Millipore (Darmstadt, Germany).

Extraction and characterization of type I collagen

Type I collagen was isolated from adult male Wistar rat tail tendon via acidic treatment, as reported previously (Rajan et al., 2007). Briefly, the bundle of collagen fibres of tendons was removed from rat tails and collected in 1X PBS. The collected fibres were immersed in acetone and then in 70% (v/v) isopropanol for 5 minutes and dissolved in 0.02 M acetic acid on a magnetic stirrer for 72 hours. The resulting mixture was blended and homogenised in ice using a house-ware blender and then centrifuged at 6,000 rpm to remove impurities. The supernatant was then collected and dialyzed against 0.02 M acetic acid for 7 days. The dialysate was changed every day. All processes were carried out at 4°C. Finally, the acid-soluble type I collagen was obtained by freeze-drying the frozen collagen solution for 48 hours.

The purity of the extracted collagen was analysed via sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) based on an established protocol (Capella-Monsonís et al., 2018). In the present study, commercially available type I collagen isolated from bovine skin (Sigma-Aldrich) was used as the reference. In addition, the chemical structure of the extracted collagen was analysed via Fourier transform infrared (FTIR), circular dichroism (CD), and ultraviolet/visible (UV/VIS) spectroscopies (Supporting Experimental Procedures).

Synthesis of methacrylated collagen (ColMA)

Type I collagen extracted from rat tail tendons (2 mg/ml) was dissolved in 5 mM dimethyl sulfoxide (DMSO). After complete dissolution, methacrylic anhydride was added dropwise to the solution in proportion to the moles of free amines of collagen. Twenty-four hours after the reaction, the solution was dialyzed against distilled water for 5 days, and ColMA was obtained via freeze-drying for 48 hours. The dialysate was changed twice a day. All processes were carried out at 4°C.

The moles of free amines presented in the backbone of collagen polymer were determined via the TNBS assay (Supporting Experimental Procedures).

Synthesis of methacrylated chitosan (ChitMA)

Chitosan (3% w/v) was dissolved in 2% (w/v) acetic acid overnight. Next, methacrylic anhydride with the ratio of 1.2 moles per mole of chitosan repeat unit was added to the solution, and the reaction was allowed to progress for 24 hours at room temperature. The solution obtained was dialyzed against distilled water for 5 days, and the purified ChitMA was obtained by freeze-drying for 48 hours. The dialysate was changed twice a day.

Determination of the degree of methacrylation (DOM)

The degree of methacrylation (DOM) of ColMA and ChitMA was calculated using proton nuclear magnetic resonance (^{1}H NMR) spectroscopy using a Bruker DRX-500 AVANCE spectrometer (Bruker BioSpin AG, Faellanden, Switzerland). Type I collagen and ColMA at a concentration of 10 mg/ml were dissolved in deuterated DMSO at room temperature (Gaudet & Shreiber, 2012). Chitosan and ChitMA at a concentration of 0.5% (w/v) were dissolved in 0.25% DCl in D_2O at room temperature (Yu et al., 2007). Then, the ^{1}H NMR spectra of samples were recorded at 25°C. The spectra were processed using MestReNova software (Mestrelab Research, Santiago de Compostela, Spain). The DOM of ColMA was calculated using the ratio of the integrated area of the lysine signal of ColMA and that of unmodified collagen (Claaßen et al., 2018). The DOM of ChitMA was determined by calculating the ratio between the integrated area of the H2–H6 peak (protons of chitosan glucosamine and N-acetyl glucosamine residues) at 2.8 to 4.0 ppm and that of the methylene peaks at 5.6 and 6.0 ppm (Yu et al., 2007; Saraiva et al., 2015; Kolawole et al., 2018).

Preparation and characterization of PRFe

Venous blood was collected from the healthy, non-smoking principal researcher, who was not taking any medication. To prepare PRFe, the blood samples were taken in 10 ml tubes without anticoagulant and immediately centrifuged by a Choukroun PRF Duo Quattro centrifuge (Nice, France) to prepare A-PRF. The red corpuscles were eliminated with a scissors and the fibrin clot obtained, which is defined as the PRF, was minced and homogenized, as described previously (Kawase et al., 2019). To extract the growth factors, the homogenized samples were allowed to stand for 20 hours at 4°C followed by stirring at 150 rpm at 37°C for 4 hours. The mixture obtained was then centrifuged at 6,000 rpm for 10 minutes. The resulting supernatant (PRFe) was sterilized using a 0.22 µm syringe filter (Suzuki et al., 2013) and stored at -20°C until use.

To characterize the prepared PRFe, concentrations of TGF-β1 and VEGF as relevant growth factors in PRF were determined using human TGF-β1 and VEGF enzyme-linked immunosorbent assay (ELISA) kits (MyBioSource, San Diego, CA, USA) according to the protocol described by the manufacturer (n = 3). The effect of the prepared PRFe on the viability of SCAP was studied *in vitro* using an MTT assay (Supporting Experimental Procedures).

