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Proceeding Paper

Decellularized Extracellular Matrix Polycaprolactone/Chitosan Composite Nanofibrous Scaffolds for Periodontal Tissue Engineering †

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Abstract: Periodontitis is an inflammatory infection caused by bacterial plaque accumulation that affects the periodontal tissues supporting the teeth. Current treatments lack bioactive signals to induce tissue repair and coordinated regeneration of all the periodontal tissues, thus alternative strategies are needed to improve clinical outcomes. Cell-derived extracellular matrix (ECM) has been used in combination with biomaterials to enhance their biofunctionality for tissue engineering (TE) applications. In this work, bioactive cell-derived ECM loaded electrospun polycaprolactone/chitosan (PCL-CTS) nanofibrous scaffolds were developed using lyophilized decellularized ECM (dECM) derived from human Periodontal Ligament Stem Cells (PDLSCs). This work's aims were to fabricate and characterize cell-derived ECM electrospun PCL-CTS scaffolds, and to evaluate their capacity to enhance the proliferation of seeded PDLSCs, envisaging future periodontal TE applications.

Keywords: cell-derived extracellular matrix; electrospinning; periodontal ligament stem cells; periodontal regeneration; tissue engineering

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1. Introduction

Periodontitis affects the periodontium, a hierarchical complex structure composed of alveolar bone, cementum and periodontal ligament, responsible for tooth support and attachment [1]. Severe cases of periodontitis are characterized by degradation of all periodontal tissues, which can lead to tooth loss [2]. In 2018, the economic burden of periodontal disease was estimated to be €158.64B in Europe [3]. Current treatments include bone grafts and membranes for guided tissue regeneration (GTR), which present limitations such as low attachment to the adjacent tissues and poor ability to promote regeneration of all periodontal tissues [4,5]. Alternative treatment strategies that lead to a coordinated regeneration of a functional periodontium are needed to improve clinical outcomes.

Tissue engineering (TE) aims to develop constructs that facilitate tissue regeneration. One strategy consists on the use of electrospun fibrous scaffolds, which can mimic the structure and architecture of the native fibrous extracellular matrix (ECM), thus

promoting cell attachment, proliferation and differentiation [6]. Electrospun nanofibrous scaffolds present suitable characteristics to be used as novel GTR membranes or to be part of multilayered biomimetic constructs for periodontal regeneration. Decellularized cell-derived ECM (dECM) has been employed in combination with scaffolds to enhance their bioactivity in several TE strategies [7–10]. dECM closely mimics the in vivo microenvironment and promotes cell proliferation and differentiation *in vitro*.

In this work, bioactive dECM loaded electrospun polycaprolactone/chitosan (PCL-CTS) composite nanofibrous scaffolds were developed using lyophilized dECM derived from human Periodontal Ligament Stem Cells (PDLSCs). The nanofibrous scaffolds were characterized in terms of their structure and elemental composition, and their biological effects on PDLSC in vitro proliferation were assessed. To the best of our knowledge, this work constitutes the first study in which dECM derived from PDLSCs was incorporated into PCL/CTS composite electrospun nanofibers to develop bioactive and biomimetic dECM loaded nanofibrous scaffolds with enhanced bioactivity for periodontal TE strategies.

2. Materials and Methods

2.1. Fabrication of Electrospun Nanofibrous Scaffolds

The materials and solvents used to prepare the polymeric solutions for electrospinning were: Polycaprolactone (PCL, Mn = 70,000–90,000 Da, Sigma-Aldrich); 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Tokyo Chemical Industry); medium molecular weight chitosan (CTS, Mn = 190,000–310,000 Da, Sigma-Aldrich); trifluoroacetic acid (TFA, Honeywell); dichloromethane (DCM, Honeywell). PCL (13% *w*/*v*) was dissolved in HFIP under agitation at room temperature. CTS (5% *w*/*v*) was dissolved in a TFA/DCM (70/30 *v*/*v*) solvent mixture and stirred for 1.5 h at 50 °C, using a magnetic stirrer. PCL and CTS solutions were blended together to obtain a 70/30 ratio *v*/*v* PCL-CTS blend solution, followed by agitation overnight. In the case of PCL-CTS-ECM electrospun fibers, lyophilized PDLSC-derived ECM (produced according to previously reported methods [7,10]) was incorporated into the CTS solution (1 mg/mL) and dispersed through agitation using a magnetic stirrer. Then, the fibrous scaffolds were fabricated by electrospinning. PCL, PCL-CTS and PCL-CTS-ECM solutions were loaded into a syringe placed in a pump and connected to a tube, which was attached to a 21G stainless steel needle. The electrospinning parameters for all solutions were: a flow rate of 0.5 mL/h, an applied voltage of 24 kV and a distance of 22 cm between needle tip and aluminum foil collector. All fibrous scaffolds were electrospun for 2.5 h, to ensure scaffold thickness, and with temperature and relative humidity varying between 23–24 °C and 30–40%, respectively.

