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Effect of the surface topographic modification on cytocompatibility of hardened calcium phosphate cement

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ABSTRACT

As cells are inherently sensitive to local nanoscale patterns of topography, the aim of this study was to determine the effect of the topographic modification of hardened calcium phosphate cement on cell response which was conducted with MC3T3-E1 cells. The results exhibited that the samples with regular blade-like crystalline structure had better cell response (cell attachment, viability, proliferation and differentiation) compared to those with irregular blade-like crystalline structure. The method of topographic modification is promising for developing a novel biomaterial of hardened calcium phosphate cement for bone repair.

1. Introduction

Calcium phosphate cement (CPC) is highly promising for wide clinical applications because of its good biocompatibility, excellent bioactivity, low heat release during the self-setting reaction, adequate stiffness, and easy shaping for any complicated geometry. Calcium phosphate cement represents an alternative to the traditional crystalline calcium phosphate ceramics prepared at high temperature, namely sintered hydroxyapatite (HA), which is hardly resorbable, or more resorbable β-tricalcium phosphate (β-TCP). In recent years, new strategies that exploit the intrinsic properties of CPCs have been envisaged, and pre-set CPC scaffolds or granules by various processing techniques have been extensively put forward [1].

The cellular response to an implant or a biomaterial is associated with its morphologic, chemical, and electrical surface characteristics [2,3]. As growing on the rough surface, the cells rearrange their cytoskeleton to better adapt to the underlying substrate, and the cell proliferation is affected by the surface profile. CPC has large specific surface area attributed to its nano-/micro-porous structure along with its intrinsic porosity, which is usually higher than 40%. Therefore, CPC can be fast resorbed, and thus enhance the osteoblast precursor cells attachment through the amount of protein adsorbed [4]. The surface with high roughness and regular topography is beneficial for cell adhesion and proliferation, and increasing cell detachment strength [5–7].

Needle-like and blade-like structures were commonly observed in CPC [5,8,9]. However, these structures did not uniformly distribute in whole sample. In the present study, polyethylene glycol 1500 (PEG1500) was used to modify the topography of the whole surface of hardened CPC. Cell attachment, viability, proliferation, and differentiation on the surface of CPC were assessed.

2. Experimental

The CPC powder used in this study was prepared by mixing partially crystallized calcium phosphate (PCCP, median diameter of 16.5 μm) and dicalcium phosphate anhydrous (DCPA, median diameter of 3.7 μm) at a weight ratio of 1:1, as described in our previous work [10–12]. 0.25 mol/L disodium hydrogen phosphate solution was used as the setting liquid. The CPC powder (P) and liquid (L) were mixed at a L/P ratio of 0.4 mL/g to form workable pastes. Cement pastes were transferred into cylindrical stainless steel moulds (diameter = 12 mm; height = 2 mm) with slight manual pressure to eliminate entrapped air bubbles. After being moulded, the samples were freeze-dried immediately to prevent the further hydration. The freeze-dried samples were designated as CPC-U. CPC-U was incubated in distilled water at 37 °C for 72 h, then freeze-dried (designated as CPC-W). CPC-U was inmersed in 40% (w/v) PEG1500 solution at 37 °C for 72 h, and then washed three times with distilled water to completely remove PEG1500 and freeze-dried (designated as CPC-P).

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X-ray powder diffraction patterns were recorded using a microprocessor-controlled X-ray diffractometer system X'Pert PRO (PANalytical, Almelo, the Netherlands) using $CuK_{\alpha 1}$ radiation. Morphology of surface of the samples was observed using a field emission scanning electron microscope (NOVA NanoSEM430; FEI, Hillsboro, USA). IR spectra of the powdered samples dispersed in KBr tablets were recorded using a Bruker Vetor 33 Fourier transform IR spectrophotometer (Bruker Biospin Corporation, Billerica, MA, USA).

Surface roughness of CPC-W and CPC-P was measured by white-light interferometer (Expert 3D, BMT). Five replicates were measured for each sample to obtain an average roughness value R_a. Statistically significant difference in the R_a values was determined by ANOVA statistical analysis. Differences were considered significant if $p < 0.05$.

Each sample was soaked in PBS at the liquid–solid ratio of 50, and the solution was refreshed every day. The calcium (Ca) and phosphorus (P) ion concentration in the solution were measured by inductively coupled plasma atomic emission spectroscopy (Optima 5300DV; PerkinElmer, Cambridge, USA). The pH value of PBS solution was measured with a pH meter (PB-10; Sartorius, Göttingen, Germany) at intervals.

MC3T3-E1 was used to examine the *in vitro* osteoblastic responses to the surface topography of the samples (CPC-W and CPC-P). The samples were sterilized by γ-radiation at 20 kGy. The samples were transferred to 24-well tissue culture plates (Greiner Bio-One; Frickenhausen, Wemmel, Belgium). An aliquot of cell suspension was seeded onto the surface of samples (6×10^4 cells/sample). The culture medium was refreshed twice a week. After 24 h incubation, specimens were taken out and washed with 1× PBS (phosphate buffer solution) for 3 times, then immobilized with 2.5% glutaraldehyde solution at 4 °C for 2 h. The immobilized samples were dehydrated with a graded series of ethanol (30%, 50%, 70%, 90%, and 100%), then air dried. The morphology of cells on CPC-W and CPC-P was observed by a scanning electron microscope (Quanta 200, FEI, USA).