Preparation of bioactive bicomponent hydrogels

Bioactive bicomponent hydrogels were formed via photocrosslinking. ColMA and ChitMA were dissolved in distilled water with a final concentration of 0.8% and 2% (w/v), respectively. After complete dissolution, ColMA and ChitMA were mixed with specific volume ratios (75/25, 50/50, and 25/75). A separate solution of EY/TEA/NVP (13.5% (v/v)) was also prepared in 10X PBX and mixed with 20% (v/v) PRFe. The ChitMA/ColMA solutions were then mixed with the PRFe/photoinitiator mixture, and after pipetting up and down several times, the precursor solution was poured into cylindrical moulds. The final concentrations of

EY, TEA, and NVP in the precursor solution were 0.1 mM, 2.25% (w/v), and 1% (w/v), respectively. The gelation occurred upon exposure to visible light using an increasing range of times (20, 30, and 40 seconds) using a LED.D curing light (Woodpecker, Guilin, China) with a wavelength of 440-490 nm and an intensity of 1000~1200 mW/cm^2. Bicomponent hydrogels without PRFe were used as the control group.

Physical characterization of hydrogels

Swelling ratio

Cylindrical hydrogels with a diameter of 6 mm and a height of 4 mm were prepared and weighed (n = 6 per group). Next, they were immersed in PBS, and the weight of the swollen gels was measured at specific time points (1, 2, 4, 6, 8, and 24 hours). The swelling ratio was defined as the ratio of the swollen weight to the initial weight (Shirzaei Sani et al., 2019).

Enzymatic degradation

The enzymatic degradation test was carried out as described previously (Unal et al., 2021). Cylindrical ChitMA/ColMA hydrogels with a diameter of 6 mm and a height of 4 mm were prepared (n = 3 per group), and their initial weight was recorded. Next, the samples were incubated in enzyme solutions at 37°C for up to 6 days. At specific time intervals (1, 7, 24, 48, 72, 96, 120, and 144 hours), the samples were removed and weighed, and the percentage of the weight loss was considered as the degradation rate. In the present study, hydrogel degradation was determined in three different types of enzyme solution including 1.5 mg/ml lysozyme solution, 5 U/ml collagenase solution, and the mixture of lysozyme and collagenase at the same concentration. Enzyme solutions were changed every 3 days.

Mechanical characterization of hydrogels

The compressive properties of ChitMA/ColMA hydrogels were evaluated using a universal mechanical testing machine (Hounsfield-H10KS, Salford, UK) with a 10 kN load cell. Cylindrical hydrogels with a diameter of 7 mm and a height of 6 mm were prepared (n = 6 per group). Samples were loaded between compression platens and compressed at a rate of 1 mm/min. Young's modulus was calculated as the slope of the linear region on the stress-strain curve.

Rheological characterization of hydrogels

Rheological characteristics of hydrogels were evaluated using an MCR301 rheometer (Anton Paar, GmbH, Graz, Austria), having a parallel plate geometry (15 mm diameter) with a desired gap height between the two plates. The disk-shaped hydrogels with a diameter of 15 mm and a height of 1 mm were prepared and placed between plates to determine the storage modulus (G') and loss modulus (G'') as a function of the frequency over the frequency range of 0.1 to 100 rad/s (n = 3 per group).

Morphological characterization of hydrogels

Gold sputtered freeze-dried hydrogels were observed by scanning electron microscopy (Philips XL30 ESEM, Eindhoven, Netherlands) to study their interior morphologies. To determine the pore size and the porosity of hydrogels, images were taken and analysed by using ImageJ software (NIH, Bethesda, MD, USA). Images from various parts of the samples were taken, and the percentage of hydrogel porosity was calculated using the built-in 'Analyze Particles' function (Haeri & Haeri, 2015; Mahdavi et al., 2020).

In vitro protein release

To evaluate total protein release from the PRFe-loaded bicomponent hydrogels, cylindrical hydrogels with a diameter of 6 mm and a height of 4 mm were prepared and immersed in 1 ml of PBS at 37°C (n = 3 per group). At specific time intervals (1, 8, 24, 72, 120, 168, and 240 hours), 200 µl of the releasing solution was removed and replaced with an equal volume of fresh PBS. The protein concentration in the collected samples was determined using the Micro-BCA™ assay according to the manufacturer's protocol. Bicomponent hydrogels without PRFe were used as the control group.

In vitro cellular studies

Cell types

Stem cells from the apical papilla (SCAP), which were supplied by Royan Biotechnology Research Institute (Iran), were cultured at 37°C and 5% CO_2 in high glucose DMEM containing 10% FBS, 1% l-glutamine, 1% NEAA, and 1% Penstrep. Cells from passages 5-8 were used for the experiments.

Three-dimensional cell encapsulation within the bicomponent hydrogels

For 3D cell encapsulation, a suspension of SCAP was prepared by trypsinization and resuspension in high glucose DMEM medium. The cell pellet was formed by centrifuging and aspirating the medium. A hydrogel precursor solution containing photoinitiators (EY/TEA/NVP) with and without PRFe was prepared and mixed with the cell pellet. Hydrogels were formed by pouring 110 µl of the precursor solution into cylindrical moulds with a diameter of 6 mm and a height of 4 mm, and photocrosslinking upon exposure to visible light for specified times (20, 30, and 40 seconds). The cell-laden hydrogels were transferred to 48-

well plates and incubated in high glucose DMEM medium at 37°C and 5% CO_2. The cell density in the hydrogels was 1×10^6 cells/ml of hydrogel.