2.2. Characterization of Electrospun Scaffolds

The morphology of electrospun fibers was characterized through Scanning Electron Microscopy (SEM) using a Phenom ProX G6 Desktop SEM (Thermo Fisher Scientific). Samples were coated with a gold/palladium layer and imaged at several magnifications. Using ImageJ software (ImageJ 1.51f, National Institutes of Health), the average fiber diameters were computed by measuring 100 individual fibers per condition from five different SEM images. The elemental composition of scaffolds was evaluated through Energy Dispersive X-Ray Analysis (EDX) using the Phenom ProX G6 Desktop SEM.

2.3. In Vitro Cell Culture on Electrospun Scaffolds

2.3.1. Scaffold Preparation and PDLSC Seeding

Before cell culture, PCL, PCL-CTS and PCL-CTS-ECM electrospun scaffolds were sterilized for 30 min under UV light exposure and washed three times (1 h each wash) with phosphate buffered saline (PBS, Gibco) + 1% antibiotic-antimycotic (A/A, Gibco) solution. The scaffolds were placed in ultra-low cell attachment 24-well plates, washed again with PBS + 1% A/A, and then incubated for 1 h at 37 °C in culture medium (low-glucose

Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS MSC qualified, Gibco) and 1% A/A).

PDLSCs were seeded on PCL, PCL-CTS and PCL-CTS-ECM electrospun scaffolds at a density of 50,000 cells per scaffold and incubated for 2 h at 37 °C and 5% CO_2 to promote initial cell attachment. The human PDLSCs used are part of the cell bank available at the Stem Cell Engineering Research Group (SCERG), Institute for Bioengineering and Biosciences (iBB) at Instituto Superior Técnico (IST). Osteogenic medium, consisted of DMEM supplemented with 10% FBS, 1% A/A, 10 mM β-glycerophosphate (Sigma-Aldrich), 50 μg/mL ascorbic acid (Sigma-Aldrich) and 10 nM dexamethasone (Sigma-Aldrich) was then added to all the scaffolds. PDLSCs were cultured on the scaffolds for 14 days and medium renewal was performed every 3-4 days.

2.3.2. PDLSC Viability and Proliferation Assay

AlamarBlue® Cell Viability Assay (Thermo Fisher Scientific) was used to evaluate the metabolic activity of PDLSCs on the different electrospun scaffolds on days 1, 7 and 14. A 10% (*v*/*v*) AlamarBlue® solution prepared in culture medium was added to the scaffolds and incubated at 37 °C in 5% CO_2 chamber for 3 h. The fluorescence intensity was measured on a plate reader (Infinite 200 Pro, Tecan) at an excitation/emission wavelength of 560/590 nm and compared to a calibration curve to determine the equivalent number of cells in each scaffold. Six independent scaffolds (N=6) were used for each condition and fluorescence was measured in triplicates. Scaffolds without cells were used as blank controls.

2.4. Statistical Analysis

Data was statistically analyzed with GraphPad Prism 9 software using one-way ANOVA, followed by Tukey post-hoc test. Data was considered statistically significant when the *p*-values obtained were less than 0.05 (95% confidence intervals, $*p < 0.05$).