The viability of MC3T3-E1 cells cultured on samples was evaluated using a Live/Dead kit (Biotium, USA) according to standard protocol provided by the manufacturer after 7 days of culture. The samples were observed with a fluorescence microscope (Zeiss Axioskop 40, Germany). The fluorescent green-colored cells denoted live cells, and the fluorescent red-colored cells were dead or compromised cells with damaged membranes. Cell proliferation on days 1, 3, and 7 were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The absorbance of produced formazan was measured at 490 nm with a microplate reader.

40 μL of cell suspension (2×10^6 cells/sample) was seeded onto the samples in 24-well plate. After 2 h, 1 mL of culture medium (α-MEM supplemented with 10% (v/v) fetal bovine serum (FBS), 10 mM sodium β-glycerophosphate, 10 nM dexamethethasone, and 82 mg/mL vitamin C) was added to the wells. The cell-constructs were cultured in a humidified atmosphere of 5% CO_2 at 37 °C. After cultured for 7 and 14 days, the cell-constructs were washed twice with PBS solution. An aliquot of 400 μL of 0.05% Triton X was added to the culture well and the mixture was incubated at 4 °C for 2 h. The supernatant was tested for alkaline phosphatase (ALP) activity. The ALP activity of MC3T3-E1 was assayed using a Laboassay™ ALP kit (Wako Pure Chemicals, Japan) in accordance with the manufacturer's instructions. 20 μL of supernatant was incubated with 100 μL of *p*-nitrophenyl phosphate (*p*-NPP) solution at 37 °C, and after 15 min, 80 μL of NaOH solution was added to terminate the reaction. The amount of *p*-nitrophenol (*p*-NP) was estimated by measuring the absorbance at 405 nm.

All data points were an average of at least four replicates and expressed as mean ± standard deviation. Statistical comparisons were assessed by one-way analysis of variance (ANOVA). Statistical significance for $p < 0.05$ was denoted by *.

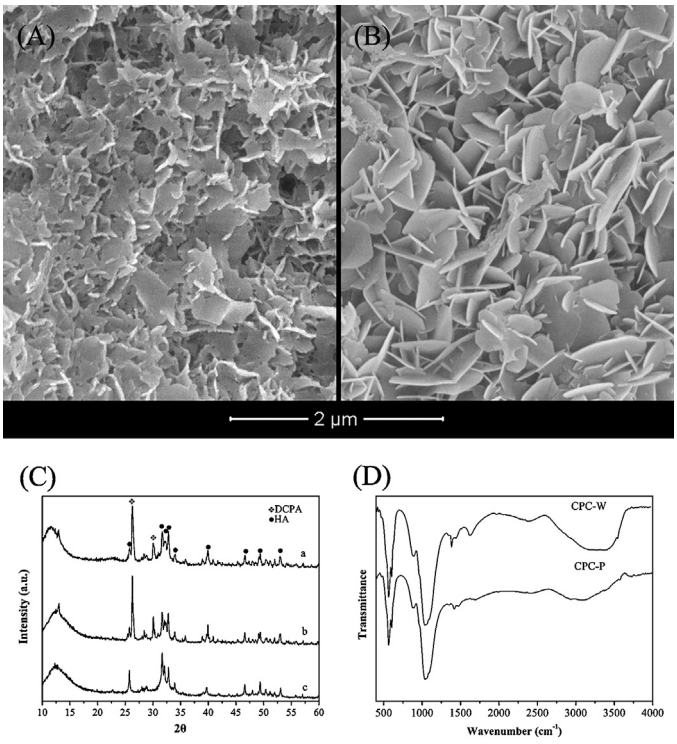

Fig. 1. SEM micrographs of the surface of CPC-W (A) and CPC-P (B) and X-ray diffraction patterns (C) of the surfaces of CPC-P (a), CPC-U (b), and CPC-W (c) as well as IR spectra (D) of the reaction products of CPC-W and CPC-P. The patterns of HA and DCPA are indexed according to JCPDS 9-432 and JCPDS 70-0359, respectively.

3. Results and discussion

As shown in Fig. 1(A), irregular blade-like HA crystals were observed on the surface of CPC-W. Regular blade-like crystals fully covered the surface of CPC-P (Fig. 1(B)). Besides the difference in

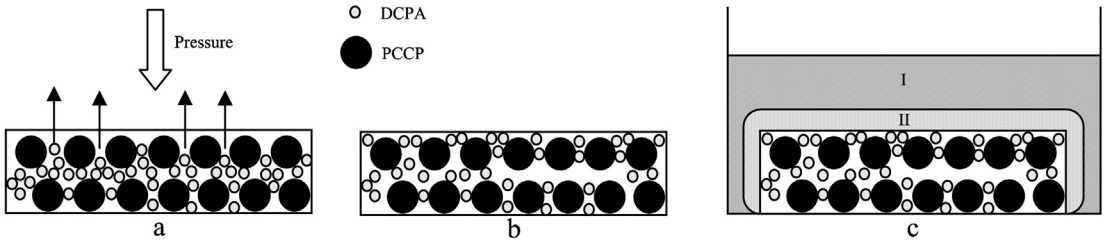

Fig. 2. The schematic diagrams for the formation of the environment for blade-like morphology: (A) under the manual pressure, the fine particles and the liquid moves through the interstice onto the sample surface; (B) as DCPA powder was finer than PCCP powder, DCPA was enriched on the sample surface; (C) ionic migration and exchange were slow in PEG1500 solution resulting in higher ionic concentration in region II than in region I.