Cell viability

An MTT colorimetric assay was used to evaluate *in vitro* cell viability. Cell-laden hydrogels with and without PRFe were prepared as described previously and incubated at 37°C and 5% CO_2 (n = 5 per group and time point). The culture media were changed every other day. After 1, 4, 7, 14, and 21 days of incubation, the culture medium was aspirated, and samples were treated with the MTT reagent (0.5 mg/ml) followed by incubation at 37°C for 4 hours. Then, the MTT solution was removed and replaced by DMSO to elute the insoluble formazan. After dye releasing, the optical density values were read in a new 96-well plate using a microplate ELISA reader (ELx800, BioTek Instruments, Winooski, VT, USA) at 490 nm.

Cell attachment, spreading, and morphology

The adhesion and cytoskeletal morphology of encapsulated cells within hydrogels with and without PRFe were assessed using DAPI and phalloidin staining (n = 3 per group). After 7 days of *in vitro* culturing, samples were harvested and fixed in 4% paraformaldehyde for 20 minutes. Then, samples were washed three times with PBS and permeabilized using 0.1% (v/v) Triton X-100. Filamentous actin (F-actin), which was expressed in encapsulated cells, was stained with phalloidin-TRITC in a dilution of 1:20 for 2 hours in the dark. Next, DAPI was applied to the samples to stain cell nuclei. Images were captured using an inverted fluorescence microscope (Olympus, Tokyo, Japan).

Cell migration

The effect of PRFe on promoting cell migration was investigated via live/dead assay. Cell-free hydrogels with and without PRFe were prepared, and cells were seeded on each sample (n = 3 per group). Forty-five minutes post seeding, the culture medium was added, and samples were incubated at 37°C and 5% CO_2. After 24 hours, samples were incubated with acridine orange at room temperature for 15 minutes, followed by incubation with propidium iodide for another 15 min. The stained samples were then washed, and the cells were visualized under an inverted fluorescence microscope (Olympus, Tokyo, Japan).

Odontogenic differentiation

To induce the odontogenic differentiation of SCAP *in vitro*, cell-laden hydrogels with and without PRFe were prepared as described above. Samples were transferred to 48-well plates and cultured in 400 µl of the odontogenic medium, composed of high glucose DMEM supplemented with 10 nM dexamethasone, 10 mM β-glycerophosphate, 50 µg/ml l-ascorbic acid, 10% FBS, and 1% Penstrep, at 37°C and 5% CO_2 (Lu et al., 2015). The differentiation media were changed every other day.

Histological analysis

A haematoxylin-eosin (H&E) staining assay was used for histological examination of cultured samples in the odontogenic medium (n = 3 per group). On day 21, cell-laden hydrogels with and without PRFe were harvested, fixed in 10% formalin solution, and embedded in paraffin. Next, cross-sections with a thickness of 5 µm were obtained from the midline of each sample and stained with H&E.

Biomineralization detection

Cell-laden hydrogels with and without PRFe were cultured in the odontogenic medium (n = 3 per group). After 21 days of culturing, the paraformaldehyde-fixed cells were stained with 2% (w/v) alizarin red S (pH = 4.2) to detect biomineralization. After several steps of washing, samples were photographed using a light microscope (Labomed, Fremont, CA, USA) to visualize calcium deposition.

Total RNA extraction and RT-PCR analysis

To investigate whether the presence of PRFe upregulated odontogenic gene expression of encapsulated SCAP, a real-time RT-qPCR was performed (n = 3 per group and time point). After 14 and 21 days of culturing gel-cell constructs with and without PRFe in the odontogenic medium, samples were pooled and total RNA was extracted using the Trizol® system (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Single strand cDNA was synthesized through reverse transcription of the extracted RNA using an oligo-dT primer (MWG. Biotech, Ebersberg, Germany). The cDNA sample was analysed using PCR master mix (Applied Biosystems, Foster City, CA, USA), SYBER Green solutions, and a StepOne™ RT-PCR system (Applied Biosystems, Foster City, CA, USA). Gene expression of alkaline phosphatase (ALP), collagen type I (COL I), dentine matrix protein 1 (DMP1), dentine sialophosphoprotein (DSPP), and vascular endothelial growth factor A (VEGFA) (Table 1) were quantified and normalized to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the relative gene expression levels were calculated using the $2^{-(\Delta CT)}$ method.

Immunohistochemistry

Expression of DSPP and COL I was investigated using immunohistochemistry (n = 3 per group). After 21 days of *in vitro* culturing in the odontogenic medium, cell-laden hydrogels with and without PRFe were fixed using 4% paraformaldehyde for 20 min and washed with PBS three times. The cells were then permeabilized with 0.3% Triton X-100 for 30 minutes. After washing with PBS, samples were incubated in 10% goat serum (G9023, Sigma-Aldrich, GmbH, Sternheim, Germany) as a blocking buffer to block the secondary antibody. Forty-five minutes later, the blocking solution was replaced with the primary antibody solution containing rabbit anti-DSPP (orb2630, Biorbyt, Cambridge, UK) and mouse anti-collagen type I (orb375159, Biorbyt, Cambridge, UK) at a 1:100 dilution. Samples were incubated at 4˚C for 24 hours followed by washing 4 times with PBS. Next, the secondary antibodies (goat anti-rabbit IgG(H+L) antibody (FITC) (orb688925) and goat anti-mouse IgG antibody (PE) (orb713456), Biorbyt, Cambridge, UK) at a 1:150 dilution were added, and samples were incubated at 37˚C and 5% CO_2 for 90 minutes in the dark. After several washing steps with PBS, nuclear staining was performed through incubation with DAPI for 20 minutes, and immunoreactivity was visualized with an Olympus fluorescent microscope (Tokyo, Japan).