3. Results

SEM images of PCL, PCL-CTS and PCL-CTS-ECM electrospun fibers (Figure 1A, B, C, respectively) showed that all the scaffolds were composed of beadless and continuous nanofibers. PCL electrospun scaffolds presented an average fiber diameter of 299 ± 169 nm, hence were more heterogenous in terms of fiber diameter, as can be seen in Figure 1A. The average fiber diameters of PCL-CTS and PCL-CTS-ECM fibers were 122 ± 21 nm and 132 ± 32 nm, respectively. Since PCL-CTS and PCL-CTS-ECM fibers showed similar diameters, the incorporation of lyophilized dECM into the PCL-CTS solution did not significantly affect the average fiber diameter or the electrospinning process.

EDX analysis showed that carbon and oxygen were the main constituents of all the scaffolds (Figure 1D–F). PCL-CTS and PCL-CTS-ECM scaffolds demonstrated similar carbon and oxygen percentages, that differed slightly from the PCL scaffolds. Both PCL and CTS polymers are composed of carbon and oxygen, however CTS also presents nitrogen in its composition. Nitrogen was only detected in scaffolds containing CTS, namely PCL-CTS and PCL-CTS-ECM scaffolds (Figure 1E,F).

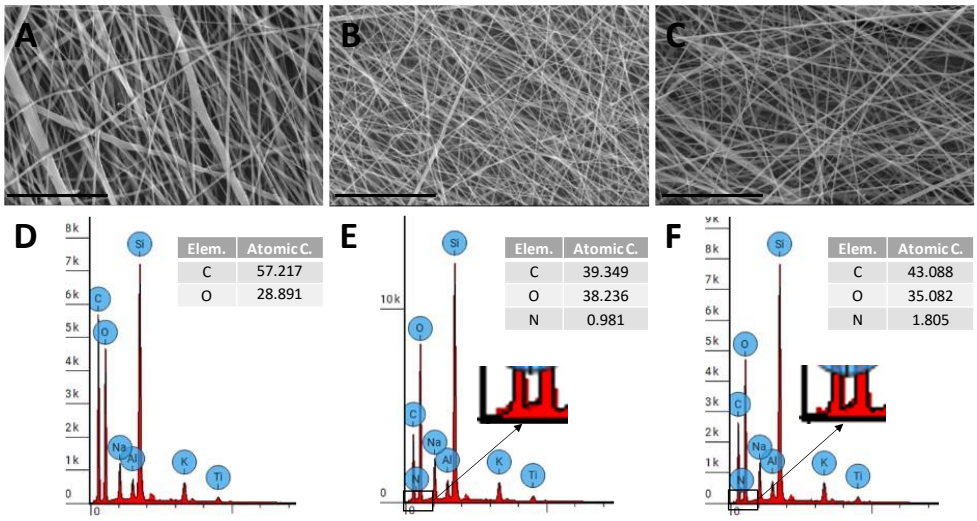

Figure 1. Scaffold characterization. SEM images of PCL (**A**), PCL-CTS (**B**) and PCL-CTS-ECM (**C**) electrospun scaffolds. Scale bar 8 µm. EDX spectra and atomic percentages of carbon (C), oxygen (O) and nitrogen (N) of PCL (**D**), PCL-CTS (**E**) and PCL-CTS-ECM (**F**) electrospun scaffolds.

The biological performance of the different electrospun scaffolds was assessed by measuring the metabolic activity of PDLSCs (Figure 2A). Interestingly, at days 1 and 7, PCL scaffolds showed a higher number of viable cells compared to PCL-CTS and PCL-CTS-ECM scaffolds. However, after 14 days, PCL-CTS ($(2.598 \pm 0.083) \times 10^5$) and PCL-CTS-ECM ($(2.833 \pm 0.070) \times 10^5$) scaffolds presented higher cell numbers compared to PCL scaffolds ($(2.439 \pm 0.100) \times 10^5$). In fact, PCL-CTS and PCL-CTS-ECM scaffolds demonstrated a statistically significant higher fold increase in the number of viable cells (Figure 2B), in comparison to PCL scaffolds. Importantly, PCL-CTS-ECM scaffolds presented significantly higher fold increases compared to PCL-CTS scaffolds, which suggests that the presence of dECM in the composition of the electrospun fibers elicits a beneficial response by PDLSCs.