Statistical analysis

Data were presented as the mean ± standard deviation (SD). The normality of the data was confirmed using the Shapiro–Wilk test ($p > 0.05$). The statistical significance of the differences between the groups was analysed using a one-way analysis of variance (ANOVA) followed by Turkey's post hoc analysis using GraphPad Prism 8.4.3 (GraphPad Software). Differences were taken to be statistically significant for a p-value of less than 0.05.

Results

Data (mean and SD) from physical, mechanical, rheological, morphological, and biological assessments are presented in the Supplementary Information (Table S1-S15).

PRFe preparation and characterization

Platelet rich fibrin (PRF) was prepared from human whole blood using centrifugation techniques. Figures 1 Ai and Aii show the blood sample immediately after centrifugation and prepared PRF located in the middle of the tube, respectively. After removal of the plasma, a translucent elastic fibrin clot, PRF, remained. The PRFe (Figure 1Aiii), which is a solution enriched with growth factors, was obtained as described previously. Quantifying the concentrations of TGF-β1 and VEGF in the PRFe as an indication of growth factors from platelets revealed that the prepared PRFe had a higher concentration of TGF-β1 (124.54 ± 36.71 pg/ml), whereas VEGF was present at a lower concentration (23.28 ± 9.51 pg/ml) (Figure 1B). The effect of PRFe on the viability and proliferation of SCAP was also investigated via MTT assays within 7 days of *in vitro* 2D culturing. The MTT analysis indicated a significant enhancement in the viability and proliferation of SCAP cultured in media supplemented with 20% (v/v) PRFe (Figure 1C). In comparison with cells in the control group, which were cultured in the growth medium without PRFe, the cell viability at day 7 was about 4 times greater in the PRFe-supplemented group ($p < 0.0001$).

Hydrogel preparation and optimization

The PRFe-loaded ChitMA/ColMA hydrogel was synthesized via photocrosslinking (Figure 2A). First, type I collagen from rat tail tendons was isolated and characterized (Figure S2). The isolated collagen and chitosan were methacrylated via covalent binding of their free amines with methacrylic moieties of methacrylic anhydride. The DOM as determined from the ^{1}H NMR spectra (Figures 2B&C) was around 59% and 32% for modified collagen (ColMA) and

chitosan (ChitMA), respectively. Bicomponent PRFe-loaded ChitMA/ColMA hydrogels were prepared through the addition of photoinitiator solution with PRFe to the precursor polymeric solution followed by exposure to visible light. To select the most suitable polymer composition and optimize photocrosslinking conditions, various combinations of ColMA and ChitMA, as well as different times of light exposure, were used, and mechanical strength, swelling ratio, and cell viability were analysed simultaneously.

As shown in Figure 3A, the increasing light exposure time, as well as the enhancement of the ChitMA/ColMA ratio improved the mechanical strength of bicomponent hydrogels. For example, for hydrogels containing 50% ColMA and 50% ChitMA, increasing the light exposure time from 20 to 40 seconds led to the enhancement of Young's modulus from 14.03 ± 1.69 kPa to 21.73 ± 3.24 kPa as a result of more crosslinks being formed. Also, at a constant light exposure time (20 seconds), Young's modulus was 20.66 ± 2.27, 14.03 ± 1.69, and 5.24 ± 1.13 kPa for ChitMA/ColMA at ratios of 75/25, 50/50, and 25/75, respectively. Comparing the swelling ratio among the bicomponent hydrogels revealed a decrease in swelling ratio by increasing light exposure time and ChitMA/ColMA ratio (Figure 3B). Evaluating the cytocompatibility of the prepared bicomponent hydrogels also demonstrated that both light exposure time and hydrogel composition affected cell viability (Figure 3C). Increasing the light exposure time at the high percentage of ChitMA significantly reduced cell viability a week after SCAP encapsulation ($p = 0.003$). Hence, the hydrogel crosslinked upon 20 seconds of light exposure with the ChitMA/ColMA ratio of 50/50 was selected as the optimal scaffold for further investigations.

Effect of PRFe on hydrogel properties

Collagen and chitosan are known to be degraded in the presence of collagenase and lysozyme, respectively. To determine the effect of PRFe on the degradation profile of optimized

bicomponent hydrogel, samples were incubated in three different enzyme solutions for up to 6 days. The degradation profile of hydrogels with and without PRFe was obtained as a function of incubation time in lysozyme (L), collagenase (C), and dual enzyme (D) solutions, as shown in (Figure 4A). The degradation of ChitMA/ColMA hydrogels in different enzyme solutions followed a similar trend independent of the incorporation of PRFe. However, the general trend of hydrogel degradation indicated that PRFe incorporation slightly increased the degradation rate. However, the difference between degraded groups in collagenase and dual enzyme solutions was not statistically significant. Comparing the swelling profile between hydrogels with and without PRFe also revealed that the presence of PRFe in the structure reduced hydrogel shrinkage (Figure 4B).

Young's modulus was also measured to evaluate the effect of PRFe incorporation on the mechanical properties of the bicomponent hydrogels. The data revealed that the PRFe-loaded hydrogels exhibited a 0.8-fold reduction in Young's modulus when compared with hydrogels without PRFe ($p < 0.001$) (Figure 4C). Such observation was consistent with the results obtained from rheological assessment, where the addition of PRFe led to a decrease in storage modulus (G') and an increase in loss modulus (G'') (Figure 4D), resulting in the lower stiffness and higher elasticity of the PRFe-loaded hydrogels. The low values (near zero) of the damping factor ($\tan \delta$), which is defined as the ratio of energy dissipated (viscous component, G'') relative to energy stored (elastic component, G'), also indicated the great elasticity of hydrogels (Figure 4E).

The influence of PRFe incorporation on morphological properties of bicomponent hydrogels was also investigated by SEM imaging. Figure 5A shows representative images of the cross sections of hydrogels. The incorporation of PRFe resulted in a relatively more compact structure with smaller pore size ($p = 0.018$) and lower porosity ($p = 0.005$) (Figure 5B).

Protein release from the PRFe-loaded ChitMA/ColMA hydrogel

The total amount of protein release from the PRFe-loaded ChitMA/ColMA hydrogel incubated in PBS is presented in Figure 5C. ChitMA/ColMA hydrogels without PRFe were used as the control group. The results indicated an initial burst of protein release during the first day of incubation, followed by a sustained release for up to 10 days. An initial protein release was also observed in control groups, which was significantly ($p < 0.0001$) lower than PRFe-loaded groups and could be due to the dissolution of uncrosslinked ColMA.

Three-dimensional cultivation of SCAP

Supporting cell viability and proliferation is a critical requirement for any scaffold including hydrogels in tissue engineering. To examine the cytocompatibility of the developed PRFe-loaded ChitMA/ColMA hydrogel, the MTT colorimetric assay was used, and the viability of encapsulated SCAP was evaluated after 1, 4, 7, 14, and 21 days of culturing. Hydrogels without PRFe were also used as the control group. As shown in Figure 6A, the incorporation of PRFe had a significant effect on the viability and proliferation of the encapsulated SCAP. Compared to those encapsulated in hydrogels without PRFe, cells encapsulated in PRFe-loaded hydrogels proliferated more than 1.5-fold after 21 days ($p < 0.0001$).

Regarding the morphology and cytoskeleton organization of encapsulated SCAP, F-actin and DAPI staining were performed after 7 days of culturing. The results indicated that the presence of collagen in the hydrogel structure supported cell attachment and elongation. However, encapsulated SCAP into the ChitMA/ColMA hydrogel with incorporated PRFe exhibited elongated shapes with more pronounced and extended actin filaments (Figure 6B).

Chemotactic properties of PRFe-loaded hydrogels

To assess the effect of PRFe incorporation on promoting SCAP migration, cells were cultured on the surface of bicomponent hydrogels with and without PRFe, and the cell migration into the hydrogels was compared using a live/dead assay. Figure 6C shows the top view and the transverse section of cell-seeded samples after 24 hours of cultivation. The results indicated the chemotactic effect of PRFe evidenced by a more than 2-fold greater number of migrated cells.

Effect of PRFe on promoting odontogenic differentiation

Cell-laden ChitMA/ColMA hydrogels with and without PRFe were cultured in the odontogenic medium for up to 21 days. Cross-sectional views of the developed hydrogels visualized with H&E staining indicated cell spreading within the hydrogels, however, the presence of PRFe led to the greater elongation of encapsulated cells (Figure 7A). To investigate the effect of PRFe incorporation on *in vitro* odontogenic differentiation of encapsulated SCAP, real-time PCR, alizarin red S staining, and immunohistochemistry were carried out. For all cell-laden hydrogels in the presence or absence of PRFe, and at different time points (day 14 and 21), the expression of odontogenic related genes, including ALP, COL I, DSPP, and DMP1, as well as the expression of VEGFA were evaluated by real-time PCR (Figure 7B). Compared to hydrogels without PRFe, the expression of odontogenic markers in the PRFe-loaded group was much greater, which indicated the positive effect of PRFe on promoting odontogenic differentiation. The expression level of VEGFA was also increased in the presence of PRFe. Gene expression in the PRFe-loaded group was enhanced from day 14 to day 21. However, no significant differences in gene expression were observed for the ChitMA/ColMA hydrogels without PRFe over time. Moreover, the incorporation of PRFe also promoted mineralized

nodule formation *in vitro* as evidenced by a significantly larger mineralized area ($p = 0.004$) defined with alizarin red S staining for the PRFe-loaded group (Figure 7C).

In addition to gene expression, the production of DSPP and COL I was visualized in ChitMA/ColMA hydrogels with and without PRFe after 3 weeks. As shown in Figure 8, the incorporation of PRFe significantly increased the production of such proteins compared to the group without PRFe ($p < 0.001$). The expressions of COL I and DSPP for the PRFe-loaded group were about 54% and 60%, respectively, while those for the group without PRFe were about 8% and 7.5% (calculated using ImageJ).