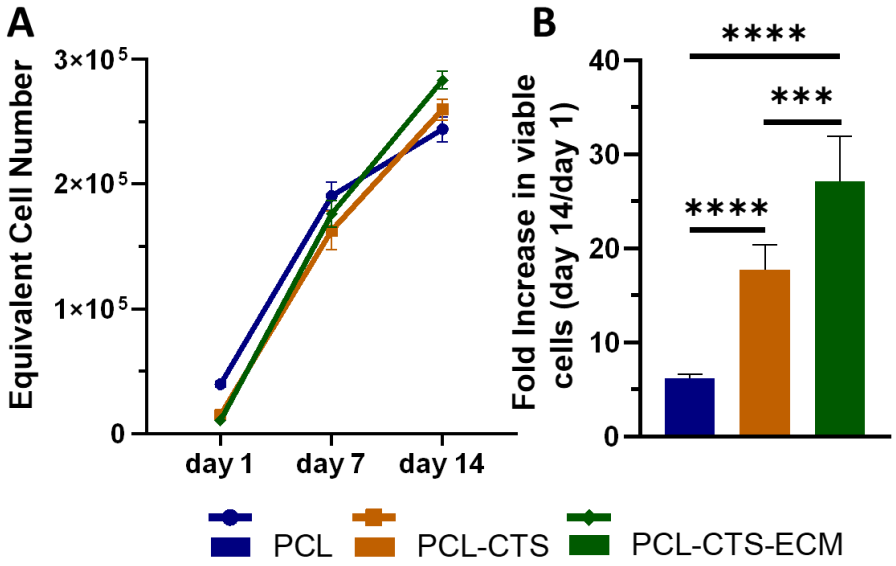

Figure 2. Effects of electrospun scaffolds on PDLSCs proliferation. PDLSCs numbers at days 1, 7 and 14 (**A**) and fold increase (**B**) in the number of viable cells at day 14 (in relation to day 1) on PCL, PCL-CTS and PCL-CTS-ECM electrospun scaffolds. For each experimental group, six different samples (N = 6) were considered in the analysis; *** $p < 0.001$, **** $p < 0.0001$.

4. Discussion

The periodontium is a complex structure and its coordinated regeneration is essential in the treatment of periodontitis. Periodontal TE strategies should have the ability to mimic the structure and composition of the periodontium and to promote the repair of all periodontal tissues. Decellularized cell-derived ECM can be employed to develop scaffolds that, in addition to the structure and architecture, also mimic more closely the composition of the native tissue microenvironment [7,9].

In this work, human PDLSCs were used for dECM production, aiming to recreate the periodontal niche. The fabricated PCL-CTS and PCL-CTS-ECM electrospun scaffolds were composed by nanofibers, thus mimicking the scale of fibers and fibrils present in the native periodontal ligament, which are of nano- to microsized order [11]. PCL-CTS and PCL-CTS-ECM fibers showed similar diameters, indicating that the incorporation of dECM did not affect the electrospinning process or alter the average fiber diameter of scaffolds, as reported in a previous study performed by our group [7]. EDX analysis showed nitrogen content only in PCL-CTS and PCL-CTS-ECM scaffolds, which confirms the presence of CTS in these scaffolds. The nitrogen content in PCL-CTS-ECM scaffolds was slightly higher compared to PCL-CTS scaffolds. This difference might be due to the incorporation of dECM, which is composed of proteins, a known source of nitrogen.

PCL-CTS and PCL-CTS-ECM scaffolds demonstrated a statistically significant increase in PDLSC proliferation in comparison to PCL scaffolds, as confirmed by higher fold increases in the number of viable cells. Increased cell viability on PCL-CTS scaffolds compared to PCL scaffolds has been previously reported in the literature [12,13]. The obtained results show that PCL-CTS-ECM scaffolds presented higher cell numbers and fold increases compared to PCL-CTS, which suggests enhanced PDLSC proliferation due to the presence of dECM in the scaffolds, which lays in agreement with previous studies [7,14].

Overall, PCL-CTS-ECM scaffolds significantly enhanced cell proliferation, while maintaining the nanofibrous structure of PCL-CTS scaffolds. The incorporation of lyophilized dECM in scaffolds is an interesting strategy to obtain a closer mimicry of the in vivo periodontal niche. Importantly, this work describes the first use of lyophilized PDLSC-derived ECM loaded electrospun nanofibrous scaffolds for periodontal TE applications and demonstrates its potential as a novel alternative strategy for periodontitis treatment.

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