Discussion

Due to the presence of a mixture of cytokines and growth factors, such as TGF-β1, VEGF, PDGF, FGF, *etc.*, in platelet granules (Qiao et al., 2017), PRF is an appropriate endogenous source for guiding odontogenic differentiation and angiogenesis in the field of regenerative endodontics. Previous studies indicated the positive effect of PRF on the fate of odontogenic stem cells and the promotion of tissue healing and regeneration (F. M. Huang et al., 2010; Hartshorne & Gluckman, 2016; He et al., 2016; Lolato et al., 2016; Bakhtiar et al., 2017; Kim et al., 2017; Panda et al., 2020). However, the use of PRF in clinical settings is associated with several limitations mainly related to its rapid degradation and non-tuneable properties. Currently, combining platelet concentrates with biomaterials to design scaffolds with tuneable properties while using their beneficial effects has received much attention (Almeida et al., 2018; Silva et al., 2018; Zhang et al., 2021; Liang et al., 2022).

Using PRF extract as a protein-rich solution combined with *in situ* forming ChitMA/ColMA hydrogels has the potential to provide a promising strategy to boost chemotactic and pro-angiogenic activity for the regeneration of pulp-like tissue. In this study, gels were formed by adding PRFe and photoinitiators to the mixture of methacrylated polymers dissolved in distilled

water, and subsequent photocrosslinking. Being injectable and photocrosslinked *in situ*, the PRFe-loaded hydrogel can easily adapt to irregular-shaped root canals and interact with the dentinal wall and preserved tissues.

Various concentrations of ChitMA/ColMA prepolymer solutions (75/25, 50/50, and 25/75), as well as different light exposure times (20, 30, and 40 seconds) with the fixed photoinitiator and PRFe concentrations, were used to evaluate the properties of bicomponent hydrogels. Herein, the PRFe concentration in the bicomponent hydrogels was fixed at 20% (v/v). Treatment of SCAP with media supplemented with different concentrations of PRFe indicated that the enhancement of PRFe concentration from 0.5 to 20% (v/v) enhanced cell viability significantly (Figure S3). Whereas, higher concentrations of PRFe facilitated collagen self-assembly and led to the formation of non-homogenous hydrogels (data not presented). Hence, at the constant concentration of PRFe, mechanical strength, swelling ratio, and cytocompatibility of the bicomponent hydrogels were assessed to optimize the most suitable hydrogel for application in regenerative endodontics. As illustrated in Figure 3, a combination of parameters affected hydrogel properties, including light exposure time and hydrogel composition. Increasing the collagen concentration improved cell-matrix interaction via providing more cell binding sites while increasing chitosan concentration improved the mechanical strength. Previous studies also indicated that a combination of surface and mechanical characteristics of scaffolds influenced the fate of SCAP *in vitro* (Lambrichta et al., 2014). Considering such observations, the hydrogel crosslinked after 20 seconds of light exposure and with the ChitMA/ColMA ratio of 50/50 was selected as the optimum scaffold for cell encapsulation.

Evaluating the effect of PRFe incorporation on hydrogels properties indicated that the PRF-loaded hydrogel had lower stiffness (Figure 4C), which aligned with the results obtained from the rheological analysis where the incorporation of PRFe led to a reduction in storage modulus, indicating higher elasticity (Figure 4D). This may be explained by the reduction of

ChitMA/ColMA macromer mobility or phase separation due to the steric hindrance provided by PRFe protein integration that impair methacrylate crosslinking after light exposure. Also, the PRFe-loaded hydrogel had a higher swelling ratio. In endodontic regenerative therapies, the shrinkage of scaffolds may adversely affect new tissue formation by creating voids that detach the cells from the dentinal wall. While an excessive swelling ratio can increase the hydrostatic pressure inside the root canal. In this study, the addition of PRFe to the ChitMA/ColMA construct made it more suitable in terms of shape retention and formation of the final tissue structure.

Platelet rich fibrin (PRF) is a natural fibrin matrix that entraps a plentiful of cytokines and growth factors. Previous studies have indicated the ability of PRF to progressively release growth factors, such as TGF-β1, VEGF, PDGF, *etc.,* during fibrin matrix remodelling (Dohan et al., 2006; Dohan Ehrenfest et al., 2009). In the present study, the presence of TGF-β1 and VEGF in the extract obtained from PRF was demonstrated using ELISA (Figure 1B). The transforming growth factor-beta1 (TGF-β1) is an important growth factor, which is essential for odontoblastic differentiation during tooth development and dentine mineralization (Tziafas & Papadimitriou, 1998; Smith, 2003; Oka et al., 2007), and VEGF is an essential factor to regulate angiogenesis. Due to the presence of various growth factors in the PRFe, the synergistic effects of these bioactive molecules are expected. To evaluate the release of PRFe-origin proteins, which also include cytokines and growth factors, the BCA assay was used. The release kinetics from the PRFe-loaded ChitMA/ColMA hydrogels after incubation in PBS was characterized by an initial burst release followed by a sustained release for up to 10 days (Figure 5C). The release profile can be explained by two processes, that is, the sequestration of proteins by the polymeric networks and electrostatic interaction. Regarding the electrostatic interaction, the elution of positively charged growth factors with the high isoelectric point was facilitated by ionic repulsion due to the positive charge of ChitMA, appearing as an initial burst release.

While, the negatively charged growth factors with low isoelectric points would bind electrostatically to positive ChitMA to be further released by ion exchange or by the degradation of the ChitMA/ColMA polymeric matrix over time.

Encapsulation of SCAP in the PRFe-loaded ChitMA/ColMA hydrogels demonstrated the positive effect of PRFe incorporation on the viability, proliferation, spreading, and morphology of SCAP. The enhanced viability of SCAP in hydrogels loaded with PRFe was observed (Figure 6A), which was due to the presence of TGF-β1 and PDGF in the PRF (He et al., 2016). Bellamy et al. (2016) reported that the controlled release of TGF-β1 significantly increased the viability of SCAP *in vitro*. PDGF is also a well-known potent mitogenic agent regulating cell growth, division, and differentiation (Kardas et al., 2020). Moreover, it has been reported that PDGF may contribute to cell attachment and facilitate cell spreading (Roberts et al., 2001). Assessment of the morphology of encapsulated cells using phalloidin/DAPI staining clearly indicated that SCAP exhibited abundant cellular extensions in the presence of PRFe (Figure 6B). The softer characteristic of the PRFe-loaded hydrogel compared to the hydrogel without PRFe, which was evidenced by lower Young's and storage moduli, might also facilitate cell spreading. The chemotactic properties of TGF-β1, PDGF, and bFGF over odontogenic stem cells, as reported previously (Smith, 2003; Takeuchi et al., 2015; He et al., 2016; Zhang et al., 2017), also led to the promotion of SCAP migration through the PRFe-loaded hydrogel, demonstrated by significantly denser migratory cells (Figure 6C). Cell migration is a key element in the success of tissue repair and regeneration, especially when using cell homing approaches. Therefore, the developed PRFe-loaded ChitMA/ColMA hydrogel may have potential to recruit resident stem cells and promote their proliferation and differentiation to regenerate pulp-dentine complex via cell homing strategies.

After investigating the effect of PRFe incorporation to support the viability of SCAP and promoting their migratory ability, the ability of the developed hydrogels to guide cell

differentiation when incubated in the odontogenic medium was studied. The data on changes in mRNA expression levels for the developed hydrogels with and without PRFe indicated a significant effect of PRFe on inducing differentiation of SCAP into odontoblast-like cells (Figure 7B). Alkaline phosphatase (ALP) plays a key role in biomineralization, and its production determines an initial event in such a process (Golub & Boesze-Battaglia, 2007). Compared to the developed ChitMA/ColMA hydrogel without PRFe, the expression of ALP by SCAP encapsulated in the PRFe-loaded hydrogels was significantly enhanced with the expression peak being observed on day 21. The overexpression of this early marker of biomineralization in the PRFe-loaded group followed by the higher deposition of calcified nodules identified with alizarin red S staining (Figure 7C) and a greater mRNA expression of COL I. Type I collagen (COL I) composes the main protein constituent of dentine matrix and plays a critical role in its biomineralization via providing initiation sites for calcification (He et al., 2019). In addition to COL I, levels of other odontoblastic markers gene, i.e. DSPP and DMP1, were also markedly increased in the hydrogels loaded with PRFe. DSPP and DMP1 are dentinal noncollagenous proteins that play a crucial role during dentinogenesis, and their expression is characteristic during odontoblastic differentiation (Qin et al., 2004). The positive effect of PRFe on the production of COL I and DSPP was also confirmed by immunohistochemistry, where the production of such proteins was significantly greater in the PRFe-loaded hydrogels (Figure 8). The incorporation of PRFe into the ChitMA/ColMA hydrogels modulates the micro-environment enrichment with growth factors, such as TGF-β1, PDGF, bFGF, etc., that may stimulate the odontogenic differentiation of SCAP. For example, previous studies determined TGF-β1 as a promoter of odontogenic differentiation (Smith, 2003). The upregulation of VEGFA, which is a proangiogenic gene, under the influence of PRFe also suggested that the developed PRFe-loaded hydrogels may facilitate angiogenesis and thus dental pulp regeneration. Due to the entrapment of dental pulp tissue within teeth and

limited access to blood supply, promoting the establishment of vascular networks is a primary requirement to regenerate pulp-like tissue throughout the entire root canal system. However, further investigations, especially *in vivo* tests, are required to evaluate the regenerative performance of the developed construct in clinical settings.

Conclusion

A bicomponent hydrogel loaded with PRFe has been developed as a potential scaffold for pulp-dentine complex tissue engineering. The new hydrogel fulfils the criteria of injectability, cytocompatibility, cell-mediated degradation, bioactive factor release, and promotion of odontogenic differentiation. The growth factor content of PRFe allows it to be used as an endogenous source of signaling molecules to support cellular activities, such as proliferation, migration, and differentiation. The developed ChitMA/ColMA hydrogel incorporating PRFe protected the viability of SCAP and promoted cell proliferation. Moreover, the addition of PRFe to the bicomponent hydrogel upregulated the expression of odontogenic markers and stimulated the deposition of mineralized nodules by SCAP. Overall, the results provide strong evidence for the potential of the developed photocrosslinkable hydrogels incorporating PRFe to be used as a bioactive scaffold in endodontic regenerative therapeutics.

Author contributions

Parisa Noohi carried out the experiments and wrote the original draft under the supervision of Mohammad J. Abdekhodaie and Mohammad H. Nekoofar. All authors contributed to the scientific discussions and read and approved the final version of the manuscript.

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Conflict of interest

The authors declare no conflict of interest.

Ethics statement

The present study was approved by the Ethics Committee of Tehran University of Medical Sciences (IR.TUMS.DENTISTRY.REC.1397.102).

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Figure Legends

Figure 1 PRFe preparation and characterization. (A) Preparation of PRFe. (i) The blood sample after centrifuging. (ii) Prepared PRF. (iii) Prepared PRFe. (B) Concentration of growth factors in PRFe. (C) The effect of PRFe on the viability and proliferation of SCAP. Data are presented as mean ± SD (**p < 0.01, ****p < 0.0001).

Figure 2 Synthesis and ^{1}H NMR analysis of PRFe-loaded ChitMA/ColMA hydrogels. (A) Schematic representation of synthesis and photocrosslinking of the PRFe-loaded bicomponent hydrogels. (B) ^{1}H NMR spectra of non-modified collagen and ColMA. (C) ^{1}H NMR spectra of non-modified chitosan and ChitMA. Red arrows show the characteristic peaks of methacrylate moieties.

Figure 3 Optimization of PRFe-loaded bicomponent hydrogels. (A) Young's modulus, (B) swelling ratio, and (c) cell viability of PRFe-loaded bicomponent hydrogels fabricated upon various light exposure times and different ChitMA/ColMA ratios. Data are presented as mean ± SD (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

Figure 4 Effect of PRFe on hydrogel properties. (A) Degradation rate of ChitMA/ColMA hydrogels with and without PRFe in lysozyme [L], collagenase [C], and dual enzyme [D] solutions. (B) Swelling ratio, (C) Young's modulus, (D) storage and loss moduli, and (E) damping factor of ChitMA/ColMA hydrogels with and without PRFe. Data are presented as mean ± SD (*p < 0.05, ***p < 0.001).

Figure 5 Morphological properties of hydrogels and total protein release. (A) Cross-sectional SEM images of ChitMA/ColMA hydrogels with and without PRFe. (B) The pore size and porosity of ChitMA/ColMA hydrogels with and without PRFe. (C) Total protein release from ChitMA/ColMA hydrogels with and without PRFe incubated in PBS using BCA protein assay kit. Data are presented as mean ± SD (*p < 0.05, **p < 0.01).

Figure 6 *In vitro* cultivation of SCAP within and on top of hydrogels. (A) Viability of SCAP encapsulated within hydrogels with and without PRFe after 1, 4, 7, 14, and 21 days. (B) Representative phalloidin (red)/DAPI (blue)-stained images of cell-laden hydrogels with and without PRFe after 7 days. (C) Representative live/dead images of SCAP seeded on hydrogels with and without PRFe after 24 hours. Data are presented as mean ± SD (*p < 0.05, ****p < 0.0001).

Figure 7 Effect of PRFe on SCAP differentiation. (A) Cross-sectional view of cell-laden hydrogels with and without PRFe visualized with H&E staining on day 21. (B) Gene expression profiles and (C) mineralized nodules formed by SCAP encapsulated within hydrogels with and without PRFe. Data are presented as mean ± SD (ns: not significant, *p < 0.05, **p < 0.01).

Figure 8 Effect of PRFe on protein expression. Visualization and quantification of DSPP and COL I protein secretion by SCAP encapsulated within ChitMA/ColMA hydrogels with and without PRFe on day 21. Data are presented as mean ± SD (***p < 0.001).

Table Legends

Table 1. Polymerase chain reaction primers.

Supporting Information

Figure legends

Figure S1 Preferred Reporting Items for Laboratory studies in Endodontology (PRILE 2021)-based flowchart.

Figure S2 Characterization of isolated type I collagen. (A) SDS-PAGE of isolated collagen from rat tail tendons and commercially available type I collagen from bovine skin (Sigma-Aldrich) as the reference. (B) UV/VIS, (C) FTIR, and (D) CD spectra of isolated collagen from rat tail tendons.

Figure S3 Effect of PRFe concentration on cell viability. Viability of SCAP treated with different concentrations of PRFe. Data are presented as mean ± SD (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

Table legends

Table S1 Effect of PRFe on the viability and proliferation of SCAP via MTT assay (Abs at 490 nm).

Table S2 Optimization of PRFe-loaded bicomponent hydrogels (Young's modulus [kPa]).

Table S3 Optimization of PRFe-loaded bicomponent hydrogels (swelling ratio [%]).

Table S4 Optimization of PRFe-loaded bicomponent hydrogels (cell viability [%]).

Table S5 Effect of PRFe on hydrogel properties (degradation [%]).

Table S6 Effect of PRFe on hydrogel properties (swelling ratio [%]).

Table S7 Effect of PRFe on hydrogel properties (Young's Modulus [kPa]).

Table S8 Effect of PRFe on hydrogel properties (storage and loss moduli [Pa]).

Table S9 Effect of PRFe on hydrogel properties (damping factor).

Table S10 Effect of PRFe on hydrogel properties (pore size [μm]).

Table S11 Effect of PRFe on hydrogel properties (porosity [%]).

Table S12 Total protein release from hydrogels (cumulative release [μg/hydrogel]).

Table S13 Cell viability via MTT assay (Abs at 490 nm).

Table S14 RT-PCR (relative expression of mRNA).

Table S15 IHC (protein expression [%]).